STUDIES ON VIROLOGIC, IMMUNOLOGIC AND OTHER HOST FACTORS CONTRIBUTING TO RESISTANCE TO HIV INFECTION IN DISCORDANT AND CONCORDANT COUPLES IN ETHIOPIA

A thesis submitted to the school of Graduate studies of Addis Ababa University in Partial fulfillment of the requirements for the Degree of Doctor of Philosophy (PhD) in Medical Microbiology

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MARCH, 2011

ADDIS ABABA

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Acknowledgments

I would like to express my sincere gratitude and respect to institutions and persons who have helped me to realize this work, and in particular to Kotebe College of Teachers Education and specially the department of biology for giving me this opportunity and being understanding and helpful during my study and bearing a burden of teaching and other assignments during all years of my study.

Addis Ababa University, Medical Faculty, Department of Microbiology, Parasitology and Immunology; Murdoch University, Perth, Australia; Center for Clinical immunology and Biostatistics(CCIB), Perth, Australia and Ethiopian Nutrition and Health Research Institute (EHNRI) for sponsoring and financial support, for allowing me to do part of my research in their respective institutes, for giving me a training and support. Their different ways of thinking was always very constructive in the many brain storming sessions.

Special thanks go to all members of CCIB for the collaboration concerning discordant couples and HIV-infected Ethiopians and for making my stay in Perth homely and comfortable. You are helping, kind, cooperative and open for fruitful discussions. Thank you very much!!

My sincere thanks are due to Oromia health Bureau, Amhara health Bureau, Tigray health Bureau, Southern Peoples nations and nationalities health Bureau and Addis Ababa health Bureau for allowing me to collect samples and for their unreserved help during my years of study. Special thanks also goes to Minilik hospital, Akaki health center, Arada health center, Gulale health center, Adama hospital, Kuyera hospital, Jimma health center and Awwasa health center for their special collaboration in counseling and advising patients and in collecting adequate number of samples from your respective institutes.

I am deeply indebted to my three advisors: Dr. Yohannes Mengistu for your generous and sincere support, and helping me through all the hardships in making this thesis a reality. I appreciate your kind help and for being with me in bad and good days. Without your help my stay at AAU would have been impossible. Dr. Dawit Wolday for your generous and sincere support and excellent supervision and knowledge. I would have been crippled and drawn in the swamp without you sending me to Australia, through your excellent ability of working with the world and your far-sighted outlook. I would have not completed this project without you helping me. Your help can not be expressed in words but you were like a good father bringing what ever I want from where ever you went. , Dr. Tsehaynesh Messele for conceiving the research idea and for allowing me to do part of my research work at EHNRI. Like my all other supervisors, without your strong support and help I wouldn't have worked this PhD research. I worked on my project not like a student but like a

legitimate worker of your institute because of you. Thank you very much for your kind help and encouragement.

Special thanks and respect goes to Nick Anderson, BD East African director for providing monoclonal antibodies worth many thousands of Birr and for preparing a template on which I work. You are generous, helpful and kind Nick! It was for your kind help that this thesis is a reality.

I appreciate the contribution of Dr. Aster Tsegaye for her unreserved help and valuables scientific discussions and for always being available when I was in need of help. Analysis of T cell subpopulations would have been impossible without your help.

My great thanks go to the laboratory staff of EHNRI, especially to Ermias Hailu for he was with me in all stages from sample collection to acquisition and analysis of my samples. He was always available when I was in need of help. So, I am highly indebted to him for his kind and genuine help.

I also appreciate Hailu Meles for his encouragement and help and material provision. I would also like to thank w/ro Alemtsehaye for her moral and material support and for being available when I was in need of help. I also thank Mulu and Asfaw for their moral and material support and encouragement.

My deepest appreciation and thanks also go to Dr. Gobena and Dr. Dawit Wolday for providing me with reagents which were not available in the country. It was a big help!

Finally, I would like to thank Dr. Almaz Abebe for facilitating my lab work by providing necessary administrative help. And last but not least, I would like to thank Geremew Tasew for always being in support of me. He was always available when I was in need of help and I can say he was my teacher of computer skill. Thus, I highly appreciate him for his encouragement and showing interest in my work. I also appreciate Mesfin Gemeda for helping me in the data analysis of viral sequences.

Overall, I wish to express my appreciation to all physicians and nurses who provided help in the different study sites in particular in the different health centers and hospitals; and to all study subjects for their willingness to participate in the studies included in my thesis.

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Abbreviations and acronyms

AAU Addis Ababa University
AIDS Acquired immunodeficiency syndrome
ART Antiretroviral treatment
CCIB Center for clinical immunology and biostatistics
CSW Commercial sex workers
CTL **C**ytotoxic T lymphocytes
CVL Cervicovaginal lavage
DC Dendritic cells
DP Dual platform
EBV Epestien var virus
EHNRI Ethiopian Health and nutrition institute
ESTC Ethiopian science and technology commission
FCM Flow cytometer
FGM Female genital mutilation
Gp Glycoprotein
HAART Highly active antiretroviral treatment
HPCO HIV prevention and control organization
HIV Human immunodeficiency virus
HLA Human leukocyte antigen
HTLV Human T cell leukemia- lymphoma virus
HSV Herpes simplex virus
HTP Harmful traditional practices
Ig Immunoglobulin
IL-7 Interleukin-7
IO Opportunistic infections
IRD Immune restoration disease
LAV Lymphadenopthy-associated virus
LTNP Long-term-non-progressors
LTR Long terminal repeat
MSM Males having sex with male
MHC Major Histocompatibility complex
MOH Ministry of health

NASBA Nucleic acid sequence based amplification

NK Natural killer cells

NRE Negative regulatory element

NSI Non-syncytium inducing

ORF Open reading frame

PBMC Peripheral blood mononuclear cells

PCR Polymerase chain reaction

PI Protease inhibitor

PIC Preintegration complex

RPR Rapid plasma regain assay

RT Reverse transcriptase

SIV Simian immunodeficiency virus

SI Syncytium inducing

SP Single platform

STD Sexually transmitted disease

STS Serological test for syphilis

TCM T central memory

TEM T effector memory

TCR T cell receptor

Th T helper cells

TPPA *Treponema palladium* agglutination assay

VCT Voluntary counseling and testing

WBC White blood cells

WHO World health organization

ABSTRACT

It was not known why some people were HIV negative and others were HIV positive despite frequent sexual relationship between couples in Ethiopia. Most of the transmission of HIV is currently between discordant couples. This is a big problem as very few people know their HIV status and protection between couples during sexual intercourse is unknown. The result obtained from the study of discordant couples is very important as it enables us to know the reasons for susceptibility and resistance to HIV infection. This is especially true for policy makers as the result obtained from this study is high quality data including behavioral, biomedical and virological factors.

The objectives of this study is, therefore, to investigate the reasons for susceptibility and resistance to HIV infection in discordant couples, which may involve behavioral, viral, immunological and other host and viral factors by comparing with concordant and healthy negative control subjects.

The study involves discordant couples who have been in marriage relationship for more than one year, concordant couples and healthy negative couples with similar marital status with discordant couples. The study was carried out in hospitals and clinics all over Ethiopia and involved discordant and concordant couples who were on the follow up for many years in the respective hospital and clinics and were not on antiretroviral treatment. The samples were analyzed with appropriate statistical package using SPSS version 13 soft ware.

To study if the difference in sexual behavior might have contributed to their serodiscordance, 325 discordant couples, 152 concordant couples and 14 healthy negative controls sexual behavior was studied. The study method involved an in-depth interview. There were known behavioral differences between discordant negatives and positives and concordant couples. There was very close similarity in behavior between discordant positives and concordant couples, showing that they shared similar risk behaviors. But the difference between discordant negatives and discordant positives was clear and big enough showing distant behavioral similarities. The healthy controls were behaviorally very much different from both discordant and concordant couples but they were similar to discordant negatives in some of their behaviors.

Almost all of them were not aware of HIV before VCT and had multiple partners before marriage and were multiply married. All had unprotected sexual intercourse before and after marriage with their partners and there was no HIV test in between marriages. The majority of them were, however, satisfied in their marriages, but the reason for the satisfaction was not based on love, faithfulness and mutual respect. The reason for the dissatisfaction was also similar. The sexual frequency and number of sexual act per each contact was higher for discordant positives and concordant couple than discordant negatives and healthy control and discordant positives and concordant couples were also involved more in traumatic sex than discordant negatives. History of STDs was also higher for discordant positives and concordant couples when compared with

discordant negatives. Discordant positives and concordant couples were exposed to more risky behavior than concordant couples. Perceived mechanisms of HIV infection was also known risk factors for HIV infection and were associated with their sexual lives, family, occupation, social evils and injustice.

When subjects were compared immunologically, discordant negative partners had adequate amount of CD4 equivalent to healthy subjects and highly significantly (P<.001) different from discordant positives. CD4 and CD8 ratio was also high indicating a healthy balance and this was also similar to healthy controls. Discordant positive partners had a significantly (P<.05) different number of CD4 cells when compared to concordant couples.

Their CD8 number was very similar to discordant negatives and there was no significant difference. Increased CD8 number was associated with decreased viral load and in some subjects even to the level of below detection level. Lower viral load in discordant positives when compared to concordant couples also indicated lower or absence of transmission to uninfected partner. CD8 T cells were responsible in decreasing viral load. The evidence for this came from the observation that concordant couples showed elevated viral load and decreased CD8 T cells number while discordant positives showed elevated CD8 and very low viral load. Their CD4 number was also closer but slightly higher than the normal boundary count and might have been capable of providing the appropriate help for CD8 cells.

Syphilis was a known risk factor for HIV transmission as it was diagnosed in many of discordant positives and concordant couples. This is possible because syphilis is a common STD in this country and its chronic nature might have accounted for its co-factor effect.

Analysis of T cell subpopulations in discordant couples showed no activation of a specific marker between discordant positives and negatives and the expression of T cell subpopulations was comparable. The only difference observed was the expression of activation markers in significantltiy (P<.05) higher proportion in concordant couples when compared with discordant positives , indicating lower immune activation in discordant positives. In discordant positives, in addition to decreased number of activation markers there were also expression of certain markers (CD4+CD45RA-CD27-) in higher proportion(>30%), which were common in long-term-non-progressors, showing that discordant positives were long-term-non-progressors.

Our study showed that there was a clear difference between discordant positives and discordant negative couples in their genetic profiles. There was also a clear difference between discordant positives and concordant couples and AIDS patients, in their genetic profiles. Ethiopian AIDS patients were different from Ethiopian concordant couples in their very significant to significant association with HLA-A*29, *18, and *41; HLA-B*0705, *1517, *4101, *5001, *7301 and *18;

HLA-C*0501, *0701, and *0740. AIDS patients were also very significantly different from discordant positives in their associations HLA-A*68, HLA-B*39 and HLA- DR*11. AIDS patient were also different from discordant negatives in their very highly significant to highly significant association with HLA-*0801, *1817, *352001 and *4901; HLA-C*7 and HLA-DR*40301. Concordant couples were also different from discordant positives in their very highly significant to significant associations with HLA-B* 0705, *0801 and *3910. Concordant couples were also different from discordant negatives in their significant association with HLA-DR*100101 and *110201. Discordant negatives were different from discordant positives in expressing HLA profiles of HLA-B*0801, *39, *41, *39; HLA-C*0716, HLA-DR*100101 and *110201. Overall, the differences between the different groups had a genetic background.

When comparisons were made between discordant positives, concordant couples and AIDS subjects, discordant positive subjects were found to be more heterozygous at all loci (HLA-A, B, C and HLA-DR) when compared with concordant couples and HIV/AIDS subjects. This showed that discordant positives better controlled HIV and maintained HIV in check and were non- progressors due to heterozygous advantage. Overall, the results for discordant positives and AIDS subjects were clear enough to show significant difference between them.

Ethiopian HIV viruses were mainly HIV type C in all discordant positives and HIV/AIDS subjects. But other subtypes such as subtype A, B and recombinant A/G subtypes were also observed. Co receptor utilization of discordant positive isolated viruses was both CCR5 and CXCR4 in equal proportion. The majority of HIV/AIDS patients used CXCR4, although about one third used CCR5 and a few also used dual co receptors. Our study showed that the majority of subtype C viruses were CXCR4/SI high/rapid subtype. And about one third was CCR5/NSI subtypes. The phylogenetic or evolutionary relationship showed that the majority of the viruses isolated from discordant positives showed subclustering in one region and those isolated from concordant couples in another region, showing that discordant positive isolated viruses were evolving independently and were related with each other but this was not seen in viruses of concordant couples and HIV/AIDS subjects.

CHAPTER 1: INTRODUCTION

1.1. General Introduction

Viral pathogens play a prominent role in human health owing to their ability to rapidly evolve creating new ways to exploit their hosts. Humans and their ancestors have also repeatedly answered their call with equally impressive adaptations. Thus, the coevolutionary race between humans and their viral pathogens is one of the most important forces in human molecular evolution, past and present (Frank, 2002). HIV was one among these viral agents which challeneged humans for more than two decades. It has costed a lot of life and its ravaging action is still persuing. The past 25 years of HIV prevention have been characterized by islands of success in a Sea of failure. Millions of people would not be newly infected each year if that were not the case. Much remains to be accomplished in the global fight against HIV. There are many more scientific and medical hurdles to be cleared and numerious logistical and operational obstacles to making therapies and other intrventions available to poor countries. Intensive and extensive research involving human behavior and biomedical parameters are still required as no vaible remedy is still at hand, of which this study is one amongest this type of attempt. Study on discordant couples is specifically important because most of the HIV transmissions currently are between married discordant couples. The result obtained from the study of discordant couples will also enable us to know about susceptable and resistant individuals.

There is no single magic bullet to fight HIV. Combination prevention is as nessary as combination treatment. Effective HIV prevention requires locally contextualized approaches that adress both individual and social norms and structures, and are grounded in human rights. This should in turn be based one evidence obtained from intensive and extensive research.

1.2. Litrature review

1.2.1. Epidimology of HIV/AIDS

HIV/AIDS, which is worldwide catastrophe, is one of the worst pandemic in human history. Although it is short-lived (25years) in the scheme of public health crises, the pandemics ranks among the most devastating microbial scourages in human history. In 25 years of HIV/AIDS, already more than 25 million people have died with 8000 deaths every day and the toll on families, communities and even entire nations has been profound (Piot *et al*., 2008).

The morbidity, mortality, and social disruption due to the global acqiured immunodeficiency syndrome (AIDS) pandemic weigh diproportionately upon resource-poor areas of the tropics (UNAIDS, 2010). Thus, the epidemic of HIV infection has not arisen in isolation, but within the context of pre-existing difficulties in disease control.

These difficulties are especially evident in Africa, where few resources and poor access to care have maintained an environoment in which many infectious diseases persist at high rates (Corbett *et al.,* 2002). What makes these societies unique from the HIV/AIDS perspective is that HIV transmission is far more diffuse than elsewhere, and occurs mostly within long-term and occasional heterosexual partenerships, often transgressing traditional concepts of high or low risk. Multiple vulnerabity and risk factors (such as mobility, gender based violence, or concurrent partenrships) , which individually exists to an even greater extent in other regions, converge to act snergistically in these societies (Piot et al., 2008) .

A quarter of a century of AIDS responses has created a huge body of knowledge about HIV transmission and how to prevent it, yet every day, around the world, nearly 7000 poeple become infected with the virus (Piot *et al.,* 2008). This gap between what is needed in HIV prevention worldwide and what has so far been achieved is huge. Because of this insufficient effort, the epidemic continues to extend its reach, and a new infection continue at a rate which puts unsustainable burdons on countries for decades to come (Marson *et al*., 2008).

HIV/AIDS is highly dynamic. Initial HIV outbreaks in highly vulnrable populations might be followed by a slower spread which could nevertheles affects a large number of people (Anderson, 1996). HIV is spreading in a very heterogenous way worldwide. Even within countries, the risk of contracting and transmitting HIV varies widely. Forexample, the risk of HIV infection varies greately with age in all countries, but countries are very different with respect to the relative risk of infection in specific subpopulation versus the general population. Thus, every prevention strategy should be flexible, given that the epidemics is dynamic, changing, and rarely stays in any one risk group.

The joint United Nations Programme on HIV/AIDS (UNAIDS) estimated that in 2007 approximately 33.2 million people worldwide were living with AIDS and HIV(UNAIDS, 2009). According to this report, people newly infected with HIV in 2008 were 2.7 million which is comprised of 2.3 million adults and 430, 000 children under 15 years of age. AIDS-related death, according to this report of December, 2009, was 2.0 million, which constituted 1.7 million adults and 280,000 children under 15 years of age.

The number of people living with HIV worldwide continued to grow in 2008, reaching an estimated 33.4 million(31.1 million-35.8 million) (UNAIDS, 2009). The total number of people living with the virus in 2008 was more than 20% higher than in 1990 (UNAIDS, 2000). The continuing rise in the population of people living with HIV reflects the combined effects of continued high rates of new HIV infection and the beneficial impact of antiretroviral therapy.

The HIV/AIDS epidemic is not uniform with global region or even within a given country. Globally, the area with the highest prevalence of HIV/AIDS is sub-Saharan Africa, where an estimated 22.4 million people-over two third of the world's total infected population and including nearly 90% of the world 's infected children – are living with HIV (UNAIDS, 2009). The next hardest hit area numerically is South and Southeast Asia, with an estimated HIV+ population of 3.8 million people (Fig1). The epidemic has also become increasingly widespread in Eastern Europe and Central Asia, where approximately 1.5 million people are infected and where prevention programs have been too limited to significantly impact the growth of the epidemic.

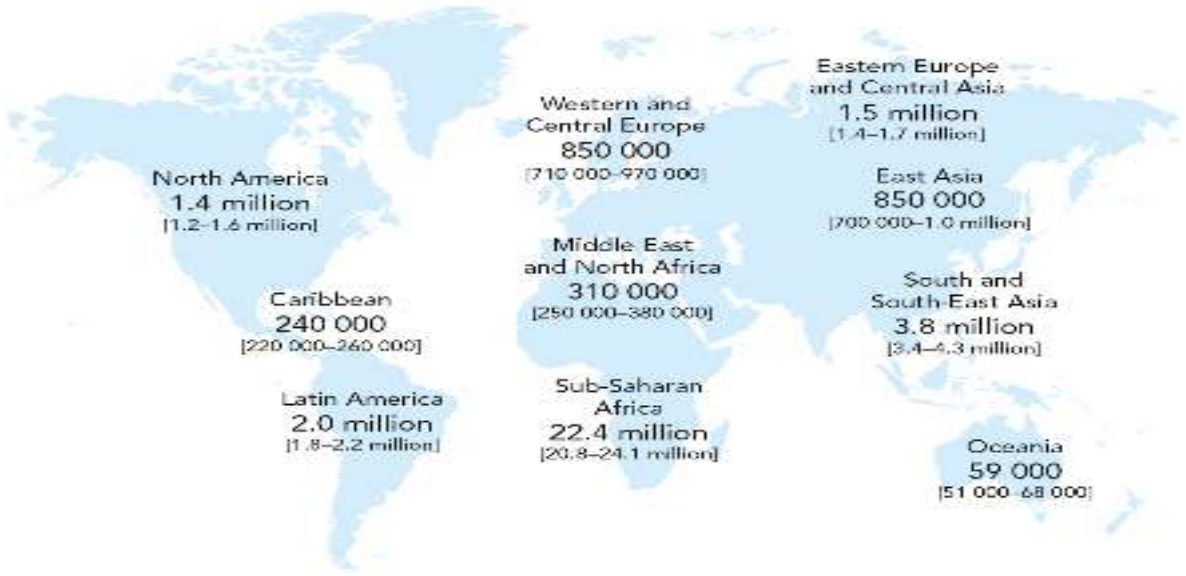


Figure 1. World wide distribution of HIV(Source , UNAIDS update, 2010)

In the broadest terms, the latest UNAIDS report finds that the generalized epidemics in many countries in sub-Saharan Africa contineu, especially in south and elsewhere the epidemic is mostly concentrated in high-risk populations ; among others these may include men who have sex with men (MSM) , intravenous drug users (IDU) and commercial sex workers. The global prevalence for men and women was approximately equal in 2009(UNAIDS, 2009). But in heavely impacted areas such as Sub-Saharan Africa a higher prevalence among women than

men have been observed. Females acount for 61% of HIV infection in Africa (Marson *et al.,* 2008).

With slightly more than 10% of the world's population, sub-Sahran Africa is home to more than two- thirds (68%) of all people living with HIV and more than three out of every four (76%) AIDS deathes globally occured here (UNAIDS, 2009) . Experts estimated that 600 000 babies become infected each year (Marison *et al*., 2008). In sub-Saharan Africa, 6000 people die daily from this deadly HIV and AIDS (Fauci, 2008). The epidemics are particularly acute in Southern Africa, where HIV prrevalence in the adult population exceeds 15% in eight countries. Nonetheless, recent evidence suggests that the epidemics in most countries in sub-Sahran Africa are stable and in a few countries may be declining(UNAIDS, 2009).

1.2.2. HIV/AIDS in Ethiopia

Ethiopia , located in Eastern Africa, is one of the sub-Saharan countries strongly hit by HIV epidemics. Like other sub-Saharan countries, it has been experiencing severe HIV/AIDS epidemics. The first two cases of HIV infection were reported in 1986, after which the disease spread at an alarming rate(Lester *et al.,* 19888; Mehret *et al.,* 1990 and Tsega *et al.,* 1998). The prevalence rate of 7.3 percent in 2000 declined, however, to 3.5 percent by 2005 after concerted prevention impact of AIDS (HAPCO, 2010). According to the calibrated single point estimate (Single point HIV prevalence estimate MOH/FHAPCO, June 2007; as cited by HAPCO, 2010) the national adult HIV prevalence, however, is reported to be 2.1 percent-7.7 % urban and 0.9% rural. The current estimate of HIV prevalence is 1.4 million(UNAIDS, 2010). The most pronounced diference in HIV prevalence between urban and rural is therfore Ethiopia where Urban dwellers are eight times more likely to be HIV infected than prople living in rural areas (Macro International , 2008; as cited by UNAIDS, 2009).

According to official publication of the FMOH and HAPCO, 2010, estimated 980, 000 people were living with HIV and AIDS in Ethiopia in 2007. Of these 260, 000 including 16,000 children-were in need antiviral treatment(ART). But testing sites increased drastically as of 2007. For example, testing rates more than doubled between 2007 and 2008 from 1000 population to 121 tests per 1000 population (UNAIDS, 2009). This was very important, although with considerable gaps, because household survey showed that previously untested men and women were more likely to be infected than their counterparts who had previously accessed testing services(Mishra et al., 2008a, as cited by UNAIDS, 2009).

1. 2.3. The human immunodeficiency virus, origin and subtypes

1.2.3.1. Origin of HIV

The treats of emerging infectious diseases are not knew in history. But no emeging infectious disease was equivalent to acquired immunodeficiency syndrome (AIDS), caused by human immunodeficiency virus, HIV.

In June 1981, Physians in New York and Cilifornia reported unusual cluster of rare diseases in previosly healthy gay men, notably *Pneumocystis carinnii* pneumonia and a form of cancer called Kapos's sarcoma (Fauci, 2008). This disease had previously affected only people with severly compromised immune system. As cases began to appear among distinctly different social groups, it became clear that the world was witnessing the unfolding of something truely novel and frightening.

Several lentiviruses cause diseases in a diverse group of animal species. For example, equine infectious anemia virus causes hemolytic anemia in horses. Similarly, lentiviruses of sheep and goats have been known for many years to be associated with a long illness and clinical symptoms. They can cause pathologic entities such as autoimmunity, pneumonitis, and brain and joint disorders in these animals (Korber et al., 1998)**.**

In spite of the observations and discoveries of retroviral diseases in animals, no infectious retrovirus had been found in human beings up to the mid-1970's . Many investigators believed that no human retrovirus would ever be found. The discovery and isolation of the first human T cell leukemia-lymphoma virus (HTLV)-1 , from immature T cell of individuals with adult T cell malignancies (Poisz, et al, 1980) only a few years before the first cases of AIDS were described were crucial steps in the identification of another human retrovirus of the lentivirus subfamilies, HIV, as the cause of AIDS.

After cases were reported originally from homosexual injecting drug users and outside the USA (in the UK), other immune – deficiency diseases were soon reported in different populations from many countries including Haiti and some African countries (Marison et al., 2008). For nearly two years, the cause of AIDS remained elusive; the scientific community was largely baffled, lacking good leaders for developing therapies or even a diagnostic test.

In 1983, Luc Montagnier's, a researcher at France's Institute of Pasteur, research team in Paris published the first paper providing evidence linking a retrovirus to AIDS (Montagnier's *et al.,* 1983). The following year, further data from Robert Gallo's group (a research group based in USA) in the United States of America provided convincing evidence that this retrovirus (later

named HIV) was the cause of AIDS (Gallo et al., 1984). This event was marked by controversy because these two teams claimed credit for the discovery. Eventually, Luc Montagnier and Robert Gallo shared recognition for the isolation and discovery of HIV (Marison et al., 2008).

HIV, originally termed human T lymphocyte virus type three (HTLV)-111, lymphadenopathy-associated virus (LAV), or AIDS-associated retrovirus (Barre-Sinoussi et al., 1983; Levy et al., 1984), was shown to be a distinct member of the HTLV group of virus. Unlike HTLV-1, however, HIV is cytopathic for its target cells and causes syncytia formation between infected and uninfected cells (Rosenberg and Fauci, 1989).

After the discovery of HIV, research moved at a breathtaking pace. A blood test to diagnose patients and to screen the blood supply quickly followed (www.aidscience.org.html), as did enormous progress in understanding the genetic and structure of HIV and its disease-causing mechanisms (Rosenberg and Fauci, 1989).

The AIDS viruses are members of the lentivirus family of retroviruses. They have been demonstrated to exhibit the remarkable properties of insidious disease induction, persistence, latency, variation, recombination, and escape from immune and drug pressure (Rosenberg and Fauci, 1989). There are two distinct types of human AIDS viruses, HIV-1 and HIV-2, which are distinguished on the basis of their genome organization and phlogenetic relationship with other primate lentiviruses (Hahn et al., 2000). Lentiviruses are also known (at least 18 or more lentiviruses) to infect primates. Humans are not the natural hosts of either HIV-1 or HIV-2 but these viruses have entered the human population as a result of zoonotic, or cross-species, transmission (Sharp and Hahn, 2008).

Although these simian lentiviruses are termed immunodeficiency viruses because of their genetic and structural similarities to the human AIDS viruses, the simian viruses have not been observed to cause diseases in their natural hosts (Hahn et al., 2000). In most instances, the infected primate species represents the natural reservoir of the viruses, and the virus is so designated (e.g. Simian immunodeficiency virus of sooty mangabeys, or SIVsm). Less frequently, primates experience incidental infection after exposure to viruses whose natural hosts is a member of a different primate species (Delves et al., 2000).

Both HIV-1 and HIV-2 which are two major groups of human immunodeficiency virus were transmitted to humans from primates through different cross-species transmission. Cross-species transmission to humans of simian viruses is not restricted to HIV strains. Monkey pox, simian T cell leukemia virus and simian foamy virus (Tchokoteu et al, 1991; Voevodin, et al., 1919; and

Heneine et al., 1998) are some examples of viruses that have been shown to jump the species barrier.

HIV-1 strains are divided into three groups, each of which was independently derived from a simian immunodeficiency virus (SIV) that naturally infect Chimpanzees (SIVcpz) and particularly *Pan troglodytes troglodytes has been implicated* (Goa, et al., 1999). This was based on the finding of a new SIVcpz sequence (SIVcpzUS) suggesting that HIV-1 epidemic had arisen as a consequence of SIVcpz transmission from a particular chimpanzee species *P. t. troglodyte* to humans. SIVcpz and HIV-1 viruses have also been found to be identical in genomic organization, containing a particular gene, Vpu, not present in other lentiviruses (Huet et al., 1990).

1. 2.3.2. Genetic diversity of HIV

There are two human immunodeficiency (HIV) sero-types (HIV-1 and HIV-2) causing human immunodeficiency syndrome (AIDS). HIV is characterized by high genetic variability and evolution rates. Both viruses are distinguished on the basis of their genomic organization and evolutionary relationships with other primate lentiviruses (Kuiken et al., 2000). HIV-1 was the first serotype of HIV isolated and the first to be recognized as the etiologic agent that is believed to be the cause of AIDS in the world. Shortly after the discovery of HIV-1, a second AIDS virus was recovered from West Africa, particularly the Cape Verde Islands and Senegal (Anderson, 1996).

The contribution of HIV-2 to the pandemic is limited and the virus is mainly localized to West Africa and India (Rubsamen - Waigmann, 1991). But HIV-1 is the predominant agent of HIV infection causing AIDS worldwide. The nucleotide sequence similarity between HIV-1 and HIV-2 is about 40 % (Kuiken et al., 2000) and there is a big similarity between HIV-2 and SIV (about 75 % nucleotide sequence similarity). Both HIV-1 and HIV-2 cause AIDS, however, in HIV-2, disease progression is slower than HIV-1, with limited impact on the survival of the majority of infected adults (Poulson et al., 1997), although it apparently manifests the same clinical spectrum (Bock and Markovitz, 2001). While the routes of transmission are the same, HIV-2 appears to be less easily transmitted than HIV-1 and the progression from HIV-2 infection to AIDS appears to be slower than in the case of HIV-1 (Kuiken et al., 2000). Despite these differences AIDS appears to be slower than in the case of HIV-1. HIV comprises three distinct virus group, believed to have arisen from at least three independent cross-species transmission events (Goa et al., 1999). These are group M (or a major group), group O (or “outlier” group) and N (non-O/ non-M) (Korber et

al., 1998). Group N appears equidistant to group M and O (Simon et al., 1998), whereas a large genetic distance separates the latter (35 to 49 %) (Lousset-Ajaka et al., 1995).

Over 95 per cent of people through out the world are infected with HIV-1belonging to group M, which is further divided into various genetic subtypes (clades), differing at the nucleotide level by as much as 30 % (Korber et al., 1999) and consists of several clades referred to as subtype A to D, F to H, J and K (Robertson et al., 2000).

Prevalence of HIV-1 group O (found in Cameroon and but not restricted to Cameroon) and N (restricted to Cameroon) is much lower than group M (Korber et al., 1998). Group O prevalence is higher than group N viruses but 2-5 % of HIV positive samples are group N when compared with group M (Peeters et al., 2000). Similarly, HIV-2 strains infecting humans have been found to comprise six distinct phylogenetic lineages, subtypes A through F (Korber et al., 1998). However, the discovery of the phenomenon of the genetic recombination has further complicated the classification of HIV-1 strains. Currently, more than 10 % HIV strains described in the data base might be recombinant forms (Fauci, 2008).

Specific subtypes tend to predominate in specific regions. That is, HIV-1 subtypes do not have a uniform distribution across the globe. Accordingly, Subtype B is predominant in the America and Western Europe, subtype “E” in Thailand, subtype A and D in East Africa, and subtype C in Ethiopia ,South Africa and the Indian subcontinent (Fig.2) (Krober et al., 1998). Extensive seroprevalence and molecular epidemiological studies by UNAIDS, WHO, and others have identified subtype A, B, C and D in different sub-Saharan countries (Osmanov et al., 2000). HIV-1 subtype C, the most predominant world epidemic constitutes more than half of all new infections worldwide (Fauci, 2008). However, due to current pattern of transmission and population migration , the proportion of subtypes in defined population are not stable but are in constant flux due to new introduction of HIV-1 subtypes, change in human behavior and mode of transmission

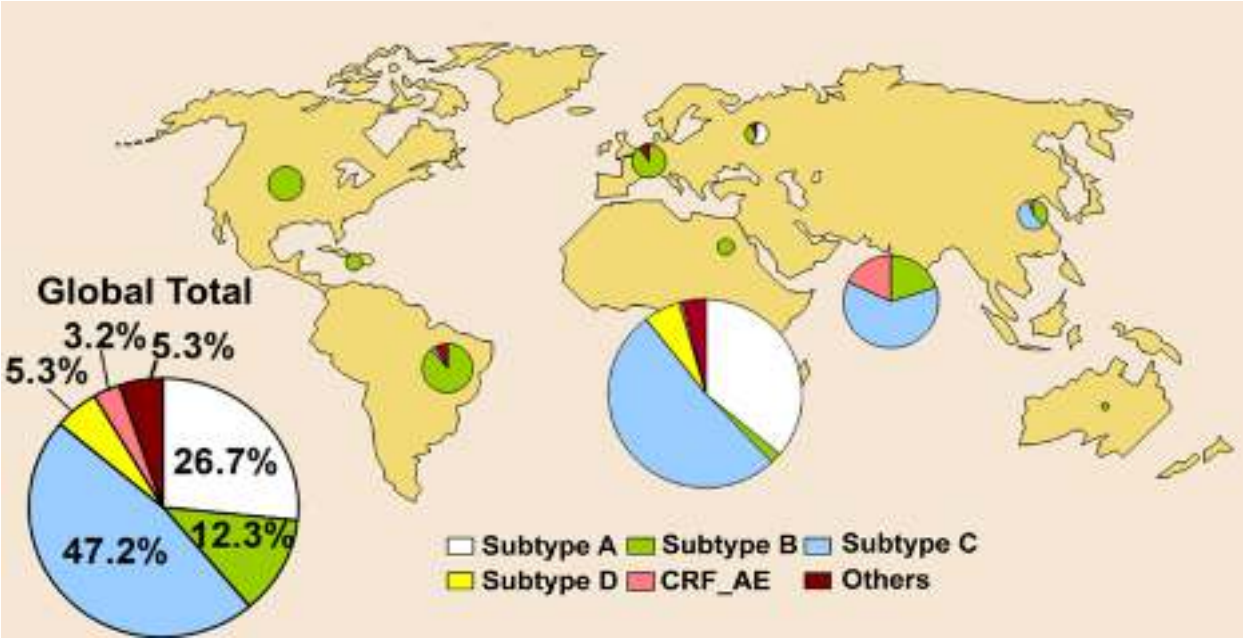


Figure 2. Distribution of HIV subtypes across the world (Source: Didier *et al.* BMC Bioinformatics 147:2105-2108)

1.2.4. HIV structure, genome and replication

The overall structure of HIV resembles that of other retroviruses. It has an icosahedral (spherical) morphology of 100-120nm in diameter (Sierra *et al*., 2005) and 72 spikes or knoblike structures are attached to the envelope glycolipids on the outside (Fig. 3. A and B)

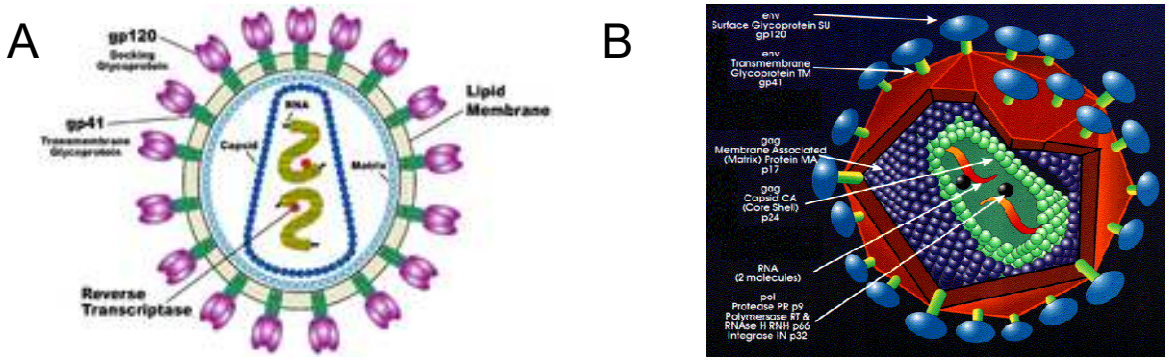


Figure 3. Diagram of HIV mature virion. A) External structure B) Internal stricture (Source: Zanetti et al., 2006)

The virus particle consists of two identical single-stranded RNA molecules and viral enzymes within a viral protein core that is surrounded by an envelope derived from a combination of the host cell membrane and virus specific glycoprotein (Rosenberg and Fauci, 1989).

The HIV envelope is made up of a lipid bilayer, captured from the host cell membrane and because the lipid bilayer originates from the host cell membrane, a number of host proteins are

embedded within it (Zanetti et al., 2006). The host proteins incorporated into viral envelope also include the major histocompatibility complex (MHC) class I and class II antigens (Sierra et al., 2005) The external spikes embedded in the lipid bilayer are of two types: glycoprotein 120(gp120)(external) and glycoprotein 41 (gp41) (transmembrane) (Zhu et al. , 2006). These proteins combine to form a mushroom-shaped complex (Fig 4 B) that protrudes from the surface of the envelope and allow the virion to bind to target cells via the CD4 receptor. As a result of its role in virus-cell attachment, the structure of the virus spike, consisting of gp120 and gp41, is of particular importance. Its structure was compiled in 2006 using cryo-electron microscope (Zanetti et al., 2006) and found to be composed a single-stalked "mushroom-shaped", with only the head, consisting of a trimer of three gp120s with a single gp41 glycoprotein, anchoring it to the envelope (Fig. 3 A).

Glycoprotein 120 is a surface structure that aids in attachment of the HIV virus to T cells and gp41 helps anchor gp120 to the viral surface (Zhu et al., 2006). The gp41 component plays a key role in the infectious process by promoting the fusion of the viral and cellular membranes so that HIV may infect the cell. Glycoprotein 41 (gp 41) is embedded in the membrane while gp120 is not. Glycoprotein 120 (gp120) is held to gp41 by non-covalent interaction. It is easily shed from the virus particle. There are a large number of sugar chains on gp120 (which may pose a problem for a vaccine). Gp120 is the protein that interacts with a receptor on the cell to be infected. Glycoprotein 41 is the fusogen that is exposed after Gp 120 has bound to the cell. The viral envelope is what encapsulates all the proteins and genetic information of the virus.

The dense viral core of HIV is bar shaped, most closely resembling viruses within the lentivirus family (Rosenberg and Fauci, 1989). The HIV-1 core encases structural proteins, enzymes and two strands of single-stranded RNA (Fig 3B). The structural protein p24, surrounding the two copies of single stranded RNA is the most abundant protein in the HIV virion (Watts et al., 2009).

In addition to structural proteins, the HIV-1 core also contains three enzymes: Reverse transcriptase proteins (RT), Integrase and Protease (Sierra et al., 2005). These three enzymes are contained within the virion, which enables them to be used as soon as the virus infects the cell. New copies of these enzymes are made for progeny virions after the DNA is integrated into the host cell. Reverse transcriptase is the enzyme that is essential for coding the viral RNA into DNA, which is integrated into the host genome (Parker et al., 2000). As RT moves along the HIV RNA, it exerts a ribonuclease activity that digests the RNA after DNA has been made (Feng et al., 1996). This allows the complementary strands of single-stranded DNA to bind and form double-

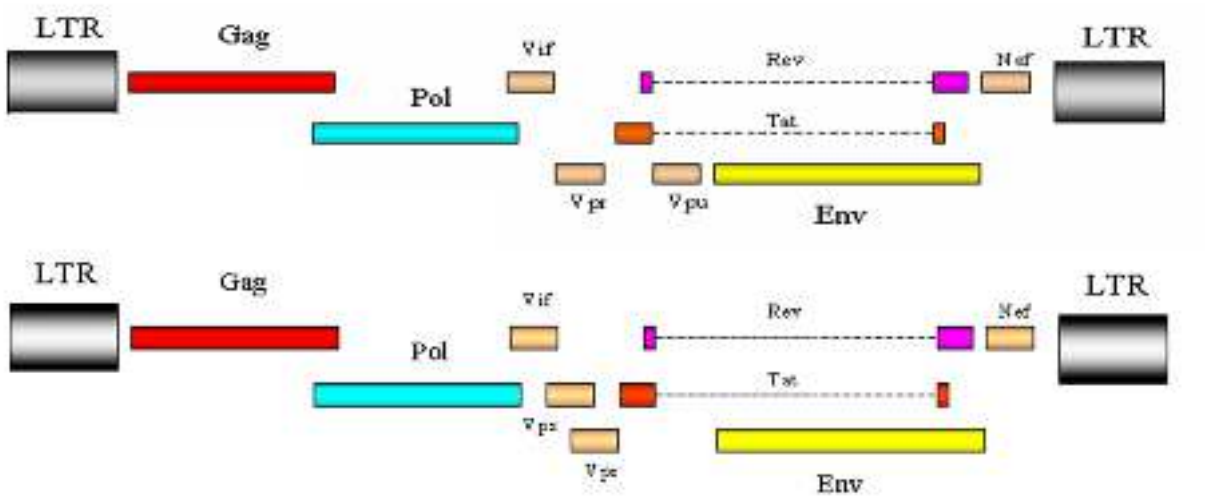
stranded. Following the action of RT, integrase participates in the insertion of the HIV genome into the cell genome (Wilk et al., 2001). Integrase is thus, the enzyme that inserts HIV's genes into the cell's genome. Due to the nature of HIV genome, cellular translation produces long strands of viral proteins that must be clipped apart to create smaller functional proteins. Protease is an enzyme that breaks down proteins into smaller proteins and /or their component peptides (Korber et al., 1998). It is also an enzyme that digests the long strands of viral genetic material (made by the host) into shorter segments to form HIV's nine genes (www.engr.uconn.edu.html). A matrix composed of an association of the viral protein p17 surrounds the virion, ensuring the integrity of the virion proteins.

The single-stranded RNA is tightly bound to the nucleocapsid proteins and enzymes. RNA is a strand of material that contains the information for the virus nine genes.

1.2.4.1. Genomic organization and protein products

A distinguishing characteristic of the lentiviruses is their unusually complex viral genome. Depending on the particular HIV isolate, the length of the genome is approximately 9749 nucleotides (Greene, 1990). The HIV genome consists of two identical single stranded RNA molecules within the virion but the persistent form of the HIV genome is proviral double stranded DNA within infected cells (Fig.4). The HIV genome has nine open reading frames (leading to nine primary translation products) but 15 proteins are made all in all, as a result of cleavage of three of the primary products (Hunt, 2008). Thus, unlike typical viral genomes where a specific set of bases encodes a single gene, the HIV genome includes several overlapping open reading frames (ORFs). The HIV genome produces 15 proteins from 9 overlapping ORFs.

A



Genome layouts of HIV-1 (upper) and HIV-2 (lower)

B

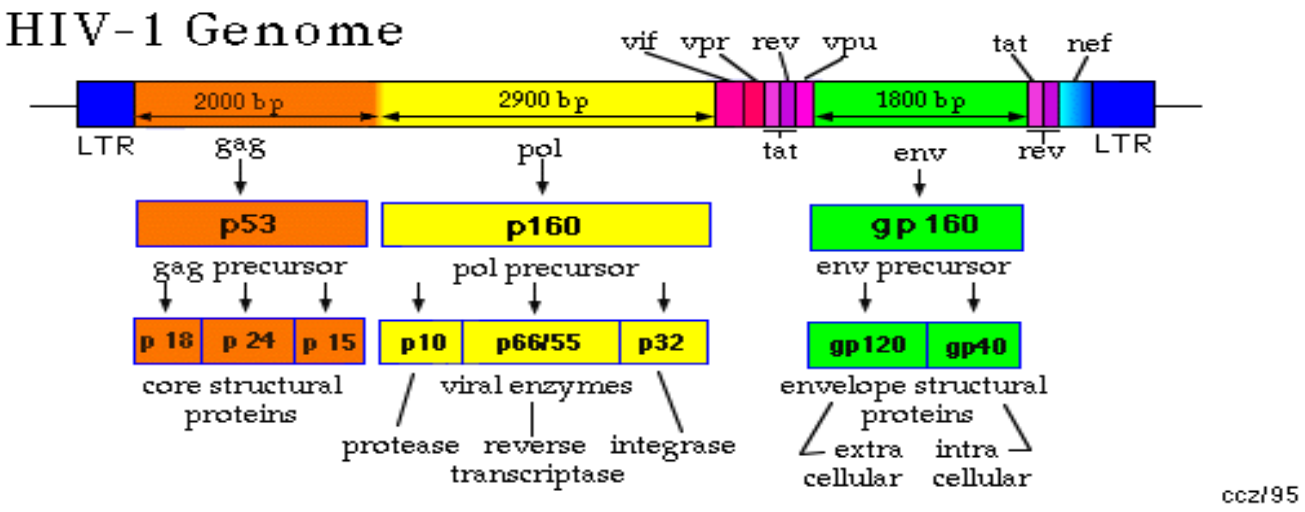


Figure 4. Structure of the HIV genome A) Genome lay out of HIV-1 and HIV-2 B) HIV genome and its proteins products (Source: Hunt, 2008)

Four main regions can be distinguished in the HIV genome (Fig. 4 A): 2 long terminal repeats (LTRs): regulatory region located at both edges of the RNA molecule, flanking the coding region. Each LTR contains an U3, R and U5 region (Starcich et al., 1985). The U3 contains binding sites for cellular transcription factors (Chen-park et al., 2002). The R region contains the trans-activation response element (TAR) (Richter et al., 2002).The region include a negative regulatory element (NRE), an enhancer region (NF-kB), Sp1 binding sites, and trans-acting responsive

(TAR) sequence (Starcich et al., 1985; Chen-park et al., 2002) encoding two polyprotein precursors; and three the *env* encoding the gp160 polypeptide precursors (Sierra et at., 2005), but also genes involved in virion maturation and morphogenesis *vpu* (viral protein V), and *vif* (virus infectivity factor) , genes involved in the regulation of viral replication : *tat* (Trans activation of transcription), *rev* (regulator of virion protein expression) and nef (Negative regulatory factor), and a gene of uncertain function : *vpr* (viral protein R)) (Greene,1990). These various gene products are translated from differently spliced mRNAs.

The three major ORFs (*gag, pol* and *env*) encode structural proteins and enzymes that are initially synthesized as multi-protein precursors. The remaining six ORFs encode regulatory proteins. In general, viral regulatory proteins (Tat, Rev, and Nef) are encoded by multiple spliced mRNA species, while the structural and enzymatic viral proteins are translated products of unspliced (Gag, Pol) or singly spliced (*Env*, *Vif*) mRNAs(Chen-Park et al., 2002).

1.2.4.2. The structural genes of HIV

These genes include the *gag*, *pol* and *env* genes common to all replication competent viruses. The gag gene and the gag and pol genes together are translated into large polyproteins which are then cleaved by a virus-encoded protease that is part of the Pol polyprotein (Veronese et al., 1988). The precursor polypeptide is processed into viral core proteins, Capsid, CA (p24), Matrix, MA (p17), Nucleocapsid, NC (p15) and p6 (Debouck, et al., 1987) (Fig. 4 B). Together these polypeptides form the nucleocapsid of the HIV virion.

The pol gene encodes three enzymes: reverse transcriptase, integrase, and protease, which is translated from an unspliced gag-pol transcript (Varmus, 1988). Reverse transcriptase, as previously indicated, copies RNA genome into double-stranded DNA. In addition to its polymerase activity, the HIV reverse transcriptase contains RNase H activity required for degradation of the RNA template during the synthesis of the double-stranded DNA (Varamus, 1988). That is, it cleaves the RNA of the RNA/DNA hybrid that is formed after the first round of polymerization.

The HIV env gene is transcribed as a single spliced viral mRNA species that when translated yields the primary env gene product polyprotein, gp160 (McCune et al., 1988). Gp160 is cleaved after translation by host enzymes in the Golgi body to form Gp120 and Gp41 proteins (Varmus, 1988). While lacking a transmembrane domain of its own, gp120 is stabilized at the cell surface by its intimate interaction with gp41 (Veronese *et al.,* 988). It has been shown that the

endoproteolytic cleavage of gp160 into gp120 and gp41 is required for viral infectivity (McCune et al., 1988). The gp41 protein appears centrally involved in fusion and syncytia formation and thus may importantly contribute to the pronounced cytopathic effects of HIV. Overall, this glycoprotein is important for the binding and infectivity of HIV (Greene, 1990).

1.2.4.3. The regulatory genes of HIV

There are at least six other genes (*tat, rev, nef, vif, vpr,* and *vpu*) that represents regulatory proteins involved in the regulation of gene expression. They also play a role in the pathogenesis of HIV disease. These genes encode small proteins and overlap with the structural genes (especially *env*) but are in different reading frame (Fig. 4).

1.2.4.4. HIV replication

There are differences between HIV-1 and HIV-2 in terms of the structure of the genome especially in that HIV -2 lacks the *vpu* gene and has *vpx* gene which is not contained in the HIV-1(Greene, 1990).

HIV as other retroviruses can exist in two forms, both as RNA within virus particles and host cell-associated DNA. HIV must enter its target cell to replicate and produce new virions, which involves transition from RNA to DNA and back again to RNA. HIV replication is completed over approximately two days and involves several key steps.

The initial step involves the attachment of the virus to cellular receptors (Fig. 5). The virus particle contains one or several glycoproteins inserted in the lipid bilayer that play essential roles for binding the cell surface receptor(s) and for fusion of the viral and cell membranes. HIV attaches to the cell surface by an interaction between the extracellular domain, gp120 and cellular receptors (Moore et al., 1993). As discussed earlier, gp 120 which is an extracellular domain is covalently associated with gp41, which is transmembrane domain and its main purpose is attachment to the cell receptors.

Glycoprotein 120 binds to several receptors on the target cell but the primary receptors on the target cell is the CD4 receptor (Clapham *et a*l., 1991). Although, CD4 is considered as the primary receptor, several studies (Valente *et al.,* 2001; Dumonceaux *et al.,* 1998) show that HIV variants may enter cells in a CD4-independent manner or use alternative surface antigens such as CD8 (Saha *et al*., 2001). CD4 surface antigen is required for productive HIV infection.

The CD4 molecule is a 55kDa T cell surface glycoprotein (Weiss, 1993) and its normal function is to participate as coreceptor in the interaction between class II major histocompatibility complex

(MHC) expression on the surface of antigen-presenting cells (APC) and the T cell receptor complex that will initiate the activation of CD4 T cells (Bour et al., 1995). CD4 molecule define the T helper (Th) phenotype and coordinates immune responses by secreting cytokines that provide stimulus to CD8 T cell (cellular) and B cell (humeral) immune responses (Bour *et al.,* 1995). CD4 markers are primarily expressed on CD4 T cells but are also expressed at lower levels on B-lymphocytes, monocytes, macrophages, dendritic cells (Clapharm, 1991), and macrophages and microglia in the central nervous system and some intestinal cells (gut epithelial cells) (Moore *et al.,* 1993; Kaul *et al.,* 2001). The expression of this molecule, thus, will determine the cell types that are susceptible to HIV infection, and therefore the extent, localization and rate of HIV replication. The hallmark of AIDS is also the decline in CD4 number to below 200 copies/ml of blood (Bour *et al.,* 1995).

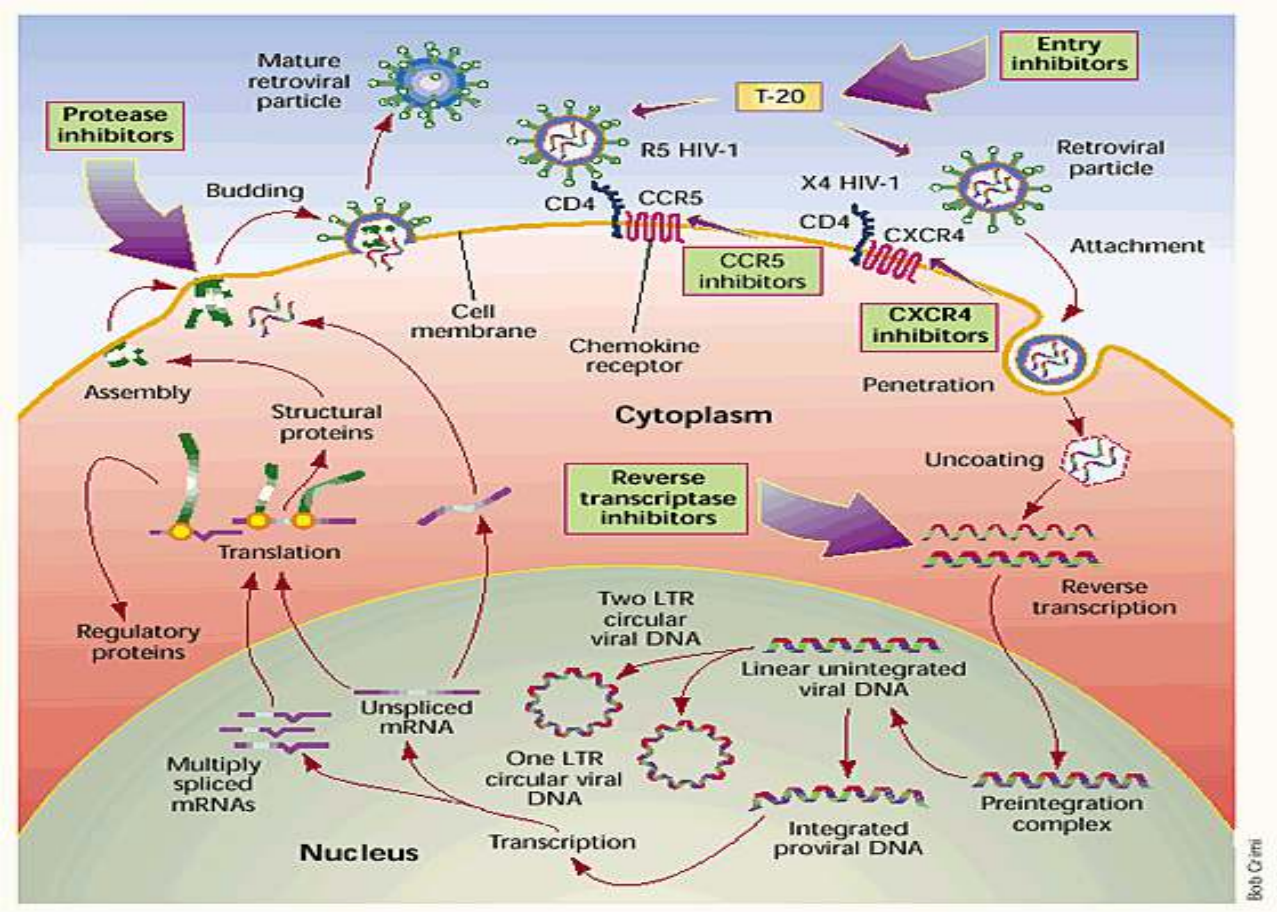


Figure 5 The life cycle of HIV (Source: Modified from Shattoock and Moore, 2003)

Several studies have demonstrated that CD4 receptor alone is not sufficient to allow HIV to fuse with the host cell membrane and gain entry (Cheng-Mayer *et al*., 1990; Clapharm et al., 1991; Fauci, 1996). Members of the chemokine receptor family, CXCR4 and CCR5, have been shown

to be necessary for entry into T cells and macrophages, respectively (Feng *et al.*, 1996; Samson et al., 1996). These coreceptor proteins belong to the super family of G- protein coupled receptors that normally bind chemokines, small molecules involved in inflammatory responses (Samson *et al*., 1996). After the binding of CD4/gp120 and gp120-coreceptors, the HIV virion is internalized by a process mainly involving cell fusion (viral membranes fuse) (Stein *et al*., 1987) and the viral core is released into the cytoplasm of the cell (Fig. 5). The envelope glycoprotein gp41 mediates fusion, which allows the HIV-1 core to enter the host cell.

The release of the core nucleocapsid (also known as Uncoating) into the host cell is helped (facilitated) by cellular factors and the viral proteins MA, Nef and Vif (Sierra et al., 2005). The viral core that is released into the cytoplasm initiates the next stage of viral replication. In this next stage, the viral RNA genome in the viral core structure is reverse transcribed by the virus encoded reverse transcriptase (also called RT) enzyme (Greene, 1990). The viral reverse transcriptase synthesizes a DNA copy of the viral RNA, the RNA template is degrade and a second complementary DNA strand is synthesized (Sierra *et al*., 2005). The double-stranded DNA copy of the viral sequence is then transported to the nucleus. But the trip to the nucleus is not an easy one.

The transport of the so-called preintegration complex (PIC), which includes the double-stranded DNA, is difficult in unactivated (non-dividing) host cells. It is generally believed that the nuclear membrane of non-dividing cells is a barrier to the transport of large complexes, such as the PIC, and that the barrier is removed when the membrane breaks down during cell division (Jacque and Stevenson, 2006). As a result, only the gene products encoded in members of the lentivirus group which includes HIV possess the ability to direct entry into the nucleus of non-dividing cells. This is due to possession of non-structural genes. The preintegration complex passes to the nuclear membrane directed by HIV-1 Vpr and enters the nucleus through the nuclear pore (LeRouzic and Benichou, 2005)

Prior to integration, the viral DNA can be found in the nucleus in three forms: linear, 1-LTR or 2-LTR circles (Wu, 2004). The 1-LTR and 2-LTR circles are formed in the nucleus because some linear DNA strands undergo recombination or end-to-end ligation. These circles appear to be dead-end products in virus replication because it is the linear cDNA that is the direct precursor of the provirus (Mark *et al.,* 2001). But others report that Nef, Tat and Rev are produced in basal amounts from these DNA forms through activation of the LTR promoter by cellular factors such as NF-kB (nuclear factor kB) (Wu, 2004; Aiken and Trono, 1995).

Assisted by another virus-specific enzyme that functions as an endonuclease, called integrase or IN, the double stranded DNA copy is integrated into the host cell DNA (now called a provirus) (Bushman, 2002). The host DNA is also cut to insert the viral DNA by integrase it self. In most retroviral systems (including HIV) unintegrated viral DNA may be seen in the nucleus. In HIV infection, a substantial amount of HIV DNA exists in an unintegrated form, potentially contribute to the cytopathicity observed in HIV infection (Shaw et al., 1984). However, for an effective virus infection to occur, HIV DNA must be integrated into the host cell (Mark *et al*., 2001).

Once the HIV proviral DNA is integrated into the host cell's chromosomal DNA, viral replication may inter a restricted latent phase (Shaw et el., 1984). The integrated provirus may be transcriptionally inactive and behaves like any other silent cellular genes. The proviral gene is transmitted to daughter cells as part of the host cell chromosome when the host cell divides (Wu, 2004). HIV infections of resting memory or naive CD4+ T cells, macrophages or mononuclear cells lead to a non-productive latent infection (Chun *et al.,* 1997).

In an activated cell, host cell signals initiate the transcription of viral DNA into genomic RNA and mRNA. Factors influencing T cell activation promote viral production. An increase in cellular activation in response to co-infection or immunizations and other factors may enhance viral replication by facilitating viral cellular entry, reverse transcription and proviral transcription (Lawn *et al.,* 2001). The presence of various inducible host transcription factors also initiate viral DNA transcription (Nabel and Baltimore, 1987). For example, the initial low level of viral gene transcription mediated by T cell activation of the LTR is markedly amplified by the activation of the HIV Tat (Transactivator) protein (Cullen and Greene, 1989). The RNA will be a genetic material for the new virion and mRNAs are spliced and translated into viral protein. At this junction a second viral transregulator (Rev) promotes expression of the structural proteins required for the assembly of infectious viral progeny (Cullen and Greene, 1989). The infected cell virtually becomes a virus-producing factory with cessation of many normal functions.

For viral assembly and budding, there must be a decrease in the number of CD4 molecules in the plasma membrane in order to avoid interactions with the newly synthesized gp120 and this is done by HIV Nef, Env and Vpu (Sierra et al., 2005). Budding triggers the activation of Protease. Protease cleaves the large precursor proteins into functional capsid, nucleocapsid, and matrix proteins (Wilk et al., 2001) and cleaves one form of the polyprotein into functional, enzymatic protein: The virion RNA and core proteins associate with viral envelope proteins that are located at the cell membrane, and the mature virion forms by budding from the cell surface (Greene,

1990). Budding of these virions from the cellular membrane promotes dissemination of the virus to other CD4+ cells and also contributes to the death of the original infected cell.

1. 2.5. Chemokine coreceptors and HIV-tropism

The primary receptor for HIV and the hallmark of HIV infection and the cause of the associated immunodeficiency is the loss of CD4. But CD4 alone is not the only target for HIV. In addition to binding the CD4 receptor, a second co-receptor is required for HIV to interact with so as to enter target cells. The notion that a co-receptor is required for HIV entry stemmed from the awareness that CD4 expression is not sufficient to explain HIV tropism from different target cells in vitro (Berger, 1997). The chemokine receptors CXCR4 (also R4) (Fourth receptor for CXC chemokines) and CCR5 (also R5) (Fifth receptor for CC chemokine), have been identified as the principal coreceptor for HIV (Dragic *et al*., 1996; Feng *et al*., 1996). CXCR4 and CCR5 are members of the CXC and CC transmembrane domain chemokine receptor subfamilies (Feng *et al*., 1996).

Chemokines are small proteins about 10Kd containing approximately 70-90 amino acid residues. They play a very important role in leukocyte activation and trafficking to sites of inflammation, a process known as chemotaxis (Baggiolini *et al.,* 1997). They show 20 to 70 per cent amino acid homology and contain four conserved cysteines forming two essential disulphide bonds. All known human chemokines fit within four classes based on these cysteine motifs near the N-terminus. CXC and CC chemokines differ by the position of the first two cysteines, which are adjacent (CC) or separated by one amino acid (CXC). Two other minor classes with two instead of four conserved cysteines (the C chemokine family) and the CX3C class with three residues separating the first two cysteines, have also been defined (Luster, 1998). Chemokines may be produced locally under pathological conditions by activated lymphocytes, leucocytes and tissue cells, whereas serum levels of chemokines are generally low (Moser, 1997). But some chemokines seem to fulfill housekeeping functions (Furie and Randolph, 1995).

Members of CC chemokines associated with HIV include RANTES (Regulated on activation, normally T cell expressed and secreted) MIP-1α (macrophage inhibitory protein-1 alpha) and MIP-1β (macrophage inhibitory protein-1 beta) and others of minor importance (Baggioline *et al*., 1997), while members of CXC chemokines include SDF-1 α (Stromal cell derived factor-1 alpha) and SDF-1 β (Stromal cell derived factor-1 beta) (Bleul et al., 1996). The CC chemokine suppresses infection by M-trophic (monocyte/macrophage-trophic) HIV strains but have little

effects on a TCL-trophic (T cell line trophic (also known as T-trophic) strains (Dragic *et al.,* 1996). SDF-1is a selective inhibitor of T-trophic HIV strain but has no effect on M-trophic cells (Bleul *et al.,* 1996).

Chemokine receptors are class of cell surface receptors that normally acts as receptors for chemokine and some chemokine receptors are co-receptors for HIV entry into target cells. CXCR4 was the first HIV co-receptor to be characterized (Bleul *et al*, 1996). CXCR4 is expressed mainly on resting, and to a lesser degree, on activated CD4 T cells (Baggioline *et al*., 1997). HIV isolates that exclusively use the CXCR4 co-receptor are referred as T cell trophic (which reflects their ability to infect T cells) or X4 strains (which reflects their ability to use the CXCR4 co-receptors).

After years of infection, CXCR4-using strains (X4 viruses) are detected in approximately 50% of infected individuals, with X4 and R5 viruses usually coexisting in the viral swarm (Bjorndal et al., 1997). X4 strains infect T-cells and induce syncytia (SI) in vitro (Koot *et al.,* 1998).The emergence of X4 variants often heralds CD4 cell depletion and accelerated disease progression (Connor *et al*., 1997), and multiple properties of X4 variants may contribute to CD4 cell decline.

CXCR4 viruses can infect immature thymocytes, precursor cells of mature CD4 lymphocytes, which express high levels of X4 but lower levels of R5 co-receptors (Baggiolini *et al.,* 1997). This may result in rapid depletion of thymus cells, which explains the rapid CD4 T cell depletion, faster progression to AIDS and higher virulence observed in patients infected with CXCR4 tropic virus (Connor *et al.,* 1997).

Subsequently, CCR5 was shown to be a co-receptor for macrophage-tropic (M-tropic) or R5 strains (Dragic et al., 1996). CCR5 expressing T cells are only a small fraction (10%) of the CD4 T cells (Grivel and Margolis, 1991). HIV isolates that use the CCR5 coreceptor for attachment to target cells and preferentially infect primary T cells and macrophages are known as M-tropic strains. HIV strains present in the early infection preferentially use the CCR5 co-receptors and thus, viruses transmitted between persons generally use CCR5 (Hoffman and Doms, 1998) and are nonsyncytium inducing in vitro (NSI) viral phenotypes. Thus, the viral isolates obtained from peripheral blood of individual's shortly after infection and during the asymptomatic phase are predominantly M-tropic. As the infection progresses to AIDS, T-tropic viruses can be isolated from many but not all patients (Bjorndal *et al.,* 1997). But both X4 and R5 strains appear to be equally cytopathic to T cell (Grivel and Margolis, 1991). Other groups also classified R5 viruses

into early R5 viruses and Late R5 viruses (Tscherning *et al*., 1998; Koning *et al*., 2003 and Karlson *et al*., 2004).

CCR5 expression has been documented on cell and tissue types that may be important targets in establishment of initial infection, including CD4+ T cells, monocyte/ macrophage, dendritic cells, Langerhans cells, and the mucosa of rectum and colon, as well as vagina and cervix (Combadierre *et al*., 1996; Ditmar *et al.,* 1997; Zhang *et al.,* 1998) and attributed to enhanced cytopathic effects, decreased sensitivity to inhibition by β-chemokines RANTES and increased levels of CD4 T cell death to late R5 viruses. These scenarios were observed from AIDS patients in the absence of X4 viruses. While there is as yet no precise understanding of the selective factors underlying early R5 restriction and the late R5 →X4 evolution in many individuals, and the contribution of these phenomena to pathogenesis, it is clear that there is an important role for CCR5 during initial viral transmission, and for CXCR4 and / or possibly other coreceptors in late stage of disease progression. It is also equally unknown why R5 strains are preferentially transmitted over CXCR4 strains at early stages. Latent reservoirs and tissue compartments express high levels of CCR5; virus reservoirs such as resting memory (CD45RO) CD4+ T lymphocytes and resting naive (CD45RA CD4+) T lymphocytes harbor replication competent but latent HIV proviral DNA and provide persistent, low –level viral replication(Skrabal *et al*., 2003; Kulkosky *et al.,* 2002). The expression of a coreceptor on CD4 T cells may be an important factor in controlling pathogenic events. In vivo, CCR5 is found in a subset of CD45RO CD4 T cells, while CXCR4 is expressed by most of memory and naive CD4 T cells (Lee *et al.,* 1999).

A third group of viruses use both CCR5 and CXCR4 as a coreceptor and are known as dual tropic (R5/X4) (Berger *et al*., 1998). These are isolates that replicate efficiently in both target cell types, and show SI phenotypes and are known as cytopatic as X4 strains. Other members of the CC chemokine receptor family may also function as coreceptors for HIV entry, although it is of minor importance (Bjorndal *et al*., 1997).

It is known that chemokine receptors are down or up regulated depending on the activation status of the cell and this may enhance or reduce susceptibility to HIV infection (Sallusto *et al.*, 1999; Bleul *et al*., 1996). For instance, a phenotype of low CCR5 expression and high secretion of β-chemokine has been associated with reduced infectability of cells by R5 HIV isolates (Paxton *et al*., 1998).

The most likely and interesting hypothesis is that SDF-1, which is constituvely expressed in the lymphoid organs where HIV replication is high, prevents the replication of X4 viruses by

blockade of CXCR4 but as lymphoid tissues are degraded SDF-1 production is lowered and X4 strains are allowed to replicate (Moore *et al.,* 1993). Disease progression, which is marked by the appearance of CXCR4-selective HIV, may be related to the extraordinary wide expression of this coreceptor.

Coreceptor tropism has been established as a determinant of disease progression; individuals with virus using the CCR5 coreceptor generally have a slower rate of progression and lower viral load (Bozzette *et al.,* 1993). However, the presence of mixed/dual-tropic CCR5/CXCR4 population or CXCR4-using virus is more common at lower CD4-cell counts and higher viral loads and aggravated disease conditions (Furrer *et al.,* 1998). Variations in coreceptor usage and preference of a specific coreceptor have also been observed associated with specific viral clade. In a cross-sectional study carried out on individuals receiving ART, a high frequency of SI and CXCR4-tropic viruses among HIV-1 subtype C were observed (Johnston *et al*., 2003). A similar study also showed, HIV-1 subtype A has been found to utilize alternative coreceptor such as CXCR6 and others (Begaud *et al.,* 2003). HIV-1 subtype D study also demonstrated a considerable predominance of X4 and R5/X4-tropic viruses (Tscherning *et al.,* 1998).

Abnormal expression of chemokine receptors and genetic variants of chemokine genes have been correlated with resistance to infection or delayed progression to AIDS (Samson, *et al*., 1996). Several polymorphisms within CCR5 gene, which have functional consequences for HIV-1 pathogenesis, have been identified (Carrington *et al.,* 1999). A32bp deletion within the exon of the CCR5 (CCR5-delta (Δ) 32) results in almost complete protection against HIV-1 infection and a slower progression to AIDS in individuals homozygous for the allele (Samson *et al.,* 1996). The mutant CCR5 gene, containing a 32 bp deletion, causes the production of a nonfunctional receptor and has been detected in individuals who remained HIV-negative despite long history of sexual contact with infected people(exposed uninfected, EU). The CCR5 Δ 32 induces a frame shift and a premature stop codon in the transmembrane domain 5, generating a truncated protein that cannot be expressed on the cell surface (Rana *et al.,* 1997). CCR5Δ 32 heterozygotes (People who inherited the CCR5-Δ 32) allele from one parent and a functional CCR5 allele from the other parent) are susceptible to HIV infection, but the progression from HIV infection to AIDS in these individuals is slower than persons with the normal CCR5 alleles (called wild-type individuals) (Katzenstein *et al.,* 1997).

Additional point mutation within the coding regions and the promoter sequence of the CCR5 gene has been identified. The phenotype of another chemokine receptor (CCR2V641) is also associated with slower progression to AIDS (Smith *et al.,* 1997). CCR2 is a minor HIV coreceptor.

Several polymorphisms in the gene encoding CXCR4 have been found, but none has proven informative. The polymorphism designated SDF1-3′A is a G → A translation at bp 809 of the 3′-UTR of the mRNA encoding one of the two known chemokine ligands for CXCR4, SDF-1β (Cohen et al., 1998). Homozygocity for the SDF-1 3′A allele has been reported to slow disease progression (Winkler *et al*., 1998).

CCR5 Δ 32 is common in Caucasians. One in every hundred Caucasians is homozygous for this deletion, and the frequency is almost threefold greater in high risk, HIV –negative individuals (Carrington *et al*., 1999); heterozygotes are approximately 20%. CCR5Δ32 is found at lower frequencies in the Middle East and India, but only sporadically among native Africans, American-Indians and East Asians (Reviewed by Berger *et al.,* 1999).

Although the frequency of CCR5Δ32 is very low in Africa, few studies in a limited countries show CCR5 and CXCR4 polymorphism exist. A study in West Africa showed that an increased prevalence of CCR5 wt/ delta 32 genotype was observed and this was found to reduce susceptibility to HIV infection (Kokkotou *et al*., 1998). A similar study showed in Cameroon, thought to be the origin of HIV-1, the allelic frequencies ranging from 10.8 to 31.3%for CCR2-641 and 0.0% to 7.1 for SDF1-3′ but no CCR5-Delta 32 alleles were found (Max, 2000). Another study carried out in sex workers showed that CCR5 polymorphism and CXCR4 expression levels do not account for HIV- resistance (Fowke *et al*., 1998). No study was conducted in Ethiopia on either CCR5 or CXCR4 polymorphism in discordant couples.

1.2.6. HIV transmission and the course of infection

1. 2.6.1. Transmission of HIV

HIV is transmitted via different routes: sexually, blood and blood products, from mother to child, and by other body fluids.Sexual transmission is the most frequent method of transmitting HIV either by heterosexual or by homosexual contacts. It accounts for more than 80% of HIV infection (Shattock and Moore, 2003). Because sexual intercourse accounts for the vast majority of HIV transmissions worldwide, it is important to understand the events that occur in the genital or rectal mucosa during transmission.

The worldwide infection rate for HIV is estimated to be 14,000 per a day and about 3-4 million people every year. HIV infection is not particularly easy to acquire sexually- the incidence of transmission between discordant couples has been estimated to be .0001- 0.0010 for each sexual contact, depending on the route of transmission (Gray, 2001). But this likelihood of sexual transmission from an infected partner of 1 in 1000 seems hard to account for current rate of transmission. In fact, one study concluded that the average probability of male- to female transmission during chronic HIV infection is between 3 in 1000 to 5 in 10000, and that these low probabilities even could not sustain an epidemic (Pao *et al*., 2005). However, the duration of HIV shedding, the high frequency with which at least some people have sexual intercourse and the rate of exacerbating risk factors influencing infectiousness and/or susceptibility means that HIV is readily transmissible over a long period of time. Thus, every sexual contact has the potential to transmit HIV infection.

HIV transmission varies widely with the phase of infection, and more than 10-fold higher during acute infection (Wawer *et al*., 2005). There is increasing evidence to suggest that infectivity is associated with viral load (Gray, 2001), and that viral load varies substantially in infected individuals over time. Within the first two weeks of HIV infection, the virus begins to replicate rapidly before the body is able to mount immune response that eventually suppresses the high burden (Pao *et al*., 2005). Although this period of high viramia is typically brief (lasting only 3-4 weeks), the per act likelihood of sexual transmission from an infected partner during this period could be as high as 1 in 25 (Coombs *et al.,* 2003).

Observational data seem to support this condition. A study in Southeastern USA found that individuals who were identified as recently infected had an almost 50% chance of infecting some one else within 3 months (Pilcher *et al.,* 2006). An investigation of a cluster of work place HIV infection in the adult film industry in Los Angeles, CA, USA, found that a newly infected male performer who had been receiving monthly PCR-based HIV tests infected 3 of 13 partners (23%) in a brief 1-2 month interval of sexual activity before actually testing positive for HIV (Taylor *et al.,* 2007). In a similar way, in Rakai, Uganda, researchers discovered that almost half of new HIV transmission they observed in couples of initially discordant HIV status was associated with the recent infection of the transmitting partner (Wawer et al., 2005). Acutely infected individuals pose a particularly profound risk, which is why epidemics usually spread explosively when they strike a new population.

Other sexually transmitted diseases (STDs) have a marked effect on both viral shedding and the risk of acquiring HIV infection (Pao *et al.,* 2005). STDs increase the amount of virus in the genital fluid of the transmitter, thereby raising transmission rates, particularly from men to women. STDs can increase the number of infected lymphocytes in the mucosa and hence virus expression through immune activation (Galvin and Cohen, 2004). The most sexually active population is also the one that is most at risk for STD- the consequence is the rapid spread of HIV infection among young and sexually active adults (Shattock and Morre, 2003).

In the genital tract of HIV –infected females, virus originates from the cervix and its associated lymphatic tissues, as well as from menstrual blood (Gupta and Klasse, 2006). Semen of HIV-positive men contains both infected macrophages and lymphocytes (Coombs and Reichelderfer, 2003). In simian immunodeficiency virus (SIV)-infected male macaque, most of the virus in the genital tract is associated with epididymidis, but ejaculates from vasectomized men, and pre-ejaculatory fluid, can be infectious (Gupta and Klasse, 2006). 10-20% of men with undetectable blood viral load due to ARV therapy have detectable virus in the genital tract (Coombs and Rrichelderfer, 2003). The explanation may be that HIV replicates in the genital tract, for example, in the macrophages that reside there and in the vagina. In addition, transmission is increased when the mucosa is made more vulnerable such as in the case of anal intercourse (Dunkle *et al*., 2004).

Transmission of HIV by blood and blood products has significantly decreased in the developed world since the introduction of HIV screening and recently by the introduction of HIV RNA testing of blood and blood units (Osmanov *et al.,* 2000). In developing countries, blood and blood products may not be screened regularly from HIV and more sensitive tests like HIV RNA to reduce the residual risk not have been implemented (UNAIDS/WHO, 2008).

Another mode of transmission is by blood or its components such as, sharing needles, syringes or unsafe practices in case of acupuncture, tattooing and piercing can lead to HIV transmission (Hunt, 2008). Medical interventions with re-used needles and syringes might also contribute to the spread of HIV (Varmus, 1988). Occupational exposure to blood components when health care workers are stuck by needles or other sharp medical instruments may also result in the transmission of HIV (Hunt, 2008). Normally, a large quantity of blood is required to result in transmission of HIV after parenteral exposure in the health care settings.

In the absence of interventions, 40-50% of exposed infants acquire HIV through mother –to child transmission (Lehmand and Farquhar, 2007). Unlike many pathogens that are vertically transmitted, HIV can be acquired *in utero*, during delivery (intrapartum), or via breast feeding.

Breast milk exposure accounts for one-third to one-half of all transmissions among breast fed infants, with intrapartum and in utero transmission responsible for the remaining cases (McIntyre, 2005).

High maternal plasma HIV RNA load has been consistently associated with increased transmission during pregnancy (Discover *et al*., 1996). HIV viremia is highest during acute HIV infection as well as in the settings of advanced disease with concomitant immunosuppressant. Women acquiring HIV post partum have two-fold increased risk of transmitting HIV via breast milk (Dunn and Newell, 1992), suggesting that primary infection increases the risk of transmission. Sexually transmitted diseases, such as syphilis and gonorrhea, are considered to increase risk of transmission. Female infants have a two-fold increased risk of infection at birth when compared to male infants (Galli *et al*., 2005).

1.2.6.2. Mechanisms of sexual HIV-transmission

The majority of the 14, 000 or more HIV daily infections are transmitted through sexual intercourse worldwide. For sexual transmission HIV has to come in contact and must cross the mucosal epithelium. Normally, the mucosal epithelium is not easy for any microorganism including, crossing and causing infection. The epithelial layer of the most exposed region of the female and male genital mucosa is composed of three multiple layers of stratified squamous epithelium. The exposed regions of male and female genital mucosa include vagina and ectocervix of women, inner foreskin, penile glans and fossa navilularis in men. In all these regions HIV can not enter and cause infection due to the fact that:

- the epithelium in these regions has limited permeability to particles that have a diameter greater than 30nm (the diameter of an HIV virion is 80-100nm) (Shettick *et al.,* 2001);
- the outermost apical surface comprises a superficial layer of dead epithelial cells that is impermeable to virus and is renewed every three days (Kreg, 1983);
- stratified genital epithelium cells are not susceptible to HIV infection and do not transcytose viral particles (but infected cells are more likely to provide a localized reservoir of virus that is external to the epithelium itself) (Miller and Shattuck, 2003);

- the mucus secreted by epithelial cells themselves can provide additional protection both by acting as a physical barrier and because it contains proteins with innate antiviral activity, such as secretoy leucocytes protease inhibitor (SLP1) (Monyama, 1999);
- some mucosal sites (endocervical cells) express high levels of natural chemokine ligands for the CXCR4 and CCR5 coreceptors, which might provide an additional innate, biochemical barrier to HIV infection (Agace, 2000) ;
- the small passages (pores) between epithelial cells known as desmosmes and abundant amorphous lipoidal materials are also important in keeping the integrity of epithelial barrier and in preventing infections (Miller and Shattock, 2003).

The endocervix of female genital structure, however, is lined with a single layered columnar epithelium and a breakdown in the integrity of such a thin barrier is more likely than of the genital stratified epithelium (Shattock and Moore, 2003) (Fig 6). Similarly, unlike its vaginal counterpart, the rectal epithelium provides little or no physical protection against potential trauma during intercourse, facilitating HIV access to the underlying target cells the systemic circulation. Moreover, the rectum, unlike the genital tract, is populated with organized lymphoid tissues that contain specialized microfold cells (M cells) that are capable of binding and presenting HIV to the underlying lymphoid tissues (Neura, 1999).

Some degree of breakdown in epithelial integrity is therefore required for HIV transmission. Anything that impairs epithelial integrity, such as physical abrasion or trauma, ulceration, inflammation, hormonal status, micronutrient levels or the use of intravaginal preparations for dry sex; can critically increase susceptibility to HIV infection (Novel *et al.,* 1984).

Epithelial micro-abrasions can be detected in 60% of women following consensual intercourse, and are also frequently observed on the inner foreskin and penile glans (Van Howe, 1999; Novel *et al.,* 1984). STDs make recipients more susceptible by breaking down the mucosal barrier in the case of genital ulcer disease and by increasing the number of susceptible cells in the mucosa through inflammation (Gupta and Klasse, 2006). Bacterial vaginosis (the colonization of the vagina by anaerobic bacteria) may further enhance the infectivity of incoming virus by raising the pH of the vaginal fluid; a higher pH would lead to slower virus inactivation and more efficient Env-mediated fusion (Barry *et al.,* 2006). High viral load, specifically in genital fluids, raises the risk of HIV transmission (Novel *et al.*, 1984).

After deposition of the virus on the recipient mucosa, the outcome is affected by properties of the virus: its retention of infectivity while traversing the mucosa and extracellular matrix, its affinity for entry receptors, the fusogenicity of its Env protein, and the efficiency of its interaction with dendritic cells (DCs) that can promote virus spread to, and amplification in, CD4+ T cells, locally and possibly at distant sites (Gupta and Klasse, 2006) (Fig 6).

Dendritic cells usually populate in the mucosa including the oral and vaginal mucosal surfaces (Liwa and KewalRomani, 2006) and because dendritic cells are located in the mucosal and the lymphoid tissues, they have been proposed to be among the first cells that encounter HIV. It has been known for more than a decade that HIV-pulsed DCs facilitate viral infection of co-cultured T cells (McDonald, 2003). Studies of DC-HIV interaction have highlighted an important role for DCs in HIV transmission at mucosal surfaces and in HIV pathogenesis (Liwa and KewalRomani, 2006; McDonald, 2003).

One of the enigmatic features of DC biology is the complexity of DC subsets. The main DC subsets include myeloid DCs and plasmacytoid DCs in the blood and Langerhans cells (LCs) in the tissue (Gupta and Klasse, 2006). The epithelial layer is populated with LCs and T cells but this varies between tissues (Miller and Shattock, 2003). Both LCs and T cell express CD4 and are targets for HIV. These sentinel cells of the immune system are primed to respond to the danger signals that are provided by tissue damage, which trigger their migration to the draining lymph nodes (Steinmann, 2003).

Since LCs do not have mannose C-type lectin receptors (CLRs), which helps to bind free virion, they can not bind free virions with these receptors , which leaves CD4 as the main HIV binding site (Shattock and Moore, 2003). Coreceptors are expressed at only low levels on human LCs, with CXCR4 usually undetectable, and CCR5 present at low levels on 24-49% of the cells (Steinman, 2003). However, compared with HIV replication in CD4 T cells, HIV replication in DCs is generally less productive (Miller and Shattock, 2003).

Genital and rectal subepithelial stromal tissues are densely populated with DCs, macrophages and T cells that express, CD4, CCR5, and to a lesser extent CXCR4 (Agace, 2000). Each type of these cell types is therefore susceptible to HIV infection. Any extensive breakdown in epithelial integrity allows HIV direct access to a rich source of target cell, allowing the establishment of infection in mucosal sites. In deed, several studies using macaque transmission models and human mucosal tissues *ex vivo* have identified subepithelial T cells, macrophages and DCs as the primary targets for infection (Agace, 2000; Steinman, 2003; Liwa and KewalRomani, 2006).

HIV crosses the epithelium barrier either because of epithelial damage, or capture by intra-epithelial DCs that convey the virus to target cells deeper in the mucosa (Pope and Haase, 2003). At mucosal surfaces during the sexual transmission of HIV, dendrtic cells are proposed to be among the first target cells to encounter the virus. The DC may merely capture X4 or R5 virus and carries it across the epithelial barrier or get infected by R5 virus and produce progeny virus (Gupta and Klasse, 2006).

HIV-infected macrophage and follicular dendritic cells in the lymph nodes easily transmit to T cells via the antigen-presentation processes. Because they interact with so many T cells, these reservoirs for HIV can infect large numbers of T cells. The long life span of some reservoirs cells, such as decades-long life of memory T cells, allows them to carry HIV for many years, providing a source of new rounds of HIV infection (De-Paoli *et al.,* 1988).

Direct tracking of HIV viral genotypes in local microenvinments indicates that cell to cell transmission of virus is a local phenomenon, wherein an infected cell releases virus that efficiently infects only nearby targets (Cheynier *et al.,* 1994). This implies that propagation of infection depends up on local cell interaction and local target cell densities, rather than on the overall number of target in the body (Grossman *et al.,* 1998). During, acute infection the mucosal tissues presumably offer, in effect, a continuum of target cells through which the virions rapidly propagates and multiplies. Ultimately, the virus disseminates via efferent lymphatic and blood to spleen, brain, liver and lungs (Gupta and Klasse, 2006).

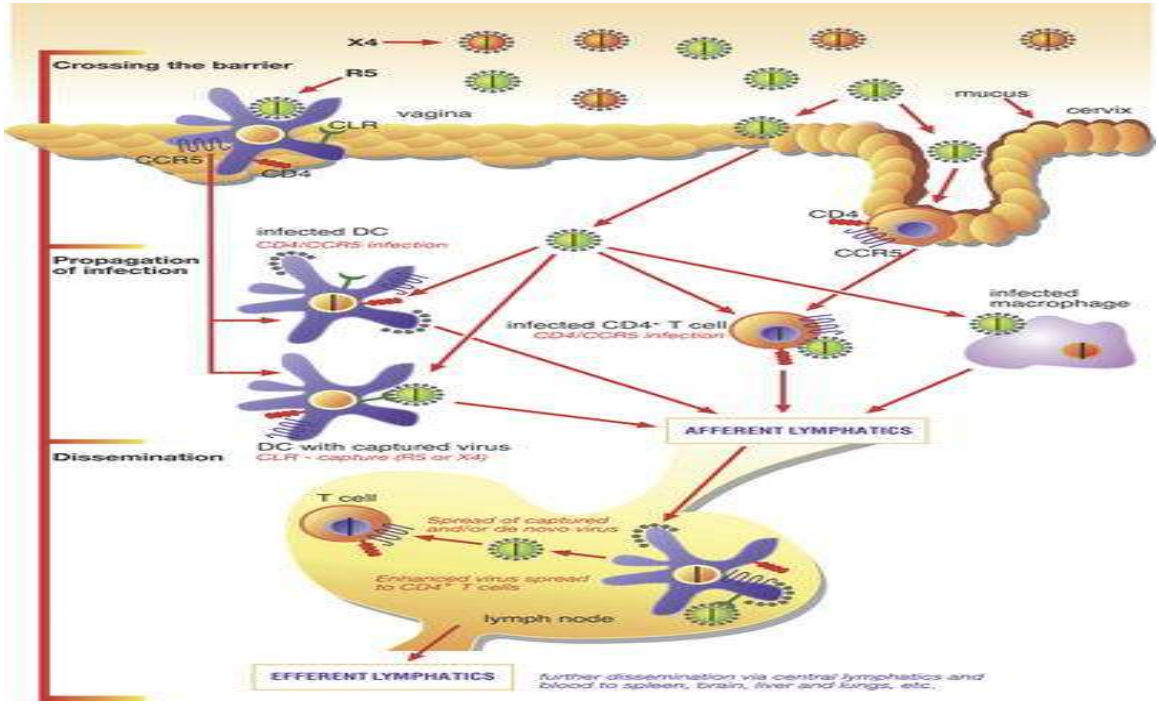


Figure. 6 Potential mechanisms for HIV transmission across mucosal epithelium (Source: Shattock and Moore, 2003)

It usually takes 4-6 days for the infection to become systemic (Pope and Haase, 2003). But this is not always the rule. Even though retroviral infection is seen as irreversible because of the integration of the pro-virus into the host cell genome, at the level of the organism, abortive HIV infections may be common (Liwu and KewalRomani, 2006).

1.2.6.3. The course of the disease

People may be exposed to HIV without becoming infected. However, once an individual is exposed to HIV, the virus travels and replicates fairly rapidly. Most of the reliable data available come from studies of genital exposure to HIV (Novel et al., 1984; Agace, 2000; Liwa and KewalRomani, 2006). These data show that within 2 to 3 days after genital exposure, the virus travels to the regional lymph nodes. After an average of 7 days (range 4 to 11 days), viremia develops. At this point, the virus is also present in the central nervous system and lymphatic system (Shattock and Moore, 2003).

As mentioned earlier, depending on the route of infection, CD4 cells in the blood or mucosal tissues may be the first cells infected or dendriric cells embedded in the epithelium layers of the skin may capture the virus and migrate to the lymph nodes. Immune activation allows the virus to spread more rapidly by increasing the pool of available cells for the virus to infect (Embretson *et*

al., 1993). As the number of activated T cells proliferates, the number of cells available for HIV virion to infect also increases. Another feature of activated immune cells is an increased rate of transcription into their proteins. An HIV-infected immune cell harbors the viral genome within its DNA. Therefore, as the rate of transcription increases in an activated immune cell, the HIV mRNA and proteins needed to make new virions also increase. Thus, viral replication only occurs in activated CD4 cells. In other words, HIV benefits from this state of immune activation (Geeraet *et al.,* 2008). The course of untreated HIV infection in the majority of individuals is shown in Figure 7 below.

It begins with an acute symptomatic illness, lasting only a few weeks, which is associated with a high viremia, sharp drop in peripheral blood CD4+ T cell count, establishment of a reservoir of latently infected CD4+ T cells (Chun *et al.,* 1997), and development of an HIV-specific immune response (Koup *et al.,* 1994). This is followed by a 100 to 1000-fold fall in viral load, a partial rise in peripheral blood CD4+ T cell count, and then a generally asymptomatic phase of chronic infection, lasting on average 10 years, which is marked by slowly falling peripheral blood CD4+ T cell counts and slowly rising viremia (Fig 7). As peripheral blood CD4+ T cells decline to less than 200 cells/ıl, when the total number of CD4+ T cells in the body has been reduced by at least half, the opportunist tumors and infection that characterize AIDS beset the individual (Haase,19991).

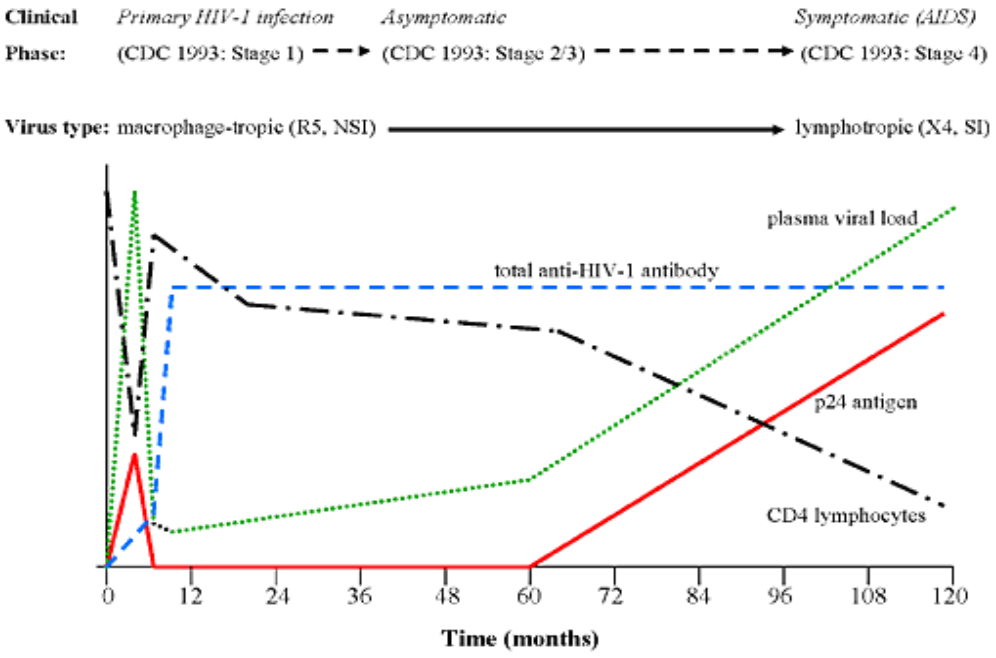


Figure 7. The natural history of typical HIV infection showing changes in plasma HIV viral load, peripheral blood CD4+ T cell count, and HIVspecific CD8+ T cell response (Source Adapted from Annu Rev Immunol, 2003.21:265-304)

In the first few weeks of infection, virus and viral antigens appear and rise often to quite high levels in the blood stream, with levels of virus in the bloodstream during initial symptomatic period typically in excess of 10 million particles per milliliter (Rosenberg *et al.,* 2000). As viremia develops, HIV infected patients develop nonspecific symptoms. These symptoms may resemble those resulting from influenza or acute mononucleosis, and usually lasts for a week to a month (Lyles *et al.,* 2000). The acute HIV syndrome occurs in approximately 50-70% of individuals with HIV infection.

The primary (acute) stage of HIV infection generally resolves ; virus and viral antigens fall to lower levels coincident with cellular immune response; and, in most infected individuals, a relatively asymptomatic, clinical latent stage ensues that lasts for several years (Koup *et al*., 1994). During this time, which generally begins at about weeks post infection and continues for a period of months to many years, the amount of virus circulating in the blood is generally stable at a level referred to as the viral load set point (Wei *et al*., 1995). During this period the lymph nodes and spleen are sites of continuous HIV replication but immune system function remains sufficient to prevent the emergence of opportunistic infection and few or no symptoms of HIV infection are evident.

Over the course of infection, the CD4+T cell count in blood slowly declines, and at the AIDS-defining levels of 200 cells/mm3, opportunistic tumors and infections supervene that eventually claim the lives of most infected individuals (Pantaleo *et al*., 1993). At this stage, the extensive T cell destruction, and infection of new CD4 cells destroys the structure and function of the lymphoid tissues and leads to substantial immune deficit (Haase, 1999).The development of AIDS in HIV-infected humans is associated with profound changes in the expression patterns of lymphocyte phenotypic markers associated with increased immune activation and decreased recall immune responses (Locher *et al*., 2003).

According to the classification system of HIV infection and expanded AIDS survey case definition for adolescents and adults, HIV is classified by clinical findings and the number of CD4 cells (WHO, 2009 update). Life threatening complications are expected if CD4+ cells drop below 200 cells/ul (Table 1).

Table 1: WHO Clinical Staging of HIV/AIDS and Case Definition

Primary HIV Infection

Asymptomatic

Acute retroviral syndrome

Clinical Stage 1

Asymptomatic

Persistent generalized lymphadenopathy

Clinical Stage 2

Moderate unexplained weight loss (<10% of presumed or measured body weight)

Recurrent respiratory infections (sinusitis, tonsillitis, otitis media, and pharyngitis)

Herpes zoster

Angular cheilitis

Recurrent oral ulceration

Papular pruritic eruptions

Seborrheic dermatitis

Fungal nail infections

Clinical Stage 3

Unexplained severe weight loss (>10% of presumed or measured body weight)

Unexplained chronic diarrhea for >1 month

Unexplained persistent fever for >1 month (>37.6°C, intermittent or constant)

Persistent oral candidiasis (thrush)

Oral hairy leukoplakia

Pulmonary tuberculosis (current)

Severe presumed bacterial infections (eg, pneumonia, empyema, pyomyositis, bone or joint infection)

Anemia (hemoglobin <8 g/dL)

Neutropenia (neutrophils <500 cells/μL)

Chronic thrombocytopenia (platelets <50,000 cells/μL)

Clinical Stage 4

HIV wasting syndrome, as defined by the CDC

***Pneumocystis* pneumonia**

Recurrent severe bacterial pneumonia

Chronic herpes simplex infection

Extrapulmonary tuberculosis

Kaposi sarcoma

Cytomegalovirus infection (retinitis or infection of other organs)

Central nervous system toxoplasmosis

HIV encephalopathy

Cryptococcosis, extrapulmonary (including meningitis)

Disseminated nontuberculosis *Mycobacteria* infection

Progressive multifocal leukoencephalopathy

Candida of the trachea, bronchi, or lungs

Chronic cryptosporidiosis (with diarrhea)

Chronic isosporiasis

Disseminated mycosis (eg, histoplasmosis, coccidioidomycosis, penicilliosis)

Recurrent nontyphoidal *Salmonella* bacteremia

Lymphoma (cerebral or B-cell non-Hodgkin)

Invasive cervical carcinoma

Atypical disseminated leishmaniasis

Symptomatic HIV-associated nephropathy

Symptomatic HIV-associated cardiomyopathy

Reactivation of American trypanosomiasis (meningoencephalitis or myocarditis)

Source: The AIDS Education & Training Centers National Resource Center (AETC). HIV Classification: WHO Staging Systems (WH), 2009, Update. Accessed March 30, 2011. Available online at http://aidsetc.org/aidsetc?page=home-00-00

1.2.7. Viral load and viral dynamics in HIV infected patients

HIV is mainly found in the blood and several kinds of body fluids. The body fluids include blood, semen, saliva, vaginal secretion, amniotic fluid, breast milk, tears, cerbro-spinal fluid and the like (Schwartlander *et al.,* 2000). The transmission of a virus can be greatly influenced by the amount of infectious virus in a body fluid and the extent of contact an individual has with the body fluid The amount of virus in a blood and body tissues differ between sexes. This difference in viral load has been ascertained after adjusting for possible confounders of the relationships of sex and viral load such as age, race, mode of virus transmission, and antiretroviral therapy use. Even after adjustment for CD4 cell count or time since seroconversion viral loads are lower in woman than in man (Gandhi *et al*., 2003).

HIV is found in both the blood and the genital tract. Viral load peaks shortly after infection and reaches an individual specific level that is probably stable within a few months of infection (Ploegh, 1998). Several studies have shown that the blood of individuals with AIDS contains more HIV RNA or infectious viral particles and more HIV-infected cells than the blood of individuals without symptoms (Roederer, 1995). Long –term asymptomatic persons had significantly less serum viral RNA than progressors (Clark *et al*., 2000). In a similar way, viral load of HIV+ve discordant partners is kept at low level for a long time (Rowland-Jones et *al.,* 1997).

1.2.7.1 HIV in the male genital tract

Studies continue to suggest that the genital tract is a distinct reservoir of HIV. Although most studies have examined HIV in semen, their results are likely to apply to the female genital tract as well. The male genital tract is generally considered to be a “sanctuary site” for HIV due to the presence of the blood testis barrier, a layer of cells connected by specialized ‘tight junctions’ that prevent drugs from passing between the blood and the areas of the testis where sperm develops

and mature (Vernazza, 2001). It is thought that the cause of low levels of antiviral drugs in semen, may allow the selection and development of drug resistant HIV strain.

Research involving a prospective longitudinal study (Gupta, 2004) found that HIV shedding in semen followed three patterns, all independent of plasma viral load: non-shedding (28%), continuous shedding (28%), and intermittent shedding (44%). Different virus populations were found in semen and plasma of intermittent shedders, while similar populations were found in continuous shedders. The same study also showed that shedding (cell-free and cell associated) in semen was significantly higher during primary infection than during chronic asymptomatic infection or after antiretroviral treatment.

A study conducted on humans following ARV regimen noted that there were higher seminal viral loads in non-adherent subjects than in those who adhere to their regimen (Vernazza, 2001). This study emphasizes the importance of appropriate ARV regimens and a good adherence in controlling viral dynamics in the genital tract, but continues to note some discordance between plasma and genital tract viral reservoirs, possibly increasing the risk for vertical and horizontal transmission in some situations.

In addition to antiviral agents, the presence of genital pathogens and inflammation are additional correlates of genital tract shedding. An investigation of HIV-1 RNA in plasma and semen during the first six months of ART in 88 patients (Gupta, 2004) showed 20 events of sexually transmitted infections and these infections were associated with transient rebounds in seminal viral load, where it had been undetectable. Levels reverted to undetectable after the infection subsided.

The presence of HIV in the genital tract has been previously reported, with several studies showing that reductions in viral load in this compartment correlate with decreases in blood for individuals on highly active ARV therapy. However, recent studies have shown that HIV is also produced in cells in the genital tract and can have unique resistance mutations compared to those in the blood (http://www projinf org). In addition viral load can sometimes be measured in the genital tract in the presence of infections or invasive procedures, even when it is not measurable in blood (Levy, 1998). This is important because many researchers have assumed that undetectable virus levels in blood, using the currently available tests, automatically means undetectable virus levels in the genital tract.

The notion that there is a low level of ARV drugs in semen, which is in turn thought to allow the selection and development of HIV strains that are resistant to the drug, however, is being challenged. This notion is being refuted on the basis of anatomical, virological and immunological

observation (Vernazza, 2001). However, the researchers acknowledge that HIV replication could occur under circumstances of inflammation or infection in the genital tract, when leukocytes may be present in the semen. They suggest that the presence of a sexually transmitted infection could underlie this, leading to the production of drug resistant virus.

1.2.7.2. HIV in the female genital tract

The concentration of HIV RNA in the blood is the best predictors of a woman's risk of transmitting HIV through heterosexual contact or during birth (http://www.Sciencedaily com.html). However, direct contact with virus in the genital tract might be the necessary key to HIV transmission. HIV-positive women may risk transmitting HIV to sexual partners and new born infants through virus released in the genital tract, even if blood levels of the virus are low (Andrea Kavaes *et al.,* 2001). Women who had little virus evident in the blood still could release infectious HIV from the cells of the uterus, cervix and surrounding tissues. This suggests separate reservoirs of HIV replication.

Studies continue to suggest that the genital tract is a distinct reservoir of HIV. In a study conducted among 311 HIV-infected women, genital tract shedding of HIV was present in nearly 80% of women with detectable plasma HIV RNA or culturable virus (presence of HIV RNA or culturable virus in genital secretions is defined as HIV shedding) but also in approximately 1/3 of women with less than 500 plasma RNA copies per ml or with negative HIV cultures of mononuclear cells (Mary and Wilson, 2001).

In another study in 5 US cities, of 247 with both culture and RNA studies of genital samples, 158(64 percent) had HIV shedding (http://www.Sciencedaily com/ html). Investigators found a positive correlation between concentrations of plasma and genital tract HIV RNA: women with high plasma concentrations were more likely than women with lower concentrations to have culturable virus and detetectable RNA in genital samples. Although HIV RNA concentration in the genital tract were about 1 log lower overall than those in plasma, in 9 of 247 women(4 percent) concentrations were higher in genital samples. These findings support the observation that the genital tract may be a separate reservoir for HIV replication in some women.

In a study conducted to investigate the effect of HIV on the hormonal milieu of the normal menstrual cycles on HIV-infected and 9 HIV-negative women, normal levels of progesterone and estradoil was observed (http:// www.Hopkins-aids edu/html). But viral load during the menstrual cycle was found to fluctuate (Deborah, 2003). In another study of viral shedding in the genital

tract through the menstrual cycle, following 55HIV-infected women with normal menses weekly for eight weeks, the same group found that the highest levels of virus were seen immediately prior to and during menses, independent of genital infections, plasma viral load, CD4 and blood mononuclear cell infectious virus titer (http:// www.Hopkins-aids edu/html).

HIV viral load in the genital tract but not plasma varies considerably throughout the menstrual cycle, suggesting that local factors may influence the genital viral load compartment independent of plasma viral load (Deborah, 2003). The highest genital tract viral levels were observed during the menstrual phase, which may point to an increase in the risk for sexual transmission at this stage of menstrual cycle.

Prior studies have indicated that sexually transmitted diseases increase the likelihood of HIV transmission and acquisition .This result may be explained, in part, by the finding that STDs associated with cervical inflammation and genital ulceration increases HIV shedding into the genital tract.

Later, other researchers examined vaginal epithelial biopsies, blood and cervicovaginal lavages from HIV-positives women in Thailand and the US and found that 84% of Thai and 14% of US women had chronic inflammatory T-cell infiltrates in the vaginal epithelium (Andrea Kovacs, 2001). Among the Thai women, fifty-fold more HIV RNA was found in the epidermis even in the absence of other genital pathogens. Inflammation may increase transmission because cells mediating this process serve as targets for initial viral infection. Thus, the presence of genital pathogens and inflammation are additional correlates of genital tract shedding.

Studies indicate that women in whom HAART has suppressed HIV concentrations in the blood might still have high concentrations of HIV in the genital tract (http://www.scienceblog.com/.html). Heterosexual women with HIV including those who have had successful antiretroviral therapy are at risk of transmitting HIV to their sexual partners and new born infants as a result of viral shedding in the genital tract (Venazza, 2001). Antiretroviral medications can reduce blood plasma viral load and genital tract viral load, but antiretroviral also lead to drug resistant HIV (Yerly *et al*., 1999). There are reports of resistant genotypic variants in the genital tract that differed from variants in the blood (Vernazza, 2001).

Two studies, one using isolates from women and one using isolates from men, show viral mutants in the genital tracts that are different to those in blood plasma, and which persisted sometimes for more than 2 years without selective pressure of treatment (Smith *et al.,* 2003; Tirado *et al*., 2003). These studies suggest that low level selective forces allow distinct viral lineages to emerge and

evolve independently in the plasma and vaginal compartment. The report that delayed clearance of drug resistance mutants was observed in the vaginal compartment and these viruses remained macrophage-tropic despite presence of T-cell tropic in the plasma and advanced HIV disease, thus, suggesting the vaginal tract could serve as a reservoir of macrophage tropic drug mutants and perhaps contribute to the transmission of drug resistance.

Studies on viral dynamics have revealed unexpectedly rapid kinetics of HIV replication (Sierra *et al.,* 2005). The virion half-life has been estimated to be six hours and the total number of virions produced per a day is at 10^{10}. Assuming a minimum burst size of approximately 100 virions per cell, the total number of productively infected cells is approximately is $10^{8.}$ Given the error rate of HIV reverse transcriptase in the order of $10^{-4 \text{ to } 10^{-5}}$ per base; and the size of the viral genome of 10^4 base pairs, the number of mutant viruses that appear each day is tremendous. This mutation rate results in a steady accumulation of random mutations in the viral quaisi species that do not significantly interferes with functional and structural constraints of viral proteins. At the same time, virus variants are continuously subjected to strong selective pressure, which results in accumulation of mutations at specific site of the viral genome (Phillips et al., 1991). And this may be the source of drug resistant variants.

1.2.7.3 Viral load and HIV transmission

Studies carried out by several authors showed that the likelihood of HIV infection depends on the selective biological phenotype of HIV isolates (Levy, 1998; Louisinrotchanaku, 2001). The eastern and southern Africa HIV epidemics, for example, is predominantly caused by HIV-1 type A, C, and D, and the rapid progression of the epidemics in these parts of Africa might, in part, be associated with greater infectivity of these HIV-1 subtypes (Schwartlander *et al*., 2001). Conversely, some have postulated that the slower evolution of the HIV epidemic in western Africa might be due to the higher population of less transmissible recombinant HIV-2 subtypes in that region (McCutchan *et al*., 2000).

But recent studies relating the probability of HIV transmission per act of intercourse and viral load similarity in the United States, Europe and Thailand with Africa showed that the rapid spread of the virus in parts of Africa is unlikely to be attributable to the characteristics of a particular subtype that facilitate transmission (Quinn *et al*., 2000).

In a study conducted in Rakai, HIV sub-typing was available for 155 infected individuals. Thirty one were infected with subtype A (20%), 103 sub-type D (66.4%), and the remaining 21 (13.5%)

had an atypical V_3 sub-type which showed complex binding patterns (Quinn et al., 2000). The transmission probabilities per act were 0.0022 with sub-type A, 0.0019 with sub-type D, and were lower with the atypical viruses (0.0012). These differences were not significant (P=0.26).

The European and U.S. epidemic are predominantly caused by HIV-1 subtype B, and Thai epidemic by subtype E and B, and the Ugandan epidemic by subtype A and D. Therefore, since the transmission probabilities per act of sexual intercourse in these populations are similar, the generalized HIV epidemic in Uganda is unlikely to be caused by a greater infectivity of subtypes A and D.

1.2.7.4. Probabilities of HIV transmission per coital act

The probability of HIV transmission per coital act plays a very important role in heterosexual transmission of HIV. The probability of HIV transmission per coital act in representative African populations per sexual act of vaginal intercourse, or infectivity, has been estimated from prospective studies of HIV-discordant partners or male contact with prostitutes (Brookmlyer, 1994). Published estimates of transmission probabilities per act vary from 0.0001 to 0.00014 in U.S. and European studies of discordant couples (Royce *et al.*, 1997) and to 0.002 in Thai couples (Duerr *et al.,* 1994). However, higher transmission probabilities 0.056-0.10 per act have been reported among men who had contacts with prostitutes in Thailand (Mastro *et al.,* 1994).

The higher transmission probabilities associated with commercial sex might be attributable to the presence of other sexually transmitted diseases (Royce *et al.,* 1997). Other factors that increase transmission include lower CD4 counts or AIDS in the HIV-positive index partner, and anal intercourse (Royce *et al.,* 1997) ; Mastro *et al.,* 1994). Male- to - female HIV transmission is usually more efficient than female-to- male transmission in U.S. and European populations (Mayer and Anderson, 1995).

There are no data on per-contact probability of transmission from representative heterosexual couples in sub-Saharan Africa, and there is little information on the efficiency of transmission associated with HIV viral subtypes. Estimation of HIV transmission probabilities rely on the accuracy of reports of the frequency of sexual intercourse.

In a study conducted on 174 monogamous couples in a village of Rakia, Uganda, the mean frequency of intercourse was 8.9 per month, which declined with age (Quinn *et al.,* 2000) and HIV viral load. Members of couples reported similar frequencies of intercourse.

Among the 97 couples in which the man tested HIV-positive first, the infected men reported a mean of 8.3 acts of intercourse per month, and their HIV-negative female partners reported 8.8 acts per month. In 77 couples in which the woman tested HIV-positive first, the infected woman reported 9.7 acts per month, and their HIV–negative partners reported 9.8 acts per month. Thus, there was relatively good agreement in the sexual frequency independently reported by both members of couples (Gray, 2001). Coital frequency was 10.0 per month for ages 15-24 compared with 7.4 per month for individuals older than 35 years.

Similar studies conducted to determine the probability of HIV transmission showed that it was 0.11% per act of intercourse (Gray, 2001). The probability was higher for individuals younger than 30 (0.13-0.17%) than for those aged 30 or older (0.06-0.09). Similarly, the probability of transmission was higher among those with genital ulcers (0.41%) than among those without (0.11%). Woman appeared to be more likely than infected men to transmit the virus (0.13- 0.09%) (Gray, 2001).

Research conducted in Rakai village also showed that the overall unadjusted probability of HIV transmission per coital act was 0.0011% (95CI 0.0008-0.0015%) (Quinn *et al*., 2000). The probability of transmission per act from HIV-positive women to their HIV-negative male partners was 0.0013, compared with a transmission probability of 0.0009 per act from HIV-positive men to HIV-negative women. The probability of transmission per act was higher if genital ulcer disease was reported by the HIV-positive partner than if it was not (0.0041 vs 0.0011) and this higher transmission probability among individuals with ulceration was seen at all viral loads (Quinn *et al.,* 2001).

Studies of discordant couples in Europe and the USA generally reported higher male- to -female transmission efficiently than female- to- male, although these investigations had few female HIV-positive index partners (Royce *et al.,* 1997). By contrast, a study (Mastro *et al.,* 1994) found a higher transmission probability per act from women-to- men than from men-to-women, which, although not significant, was consistent with previous incidence data from Rakai among HIV discordant couples (Quinn *et al*., 2000). Similarly, higher female-to- male transmission has been reported from other less developed countries (O'Farrell, 2001). Several African studies show that HIV prevalence and incidence are higher in young women than in young men (Schwartlander *et al*., 2000), but these data suggest that the sex specific differentials in HIV prevalence are unlikely to be caused by sex differences in the efficiency of HIV transmission.

Other factors that influence transmission included age (which decreased risk) and circumcision (especially if the male was HIV-negative partner). Previous studies among high-risk populations have shown that uncircumcised men have an increased risk of heterosexual acquisition of HIV compared to circumcised men. The finding , in the rural Rakai district, that circumcision afforded protection against HIV infection, with no infections among 50 HIV negative circumcised men as compared to 40 infections among 137 men (Quinn *et al*., 2000), suggested another potential biological method of HIV prevention. This is probably due to the biological characteristics of the foreskin of uncircumcised men, which is prone to microulceration, is associated with an increased frequency of STDs, and provides an increased surface area of epithelial tissue that is susceptible to HIV.

High maternal plasma HIV RNA load has also been consistently associated with increased transmission during pregnancy (Discover *et al.,* 1996), and studies have demonstrated that reduction of HIV viral load with antiviral drugs decreases rates of in utero transmission (Mofenson *et al.,* 1999). Moreover, it has been shown that women acquiring HIV post partum have two-fold increased risk of transmitting HIV via breast milk because of viral load during acute infection (Dunn and Newell, 1992).

1.2.7.5 Viral load and resistance

There may be compartmentalization of viral levels in the genital secretions that may not necessarily correlate with the reduction in the peripheral blood. However, a large survey to examine the link between the concentration of a virus in a person's blood – known as viral load- and other risk factors for HIV heterosexual transmission was conducted (Gray, 2001).

The finding suggests viral load is the most important predictor of HIV transmission between men and women, regardless of the transmitting individual. The two-and-half-year survey followed more than 400 heterosexual couples, in each of which only one person was HIV-positive. The study found that the more virus individuals carried, the more likely they were to infect their sexual partners. Conversely, no one who had fewer than 1500 copies of HIV per milliliter (ml) of blood transmitted the virus to his or her partner. According to this study, with every 10-fold rise in the concentration of HIV in the blood stream, transmission more than doubled (Gray, 2000). Nearly 80 percent of the cases of new infections resulted from exposure to HIV-positive partners with more than 10,000 copies of HIV /ml of blood.

Overall, the probability of HIV transmission was 0.11% per act of intercourse. The risk of transmission climbed sharply and steadily as viral load increased compared with men and women whose viral load was less than 1700 copies/ml, those with a viral load of 1700-38500 copies /ml were 16 times as likely to transmit the virus (rate ratio, 16.1); the rate ratio rose to 27.7 for individuals with a viral load of more than 38000 copies/ml. An infected individual with genital ulcer disease was at an increased risk of transmitting the virus, but not other STD-related factors were associated with the risk (Gray, 2001).

In another study where 1022 HIV-discordant couples were followed between 1994 and 2000, a total of 162 initially HIV-seronegative individuals seroconvert during follow-up. Plasma HIV RNA was significantly higher among transmitters, at a median of 123,507 copies/ml and 51,310 copies/ml ($P<0.001$). The risk ratio for transmission was 7.6 among woman with HIV RNA of at least 100,000 copies/ml, and 4.1 for viral loads between 10,000 and 100,000 copies/ml, with women with lower viral counts (Grace, 2001). According to this study, at plasma viral loads exceeding 100,000 copies/ml, HIV infected women are more than three times as likely to transmit the virus to their partners than are men with the same viral load. In this study, conducted in Zambia, the relationship between viral load and transmission was much marked among men, with corresponding risk ratio of 2.1 and 1.2, respectively (Grace, 2001).

On a previous study conducted to determine frequency of coital intercourse and risk of HIV transmission among HIV infected individuals, coital frequencies declined with viral loads; the mean frequency of intercourse was 10.4 acts per month in individuals with viral loads of less than 1700 copies/ml, where the frequency was 7.9 among those with viral loads of less greater than 48500 copies/ml ($P=0.10$).

Another previous study carried out to investigate risk factors for homosexual transmission of HIV where comparison between 10 monogamous homosexual couples between whom HIV transmission had not occurred showed that peripheral cellular infectious load was lower in the group of transmitters (Blaak *et al*., 1998). These results suggest that a combination of susceptibility of target cells and inoculum size upon homosexual exposures largely determine whether HIV infection is established.

In a longitudinal cohort study carried out on 160 patients, 70 percent of the men whose viral load was greater than 30,000 c/ml died within six years of the test, the average survival tome being 4.4 years. In contrast, less than 1 percent of patients whose viral load was below 500 copies /ml died

in six years, and the average survival time was more than 10 years, indicating that HIV prognosis varying with viral load (Mellors, 1998).

1.2.7.6. Applications of viral load measurement

Viral load measures have several applications in medical practice. Viral load measures, for example, have replicated assessment of clinical outcome in therapeutic trials and drug treatment guidelines aimed to maintain viral load below 50copies/ml. Failure to reach that level is associated with breakthrough of drug-resistant virus and loss of control over HIV replication (drug resistance). Even some authorities have come to consensus that those with undetectable plasma HIV RNA (<40 copies/ml) are sexually non-infectious (Wilson et al., 2008), although with many critisms.

At all stages of disease, plasma RNA viral load remains the most potent predictor of outcome in HIV infected individuals. Cross-sectional stratifications of RNA loads provide a highly significant indicator of the likelihood of progression to AIDS and mortality. Even shortly after seroconversion the virologic set point is significantly associated with prognosis, suggesting that in most individuals the determination of progression are present early in the course of infection (Graham *et al.,* 1998). Measurement of viral load in infected individuals is also important because HIV is directly cytopathic and cell destruction may underlie the development of disease in infected individuals (Finzi and Siliciano, 1998).

1.2.8 Immunology of HIV

As is generally the case in human viral infections, individuals infected with HIV develop both humoral and cellular immune response (Pope and Haase, 2003). Similar to other viral diseases, HIV disease can be best explained as a dynamic and long-lasting struggle between the virus and the immune system of the host. Particularly, HIV-specific humoral and cellular immune responses localized at the mucosal surfaces play an important role, as HIV infections occur predominantly through sexual contact at mucosal surfaces.

1. 2.8.1 Humoral immune responses to HIV

Antibodies to HIV proteins appear shortly after infection, usually within1-4 weeks, although cases of seroconversion have been described up to six months after infection (Horsbugh, 1989). Genital tract immunity is mediated by monomeric IgG and IgA and secretory IgA (sIgA) (Parr, M and Parr, E, 1996). Studies of anti-HIV IgG and IgA in cervicovaginal secretions have demonstrated predominance of IgG, with 63-100% of HIV-infected woman having measurable IgG levels and

8-94% having measurable IgA levels in cervicovaginal lavage (CVL) (Haimovici et al., 1997). Studies that have compared the ratio of anti-HIV antibody to total antibody (specific activity) in serum have demonstrated the presence of locally produced anti-HIV IgG and IgA in the genital tract (Belec *et al.,* 1995). Thus, semen and CVL fluids contain more IgG than IgA. HIV-neutralizing imunoglobulins of both classes and of IgA in particular have been associated with HIV non-acquisition (Hirbod *et al.,* 2008).

Crucial for antibody-mediated reduction of HIV viral particles is the amount of neutralizing antibodies rather than the amount of total specific antibodies. Neutralizing activity can be detected shortly after the appearance of the first specific antibodies (Poignard *et al.,* 1996). However, sera obtained from HIV infected patients are not capable to reduce significantly viral infectivity in vitro (Kostrikis *et al.,* 1996); the neutralizing titer is initially weak and tends to have a relatively narrow window of specificity. Additionally, no correlation between the amount of maternal neutralizing HIV specific antibodies in infected pregnant woman and the rate of vertical HIV transmission could be observed (Hengel *et al.,* 1996).

The passive transfer of potent HIV-neutralizing antibodies protect against mucosal viral challenge in macaques (Finizi and Siliciano, 1998). And passive transfer to HIV-infected individuals delayed viral rebound in some patients interrupting antiviral therapy (Hirbod et al., 2008). Besides, high levels of neutralizing antibodies have been described in long-term nonprogressive infection (Adalid-peralta *et al.,* 2006).

Despite all these antiviral mechanisms both humoral and cellular immune systems have no ability to prevent HIV infection. The antibody response does not appear to contribute significantly to the early control of HIV replication: ultimately the course of HIV infection is almost universally fatal. In general, HIV specific hmoral immune response appears to play a minor role in controlling HIV infection. More than its preventive roles its diagnostic and prognostic roles is higher. Besides, occasions that it contributes to the pathogenesis of AIDS are also higher than its protective roles.

1 .2 8.2 Cell-mediated immune response to HIV

It is a well established fact that HIV depletes CD4 cells starting early from acute infection. HIV infection results in a selective depletion of the CD4 T cells population, and in particular HIV-specific CD4 T-cells (Douek, 2002). Cellular immune responses are critical part of the host's defense against viral infections.

1. 2.8.2.1. CD4 cells

Normal CD4 count in adult ranges from 500 to 1200 cells per cubic milliliters (mm^3) of volume (Kalams *et al*., 1998). As the virus reproduces it destroys CD4 cells and reduces the count. The maintenance of general systemic immunological integrity is dependant on a balanced population of functionally competent CD4 cells. In a 'steady state' of normal health, there is a dynamic process of response, selection, expansion, loss and renewal. In HIV-infection, however, the sinario is different.

Huge numbers of CD4+ T cells are destroyed daily and since in most individuals the decline in CD4+ T cells number over time is gradual, an almost equivalent number must be generated to reconstitute the peripheral pool (Douek, 2002). During HIV infection, the mean HIV-associated CD4+ T cell loss in the peripheral blood of seropositive patients amounts to about $35x10^6$ T cells per day (Ho *et al*., 1995). Since CD3+ T cells counts remain stable for a long period after seroconversion (Finizi, 1999), it must be assumed that an equal number of T cells will be released daily by the thymus to restore the peripheral CD3+ T cell pool. However, since both CD4 and CD8+T cells are produced, while only and mostly CD4+ T cells are lost, it follows that a net loss of CD4+ T cells will result.

CD4+ or helper T cells can be viewed as the orchestrators of the adaptive immune responses. The CD4+ T cells recognize foreign antigens bound to host proteins and aids B cells (e.g., through production of various cytokines) in the production of antibodies (Delves and Roitt, 2000). CD4+ T cells also aid in the stimulation and recruitment of another subset of T cells (e.g., through production of cytokines) (Von Andrian and Mackay, 2000). Since the CD4+ T cells plays a pivotal role in the induction of most immunological responses, HIV-induced damage to the CD4 T cell population results in the destruction of a wide range of immune functions, ultimately leading to extreme immunosuppression and opportunistic diseases (Ho *et al.,* 1995). Measurement of peripheral blood CD4 T cell lymphocytes is probably the most important laboratory assay for evaluation and monitoring of patients with HIV. The CD4 count is critical for determining the clinical stage of HIV infection, for deciding when to start ARV therapy, for evaluating the efficiency of treatment, and for changing the medication when necessary. Most HIV treatment decisions are therefore based upon the CD4 count (Mellors *et al.,* 1997; Fehey *et al*., 1998).

Both CD4 and CD8 T cells play a very important role in immunity to viral infections, although CD8 T cells especially the CTLs are the major contributors to the antiviral T cell immune

response (Delves and Roitt, 2000). As a result, a ratio of CD4 to CD8 is examined frequently during HIV infection.

The human CD4:CD8 ratio appears to be under the control of a major gene, i.e. some individuals are genetically predisposed to a high CD4:CD8 ratio, while others will be predisposed to a low ratio-a ratio of circulating CD4:CD8 T cells lower than 1 is found in about 5% of healthy subjects (Amadori *et al.,* 1995). The evidence suggests that seropositive individuals predisposed to a high CD4:CD8 ratio would be more efficient in replenishing CD4+ T cells losses than those predisposed to a low CD4:CD8 ratio: the latter group would arguably suffer a more profound and persistent CD4+ T cell lymphopenia and CD8+ T cell lymphocytosis (Hellerstein, 1999). Thus, it would be more likely that seropositive individual with a genetically high CD4:CD8 ratio progress more slowly to full-blown AIDS compared with low-ratio seropositve subjects.

Despite decades of intensive research, the mechanisms underlying CD4+ T cell depletion remain widely debated. It was initially proposed that CD4+ T cells disappear by viral infection and subsequent cytolytic effects, and/or by the removal of infected CD4+ T cells by the immune response (Ho *et al.,* 1995). Later, "immune activation" hypothesis has gained popularity (Grossman *et al.,* 2002). HIV infection in humans, and Simian immunodeficiency virus (SIV) infection in rhesus macaques, is characterized by increased rates of cell division in CD4+ and CD8+ T cells, natural killer cell; and B cells (De Boer *et al.*, 2003), and up-regulation of various activation markers. Thus, the virus (HIV) has evolved strategies to increase the availability of suitable target cell and this would be like "fueling the fire," resulting in more infection and runaway depletion of CD4+ T cells. Thus, it is widely accepted that chronic, infection-induced immune activation is the force driving the progressive decline in CD4 cell number and other detrimental effects that result in AIDS (Sousa *et al*., 2002). Others also proposed increased anergy or apoptosis, or loss of mature MHC-restricted memory Th cells, down-regulation of MHC receptors, dysfunction of thymus and other lymphoid organs (reviewed by Heeney, 1995).

Several studies have been carried out to characterize the immunologic status of Ethiopians following the observation of CD4 T-lymphocytopenia without opportunistic infection in HIV seronegative Ethiopian immigrants to Israel and a case report of idiopathic CD4+ T lymphocytopenia in an HIV-negative Ethiopian with AIDS-like symptoms, in Italy (Montella *et al.,* 1994). A similar comparative study carried out in Israelis and Swedish demonstrated a perturbed immune system in Ethiopians (Work *et al.,* 1997; Kalinkovich *et al*., 1998). Overall these findings indicated low CD4+ and high CD8+, low Naive CD4+ T cell counts and increased

expression of activation markers such as HLA-DR and CD38. All these showed a persistently activated state of the immune system of Ethiopians.

To assess possible differences in immune status, proportions and absolute numbers of subsets of CD4+ and CD8+T cells were also compared between HIV-negative healthy Ethiopians and HIV-negative Dutch (Messele *et al*., 1999). The result showed that both proportions and absolute numbers of CD4+ and CD8+ T cells expressing CD28 were significantly reduced in Ethiopians versus Dutch. Also, both proportions and absolute numbers of the effector CD8+ T cell population as well as the CD4+CD45-CD27- and CD8+CD45RA-CD27- T cell populations were increased in Ethiopians. In addition, the possible association between the described subsets and HIV status showed a gradual increase of activated CD4+ and CD8+ T cells, a decrease of CD8+ T cells. Furthermore, a decrease of naive CD8+ T cells and an increase of memory CD8+ T cells in AIDS patients were observed. Later on, a larger study carried out on factory workers confirmed that Ethiopians have significantly decreased CD4+ T cell counts and highly activated immune status (Kassu *et al.,* 2001). Another study also confirmed that most Ethiopians develop multiple characteristics of chronic immune activation characterized by low naive T cell numbers at early age (Tsegaye *et al*., 2003).

1. 2.8.2.2. Cytotoxic T lymphocytes (CTLs)

Lymphocytes bearing the CD8 cell surface glycoprotein (CD8 T cells) are classified into suppressor and cytotoxic T lymphocytes, There is no difference between the CD8 marker in suppressor and cytotoxic regarding molecular structure (reviewed by Douek *et al.,* 2003).

CTLs are major contributors to the antiviral T cell immune response. CTLs recognize peptide antigens presented by class I major histocompatiblity (MHC) molecules of immune system. CTLs recognize target cells carrying non-self antigens via the T cell receptor (TCR), which interacts with non-self antigen-derived peptides accommodated into MHC binding sites on the target cell. In the absence of foreign antigens, MHC molecules are occupied by self-peptides that generally are not polymorphic and therefore can be recognized by allospecific CTL (reviewed by Sierra *et al.,* 2005). When these cells make contact with antigens through their specific T cell receptors, provided that this is accompanied by certain important co-signals the T-cell is activated to divide, differentiate, and mediate lysis of infected cells.

The number of CD8+ cells in a blood is about 1000 cells/ul and the total number is approximately 10^{11}, which is 2.5 fold less than that of the CD4 T cells (Clark *et al.,* 2000). In the normal situation the total production of CD8+ T cells seem to be lower than CD4+ T cell production.

There is subsequent change in the proportions of T cell subsets during HIV-infection. Overall, CD4+ T cells decline in number while the CD8+ T cell population increases overtime (Brander and Riviere, 2002). The increase in the CD8+ T cell pool is the result of massive peripheral expansion of memory cells. This subset only begins to decline shortly preceding AIDS diagnosis (Ho *et al.,* 1995). The CD4+ memory compartment also initially expands due to peripheral expansion, but memory cells are then progressively lost. Interestingly, in both CD4+ and the CD8+ subsets, the naive compartment begins to decline soon after infection (Clark *et al*., 2000). Thus, the T cell depletion observed in HIV infection consists of naive cells of both CD4+ and CD8+ subsets, and memory cells of the CD4+ subsets.

In HIV-infected persons, the distribution of CD4+ and CD8+ lymphocytes differ in blood and lymphoid tissue. In a group of HIV positive patients in early stage of disease, the total body numbers of CD8+ T cells in the peripheral blood increase approximately 2 fold, and in the lymphoid tissue approximately 3-fold (Brander and Riviere, 2002). As a consequence, the percentage of CD8+ T cells residing in the blood increases from 5.6% to approximately 11% (Clark *et al.,* 2000), and there seems during HIV infection, an increased trapping of CD8+ T cells in the lymphoid tissue.

CD8+ MHC class1-resrticted cytotoxic T lymphocyte responses are essential in controlling most non-lytic virus infection (Clark and Deboer, 2000). On recognition of viral antigens, CD8+ T cells are activated to kill virus-infected cells by excretion of perforin and grenzymes or by Fas receptor triggering or they inhibit viral replication by releasing cytokines, most notably interferon-gamma (reviewed by Douek *et al*., 2003).

CTLs are major contributors to the antiviral T cell immune response. Infection by the human immunodeficiency virus leads to the development of CTL responses against a variety of viral antigens, particularly to epitopes within the Gag, RT, Nef and Env proteins (Levy *et al.,* 1996). Some infected individuals have extremely high levels of *in vivo* activated HIV-specific CTL (Appay *et al.,* 2002). The magnitude of the response in some HIV-infected person was unprecedented, in that antiviral CTLs are observed even without the need for *in vitro* stimulation and expansion in PBMC (Mollet *et al*., 2000).

CTL may contribute to protection or the delay of disease progression. A correlation has been shown between the decline in ability of CD8+T cells to inhibit HIV–replication and the progression of disease (Brander and Riviere, 2002). A very good correlation between long-term survival and CTL activity has been reported in pediatric HIV-infected patients (Clark *et al.,* 2000). Thus, HIV-specific CTLs contribute to controlling viral replication and delaying the onset of disease.

The response by CTL is the most relevant to antiviral activity. A striking feature of HIV infection is that the HIV-specific CTLs of an infected person are derived toward multiple epitopes (Rowland-Jones *et al.,* 1997). This is different from most virus infections previously studied, in which a dominant CTL response has been identified for a given restriction element and most people (or mice) with that MHC type respond through that allele to a single epitope.

HIV-specific CTLs have been observed as early as a few days following the onset of acute infection and in general before (neutralizing) antibody responses could be detected (Borrow *et al.,* 1994). CTL responses against HIV can be generated very rapidly and may in deed be crucial in limiting the initial hit on the immune system, especially by preventing on-going infection of HIV-specific CD4 T cells (Brander and Riviere, 2002).

The appearance of HIV-specific CTLs usually parallels a striking diminution of the viremia in infected patients. Thus, virus CTL activity is associated with control of viremia in primary HIV infection (Borrow *et al.,* 1994). Interestingly, some individuals were observed who remaining HIV-seronegative despite frequent exposure to the virus, and seem to harbor HIV-specific CTL responses (Brander and Riviere, 2002). These CTL responses may be coincidental, demonstrating that some degree of viral replication has been repelled because of efficient HIV-specific CTL response.

After the initial phase, the CTL response stabilizes at a lower level that is related to the viral load. High CTL levels may correlate with low virus load and vice versa (Epstein, 1993). In the asymptomatic mid-phase of HIV infection as many as 1% of peripheral blood cells can be effecter CTLs (Brander and Riviere, 2002), whereas estimates of memory CTL range from 1 in 10^3 to 1 in 10^4. This discrepancy between effector and memory CTLs numbers is consistent with some degree of terminal effector CTLs, possibly as a result of over stimulation, which could leave the CTLs vulnerable to depletion from clonal expansion.

The high levels of CTL are unusual feature of HIV infection (Douek *et al.,* 2003). For patients with strong CTL activity the majority of infected CD4 T cells could be killed by CTL rather than

the virus. If most infected cells are lysed in the window between expression of viral proteins and production of virus, the amount of virus produced will be limited. Low antigen loads might stimulate strong CTL response if helper T cell function is good and that the same levels of CTLs might require far more antigen when helper T cell functions are impaired (Rowland-Jones *et al.,* 1997).

Generally, the anti-HIV CTL response is considered to be stable throughout the asymptomatic period. However a stable total CTL response may conceal an unstable pattern of shifting immunodominant responses in response to variation in dominant virus mutant (Brander and Riviere, 2002). The implication is that the stability might be an illusion, at least in some patients, it is also likely, and that good control of HIV in this phase might be achieved at the cost of a gradual decline in CD4 T cells.

CD8+ T cells can also control HIV infection without killing the infected cell (Walker et al., 1986). This noncytotoxic cellular immune response involves suppression by CD8+ cells of HIV replication in CD4+ T cells and macrophages. The antiviral activity is not MHC restricted and is mediated, at least in part, by a soluble factor.

The functional competence of HIV-specific CD8+ T cells could eventually fail during the late stages of infection, studies have shown that this occur when impaired cytokine production (Kostense, 2002) and reduced CD3 expression (Trimble and Lieberman, 1998) by these cells takes place. However, this coincides with the general collapse of the immune system during the development of AIDS. Thus, overall, HIV-specific CD8+ T cells appear in a functional state throughout most of the course of infection.

HIV has developed numerous strategies to evade host immunity (Reviewed by Appay and Rowland-Jones, 2002), which clearly diminish the ability of the host to fight the virus. The combination of CD4 T cell depletion and immune escape could be particularly significant, a situation in which the action threshold for HIV specific CTLs is effectively raised, so that an optimal CTL response is not triggered by low levels of viral replication around the viral set point. Thus, even if their function is not directly impaired, HIV-specific CTLs might be unable to mount an effective response to eradicate the virus.

Virus-specific helper T cell may be crucial to the function of antigen specific cytotoxic T lymphocyte in virus infection. In the case of HIV infection they may contribute critically to suppression of virus replication in the infected individual (Brander and Riviere, 2002). Generation of CD8+ response can either depend upon help from CD4+ T cells or can be helper-

independent. In some systems, CD8+ T cells can make an initial, limited response in the absence of CD4+ T cell help, but the response then declines rapidly unless T cell help is available (Deeths *et al.,* 1999).

1. 2. 8. 2. 3. Naive, memory and effector T cells

According to the expression of cell surface markers, both CD4+ and CD8+ T cell populations, exhibit different functional characteristics and homing capacities. These surface markers include immune activation markers such as HLADR and CD38 (Kesten *et al.,* 1992; Kestens *et al.,* 1994), adhesion and homing markers such as CD62L (also Known as L-selectin and CC-chemokine receptor 7(CCR7) (Tripp *et al.,* 1995; Sallusto *et al.,* 1999), costimulatory molecules such as CD27, CD28, CD45RA/RO (Hintezen, 1993: Hamann, 1997), and Ki67 which is a recent proliferation marker (Clark *et al.,,* 2000).

CD28 and CD27 are costimulatory molecules, which provide signals needed for the correct activation of specific T cell after T cell receptor ligation. After the interaction of CD27 and CD28 with their ligands, T cells will expand and become “effector” cells with the ability to respond directly to pathogens (Hamann, 1997). Because these ‘effector’ T cells do not need another signal from these molecules (they are already functionally fully matured), these molecules are down regulated. Down regulation of CD27 is irreversible (Hintzen, 1993) and relates to the differentiation status of antigen specific T cells.

CCR7 is a homing molecule, therefore, CCR7 loss is related to altered migratory capacities (Sallusto, 1999) and CCR7 expression might, therefore, provide insight into homing potential, which is probably related but not identical to the cellular differentiation status. In addition, as expected from its function, CCR7 can be re-induced on CCR7- cells upon stimulation (Champagne, 2001); to enable migration of the antigen activated cells to lymph nodes. Reactivation, therefore, results in T cells with a central memory CCR7+ phenotype but with functional characteristics of full-blown effector cells with the corresponding CD27-phenotype. CCR7 and CD62L are homing molecules. CCR7 and CD62L are essential for lymphocytes to traverse high endothelial venules and therefore to enter lymph nodes (Lefrancois and Marzo, 2006).

Upon stimulation, T cells switch from expression of CD45RA to its smaller isoform CD45RO (De Rosa, 2001). These observations form the basis of the widely held view that CD45RA+ cells are immunologically ’Naive’, and CD45RO+ cells are “memory cells”. The naive/memory hypothesis postulates that naive T cells respond to specific antigen by switching expression of CD45RA to

RO isoforms. The lower activation threshold of CD45RO+ T cells constitutes an obligatory feature of immunological memory.

HLA-DR and CD38 are surface phenotypes which increase with increased state of immune activation (Kestens, 1992), and therefore, their status indicates whether there is immune activation or not. Human proliferating cells express the Ki67 nuclear antigen during the late G1 (growth-1), S (synthesis), G2 (growth-2) and M (mitotic) phase of cell cycle (Clark, 2000) and the number can be quantified with Ki67 mAb by flow cytometer.

In a healthy individual over the course of a lifetime sufficient CD4+ and CD8+ T cell numbers are maintained to ensure immune competence. However, the proportions of the various pools change dramatically and differentially with age: naive T cells decrease with respect to CD8+ T cells (reviewed by Douek *et al*., 2003).

Thus, T cell differentiation or post-thymic development involves sequential down regulation or up regulations of cell surface molecules. Of interest is the apparent similarity between both CD4+ and CD8+T cell subsets in their patterns of differentiation. Late differentiated CD8+ T cells show increased cytotoxic potential, although CD4+ T cells at this stage acquire cytotoxicity for the first time (Appay, 2002). Chronic activation probably has an important role in further driving T cell differentiation, although other factors, many still unclear, could be involved. As T cells further differentiate, they tend to lose some proliferative capacity, so that late differentiated cells are CD28-CD27-. Accordingly the T cell differentiation pathway might be related to a process of T cell senescence (Appay and Rowland-Jones, 2002).

Naive, effector and memory T cells are not immune cells belonging to different lineages. Rather they are different maturational stages (Clark *et al*., 2000). Memory cells differ from naive cells in that they have been activated (typically by antigen) at some time after export from the thymus. Effector T cells are cells which are stimulated by antigen and are able to proliferate and carry out immune functions. Naive, effector and memory cells occur in both CD4+ and CD8+ subsets in adults (Hamann, 1997).

Naive T cells are long-lived resting cells that reside in the recirculating lymphocyte pool and migrate continuously from blood to lymph through specialized T cell zones in the secondary lymphoid tissues, the spleen, lymph nodes, and Peyer's patches (Picker and Siegelman, 1999). The survival of naive T cells requires continuous contact with self peptides bound to MHC molecules combined with exposure to a cytotokine interleukin 7 (IL-7). Together, the two ligands

are presumed to induce a form of low-level signaling that is sufficient to keep the cells alive but does not induce them to enter the cell cycle.

Naive T cells express CCR7, CD62L (Lefrancois and Marzo, 2006), CD45RA, CD28, CD27 cell surface markers in both CD4+ and CD8+ cells (Appay and Rowland-Jones, 2002). Primary T cell responses are initiated in secondary lymphoid organs by mature antigen-presenting cells, i.e., dendritic cells (DCs) (Clark and Deboer, 2002). Recognition of immunologic peptides bound to cell surface MHC molecules on DCs in the T cell zones causes selective sequestration of antigen-specific recirculating T cell entering lymphoid tissues from the blood (Picker and Siegelman, 1999); the trapped cells are then induced to proliferate to CTL.

In healthy blood donors, about 30% of CD4 T lymphocytes are of naive phenotype, expressing CD45RA, and 50% of memory type, expressing CD45RO. In the CD8+ T lymphocytes population approximately half the cells express CD45RA and 25% express CD45RO. When CD45RA+ cells are activated, there is transition to CD45RO during which time both markers are expressed (Hamann, 1997). A cycle of activation and quiescence maintain the size of the circulating T cell pool and keeps the ratio of CD45RA+:CD45RO+ T cells at approximately 1:1 throughout adult life (Helber *et al.,* 1993). That is, there is normally a kinetic equilibrium between CD45RA+ and CD45RO+ T cells, driven by antigen presentation.

HIV infection is characterized by a high turnover of naive and memory lymphocytes and by a selective depletion of memory CD4+CD45RA- T lymphocytes in the early asymptomatic stage (Helbert *et al*., 1993). Functional and phonotypical evidence shows that memory T cells functions are specifically lost. Studies have established that the CD45RA+ and CD45RO+ CD4+ T cells are lost at different stages of HIV infection. Around the same time of the onset of symptoms, when total CD4+ T cell counts fall below 400/mm^3 and HIV antigenaemia occurs, CD45RA+CD4+ T cells are also lost (De-paoli et al., 1988). Thus, in the advanced disease stage, the naive T lymphocytes are lost. The decrease in naive CD8+CD45RA+CD45RO- T cells parallels the decline in CD4+CD45RA+CD45RO- T cells (Helbert *et al.,* 1993). Thus, both CD4+ and CD8+ naive T cells are gradually depleted during HIV infection, and increased age is an important risk factor for HIV disease progression.

Most T cells are direct descendants of naive T cells that have encountered antigen in the context of appropriate co- stimulatory signals (Clark and Deboer, 2000). Memory T cells in both CD4+ and CD8+ express CD45RO+CD28-CD27-CCR7- cell surface markers (Baarle et al., 2002).

Increasing cell division rates of memory T cells by immune activation could increase their capacity of self-renewal, and lead to increased cell count. Estimates of the lifespan of memory T cells vary considerably, but are typically estimated to have a time scale of months (Borghans and DeBoer, 2007). Depletion by immune activation should therefore be completed in a few months. CD4+ and CD8+ T cell subsets are classified into central and effector memory T cell (Lefrancois and Marzo, 2006). The broad categorization of central memory (T_{CM}) and effector memory T cell (T_{EM}), although imprecise in some aspects, nevertheless provides a useful framework for theoretical and experimental designs. T_{CM} cells generally express both CCR7 and CD62L receptors, whereas T_{EM} cells express neither (Sallusto *et al.,* 1999). Low-level expression of CD27 has also emerged as an indicator of constitutive effector function in human and mouse memory CD8+ T cells (Hamann, 1997).

Central memory CD4+ T cells express CD45RO+CCR7+ and effector memory CD4+ T cells also express CD45RO+CCR7+. Phenotyping of CD8+ T cells showed a similar pattern, with an additional CD45RA+CCR7- subset that was considered to be a differentiated effector population, consistent with their lacking CD27(Seder and Ahmed, 2003). This is in agreement or compatible with a linear pathway of antigen-dependent T cell development from naive T cells to central memory T cells (both expressing CCR7) to effector memory T cell, to effector cells having a reversion to the CD45RA isoform lacking CCR7.

Although HIV-specific CD8+ T cells do not express CD28, the majority of HIV-specific T cells appear to be of the CD27+ memory phenotype (Hamann, 2002). Moreover, we do not observe an enrichment of HIV-specific T cells in the highly differentiated CD27-subset during the course of HIV infection. A low CD27- : CD27+ ratio is observed for HIV-specific T cells suggesting that impaired differentiation of virus-specific T cells is associated with disease progression (Hintzen, 1993). Individuals co-infected with HIV and EBV persistently had low numbers of HIV-specificCD27- T cells, despite persistent active viral replication (>100,000 viral RNA copies/ml), which one would expect to drive HIV-specific T cells to the CD27-CD8+ phenotype (Appa and Rowland-Jones, 2002).

In the majority of HIV-infected individuals, most HIV specific T cells are of the less differentiated CD27+ phenotype, in HIV-infected long- term asymptomatic, who have been able to control virus replication for a longer period, a relatively high proportion(>20%) of HIV-specific T cells are CD45RO+CD27- cells (Hintzen, 1993). In addition, HIV-specific T cells in these individuals respond better to antigenic stimuli than HIV-specific T cells in individuals that

progressed to AIDS. Besides, CD27- T cells contain more granzyme B and perforin and exert stronger direct cytolytic activity compared to CD27+cells (Baarle *et al.,* 2002). These data suggests that HIV-specific CD8+ T cells that have differentiated to the CD27- stage are associated with delayed disease progression.

In Ethiopia, a study carried out by Messele et al in 1999, comparing immune status between HIV-Ve Ethiopians and Dutch, showing both proportions and absolute numbers of the effector CD8+ T cell population as well the CD4+CD45RA- and CD8+CD45RA-CD27- T cell populations , were increased. The same study showed a decrease of naive CD8+ T cells and an increase of memory CD8+ T cells in AIDS patients (Messele *et al*., 1999). These results suggest a generally activated immune system among HIV- Ethiopians.

A similar study by Kassu *et al.,* 2001, on 562 sugar factory workers, showed a significantly higher CD8+ T cell counts, resulting on a proportional increase on each of the CD8+ T cell compartments. These included naive (CD45RA+CD27+), memory (CD45RA-CD27-), activated (HLA-DR+CD38+) CD8+ subpopulations (Kassu *et al.,* 2001). But no study was carried out on discordant couples in Ethiopia

1.2.9. Human leukocyte antigen (HLA) and resistance and susceptibility to HIV

As we mentioned earlier, CD8+ CTLs have been shown to protect against a variety of viral infections in animals and humans. Therefore, it is possible that such a response will afford protection against retroviral infections as well. Several lines of experimental evidence support this supposition. First, a strong CTL response has been implicated in suppressing HIV infection in humans (Rowland-Jones *et al.,* 1995) and SIV infection in macaques (Gallimore et al., 1995). Second, there is an inverse correlation between viral load and virus specific CTL precursor frequencies in HIV-infected patient (Klein *et al.,* 1995). Third, a selective reduction of HIV-specific CTL precursors occurs in the advanced stage of HIV-infection (Kersten *et al.,* 1993). Finally, there is also a correlation between the level of plasma viremia and the number of effector CTLs (Brander and Riviere, 2002).

If CTLs are important in controlling HIV infection, HLA class I type should play a major role in determining disease progression. HLA class I molecules have a direct and special connection to viruses: they play a central role in the task of alerting CTLs to cells that have been breached by virus and are an unambiguous antiviral adaptation (Reviewed by Worobey *et al.,* 2007).

Cell mediated immune responses require that the T cell be presented with foreign antigen bound to host proteins. These host proteins are known as the major histocompatibility complexes

(MHCs), or human leukocyte antigens (HLAs). They are expressed on the surfaces of a large variety of cells and translated from a region of highly polymorphic genes (Klein and Sato, 2000). The HLA is extremely gene-dense region of chromosome 6, housing approximately 130 expressed genes, almost half of which have immune system function (Reviewed by Worobey *et al*., 2007). These included the diverse and rapidly evolving class I and class II genes, as well as many other genes with immune function. These genes are necessary for the production of T-dependent antibody. T cells do not recognize free or soluble antigens, but rather peptides of antigen that are bound to HLA.

HLA class I consists of Transmembrane heavy chain, which is complexed to soluble-β_2-microglobulin. All nucleated cells except red blood cells express HLA class I (Sierra *et al.,* 2005). They bind and transport intracellular cytosolic viral peptides of about 9 to 10 amino acids in length. The T cells that recognize foreign peptides complexed to class I HLA activate cytotoxic CD8+ T cells, which specifically lyse virus-infected cells (Finzi and Siliciano, 1998). Because, potentially, any nucleated cell could be infected with a virus, all cells in the body, except red blood cells, express class I HLA and are thereby scrutinized by T cells for evidence of a foreign peptide. HLA class I molecules are mainly involved in a fight against viruses.

Class II HLA types are found only on antigen-presenting cells, such as macrophages, dendritic cells, or lymphocytes. Although class II molecules are expressed on these cells, they can be induced on a variety of other cell types (Reviewed by Tortorella *et al*., 2000). CD4+T cells recognize peptides of about 11 to 12 amino acids in length bound to HLA class II molecules, which is composed of an α and β transmembrane chain. CD4+ T cells recognize antigen which are endocytosed and processed by antigen presenting cells and expressed on the surface of antigen presenting cells. Therefore, as a general rule, CD8+ T cells respond to foreign antigens synthesized within a cell (requiring infection of that cell), while CD4+ T cells respond to antigens encountered outside of the cell (Klein and Sato, 2000).

There is a large degree of specificity between the binding of MHC and viral peptides. A given HLA variant will only bind one or few short peptide fragments (Frank, 2000). This puts a strong selective pressure on humans to evolve and maintain a diverse array of MHC molecules. Accordingly, HLA are by far the most polymorphic of any in our genome. There are three major polymorphic genes for class I (A, B, and C) (Janeway *et al*., 2004). Because these genes are codominant, a cell will commonly express six different HLA class molecules (three from each parent). Heterozygosity at HLA loci can lead to powerfully diverse immune response (Carrington

et al., 1999). The class II polymorphic genes are DR, DP, and DQ, all composed of α and β chains. Many individuals also have a gene for an extra DRβ chain, either of which can combine with the DRα chain (Janeway *et al.,* 2004). Thus, class II HLA heterozygous individuals can express eight different polymorphic alleles (four from each parent).

Allelic variation among these genes of both HLA classes is usually very high. HLA-B, leads the way with 851 alleles, followed by HLA-A, with 506, and HLA-DRB1, with 476 (Reviewed by Worobey *et al.,* 2007). The reason an individual needs so many possible HLA proteins is to be able to generate a diverse array of grooves in the HLA molecules, such that there would be a groove to fit at least some antigens from each potential pathogen (Janeway *et al.,* 2004). This is especially significant for CD8+ T cells than others. The extreme polymorphism in HLA class I region of the human genome is suggested to provide an advantage in pathogen defense mediated by CD8+ cells (Parham, 1996).

Although this strategy works most of the time, there are some HLA types that have been linked to an increased frequency of resistance, or increased frequency of certain immunological diseases. HLA class I molecules present pathogen-derived peptides on the surface of infected cells for recognition by CD8+ T cells. The relative contributions of HLA-A, B and C against HIV, immune control of which is dependent upon virus-specific CD8+ T-cells activity is not the same (Kleplela *et al*., 2004).

It is known that HLA-B is the most polymorphic allele among all HLA alleles. HLA-B also plays a dominant role in influencing HIV disease outcome. In a study carried out in south Africa on 375 HIV-infected subjects a significantly greater number of CD8+ T cells responses were HLA-B restricted (2.5 fold) when compared to HLA-A (Kleplela *et al.,* 2004). The same study demonstrated that variation in viral set point, in absolute CD4 count and, in rate of disease progression is strongly associated with particular HLA-B but not HLA-A allele expression. Thus, substantially greater selection pressure is imposed on HIV-1 by HLA-B alleles than by HLA-A.

Another study carried out later to determine protein specification of HLA alleles showed that, the HLA-B-restricted Gag-specific responses were most strongly associated with the effective control of viremia, and the HLA-B-restricted Env-specific responses with a lack of control (Kiepiela *et al*., 2007). The observation that Gag-specific CD8+ T cell responses are associated with effective control of viremia, whereas Env-specific responses are associated with ineffective control show that greater allele-specific HIV amino acid sequence variation should be observed in Gag compared to Env, despite the fact that Gag is more conserved than Env and therefore less likely to

accommodate sequence change without significant fitness cost (Martinez-picado, 2006). In contrast, HLA-C-restricted Gag specific responses were most strongly associated with viremia, even when within Gag (Kiepiela *et al*., 2007). These data indicate that within-protein differences exist between CD8+ T cells specificities that are related to the HLA-A, HLA-B or HLA-C restriction of the responses. In addition, the study also suggests a dominant role for HLA-B alleles in successful or unsuccessful immune containment of HIV infection.

In HIV, both survival and transmission risk depends substantially on viral load, and therefore on the HLA-B expressed (Gercia, 1999). Strong evidence for this comes from the studies showing the association between B*57 and B*5801 with low viremia, and between B*18 and B*5802 with high viremia. These conclusions are supported by previous studies in B-clade infected Caucasians which show that HLA-B alles are most closely associated with non-progression/low viral load (Kaslow, 1996) or, progression/high viral load (Moore, 2002). The dominant effect of HLA-B-restricted CTL responses on HIV is also supported by work that shows HLA class I homozygous disadvantage in HIV infection (Carrington, 1999). The study showed that the relative hazard of progression to AIDS or death was two-fold-to-three fold higher for homozygosity at the HLA-B versus HLA-A. And in addition, studies of rare supratype advantage demonstrated an HLA-B effect only (Trachtenberg, 2003).

Although the remarkable diversity of the HLA system has provided an evolutionary advantage to human populations against many ancestral and contemporary pathogens, the combination of extreme mutability, rapid replication, recombination and plasticity enables HIV to adopt in hosts with diverse HLA genotypes (John and Mallal, 2005). The great disparity of evolutionary time scale underpins HIV's success in combating HLA diversity: human evolution plays over many thousands of years, while HIV evolution can proceed over days to weeks, observable within the life of a single host and during transmission between hosts. However, an individual's HLA genotype can have far-reaching effects on the outcome of a variety of viral infection and predictive, for example, of whether HIV is likely to kill you quickly or slowly, should you become infected.

Several studies have shown a direct association between class I HLA types and rates of HIV disease progression. For example, heterozygocity for HLA-A, B, and C is known in delaying onset to AIDS (Carrington *et al*., 1999). A study in South Africa also showed that HIV infected infants had a much higher frequency of deleterious HLA-B alleles (B*57 and B*5801) and a

much lower frequency of protective alleles (B*57 and B*5802) than the population at large (Klepela *et al.,* 2004).

Studies with HIV infected long term non-progressors (LTNP) showed increased frequency of specific HLA class I (HLA-A1, HLA-A2, HLA-B14, HLA-B17) and Class II (HLA-DR5, HLA-DR6) alleles (Margerowska *et al.,* 1999). In contrast, the presence of HLA-B35, HLA-DR1, HLA-DR3, and HLA-DQ1 were more frequent in rapid progressing patients (Margerowska *et al*., 1999 and MacDonald *et al.,* 2000).

HLA-B35 has been shown in five studies to be associated with rapid progression to AIDS (Reviewed by Tortorella *et al.,* 2007). It has been shown to present epitopes that are conserved between HIV-1 or HIV-2 and the different HIV-1 subtypes (Reviewed by Rowland-Jones *et al.,* 1995). The HLA haplotype HLA A1,-B8 and HLA class II -DR3 has also been found to be associated with rapid progression (MacDonald *et al*., 2000). Other HLA types associated with rapid progression were B37, B49, and certain combinations of TAP alleles (McMichael *et al.,* 1994).

Highest ranked of HLA class I types associated with protection were HLA-B27 and HLA-B57 both of which tend to select conserved epitopes (McMichael *et al.,* 1994). The TAP2.3 allele associated with HLA-A25,-26, and -32, and B18 also appeared to offer protection (Rowland-Jones *et al.,* 1995). In a cohort of prostitutes in Nairobi, a small number of women have been identified who show resistance to HIV infection despite repeated exposure. HLA-A*6802 and HLA*B18 appear to be more frequent than expected (Fowke et al., 1996) in these subjects.

Some associations of HIV infection with class II HLA type have been described. HLA-DR13 and DR2 were found to reduce transmission from mother to baby and HLA-DR5 to delaying progression to AIDS (Reviewed by Rowland-Jones, *et al.,* 1995). In an investigation carried-out on Thai HIV discordant couples to determine the association of HLA-DRB1, -DQA1 and -DQB1, a significantly lower frequency of DRB1*14, and DQA1*0103 alleles were found in the seropositive individuals when compared with HIV-negative controls. In contrast, there was no significant difference in HLA-DQB1* allele frequencies (Kitayaposon *et al*., 2004).

HLA class I surface expression is essential for antiviral immunity. HIV expresses two proteins that down regulate the expression of surface HLA class I molecules, *Ne*f and *Vpu* (Fellay *et al.,* 2007). These HIV accessory proteins are not required for viral replication, but are important for viral pathogenesis. Both Nef and Vpu are multifunctional; they aid in virion release from the infected cell and down regulate immunologically relevant molecule such as HLA class I

molecules (Frank, 2002). Nef accelerates endocytosis of class I complexes and targets to the lysosomes while Vpu prevents the cell surface expression of class I molecules (Reviewed by Tortorella *et al*., 2000). Vpu attacks only newly synthesized class I molecules and induces their destabilization.

HIV Nef targets HLA-A and –B locus products but not the –C locus products. It is known that HLA-A and –B class loci are mostly associated with a vigorous CTL response, and HLA-C is much less likely to stimulate a strong CTL response but is expressed at high levels (Collins and Baltimore, 1999). In so doing, it hobbles the host's CTL response but leaves enough important HLA molecules on the cell's surface to inhibit NK-cell activity. This way it also thwarts the innate immunity.

HIV-Nef also interacts with CD4 at the cell surface and induces a 5-to-10-fold increase in CD4 endocytosis, followed by transport of CD4 to the lysosomes (Ploegh, 1998). Down regulation of CD4 may also prevent activation of infected T-helper cells via the MHC class II antigen presentation pathway and thus help the virus evade immune detection.

Few studies have been conducted to determine HLA class I allelic frequency in Ethiopia. One amongst this was a study carried out on 50 HIV positive and 50 HIV-negative subjects. The study found 16 different HLA-A, 23 HLA-B and 12 HLA-C types (Tsegaye et al., 2004). When analyzed by HIV status, HLA-A2, B44 and B57 tended to be over-represented in HIV-positive, whereas HLA-A30, B13, B14 and B41 were over-represented in HIV-negative. This study was carried out on heterosexual couples and no study was carried out on monogamous discordant couples. The study also involved few subjects and further research was not carried out.

1.3. Treatment of HIV/AIDS and its challenges

After the discovery of HIV, research moved at a breathtaking pace. A blood test to diagnose patients and to screen the blood supply quickly followed (www.aidscience.org.html), as did enormous progress in understanding the genetic and structure of HIV and its disease-causing mechanisms (Rosenberg and Fauci, 1989). The rapid clinical testing and licensing in 1987 of the first effective drug against HIV, Zidovudine (AZT), caused great excitement (Fauci, 2008). In retrospect this was unfounded, as the molecular characteristics of HIV, notably its propensity to replicate and mutate rapidly, made any single drug unlikely to hold the virus in check. HIV quickly developed resistance to AZT and the benefit of the drug rapidly waned (Marison et al., 2008).

Gradually, the fruit of cutting –edge drug development began to appear. In late 1995, the first of a new class of antiretroviral drugs-Protease inhibitors- reached the market (Fauci, 2008), Other new drugs that attacked the virus in different ways followed and we soon had a greater number of effective drugs for HIV than all other viral disease combined. The new therapies used in combination with older medicines rapidly improved the prognosis for vast numbers of HIV-infected patients. Although with many limitations, these drugs launched a new era of optimism.

But HIV/AIDS is predominantly a disease of the poor world, where access to scientific advances and therapies is difficult. Less than one-third of the people who need antiretroviral therapy are currently receiving it (WHO, 2004), and new infections are outstripping the ability to treat everyone infected with the virus and current HIV therapy is a life-long commitment. Thus, the undefeatable virus is evolving and expanding despite all efforts made by human beings.

Highly active antiretroviral therapy (HAART) has dramatically decreased HIV-1 assosiated mortality and morbidity (Touloume *et al*., 2006). The goal of HAART in HIV-infected patients is to reduce plasma HIV viral load (HIV RNA) to undetectable levels and to increase the CD4 cell count (Sungkanupaph *et al.,* 2006). Achievement of this goal reduces the rate of disease progression and death.

This initial enthusiasm has been dampened because of many problems associated with HAART. Prolonged suppresion of viral load does not eradicate HIV-1 infection because latent HIV-1 reserviors establish early during infection and prevents sterilizing immunity and poses a major obstacle to virus eradication (Geeraert et al., 2008). The discovery of a pool of latently infected, resting CD4 T cells identifies one element likely to be promoting the observed reservior pool (Chun *et al.,* 1997). Moreover, approximately 25% of patients initiating HAART either do not achieve viral suppression or lose it within 2 to 3 years (Bertlett et al., 2001). Some patients also experience isolated episodes of transiently detactable HIV RNA or viral rebound (Sungkanupaph *et al.,* 2006). Rates of viral rebound of 25-33% have been reported among patients on HAART who have achieved undetectable HIV RNA (Le Moing *et al.,* 2006). The same study also demonistrated that some virological failures have not been associated with risk but in some viral rebound has been associated with greater risk of viral failure. Long-term administration of HAART is associated with poor adherence leading to an increased risk for developing drug resistance (Touloumi *et al.,* 2006). Incomplete adherance to treatment plans account for about half of treatment failures (Smith *et al.,* 2005). Drug resistance is a major problem in antiretroviral treatment (ARV) and has been observed to be transmitted from mother

to fetus during pregnancy (Bauer *et al.,* 2006) and drug resistant HIV can be transmitted to a chronically HIV- infected parteners (Frederick *et al*., 2007). Prior reports of HIV superinfection have documented transmission of drug-resistant HIV and potentially rapid disease progression in early HIV infection (Smith *et al.,* 2005).

Many metabolic and physiological abnormalities are also common during antiretroviral treatment. The first systematic study of these phenomena, in a large Australian cohort, found subcutaneous fat wasting in the face, limbs, buttocks and upper trunk (termed "Peripheral lipidystrophy") , associated with abnormal visceral obesity, dyslipiaemia and insulin resistance (Mina, *et al.,* 2001). Prior studies also described benign symmetric lipomatosis, localized lipomas, 'buffalo humps', intra-abdominal fat accumulation and breast enlargement in HIV-infected men and women on various antiretroviral combinations, predominantly (but not exclusively) including protease inhibitors (PIs) (Barrlett, *et al*., 1998). A similar study also showed that use of PIs is associated with an increased risk of myocardial infraction in patients on treatment (Holmberg, *et al*., 2002). Patients experience psychological morbidity and increased barriers to good adherence to therapy becuase of these problems.

Suppression of HIV replication by HAART often restores protective pathogen-specific immune response, but in some patients the restored immune response is immunopathological and causes disease, immune restoration disease (IRD) (Martyn, *et al*., 2004). The patients may occasionally experience opportunistic infections (IOs) such as infections by mycobacteria, cryptococci, herpesviruses, hepatitis B and C virus and JC virus (French *et al.,* 2007).

There has been an increase of more than ten times over the past 5 years in the number of people placed on antiretroviral drugs. But for every two patients placed on antiretroviral drugs during 2007, five new HIV infections occurred (Lay, 2008).

1.4. Discordant couples

About 85% of HIV transmission is sexual. If the pandemic has proved nothing else, it is that a diverse sexual life is part of being human. But despite the vast increase in the awareness of sexual diversity which has come in the wake of HIV/AIDS-driven research and community action, programming responses still find it hard to tackle sexual transmission in the right way or in the right population (Barker *et al.,* 1998). Much of the history of preventing sexual transmission of HIV has concentrated on reducing multiple partnerships or adopting condom use in casual sex of HIV.

In the late 1980s AIDS researchers began to notice that some of their patients just weren't getting AIDS-despite the fact that they had been infected for roughly 10 years with the human immunodeficiency virus (HIV) (Buchbinder *et al.,* 1994). The scientists started to hope that such "long-term non-progressors" (LTNP), some of whom happened to have strains of HIV that were missing some genetic information, might hold the key to developing an AIDS vaccine.

The clinical course and outcome of HIV infection are highly variable. A full spectrum of pathology has been observed, from rapid progression to AIDS within months of HIV seroconversion, to asymptomatic survival for more than a decade. This phenomenon probably reflects the multiphasic and multifactoral nature of the virus-host interactions (Barker *et al.,* 1998). In general, HIV-infected individuals develop AIDS within several years, but about 5% remain healthy over an extended time (and are known as long-term nonprogressors, LTNP).

Only low level HIV-specific CD4+ Th responses with stimulant indices rarely above 5 or the complete lack of HIV-specific proliferative activity was reported in chronically infected AIDS patients (Zhang et al., 2001). In long-term non-progressors, however a vigorous proliferative response to HIV antigens with stimulation indices up to 200 was reported (Rosenberg et al., 1997). These indices however constitute a small subpopulation of infected individuals who maintain normal Th cell count and an undetectable viral load despite infection for many years. It was further observed that the degree of the proliferative response to the HIV Gag p24 protein was negatively correlated with the viral load. This association suggested that virus-specific helper T-lymphocytes contribute to containing HIV replication. Long-term-non-progressors remain clinically healthy, displaying stable CD4 T-lymphocyte counts and low levels of viral replication for many years (Barker *et al.,* 1998). Studies also showed that broadly neutralizing antibody responses are stronger and more frequent in LTNP than in other HIV-infected patients (Buchbinder *et al*., 1994).

Rare subsets of highly exposed persons who resist HIV infections have been identified in a variety of settings (Beyrer *et al.,* 1999; Benzle *et al*., 2000). These apparently HIV-resistant persons are HIV negative by all standard serological and polymerase chain reaction (PCR) assay. Several nomenclatures and classifications have been used to identify such persons: highly exposed persistently seronegative (HEPS), exposed uninfected (EU), exposed seronegative (ES), discordant couples (DS) and positive – negative couples (PNC). The term discordant couples are used in this study, as the acronym encompasses people of mixed HIV status. In a similar way, the term concordant couple is also used to indicate seroconcordance (of similar HIV status).

As the AIDS epidemics mature, a higher proportion of HIV infections take place within marriages or other long-tem partnerships (Dunkle et al., 2008). In Uganda, for example, HIV is present in 8% of all married or cohabiting couples, and in only about half the cases are both the partners infected with HIV (Wabrire-Mangen, 2006). Similarly, across Burkina Faso, Cameroon, Ghana, Kenya, and Tanzania, two-third of couples in which HIV is present are serodiscordant, and in 30-40% of these cases the female partner is infected (de Walque, 2006). A large scale HIV-prevention trail focusing on serodicordant couples in eastern and southern Africa reported that serodiscordance varied between 85 and 31% (Wabrire-Mangen, 2006). Some estimates suggest that 60-95% of new HIV infection in Rwanda and Zambia occur between married couples living together (Dunkle *et al.,* 2008).

Although these people were expected to be rare, resistant individuals are a more widespread and significant phenomenon than first realized. It has been observed in Thailand (Beyrer et al., 1999); Uganda (Wabrire-Mangen, 2006); Zambia (Dunkle *et al.,* 2008) and in many other countries worldwide. Many human HIV transmissions in sub-Saharan Africa are believed to occur between married adults who are discordant for their HIV status.

The discordant status of couples, however, is not permanent and seroconcordance has been observed in some couples. For example, in a study conducted on 77 couples, 27% of infected women and 18% of infected men had transmitted the virus to their partners (http//www.agi.USA.org/pubs/html). The rate of transmission from male to female was identical to the rate of transmission from female to male with one exception. If the seronegative male partner circumcised, no seroconversion occurred, in contrast to an incident rate of 16.7% among the 137 uncircumcised male partners. Many however have remained discordant despite a long time unprotected sexual intercourse.

Resistance to HIV infection and AIDS can occur in a minority of individuals and at several stages (steps). First, resistance can occur during transmission of HIV via parentral, vertical or sexual routes (Dunkle *et al*., 2008). Among high-risk population of health-care workers, children born to HIV seropositive mothers, prostitutes, homosexual men and heterosexual people, individuals have been identified who did not become infected with HIV despite frequent exposure to the virus. Second, resistance to HIV-induced disease can occur during different clinical stages of HIV infection. Resistance to severe acute viral syndrome is observed in the majority of HIV-infected subjects, who experience only very mild clinical symptoms during primary HIV infection. Resistance to HIV-infection in people exposed to the virus may be quite different from

the resistance to disease progression or death that is seen in a small fraction of HIV-infected people (Beyrer, 1999).

The lack of knowledge about discordance can increase its prevalence, while poor use of condoms within discordant couples increases the seroprevalence of HIV/AIDS. That is, the lack of knowledge increases the risk of contamination among the healthy partner. However, the knowledge of discordance can also lead to poor relations in the couples, separation and even divorce (Naandali *et al.,* 2004). Thus, multifaceted problems are associated with HIV-discordance.

Variability in susceptibility to infection and disease caused by infectious agents is a characteristic of all population. Among susceptible individuals exposed to an infection, not all develop disease. It seems logical that variability in susceptibility to infection and disease with human immunodeficiency virus also exists.

Resistance to HIV infection is likely to depend on a complex array of features contributed by the pathogen and the host (Clerici *et al*., 1997). A combination of factors including cellular and humeral immunity, genetic factors, viral characteristics, coreceptor integrity and others may be involved in the persistent HIV- resistance.

1.4.1. Mechanisms of discordance

1.4.1.1. Immunological factors

Evidence suggests that a number of host immunologic factors play critical roles in protection against sexually transmitted HIV infection in HIV-discordant partners. Both mucosal(genital tract) and systemic HIV-specific immune responses have been described in discordant groups, including HIV-specific CD4+ and CD8+ T cells, and HIV-neutralizing IgA antibodies (Devito *et al*., 2000; Kaul *et al.,* 2000 and Devito *et al.,* 2002).

HIV-neutralizing immunoglobulin A (IgA) and HIV-specific cellular immunity has been described in HIV-discordant couples (Devita *et al.,* 2000). For HIV-neutralizing antibodies to protect against sexual HIV acquisition, such antibodies would likely need to be presented at the level of the genital tract mucosa.

Both male and female genital tract tissues lack inductive mucosal sites analogous to intestinal peyer's patchs. Consequently, local humeral and cellular immune response stimulated by HIV is weak or absent. In contrast to typical external secretions such as intestinal fluid that contains secretory immunoglobulin A(S-IgA) as the dominant isotype, semen and cervico-vaginal fluid

contain more IgG than IgA (Mestecky *et al.,* 2005). But virus specific IgA has been described in the genital tract of highly-exposed, HIV-uninfected Kenyan prostitutes. It is also present in a minority of lower risk seronegative controls, where it is highly associated with HIV risk taking behavior. These data suggest a role for mucosal virus-specific humoral responses in resistance to HIV-1, independent of host cellular response.

Humoral immune responses to HIV may be significant in controlling plasma viraemia. Antibody titers in long term survivors are usually higher than those in individuals who have progressive diseases, and contain antibodies directed against several antigens (Klein and Miedema, 1995). HIV-specific antibodies may neutralize cell-free virus particles and thus prevent virus progeny from infecting new cells.

Natural infection with HIV generates many virus specific antibodies, but neutralizing antibodies able to inhibit infection by a broad range of primary isolates *in vitro* are rarely detected in sera from HIV-infected patients. Only a few broadly neutralizing monoclonal antibody have been generated from such individuals (Binley *et al*., 2004).

The mucosal tissues are the protective linings in the body's main cavities, including the urogenital tract. It was known that some individuals who are high risk for HIV could remain seronegative, while showing local immune response in their urogenital tract. In these people HIV-specific IgA are present in the urine but absent in the blood (http://www.scienceblog.com/community/older/199802110.html). This may imply that some people's immune system can keep HIV contained and silent within certain body compartments. As most HIV tests involve serum HIV antibodies, it is possible that in HIV-discordant couples HIV is arrested in the tissues but never moves to other lymphoid tissues carried by blood and blood cells.

It is known that the course of HIV infection is characterized by a gradual loss of CD4+ T-lymphocytes. However, few individuals who are resistant to HIV and the HIV infected non-progressors remain clinically healthy displaying stable CD4 T-lymphocyte counts (Barker *et al.*, 1998). The presence of neutralizing antibodies (Devito *et al.,* 2002); high CD4+ T cell count, and low level of HIV-1RNA-in serum (Paxon *et al.,* 1996) in concert or in isolation can account for non progression.

Although in African settings increased number of CD4 cells is associated with immune activation; it is possible that differences in CD4 number between discordant couples may confer resistance. The provision of an important help to CTL may also offer resistance to discordant couples. CD8

T-cell immunity is thought to require helper activity derived from CD4 T-cell. However, under some circumstances, effective CD8-dependent T cell responses occur in vivo without CD4 T cell help (Malek, 2002).

It has been established that both acute human immunodeficiency virus and simian immunodeficiency virus infections are accompanied by a dramatic and selective loss of memory CD4+ T cells predominantly from the mucosal surfaces (Mattapallil *et al*., 2005). But in HIV resistant and LTNP CD45RO CD4+ T cells and responses to recall antigens are well maintained (Simone *et al.,* 1996). Furthermore, in these subjects, the number of activated lymphocytes, the amount of aberrant cytokine production and the rate of HIV replication were all lower. Similar mechanisms may operate between discordant couples.

It is a well established fact that HIV causes damage to the lymphoid gland and changes their architecture. Long-term clinically healthy HIV infected subjects are usually characterized by relatively little observable damage to the immune system. The lymph nodes of long-term survivors do not show involution, fibrosis or lymphocyte depletion (Klein and Miedema, 1995) as in disease progressors. This shows that T cell function is stable and relatively preserved in long-term survivors compared with those who progress to disease. A similar mechanism may also exist between discordant couples.

Virus-specific CTLs eliminate virus-infected cells via MHC class I restricted killing or mediate inhibition of viral replication via soluble factors. CTLs are therefore considered to be the major defensive arm of the adaptive immune system in the battle against viral infections. In deed, increased CD8+ T cell numbers and strong and persistent HIV specific CTL responses against several HIV proteins, together with low numbers of HIV infected cells, have been observed in HIV resistant and long-term survivors (Barker *et al.,* 1998). HIV-specific CTLs from resistant and long-term survivors may differ from the CTLs of those who progress to disease in their epitope specificity or T cell receptor repertoire (Klein and Miedema, 1995).

Studies indicate that CTLs are recruited very early during encounter with HIV and HIV-specific CTLs have been observed as early as a few days following the onset of acute symptoms and in general before neutralizing antibody response could be detected (Borrow *et al.,* 1999). The appearance of HIV-specific CTLs usually parallels a striking diminution of the viremia in infected patients.

Interestingly, some individuals were observed who remained HIV-negative despite frequent exposure to the virus, and seem to harbor HIV-specific CTL responses (Greenough *et al.,* 1997).

Further, in a group from Gambia who seemed to have escaped HIV-infection despite several years of high risk sexual behavior three out of six patients had CTLs that recognize HIV-1 and HIV-2 cross reactive epitopes (Roland-Jones *et al*., 1995). Similar study showed absence of seroconversion in Kenyan prostitutes exposed heavily and repeatedly to HIV was also due to HIV-specific CTL (Shearer and Clerici, 1996) and similar results have been reported from many other countries.

In some of these experiments CD8+T cells suppressed HIV replication in a dose dependent manner. The magnitude of the response in some HIV-infected persons was unprecedented, in that antiviral cytotoxic activity could be detected in freshly isolated PBMC without the need for an *in vitro* stimulation and expansion (Effros and Pawelec, *1997)*. It has also been demonstrated that cytotoxic T lymphocytes are also present in both cervical and peripheral blood PBMC from a subpopulation of highly-HIV-exposed but persistently seronegative individuals (Henrard *et al.,* 1995).

CD8+ CTLs have been shown to protect against a variety of viral infections in animals and humans. It is possible that such a response will afford protection against retroviral infection as well. It is therefore, highly likely that CTLs may play a great role in the prevention of transmission of HIV infection in discordant couples.

Evidence suggests that CD8+ T cells are involved in the control of HIV infection by the release of HIV –suppressive factors. The human chemokine RANTES and the macrophage inflammatory protein-1 alpha (MIP-1α) and macrophage inflammatory protein-beta (MIP-1β), were identified as the major component of HIV-suppressive factors (Cocehi *et al.,* 1995) and they are produced by both immortalized and primarily patient CD8 T cell.

Chemokines potentially inhibit infection by primary and macrophage tropic isolates while T cell – line adapted viral strains tend to be insensitive to their suppressive effects (Ackhatith *et al.,* 1996). The beta-chemokine MIP-1 α, MIP-1 β and RANTES have been implicated in the suppression of viral replication by CD8+ T cell from HIV-infected individuals (Robert *et al.,* 1997). In an experiment involving viral isolates of both non-syncytiums inducing (NSI) and syncytium-inducing (SI) biological phenotypes recovered from patients at various stages of HIV-infection exhibited that only the isolates with the NSI phenotype were substantially initially inhibited by the beta-chemokine (Jonson *et al.,* 1996). More important to note, these data demonstrate that resistance to inhibition by beta-chemokine RANTES, MIP-1 α and MIP-1 β is restricted to T-cell line adapted SI isolates. Analysis of isolates obtained sequentially from NSI to SI phenotype

during clinical progression exhibited a parallel loss of sensitivity to β –chemokine (Robert *et al.,* 1997).

Similar studies also showed that the beta-chemokines MIP- 1α and MIP-1 β inhibit HIV replication in an anti-CD3 or recall antigen-stimulated peripheral blood mononuclear cells of asymptomatic HIV-infected subjects (Kinter *et al.,* 1996). Both CD4+ and CD8+ PBMC subjects from HIV-infected individuals produced significant levels of beta chemokines. These data suggest that the levels of HIV replication in CD4+ PBMC reflect the balance of the opposing effects of endogenous suppressive factors and HIV-inducing cytokines.

In general, activated CD8+ T lymphocytes from HIV-infected individuals produce a soluble noncytotoxic activity that suppresses infection by HIV, an effect thought to be specific for the virus. The production of suppressive activity correlates with immune status and decreases gradually in parallel with disease progression (Ackhatith *et al*., 1996). These observations indicate that β-chemokines are responsible for a major proportion of HIV-specific suppressor activity produced by primary T cells. Each of these factors could contribute to HIV resistance. As most discordant couples have one or more of these factors they may prevent HIV infection synergically by blocking HIV cell entry, delaying its dissemination, or killing HIV-infected cells. Thus, the possible sero-status difference between HIV-discordant couples may be due to the presence of suppressive beta-chemokine in the negative partner.

1.4.1.2. Genetic factors

The characteristics of the host particularly the host genetic factors also play a very important role in preventing HIV infection. HIV-specific CTLs are present in the individual of the resistant discordant couples and these are HLA-restricted. Selection of epitopes is almost entirely determined by HLA type and selection of conserved compared to variable epitopes as targets for CTL response could be a major contributing factor to the rate of disease progression.

Specific allele of HLA locus is associated with different rates of progression from infection to an AIDS diagnosis (Kaslow, 1996). HLA-B27, HLA-B57, HLA-DQ4 and HLA-B53 and HLA-B55 are found increased in seronegative in comparison to the seropositives (Kaslow 1996; Rohowsky-Kochan et al., 1998). In contrast HLA-A*0201, HLA-DQ7, HLA-CW7, HLA-B44, HLA-A26 are markedly increased in HIV+ partners (Rohowsky-Kochan *et al.,* 1998; Makedonas *et al*., 2005). A study also showed that other HLA haplotypes such as HLA-B53 conferring resistance to HIV-1 and HIV-2 (Rowland-Jones et al., 1999).

A study in Ethiopia showed HLA-A2, HLA-B44 and HLA-A30, HLA-B13, HLA-B14 and HLA-B41 were over-represented in HIV-negative Ethiopians (Tsegaye et al., 2004). But this study was not about monogamous discordant couples. However, differences like these may be responsible for discordance. It is also possible that unknown genetic factors may be responsible for the relative resistance and susceptibility to HIV infection.

1.4.1.3. Viral characteristics

The likelihood of HIV infection depends on the route of exposure, the level of infectiousness, and the frequency of exposure. Highly HIV exposed persons, such as sex workers, injection drug users, partners of HIV-positive persons, and children of HIV-infected women, have particularly high incidence rates (Frank, 2002). Because of the viral characteristics the cells of some resistant individuals cannot be infected. And even if infection is caused it is transient infection that is cleared and may be localized to certain tissues (compartmentalized) (Kaslow, 1996).

The possible reason for the induction and maintenance of adoptive immune response (if it is present) is due to low level continuous viral replication in mucosal tissue below the level of clinical detection (Zhu *et al*., 2003), single rounds of HIV replication followed by viral clearance and immune processing of nonreplication competent viruses or viral fragments (Larsson et al., 2002). All these are associated with the competence and viral characteristics.

Dose of HIV RNA play a significant role in determining whether transmission occurs or not (Quinn *et al.,* 2000). The most important variable that was associated with both transmission and acquisition was the viral level of HIV in the infected partner. A significant dose response effect with respect to both male-to-female transmission and female-to male transmission was also observed. The rate of transmission rose from 2.2% among individuals with viral levels <3500c/ml to 23.0% at levels>50,000c/ml. There was also a threshold below (<1500c/ml) which no transmission occurred. Thus, lower viral load may be the reason for absence of transmission between discordant couples.

1.4.1.4. Coreceptor integrity

The number or amounts of CCR5, the major coreceptor for M-tropic and CXCR4 for T-trophic HIV isolates are important for the infection of these cells by HIV. The on average lower proportion of CCR5 expressing CD4+Tcells and lower CCR5 surface expression levels on PBMC, may result in slower spread of the virus and hence explains reduced HIV-susceptibility of PBMC and slower progression (Kozak *et al.,* 1997). Changes in cell surface concentration of

coreceptors may control infection by HIV CD4 and CCR5 required for efficient infections by macrophage trophic HIV. Both coreceptors are interdependent and that the requirements for each are increased when the other component is present in a limiting amount (Emily *et al.,* 1998).

Chemokine receptor polymorphism and beta-chemokine overproduction have been among the mechanisms suggested to be responsible for resistance to HIV infection. Expression levels of CCR5 among resistant women were shown to be equivalent to that found in low-risk seronegative (negative) controls, while CXCR4 expression was greater among some of the resistant women (Fowke *et al.,* 1998). One mechanism by which these and other chemokine receptors are altered functionless is by mutation(s) in the genes encoding these receptors. Typical example for this is the mutation of the homozygous deletion that affects the structure and membrane expression of CCR5 (CCR5 delta32) (Fellay *et al.,* 2007). As the resistance conferring homozygous conditions is present only in 1% of Caucasians, and the percentage of resistant individuals that do not become infected is high, it is clear that this mutation represents only part of the picture. Overall, chemokine receptor concentration and chemokine receptor polymorphism is one of the possible reasons for serodiscordance in discordant couples.

1.4.1.5. Mucosal immunity

Most HIV transmission is sexual and immune responses in the genital mucosa may be important in mediating protection against HIV infection. In a study conducted in Kenyan female sex workers, HIV-specific IgA was present in the genital tract of 16 out of 27(76%) HIV-resistant sex workers (Kaul *et al.,* 2000). These data suggest a role for mucosal HIV-specific IgA responses. In a similar study conducted in the sera of 15 sexually exposed seronegative persons and their HIV-infected partners; the HIV positive subjects had HIV-specific serum IgG (Mazzole *et al.,* 1999). This shows that immunological picture for resistance to HIV infection should include HIV-specific cell mediated immunity as well as HIV-specific IgA mediated mucosal and systemic immunity. Similarly, a study conducted in Thai female sex workers with epidemiological evidence of exposure to HIV showed significant differences in humoral immune response (Beyrer *et al.,* 1999). In this study, gp160-specific IgA responses were detected in cervicovaginal lavage fluids in 6 of 13 persistently seronegative commercial sex workers but 0 of 20 seronegative subjects. These and several other studies show that strong mucosal immunity is a contributing factor for HIV resistance.

In general, cellular resistance to HIV is a concerted effort of several factors involving humoral and cellular immunity, including mucosal immunity; viral and genetic factors and chemokines and the abundance or absence of chemokine receptors.

1.4. 2. Sexual behavior and risk factors associated with HIV infection

Heterosexual transmission remains the most common mode of transmission of HIV throughout the world. Over 85% of new infections are acquired heterosexually, with the greatest predominance in sub-Saharan Africa. Studies of HIV discordant couples (given recent data that approximately 70% of incident HIV cases are transmitted from regular partner) provide perhaps the best information on the efficiency of transmission and the biological and behavioral variables that influence infectiousness of and susceptibility to HIV. A study of HIV discordant couples shows inter-partner reliability for risk factors of HIV transmission. Agreement among couples was good for common sexual practices, especially vaginal intercourse and time since last intercourse but there was greater disagreement for the occurrence of anal and oral sex (Melanie *et al.,* 1998). Thus, the study of sexual behavior of HIV discordant couples gives good and reliable results.

Discordance is a relatively new phenomenon and the public is still struggling to understand how it occurs and why one would remain negative while the partner is positive. Most men test by proxy with the assumptions that their partner's HIV results are the same as their own. But discordance is also a source of dispute among couples, as knowledge of discordance has led to poor relations in the couples, separation and even divorce. The lack of knowledge of discordance has also increased the risk of contamination among the healthy partners (Ndandali *et al*., 2004).

1.4.3. Sexual behavior

Sexual behavior is diverse and a diverse sexual life is part of being human. Sexual behavior is not just determined by instinct but is socially determined and socially controlled to a greater extent. Friends, family, neighborhoods, religious beliefs and education dramatically influence who one chooses for sexual partner(s) and how one behaves sexually (Coates *et al.,* 2008). Sexual behaviors that cause most HIV infections worldwide occur for many motivations (eg. reproduction, desire, peer pressure, pleasure, physical or psychological dependence, self-esteem, love, access to material goods, obligations, coercion and force, habit, gender roles, custom, and culture (Coates *et al*., 2008). Sexual behavior typically does not occur in public, making it

difficult to motivate protection when potential transmission occurs, and making it almost impossible to verify reports of what people say have or have not done.

Sexual behavior and number of partners are major determinants of HIV transmission. Personal factors and environmental domains together determine any of the subsequent behavioral change. Demographic characteristics such as age and sex, and education may influence one's belief and attitudes towards a particular behavior. On the other hand, cultural differences, gender and spirituality and accessibility to prevention mechanisms constitute the environmental domains.

Fundamental goal of HIV prevention is to change the behavior that puts individuals at risk of infection. For the past two and half decades, HIV prevention has been dominated by individual-level behavioral interventions that seek to influence knowledge, attitudes, and behavior, such as promotion of condom use or sexual-health education ,and education of injecting drug users about the dangers of sharing equipments. Although some individually oriented interventions have shown results in reducing risk behavior their success is substantially improved when HIV prevention addresses the broader structural factors that shape or constrain individual behavior, such as poverty and wealth, gender, age, policy, and power (Gupta *et al*., 2006).

Behavioral change has been responsible for the prevention success to date. Strategies to modify risk behaviors need to remain a main priority to HIV prevention. Behavioral strategy aims might involve increased knowledge about how to protect oneself from HIV infection; stigma reduction; encouraging access to services (HIV counseling and testing, diagnosis and treatment of sexually transmitted infections, use of antenatal and reproductive health services) ; improving attitudes toward safer sexual practices; delaying onset of intercourse; decreasing number of partners; reducing use of sex workers; increasing condom use and sales; recognition of early symptoms of sexually transmitted infection or HIV; recognition of the benefit and limitations of male circumcision for protection against HIV; disclosure of HIV serostatus; harm reduction strategies; how to access treatment for HIV; the importance of adherence to ART; and so on (Reviewed by Coates *et al*., 2008).

Behavioral strategies to accomplish these goals can focus on individuals, couples, families, peer groups or networks, institutions, and entire community.

1.4.4. Risk factors associated with HIV infection

There are several risk-factors responsible for HIV acquisition. The simplest fact that 90% of the world's HIV infections occur in developing countries (predominantly in the sub-Saharan Africa)

is evident that social, economic, and political structures drive risk behaviors and shape vulnerability.

Some studies show an association between structural factors and HIV risk. These include studies of the macro level correlates of infection, such as income per head, gender inequalities, and social marginalization (Barnett and Whiteside, 1995) ; cross-sectional studies examining the relation between HIV prevalence and factors such as migration or location of residence (Lurie *et al.,* 2003 ; Serwadda *et al.,* 1992); and cross-sectional studies associating risk behavior with factors such as past exposure to domestic violence, school enrolment, and being orphaned (Rwenge, 2003).

Other studies have more explicitly described the mechanism by which structural factors can affect HIV risk. For example, sexual violence, a manifestation of gender inequality, has been linked to an increased risk of HIV transmission (Rwenge, 2003). Fear of HIV/AIDS-related stigma and discrimination discourages people from seeking HIV counseling and testing and from disclosing their status to their sexual partners (Barnett and Whiteside, 1995). And women who regularly experience gender-related violence might be unable to negotiate condom use

The other forms in which structural factors affect HIV vulnerability include poverty and education. There is strong association between economic status and HIV prevalence (Rwenge, 2003). A common assumption is that poor people are most vulnerable to HIV. For example, the bulk of the world's HIV infections have been in sub-Saharan Africa (the poorest region of the world) (Barnett and Whiteside, 1995). But this work is also criticized on the basis that within sub-Saharan Africa, the wealthiest nations are those affected by HIV/AIDS (Reviewed by Gupta *et al.,* 2006).

There is also a direct association between education and vulnerability to HIV. Before 1995 studies showed that higher rates of HIV-infection in educated women, possibly linked to higher socioeconomic status and mobility and having more sexual partners than less educated women (Reviewed by Coates *et al*., 2008). But structures affecting risk are not static and may change both in their form and their effect as an epidemic evolves. This had been witnessed by the observation that as the epidemic developed overtime education became more protective and the number of HIV positive uneducated men and women increased overtime (Hergreaves *et al.,* 2007).

There are also several other risk and vulnerability factors at an individual behavioral level. Much of the history of preventing sexual transmission of HIV is on reducing multiple partnerships or adopting condom use in casual sex. One major source of HIV transmission is concurrent partnerships. High rates of concurrent partnerships (which include concurrent partnership, serial

partners, and sex with many casual partners) vary widely in different countries. At an individual level, relationship with multiple partners remains a strong predictor of HIV infection (Garnett, 2007). Effective responses to HIV risk within established intimate relationships have always been the hardest of prevention challenges because most people simply will not choose celibacy.

Formative research conducted in nine countries of Southern Africa in the second half of 2007 reported very similar reasons across the sub region for multiple and concurrent partnerships: dissatisfaction with main relationships; social norms (cultural, gender, and peer-pressure issues); poverty and materialism; male domination; and alcohol use (Reviewed by Gupta *et al.,* 2006). HIV risk has been strongly associated with the use of illicit drugs and with alcohol use, especially in settings where sexual contacts are also made. Alcohol and drug use might not be the straightforward causes of risky behavior, but they do seem to sustain subcultures of risk. To reduce sexual risk inherently requires, at a minimum, engaging the sexual dyad, whether heterosexual or homosexual

1.4.5. Risk factors associated to HIV transmission in Ethiopia

In the Ethiopian context several factors suggest that there is real danger of HIV spreading to government employees. According to Girma, 2007, there are three reasons for this: economic loss caused by the decline of labor force; the presence of most government workers in the main urban centers where there is high prevalence of HIV; and the employment of many workers routinely on job transfer or field work spending many days away from home, with the likelihood of sexual relationships with casual partners.

Among specific population groups that are disproportionately affected by the pandemic are sex workers, people with sexually transmitted diseases and some occupational groups such as military personnel (Max, 2000). Sexual behavior is undoubtedly the most important detrimental of the spread of HIV and has been a major area of focus for the prevention and control of the epidemic.

Different studies in Africa and elsewhere show that the epidemiology of HIV/AIDS is highly related to migration. In most sub-Saharan African countries mobility and HIV/AIDS are linked to heterosexual transmission; fueled by rampant STDs, multiple and commercial sexual relations, low condom use, poor access to health services and other socio-cultural and economic factors related to population and movement (Belay, 2008).

In sub-Saharan Africa the main risk factors for high HIV transmission include, population movement, poverty and or unequal distribution of wealth, gender inequality and various cultural influences (UNAIDS, 2002).

The most significant socio-cultural and economic factors which place migrants in a high risk situation are the movement of people from their rural areas without a stable sexual partner, fragmented social networks, reduced availability and accessibility of health and services, economic transition and urban life style which exposes migrants to practice sexual risk-taking behavior (Max, 2000).

Sexual behavior, number of partners and use of condoms are major determinants of HIV transmission. In Ethiopia, heterosexual contact and pre-natal transmission are the primary modes of transmission for HIV (EPHA, 2005). There are also several kinds of risk factors fueling HIV transmission in both rural and urban areas. In a country where millions are at risk and the issue of curative treatment is a long-term agenda, working and aiming at behavioral change is still unavoidable strategy. Despite intense efforts by different sectors and high levels of awareness among people living in towns, the desired behavioral changes have not been observed and there are still fertile grounds for the spread of the virus both in cities and rural areas.

A study carried out in Addis Ababa to identify risk factors for HIV infection, in males, reported sexual behaviors and past history of syphilis were strongly associated with HIV infection. In females, socio-demographic characteristics (e.g., low income, low education, and living alone), rather than sexual behaviors, were associated with HIV infection (Arnaud Fontanet and Tilahun W/Michael, 1999). But other studies carried out later did not prove this.

The 5th AIDS in Ethiopia report document showed that 17.4% of young people reported risky sex with commercial or non-commercial partner. Young males were engaged more in risky sex than young females 19.4% of male's vs 16.1% of females. Most (93.5%) young people did not consider themselves to be at risk of HIV infection.

In most of the study areas, females started sex at an earlier age than males. The most productive forces of the society: the youth workers, CSW (commercial sex workers), students, and those between the ages of 15-49 were considered to be most affected by HIV/AIDS. The major factors that exposed these groups of people to the virus were: premarital sex, extramarital sex, promiscuity, adventure, excessive alcoholic intake, mobility, rape, abduction, inheritance marriage, skin cutting and harmful traditional practices (EPHA, 2005).

Of the behaviors known to place individuals at risk of HIV infection, having multiple sexual partners is probably the key concern in much of sub-Saharan Africa. The study carried out on government employees of whom 80% has been sexually active 13.2% had multiple sexual partners (Girma, 2007). Being single, Chat chewing and field work was significantly associated with multiple sexual partners. Premarital and extramarital sex is an adventure for young and even married woman. Some woman who has no "wedaj" or extra lover are considered as unwanted and thus as an outcast (EPHA, 2005).

A study carried out on sugar factory workers showed that 66.2% of them acknowledged more than five sexual partners in their lifetime; 10.2% reporting a history of genital discharge; and 2.1% reporting a genital ulcer (Mekonen, 2003). Only 9.7% reported having had casual sexual partners and 43.4% acknowledged having sex with CSW; when going to bars. A similar study carried out on male daily laborers showed that 43.4% and 55.9% practiced sex with multiple sexual partners and commercial sex workers, respectively (Belay, 2008). Having multiple sexual partners was positively associated with longer duration of stay in the town.

Although HIV prevalence is 8 times lower in rural areas of Ethiopia, the behaviors related to HIV/AIDS are increasing in rural Ethiopia. Massive in and out movements contribute to the spread of the virus in the rural areas. Other factors responsible for the spread of HIV/AIDS in rural communities are: premarital sex, extramarital sex, , drinking alcohol(34.1%) and chewing chat (29.9%), harmful traditional practices, low treatment seeking behavior by STD (EPHA, 2005). According to this report, demobilized soldiers, merchants, people who visit local towns for social and business reasons have been associated in transmitting HIV from towns to villages. A study conducted on the sexual behavior of farmers, ex-soldiers, merchants and students has shown that these groups of people had frequent sexual contacts with female sex workers in urban as well as in rural areas (Shabir and Larison,1995).

Mobility, unprotected sexual contact, harmful traditional practices, sharing contaminated sharp instruments, drinking alcohol (Intoxication), having mistress (Wushima in Amhara, Segno in Oromo) in towns and villages themselves, inheritance marriage, marriages without knowing HIV status and poor health seeking behavior were considered to contribute to the spread of HIV/AIDS. In all these areas, there was an increase in poverty because of disease, unemployment, and bad working culture and that pushed females to prostitution. Population growth, overcrowding, low productivity and drought were reported to be causes of poverty *(EPHA, 2000).*

Divorce and remarriage without testing for HIV was common in most towns and rural communities. Inheritance marriage was accepted culture among certain ethnic groups. Lack of openness on the issues of sex was a common problem across regions. Uvulectomy, milk-teeth extraction, cupping, female genital mutilations (FGMs), abduction, rape, and inheritance marriage were major routes for the transmission of HIV/AIDS (Shabir and Larson, 1995).

Pre-marital and extra-marital sex, within villages and outside villages was common in most of the regions. Involvement in premarital and extramarital sex, and abduction were considered as adventurous act by young people in some regions. Marriage to the wife of the late brother "Warsa" and inheritance marriage was common in Oromia and Afar compared to other regions (EPHA, 2005).

According to these studies, the degree of major risk behaviors aggravating the transmission of HIV in rural communities varies by region and even by district based on culture, education and other conditions. At the village level, extramarital sex is the major factor for the transmission of HIV/AIDS. Harmful traditional practices (HTP) constitute other important risk factors for the spread of HIV/AIDS in the rural areas. Common HTPs identified in this study included: FGM, inheritance marriage, early marriage, abduction, uvulectomy, tonsillectomy, milk-teeth extraction, tribal marks, blood letting, cupping and skin cutting. Harmful traditional practices related to marriage such abduction and inheritance marriage aggravated the transmission of HIV through sexual intercourse.

All these show that there is a favorable ground for HIV transmission and spreading throughout Ethiopia. Thus one can easily understand that contextual factors (socio-cultural and politico-legal issues) lie behind the development and existence of risk behaviors to HIV/AIDS (Hagos, 2007). These contextual factors operate through mediators such as individual behavioral causes and immediate causal factors.

HIV/AIDS programs have demonstrated a high level of awareness among people living in towns. However, the paradox is that the high level of awareness and knowledge about HIV/AIDS has not led to positive behavioral change. For example, despite the prevalence of high risk sexual behavior, the usage of condom has never been sufficient enough and consistent. In one study, only 57.9% of sexually active respondents had experienced condom use and from these only 34.9% were consistent users (Belay, 2008). In another study employing government employee only 47.3% used condom of which 13.2% had experienced sexual intercourse with multiple sexual partners. More men than women reported using condoms in this study. One important prediction

of condom use is marital status. Married or cohabiting men and women were less likely to use condom (thus condom use within marriage is uncommon) in Ethiopia. The main reason given for not using condoms was trust in the partner (Girma, 2007). Perceived susceptibility to HIV was the most powerful predictor of intension to use condoms.

In rural areas of the country, few people ever used condoms and some have never seen condoms. And many had negative attitudes towards condom use. Some considered condom as a means of encouraging promiscuity and believed that the use of condom was against religion (EPHA, 2005). Some even perceived condom use as a means of reducing sexual pleasure, and misconceptions have been reported that condoms themselves contain the HIV virus.

The effectiveness of male latex condoms for prevention of HIV transmission is up to 85% based on the data from several longitudinal cohort studies of serodicordant heterosexual couples (Weller and Davis, 2002). But when male condoms are used consistently, their effectiveness can be as high as 95% (Pinkerton and Abramson, 1997). However, the effectiveness of condoms at the population level is not well established. Many people with HIV in Ethiopia do not know that they are infected up to now, only a small percentage of those with HIV/AIDS have had access to reliable voluntary counseling and testing (VCT) services. VCT is one of the strategies to control HIV and its services are expanding in Ethiopia. However, a study (Ermias, 2003) revealed that only 2% of men had been HIV tested, indicating that many people with HIV are not using the VCT services. Another study showed that less than 4% of those reporting risky sex had accessed VCT services (MOH, 2004).

1.4.6. Sexually transmitted diseases as a risk factor for HIV transmission

The AIDS pandemic is best described as the sum of discontinuous and overlapping epidemics of disease among populations of variable and varying risk. This provides that the epidemic of HIV infection has not arisen and does not act in isolation. It is rather a unique disease where probably for the first time in history diseases have united towards weakening and finally eliminating their hosts.

Diseases due to coinfecting pathogens may be due to primary infection, recurrent infection, or the reactivation of latent infection (Karp and Clebunders, 2007). For some pathogens, the risk factor responsible for the acquisition of the confecting pathogen is similar for the acquisition of the pathogen. Typical example for this is HIV and other sexually transmitted diseases (STDs). As a

result of this epidemiologic complexity, both the prevalence and expression of coinfection are likely to be variable across ecological, economic, political, behavioral, and cultural dividends.

These interactions affect the control of HIV and associated infections and their prevalence will increase and this will lead to increased transmission and accelerated HIV progression in the regions with intense exposure to infections (Corbett *et al.,* 2002).

Conventional STDs act as cofactors in HIV transmission by increasing HIV susceptibility and infectivity. Sexually transmitted diseases increase the amount of virus in the genital fluids of the transmitter, thereby raising transmission rates particularly from men to women (Gupta and Klasse, 2006). STDS can increase the number of infected lymphocytes in the mucosa and enhance virus expression through immune activation (Galvin and Cohn, 2004). STDs make recipient more susceptible by breaking down the mucosal barrier in the case of genital ulcer disease and increasing the number of susceptible cells in the mucosa through inflammation. STDs can also affect HIV transmission in other ways. Bacterial vaginosis (the colonization of the vagina by an aerobic bacteria), for example, may further enhance the infectivity of incoming virus by raising the pH and would lead to slower virus inactivation and more efficient Env-mediated fusion (Coombs *et al.,* 2003). In a minority of men STDs are also known in increasing seminal plasma HIV RNA to detectable levels. In their study, Barroso and coworkers demonstrated that a minority of men (2/13) with seminal plasma RNA suppressed by ARV therapy who acquired an STD had an increase in seminal plasma HIV RNA to detectable levels (Barroso *et al.,* 2000). These increases were transient; however, this observation raises two major concerns: 1) HIV suppression in the genital tract can be over-ridden by the inflammation of STDs, and 2) men with HIV are continuing to acquire STDs, a marker for unsafe sexual practices.

The sexual transmission of HIV infection within partnerships seems to be facilitated by several sexually transmitted infections(Fleming *et al.,* 1999).Longitudinal epidemiological studies have provided direct evidence that sexually transmitted infections in HIV-uninfected men and women increases their susceptibility to HIV infection, with genital ulcerative diseases, such as syphilis, chancroid, and genital herpes having larger effects on susceptibility than gonorrhea, chlamydial infection, and trichomoniasis in women (Rottingen *et al.,* 2001).

A further major concern is the potential effects of HIV on transmission rates and dynamics of HIV-associated infections that are directly or indirectly transmitted from person to person. Symptoms of STDs in the HIV-infected partner (such as vaginal or urethral discharge or dysuria, were associated with a greater risk of transmission to the uninfected partner (Flemming *et al.,*

1999). Similarly, the presence of AIDS-defining symptoms or signs was also significantly associated with an increased rate of transmission. It is a well established fact that transmission of HIV between adults in sub-Saharan Africa is mainly through heterosexual intercourse, and is enhanced in the presence of other STDs (Rottingen *et al.,* 2001). The rate of incidence of curable STDs in sub-Saharan Africa, which includes Ethiopia, is the highest in the world, with 69 million new cases per year in a population of 269 million adults aged 15-49 years (Corbett *et al.,* 2002). Data obtained from antenatal clinics shows that active syphilis range from2.5% in Burkina Faso to 17.4% in Cameroon (WHO, 2001), which is several orders of magnitude higher than Western Europe. Because HIV and other STDs share the same mode of transmission, they tend to cluster in the same subpopulations, and surveillance data from STD clinics generally show much higher HIV rates than in the general population. The rates of STDs in Africa results from a combination of behavioral factors and poor health-service delivery (WHO, 2001). Risky sexual behavior is affected by a mix of social, economic, and cultural factors, and its effects are compounded by poor access to preventive measures such as condom use.

Sex workers and their clients as core groups of high frequency transmitters play a dominant role in the transmission of HIV and other sexually transmitted disease. In a research carried out in Surabaya, Indonesia, on sex workers, the prevalence of other STDs

(Chlamydia, gonorrhea, serological test for syphilis positive, and/or trichomoniasis) in female sex workers were 48% in brothels, 42% on the streets, 16% in marriage parlours, 25% in barber shops, 17% at call-girl houses, and 10% in night clubs (Joesoef et al., 1997). Sex workers from the brothels had the highest prevalence rates of gonorrhea (24%) and trichomoniasis (8%), while sex workers from the streets and the barber shop had the highest rates of serological test for syphilis(53%) positive (30%) and Chlamydia (18%). STD rates decreased with an increase in age (except for syphilis test serum positive), an increase in education, a decrease in the number of sex partners, and condom use in the previous week.

Increases in the prevalence of syphilis, gonorrhea, and Chlamydial infection have been observed among males having sex with male (MSM) in the United States and Europe (Rottinger *et al.,* 2001). Of the HIV-positive men, 45% reported having HIV-negative sex partners and 42% reported having sex partners with unknown serostatus, whereas 14% and 57% of HIV-negative men reported having sex with HIV-positive and unknown-serostatus sex partners, respectively. Gonorrhea, chlamydia, or syphilis was diagnosed in 12% of HIV-positive and 13% of HIV-negative MSM, and the rates did not differ between men with HIV-concordant and HIV-

discordant partnerships (William *et al*., 2002). A previous study carried out in New York City, however, demonstrated decreases in prevalence both overall and among men who have sex with men, from 9% to 5% and from 47% to 19%, respectively (Torian *et al*., 1999). But despite this decline, the presence of syphilis or gonorrhea in MSM was a predictor of HIV infection.

In a study carried out in Ethiopia, STDs occurrences were found to be statistically associated with the practice of multiple sexual partner and inconsistent condom use (Belay, 2008). In this study, 48.2% of the study participants admitted having either the problem of genital discharge or ulcers during their stay in the town. This same study showed 35.5% of the currently sexually active respondents were diseased by one of the STDs during their stay in the town. This is the rate of self reported sexually transmitted diseases. Another study also showed that STDs are known risk factors in Ethiopia (EPHA, 2006). Still another study in Ethiopia showed self reported STD is only 13.7% (EPHA, 2005). This may be an understandable; since matters related to sex are personal and may not be reported because contracting such a disease is an unacceptable practice in the society. Of great importance in this study is that a significant proportion (24.3%) reported that they had not taken treatment, while 7.25% visited local injectors and 11.5% approached traditional healers. Only half of these infected (48.7%) visited health facilities.

Previously, a study carried out on a cohort of sugar factory workers showed that the prevalence of HIV and syphilis antibodies at intake in the cohort was high, 11.8% and 27.8%, respectively, with no difference by gender for HIV serological status (Arnaud Fontanet and Tilahun W/Michael, 1999). This study concluded at the end that what is true for other sub-Saharan countries is also true for Ethiopia.

Being the major health problem little studies have been carried out on STDs in Ethiopia in association with HIV and virtually no study was carried out on discordant couples. Both rates of self- reported sexually transmitted diseases and serological test results are missing on discordant couples in Ethiopia.

1.5. Diagnostic methods of HIV infection

The etiologic agents of AIDS, HIV-1 and 2, can be isolated from patients with AIDS, AIDS related complex (ARC) and from healthy individuals at high risk from AIDS Serological evidence of HIV infection may be obtained by testing for HIV antigen or antibodies in serum or plasma of individuals suspected of HIV infection. The diagnostic methods in HIV infection serve different purposes (www. Roche. Com). These are:

1. the identification and characterization of HIV infection;
2 analysis of the impact of HIV infection on the immune system;
3. the assessment of infection that are transmitted via the same route as HIV; and
4. the assessment of complications of HIV infection or its treatment.

There are several different kinds of methods to diagnose HIV infection. These are the following.

1.5.1. Diagnosis of HIV infection

The cornerstones for the diagnosis of HIV infection is an immunoassay which recognize antibodies to HIV-1, HIV-2 and to the HIV-1 group O. Antibodies to HIV are detectable within four to six weeks of infection by commonly employed tests and in virtually all infected individuals within six months (Fields, 1990). Ones antibodies appear in the blood, they persist for the life time.

Diagnosis of HIV infection can be carried out by detecting any of the following: Antibodies to HIV, p24 HIV antigens, HIV nucleic acid (RNA/DNA) and HIV in clinical samples.

In primary infection with human immunodeficiency virus, the virus in the blood can be demonstrated by nucleic acid-based test (PCR for pro-viral DNA and RT-PCR for viral RNA), p24 antigen testing or culture. The addition of p24 antigen detection into the immunoassay has the advantage of improved sensitivity in seroconverted samples (WHO, 2004).

The most commonly used test for the diagnosis of HIV infection is by serological tests detecting anti- HIV antibodies. It is economical, rapid and can be performed easily in most laboratories.

1.5.2. Detection of antibody to HIV

ELISA (Enzyme linked immunosorbent assay) is the most widely used technique for the detection of antibody to HIV. HIV antibody tests have been classified as first to fourth generation tests based on the principle used in the assay as well as the type of antigens used in the assay (WHO, 2004). The first generation tests used viral lysates as antigens and the second used recombinant HIV proteins and /or synthetic peptides as antigens. The second generation kits used recombinant proteins/peptides since antigens were used in the third generation assays. The fourth generation kits are based on simultaneous detection of HIV antibodies and immune complexes and have very high sensitivity and specificity.

The fourth generation kits are currently used frequently as antibodies to HIV-1 and /or HIV-2 can be detected throughout virtually the entire infection period, starting at or shortly after the acute phase and lasting till the end of AIDS. Progressive improvements in assay sensitivity has reduced

the so called window phase, i.e. the time between infection with HIV virus and the moment that antibodies to HIV can be detected by sensitive HIV antibody tests (www.Roche.Com). It is based on a one –step "Sandwich" principle. A mixture of HIV antigens and HIV antibodies coupled to horseradish peroxidase serves as the conjugate with tetramethylbenzidine and peroxide as the substrate. Upon completion of the assay, the development of color indicates the presence of HIV antibody or HIV antigen, while no or low color development suggests the absence of HIV antibodies or antigens (www.Roche.Com). ELISA techniques require an ELISA washer and a reader and are suitable for use in laboratories where more than 30 samples are tested each time. The results of ELISA as well as other immunoassays are expressed as positive (highly reactive), negative (non-reactive), or indeterminate (partially reactive).

This highly sensitive immunoassay lacks however a very high levels of specificity, especially in low risk individuals. Because of the limited specificity of HIV screening tests, a positive ELISA needs to be repeated and then confirmed by a more specific method which is the western blot, HIV proteins are separated by their molecular weight, and reactivity of the patient serum with the different proteins is assessed. Positive western blot needs to have positive bands of at least two or three HIV proteins p24, gp41 and gp120/160 (WHO, 2004). If these criteria are fulfilled the individual needs to be considered HIV positive. If the western blot is not considered to be positive or negative it is categorized as indeterminate. The western blot should be repeated after one month to determine whether or not the indeterminate pattern is a pattern in evolution.

Using antigens employed in the third generation ELISA systems, several rapid tests have been developed and are widely used. The commonly employed rapid anti-HIV tests are based on the principle of immunochromatography, dot immunoassay, or particle agglutination (e.g.-gelatin or latex). These tests are available in smaller test packs and each test has independent controls. Therefore, these are suitable for a laboratory that tests smaller sample numbers as well as for stand alone samples. They are technically simple to perform and most of them have sensitivity and specificity comparable to ELISA (WHO, 2004).

In Ethiopia testing algorithm in use included determine, capillus and unigold. But currently this has been replaced by testing algorithm which includes HIV1/2 Stat-pak assay, 2KHB and unigold. Assays for the detection of anti-HIV antibody in whole blood, saliva/ oral fluid, urine and dried blood spot have also been developed.

Diagnosis of HIV infection in babies born to HIV-infected mothers cannot be established by conventional antibody tests. The presence of anti-HIV antibody in the newborn may not

necessarily indicate primary infection. It may be due to passive transmission of anti-HIV antibody from mother to uninfected child. These maternal antibodies may persist even up to 18 months. Hence, diagnosis in children less than 18 months of ages is possible only by the detection of HIV nucleic acids, viral culture, or detection of the p24 antigen.

1.5.3. HIV RNA Quantification (Viral load)

HIV RNA can be found in the plasma of HIV-infected individuals in the early phase of infection and in chronic HIV infection. The quantification of HIV RNA is also known as viral load. HIV viral load refers to the number of viral particles found in each milliliter of blood. The more HIV viral load particles you have in your blood, the faster your CD4 cells are likely to be destroyed and the faster you will progress toward AIDS.

HIV viral load is determined with tests using advanced technology that are extremely sensitive for measuring the amount of HIV genetic material present in the blood. A reliable and sensitive viral load levels down to 50 copies/ml, have a high specificity (i.e., positive only when the virus is present) and provide reproducible results from one test to the next.

HIV RNA concentration allows an estimate of infectivity and represents a biomarker for the efficacy of ART, HIV-viral load measurement has specifically been important currently for monitoring treatment. It requires the establishment of a baseline plasma viral load before starting ART. The viral load in the case of successful ART becomes undetectable in 4 to 6 months of therapy (WHO, 2004).

HIV RNA can be measured using a variety of commercial kits. In all cases it is based on the principle that the conserved regions of HIV are amplified after reverse transcription and after amplification the amount of DNA is measured (Schupbach, 2003).

This method was developed in 1994 and was the first commercially available test under the name of “Roche’s AMPLICOR HIV-1 MONITOR” (www. Roche. Com). Nuclisens Easy Q HIV-1 is a nucleic acid amplification assay for the quantitative determination of HIV RNA in human EDTA plasma of HIV infected individuals using the Nuclisens Easy Q analyzer. This test quantifies HIV with a linear dynamic range from 50-3 000,000 copies/ml. Plasma samples containing HIV group M subtypes A to J have been validated for quantification by the assay. It is intended to be used for the NASBA-based amplification and real time detection of isolated HIV RNA. Nuclisens Easy Q HIV-1 consists of two step process, namely nucleic amplification combined with a homogeneous detection step. This process requires isolated nucleic acids starting material.

HIV viral load is usually reported as the number of HIV viral particles of each milliliters of blood plasma (reported as “copies/ml”). Viral load levels can range from less than 50 copies/ml to more than 20 million copies/ml. Changes in viral load are often reported as logarithmic or “log” changes. This mathematical term refers to a change in the value of what is being measured by a factor of 10.

Using appropriate standards HIV RNA can be quantitated quite precisely; however, different states such as concomitant infection or state of immune activation might affect viral replication. Therefore, single determination and changes in HIV RNA should not be used for decision making; a second HIV RNA test is required to confirm the initial finding. HIV RNA determination is recommended at the time of HIV diagnosis and at three to four months intervals. Once the treatment is initiated HIV RNA should be monitored every four weeks until steady state levels of HIV RNA are reached (WHO, 2004).

Proviral DNA can also be measured in peripheral blood mononuclear cells using a DNA PCR test. This is currently done for research use only and might be of use if serum HIV RNA becomes undetectable (www.Roche.Com). The sensitivity of the proviral DNA test is extremely high and can measure 1 copy/10,000 to 100,000 cells. Serum levels of p24 can be an important marker of newly infected HIV patients but the utility of this procedure is hampered by the existence of endogenous p24 antibodies that bind to p24 (Sierra et al., 2005). Since serum p24 levels may also correlate with viral load and this cannot be affected by the presence of endogenous antibody, it can be used as a most sensitive method to measure viral replication (Watts *et al*., 2009).

1.5.4. CD4 and T cell counts

CD4 positive cells are the major target for HIV and are reduced over time to present a marker for the state of the immune system (Borghans and DeBoer, 2007). The utility of CD4 T cell measurement involves clinical considerations for HIV disease classification and AIDS definition, assessment of prognosis, and the design of clinical trials (WHO, 2004). It is well recognized now that accurate and reliable enumeration of CD4 T cell counts is very crucial for monitoring the rate of progression to AIDS, both for initiating prophylaxis for opportunistic infection as well as monitoring the impact of ART.

CD4 measurnents is thus of vital importance in HIV/AIDS infection. Flowcytometery is the gold standard for CD4 T cell measurements and also the first choice if a large throughput of samples is required (WHO, 2004). To measure an absolute count of CD4, usually two concepts (Dual-

platform (DP) and single- platform (SP) are employed. Dual-platform approach uses two instruments to measure absolute count of CD4, while single –platform approach uses one instrument to count absolute count of CD4. In the previous approach FCM is used to generate a percentage of CD4 among lymphocytes and a hematological analyzer to enumerate the absolute lymphocyte counts and the absolute CD4 count is calculated by multiplying percentage CD4 T cells with absolute lymphocyte count. In the latter approach absolute CD4 T cell counts are derived directly without the need for a hematological analyzer by the addition of a known density of reference beads to the sample (FACSCount).

The principle of flowcytometer is based on the emission of light by fluorescence conjugated antibody stained cells (www. bdbioSCiences.Com). Flowcytometer operates by introducing cells stained with fluorescence conjugated antibody or absorption dyes in a fluid stream under a slight pressure to pass through nozzle into the beam of light, usually generated by a laser. Light that is scattered and emitted by cells is then separated into constituent wavelengths by series of optical filters and mirrirors. This separated optical light falls on individual photo detectors and then is translated into electrical pulse, or analog signals, proportional to the amount of incident light detected by the detectors. Each analog signal is finally converted into a digital signal. The magnitude of digital signals is then processed by the data processing and analysis unit. The numbers are proportional to the amount of light emitted from, or scattered by, individual staining cells.

The process of preparing the cells for flow cytometric observation is known as immunophenotyping. Immunophenotyping refers to the detection of antigenic determinants (which are unique to particular cell types) on the surface of WBCs using antigen-specific monoclonal antibodies that have been labeled with a fluorescent dye or fluorochrome (e.g.Phycoerythrin (PE), Flourescein isothiocynine (FITC), Allophycocyanine (APC) and Flourescein Isothiocynate (PerCP). The fluorochrome-labeled cells are analyzed by using a flow cytometer, which categorizes individual cells according to size, granularity, fluorochrome, and intensity of fluorescence. Size and granularity, detected by light scattering, characterize the types of WBCs (i.e., granulocytes, monocytes, and lymphocyte. Fluorochrome-labeled antibodies distinguish populations and subpopulations of WBCs.

Different kinds of immunophenotyping antibody panels and different color analysis of flow cytometer counts have been in use up to now. Accordingly two-color immunophenotyping, three-color immunophenotyping, four-color immunophentyping, etc. are in use today (www.

bdSciences, Com). Not only CD4 and CD8 T cells can be analyzed by different color parameters. Other subpopulations such as activated, memory, naive and others can be tested using flow cytometer (as indicated under material and methods).

1.6. Relevance of the study

In Ethiopia little is known about discordant couples and how HIV is transmitted from healthy to infected person and the vise versa. Sexual frequencyin realtion to viral load, cofactors associated with HIV transmission and other behavioral factors exposing subjects (couples and others) to HIV are not clearly understood. Immunologic and other host factors responsible for resistance and susceptiblity to HIVis totally unknown. Thus, there is wide gap of knowledge about HIV in Ethiopia. These types of studies are, therfore, very important to adress these isues.

An alternative approach to long-term control of viral replication and disease progression are still needed to control and prevent HIV/AIDS. One such promising area is the study of immunological, behavioral, virological and other host factors contributing to resistance to HIV infection in discordant couples.

As HIV epidemics get stabilized it is clear that the number of resistant individuals increase although in much lower frequency. Most of the resistance and transmission of HIV develops or occurs in stable and permanent relationships currently. Thus, the knowledge of discordance itself can increase its prevalence, while poor use of condoms within discordant couples increases its sero – prevalence of HIV/AIDS. Knowledge of discordance can also lead to poor relations in the couples, separation and even divorce. This study helps to address such new events in HIV prevention. Heterosexual transmission remains the most common mode of transmission of HIV and most of the transmission currently occurs between married couples. The result of this study has important implications for further understanding of heterosexual HIV transmission and for the development of means to prevent such transmission and to hopefully slow the spread of HIV.

The data generated in this study is high quality data involving behavioral, immunological, molecular biological, virological and other host factors altogether. As a result, this study would enable us to understand the unknown events in HIV transmission and dynamics. Thus, it has a practical application in solving HIV problems. Factors that affect the transmission of large number of HIV have a paramount importance in understanding the spread of HIV in a population. Understanding efficiency of transmission will inform public health policy makers, vaccine development, and the impact of intervention on the likelihood of transmission within a population. The result of this study helps immensely towards this end.

This study elucidates for the first time (only 5 full genome sequences have been sequenced up to now) the sequence of full HIV genome of Ethiopian HIV and human leukocytes antigen (HLA) in AIDS patients in adequate number. The association of HLA with the sequence of the full HIV genome helps us to identify polymorphisms that explain the variations among individuals who are susceptible to HIV. Thus, an understanding why some people establish and maintain effective control of HIV and others don't is a priority, the result of this study is of relevance to the country as it elucidates why humans show remarkable variation in vulnerability to infection by HIV and especially in the clinical outcome following infection.Many of the results of the study on discordant couples in Ethiopia are new and unknown up to now. As a result, it fills the knowledge gap existing in HIV transmission and prevention in discordant couples in Ethiopia. It also initiates further study on HIV/AIDS in Ethiopia and base every prevention intervention on the results obtained from the research.

1.7. Hypothesis

It is knwn that in all diseases there are resistant and susceptible individual. The mechanisms of resistance are not the same in all individuals. HIV is aslo a disease in which all people may not show the same type of resistance. Thus, we hypothesise that both resistance and susceptability to HIV infection is not due to a single factor but due to a concerted activitivities or factors. Resistance to HIV, thus, may be due to behavioral, immunolgic, virologic and other host factors acting in concert. The mechanisms of resistance may not be the same for all but involves several factors in all. Sceptabilty to HIV may also involve exposure to cofactors in the envirnoment such as infection by other envirnomental pathogens and due to involvement in several risky behaviors and host factors.

1.8. Objectives of the study

1.8.1 General objectives

- To determine factors associated to resistance to HIV-infection between discordant couples despite frequent exposure to HIV.
- To identify HLA alleles frequently associated with HIV infection susceptibility and to describe the full HIV genome sequence of Ethiopian HIV virus.

1.8.2. Specific objectives

- to study sexual behavior and other risk factors associated with transmission and infection with HIV;

- to determine cellular and immunological factors contributing to susceptibility or resistance to HIV;
- to examine virological factors contributing to susceptibility or resistance to HIV;
- to identify HLA alleles frequently associated with HIV susceptibility; and
- to sequence and characterize full genome of HIV isolate(s) in Ethiopian HIV.

CHAPTER II: MATERIAL AND METHODS

2.1. Study area

The study was carried out on HIV discordant, concordant and HIV-seronegative (as a control) couples from January 2007- January 2009 in five Administrative Regions and Addis Ababa, the capital city of Ethiopia. The five administrative regions were Oromia, Amhara, Tigria, and Southern Peoples Nations and Nationalities and Administrative Regions and Addis Ababa. The study involved Jimma health center, Kuyera hospital (Shashamane), Adama hospital, Fiche hospital in Oromia region; three health centers (Bahirdar health center, Abay health center, and Han health center) and Felege Hiwot hospital in Amhara region; three health centers (Kasech health center, Mekele health center, and Semen health center) in Tigria region and; Awassa health center in Southern peoples Nations and Nationalities Admistrative Region. Twenty two health centers and four major hospitals (Black Lion Referral and teaching hospital, Minilek hospital, Prince Zewditu hospital and Yakutat 12 hospital) were involved in Addis Ababa. The study was all in all carried out in government health center and hospitals. Samples obtained from these areas were analyzed in Ethiopian health and nutrition research institute (EHNRI) and Murdoch University, Australia, laboratories.

2.2. Study design

The study design was a single spot prospective cross sectional study involving comparisons of behavioral, immunological, virological and other host factors contributing to resistance and/or susceptibility to HIV infection in discordant couples. It also involved HLA class I and II typing and full genome sequencing of Ethiopian HIV.

2.3. Study population and sample size

Sample size for discordant couples has been determined for the study using a probability of 0.30(30%) and a difference of 0.05 (5 %), which the study of Kenya has showed (Okoth, 2004). Accordingly, a total of 325 discordant couples (95%CI=323), 75 concordant subjects and 5-10

low risk seronegative subjects were determined to be the sample size. The sample size was determined using the formula for single population and considering the above mentioned assumptions (Okoth, 2004).

A total of 330 discordant couples, 75 concordant couples and 7 low risk seronegative couples were investigated in this study. The inclusion criteria involved:

- willingness to provide individual informed consent, on the part of either or both members of the couples;
- acceptance of couples counseling on the part of one or both partners regarding HIV results;
- age greater than 18. Children were not included in this study since it specifically focuses on permanent sexual relationships;
- having permanent monogamous (marriage) relationships for more than one year;
- being HIV serodiscordant or seroconcordant couples; and
- being treatment (ARV) naive.

Most of the subjects were counseled, tested and registered as HIV discordant or concordant couples and were on follow up by the respective health institutions (health centers and hospitals). That is, they were identified, counseled, tested and registered as discordant or concordant couples by the nurses and doctors of the respective health centers and/or hospitals

2.4. Ethical considerations

The study was conducted in accordance with the ethical principles stipulated in the last revised version of the Helsinki declaration, the operational guideline for ethical committees of Ethiopia. The study was conducted after obtaining the national ethical clearance from the then Ethiopian Science and Technology Commission (ESTC) and the now Science and Technology and the institutional clearance from Ethiopian Health and Nutrition Institute (EHNRI) and Addis Ababa University (AAU). An official letter of cooperation attached with ethical clearance copy from EHNRI and AAU was written to regional health Bureaus and a similar letter was written from regional health Bureaus to the respective health centers and hospitals.

Participation in the study was voluntary. Detailed information about the study was made available for all patients in their language. Only patients who gave informed consent were included in the study. The consent form was completed only after the patient had understood the points

enumerated in the information sheet. All study participants were able to withdraw from the study at any point without any consequence to his/her care and clinical management.

Data on HIV status was dealt with due care for respect of anonymity. This was achieved by identifying blood samples and test results by code, not by name, with no personal identifier to link the samples to the client.

2.5. Sample collection, transportation and analysis

After the patients were identified and their willingness to participate in the research was approved, patients were asked to give samples (blood, urine and vaginal wash) and willingness to be interviewed for behavioral study. Blood was collected by trained and experienced nurses while vaginal wash with PBS was carried out by the subjects themselves after appropriate orientations were given. In a similar way, urine was also collected by the subjects themselves in a sterile urine tubes.

Twenty milliliter whole blood was collected from each study subject in vacationer tubes in EDTA and transported to the laboratory on the same day it was collected for analysis. Blood samples were always collected at the same time starting early in the mornings from 8:00 AM to 11:30 AM and was analyzed within 24 hours

The blood sample was rejected if it was haemolysed, turbid or had not been stored and transported properly, didn't carry appropriate label, and the container had leaked.

Vaginal wash and urine were collected in sterile tubes and transported to the laboratory and stored at - 80 ^{O}C until analysis.

Laboratory analysis was carried out at EHNRI and Center for Clinical Immunology and Biostatistics (CCIB) research institute attached to Murdoch University.

2.6. Questionnaire

Using structured questionnaires (See Apendex-1) interview was used to collect data.

The questionnaire contained questions related to demographic characteristics, knowledge about HIV/AIDS and VCT, previous sexual behavior, risk factors associated to HIV transmission, marriage satsfaction/disatifactiion, and sexual intercourse frequency.

The data collection instrument format (the questionnaire) was first developed in English and translated to Amharic and translated back to English by different individuals for its accuracy and desired result.

The questionnaire was structured and standardized. A pre-test was conducted on a community group who were not part of the study group. Each questionnaire was checked for completeness, missed values and unlikely responses, and then manually cleaned up on such indications.

The subjects were interviewed face-to –face using pre-tested structured questionnaire. The interviews were carried out on sample collection days in a separate and private room. The in-depth interview was check-list guided. The completeness of the data was checked every time after the data were collected by the principal investigator. Data entry (double entry), cleaning and analysis were done using the appropriate statistical software.

2.7. Data analysis

The collected data was entered and analyzes using SPSS version 13 soft ware. Mean, median, mode and standard deviation were collected for many parameters in the study. Results were compared in discordant, concordant and negative control. When the comparisons involved two groups, non-parametric (Mann-Whitney U-test) method was used. But when comparisons were made between three groups or more groups, the level of significance (α) was adjusted using Boferroni corrections ($\alpha = 0.033$). This association between several parameters was determined using a multivariate regression analysis. Correlation coefficients were calculated by the spearman's test.

2.8. Methods

2.8.1. HIV-testing

HIV testing was performed by using a combination of HIV rapid assays (according to the National HIV testing algorithm) using Determine (Abbott, Japan), Capillus (Biotech, Ireland) and Uni-gold (Biotech, Ireland) and enzyme-linked immunosorbent assay (Vironostica, HIV Uni form Ag/Ab, Boxtel, The Netherlands). The testing involved serial testing algorthim and this was done to re-test subjects which were already tested in their respective health institutions to prove whether the subjects were truly HIV positive or not and hence truly discordant or concordant couples.The enzyme-linked immunosorbent assay was carried out first and samples which were both positive and negative were re-tested by serial testing algorithim and categorized as positive and negative after the completion serial testing algorthim. Results were interpretted as positive when the test was positive by ELISA and by two successive tests of serial algorithim and negative when it was negative by ELISA and the two successive serial algorithim tests. Thus, the results were confirmed by many times testing and re-testing.

2.8.2. Syphilis serology

Syphilis serology was performed by *Treponema palladium* particle agglutination assay (TPPA) (Serodia-TPPA, Fujirebio, Japan) and rapid plasma reagin assay (RPR) (RPR-nosticon II; Organnon Teknika, Boxtel, The Netherlands), according to the manufacturers instruction. Serum samples isolated and kept frozen at -80^{0}C and tested with RPR (according to the maufaturers instruction) and samples which were positive for RPR were re-tested by TPPAand results were accepted only when were found positive by TPPA and rejected when found negative by TPPA.

2.8.3 Peripheral blood mononuclear cell isolation

Venous blood was collected from the study subjects in EDTA vacutainer tubes and serum and blood cells were separated by centrifugation. The serum was separated and stored at -80 ^{O}C until further analysis was carried out. The remaining blood cells were diluted with PBS and layered over Ficoll-Hypaque. After density gradient centrifugation on Ficoll-Hypaque, PBMC was collected and viable frozen in liquid nitrogen until further analysis was carried out.

2.8.4 Determination of viral load

Viral load was determined by quantifying the amount of HIV RNA in plasma samples stored at -80 ^{O}C using Nucleic Acid Sequence Based Amplification (NASBA) assay (NUCLISENS, Organon Teknika, The Netherlands) . The minimum detection limit of this assay was 50 copies/ml. It was known that NASBA methodology gives quantitatively reliable results on HIV subtype C plasma samples (Alaeus et al., 1997)

2.8.5 Cell surface and intracellular staining and analysis

Surface and intracellular staining and analysis was performed using standard flow cytometry procedure by FACSCalibur (BD, San Jose, CA). All straining were carried out by monoclonal antibody (mAb) to which is conjugated four different kinds of florochromes: Allophycocyanine (APC), Peridinin chlorophyll protein (PerCP), Flourescein Isothiocyanate (FITC) and Phychoerythrin (PE) (all from BD, San Jose, CA). Absolute CD4+ and CD8+ T-cells count was carried out by three color surface staining involving the following flourochrome conjugated monoclonal antibodies: CD3FITC-CD45RAPerCP-CD4PE, CD3FITC-CD45RAPerCP-CD8PE (BD, San Jose, CA). Four color surface staining was carried out by staining with four florochrome conjugated monoclonal antibody as follows: CD8PerCP-CCR5PE-CXCR4FITC-CD4APC, CD8PerCP-HLADRPE-CD38FITC-CD4APC, CD4PerCP-CD45RAPE-CD27APC-Ki67FITC, andCD8PerCP-CD45RAPE-CD27APC-Ki67FITC. PBMC were thawed (RPMI with 10% fetal

calf serum, FCS), washed with PBA (phosphate Buffer Saline with 0.05 Bovine Serum Albumin (BSA) and stained for CD3, CD4, CD8, and CD45RA for three color-testing and CD4, CD8, CD45RA, CD38, CCR5, CXCR4, CD27, HLADR monoclonal antibodies in dark for 20 minutes. After a second washing step with PBS, the cells were fixed and permeablized by incubating cells with permeabilization buffer and FACS lysing solution (BD) for 10 minutes at room temperature. Cells were then washed and stained with Ki-67 FITC for intracellular staining for 20 minutes at $4^{O}C$ in the dark. After a final washing step with permeabilization buffer, analysis was performed using three colors or four colors FACSCalibur (cellquest software, BD). In the lymphocyte gate, 50,000 to 100,000 events were acquired and results were expressed in terms of absolute number (three-color analysis) and as percentage if cell surface markers in four- color analysis. In all cases a control was set up using IgGγ APC and TriTEST (IgGγ1FITC/γ2PE/CD45PerCP. The FASCan/FACSCalibur was calibrated with CaliBRITE fluorescent beads on weekly basis.

2.8.6 Sequence Based HLA typing:

HLA-typing was determined using PCR amplification of exons 2 and 3 of the genes HLA-A, HLA-B, HLA-C and HLA-DR followed by DNA sequencing methods. Briefly, genomic DNA was extracted from Buffy coats, whole blood or plasma manually with the QIAamp DNA Blood Mini Kit (Qiagen) or the Agencourt Genfind DNA extraction kit (Beckman Coulter) with a method adapted for the Biomek FX robotic workstation. Exons two and three were amplified from each HLA gene and products were then purified using Agencourt Ampure (Beckman Coulter), sequenced with Big Dye terminator v3.1 methods (Applied Biosystems) and then cleaned up with Agencourt Cleanseq (Beckman Coulter). Finally, sample data was collected on an ABI PRISM 3730 Genetic Analyser. Applied Biosystems 3730 Data Collection software v5.0 was used to collect electropherograms which are then analysed with Applied Biosystems Software v5.2 and Assign v4.0.0.11 (Conexio Genomics Pty Ltd). The Assign program compared collected sample data against a database of known HLA sequences and assigned alleles accordingly.

2.8.7 Full Length sequencing of the HIV genome.

Methods for each step of sample preparation, including reverse transcription, PCR reactions, and sequencing were developed on high-throughput automated liquid handling instruments (Biomek FX, Beckman Coulter) with 96/384 well capacity. Briefly, depending on viral load, HIV-1 RNA was isolated from 0.5-1mL of plasma, RNA was then reverse transcribed to generate a template for full length amplicons of the HIV genome. Nested PCRs were performed to generate 2-3 amplicons

spanning the genome. Both strands were sequenced and sequencing data was collected on an ABI prism 3100XL Genetic Analyser. Sequencing software Analysis Software Assign Viral (Conexio Genomics Pty Ltd) was used for the data analysis. Alternate primers were used where necessary to fill gaps remaining in the sequence. A software program "Primer Sleeper" automated selection of alternative primers was used based on the sequence already generated.

Full and near-full genome sequencing was carried out by comparative analysis by aligning sequences to HIV reference sequence, HXB2 (Ratner et al., 1985). Where anomalies have been identified in each region they were listed along with the position. All anomalies have been checked against the sequence data and verified and checked as present. All sequence data has been verified and checked against HXB2. All data were generated by using proviral DNA and by sequencing from RNA.

Genomic DNA was obtained directly from the patients peripheral blood mononuclear cells (PBMCs) –buffy coats. Purified HXB2 DNA served as a template for sequencing; taking into consideration that the profiles of viral variations across the HIV-1genome were similar among subtype B and C. Both strand sequencing was combined with a strategy involving overlapping sequences. Dye terminator sequencing on an automated DNA sequenator (model 373A; Applied Biosystems, Inc., Foster city, Calif) was used. Thus, overlapping polymerase chain reactions (PCRs) were done to obtain the full- length and the near-full- length genome sequence of each strain.

Near full-length proviral genomes were amplified as previously reported by Gao *et al.,* 1998. The complete genome sequences were determined by the primer walking method on both strands of DNA and aligned with a set of reference sequences, using the profile alignment option of CLUSTAL W (Gao et al., 1998).

Each sample was amplified by a two-round PCR amplification reaction and all PCR fragments were detected by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. The amplified products were purified using a QiaQuik gel extraction (Qiagen, Qiagen SA, France).

Nucleotide sequences were obtained by direct sequencing of the amplified DNA, using the inner primer of the *nef* gen*e,* the inner primers of the accessory genes, and several primers encompassing the *gag* and the *pol* genes. Cycle sequencing was performed using fluorescent dye-terminator (dye terminator cycle sequencing with Ampli Taq DNA polymerase FS, Perkin Elmer, Roissy; France) according to the instructions of the manufacturer. Electrophoresis and data collection were done on an Applied Biosystems 373A (stretch model) automatic DNA sequencer. The sequence fragments

were assembled into contiguous sequencer and a consensus of the two strands was formed by using the Seqed program (Applied Biosystems, Branchburg, NJ).

The PCR conditions for each round of amplifications were as follows: a first denaturation step for 5 min at 94^0C, 30 sec at 55^0C, and 2 min at 72^0C, with a final extension for 5 min at 72^0C, in a final volume of 50ul; 20Pmol/lit of each primer, 20mmol/lit of dNTPs, and 2.5Uof Taq polymerase were used. Of this round 5ul was used for the second round, which used 32 cycles with 50^0C as the annealing temperature. This protocol was used for all viruses. Finally, these were separated by electrophoresis in 5% acryl amide gels under non-denaturing conditions at 150V for 4 h, and then visualized by autoradiography.

2.8.8.1. Blast subtyping

A web-based HIV-1subtyping system that uses the BLAST algorithm was done on our envelope gene sequence to identify subtypes (http://www.ncbi.nlm.nih.gov/retroviruses/HIV-1/). The subtyping method employs a BLAST comparison between the HIV-1 sequence to be sub typed, input sequences, and a panel of complete genome references for the subtypes A, B, C, D, E, F, G, and H of group M, as well as for group O and N available in the GenBank. This program detects the best local similarities between a query sequence and a set of HIV-1 subtype reference sequences without performing a global alignment. A sliding window along the query sequence allows the detection of possible intersubtype recombinants with interspersed regions from two or more subtypes.

2.8.8.2. Phylogenetic tree analysis

The phylogenetic neighbour-joining tree was generated establishing distances between sequences using the phylogentic computerized program. Nucleotide sequences for each of the genes were aligned using CLUSTAL W (Thompson *et al.,* 1994) with minor manual adjustments, bearing in mind the gene sequence sites where there was a gap in any of the sequences, as well as areas of uncertain alignment, were excluded from all sequence comparison. The phylogenetic tree was constructed from nucleotide sequences, using the neighbour-joining method and the Kimura two-parameter model. That is, genetic distances were calculated with Kimura's two parameter method (Kimura, 1980). The bootstrap values at each node represent the percentage of 1000 bootstrap replication that supports the branching order.

Analyses of envelope C2V3 hypersensitive loop was also carried out to analyse co-receptor usage and percentage of viruses using CCR5 and R4 was determined for HIV positive discordant couples and for HIV positive concordant couples

CHAPTER III: RESULTS

3.1. Study population and Demographic and epidemiological profile

A total of 650 discordant, 150 concordant and 8 healthy control subjects responded to the in-depth interview from 824 proposed study participants (98% response rate). All the study participants were sexually active and were from urban areas with a mean age of 32(15-65). Most of them (83%) were between 20-37 years of age (Fig 8 A). As shown in Table 6 the majority (787(95%) were Orthodox and 25(3%) Protestant Christians, and 16(2%) were Muslims. 353 (43.5%) had elementary level of education, 239(29.5%) secondary level of education, 27(3.3%) college and above level, 32(3.95%) read and write and 159(19.6%) were illiterates (Figure8 C). As presented in figure 8 B many of the subject studied were house wives 246(30%), laborers 228(28%) followed by government employees 162(19.8%) and merchants 77(9.4%). A large proportion (76.8%) of the subjects had two or more number of live children (Table 2). The average number of children was 2.6The demographic characteristics of the study population were as shown in Table 2.

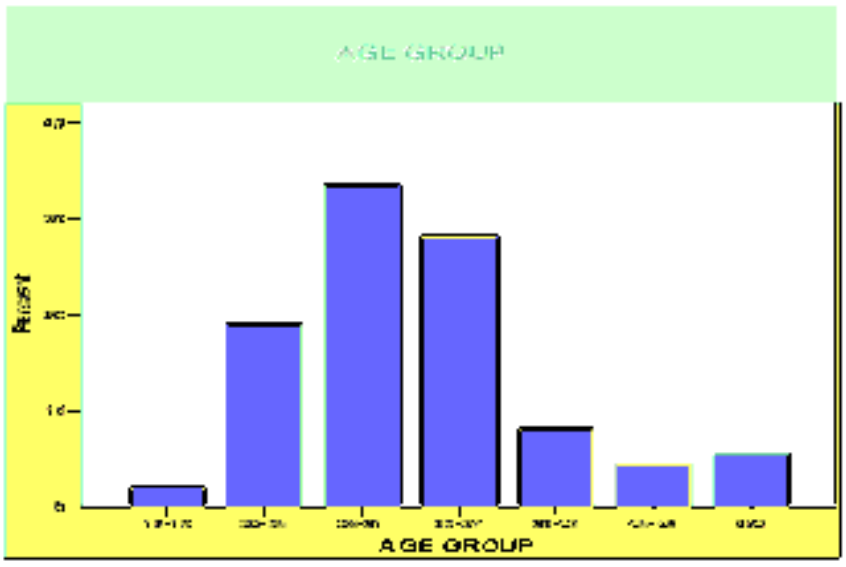
Table 2: The demographic characteristics of the study population. NGO, Non-government organization; **SPNNR,** Southern peoples nations and nationalities region

Variable		Discordant couples	Concordant couples	Negative control	Frequency	100%
Sites collected	Oromia	102	26	1	258	31%
	Amhara	30	5	1	72	8.7%
	Tigria	25	5	1	62	7.5%
	SPNNAR	42	5	1	96	11.7%
	Addis Ababa	130	35	3	336	40.8%
Gender	Male	329	75	7		
	Female	329	75	7		
Age(years)	15-19	178	3	2		
	20-25	305	28	2		
	26-31	112	51	2		
	32-37	25	42	4		
	38-43	30	12	4		
	45-49	-	8	-		
	>50	-	8	-		
Mean age(range)	32(15-65)					
Marital status	Married	650	152	14		
	Single	-	-	-		
Number of years in marriage	1-3	178	51	2		
	4-9	305	62	2		

	10-15	112	27	4		
	16-20	25	2	6		
	>20	30	10	-		
Religion	Orthodox				783	95%
	Protestant				25	3%
	Muslims				16	2%
Educational status	Illiterate	132	25	2	159	19.6%
	Read and write	30	2	-	32	3.95%
	Elementary	269	79	5	353	43.58%
	Secondary	193	41	5	239	29.50%
	College and above	20	5	2	27	3.33%
Occupation	Farmer	15	7	-	22	2.79%
	Merchant	71	6	-	77	9.55%
	House wife	203	43	-	246	30.52%
	Gov. employee	116	40	6	162	20.09%
	NGO	20	7	1	28	3.47%
	Laborer	188	34	6	228	28.28%
	Broker	10	3	-	13	1.61%
	Driver	20	3	-	23	2.53%
	Soldier	5	1	1	7	.86%

Variable		**Discordant couples**	**Concordant couples**	**Negative control**	**Frequency**	**Percentage**
Numberof children	One child	167	33	1		
	Two children	426	44	1		
	Three children	333	41	6		
	Four children	333	15	-		
	Five children and above	300	14	-		

A



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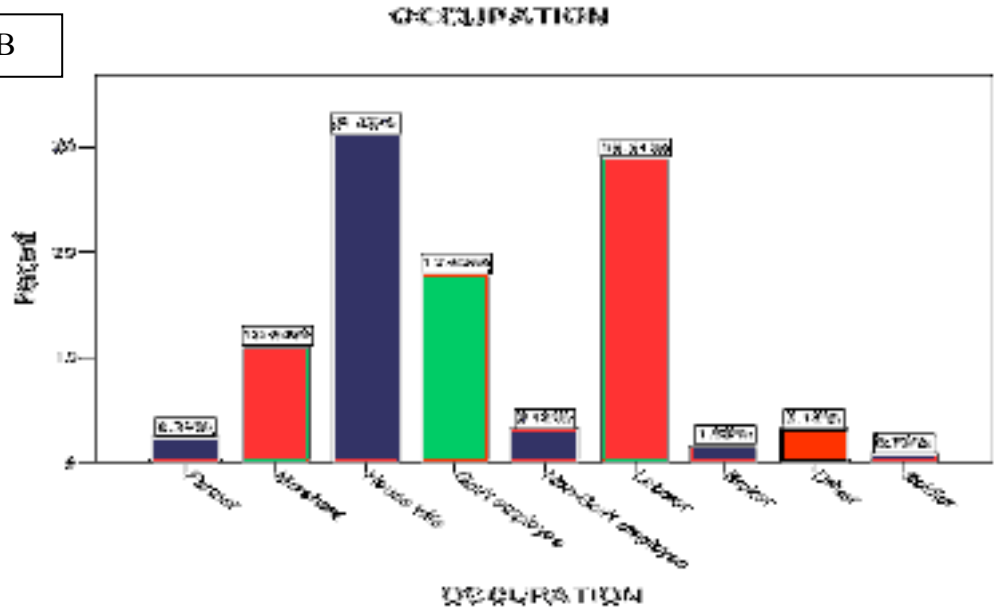

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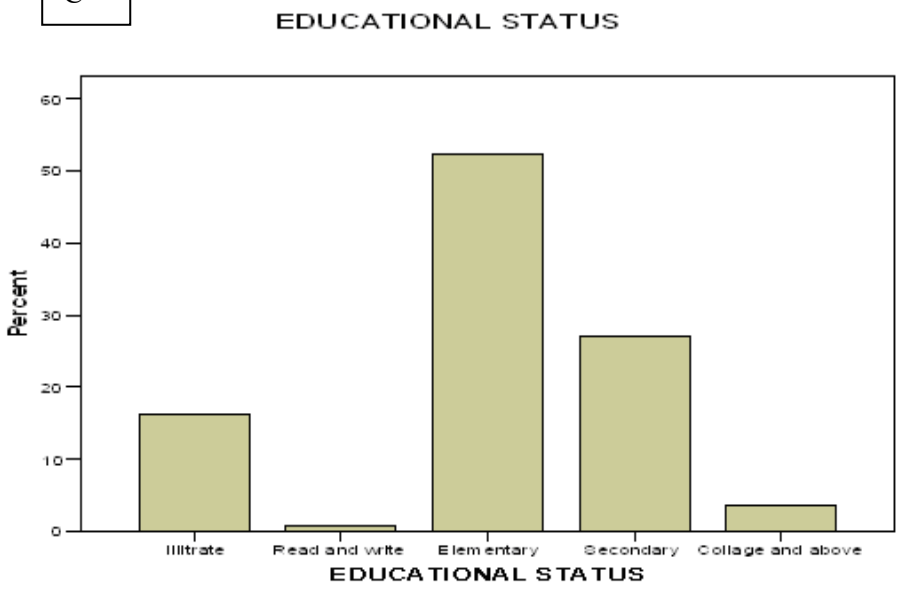
EDUCATIONAL STATUS
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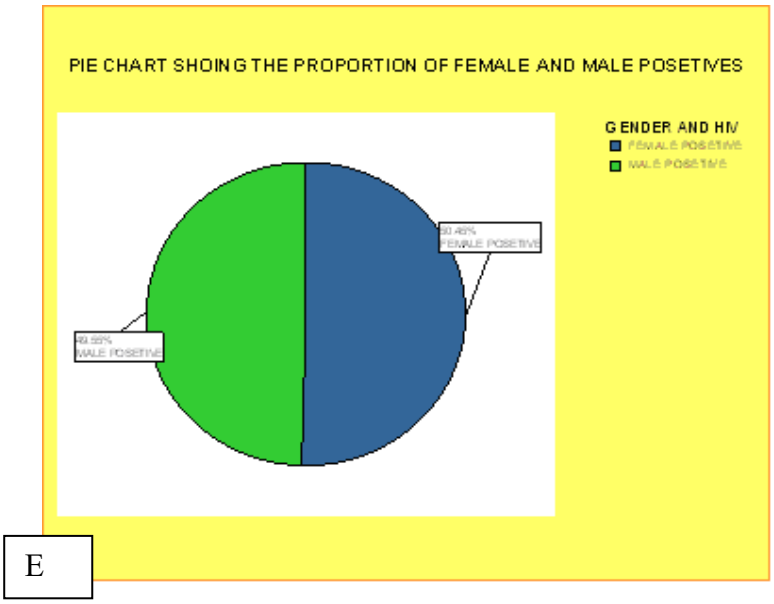

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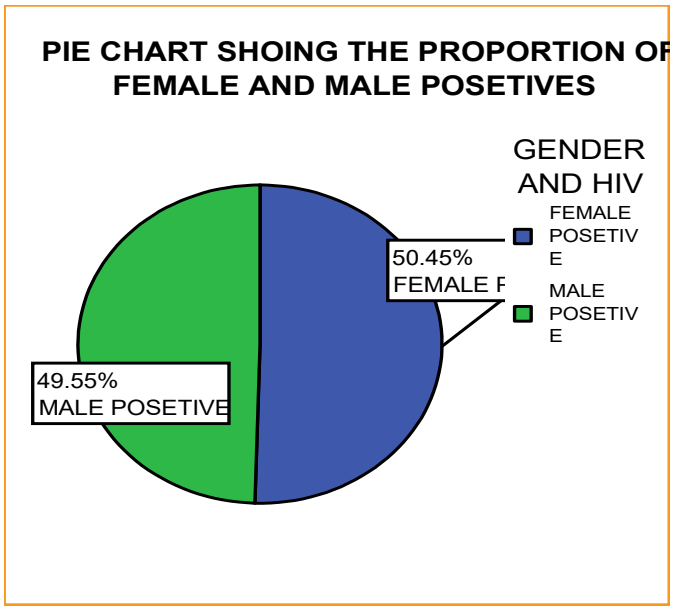


Figure 8. Epidemiological features, A) Age group B) Occupation C) Educational status D) proportions of male and female positive in concordant couples E) Proportions of male and female HIV positive in discordant couples

3.2. Sexual behavior study

All subjects were couples on permanent marriage relationship for more than one year. The proportion of male and female positives were 323 (49%) for male and 328 (51%) for females, OR=.527 (95% CI .324-.856) showing that females were slightly more HIV positive than males in discordant couples (Fig 8 E). For concordant subjects the proportion of negatives and positives was

the same in both sexes (Fig. 8 D). When educational status was compared with serostatus, there were more positives in the illiterate, elementary and secondary education level than in those who can read and write and those with college and above educational background. But the difference was not significant (p>.057). The positives were common in the age range of 20-30, where as the negatives were common in the age range of 32-50.

All subjects were engaged in sex before marriage and had multiple partners. Only 106(33%) discordant negatives (DSCN), 91(28%) discordant positives (DSCP), 28(25%) concordant couples (CONC) and 6(75%) healthy control subjects had one partner in their life time (Table 3). The majority 227(67%) discordant negatives, 234(64%) discordant positives, 83(75%) concordant couples, and 2(25%) healthy controls had two or more sexual partners before marriage. Multiple partner number was greater in concordant couples than in discordant couples. The average number of sexual partners was 2.52(1-5) (95%CI 2.37-2.67) for discordant couples and 2.62 (2-7) (95%CI 2.37- 2.69) for concordant couples. The difference between discordant and concordant couples was however not significant.

Frequency of sexual intercourse per week was only one for 62(19%) discordant couples and 25(22%) of concordant couples (Table 3). It was normal (two times) for 142(43%) discordant negatives, 127(39%) discordant positives and 39(35%) concordant couples. Discordant negatives had more normal sexual desire when compared with discordant positives (43% vs 39%) and concordant couples (43% vs 35%); discordant positives had more normal sexual frequency when compared to concordant couples (39% vs35%). Sexual frequency was normal for 43% discordant couples, 35% concordant couples and the majority (75%) of healthy controls (Table 7). Sexual frequency was greater than three times per a week for122 (38%) discordant negatives, 135(42%) discordant positives, 47(42%) concordant couples, and 2(25%) healthy controls. In all cases, i.e. in both discordant and concordant couples, the percentage of people involved in more than three times sex per a week was greater than normal control.

The number of sexual intercourse per each time was one in 142(44%) discordant negatives, 140(43%) discordant positives, 45(40%) concordant couples and 1(12.5%) healthy control. It was two times in each contact in129 (40%) discordant negatives, 137(42%) discordant positives, 49(44%) concordant couples and 7(87.5%) negative control. The number of sexual act per each time was greater than two times in 18(32.5%) discordant negatives, 155(56.5%) discordant positives, and 60(65%) concordant couples The number of times sexual intercourse was done in each contact was highest for concordant couples(65%) when compared to both discordant

positives(56.5%) and discordant negatives(32.5%). There was also a big difference between discordant negatives (32.5%) and discordant positives (56.6%). The number of people involved in three sexual acts per each time was less than 16% in all cases but highest for concordant couples and discordant positives but lowest for discordant negatives. For the vast majority, the number of intercourse in each time was between one and two times (Table 3).

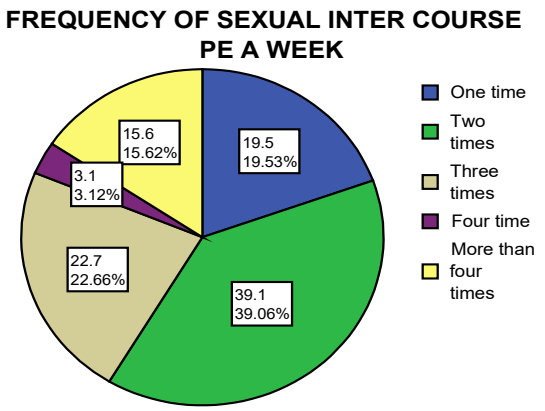
Table 3: Behavioral characteristics of HIV discordant, concordant and normal control subjects. DSCN= discordant negative, DSCP= discordant positive, CONC concordant couples, n= sample size

Variables		DSCN (n=325)	DSCP (n=325)	CONC (n=152)	Healthy controls (n=14)
Number of previous sexual partners	Only one partner	106 (33%)	91(28%)	28(25%)	6(75%)
	Two partners	112(34%)	112(34%)	42(38%)	2(25%)
	More than two partners	107(33%)	122(30%)	41(37%)	
Frequency of sexual intercourse/week	One time	61(19%)	63(19%)	25(22%)	
	Two times	142(43%)	127(39%)	39(35%)	6(75%)
	Three times	74(23%)	74(23%)	32(28%)	2(25%)
	Four times	7(2%)	10(3%)	4(4%)	
	More than four times	41(13%)	51(16%)	11(10%)	
Frequency of sex / time	One time	142(44%)	140(43%)	45(40%)	1(12.5%)
	Two times	129(40%)	137(42%)	49(44%)	7(87.5%)
	Three times	46(4%)	11(12%)	14(13%)	
	More than three times	8(2%)	7(2.5%)	3(3%)	
Protected or unprotected sex	With condom always	18(6%)	13(4%)	1(1%)	7(87.5%)
	With condom sometimes	6(2%)	5(2%)	4(4%)	1(12.5%)
	Without condom always	298(92%)	307(94%)	106(95%)	
Type of sex involved	Soft	190(58%)	109(34%)	25(23%)	6(75%)
	Aggressive	135(42%)	216(66%)	86(77%)	2(25%)
HIV awareness	Previously aware	106(33%)	91(28%)	17(15%)	8(100%)
	Aware after VCT	213(66%)	292(90.7%)	94(85%)	

Of those with sexual frequency greater than three times per a week 36.5% were in the ages between 20-43 years of age. Sexual frequency of more than three times a week was highest for age ranging from 26-31. When education was compared with sexual frequency, sexual frequency of more than three times was highest (17.4%) for those with elementary level of education followed by those

with secondary (13.7%) level of education and illiterates (5.8%). Sexual frequency of more than three times was lowest for those with college and above and those with the ability to read and write. Sexual frequency increased as the number of previous sexual partners increased. A frequency of more than three times was observed in 25(9.8%) of those with one previous sexual partner, in 34(13.3%) of those with two previous sexual partner, and in 41(16%) of those who had three previous sexual partners.There was an association between sexual frequency, educational status and previous sexual partners (r=.168, p>.087) but the difference was not significant. There was a significant association between number of previous sexual partners and sexual frequency(r=.551, p=.026). The following Pie chart (Fig 9) shows frequency of sexual intercourse per a week (A), frequency of sexual intercourse per time (B) and number of previous sexual partners (C).

A



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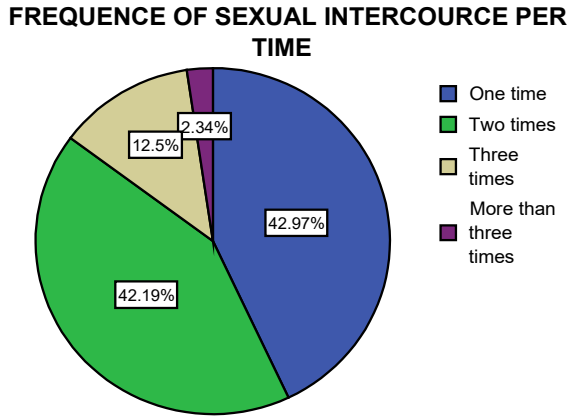
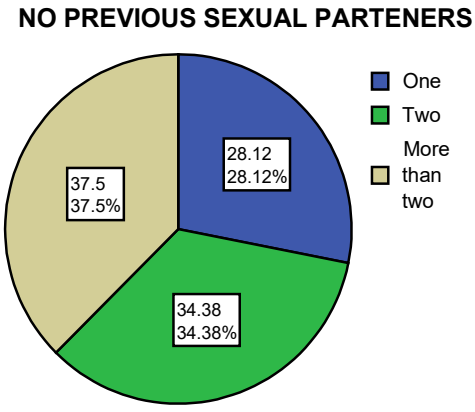




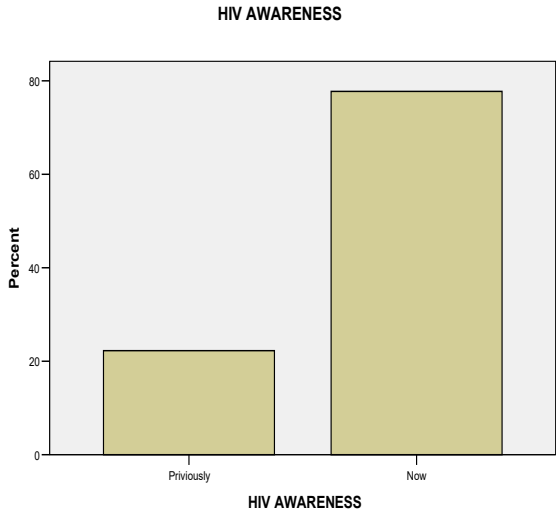
C

Figure 9. Frequency of sexual intercourse per week (A) and frequency of sexual intercourse per time (B) and number of previous sexual partners(C)

The type of sex involved by the subjects were classified into soft sex (not associated with cut, aberrations, traumas and bleeding and aggressive sex (associated with traumas, cuts, aberrations and bleeding most of the time). The discordant negatives were involved more in soft sex 190 (58%) when compared with aggressive sex 135(42%) (Table 7). Discordant positives (Table 7) and concordant couples were involved more in aggressive sex than soft sex (66% vs 34%) and (77% vs23%), respectively. The majority (75%) of the healthy controls were involved in soft sex (Table 7). HIV positive partners in both discordant and concordant couples were involved in aggressive sex most of the time when compared with HIV negative partners. Concordant couples were also involved in aggressive sex most of the time than discordant positives.

When HIV serostatus was compared with the type of sex, it was found that those who were involved in soft sex were less likely to be exposed to HIV (OR=. 280) (95%CI= .171 - .436) than those who were involved in aggressive sex. This implied that the odds of contracting HIV were between .171 - .436 times lower among those who were involved in soft sex than those who were involved in aggressive sex.

A



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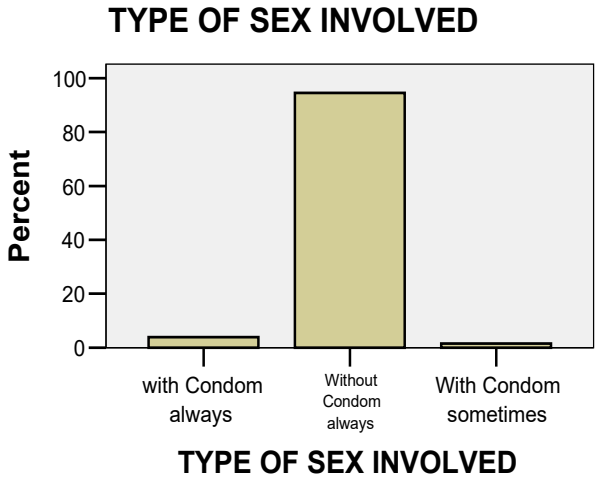


Figure 10 A) HIV awareness B) Types of sex involved (with/without condom)

Despite being involved in aggressive sex, most of the study subjects were also involved in unprotected sex (Table 10) (Fig 10 B). Only 18(6%) of discordant negatives, 13(4%) of discordant positives, 1(1%) 0f concordant couples were involved in safe sex before marriage. But most of the negative controls 7(87.5%) used condoms repeatedly before marriage. The majority, 298(92%) of discordant negatives, 307(94%) of discordant positives and 106(95%) of concordant couples were involved in unprotected sex before marriage (Table3). Concordant couples were involved more in unprotected sex than discordant couples (95% vs 93%) The majority of the subjects were involved in unprotected aggressive sex before marriage (Fig 10 B). Only very few of the subjects 106(33%) of discordant negatives, 91(28%) of discordant positives, 17(15%) of concordant couples were

aware of HIV before VCT (Fig 10 A) when compared with healthy control 8(100%) (Table 3) .The majority 213(66%) discordant negatives, 292(90.7%) discordant positives, and 94(85%) of concordant couples were aware of HIV after VCT (Fig 10 A). The majority of both discordant and concordant couples (Fig 10 A) were not aware of HIV before VCT.

All subjects were multiply married and there was no HIV testing in between each marriage. Only 154(47%) discordant negatives, 104(32%) discordant positives, 37(33%) concordant couples were married once (Table 4).

Table 4: The number of times Married and problems of marriage associated with risk factors. DSCN (discordant negative), DSCP (discordant positive), CONC (concordant negative), and NC (negative control)

Variable		DSCN (n= 325)	DSCP(n=325)	CONC(n=152)	NC (n=14)
Number of times married	Only one time	154(47%)	104(32%)	37(33%)	5(63%)
	Two times	140(43%)	160(49%)	46(42%)	3(37%)
	Three times	23(7%)	51(16%)	20(18%)	
	More than three times	8(3%)	11(4%)		
Number of years in marriage	1-3 years	89(27%)	81(24%)	37(33%)	2(25%)
	4-9 years	153(47%)	155(48%)	46(42%)	2(25%)
	10-15 years	56(17%)	56(17%)	20(18%)	2(25%)
	16-20years	13(4%)	15(5%)	1(1%)	2(25%)
	More than 20 years	15(5%)	18(6%)	7(6%)	
Satisfaction with marriage	Yes	302(93%)	289(89%)	101(91%)	8(100%)
	No	23(7%)	37(11%)	10(9%)	
Reasons for satisfaction in marriage	Sexual satisfaction	10(3%)	25(8%)	13(12%)	
	Presence of children	33(10%)	6(2%)	10(9%)	
	Handling (caring)	14(4%)	12(5%)		
	Honesty and faithfulness	10(3%)	11(5%)		2(25%)
	Love	24(7%)	5(2%)	7(6%)	2(25%)
	Religion	14(4%)	3(1%)	1(1%)	
	Economic	10(3%)	20(6%)	15(13%)	
	More than one of the above factors	210(66%)	248(76%)	60(54%)	4(50%)
Reasons for dissatisfaction in marriage	Economic	46(14%)	13(4%)	17(15%)	
	Handling	31(10%)	33(10%)	11(10%)	
	Absence of children	46(14%)	13(4%)	20(18%)	
	Disagreement	46(14%)	22 (7%)	23(21%)	
	Sexual incompatibility	46(14%)	76(23%)	8(7%)	
	Dishonesty	32(10%)	86(26%)	18(16%)	
	More than of the above	78(24%)	8(26%)	14(13%)	

One – time marriage was greater (47%) for discordant negatives when compared with discordant positives (32%), concordant couples (33%) and less than the healthy control (63%).

More than 50% of discordant negatives (171(53%)), 221(68%) discordant positives and 67(61%) concordant couples were married for more than two times. The percentage of more than two times marriage was greater (68% and 61%) for HIV positive partners when compared with HIV negative partners (53%) (Table 4). The average number of marriages was 1.90 for HIV positive partner and 1.65 for HIV negative partners (p<.009). Overall, the average number of marriage for all was 1.77 (1-5). There was a weak positive relationship between numbers of times married and sexual frequency (r=.107, p>.663).

Multiple marriages without testing for HIV are a known risk factor for HIV transmission. The majority 242(74%) discordant negatives, 236(72%) discordant positives, 83(75%) concordant couples divorced in less than 10 years of marriage life. The chance that the marriage could survive for more than 10 years was less than 30% for all. Although divorce rate was higher and multiple marriages was frequent, the majority 302(93%) discordant negatives, 289(89%) discordant positives, 101(91%) concordant couples and 8(100%) negative couples were very much satisfied with their marriage when compared with 23(7%) discordant negatives, 37(11%) discordant positives and 10(9%) concordant positives (Table 4).

The reasons for satisfaction and dissatisfaction in marriage were found to be diverse and variable as indicated in Table11. Only 24(7%) discordant negatives, 5(2%) discordant positives, 7(6%) concordant couples and 2(25%) healthy controls attributed success in their marriage to love, which is a a strong power in marriage life.

3.3. History of STDs and perceived mode of HIV infection

3.3.1. History of STD

Sexually transmitted diseases (STDs) are one of the major co-factors in facilitating HIV transmission. Gonorrhea, syphilis, Chlamydial infections and chancroid are the most common STDs in Ethiopia. To assess the role of STDs as a facilitator of HIV infection, study subjects were asked for the history of the symptoms of STDs. Fifty six percent of discordant positives and 54% of concordant couples and 43% discordant negatives disclosed the history of STDs in their life times, while 57% od discordant negatives, 44% of discordant positives, 46% of concordant couples and 100% of the negative controls disclosed that they have not observed any kind of symptoms of STDs in their life time (Table 5). The prevalence of the history of the symptoms of STDs was not the same between HIV positives and negatives. It was 43% yes and 57% no for discordant negatives; 44% no and 56% yes for discordant positives couples. With a mean difference of 1.57 between

discordant and concordant couples, when an independent T-test was calculated, the difference was found to be significant (p<.046).

Table 5: History of STD and perceived mode of HIV infection. DSCN (Discordant negatives), DSCP (Discordant positives), CONC (concordant couples)

Variable		DSCN	DSCP	CONC	NC
Encounter with STD	Yes	140(43%)	181(56%)	60(54%)	
	No	185(57%)	144(44%)	51(46%)	8(100%)
Types of STDs suspected	Gonorrhea	48(34.4%)	79(43.6%)	19(31.6%)	
	Syphilis	46(32.8%)	23(12.3%)	25(41.6%)	
	Others	46(32.8%)	79(43.6%)	16(26.6%)	
Perceived HIV infection mechanism	Infection in a family		41(13%)	7(6%)	
	Promiscuity		69(21%)	23(20%)	
	Multiple marriage		3(1%)	4(4%)	
	Extramarital sexual intercourse		10(3%)	6(5%)	
	Occupational risk		33(7%)	4(4%)	
	Contact with risk group(sexual)		74(23%)	24(21%)	
	contact with risk group		23(7%)	4(4%)	associated wth livilihood
	Infection from a partner		48(15%)	32(29%)	
	Rape		3(1%)	5(5%)	
	More than one of the above		39(9%)	2(2%)	

The history of acquisition of STDs were also associated with HIV seropositivity (r=.256) and odd ratio (OR= 1.071). The odd ratio (OR= 1.070> 1) implied that among subjects with previous STD symptoms the chance of being positive was higher than those without symptoms (95% CI= .664-1.725). This showed that the odds of contracting HIV were between .664 and 1.070 times higher among those with symptoms than without symptoms.

The most common STDs encountered were (Table 5) Gonorrhea 48(34.4%) in discordant negatives, 79(43.6%) in discordant positives, 19(31.6%) in concordant couples; syphilis 46(32.8%) in discordant negatives, 23 (12.3%) in discordant positives and 25(41.6%) in concordant couples. Other STDs such as chancroid and the like were also reported as 46(32.8%) in discordant

negatives 79(43.6%) in discordant positives and 16(26.6%) in concordant couples (Table 5). No history of STDs was reported in negative controls.

3.3.2. Perceived mode of HIV infection

To determine how HIV was transmitted from one person to another in discordant couples and to learn from this mode of transmission and to avoid future transmission by learning from the past history of HIV positive discordant partners based on the known HIV risk factors, subjects were asked how they were infected with HIV. Naive views of HIV transmission and transmission methods which were not associated with the known risk factors were discarded and the perceived HIV infection mechanisms were found to be **Promiscuity**(69(21%) discordant positives, 23(20%) concordant couples), **infection within a family (** 41(13%) in discordant positives and 7(6%) in concordant couples), **multiple marriage with infected partner** (3(1%) in discordant positives and 4(4%) in concordant couples) , **extramarital sexual intercourse** (10(3%) in discordant positives 6(5%) concordant positives) , **occupational risk** (33(7%) in discordant positives and 4(4%) in concordant couples), **contact with risk group (associated with livelihood)** (23(7%) in discordant couple and 4(4%) in concordant couples), **contact with risk group (sexual)** (74(23%) in discordant positives and 24(21%) in concordant couples), **infection from a partner**(48(15%) in discordant positives and 32((29%) in concordant couples), **rape** (3(1%) in discordant positives and 5 (5%) in concordant couples) and **more than one of the above factors**(in 39(9%) in discordant positives and 2(2%) concordant couples) (Table 5). The most common perceived HIV infection mechanism(s) were infection from a partner, contact with risk groups and promiscuity.

3.4. Imunological Profile: CD4/CD8, other subpopulations, Coreceptors and cofactors

To asses if the difference in the amount of CD4 and CD8 T cells were responsible for the susceptibility and/or resistance to HIV infection in discordant couples, absolute counts of CD4 and CD8 T cells were measured using a three color – flow cytometer. The result showed that the median average number of CD4 in discordant negative partners was 749(95% CI 706-792) and 570(95% CI 483-658) (Table 6) in discordant positive partners and the difference was very highly significant ($p < .001$). Similar result was also obtained for CD8 T cells. The median average number of CD8 T cells in discordant negative partners was 921(95% CI 825-1017) and in discordant positive partners it was 850(95% CI 798-904) and the difference was not significant ($p > .05$) (Table 6). The ratio of CD4 to CD8 in discordant negative partners was 0.81 and 0.67 for discordant positive partners and

0 .94 for the negative control (Table 6). There was a positive correlation (r =.520) between CD4 and CD8 in discordant negative partners although this was not significant (p > .05) (Table 7). Comparison of the difference between CD4 and CD8 in discordant positive partners, however, showed a negative correlation(r = -.468) and the difference was very highly significant (p <.002) (Table 6). The median average number of CD4 and CD8 for healthy control subjects (CD4 (879(95% CI 762-996); CD8 (934(95% CI 854-1013) was very similar to the discordant negative partners and the difference was not significant (p> .05). But there was a big difference (p < .05) between the negative control and the positive discordant partners. The ratio of CD4 to CD8 (0.94) was also very close to discordant negatives than discordant positives (Table 6).

The median average of CD4 count for concordant couples was 261 (95% CI 278-325) and CD8 673(95% CI 455-779) and the ratio of CD4 to CD8 was very low (0.37) (Table 6). There was a week negative correlation (r = -.014) between CD4 and CD8 but the difference between CD4 and CD8 in concordant couples was very highly significant (p<.0001) (Table 6 and 7).The difference in CD4 between concordant couples and discordant partners was two-fold and significant (p<.05) (data not shown). Similarly, the difference in CD8 numbers between both groups was also highly significant (p<.05) (Table 6).

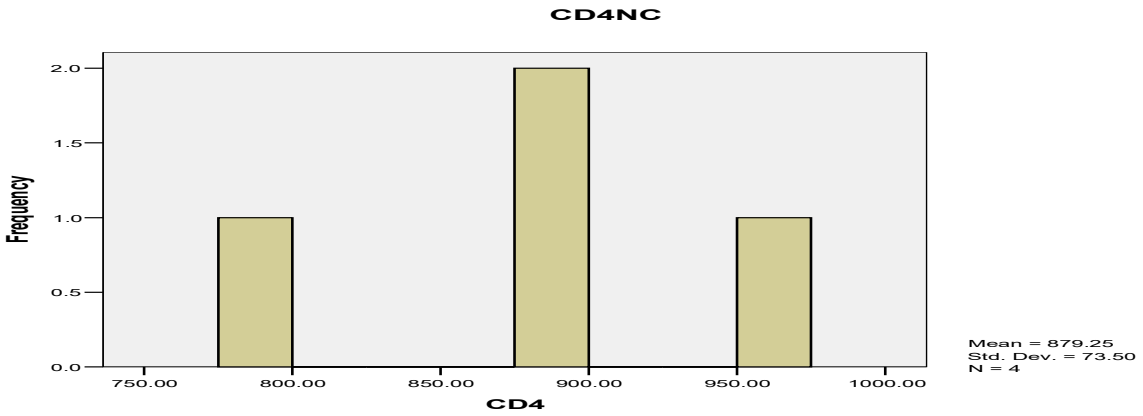
Table 6: CD4, CD8, CD4/CD8 ratio and percentage of syphilis serology test result of discordant, concordant couples and the negative control

parameters	variables	Median(Range)	95%CI	percentage	Ratio	p-value
Discordant negatives (n = 46)	CD4	749(603-792)[a]	706-792	45		a and b .001
	CD8	921(720- 1200)[c]	825-1017	55		c and d >.05
	CD4/CD8 ratio				.81	
	Syphilis (n = 79)			8.3		
Discordant positives (n = 46)	CD4	570(297-790)[b]	483-658	40		b aand d <.002
	CD8	850(720- 997)[d]	798-904	51		
	CD4/CD8 ratio				.67	
	Viral load	4733(130-32000)				
	Syphilis			44.4		
Concordant couples (n=46)	CD4	261(81-446)[e]	278-325			e and f P<.0001, b and e <.05
	CD8	673(455-779)[f]				
	CD4/CD8 ratio				.373	
	Viral load	272480(200,000-290,000)				
	Syphilis(n=232)			33.3		
Negative control couples (n= 4)	CD4	879(790-970)[g]	762-996	48.5		b and g and d and t <.05
	CD8	934(883-1002)[h]	854-1013	51.5		d and h >.05
	CD4/CD8				.94	
n = 232	Syphilis(n=232)			0		

For the majority of discordant negatives CD4 was greater than 700 (Fig. 11 A and B) while CD8 was for all greater than 700. CD4 count was between 400 and 800 and CD8 was between 700 and 1000 for the majority of discordant positive partners (Fig 11 C and D). For concordant couples, however, CD4 was between 200 and 450 and CD8 was 600 to 800 for the majority of the subjects

(Fig 11 G and H). The result of the negative controls was very similar to discordant negatives, although was slightly higher (Fig 11 G and H).

A



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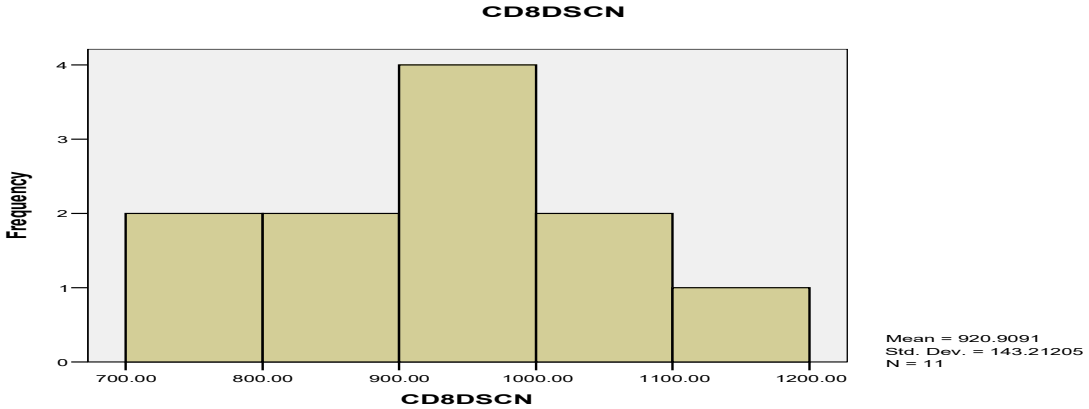

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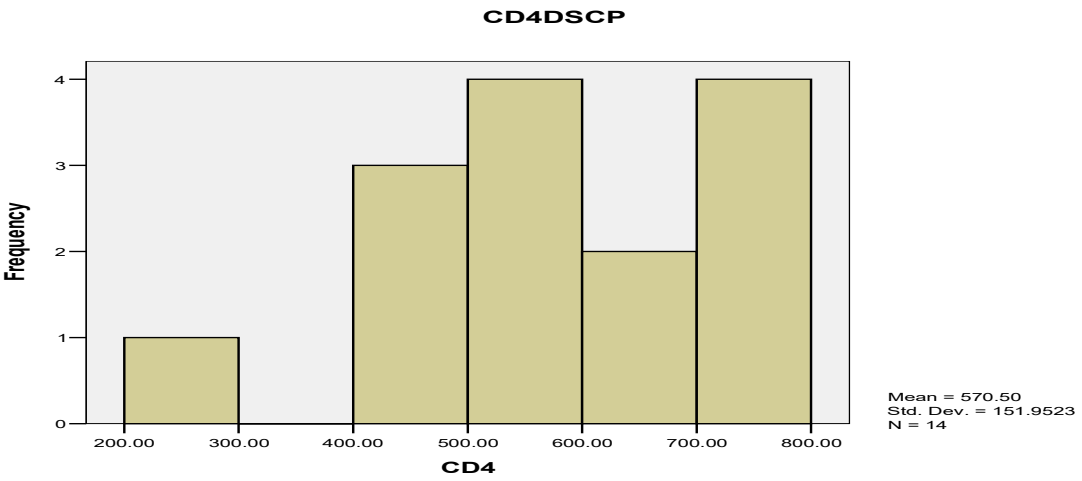
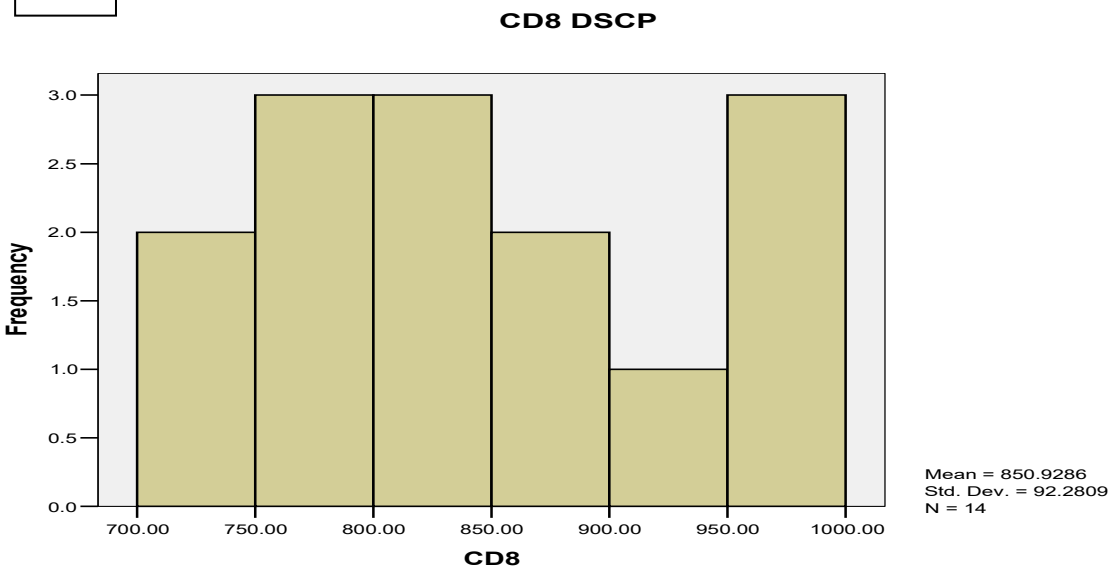
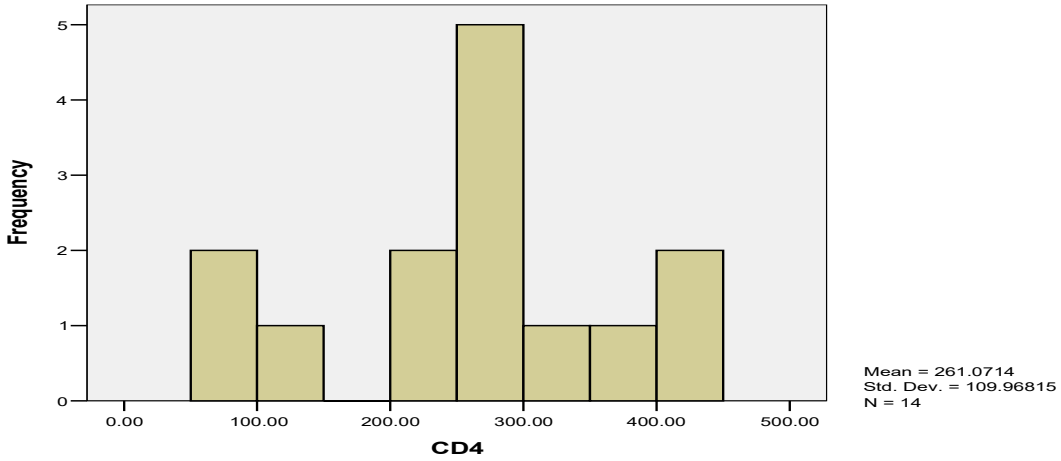

CD4DSCP
Frequency
0
1
2
3
4
200.00
300.00
400.00
500.00
600.00
700.00
800.00
CD4
Mean = 570.50
Std. Dev. = 151.9523
N = 14

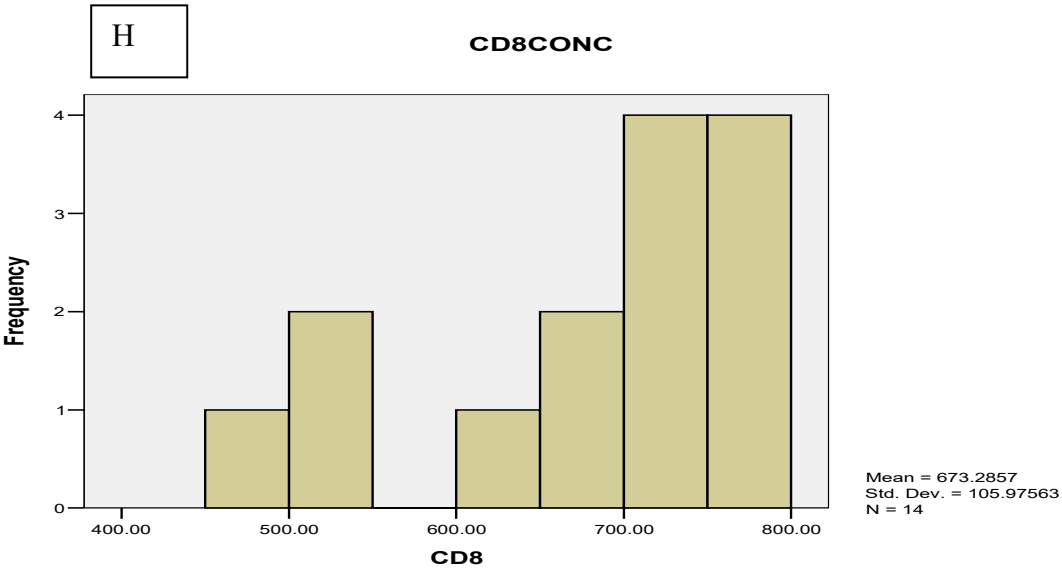
D


CD8 DSCP
Frequency
0.0
0.5
1.0
1.5
2.0
2.5
3.0
700.00
750.00
800.00
850.00
900.00
950.00
1000.00
CD8
Mean = 850.9286
Std. Dev. = 92.2809
N = 14

G


CD4CONC
Frequency
0
1
2
3
4
5
0.00
100.00
200.00
300.00
400.00
500.00
CD4
Mean = 261.0714
Std. Dev. = 109.96815
N = 14

H


CD8CONC
Frequency
0
1
2
3
4
400.00
500.00
600.00
700.00
800.00
CD8
Mean = 673.2857
Std. Dev. = 105.97563
N = 14

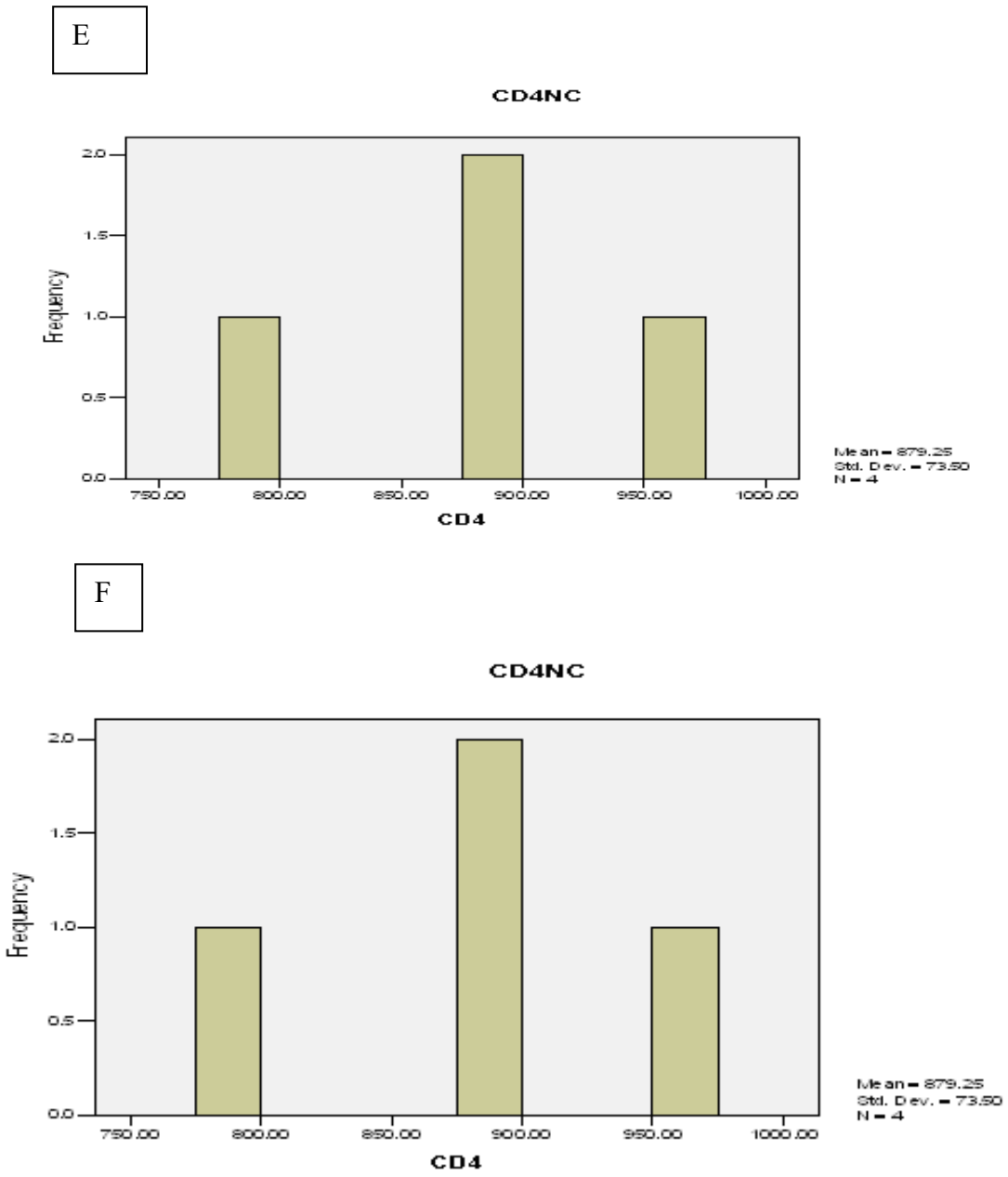


Figure 11 Frequencies of CD4 and CD8 in discordant negatives (A and B), discordant positives (C and D), concordant couples (E and F), and negative control (G and H)

When syphilis serum antibody was tested to determine co-factor effect of STDs, 34 %(79 out of 232) were positive for syphilis. This included 8.3% discordant negatives, 44.4% discordant positives and 33.3% concordant couples (Table 7). This result was greater than what has been reported in the report of the history of STDs during the behavioural study. The average number of viral RNA count/ml of blood was 4733 (130- 32000) c/ml for discordant positives and 272480(200 000- 290 000) c/ml for concordant couples (Table 6).

There was about 60- fold difference in viral load count between discordant positives and concordant couples (Fig 12). The difference between viral load of discordant positives and concordant couples was also very highly significant (p<.001).

Among discordant positives 15 out of 61 (24.5%) were serologically positive for HIV but viral load was below detection level (BDL) (Fig 12). The difference between those serologically positive with detectable viral load and those who are serologically positive but without detectable viral load was also very highly significant (P<. 001) (Fig 12). These subjects had elevated number of both CD4 and CD8 and were negative for syphilis.

Viral load was also found to be closely associated with CD4, CD8 and serological syphilis positivity (Table 7). There was a negative correlation (r = -.662) between viral load and CD4 and a weaker negative correlation (r = -.244) between viral load and CD8 in discordant positives. The relationship was not however significant (p> .05) in both cases in discordant positives. There was however, a weak positive correlation(r = .125) between viral load and CD4 and viral load and CD8 and the relationship was not significant (p> .05) in concordant couples (Table 8).The association of viral load and syphilis was a weak positive correlation for discordant positives(r=.085) with high significance (p < 0) and a weak negative correlation (r= -.198) correlation and very high significance (p< .002) for concordant couples (Table 8).

The relationship of CD4 between discordant negative partners and discordant positive partners was an inverse relationship (r = -.671) (p< .05) and the difference between them was significant (p<. 05) (Table 8) (Fig 13).There was a positive but a weak correlation (r = .299) between CD4 of discordant negatives and concordant couples (p >.05) but the difference was highly significant (p< 0). There was no difference between CD4 of discordant negatives and the negative controls (p> .05). However, there was a direct relationship between CD8 of discordant negatives and discordant positives (r = .432) (p> .05) and their difference was not significant (p > .05). But the difference in the number of CD8 T cells between discordant negatives and concordant couples was highly significant (p< 0).

Table 7: Correlations and Paired T-test of CD4, CD8, viral loads and Syphilis of Discordant, Concordant and Negative controls

Parameters	Variables	Correlations	Paired sample t-test
Discordant negatives (n = 46)	CD4-CD8	.522	.099
	CD4- Syphilis	.382	.246
	CD8- Syphilis	.53	.099
Discordant positives (n = 46)	CD4- CD8	- .468	.000
	CD4-Viral load	- .662	.054
	Viral load- CD8	- . 244	.047
Concordant couples	CD4-CD8	-.54	.000
	Viral load-CD4	.125	.075
	Viral load-CD8	-.125	.075
Negative control (n = 4)	CD4- CD8	.595	.405

But there was no difference in the number of CD8 T cells between discordant negatives and the healthy control. Figure 13 summerizes the relationship between CD4 and CD8 in discordant positives, discordant negative, concordant couples and the negative control.

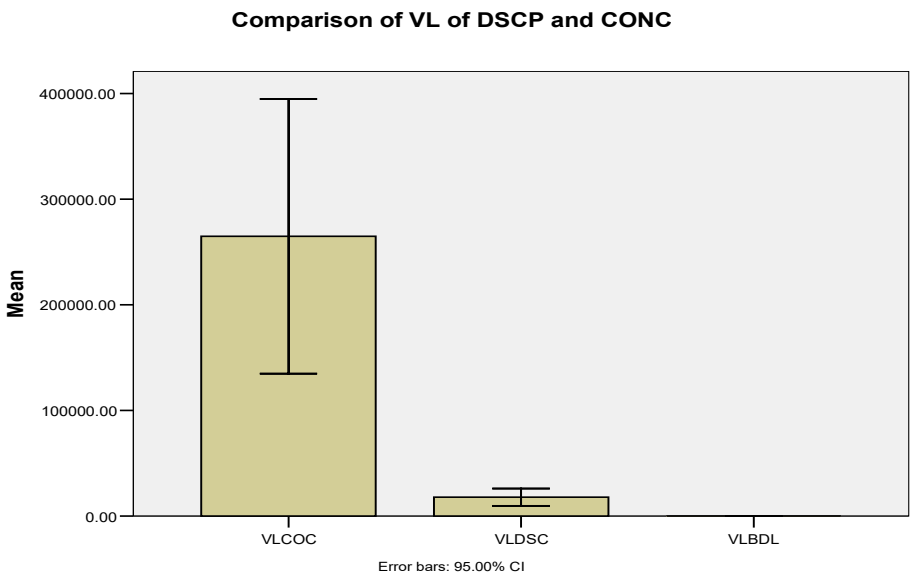


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Figure 12 Comparison of Viral loads of discordant positives (VLDSC), concordant couples (VLCON) and discordant positives below detection level (VLBDL)

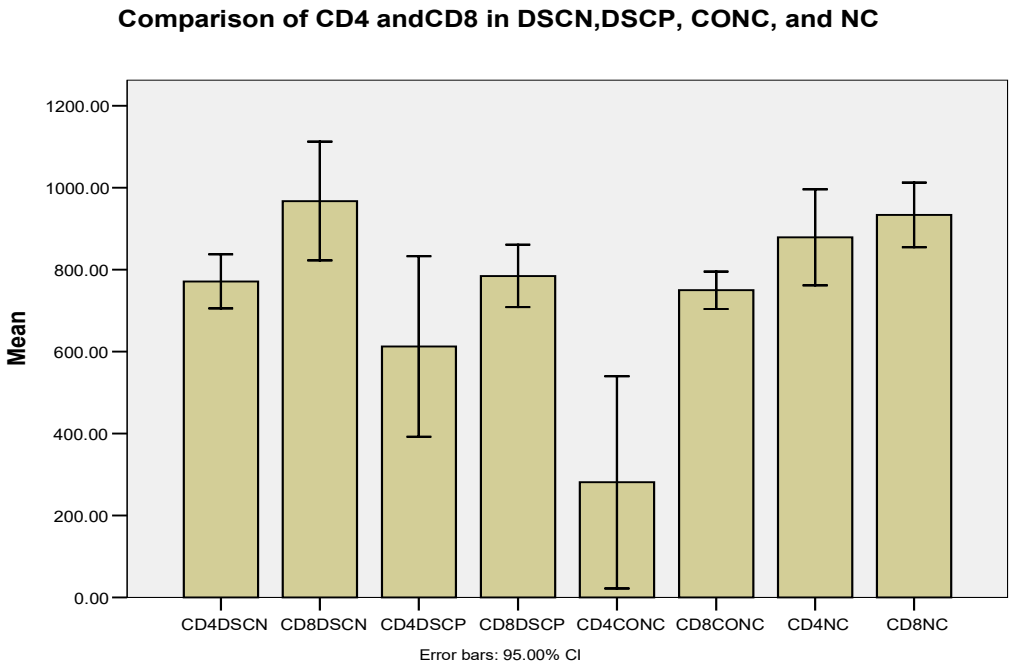

Figure 13 Comparisons of CD4 and CD8 in discordant negatives (DSCN), discordant positives (DSCP), concordant couples (CONC), and Negative controls (NC)

Table 8: Correlations and paired sample T-test of CD4, CD8 and viral loads of DSCP (discordant positive) and CONC (concordant couples)

Variables	Parameters	Correlations	sig	Paired sample t-test
CD4	CD4DSCN-CD4DSCP	-.671	.024	.033
	CD4DSCN-CD4CONC	.299	.372	.000
	CD4DSCN-CD4NC	.217	.783	.066
CD8	CD8DSCN-CD8DSCP	.432	.184	.082
	CD8DSCN-CD8CONC	.199	.558	.000
	CD8DSC-CD8NC	.352	.648	.498
CD4 and CD8	CD4DSCN-CD8DSCN	.552	.099	.001
	CD4DSCP-CD8DSCP	-.443	.149	.002
	CD4CONC-CD8CONC	.189	.573	.000
	CD4NC-CD8NC	.595	.405	.162
Viral load	VLCONC-VLDSCP	-.110	.610	.000
	VLCONCO-VLBDL			.001
	VLDSCP-VLBDL			.000

The relationship between CD4 and CD8 was also variable. There was a direct correlation (r = .552) between CD4 and CD8 in discordant negatives and there was also a significant difference between them ($p < .001$) (Table 8). But there was a negative correlation (r = -.443) between CD4 and CD8 of discordant positives and negatives, although was not statistically significant ($p > .05$). The difference between CD4 and CD8 of discordant positives and negatives was however very significant ($p < .002$). The difference between CD4 and CD8 of concordant couples was also very highly significant ($P < 0$) (Table 8). The difference between CD4 and CD8 of the negative controls was however not significant ($p > .05$).

To investigate if the difference in serostatus to HIV was due to the difference in the expression of certain markers in both CD4 and CD8 T cells, an in depth analysis of proportions of T cells subpopulations was carried out on thawed cryopreserved PBMC using a four-color flow cytometric staining as indicated under material and method. To investigate the proportions of naive, memory and effector T cell subsets, T cell subsets were measured using a combination of CD27 and CD45RA monoclonal antibodies. Activated and resting T cell subsets were measured using a combination of HLA-DR and CD38 monoclonal antibodies and the expression of CCR5 and

CXCR4 was measured using a combination of CCR5 and CXCR4 monoclonal antibodies as indicated under material and method. In a similar way, recently proliferating T cell subpopulations of memory, naive and effector T cells were measured by nuclear staining of Ki67 antigen using Ki67monoclonal antibodies. Table 9 summarizes the observation.

Table 9: Proportions of Tcell subsets of discordant negatives, discordant positives, concordant couples and the negative control (% of mean + SD, Median percentile (95%percentile range)). Top value = mean ± SD (percentile), bottom = median percentile (range)

T cell subsets	Discordant negative (n = 27)	Discordant positives (n=27)	Concordant couples (n= 10)	Negative control (n = 4)	P value
CD4	18.7±9.5 17(4-38)	11.6±7.7 10(2-36)	4.0±0 4.0(4-4)	15.8±8.9 15.5(8.24)	P<.05
CD8	26.8±13.2 27(0-62)	20.2±14.6 21(1-50)	78.1±28.5 91.5(20-90)	23 ±5.8 22.5(4-33)	P<.01
CD4+CCR5+CXCR4-	9.9 ±15.4 6(0-82)	7.4±13.5 4(0-7)	8.1±6.5 8(0-18)	8.0±3.4 9.5(3-10)	p<.05
CD4+CCR5+CXCR4+	1.1±2.1 0(0-9)	1.6± 4.7 1(0-25)	8.4±11.7 6(0-40)	0.5±0.57 0.5(0-1)	P<.01
CD4+CCR5-CXCR4+	9±23.5 2(0-82)	3.9±8.6 1(0-44)	4.8±12.3 1(0-25)	1.3±0.5 1(1-2)	p<.05
CD4+CCR5-CXCR4-	69.8±32.7 85(10-99)	82±3.1 90(18-100)	9.4±2.1 2(0-40)	90.3 ±4 89(87-96)	P<,01
CD8+HLADR+CD38- (activated)	15.7±14 14(0-62)	20.2±17.3 16(0-62)	25.5±16.5 26.5(0-48)	35±8.9 31.5(29-48)	P<.05
CD8+HLA-DR+CD38+ (activated)	1.5±2 2(0-3)	1.9(0-2) 1(0-11)	31.3±28.6 25.5(3-10)	1.3±0.95 1.5(0-2)	P<.01
CD8+HLA-DR-CD38+ (activated)	2.3 ±2 .31(0-16)	5.5±10.2 2.0(0-15)	2.4±2.9 1(0-10)	4.0±3.5 4(1-7)	
CD8+HLA-DR-CD38- (resting)	77.9±8.8 84(35-99)	67.9 ±19.4 74(36-98)	4.3±7.6 1(0-20)	59.5±5.8 61.5(51-	P<.01

T cell subsets	Discordant negative	Discordant positive	Concordant couples	Negative control (64)	P-value
CD4+HLA-DR+CD38- (activated)	14.2± 4 12.0(0-58)	21.0±6.0 18(2-62)	25.5±16.5 26.5(0-48)	34.3 ±8.8 31(28-47)	
CD4+HLA-DR+CD38+ (activated)	2.1±1 2(0-5)	2.6±1 2(0-10)	31.3±28.6 25.5(3-100)	1.8±0.5 2.0(1-2)	P<.01
CD4+HLA-DR-CD38+ (Activated)	2.9 ±3 3.0 (0-8)	7.8±2 4(0-65)	2.4±2.9 1(0-10)	2.8±2.9 1.5(1-7)	
CD4+HLA-DR-CD38-(resting)	70.6±40 76(40-98)	60 .8±16 60(36-89)	62±25.9 67(0-96)	60.8±7.9 62(50-69)	
CD4+CD27+CD45RA- (memory)	9.7±1 4(0-51)	9.2±14.3 5(0-54)	4.3±7.6 1(0-20)	7.8±0.5 4(1-2)	P<.05
CD4+CD27+CD45RA+(naive)	13.3± 0 10(0-50)	11.9±13.0 8(0-53)	20.8±20.6 10.5(0-58)	12.5±3.1 12.5(9-16)	P >.05
CD4+CD27-CD45RA+ (effector)	24.1±1 18(0-68)	21.2±20.6 18(0-62)	18.2±22.5 9.5(1-65)	52.8±14.7 52(37-70)	P<.01
CD4+CD27-CD45RA- (memory/effector)	36.5±14 19(1-94)	40.1±31.3 30(0-54)	17.4±13.4 16(1-35)	26.8±10.2 26.5(17-37)	P<.05
CD4+CD27+CD45RA-Ki67+	15.4 ±9 9(0-85)	20.3±28.3 7(0-50)	41.6±31.5 40(0-78)	6.0±3.6 7(1-9)	P<.001
CD4+CD27+CD45RA+Ki67+	7.5±0 6(0-20)	9.7±11.3 8(0-50)	10.8±21.4 4(0-70)	5±3.2 4.5(2-9)	P<.05
CD4+CD27-CD45RA+Ki67+	5.8±0 4(0-34)	5.7±6.7 4(0-24)	12.6±19.8 4(0-65)	2.3±1.7 2.5(0-4)	P<.05
CD4+CD27-CD45RA-Ki67+	4.3±0 2(0-19)	5.1±5.1 4(2-6)	1.9±4.4 0(0-11)	3.8±2.6 4.5(0-6)	

Comparisons between subpopulations were not significantly different in most cases between discordant negatives and discordant positives and the results were also similar with the results of the

negative control with some exceptions. But the results of concordant couples were in many cases different from both discordant couples and the negative control.

CD4+ memory T cells (CD4+CD27+CD45RA-) were almost equal with the negative control in discordant positives and discordant negative and was significantly ($p<.05$) different from concordant partners (Table 9). The difference in the proportion of memory T cells between discordant couples was not significant ($p >.05$) and it was almost equal. The proportions of naive CD4+(CD4+CD27+CD45RA+) T cell subsets were comparable between all groups although it was lower than the negative control in all cases but was not significant($p >.05$). The proportion of naive and memory T cell subset was almost 1:11 in concordant couples, showing that memory T cell were very much depleted in concordant couples. The ratio of memory to naive T cells (0.5) was highest for discordant positives when compared with discordant negative(0.4) and the negative control(0.32).Thus, there were more memory T cells in discordant negative and positive partners(although naive T cells were greater than memory T cells), showing a differently operating mechanisms of immunity between discordant and concordant couples.

Effector T cell subpopulation (CD4+CD27-CD45RA+) was comparable between discordant partners (almost 2X of concordant couples) (Table 9) but the proportion was more than 2-fold greater in the negative control subjects and was very highly significant ($p< .01$) when compared with discordant and concordant partners.

Memory/effector T cell subpopulation was highest in discordant positives (30%) (Table 9), followed by discordant negatives (19%) and was lowest (16%) in concordant couples. The median percentage of memory/effector T cell subpopulation in discordant couples was also greater than the negative control and the difference between discordant and concordant couples was significant ($p<.05$). In all groups, memory/ effector T cell subpopulation was highest when compared to naive, memory or effector T cell subpopulations independently. Rate of T cell subpopulation proliferation, as assessed by nuclear Ki67 antigen measurement was highest (40%) in concordant couples and these were memory T cells. In the remaining subpopulations rate of recent proliferation ranged from 0-11%, indicating slow rate of recent proliferation. The rate of proliferation was very similar between discordant partners, showing that their rate of proliferation was not different. Overall, currently proliferating T cell subpopulations were highest in concordant couples than in others. For the remaining groups of T cell subpopulations recently proliferating cells were less than 10% but in all the proportion of recently proliferating T cell subpopulations were greater than the negative contro (Table 9).

The ratio of CD27+:CD27- was lowest for concordant couples (.36) highest in discordant positives followed by discordant negatives (0.43, 0.40), respectively. In discordant positives the ratio was greater than the negative control and in discordant negatives it was equal to the negative control.

The majority (60- 78) of the T cell subpopulations were resting T cells (Table 9) , as assessed by the measurement of CD4+HLA-DR-CD38- expression. The remaining subpopulations were activated, although T cells from the negative control were most activated when compared with discordant and concordant couples. Most of the activated T cells expressed CD4+HLA-DR+CD38- markers (Table 9). But in concordant couples equal amount of CD4+HLA-DR+CD38- and CD4+HLA-DR+CD38+ (26.5% vs 25.5%, almost 1:1) markers were expressed. T cell subpopulations from discordant positive (18%) were more activated than T cell subpopulations from discordant negatives(12%) but the difference was not statistically significant ($p>.05$).But this was highest in concordant couples and the negative control, showing that concordant couples and the negative control subjects were significantly more activated than DSC couples.

Like CD4+ T cells, CD8 T cells from discordant positives and discordant negatives were largely resting T cells (74-84%) and this was also similar to the negative control (61.5%). But here the negative control is less than discordant partners. The proportion of resting T cells in concordant couples was much lower (1%) than discordant partners and the negative control and this was highly significant ($p<.01$) (Table 9). There were many activated CD8+T cells in concordant couples than in discordant partners. Like activated CD4+ T cells, the healthy negative controls had also more activated CD8+ T cells than all of them. The difference between the negative control and discordant negatives and concordant couples was also significant ($p<.05$). Overall, discordant negatives were the least activated, which were followed by discordant positives. The activated T cells in both CD4+ and CD8+ expressed more HLA-DR+CD38- markers than HLA-DR-CD38+ subpopulations.

The majority of T cell subpopulations (80-90%) didn't express either CCR5 or CXCR4 receptors except in discordant couples (Table 9). CCR5 expression was higher in concordant couples (8%) when compared with discordant positives (4%) and discordant negatives (6%). The negative controls expressed more CCR5 (9.5%). In all CXCR4 expression was lowest (1-2%) including the negative control. The double positives were also lowest in all except in concordant couples (6%), overall the expression of both receptors was comparable between discordant positives and negatives and could not account for sero-discorance, although lower expression of CCR5 in discordant negatives and positives may indicate slower or non transmission in these subjects.

3.5. Viral gentyping

To investigate whether the difference between discordant positives and concordant couples was due to viral genetic factors or not, partial and full genome sequencing was carried out on HIV isolates from discordant positives, concordant couples and HIV/AIDS patients as indicated under material and method (Appendex-2). Full or near- full length genome sequencing was carried out from proviral DNA and from viral RNA, by comparative analysis by aligning sequences to HXB2, and by amplifying using PCR techniques, as indicated under material and method.

Out of the total full and near-full length viral sequences, 9 full genome sequence of viruses isolated from discordant positive, 2 full genome sequence of viruses isolated from concordant couples, and 24 full genome sequences of viruses isolated from HIV/AIDS patient isolated viruses were analyzed using different methods for their viral subtype, co-receptor usage and geographical similarities with other viruses from different countries. Table 10 summarizes the results obtained from these analyses.

Table 10: HIV subtypes and Co-receptor utilization by these viruses. DSCP = discordant positives, CONC = concordant couples, HIV/AIDS = HIV AIDS patients. All samples were obtained from Ethiopian regions (ET = Ethiopia, AWA = Awassa, BCT = Bahir Dar central clinic, AKC = Akaki Kaliti clinic (Addis Ababa). ARC = Arada clinic (Addis Ababa)

No.	Id	DSCP, CONC and HIV/AIDS	Subtype	Co-receptor	Lanl Geography	Remark
1	AKC14	CONCP	C	Ambiguous	DJ, IS	
2	AKC139	DSCP	Ambiguous			
3	ARC15	DSCP	C			
4	AWA3	DSCP	C		BW, MW	
5	AWA7	DSCP	C			
6	AWA9	DSCP	C			
7	AWA10	DSCP	C			
8	AWA18	DSCP	C			
9	AWA25	DSCP	C	CCR5		
10	AWA32	CONC	C			
11	BCT10	DSCP	A	CXCR4	Ug, KE, TA	
12	ET002	HIV/AIDS	B	CXCR4		
13	ET003	HIV/AIDS	C	CXCR4		
14	ET004-1	HIV/AIDS	C(wt)	R5X4	C, IN	very similar to HXB2
15	ET004-2	HIV/AIDS	C	CXCR4	DJ, FL, ZA	
16	ET005	HIV/AIDS	C	CXCR4	ZM	
17	ET006	HIV/AIDS	C	CXCR4		
18	ET007	HIV/AIDS	C		ZM,MW, BW	
19	ET008	HIV/AIDS	A or A/G	CXCR4		
20	ET009	HIV/AIDS	C			
21	ET010	HIV/AIDS	C			
22	ET011	HIV/AIDS	C			
23	ET012	HIV/AIDS	C			
24	ET013	HIV/AIDS	C	CXCR4		
25	ET014	HIV/AIDS	C	CXCR4		
26	ET016	HIV/AIDS	A or A/G	CCR5		
27	ET018	HIV/AIDS	A or A/G	CXCR4		
28	ET022	HIV/AIDS	C	CXCR4		
29	ET024	HIV/AIDS	C	R5X4		
30	ET031	HIV/AIDS	C			
31	ET052	HIV/AIDS	C	CCR5		
32	ET060	HIV/AIDS	C	CCR5		
33	ET062	HIV/AIDS	C	CCR5		
34	ET064	HIV/AIDS	C	CCR5		
35	ET068	HIV/AIDS	C	CCR5		

HIV *Env* gene sequence based sub typing was carried out using three web-based: Seq Locator Ianl (www.hiv. Ianl. .gov/contents/sequence/LOC), Raga HIV-1 sub typing Tool-version 2.0 (hivdb.stanford.Edu/Rega subtyping/) and HIV-1 automated HIV blast Ianl (www.hiv.Ianl.gov/content/sequence/BAS). Results were accepted when there was agreement between the results of the three and rejected or considered as ambiguous when there was disagreement between the three results. HIV blast Ianal was also used to locate the geographical similarity of the subtypes with other countries' or continent's subtypes.

The result was as shown in Table 10 and Table 11. In discordant positives 77% (7/9) were subtype C while 11 %(1/9) was subtype A. Recombinant forms were not observed in discordant positive subjects. In concordant couples all, 100% (2/2) were subtype C, a virus subtype which is a predominant form in Ethiopia. In HIV/AIDS patients the majority79% (19/24) were subtype C subtypes. But other subtypes such as subtype B (4.1%; 1/24) and recombinant A or A/G forms 12.5% (3/24) were also found (Table 11).

Lanl geographical distributions showed that many of these viruses were very similar to other African country's subtypes (Table 10). Some of these countries were neighboring countries such as Djibouti, Kenya, Uganda, and many East and Central African countries such as Tanzania, Zambia, Zimbabwe; and South African countries such as Malawi and Botswana. Similarity was also observed between Israel and Ethiopian subtype sequence in one patient (Table 10). In one case the wild type viral sequence was also observed.

The observation of other subtypes such as subtype A and B and the recombinant forms than were previously observed subtype C and C' showed that other viruses, other than type C were introduced or being introduced to Ethiopia. The emergence of recombinant forms also showed that people are acquiring multiple viruses and new forms are still appearing.

A. Aligned V3 region Type CCR5

Consensus B:

```
TGT ACA AGA CCC AAC AAC AAT ACA AGA AAA AGT ATA CGT ATA GGA CCA GGA CAA GCA TTC TAT GCA ACA GGA GAC
C   T   R   P   N   N   N   T   R   K   S   I   R   I   G   P   G   Q   A   F   Y   A   T   G   D
ATA ATA GGA GAT ATA AGA CAA GCA CAT TGT
I   I   G   D   I   R   Q   A   H   C
```

Query:

TGT ACT AGG CCC AAC AAT AAT ACA AGG AAA AGT GTG AGG ATA GGA CCA GGA CAA ATG TTC TAT GCA ACA -- GGA ATA

C T R P N N N T R K S V R I G P G Q M F Y A T -G I

ATA GGA AAT ATA AGA CAA GCA CAT TGT

I I G N I R Q A H

B. Aligned V3 region –CXCR4

Consensus B

TGT ACA AGA CCC AAC AAC AAT ACA AGA AAA AGT ATA CGT ATA GGA CCA GGA CAA GCA TTC TAT GCA ACA GGA GAC

B C T R P N N N T R K S I R I G P G Q A F Y A T G

ATA ATA GGA GAT ATA -- -- AGA CAA GCA CAT TGT

D I I G D -- R Q A H C

Query

TGA ACA AGG CCC AAC AAT AAT ACA AGA AAA AGT AGT GAA AGG GAC TGG AAT AAA ACT TTA ACA AGT GTA AGT GAA

C T R P N N N T R K S S E R D W N K T L T S V S E

AAA CTA AAA GAA CTC TTC CCT AAT AAG ACA -- --

K L K E L F P N K T -- -

Figure 14 Aligned V3 region A) Type CCR5 B) Type CXCR4

Viral coreceptor usage was also analyzed by using web-based, WebPSSM, gene2pheno (genotype (version.0)), as previously described by Fouchier et al., 1992,,aimed at revealing the relationship between V3 loop sequence and viral coreceptor usage. Ethiopian HIV viruses used CCR5 (Fig 13 A) and CXCR4 (Fig 14B) coreceptors. Others which used R5X4 were also observed. Table 10 and 11 shows the types of viruses using CCR5, CXCR4 and R5X4 coreceptors and their proportions. HIV discordant positive subjects used CCR5 and CXCR4 coreceptors in equal proportion (Table 11). Their ratio was 1:1. Significant proportion of HIV/AIDS viruses (61% (11/18) used CXCR4 and about a third, 33.3(6/18) used CCR5 coreceptors (Table 11). About 5.7% (2/18) used dual coreceptors (Table 11). The ratio of CXCR4 to CCR5 was almost 2:1.

Table 11: percentages of different subtypes and Co-receptor types. DSCP= discordant positive, CONC= concordant couples, HIV/AIDS= HIV AIDS patients

Parameters	DSCP (%)	CONC (%)	HIV/AIDS (%)	TOTAL ISOLATES
Subtypes				
A	11(1/9)			9
A orA/G(recombinants)	0		12.5(3/24)	24
B	0		4.1(1/24)	24
C	77(7/9)	100(2/2)	79(19/24)	35
Co-receptors				
CCR5	50(1/2)		33.3(6/18)	20
CXCR4	50(1/2)		61(11/18)	20
R5X4	0		5.7 (2/18)	18

To see the phylogenetic relationship, or their evolutionary relations, of viruses isolated from discordant positives, concordant couples and HIV/AIDS patients, a complete genome nucleotide sequence tree of 49 isolates was constructed, as indicated under material and method. The phylogenetic tree was constructed from the nucleotide sequence of 6 discordant positive, 5 concordant couples and 38 HIV/AIDS subjects isolated HIV viruses (Fig 15).

As indicated in Figure 2.6, the discordant positive HIV isolates (AKC 139, KKK-6, AWA-7 and AWA-3) subclastured in one region. The other two isolates of discordant positives (ARC-15, AWA-18 and AKC139) diverged early from a common stock and are evolving independently without branching and rebranching from others. Three isolates (KKK-6, AWA-6, and AWA-7) formed a branch from a common stock and diverged from HIV/AIDS patient's isolated stocks. KKK-6 diverged from a common stock and separated from ET021 at a time. Similarly, AWA-7 diverged from a common stock with ET122 and separated from it at a certain time and is evolving on its own. AWA-3 also diverged from a common stock with ET031 and is evolving on its own.

A similar sub clustering of another DSCP isolates was observed in discordant positives at opposite pole, showing that discordant positive isolates were diverging very far from each other. There was a sub clustering by isolates FTT-19, FTT-6, AWA-32, AWA-16 and AWA-18. AWA-32 and FTT-16 diverged early from a common stock and are evolving independently. AWA-32, FTT-10 and FTT-19 separated or rebranched recently from a common stock and are evolving separately from HIV/AIDS isolates. AWA-18 and FTT-8 evolved independently and are evolving in parallel with each other. In CONC isolates (AKC14 and AWA32) no tendency of subclustering was observed as in HIV/AIDS subjects

HIV/AIDS isolated viruses were distributed all over and did not show sub clustering. Vey few (4 out of 38) evolved independently, while 4 out of 9 separated from the main line and were evolving independently in discordant positive isolated HIV. Like HIV/AIDS isolated viruses fewer (2 out of 6) were evolving independently in concordant couples. More recent branching from a common stock and rebranching was observed in HIV/AIDS isolated viral stocks, showing more divergence still in this group.

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 70% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 71 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

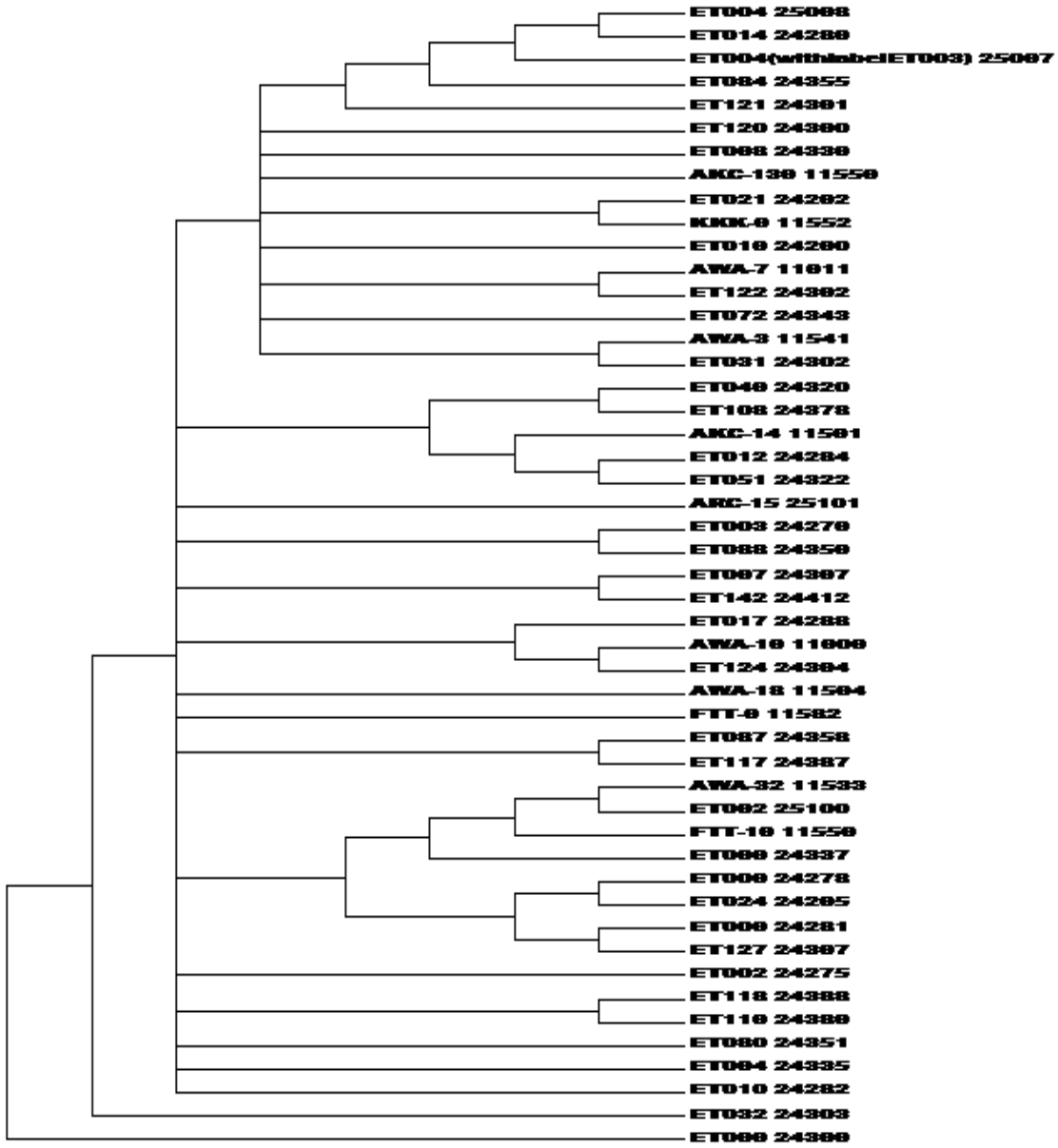

Figure *15* Evolutionary relationships of 49 taxa (linearized)

3.6. HLA typing

HLA genotyping has been performed by DNA sequencing of the exon 2-3 region of HLA class I and exon 2 region of HLA-DRB1. To determine HLA class I and II frequencies and to investigate if the differences in HLA types and frequencies were associated with resistance or susceptibility to HIV, DNA based (molecular) HLA typing was carried out and the results of 10 discordant negatives, 52 discordant positives, 49 concordant couples and 138 HIV /AIDS subjects HLA frequencies were obtained. Five different types of HLA-A1, 6 different types of HLA-A2, 7 different kinds of HLA-B, 5 different kinds of HLA-B2, 3 different kinds of HLA-C1 and HLA-C2,

3 different kinds of HLA-DR1 and 2 different kinds of HLA-DR2 were found in discordant negatives (Table12 and 13). In discordant positives 16 different kinds of HLA-A1, HLA-A2, HLA-B1, 13 different kinds of HLA-B2, 6 different kinds of HLA-C1, 5 different kinds of HLA-C2 and HLA-DR1 and 4 different kinds of HLA-DR2 were found. In a similar way, 12 different kinds of HLA-A1, 14 different kinds of HLA-A2, 24 different kinds of HLA-B1 and 16 different kinds of HLA-B2, 4 different kinds of HLA-C1 and C2, 3 different kinds of HLA-DR1 and 1 type of HLA-DR2 was found in concordant couples (Table 12). The results for HIV /AIDS were: 25 different kinds of HLA-A1 and A2, 31 different kinds of HLA-B1, 28 different kinds of HLA-B2, 18 different kinds of HLA-C1 and HLA-DR2 and 17 different kinds of HLA-DR1. In almost all cases more different kinds of HLA-B1 and B2 followed by HLA-C1 and C2 were found among HLA class I types. Similar results were obtained for HLA class II.

Table 12: The different kinds of HLA class I and II types in discordant negatives, discordant positives, concordant couples and HIV/AIDS subjects (HLA-A1= HLA-A allele1, HLA-A2=HLA-A allele 2, HLA-B1= HLA-B allele1, HLA-B2=HLA-B allele2, HLA-C1= HLA-C allele1, HLA-C2=HLA-C allele2, HLA-DR1= HLA-DR allele1, HLA-DR2=HLA-DR allele 2)

	HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-C1	HLA-C2	HLA-DR1	HLA-DR2
DSCN	**5**	**6**	**7**	**5**	**3**	**3**	**3**	**2**
DSCP	**16**	**16**	**16**	**13**	**6**	**5**	**5**	**4**
CONC	**12**	**14**	**24**	**16**	**4**	**4**	**3**	**1**
AIDS	**25**	**25**	**31**	**28**	**18**	**13**	**17**	**18**

DSCN= discordant negative, DSCP= discordant positive, CONC= concordant couples, AIDS= AIDS patients

The HLA alleles were then pooled out to determine the frequency, proporttion and X^2 –based p-value to see the associations between the different groups. Frequencies and proportions were compared between AIDS, concordant couples, discordant negativea and discordant positives. The results were as shown in Table 13.

Table 13 Comparison of HLA-A, HLA-B, HLA-C and HLA-DR aggregate subtypes in AIDS, DSCP (discordant positive), DSCN (discordant negative), and CONC (concordant couples) subjects. HST (HLA subtypes)

HLA-A	AIDS			CONC			DSCN			DSCP			p-value
HST	N	P	%	N	P	%	N	P	%	N	P	%	
1	99	32	14.1	40	7	3.1	7	2	0.9	34	6	2.6	0.39
2	89	42	18.5	27	20	8.8	4	5	2.2	27	13	5.7	0.34
3	102	29	12.8	36	11	4.6	7	2	0.9	26	14	6.2	0.44
26	130	1	0.4	46	1	0.4	9	0	0	38	2	0.9	0.40
29	125	6	2.6	47	0	0	9	0	0	40	0	0	0.08
30	88	43	18.9	33	14	6.2	8	1	0.4	30	10	4.4	0.42
34	128	3	1.3	46	1	0.4	8	1	0.4	39	1	0.4	0.67
66	117	14	6.2	44	3	1.3	9	0	0	36	4	1.8	0.45
68	111	20	8.8	34	13	5.7	5	4	1.8	27	13	5.7	0.02
HLA-B	AIDS			CONC			DSCN			DSCP			p-value
HST	N	P	%	N	P	%	N	P	%	N	P	%	
7	122	20	8.5	38	7	3	7	3	1.3	32	7	3	0.63
14	121	21	8.9	40	5	2.1	9	1	0.4	31	8	3.4	0.64
15	106	37	15.7	37	8	3.4	8	2	0.9	28	11	4.7	0.62
18	139	3	1.3	40	5	2.1	8	2	0.9	38	1	0.4	0.02
27	137	5	2.1	45	0	0	10	0	0	37	2	0.9	0.26
35	137	5	2.1	44	1	0.4	9	1	0.4	38	1	0.4	0.75
39	130	12	5.1	40	5	2.1	10	0	0	39	0	0	0.03
41	115	27	11.4	44	1	0.4	9	1	0.4	34	5	2.1	0.01
44	130	12	5.1	40	5	2.1	9	1	0.4	35	4	1.7	0.95
51	136	7	3	42	3	1.3	10	0	0	37	2	0.9	0.73
57	115	27	11.4	35	10	4.2	8	2	0.9	30	9	3.8	0.93
HLA-C	AIDS			CONC			DSCN			DSCP			p-value
HST	N	P	%	N	P	%	N	P	%	N	P	%	
3	127	6	3.2	22	2	1.1	4	0	0	23	3	1.6	0.49
4	99	34	18.2	19	15	2.7	3	1	0.5	20	6	1.6	0.96
7	51	82	43.9	14	10	5.4	0	4	2.1	9	17	9.1	0.05
8	125	8	4.3	21	3	1.1	4	0	0	24	4	1.1	0.63
HLA-DR	AIDS			CONC			DSCN			DSCP			p-value
HST	N	P	%	N	P	%	N	P	%	N	P	%	
1	110	20	10.8	19	5	2.7	5	0	0	22	4	2.2	0.53
4	104	26	14.1	21	3	1.6	4	1	0.5	24	2	1.1	0.37
11	117	13	7	23	1	0.5	4	1	0.5	26	0	0	0.09
13	116	14	7.6	21	3	1.6	5	0	0	22	4	2.2	0.64
15	108	22	11.9	16	8	4.3	4	1	0.5	18	8	4.3	0.19

For the subjects in which 20% of the expected count was less than 5, p-value was calculated by Fishers Exact Test method and the result was as shown in Table 14

Table 14 Aggregate HLA subtypes Fishers Exact Test calculated p-value of those in which 20% of cells have expected count less than 5 (A = p<.001(very highly significant), B = p<.01 (very highly significant), C = p<.05 (significant) AIDS(subjects at AIDS stage ,CONC(concordant couples), DSCP(discordant positive subjects), DSCN(discordant negative Subjects)

HLA/subtype	AIDS vs CONC	AIDS vs DSCP	AIDS vs DCSN	CONC vs DSCN	CONC vs DSCP	DSCN vs DSCP
HLA-A	29[C]	68[B]				
	18[B]					
	41[A]					
HLA-B						
	18[B]	39[B]				39[B]
	41[A]					41[C]
HLA-C						
			7[B]			
HLA-DR		11[B]				11[C]

HLA-B*49 (p<.01), HLA-A*68 (p<.01), and HLA-B41 (p<.001) (Table 14) were found strongly associated with AIDS patients when compared with all others. The strongest association was observed for HLA-B*41(p<.001) in AIDS patients. When AIDS patients were compared with concordant couples, HLA-A*41 (p<.001), HLA-A*18 (p<.01), HLA-A*29 (p<.05) were found to be significantly associated with AIDS patients. HLA-B*41 (p<.001) and HLA –B*18 (p<.01) were also found to be strongly associated with AIDS patients (Table 14).

When AIDS patients were compared with discordant positive subjects, three HLA subtypes: HLA-A*68 (p<.01), HLA-B*39 (p<.01) and HLA-DR*11 (p<.01) were found to be very strongly associcated with AIDS subjects, showing that discordant positive subjects were different from AIDS subjects. When AIDS subjects were compared with discordant negatives, the only HLA type found to be associated with AIDS subjects was HLA-C*7 (p<.01) (Table 14).

Comparisons of associations of HLA subtypes between discordant negative and discordant positive showed that three HLA subtypes were strongly associated with discordant negative subjects. These were HLA-B*39 (p<.01), HLA-B*41 and HLA-DR*11 (p<.05) (Table 14). The subtypes which were found associated with resistance to HIV in other studies were not observed in our study.

To investigate the frequency and proportions of the different forms within the subtypes and their relationships with the different groups, the different forms of the subtypes were analysed and the result was as shown in Table 15(HLA-A), Table 16 (HLA-B), Table 17 (HLA-C), and Table 18 (HLA-DR).

Table 15 proportions and X^2 values (likelihood ratios and Pearsons p-values) of HLA-A subtypes in AIDS, concordant (CONC), discordant negative (DSCN) and discordant positive (DSCP) study subjects. HST (HLA subtypes)

PID	AIDS			CONC			DSCN			DSCP			X^2 Value	
HST	N	P	%	N	P	%	N	P	%	N	P	%	LR	Pearson
0101	110	21	9.3	44	3	1.3	8	1	0.44	37	3	1.3	0.23	0.25
0103	119	12	5.3	44	4	1.8	8	1	0.44	37	3	1.3	0.98	0.98
0109	130	1	0.4	47	0	0	9	0	0	40	0	0	0.77	0.86
0201	130	1	0.4	47	0	0	9	0	0	40	0	0	0.44	0.36
0202	121	10	4.4	41	6	2.6	7	2	0.9	38	2	0.9	0.33	0.27
0205	125	6	2.6	43	4	1.8	9	0	0	38	2	0.9	0.58	0.66
0214	131	0	0	46	1	0.4	9	0	0	40	0	0	0.36	0.28
0222	129	2	0.9	46	1	0.4	9	0	0	38	2	0.9	0.68	0.59
0301	104	27	11.9	37	10	4.4	7	2	0.9	27	13	5.7	0.49	0.46
0302	129	2	0.9	46	1	0.4	9	0	0	39	1	0.4	0.92	0.95
0308	130	1	0.4	47	0	0	9	0	0	40	0	0	0.78	0.86
2301	123	8	3.5	40	1	0.4	9	0	0	37	3	1.3	0.44	0.57
2402	123	8	3.5	42	5	2.2	9	0	0	40	0	0	0.06	0.16
2601	130	1	0.4	47	0	0	9	0	0	40	0	0	0.77	0.86
2612	131	0	0	46	1	0.4	9	0	0	38	2	0.9	0.09	0.09
2901	126	5	2.2	47	0	0	9	0	0	40	0	0	0.13	0.29
290201	130	1	0.4	47	0	0	9	0	0	40	0	0	0.77	0.86
3001	110	21	9.3	40	7	3.1	8	1	0.4	37	3	1.3	0.54	0.59
3002	121	10	4.4	46	1	0.4	9	0	0	37	3	1.3	0.32	0.47
3004	120	11	4.6	42	5	2.2	9	0	0	37	3	1.3	0.59	0.76
3010	127	4	1.8	45	2	0.9	9	0	0	40	0	0	0.37	0.59
301102	131	0	0	47	0	0	9	0	0	39	1	0.4	0.32	0.19
3104	129	2	0.9	46	1	0.4	9	0	0	40	0	0	0.67	0.82
3202	118	13	5.7	45	2	0.9	9	0	0	39	1	0.4	0.16	0.24
330301	128	3	1.3	44	3	1.3	9	0	0	39	1	0.4	0.52	0.51
3402	128	3	1.3	46	1	0.4	8	1	0.4	40	0	0	0.32	0.24
3404	131	0	0	47	0	0	9	0	0	39	1	0.4	0.32	0.19
6601	120	11	4.6	44	3	1.3	9	0	0	37	3	1.3	0.65	0.81
6603	128	3	1.3	47	0	0	9	0	0	39	1	0.4	0.51	0.72
680101	124	7	3.1	43	4	1.8	7	2	0.9	35	5	2.2	0.24	0.18
6802	117	14	6.7	38	9	4.0	7	2	0.9	31	9	4.0	0.20	0.20
7401	123	8	3.5	46	1	0.4	9	0	0	40	0	0	0.12	0.26

Table 16 proportions and X^2 values (likelihood ratios and Pearsons p-values) of HLA-B subtypes in AIDS, concordant (CONC), discordant negative (DSCN) and discordant positive (DSCP) study subjects. HST (HLA subtypes). HST (HLA subtypes)

	AIDS			CONC			DSCN			DSCP			X^2 values	
HST	N	P	%	N	P	%	N	P	%	N	P	%	LR	Pearsons p-value
0702	131	11	4.7	38	7	3	8	7	0.85	35	4	1.7	0.38	0.33
0705	133	9	3.8	45	0	0	8	2	0.85	35	4	1.7	0.11	0.33
0801	137	5	2.1	39	6	2.5	6	4	1.7	38	1	0.4	0.001	.0001
130201	126	16	6.8	35	10	4.2	9	1	0.4	32	7	3.0	0.29	0.27
1401	139	3	1.3	44	1	0.4	10	0	0	38	1	0.4	0.92	0.97
140201	124	18	7.3	42	3	1.3	9	1	0.4	32	7	3.0	0.45	0.47
140602	142	0	0	44	1	0.42	10	0	0	39	0	0	0.34	0.23
1501	140	2	0.9	45	0	0	10	0	0	39	0	0	0.56	0.72
1503	116	26	11	39	6	2.5	9	1	0.4	30	9	3.8	0.60	0.61
1510	140	2	0.9	44	1	0.4	10	0	0	38	1	0.4	0.9	0.9
1516	142	0	0	44	1	0.4	10	0	0	39	0	0	0.32	0.23
1517	136	6	2.5	45	0	0	9	1	0.4	37	2	0.9	0.21	0.38
1518	141	1	0.4	45	0	0	10	0	0	39	0	0	0.80	0.88
1531	141	1	0.4	45	0	0	10	0	0	39	0	0	0.80	0.88
1801	139	3	1.3	40	5	2.1	9	1	0.42	38	1	0.4	0. 90	.05
1807	142	0	0	45	0	0	9	1	0.4	39	0	0	0.09	0.0001
2703	140	2	09	45	0	0	10	0	0	37	2	0.9	0.29	0.29
2705	139	3	1.3	45	0	0	10	0	0	39	0	0	0.38	0.57
3501	139	3	1.3	45	0	0	10	0	0	38	1	0.4	0.35	0.73
350201	142	0	0	44	1	0.4	10	0	0	39	0	0	0.34	0.23
380801	140	2	0.9	45	0	0	10	0	0	39	0	0	0.56	0.72
352001	142	0	0	45	0	0	9	1	0.4	39	0	0	0093	.0001
370101	139	3	1.3	44	1	0.4	10	0	0	37	2	0.9	0.69	0.69
380101	141	1	0.4	44	1	0.4	10	0	0	39	0	0	0.66	0.69
3910	136	6	2.54	42	3	1.3	10	0	0	39	0	0	0.19	0.39
3924	136	6	2.5	43	2	0.9	10	0	0	39	0	0	0.28	0.54
4001	139	3	1.3	45	0	0	10	0	0	39	0	0	0.38	0.57
4002	139	3	1.3	45	0	0	10	0	0	39	0	0	0.38	0.57
4101	124	18	7.63	44	1	0.4	9	1	0.4	37		0.9	0.38	0.57
4102	132	10	4.2	45	0	0	10	0	0	36	3	1.3	0.07	0.15
4201	140	2	0.9	44	1	0.4	10	0	0	36	3	1.3	0.07	0.24
4202	141	1	0.4	44	1	0.4	10	0	0	39	0	0	0.67	0.81
4402	141	1	0.4	44	1	0.4	10	0	0	39	0	0	0.67	0.69
4403	132	10	4.2	41	4	1.7	9	1	0.4	36	3	1.3	0.97	0.97
440302	142	0	0	45	0	0	10	0	0	38	1	0.4	0.30	0.17
4415	141	1	0.4	45	0	0	10	0	0	39	0	0	0.79	0.88
4501	142	0	0	44	1	0.4	10	0	0	39	0	0	0.34	0.23
4701	137	5	2.1	45	0	0	10	0	0	39	0	0	0.16	0.33
4901	105	37	15.7	40	5	2.1	10	0	0	30	9	3.8	0.02	.06
5001	142	0	0	42	3	1.3	10	0	0	38	1	0.4	0.02	0.03
5101	136	6	2.5	42	3	1.3	10	0	0	38	1	0.4	0.63	0.71
5108	141	10	0.4	45	0	0	10	0	0	38	1	0.4	0.60	0.59
530101	134	8	3.4	43	2	0.9	9	1	0.4	35	4	1.7	0.69	0.65
570101	135	7	3.0	41	1	0.4	10	0	0	39	0	0	0.21	0.40
570301	129	13	5.5	38	7	3	9	1	0.4	34	5	2.1	0.68	0.66
5801	132	10	4.2	42	3	1.3	10	0	0	37	2	0.9	0/67	0.82
7301	139	3	1.3	41	4	1.7	10	0	0	38	1	0.4	0.21	0.15

Table 17 proportions and X^2 values (likelihood ratios and Pearsons p-values) of HLA-C subtypes in AIDS, concordant (CONC), discordant negative (DSCN) and discordant positive (DSCP) study subjects. HST (HLA subtypes)

	AIDS			CONC			DSCN			DSCP			X^2 value	
HST	N	P	%	N	P	%	N	P	%	N	P	%	LR	P p-value
0202	128	5	2.7	24	0	0	4	0	0	24	2	1.1	0.39	0.53
0302	128	5	2.7	22	2	1.1	4	0	0	23	3	1.6	0.38	0.35
0304	32	1	05	24	0	0	4	0	0	26	0	0	0.88	0.94
30402	130	3	1.6	23	1	0.53	4	0	0	26	0	0	0.64	0.77
33802	132	1	0.5	23	1	0.5	4	0	0	26	0	0	0.56	0.45
0401	109	24	12.8	21	3	1.6	3	1	0.5	21	5	2.7	0.88	0.88
0407	123	10	5.4	22	2	1.1	4	0	0	25	1	0.5	0.76	0.84
0501	133	0	0	23	1	0.5	4	0	0	26	0	0	0.25	0.07
0602	105	28	15	16	8	4.3	4	0	0	23	3	1.6	0.14	0.19
0701	64	69	36.9	19	5	2.7	2	2	1.1	13	13	7	0.04	0.04
0702	123	10	5.4	20	4	2.1	3	1	0.5	22	4	2.1	0.32	0.27
0704	128	5	2.7	23	1	0.5	4	0	0	26	0	0	0.54	0.75
0716	133	0	0	24	0	0	3	1	0.5	26	0	0	0.04	.001
0739	132	1	0.5	24	0	0	4	0	0	26	0	0	0.87	0.93
0740	133	0	0	23	1	0.5	4	0	0	26	0	0	0.24	0.07
0802	112	21	11.2	21	3	1.6	4	0	0	20	6	3.2	0.47	0.58
1203	128	5	2.7	22	2	1.1	4	0	0	25	1	0.5	0.75	0.74
1403	132	1	0.5	24	0	0	4	0	0	26	0	0	0.88	0.93
1505	115	18	9.3	20	4	2.1	3	1	0.5	24	2	1.1	0.69	0.7
160101	130	3	1.6	23	1	0.5	4	0	0	25	1	0.5	0.89	0.91
1602	132	1	0.5	24	0	0	4	0	0	26	0	0	0.88	0.94
160401	129	4	2.1	22	2	1.1	4	0	0	25	1	0.5	0.67	0.62
1701	106	27	14.4	22	2	1.1	3	1	0.5	21	5	2.7	0.5	0.5
1801	123	10	5.4	22	2	1.1	3	1	0.5	24	2	1.1	0.77	0.65

Table 18 proportions and X^2 values (likelihood ratios and Pearsons p-values) of HLA-DR subtypes in AIDS, concordant (CONC), discordant negative (DSCN) and discordant positive (DSCP) study subjects. HST (HLA subtypes)

	AIDS			CONC			DSCN			DSCP			X^2 value		
HST	N	P	%	N	P	%	N	P	%	N	P	%	LR	P-value	
1102	130	0	0	24	0	0	5	0	0	25	1	0.5	0.27	0.10	
80401	130	0	23	1	0.5	5	0	0	25	1	1	0.5	0.15	0.14	
10101	129	1	0.5	24	0	0	5	0	0	26	0	0	0.87	0.93	
10201	111	19	10.3	18	5	2.7	5	0	0	22	4	2.2	0.37	0.21	
30101	107	21	11.4	21	3	1.6	4	1	0.5	21	4	2.7	0.93	0.95	
40101	127	3	1.6	23	1	0.5	5	0	0	26	0	0	0.63	0.76	
40301	123	7	3.8	22	2	1.1	23	2	1.1	24	2	1.1	0.17	0.03	
0404	117	13	7.0	23	1	0.5	5	0	0	26	0	0	0.09	0.26	
0405	120	10	5.4	22	2	1.1	4	1	0.5	24	2	1.1	0.86	0.80	
0406	127	3	1.6	24	0	0	5	0	0	26	0	0	0.54	0.73	
0408	129	1	0.54	24	0	0	5	0	0	26	0	0	0.87	0.93	
0701	128	2	1.1	24	0	0	5	0	0	26	0	0	0.7	0.84	
70101	94	30	16.2	15	7	3.8	3	2	1.1	17	9	4.9	0.49	0.63	
80401	103	25	13.5	23	1	0.5	5	0	0	23	2	1.1	0.17	0.29	
0808	130	0	0	24	0	0	5	0	0	25	1	0.5	0.27	0.10	
100101	123	7	3.8	24	0	0	4	1	0.5	26	0	0	0.09	0.13	
1101	125	5	2.7	23	1	0.5	5	0	0	26	0	0	0.52	0.74	
110102	125	5	2.7	24	0	0	5	0	0	26	0	0	0.30	0.53	
110201	128	2	1.1	24	0	0	4	1	0.5	26	0	0	0.17	0.001	
110401	129	1	0.5	24	0	0	5	0	0	26	0	0	0.87	0.93	
130101	129	1	0.5	22	2	1.1	5	0	0	25	1	0.5	0.20	0.11	
1401	126	4	2.2	24	0	0	5	0	0	26	0	0	0.41	0.63	
150101	128	2	1.1	23	1	0.5	5	0	0	23	3	1.6	0.14	0.06	
1503	108	20	10.8	16	7	3.8	4	1	0.5	20	5	2.7	0.70	0.67	
16601	129	1	0.5	24	0	0	5	0	0	26	0	0	0.87	0.93	
130201	93	34	18.4	19	5	2.7	4	1	0.5	18	8	4.2	0.82	0.91	

To look at the associations of the different forms of the subtypes to AIDS, concordant couples, discordant positive and discordant negative subjects, p-values were calculated from their X^2 s and for those in which 20 % cell count was less than 5, p-values were calculated by correctiong with Fishers Exact Test. The values for these associations were shown in table 19.

Table 19 HLA subtypes Fisher's Exact Test calculated p-value of those in which 20% of cells have expected count less than 5 (A= p<.001(very highly significant), B= p<.01(very significant), C= p<.05(significant).

HLA/subtype	AIDS vs CONC	AIDS vs DSCN	AIDS vs DCSP	CONC vs DSCN	CONC vs DSCP	DSCN vs DSCP
HLA-A						
HLA-B	0705[B]	0801[A]			0705[B]	0801[A]
	1517[C]	1817[B]			0801[C]	4901[C]
	4101[B]	352001[B]			3910[C]	
	5001[C]	4901[B]				
	7301[C]					
HLA-C	0501[C]					0716[C]
	0701[A]					
	0740[C]					
HLA-DR		40301[B]		100101[C]		100101[C]
				110201[C]		110201[C]

HLA-A*0101(9.3%), *3001(9.3%) and HLA-A*0301(11.9%) were found in higher frequency and percentage in AIDS subjects (Table 15). In concordant couples, discordant positives and discordant negative subjects the percentage and frequency of the different HLA-A subtypes was less than 5%. Relatively, higher frequencies of HLA-B subtypes were observed in AIDS and concordant couples (Table 16). HLA-B*4901 (15.7%), HLA-B*1503(11%) and HLA-B*2703(9%) were found in higher proportions when compared with other subtypes in AIDS subjects. In concordant coulpes, HLA-B*0702(3%), *5703(3%) were found in highest proportions among the members o fthe group. Among the subtypes analysed the highest proportions and frequencies were observed In HLA-C subtypes. HLA-C*0701(36.9%), *1701(14.4%), *0401(12.8%) and *1701(14.4%) (Table 17) were observed in higher proportions in AIDS subjects. HLA-C *0602(4.3%), and HLA-C *0701(2.7%) were relatively found in higher proportions in concordant couples. In discordant positive subjects, HLA-C*0802(3.2%) and HLA-C*0401(2.7%) were alos observed in highest proportions among the group members.

In AIDS patients HLA-DR*130201(18.4%), HLA-DR*70101(16.2%), *80401(13.5%), *30101 (11.4%), *10201(10.3%) and *1503(10.8%) (Table 18) were observed in greater than 10% when compared with other subtypes. In concordant couples, HLA-DR*70101(3.8%), *1503(3.8%) and HLA-DR*10201(2.7%) were observed in higher proportion. In discordant positives HLA-DR*70101(4.9%), HLA-DR*130201(4.2%) and HLA-DR*30101(2.7%) and *1503(2.7%) were observed in higher proportions (Table 18).

HLA-A subtypes were not found to be significantly associated with any of the clinical group. But when AIDS subjects were compared with concordant couples (Table 19), HLA-B*0705(p<.05) and HLA-B*4101(p<.01) to be significantly associated with AIDS subjects. A similar omparison also showed that HLA-B*1517, *5001, *7301(p<.05) were found to be significantly associated with AIDS subjects (Table 19).

Similarly, HLA-C*0701(p<.01), *0501, *0740(p<.05) were found to be associated with AIDS subjects when compared with concordant subjects (Table 19). No HLA – DR subtype was observed to be assosiacted with AIDS. Comparison of AIDS subjects with discordant negative subjects

showed that HLA-B*0801(p<.001), HLA-B*1817, *352001(p<.01), *4901(p<.01) were strongly associated with AIDS subjects. HLA-DR*40301 (p<.01) was also found to be very hihly significantly with AIDS subjects when compared with discordant negatives (Table 19).
When concordant couples and discordant negative subjects were compared, only HLA-DR*100101 and HLA-DR*110201 (p<.05) were found to be significantly associated with concordant couples (Table 19). Similar comparisons between concordant and discordant positive subjects showed that HLA-B*0705(p<.01), *0801(p<.05) and *3910(p<.05) were found to be associated with concordant couples (Table 19).
Discordant negative and discordant positive subjects showed strong associations with different HLA groups. HLA-B*0801(p<.001), *4901(p<.01) were strongly associated with discordant negatives than discordant positives subjects (Table 19). Among HLA-C, HLA-C*0716(p<.05) was found to be associated with HIV negativity. HLA-DR*100101 and *110201(p<.05 were also found to be significantly associated with discordant negatives (Table 19).

To investigate whether heterozygous advantage was present in HIV discordant couples or not homozygosity and heterozygosity was studied in the different subjects. The result as is shown in Table 20, indicated that discordan positive subjectys were more heterozygous in HLA-A, HLA-B, HLA-C and HLA-DR loci when compared with concordant couples and AIDS subjects, showing that discordant positive subjects had clear heterozygous advantage when compared with all others.

Table 20: proportions of heterozygous and homozygous HLA types in discordant negatives (DSCN), discordant positives (DSCP), concordant couples (CONC) and HIV/AIDS subjects. (HNZ= homozygocity, HTRZ= heterozygocity)

Proportions	HLA-A				HLA-B				HLA-C				HLA-DR			
	DSCN	DSCP	CONC	AIDS	DSCN	DSCP	CONC	AIDS	DSCN	DSCP	CONC	AIDS	DSCN	DSCP	CONC	AIDS
n		10	41	138		10	41	138		10	41	138		10		138
frequency		1	5	18		1	4	7		1	1	19		2		5
% HMZ		1.1	12.1	7.3		1.1	9.8	5.07		1.1	2.5	13.8		1.44		3.62
% HTRZ		98.9	87.9	92.7		98.9	91.2	94.9		98.9	97.5	86.2		98.6		96.4

CHAPTER IV: DISCUSSIONS

4.1. Sexual Behavior study

Studies carried out on discordant couples provide perhaps the best information on the efficiency of transmission and the biological and behavioral variables that influence infectiousness of and susceptibility to HIV. It is also important because more than 70% of current HIV transmission is between discordant couples. For example, in Uganda, Kenya and Zambia, married women were the population group in whom HIV transmission is increasing most rapidly and sexual health benefits of marriage is being compromised (Welling et al., 2006). This study provides information on the sexual behavior of HIV discordant, concordant and HIV negative healthy control adults accessing randomly selected clinics and hospitals all across Ethiopia. All were sexually active and married (monogamous) partners and family holders with an average number of 2.6 children each. Most of them were below secondary level of education. Occupationally most of them were house wives, laborers, government employees and others. Almost all of them were young adults living in urban areas.

The vast majority of them (greater than 85% of HIV positives and 66%of the discordant negatives) were not aware of HIV until they were counseled and tested in their respective clinics and hospitals. But their awareness was dramatically increased after voluntary counseling and testing and was permanently protected from HIV and additional risks .This clearly shows that VCT plays a very important role in reducing risk of HIV transmission as the fundamental goal of HIV prevention is to change the behavior that puts individuals at risk of infection. Thus, allowing people to know their HIV status creates unshakeable awareness and should be enhanced to curb the current HIV transmission in regular partners. This is very important for the prevention of HIV transmission as only very few people know their HIV status in this country, particularly the married ones. This finding informs policy makers to design the right strategy and give priority to the strategy based on research findings. Ignorance about ones serostatus regarding HIV is thus the major problem in addressing the issues of HIV in Ethiopia.

The majority of them were involved in several risky activities exposing themselves to HIV transmission. When condom use was taken as a marker of safe sex, almost all of them were engaged in sex without condom before voluntary counseling and testing. No other protective device was known and used by these couples either. Only a very small number of subjects (<7%) used condom persistently. This was the direct reflection of lack of awareness of HIV and their view that sex is

never associated with any risk. The proportion of subjects being involved in unsafe sex was greater in HIV positives of both discordant and concordant couples.

Discordant negatives were also involved in unsafe sex more than the negative controls. It was not clear why discordant negatives were not infected with HIV despite high risk sexual activity. But other biological and host factors may be protecting them from getting HIV infection.

Among the discordant couples groups the proportion of the male and female positives were not similar. There were more negative males than positive males and more positive females than positive males and negative females. This shows that females were exposed to more risky behaviors than males since HIV is not a normal micro flora of any one group. When age was compared to serostatus, most of HIV positives were found in the age groups of 20-30 years which were potentially young and victims of AIDS as in all other studies. It is known that HIV mainly infects young groups. But the negatives were mainly in the age group of 32-50 years. These are potentially young adults within the reach of HIV infection but might have been protected because of their relative maturity and involvement in less risky activities (protected behaviorally), or by other immunological and host factors.

Education is one of the factors that predispose one self to HIV infection. In the early stages of HIV infection, learned people were infected more but in the later stages of the epidemics the less educated groups were infected more(de Walque, 2006) . Our data showed that those with lower level of education were infected more than those with college and above level of education. These might have been school drop-outs or out of school youth which might have been chosen marriage as a means of livelihood and involved in low income generating work. Those who can read and write but have not joined any regular schools were also equally protected as learned groups. The reason for this was not clear but these people might have been beeen culturally strong religious people who were protected by social and religious norms, or else might have been isolated and marginalized groups because of low educational standard and low economic position from the risky groups. This may also show that schools might have been places where risky behaviors are acquired and fostered. The fact that the better learned groups are less infected with HIV was in agreement with the previous studies (Schwartlander *et al*., 2000; de Walque, 2006).

Both concurrent partnerships and serial monogamous sexual relationships are known risk factors for HIV transmission. More than 65% of the discordant couples and 75% of concordant couples had multiple partners before marriage. In all relationships there was unprotected sexual intercourse. Testing for HIV was also not known when one changed his or her partner from one to another.

Many of these relationships were never broken up completely but loosen or weakened when new friends were obtained.

The type of sex carried out by subjects also varied as in other behavioral aspects between discordant couples and between discordant positives and concordant couples compared to healthy controls. Subjects who were not exposed to HIV frequented a type of sex which was less traumatic(soft sex) while subjects who were exposed to HIV frequented the type of sex which was associated with trauma, cut, damages and bleeding(aggressive sex). For discordant positives the rate of aggressive sex was almost two-fold when compared to soft sex. For concordant couples the rate of aggressive sex was more than three-fold higher when compared with soft sex. For discordant negatives and healthy controls the rate of soft sex was much greater than aggressive sex. These results showed clear and sharp differences in the type of sex between HIV negatives and positives of discordant couples and concordant couples. There were more chances and favorable conditions to be exposed to HIV in discordant positives and concordant couples than discordant negatives due to this kind of truamatic sex. Since our study subjects were young people one can obseve how damaging this could be as the first sex is always truamatic and this people might have been HIV positive for a long time.

In general, not only the number of partners, sexual frequency per a week and number of sex per time but the type of sex may also play an important role in HIV transmission and propagation. The result of the association of HIV positivity with the type of sex also showed that people with aggressive sex were more HIV positives than HIV negatives (OR = .280). The chance of contracting HIV for people involved in soft sex was .171- .436 times lower than those involved in aggressive sex. This was a clear indicator that aggressive sex was a marker of HIV positivity in this study.

Taking African (particularly sub-Saharan Africa) conditions into consideration, it cannot only be concurrent or serial partnership that contributed to the prevalence of HIV in the region but also the existence of known risk factors associated with sex. Under socio-economic and political uncertainties and the existence of co-infecting diseases such as tuberculosis, malaria, Schistosomiasis, STDs and others; immune activation caused by these and other diseases; elevated viral load due to immunosuppressive diseases and co- infection with several kinds of diseases and lower CD4 count when compared with European counter parts; it is very difficult to believe that the rampant spread of HIV in Africa is only due to concurrent partnerships. Only concurrent relationship cannot also answer why about 11% of discordant couple seroconverts every year (Okoth, 2004; Quinn, *et al*., 2000).

Based on these unique sub-Saharan African conditions and the results of our studies, it is very difficult to believe that only concurrent partnerships are the major cause of HIV transmission, as even some of the relationships are difficult to categorize as serial or concurrent partnerships. Although in primary stage of HIV viral load increases, there are many other conditions (some mentioned above) which can increase and maintain viral load at an elevated level in Africa. When the general population is at a higher risk of HIV, it is difficult to believe that one single factor alone (concurrent partnership) is responsible for the rampant spread of HIV in the population. Thus, our result does not agree with the view of Vernazza et al. (1991) and Halpern and Epstein's (2004) work that concludes concurrent partnership is the major reason for the spread of HIV in Africa.

The existence of series of risk behaviors such as having multiple partners, higher sexual frequency per week and per time and involvement in risky type of sex helped by the existence of adverse environmental conditions fans HIV propagation in a population.

For several reasons but mainly for the desire to get and bring up children both discordant and concordant couples were led to permanent marriage relationships. But only less 35% of discordant positives and concordant couples were married only once in their life time. Approximately about 50% of discordant negatives were, however, married only once in their life. Discordant negatives were in a better behavioral position of having one marriage partner in their marriage lives. On average both discordant positives and concordant couples were married 1.9 times and discordant negatives 1.65 times and the difference was very significant ($p < .009$) showing that the HIV positives were married many times than the HIV negative partners. Between each marriage and divorce there was no HIV testing, as a result, the risk of HIV cannot be ruled out. Although these relationships were serial monogamy, due to several reasons such as divorce from previously infected partner and re-marriage to a new healthy partner, or re-marriage as a result of death of previous husband, the risk of HIV infection is very high. Because multiple marriages without testing for HIV is a known risk factor, these types of permanent relations may not be protective from HIV and the risk of being infected with HIV is, thus, increased. For many of them, the marriage could not last for more than 9 years and extramarital sexual intercourse was common.

The reason for multiple marriage and divorce was not clear as 93% of discordant negatives, 89% of discordant positives and 91% of concordant couples were happy with their marriage. All (100%) of healthy controls were happy with their marriage and their marriage lasted significantly ($p < .02$) longer than both discordant and concordant couples.

The reason for satisfaction in marriage was not the same for all subjects and varied between subjects. For some it was satisfaction of physical needs (sexual satisfaction and handling during marriage), for others it was fulfillment of life goals (the desire to get and bring-up children), and still for others it was religious affiliation and material benefits (economic). But love and the associated honesty and faithfulness which make couples tolerant and respect each other were almost missing from the marriage. It is known that love, honesty and faithfulness are corner-stones for true and lasting marriage. In absence of love and faithfulness, any thing under the sun can easily shake the marriage and break the already week and loose bond between married couples. Love and faithfulness are also important tools for HIV prevention. For many of the subjects one or more of these factors were responsible for the satisfaction in marriage although love and faithfulness are still in small dose.

Directly the opposite reasons were responsible for the dissatisfaction in a marriage and probably the breakage of the marriage. Sexual incompatibility, dishonesty and absence of children were the major reasons for the dissatisfaction in marriage. In general, the marriage for most of them was not true marriage but an association to fulfill certain life goals and it was simply living together. Love, faithfulness, honesty, tolerance and mutual understanding and respect (all of which are true signs of love) were missing in most of these marriages. These types of marriages were not protective from HIV.

4.2. Hitory of STD and perieved HIV infection mechanisms

It is known that in patients with genital ulcers and mucosal inflammatory STDs and in patients with a history of STDs, HIV is considerably greater (Vernazza et al., 1991). Chlamydial infections, gonorrhea and syphilis on top of being facilitators of HIV transmission, and having overlapping epidemiological mode of transmission, are known to increase HIV replication (Ho et al., 1995; Ramsey et al., 1995; Theus et al., 1998). Gonorrhea, syphilis and Chlamydial infections are very common STDs in Ethiopia. Thus, these STDs are the common risk factors for HIV in this country.

When subjects were asked for the history of STD, 56% of discordant positives and 54% of concordant couples and 44% of discordant negatives reported the history of one of the STDs. The reported history of STDs was highest in discordant positives and concordant couples. The difference between HIV negative discordant couples and the negative control was also very big (40% vs 0%). This showed that discordant negatives were also exposed, although not in equal

magnitude, to high risk activities. The difference between discordant couples and concordant couples was also significant ($p < .046$).

The most frequently reported STDs were gonorrhea, syphilis, and chancroid and chlaymiadal infection (categorized as others). All are known risk factors for HIV and this indicated that these were among the most important factors which exposed them to HIV. Although history of STDs was also higher in discordant negatives, it was not clear why they were not infected with HIV. But it is expected that strong host and viral factors might have protected them from HIV infection despite the occurrence of co-factors of HIV transmission.

When HIV discordant positives and concordant couples were asked how they were infected with HIV, almost all of them attributed their HIV infection to known HIV risk factors. Many subjects reported as a cause of HIV infection their sexual lives: Promiscuity, multiple marriages with HIV infected partners, extramarital sexual intercourse, and sexual intercourse with risky groups and infection from a partner.

Many of the subjects described the cause of their infection with HIV due to their promiscuity. These subjects mentioned that they were never satisfied with one man or woman even under permanent marriage relationships. Thus, until they were tested and counseled they used to have sex indiscriminately with any body. These people complained that they might have some problems with themselves because of the behavior they are showing.

Multiple marriages without testing for HIV are very common in this country. It is possible that the would be married partner's previous husband or wife might have died of HIV and migrated to some other place to avoid stigma and discrimination, or might have abandoned her previous husband or wife because of disclosure of HIV status of one of the partners, or it may be inheritance of the late brothers wife, leading to remarriage.

It is also possible that either one or both of the would be married couples might have been a prostitute, demobilized infected soldiers, displaced refugee and now settled but infected previously during migration or marriage. In addition, in a country where only very few people know about their HIV status, the seemingly healthy persons may be HIV carriers, marriage with these kinds' people can result in HIV infection. As a result, multiple marriages without knowing HIV status it self would be one of the ways in which HIV may be transmitted. Extramarital sex and contact with risk groups (mainly with prostitutes) are the known risk of HIV infection in Ethiopia. Sexual contact with more than one partner will increase the risk of HIV infection. Prostitutes (about 72%) are HIV infected in Ethiopia and are the major core groups which propagate HIV to all members

who come to visit them. It is therefore possible that any one who had visited prostitutes can carry on the virus from them to other partners. As a result if one of partners had been involved in a risky behavior, the other innocent partner would be infected with HIV. Thus, unless both partners are honest and faithful, HIV creeps in to the family through one of the cracks. That was what the study subjects described as an infection from a partner.

In a family where health literacy is very low and HIV awareness is minimal, if one member of the family is infected, the chance that other members of the family are infected due to ignorance is very high. This may be by directly coming in contact with blood and blood products or other body secretions of the infected person, or by sharing sharp objects which has been used by the infected person or by some other mechanisms. Because HIV/AIDS is a chronic infection and during the early stages symptoms are not apparent, both the infected person and the whole family are not aware of it and the whole family may be infected unknowingly.

Many attributed the cause of their HIV infection to occupational risk. Some of these were traditional midwifery, those who wash and settle dead bodies of HIV infected patients (Genagh in Amharic), maid servants and people who take care of AIDS patients at home and health institutions, Police forces dealing with wounded criminals, health personnel and traditional injectors and those servered by them. It is possible that HIV can be transmitted under all such situations due to poor health literacy and negligence. Because most diseases and deaths are due to HIV/AIDS, the likelihood that HIV would be transmitted in this way may not be undermined although litratures on these are not currently available in this country.

A social evil such as being raped by HIV infected individuals was also reported by 1% discordant positives and 4% concordant couples. Rape is known to be carried out to show dominance and is usually conducted to revenge somebody or something by force against the will of the subject to be raped. As a result, the chance that it is associated with high risk behavior is very high. The subjects were raped by demobilized soldiers, crime suspects and drivers. The rapists belonged to the high risk groups and the chance that they might have infected them was very high.

4.3 Immunological profile, coreceptors, and cofactors

Cellular immune responses are critical part of the host's defense against viral infections. Both CD4 and CD8 T cells play a very important role in immunity to viral infections. The major damage caused by HIV to the immune system is a depletion of CD4 T cells. CD4 T cells loss leads to an irreversible breakage or weakening of the immune system and to an inevitable AIDS and finally to

the demise of the infected person. On the other hand, strong immunity including an appropriate help provided by CD4 T cells is the major contributor of an aborted HIV infections and a delayed progression to AIDS (Rosenberg *et al.,* 1997).

In HIV discordant couples the major reason why HIV was not transmitted to HIV negative partners is due to strong cellular immunity. In subjects who have been living together as a husband and wife for more than three years and up to 14 years with a different HIV serostatus, a CD4 count similar to healthy uninfected people was observed in discordant negative partners. There was no difference between healthy uninfected subjects ($p > .05$) and HIV negative discordant partners. There was not only normal number of CD4 cells, but also the number of CD8 T cells was also normal and similar to healthy controls($p > .05$). The ratio of CD4 to CD8 was higher (0.81) in discordant negatives when compared to the ratio of discordant positive partners (0.61) and was very similar to healthy controls (0.94), indicating that CD4 and CD8 T cells were as potent as in healthy individuals in discordant negatives. Healthy CD4 and CD8 count with higher CD4 to CD8 ratio is a characteristic of resistant individuals (Von Andrian and Makay, 2000). This had also been observed in discordant couple's studies in other countries (Devito *et al.,* 2000; Kaul *et al.,* 2000). Thus, our result is in agreement with other studies, as higher CD4 count is known in reducing or completely clearing HIV from the body when one is infected. CD4 count is an indicator of efficient immunity and lower CD4 count with progression of disease (Barker *et al.,* 1998).

The difference in the number of CD4 T cells between discordant negative partners and positive partners was very highly significant ($p = 0$), although the difference in the number of CD8 T cells between these partners was not significant ($p > .05$). The relationship between CD4 and CD8 in discordant negative partners was a direct relationship, which is another sign of a healthy immune system, and there was an inverse relationship between CD4 and CD8 T cells in discordant positive partners. Although the inverse relationship was weaker ($r = -.468$, $p < 0$), the relationship was very highly significant indicating a different relationship between CD4 and CD8 in discordant positives when compared to discordant negatives.

The number of CD4 T cells of discordant negative partners was more than 3-fold higher than CD4 of concordant couples and was significant ($p < .05$). The difference in CD8 number between discordant negative and concordant couples was also significant, although it was not many-fold different. For the majority of the discordant negative partners CD4 count was greater than 700 and CD8 count was greater than 700 for all, indicating similar pattern in all subjects.

A clear pattern of lower counts of CD4 and CD8 was also observed for discordant positive partners and concordant couples, consecutively. The higher CD4 to CD8 ratio; the close similarity of CD4 and CD8 to healthy subjects; the big difference between discordant negatives and discordant positives and concordant couples, clearly showed that discordant negative partners had a normal and potent CD4 and CD8 T cells. This may be one of the reasons why discordant negative partners were protected from HIV despite frequent exposure to HIV.

The CD4 count of Discordant positives, although significantly different from discordant negative partners, was not below the normal range (>500) of healthy CD4 count; and it was significantly ($p<0$) different from concordant couples with a higher CD4 to CD8 ratio (.61 vs .37). The observation that the CD4 count was between 400 and 800 for the majority of these subjects also indicate that it was not abnormally low, although it was on the boundary between healthy and HIV infected individuals. This cannot also occur by chance as it involved many subjects.

However, the CD8 count of discordant positive partners was not significantly different from discordant negative partners but was very highly significantly ($p<0$) different from concordant couples. This indicated that CD8 T cells which are potent antiviral agents may be keeping the viral load lower and preventing abrupt decline of CD4 T cells. This was clearly seen when viral load of discordant positive partners was compared with concordant couples. The viral load of concordant couples was 60-fold higher than discordant positive partners and the difference was very highly significant ($p<0$). CD4 count of discordant positive partners was more than 2-fold higher than concordant couples; and CD8 count of discordant positives were also very highly significantly ($p< 0$) different from concordant couples.

There was also an inverse relationship between CD4 and CD8 in both discordant positives and concordant couples, although this was much weaker in concordant couples. Discordant positives showed the characteristics of long term non progressors (Mattapallil et al., 2005). CD4 count did not decline drastically and was capable of providing help to CD8 T cells. CD8T cells are strong antiviral agents and this could maintain the viral load at lower level (Simone et al., 1996). Our results thus were not different from the study of long- term- non- progressors.

This was also supported by the evidence from the presence of certain kinds of T cell subpopulations and HLA subtypes. The fact that both CD4 and CD8 count and the ratio was different from concordant couple also clearly showed that discordant positives were different from concordant positives.

Some of the discordant couple, who were serologically positive for HIV, had no detectable viral load. These subjects had higher CD4 and CD8 (particularly CD8 T cells) than others with detectable viral load. Since the only difference we observed was number of CD4 and CD8 T cells, it could be explained that potent cytotoxic T lymphocytes in these subjects might have controlled the viral load to the level undetectable in the blood. These may also be HIV-specific cytotoxic T lymphocytes capable of clearing HIV from the body tissues.

The stronger negative correlation between viral load and CD4 (r = - 0.66) also indicated that CD4 played an important role in reducing viral load such as by providing appropriate help to CD8 or by other mechanisms. The weak negative correlation between CD8 and viral load also indicated that as viral load decreased CD8 T cells increased. The fact that there was a potent immune response involving CD4 and CD8 (may also include others) can further be substantiated by the fact that this was not observed in concordant couples.

Cytotoxic T lymphocytes (CTL) have been suggested to play an important role in the control of HIV infection (Shearer and Clerici*, et al*., 1997; Malek *et al.,* 2002). It is possible that CD8+ T cells may have the same or different roles in discordant negatives (probably by protecting HIV infection) and discordant positives (probably by delaying progression) and in concordant couples having destructive roles. It remains likely that phenotypic differences may reside in the ability to CD8+ T cells to mediate cytolysis, secrete suppressive factors, or proliferate *in vivo* (Rowland-Jones et al., 1995). The inverse relationship between viral load and CD8+ T cells (Table 14)(r = -.24, p< .05) indicated that CD8+ T cells could suppress HIV progression , in presence of CD8+ T cells the viral load was found to be lower. As it is known from previous studies (Malek, 2002; Barker *et al.,* 1998), strong CTL could clear or suppress HIV virus completely. These may be strong HIV specific CTLs capable of clearing HIV and maintaining HIV at lower level (Malek, 2002). Thus further characterization of these CTLs could explain the mechanism of actions and type of help provided by CD4 T cells to CTLs.

Syphilis as a co-factor for HIV transmission was observed in both discordant positives (44.4%) and concordant couples (33.3%) when compared with discordant negatives (8.3%), indicating that syphilis was a strong co-factor in HIV acquisition and highly related to HIV transmission. The result was much higher than that had been reported in the behavioral study report, indicating that many people did not know that they had been infected with syphilis in their lives or might have under-reported due to the negative charisma associated with the disclosure of their status.

It is known that syphilis is the most common ulcerative STD in this country (Belay, 2008). Syphilis can remain silent in the body for a long time. Due to this chronic nature, it might have aggravated the transmission and progression to AIDS. Since syphilis positivity was much higher in concordant couples and discordant positives than in discordant negatives, syphilis and other STDs could be the major facilitators of HIV transmission and progression. The behavioral data obtained in this study also showed the history of syphilis and other STDs, supporting our laboratory data. All these indicated the extent of exposure to risky sexual activities.

All the subjects who had undetectable viral load were negative for syphilis serology, indicating reduced co-factor effect of other sexually transmitted diseases including syphilis. There was a weak positive correlation ($r = .085$) between syphilis and viral load, indicating the increase of viral load with an increase of syphilis positivity ($p < 0$). It was not clear why there was a very significant association between syphilis positivity and viral load, although there was a weak negative correlation ($r = -.198$) between them. However, it was clear that the associations were weak but very highly significant in both discordant positive partners and concordant couples. This result also showed that sexually transmission of HIV was associated and facilitated by syphilis and other STDs in both discordant positive and concordant couples than discordant negative partners.

Several studies have shown that healthy Ethiopians are characterized by reduced total CD4 count (both proportion and absolute number), increased CD8 T cell and altered T cells subpopulation, particularly reduced naive T cells and elevated CD4 memory T cells (Messele *et al*., 1999; Kassu *et al*., 2001). These immune alterations, as has been observed in children, were not natural, but acquired one (Tsegaye *et al.,* 2003). The reasons for these were mostly environmental although genetic factors could also never be ruled out. Similar studies also showed that Ethiopian HIV infection is characterized by low total and naive CD4+ T cell count, high T cell proliferation and increased memory T cells (Tsegaye *et al*., 2003). The early erosion of the naive T cell pool has resulted in an increased proportion of cells with a more differentiated CD27- memory phenotype.

Immune activation is responsible for these differences of Ethiopians from others. Both immunological differences of healthy Ethiopians and Ethiopian HIV infection are attributable to immune activation prevalent in this country (Messele *et al.,* 1999). To demonstrate that if these kinds of factors were responsible for resistance and susceptibility to HIV in Ethiopian discordant couples T cells subpopulations were investigated.

The absolute number and proportions of CD4 and CD8 T cell of discordant negative partners was normal. In Discordant positive partners, although absolute number and proportions of CD4 T cells

was relatively lower than discordant negatives, it was stable and significantly different form concordant couples. The proportion and the number of CD4 T cells were also in the lower boundary of the normal count.

In their immune status, the discordant positives occupied an intermediate position between discordant negatives and concordant couples as can be deduced from both the absolute number and proportions of both CD4 and CD8 counts and the different subpopulation's counts and proportions. These clearly demonstrated that an equal balance of power where one cannot defeat or be defeated by the other, existed between the immune system and the HIV infection in discordant positives.

The evidence for this was lower viral load even to the extent of undetectability maintained by the immune system, elevated CD8 count, intermediate count of CD4, reduced activation markers and expression of efficient subpopulations of CD4+ effector/memory T cells. The CD4/CD8 ratio also reflected this pattern, slightly lower than discordant negatives and about 2X of concordant couples (Table13). The proportion of CD4 and CD8 was also in favor of this notion.

Specific subpopulations were not selectively expressed in both discordant negatives and discordant positives, capable of showing marked significant difference between them. The difference between discordant negatives and discordant positives in T cell subpopulations proportions were insignificant and could not account for their differences. Differences between discordant partners and the negative control were also insignificant and were almost similar, showing similar patterns and efficiency of action of T cell subpopulations between them. But the differences between discordant partners and concordant couples were marked and very significant, showing differences in subpopulations and activity. The profiles of subpopulations of T cells in concordant couples reflect features of AIDS.

As can be deduced from the median percentile (Table 13) of CCR5 and CXCR4, both CCR5 and CXCR4 were expressed in lower amount in both discordant positives and discordant negatives and these were not significant to account for their differences. This is also in agreement with previous studies (both domestic and African) which showed that CCR5 and CXCR4 are lower than other parts of the world (Messele *et al.,* 1999; and Fort *et al.*, 1998). In all groups, including the negative control, the double negatives were the majority (85-90%) (Table 13), showing that these receptors were no expressed or expressed in lower amount in the study subjects. Relatively CCR5 receptors were expressed more in concordant couples and the negative control (8-10%).

The reason why these receptors were expressed more (although not significant) in concordant couples and the negative control was not clear from this study. Although it is known that memory

cells express these receptors (Baggioline et al., 1996), memory T cells were also very small in the group. But cells expressing HLA-DR were found in higher number in concordant couples and the negative control. For concordant couples HLA-DR expression could account for CCR5 expression, since CCR5 expression is associated with increased HLA-DR expression and disease progression (Gupta and Klasse, 2006).

CD4 and CD8 count as well as CD4/CD8 ratio and other immunological profiles all indicated that concordant couples were at AIDS stage and this may support the idea that increased CCR5 expression may be due to increased expression of HLA-DR. Moreover, there was also a week positive association ($r = 0.5$, $p > .05$) between CCR5 and HLA-DR expression, although it was not statistically significant. But the reason for the expression CCR5 in the negative control was not clear from the results we obtained in this study.

In general, the difference in the expression of CCR5 and CXCR4 receptors was not significant between discordant partners and concordant couples and could not account for their differences.

There was no significant difference between discordant couples in the percentage of CD4 HLA-DR+, CD38+, and HLA-DR+ CD38+ markers, which are activation markers, showing that differences in activation markers could not account for the difference between discordant negatives and discordant positives. These subpopulations were almost comparable. The majority of these subpopulations (60-70%) were resting T cells. But the percentage of resting T cells of discordant negative partners was significantly ($p < .05$) greater than concordant couples, showing less antigenic challenge in discordant negatives (De Rosa, 2001).

The proportions of resting CD4+ T cells were comparable between discordant positives and concordant couples (Table 13) and were not significant ($p > .05$). The negative control was also similar with the HIV positives. The reason for this is unknown, although it known that there may be antigenic challenge by other infectious agent which may be viral or other pathogenic agent with similar immune profiles.

Concordant couples were more activated than either discordant positives or discordant negatives and the difference was significant ($p < .05$) as can be deduced from the expression of activation markers. This showed that both discordant positives and negatives were less activated than concordant couples. It is known that immune activation increases rate of HIV infection and it is the major cause of intensified HIV infection in Ethiopia (Messele et al., 1999). It is possible that particularly discordant negatives were less immune activated, probably due to genetic or other factors and could avoid or abort HIV infection, if any infection had ever occurred. For example,

genetic factors which may reduce CCR5 expression can make immune cells less susceptible to HIV infection.

Host and environmental factors (like less exposure to environmental pathogen or resistance to infection to environmental pathogens) may also decrease immune activation. Similar mechanism may also operate in discordant positives, enabling them to fight HIV by reducing activation markers and keeping them in check, as activation markers were not significantly different from concordant couples.

Concordant couples showed typical features of people with AIDS (increased immune activation markers and lower resting T cells) and were completely different from discordant couples. The negative controls also showed more activation markers than discordant negatives, giving evidence for the differently functioning of the immune system of discordant couples. Discordant negatives in particular may be resistant to HIV due to their lower activation markers and discordant positives might have kept HIV at lower level by being less immune activated. This was observed only in our study as no comparisons were made between discordant positive and concordant couples up to now.

Similar profile was observed in CD8+T cells. There was no difference in activation markers between discordant negatives and discordant positives and there was a marked significant difference from concordant couples. The number of activated CD8+T cells was again significantly increased in concordant couples when compared with discordant couples. The increase of activation markers in CD8+ T cells could also be associated with immune activation and susceptibility of T cells to HIV in concordant couples, as activated CD8+ T cells in AIDS stage are known even to destroy the would be fighter immune cells and cause more damage than help (Sousa et al., 2002) . Thus, the presence of less activated T cells might have benefited discordant couples by reducing immune activation and strengthened the immune system to fight HIV. Although immune activation is prominent in this country (Messele et al., 1999), these subjects might have less immune activated due to host or environmental factors.

Differences in the proportion of naive, memory and effector T cell subpopulations were not significant ($p > .05$) between discordant positives and negatives and was very similar to the negative control. As a result, differences in these subpopulations could not account for the difference in susceptibility/ resistance to HIV. Memory T cells expressing CD4+CD27+CD45RA- were depleted in concordant couples but were almost equivalent to the negative control in discordant couples. Naive T cell subpopulations were comparable in all groups, including the negative control. The ratio of CD27- to CD27+ was relatively higher in discordant positives than in concordant couples,

showing unimpaired and actively differentiating virus-specific T cells inhibiting or retarding disease progression (Hintzen, 1993). Thus, this is additional evidence that discordant positives showed long- term- non- progressor's profile and this in agreement with previous features of long-term-non-progressors.

A relatively higher proportion of CD4+CD27-CD45RA- memory/effector T cell subpopulation, which were significantly different from both discordant negatives and concordant couples, was observed in discordant positives, a scenario which is frequently observed in long-term –non-progressers. This T cell subpopulation is known in delaying disease progression in HIV positives but with no progressing disease. The presence of CD27- effector /memory T cell subsets in higher proportion in discordant couples (particularly in discordant positives), showed repeated antigenic challenge and hence efficient immune system capable of keeping the viral load to a minimum, preventing further depletion of CD4 and disease progression. The observation that proliferation increased in CD4+CD27+CD45RA- memory T cells might be a haemostatic mechanism to replace the depleted memory T cells in an effort to maintain the immune system in concordant couples, although the final fate of these cells would also not be different. Therefore, one could suggest that this was repeated division in an attempt to compensate for the loss of depleted memory T cell but would finally lead to T cell wastage.

The reason why effector T cells were higher in the negative control was not clear from this study. But since immune activation is the characteristics of healthy Ethiopians, this may be the reason for the elevated number of effector T cells (Messele *et al.,* 1999). Repeated antigenic challenge from the environment may be the underlying reason for this (Tsegaye *et al*., 2003)

In general, healthy T cell subpopulations similar to the negative control (even sometimes better), reduced activation markers, and hence reduced immune activation, and others might have enabled the discordant negatives to eradicate HIV and develop resistance. Discordant positives might have controlled viral load by reducing the activation markers and hence immune activation and using their immune system to fight HIV. The presence of CD4+ CD45RA-CD27- memory/effector T cells in higher proportion (30%) might have helped in delaying disease progression and further decline of CD4 T cells (Hintzen, 1993).

Higher CD27- to CD27+ ratio also indicated a relatively efficient immunity in discordant positives. But these factors alone could not be responsible for the resistance/ susceptibility to HIV, as HIV/AIDS is more complicated than this. But these might be a share contributed by the immune system, helping other known or unknown host and genetic factors responsible for resistance and

susceptibility to HIV. HIV resistance/susceptibility could not be caused by a single factor but a concerted activity of host, immunological, virological and genetic factors. Hence, further characterization and extended studies might elucidate how these complicated factors might prevent HIV.

4.4. Viral genotyping

HIV is characterized by its enormous plasticity and non-stoppable diversity due to error-prone reverse transcriptase and absence of exonulcease editing the errors occurred. Uniformity in genetic make-up is never the rule in HIV. HIV clades are phylogentically classified on the basis of the 20-50% differences in envelope *(env*) nucleotide sequences into three groups; group M being one of them. Within M subgroups, inter-clade *env* variation differs by 20-30%, whereas intra-clade variation of 10-15% is observed (Fields, 1990).

Geographical distribution of the different subtypes is very heterogeneous and in Africa all subtypes have been extensively characterized by genetic analysis. The various genetic subtypes differ in their geographic spread and so the subtype designations have been powerful molecular epidemiological markers for tracking the course of the global pandemic.

HIV-1 subtype has been estimated to account for 48% of HIV-1 infection worldwide and 51.5% of HIV-1 infection in Africa (Essex, 1998), where the main mode of transmission is heterosexual. The predominant subtype of HIV in Ethiopia is also subtype C since the start of the epidemics over two decades of age. But sequence analysis demonstrated the presence of distinct subcluster (C prime C') within the main subtype group of viruses in Ethiopia (Abebe et al., 2000). A number of recent findings (Fahey *et al.,* 1998; Novitska *et al*., 1999) argue that one possible cause of the high viral diversity in Sub Saharan Africa could be higher flexibility of subtype C virus and its altered ability to diversify. The arguments as cited by Novitska et al., 1999 include:that Subtype C is predominant in most recent HIV-1 epidemic worldwide; the highest prevalence of HIV-1 infection in various epidemics is caused mainly by subtype C; Subtype C virus may have a faster disease progression; patients infected with HIV-1 subtype C develops to AIDS earlier than patients with subtype A virus; and ˙the viral load of subtype C infections may be higher in different compartments that might cause an increased level of viral transmission.

In deed, clade C has created the recent epicenter of HIV pandemic by its uncontrolled spread throughout Botswana, Zimbabwe, Malawi, Zambia, and Namibia, Lesotho, South Africa, India, Nepal and China. Ethiopian clade C isolates differ (with respect to RT) from clade B by 6.8- 10%,

and intra-clade differences of 3.5-5.8% have been reported for strains from Africa, India and South America (Rodenburg *et al.,* 2001).

In light of all these features of subtype C, the Ethiopian HIV is characterized by the predominance of this subtype C as observed from our findings. In all, discordant positives, concordant couples and HIV/AIDS patients the majority of the viruses isolated were subtype C, although there was an introduction of subtype A, B and recombinant forms. It was not clear from this study whether the subtype C from discordant positives, concordant couples and HIV/AIDS were the same subtype C. Analysis of the *gag* and *pol* genes in previous studies showed that Ethiopian HIV virus can be classified into C and C' (Abebe et al., 2000). It is also not known whether these viruses have different biological properties or not. It is possible that the viruses from discordant positives might have been Subtype C' as our previous results indicated that discordant positive subjects were not the same as HIV/AIDS patients or concordant couples in many of their profiles including lower CD4 count and higher viral load and others.

The predominance of subtype C in the Ethiopian epidemics was still maintained as indicated by the C subtype observed in this study and other previous reports. Thus, our result agrees with previous findings that Ethiopian HIV is predominantly subtype C. What was different from previous study was that in discordant positives the same virus which caused AIDS epidemic was found in large number. But further characterization of *pol* and *gag* and amino acid sequences should be carried out to show whether these viruses were subtype C' and were different in biological properties from HIV/AIDS isolated viruses.

Another difference in our study from previous studies was the observation of subtypes A and B and recombinant forms from Ethiopia. This is possible because as HIV/AIDS pandemic grows, viral strains become more geographically dispersed and simultaneous presence of multiple subtypes all over the world is becoming vey common. It is expected that this will increase due to population migration and the expansion of trade and investment. Since subtype A is very common in neighboring and sub-Saharan countries and sub-type B is common in European and American, these might have been introduced to Ethiopia.

The recombinant forms observed were the ones which were vey common in Africa, A/G, and the group which also used CCR5 and CXCR4 coreceptors (Sierra *et al.,* 2005; Worobey *et al.,* 2007)).

Recombinant events among sequences of different genetic subtypes of HIV group M have been frequently identified and are becoming of epidemiological importance. The observation of recombinant forms in Ethiopia indicated that different subtypes were circulating in the population

sumaltinaneously. Thus, the finding of subtype A or A/G indicated that either than type C type A and G were also circulating in the population. The number of different subpopulations and recombinant forms circulating in Ethiopia might be greater than that reported and further molecular epidemiological studies are needed to show the real situation. Overall, although subtype C virus is the predominant form, other subtypes are also creeping in to the country and recombinant forms are also being observed.

The V3 loop of HIV-1 is critical for coreceptor binding and is the main determinant of which of the cellular coreceptors, CCR5 or CXCR4, the viruses use for cell entry (Fouchier, et al., 1998). Most HIV clades cause disease by assuming the CCR5+/NSIphenotype during early disease and the CXCR4/SI phenotype during the end stage of the disease (Peeters et al., 1999). Thus, it has been shown that viruses binding to CCR5 or CXCR4 are almost exclusively present during the early asymptomatic stage of infection, whereas CXCR4-binding viruses may emerge in later phases of the infection and are associated with a CD4+ T cell decline and progressing towards AIDS. Relatively CCR5/NSI strains are more conserved when compared to more diverse CXCR4-trophic and SI strains evolving in an apparently unconstrained manner.

The view that early viruses use CCR5 and late viruses use CXCR4 depends up on the type of the clade (peeters *et al..,* 1999) and may not hold true for clade A, C or D. Clade A viruses tend to favor CCR5 even at later stages, while clade C strains rarely become CXCR4/SI even in the stage of the disease (Tsherning *et al.,* 1998). The reason for this was attributed to persistent immune activation experienced by many Africans (Fahey *et al.,* 1998), as persistent immune activation constantly trigger CCR5 over-expression. Thus, the CXCR4-positive rapid/high phenotype is underrepresented among subtype C isolates and syncytium--inducing phenotype is rare among subtype C-infected patients (Novetsky *et al.,* 1999).

HIV subtype C was the predominant subtype and accordingly CCR5 would have been used as a coreceptor for viral entry into the cell. However our result showed even in discordant positives CCR5 and CXCR4 were used equally (50% each). In HIV/AIDS patients about 61% (22/18) used CXCR4 as a coreceptor and only 33.3 %(6/8) used CCR5 coreceptor, the remaining 5.7% (2/18) used dual coreceptors. Our result does not agree with the previous work that subtype C viruses never use CXCR4 even in late stage of the disease (Peeters *et al.,* 1999). Our concordant ouples and HIV/AIDS patients (subjects which were not couples but AIDS patients for which we did HIV sequencing) showed the characteristic AIDS feature with devastating diseases and hence were CXCR4/SI rapid/high type. But discordant positive subjects showed normal CD4 count and lower

viral load and used both CCR5 and CXCR4 equally. The coreceptor usage in HIV/AIDS patients was not homogeneous but both coreceptors were used in a ratio of 2:1 of CXCR4 to CCR5. The reason for this was not clear from this study but it was obvious that the Ethiopian subtype C isolates used (probably converted from CCR5 to CXCR4) CXCR4 during AIDS stage. The molecular biological identification of these receptors was also different from our immunological staining, which showed about 8-10% CCR5 coreceptor only, probably indicating the staining method might have not been a method of choice when compared to this novel molecular method. Ethiopian HIV is the worst HIV and its progress to AIDS is rapid as was shown by previous workers. This is the characteristics of CXCR4/SI using rapid/high strains and our result reflected this feature of HIV virus. But the fact that about a third of these viruses used CCR5 coreceptors indicated that Ethiopian HIV viruses were/are not homogeneous in their coreceptor utilization. Thus, it is tempting to stress that further molecular epidemiological studies involving larger sample size should be conducted.

To understand more fully if there was genetic variation among isolates obtained from discordant positives, concordant couples and HIV/AIDS subjects, phylogenetic trees were constructed using neighbor-joining algorithm and the Kimura two-parameter model. A complete genome nucleotide sequence tree depicting the phylogenetic positions of the 49 characterized HIV-1 strains were used (Fig 14).

Phylogenetic analysis showed that most viruses identified from discordant couples formed subcluster around the same region, showing that these viruses were more closely related to each other and were of recent origin or emerged together. Two isolates from discordant group (AWA-18 and ARC-15) diverged early and were evolving independently without divergening to any kind, showing independent evolution. But AWA-18 was far from AR-15 on the evolutionary tree. AWA-18 evolved in parallel with FTT-8 which was a concordant subject. Some of the viruses of discordant positives diverged from a common stock, which gave rise to both discordant positives and HIV/AIDS derived viruses, showing that different lines of evolution being pursued probably one to a mild form and the other to an aggressive one.

Similar subclustering was also observed in viruses isolated from concordant couples, although at a distant site on the evolutionary tree, showing viruses isolated from discordant couples were different from concordant couples on the evolutionary tree. HIV /AIDS isolated viruses did not form subclustering and were highly divergent groups, suggesting a more long-standing evolution of

these groups sometimes giving rise to discordant positive viruses. Many of them started to evolve independently early and were evolving in to a separate line.

4.5. HLA typing

Susceptibility or resistance to any infection is determined by the highly polymorphic region of the human genome (HLA). It is extremely polymorphic to cope up with ever evolving pathogenic agents, although it can not cope up with the speed pathogenic agents evolve, which is measured in days , week and months while human HLA gene evolution requiring hundreds of years. HLA typing has practical application in identifying genes which makes us susceptible or resistant to any infection and this is very important for vaccine design and gene therapy.

As the cause for resistance or susceptibility to any infection has its roots in HLA genes, the reasons for acting differently to HIV as in the case of HIV discordant couples may be due to the presence of different HLA subtypes offering resistance or making susceptible to infection. Thus, in order to analyze the effect of host HLA types on resistance or susceptibility to HIV infection and to study the HLA profile of Ethiopian HIV/AIDS subjects, DNA based HLA typing was carried out and result was collected from 239 subjects (discordant negative, discordant positive, concordant couples and HIV positive subjects).

HLA genes carry out their functions through the immune system. Whether the immune system is a predisposing or a protective one is determined by HLA genes. Reduction in CD4 number, increased viral load and other immunological abnormalities caused by HIV are determined by host genetic factors, as resistance and /or susceptibility to an infection has host genetic regulation at its back. As CD4 T cells are the foremost targets of HIV, they are gradually lost as the disease progresses. CTLs do not have CD4 receptors; as a result, they are expected to be the major player in HIV regulation.

HIV infection and intrusion of viral particles are counteracted by CTL-mediated immune responses both during acute and chronic HIV infections (Tripathi and Agrawal, 2007); and the high concentration of CTL results from continued antigenic stimulation during chronic infection. CTLs recognize and kill HIV infected cells through the recognition of self HLA molecules on antigen presenting cells by different mechanisms. The presences of enormously large number of different HLA subtypes help to present different peptides of HIV to CTL.

Many different HLA subtypes of both class I and II were found. The finding was proportional to the diversity of HLA class I alleles (according to IMGT-HLA database approximately 1178 HLA-B alleles compared with 767 HLA-A and 439 alleles in HLA-C (IMGT database, 2009; as cited by

Huang et al., 2009 are known). Thus, proportionally many different kinds of HLA-B alleles, followed by HLA-A and HLA-C alleles were found in all study subjects. The number of the different subtypes was highest for all classes of HLA in HIV /AIDS subjects and concordant couples. This might have been due to the higher number of sample size typed and analyzed, although the diversity of HLA alleles and subtypes could not be ruled out. Relatively fewer subtypes were observed in discordant negatives and positives, the reason being similar to the above. HLA profile of Concordant couples and HIV /AIDS subject was similaras were many other similarities between them. But some subtypes were significantly associated to either of them and may not be identical.

Many different subtypes were discovered in our study when compared with previous workers (Tsegaye, *et al*., 2004; Ferrari *et al.,* 2004), which analyzed only 50 HIV /AIDS subjects and 50 HIV negative and 36 HIV positive subjects, respectively. Moreover, their study did not include HLA class II alleles and did not involve discordant couples beyond testing and analyzing small sample size.

Our findings clearly indicated that HLA subtypes in AIDS, concordant couples, discordant positives and discordant negative subjects were not identical. Specifically, all AIDS patients, as were in other parameters, were significantly different from concordant as well as discordant couples. AIDS patients were not the same as concordant couples, as many HLA subtypes were found to be significantly associated with AIDS subjects when compared with concordant couples. Many of the subtypes significantly associated with AIDS patients when compared with concordant couples were HLA class I types. Our previous studies indicated that CD4 and CD8 T cells were significantly different in AIDS patients when compared with both concordant and discordant couples. Hence, many of the HLA subtypes such as HLA*29, *18 and *41; HLA-B*18 and *14 were only strongly associated with AIDS patients but not in others. These subtypes were subtypes which made AIDS patients to succumd to AIDS quickly and were also associated with worsened and aggravated clinical conditions. Comparisons between AIDS and discordant positive subjects also indicated that AIDS subjects were significantly different in their associations with HLA subtypes. HLA-A*68, HLA-B*39 and HLA-DR11 were very significantly associated with AIDS patients when compared with discordant positive subjects, indicating that AIDS patients were also different from discordant positives as was also observed in other difference in our previous studies. As a resutl, it is tempting to conclude that the difference between discordant positives and AIDS patients had genetic background.Only HLA-C*7 were significantly associated with AIDS patients when compared with

discordant negative subjects among HLA-C subtypes. Many of these subtypes were also very closely asssociated with AIDS in many studies. Thus, it is clear that Ethiopian AIDS patients, according to our study, are rapid progressors exhibiting worsened disease conditions baecause of these specifically AIDS associated HLA subtypes, proving that there was a genetic background behind these scenarios.

Discordant positive and discordant negative subjects were aslo diferent in their HLA subtypes, as in all other parameters.HLA-B*39, *41 and HLA-DR*11 were significantly associated with discordant negatives, indicating that resistance to HIV had a genetic background. Similar difference s were also observed beteen discordant negatives and AIDS patients in that HLA-C*7, had signifacant association with discordant negative subjects. Hence, the difference between discordant negative subjects and discordant positive, as well as AIDS patients, had a genetic bacckground due to the significant associations of these HLA subtypes. Thus, these subtypes are resistance determining subtypes. HLA-B*39 and HLA-DR*11 were significantly asscociated with discordant positives when compared with AIDS patients. Although this indicated the difference of discordant positves from AIDS patients, it was not clear from this study why these subtypes differentiated discordant positves and AIDS subjects as well as discordant positives and discordant negative subjects. But it is highly likely that in discordant positive subjects these subtypes might be the subtypes determining the long-term- non-progression, maintaining both CD4 and CD8 count at normal level and providing absolute protection for discordant negatives. Other genetic and host factors might have also caused this disparity. Thus, our evidence strongly indicated that HLA-B*39, *41 and HLA-DR*11 were subtypes associated with resistance to HIV.

When these pooled subtypes were analyzed for their different forms, the different forms (as indicated in Table 19) were mainly HLA-B, C and HLA-DR subtypes. This was in agreement with our previous studies in that CD8 T cells were significantly different in different groups and were associated with different clinical out-comes.This proved that HLA class I subtypes were very important in detrmining the susceptability and/or resiastance of subjects to HIV infection and rapid progression to AIDS. HLA-B*0705, *4101 were very strongly ($p<.01$) associated with AIDS patients and HLA-B*1517, *5001 and *7301 were also significantly associated with susceptability to AIDS (Table 19).

HLA-C plays a very important role in determining the fate of HIV in many studies next to HLA-B. The strong and very significant association of HLA-C*0701 and significant association of HLA-C*0740 with AIDS could have not occurred by chance. Thus, the association of these forms with

AIDS might have been responsible for the rapid progression and worsened disease conditions of these subjects.

As there were many forms associated with susceptability to AIDS, there were many forms significantly associated with resiatance in discordant negatives. These included HLA-B*0801, *4901; HLA-C*0716 among HLA class I subtypes and HLA-DR*100101 and *110201. Thus, the persistently constant and normal CD4 and CD8 count making these subjects resistant to HIV was associated with these subtypes and forms, indicating that resistance to HIV had a genetic background.

Discordant negatives were aslo different from AIDS subjects because of the strong associations of HLA-B*0801, *1817, *352001 and *4901 and HLA-DR*40301 with AIDs when compared with dsicordant negatives. Thus, in absence of these HIV susceptablity HLA subtype forms, dsicordant negatives would be in a better position to combat HIV as they were not naturally susceptable to HIV. As a result, the absence of these forms in discordant negatives might have aslo contributed to their resistance to HIV. Discordant negatives were also different from concordant couples because of the significant associations of HLA-DR*100101 and *110201 to concordant couples when compared with discordant negatives. Thus discordant negatives were different from discordant positives, concordant couples and AIDS patient in their HLA profiles. Similarly, AIDS patients were also very signicantly differen from concordant couples and discordant positves. Discordant positives were also diferent from concordant couples in their HLA profiles. Thus, differnet genetic mechanism operated in all groups in determining susceptability and resistance to HIV. Our findings are therfore in agreement with the previuos behavioral, immunological and other host factor(s) differnces in the different groups.

Homozygocity and heterozygocity of alleles and subtypes are known to make rapid or delay disease progression in HIV. Heterozygocity for HLA-A, B, and C is known in delaying onset to AIDS. It has been shown that homozygocity at the class I loci is associated with relatively rapid progression to disease compared with heterozygotes (Carrington et al., 1999). The heterozygote advantage probably stems from the ability of such individuals to present a wider array of virus-derived epitopes to a more diverse CTL. Hence, heterozygocity may be associated with delayed progression to AIDS.

When comparisons were made between discordant positives, concordant couples and HIV positive subjects, discordant positive subjects were found to be more heterozygous at all loci (HLA-A, B, C and HLA-DR) when compared with concordant couples and HIV/AIDS subjects. At HLA-A loci

the proportion between HIV/AIDS and CONC was (98.9% vs 87.9%) and between DSCP and HIV/AIDS was (98.9% vs 92.7%). Similarly, the proportion between DSCP and CONC and DSCP and HIV/AIDS at HLA-B loci was (98.9% vs 91.2%; 98.9% vs 94.9%), respectively. At HLA-C loci this was 98.9% vs 97.5%; 98.9% vs86.2%, and between DSCP and CONC and DSCP and HIV/AIDS, respectively. Similar result was also obtained when DSCPs were compared with HIV/AIDS (98.6% vs 94.4 %,) at HLA-DR loci. These showed that discordant positives were at an advantageous position by being more heterozygous and were capable of delaying progression to AIDS, when compared with concordant couples and HIV /AIDS subjects, which had more homozygous subtypes than discordant positives. The difference between concordant couples and HIV /AIDS subjects was not significantly different from each other, showing that both were equally homozygous.

4.6. Limitations of the study

The study has a limitation in that it is a one-shot cross-sectional study. Although 50 discordant negative subjects were re-tested for HIV status and did not seroconvert even after one year, larger longitudinal study could give thorough and meaningful result. Many parameters like vaginal soluble IgA and urine IgG and IgM level, chemokine production, HIV-peptide stimulated CD8 T cell activation, the study of purified CD4 and CD8 and cytokine production by discordant couples have not been studied due to logistic and resource problems, although samples for these parameters have been collected and kept frozen. Thus, absence of the results from these parameter's studies have created a small gap in the study. Lack of time and training for the analysis of the obtained data such as HIV full genome sequence analysis is also the limitation of the study. A small number of samples were analyzed for HLA in discordant negatives. Although this can give us clue about HLA subtypes, large sample size should be typed to get a meaningful result

4.7. Conclusions

There were known behavioral differences between discordant negatives and positives and concordant couples. There was very close similarity in behavior between discordant positives and concordant couples, showing that they shared similar risk behaviors. But the difference between discordant negatives and discordant positives was clear and big enough showing distant behavioral similarities. The healthy controls were behaviorally very much different from both discordant and concordant couples but they were similar to discordant negatives in some of their behaviors.

Almost all of them were not aware of HIV until they were voluntarily counseled and tested and were involved in unprotected sex before marriage. All of them had multiple partners and many times married. Discordant positives and concordant couples had more partners and were more times married when compared to discordant negatives and healthy controls. Their sexual frequencies and the number of sexual intercourse per a week was higher than discordant negatives

Although many of them were multiply married and never stuck to one marriage partner, the vast majority of them were satisfied in their marriage. The reason why they were satisfied in their marriage was varied. Love, faithfulness, and honesty were not the major reasons for their satisfaction in marriage. Some of them were dissatisfied in their marriages and the reason for this revolved around absence of love, faithfulness, honesty and mutual respect to each other and tolerance all resulting from absence of true love.

Many of the discordant positives and concordant couples had history of symptoms of STDs. But in discordant negatives fewer members reported the history of STDs. The major STDs reported were gonorrhea, syphilis, and chancroid and chlaymiadial diseases, in decreasing order.

The HIV positive partners perceived HIV infection mechanisms were known risk factors of HIV transmission in the country. These were associated with their sexual lives, family, occupation, social evils and injustice.

When subjects were compared immunologically, discordant negative partners had adequate amount of CD4 equivalent to healthy subjects and highly significantly different from discordant positives. CD4 and CD8 ratio was also high indicating a healthy balance and this was also similar to healthy controls. Discordant positive partners had a significantly different number of CD4 cells when compared to concordant couples. Their CD8 number was very similar to discordant negatives and there was no significant difference. Increased CD8 number was associated with decreased viral load and in some subjects even to the level of below detection level. Lower viral load in discordant positives when compared to concordant couples also indicated lower or absence of transmission to uninfected partner. CD8 T cells were responsible in decreasing viral load. The evidence for this came from the observation that concordant couples showed elevated viral load and decreased CD8 T cells number while discordant positives showed elevated CD8 and very low viral load. CD8+T cells may have different roles in discordant positives and concordant couples as there was an inverse relationship between viral load and CD8+ T cells in discordant positives but not in concordant couples. Phenotypic difference of CD8+ T cells may underlie these differences in function as phenotypic differences may reside in the ability to CD8+ T cells to mediate cytolysis,

secrete suppressive factors, or proliferate *in vivo*. Their CD4 number was also closer but slightly higher than the normal boundary count and might have been capable of providing the appropriate help for CD8 cells.Syphilis was a known risk factor for HIV transmission as it was diagnosed in many of discordant positives and concordant couples. This is possible because syphilis is a common STD in this country and its chronic nature might have accounted for its co-factor effect. Analysis of T cell subpopulations in discordant couples showed no activation of a specific marker between discordant positives and negatives and the expression of T cell subpopulations was comparable. In discordant positives the immune system was perfectly healthy and there was no indication of any abnormality. Rather, activation markers, which are indicators of immune activation, were significantly lower than concordant couples. These less activated immune cells, with others, might have enabled them to fight HIV. In discordant positives, in addition to decreased number of activation markers there were also expression of certain markers (CD4+CD45RA-CD27-) in higher proportion, which were common in long-term-non-progressors. The presence of this marker in higher proportion (30%), with others, might have enabled them to fight HIV and prevented further spread, but might have not been able to clear HIV completely due to some unknown factors.

Our study showed that there was a clear difference between discordant positives and discordant negative couples in their genetic profiles. There was also a clear difference between discordant positives and concordant couples and AIDS patients, in their genetic profiles. Ethiopian AIDS patients were different from Ethiopian concordant couples in their very significant to significant association with HLA-A*29, *18, and *41; HLA-B*0705, *1517, *4101, *5001, *7301 and *18; HLA-C*0501, *0701, and *0740. AIDS patients were also very significantly differen from dsicordant positves in their associations HLA-A*68, HLA-B*39 and HLA- DR*11. AIDS patient were also different from discordant negatives in their very highly significant to highly significant association with HLA-*0801, *1817, *352001 and *4901; HLA-C*7 and HLA-DR*40301. Concordant couples were also different from discordant positves in their very higly signifcant to significant associations with HLA-B* 0705, *0801 and *3910. Concordant couples were also diferrent from dsicordant negatives in their significant association with HLA-DR*100101 and *110201. Discordant negatives were different from discordant positives in expressing HLA profiles of HLA-B*0801, *39, *41, *39; HLA-C*0716, HLA-DR*100101 and *110201. Overall, the differnces between the different groups had a genetic background.

When comparisons were made between discordant positives, concordant couples and AIDS subjects, discordant positive subjects were found to be more heterozygous at all loci (HLA-A, B, C

and HLA-DR) when compared with concordant couples and HIV/AIDS subjects. This showed that discordant positives better controlled HIV and maintained HIV in check and were non- progressors due to heterozygous advantage. Overall, the results for discordant positives and AIDS subjects were clear enough to show significant difference between them.

Ethiopian HIV viruses were mainly HIV type C in all discordant positives and HIV/AIDS subjects. But other subtypes such as subtype A, B and recombinant A/G subtypes were also observed.. The observation of other subtypes and recombinant forms indicated that other subtypes are circulating in the population and the possibility of co-infection by different subtypes. Coreceptor utilization by Ethiopian HIV subtype C was different from the previous coreceptor utilization by subtype C viruses. Coreceptor utilization of discordant positive isolated viruses was both CCR5 and CXCR4 in equal proportion. The majority of HIV/AIDS patients used CXCR4, although about one third used CCR5 and a few also used dual co receptors. Our study showed that the majority of subtype C viruses were CXCR4/SI high/rapid subtype. And about one third was CCR5/NSI subtypes. The reason for this mosaic utilization of co-receptors was unknown from this study although the majority of our subjects showed a typical AIDS profile. The phylogenetic or evolutionary relationship showed that the majority of the viruses isolated from discordant positives showed subclustering in one region and those isolated from concordant couples in another region, showing that discordant positive isolated viruses were evolving independently and were related with each other. HIV/AIDS isolated viruses did not show subclustering and were highly divergent. Some also segregated early and were evolving independently.

4.8. Recommendations

Due to the above indicated shortcomings of the study, the following further studies are highly recommended.

Further longitudinal studies involving larger sample size on study subjects as well as new study subjects.

Further studies on purified CD4, CD8 and their subpopulations to further investigate the role of these T cells in clearing HIV and their interactions in doing so.

Further analysis of samples collected and stimulated and stored at -80 such as PHA stimulated PBMC supernatants for chemokine and cytokine production, testing vaginal wash and urine for soluble IgA and other immunoglobulin such as IgM and IgG; testing for serum antibodies for HIV co-factors such as herpes simplex type-2 and others.

Further analysis of full viral genome sequence and their amino acid sequences to further characterize Ethiopian HIV genes and the similarity and differences with other African countries and the rest of the world.

Analysis of full genome sequences of HIV subtypes to study whether Ethiopian subtypes are C or C' and their biological properties, the introduction of other subtypes of HIV to Ethiopia and the emergence of recombinant forms, and drug resistance in discordant and concordant couples.

More samples should be typed for HLA in discordant negatives as fewer samples were analyzed.

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6. Appendixes

Appendix- one

Questionnaire

Structured in-depth interview Questionnaire for the Mechanism of Resistance to Discordant Couple PhD project

Identification Code----------

Part I Socio demographic characteristics

1. Age-------- Years

2. Gender A) Male -------- B) Female--------

3. Marital statuses

A) Single------ B) Married------C) Separated ------- D) Divorced----

E) Widowed-------

4. How many times are you married?

A) One--- B) Two---- C) ---- Three---- D) more than three times-----

5. For how many years have you been together? (Number of years in marriage)

A) For one year----- B) For two years----- C) For three years----- D) For more than four years----------

6. What is your educational status? (Educational status)

A) Illiterate--------

B) Read and write------

C) Elementary-------

D) High school completed----

E) College and above------

7. Occupation/Work

A) Farmer

B) Merchant

C) House wife

D) Government Employee

E) Non-government Employee

F) Laborer

G) Broker

H) Driver

I) Solder

8. Number of children

A) One

B) Two

C) More than two

Part II History of HIV infection

9. Are you aware of HIV? When?

A) Before VCT

B) After VCT

10. If you are infected with HIV, how are you infected with HIV? (Perceived mechanism of HIV infection)

A) Infection within a family

B) Promiscuity

C) Multiple marriages

D) Extramarital sexual intercourse

E) Occupational risk

F) Contact with risk group (prostitution)

G) Infection from a partner

F) Rape

H) More than one of the above factors

11. Number of previous sexual partner

A) Only one

B) Two

C) Three

D) Four

E) More than four

12. Type of sex involved

A) With Condom always

B) With Condom sometimes

D) Without condom

E) Others

13. Frequency of sexual intercourse per a week

A) One time

B) Two times

C) Three times

D) Four times

E) More than four times

14. Frequency of sexual intercourse in one time contact

A) One time

B) Two times

C) Three times

D) More than Three times

15. The type (nature of the sex) involved

A) Soft sex

B) Aggressive sex

16. Are you satisfied with your marriage?

Yes

No

17. Reasons for satisfaction

A) Sexual satisfaction (Compatibility)

B) Presence of children

C) Handling (Caring, with good behavior, not nagging, and the like)

D) Economic

E) Dedicated for marriage (Working for the good of the family, relatives; solving all problems in the right way and paying the necessary scarifications when necessary)

F) Honest and faithfulness

G) Love

H) Religion

I) More than one of the above

18. Reasons for dissatisfaction in marriage

A) Economic

B) Mishandling

C) Sexual incompatibility

D) Absence of children

E) Disagreements

F) Joulesy

G) Not dedicated for marriage

H) More than one of the above

Part III History of sexual transmission of disease

19. Did you have symptoms of sexually transmitted diseases? (Like burning sensation after urination, ulcers and swellings on the genitalia, discharge of fluids from the genitalia and the like)

A) Yes

B) No

20. What type of sexually transmitted disease was it?

A) Syphilis

B) Gonorrhoea

C) Others

21. Did you get any treatment for this?

A) Yes

B) N0

Appendex-2 Viral sequence summary data

REGION AMPLIFIED	SEQUENCE FROMP ROVIRAL DNA ONLY	SEQUENCE FROM RNA ONLY	TOTAL
Full Genome	27	98	125
gag-vif	6	13	19
gag-pol	11	13	24
gag and vif-nef	1	1	2
gag-vif and nef	0	3	3
vif-nef	5	2	7
only small region on *pol*	15	1	16
only *gag*	9	17	26
sample for which PCR was run	74/161	136/145	210
Samples for which sequence data was obtained	47/161	110/145	157