THE ROLE OF INTERLEUKIN-10 PROMOTER POLYMORPHISMS IN HIV-1 SUSCEPTIBILITY AND PRIMARY HIV-1 PATHOGENESIS

by

Dshanta D. Naicker

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PREFACE

The experimental work described in this dissertation was carried out in the Hasso Plattner Research Laboratory, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, from April 2006 to December 2007, under the supervision of Professor Thumbi Ndung'u and the co-supervision of Professor Emil Kormuth.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any other University. Where use has been made of the work of others, it is duly acknowledged in the text.

Signed:
Dshanta D. Naicker (candidate)

Signed:
Professor Thumbi Ndung'u (supervisor)

Signed:
Professor Emil Kormuth (co-supervisor)

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I would like to express my appreciation and sincerest thanks to the following for their contribution to this project:

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My family, thank you for always being there for me, your love, encouragement and understanding are immensely appreciated, thank you for believing in me.

In Loving Memory of my Uncle Sounds (1959 – 2007)

ABBREVIATIONS

AIC	:	Acute Infection Cohort
AIDS	:	Acquired Immunodeficiency Virus Syndrome
ARG	:	AIDS Restricting Gene
ARMS-PCR	:	Amplification refractory mutation system-polymerase chain reaction
Bp	:	base pairs
CAPRISA	:	Centre for the AIDS Program of Research in South Africa
DNA	:	Deoxyribonucleic acid
G	:	gram(s)
Het	:	Heterozygote
HIV	:	Human Immunodeficiency Virus
Hom-P	:	Homozygous for polymorphism
Hom-WT	:	Homozygous Wild Type
IL-10	:	Interleukin-10
kDa	:	kiloDaltons
mg/ml	:	milligrams per millilitre
ml	:	millilitre
ng	:	nanograms
pg/ml	:	Picograms per millilitre
RNA	:	Ribonucleic acid
Th1	:	T Helper Cell Type 1
Th2	:	T Helper Cell Type 2
WHO	:	World Health Organisation
µl	:	microlitre

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ABSTRACT

Host genetic factors may partially account for the uneven distribution of HIV infection worldwide. In addition to influencing relative susceptibility to HIV, host genetic factors may also affect the rate of disease progression in persons who are already HIV infected. IL-10 was previously identified as an AIDS restricting gene (ARG), i.e. human genes with polymorphic variants that influence the outcome of HIV-1 exposure or infection. IL-10 is a Th2 cytokine, with anti-inflammatory properties, and plays a significant role in the regulation of immune responses; this cytokine may also directly influence viral replication. This study focused on the role of genetic polymorphisms in the proximal promoter region of the IL-10 gene on HIV-1 susceptibility and primary HIV-1 pathogenesis in a South African cohort comprising of women at high risk of HIV-1 infection.

In this study 228 black females from the CAPRISA Acute Infection cohort were genotyped for two polymorphisms that naturally occur within the proximal region of the IL-10 promoter, at positions -1082 and -592 (tracking -819) relative to the transcription start site. DNA samples from study participants were genotyped using the amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) method, which utilises specifically designed primers to detect single nucleotide polymorphisms. The allele frequencies for the mutant -1082G and -592A variants were 0.3203 and 0.333 respectively.

Individuals homozygous for the mutation at the -592 position (AA genotype) were 2.78 times more likely to become HIV infected, compared to those who were homozygous wild type (CC genotype) at the same position (*p*-value=0.0237). Among those who became HIV infected, we found a hierarchical association between IL-10 promoter variants and HIV-1 plasma viral load or $CD4^+$ T cell counts over the course of the first year of HIV-1 infection. At earlier time points, i.e. 0-3 months post-infection, the -1082GG group had significantly higher median viral loads than the -1082AA or -1082AG groups (*p*-values= <0.0001 and 0.0003 respectively); and the -1082AA group had the highest median $CD4^+$ T cell count compared to the -1082AG or -1082GG groups and this was significant (*p*-values= 0.0194 and 0.0122 respectively). At 6-12 months post-infection the median viral load of the -1082GG

group was lower than -1082AA group, however this was not significant (*p*-value=0.6767). Analysis of the effect of the -592 polymorphism showed that the -592AA group had a lower median viral load at 0-3 months post-infection compared to the homozygous wild-type group (i.e. -592CC p-value=0.0093); and the median $CD4^+$ T cell count for the -592AA group was significantly higher than the -592CC group (*p*-value= 0.0198). At 6-12 months post-infection, the median viral load as well as the median $CD4^+$ T cell count of the -592AA group were both no longer significantly different to the -592CC group (*p*-values= 0.6441and 0.6461 respectively). Plasma IL-10 expression was not significantly different between the IL-10 genotypes for any of the polymorphic positions.

Overall, these results suggest that polymorphisms within the IL-10 promoter may influence the risk of HIV infection and that they may affect primary HIV-1 pathogenesis. Interestingly, our data suggests that the effect of these polymorphic variants on viral and CD4+ T cell counts may vary according to time post-infection. To our knowledge, this is the first study to suggest that an ARG may have a differential effect on markers of disease progression depending on the phase of infection studied. The mechanisms underlying these observations require further studies and may have important implications for HIV/AIDS pathogenesis and the development of effective vaccine and immunotherapeutic strategies.

ETHICAL APPROVAL

Full ethical approval, from the Ethics Committee of the Nelson R. Mandela School of Medicine, University of KwaZulu-Natal (ref E013/04), and informed patient consent was obtained for this study. This study also received ethical approval from the University of Cape Town and The University of Witwatersrand.

Chapter 1

Literature Review

Chapter 1

Literature Review

1.1. INTRODUCTION

In 1994, Dr Samuel Broder of the World Health Organization (WHO), said: "In June of 1981 we saw a young gay man with the most devastating immune deficiency we had ever seen. We said, 'We don't know what this is, but we hope we don't ever see another case like it again' ". We have come along more than two decades since and the global acquired immune deficiency syndrome (AIDS) epidemic has risen to pandemic proportions. Despite the broad and substantial increase in knowledge of this disease since the description of the initial cases, there is still no preventive vaccine or cure available.

AIDS was first described in 1981 in the United States as a cluster of unusual immunodeficiencies affecting young homosexual men (Selik *et al.*, 1984; Cameron, 2006). The human immunodeficiency virus (HIV) was isolated in 1985, and strong epidemiological evidence suggested that HIV is the agent that causes AIDS. According to the 2007 Report on the Global AIDS epidemic (UNAIDS, 2007) 32 200 000 individuals world-wide are now living with HIV. Of this total approximately 22 500 000 HIV-infected individuals are from sub-Saharan Africa, and this includes South Africa. Figure 1.1 below is a map of the global view of HIV infection. These data demonstrate that almost two-thirds of the world's HIV-infected individuals are from sub-Saharan Africa.

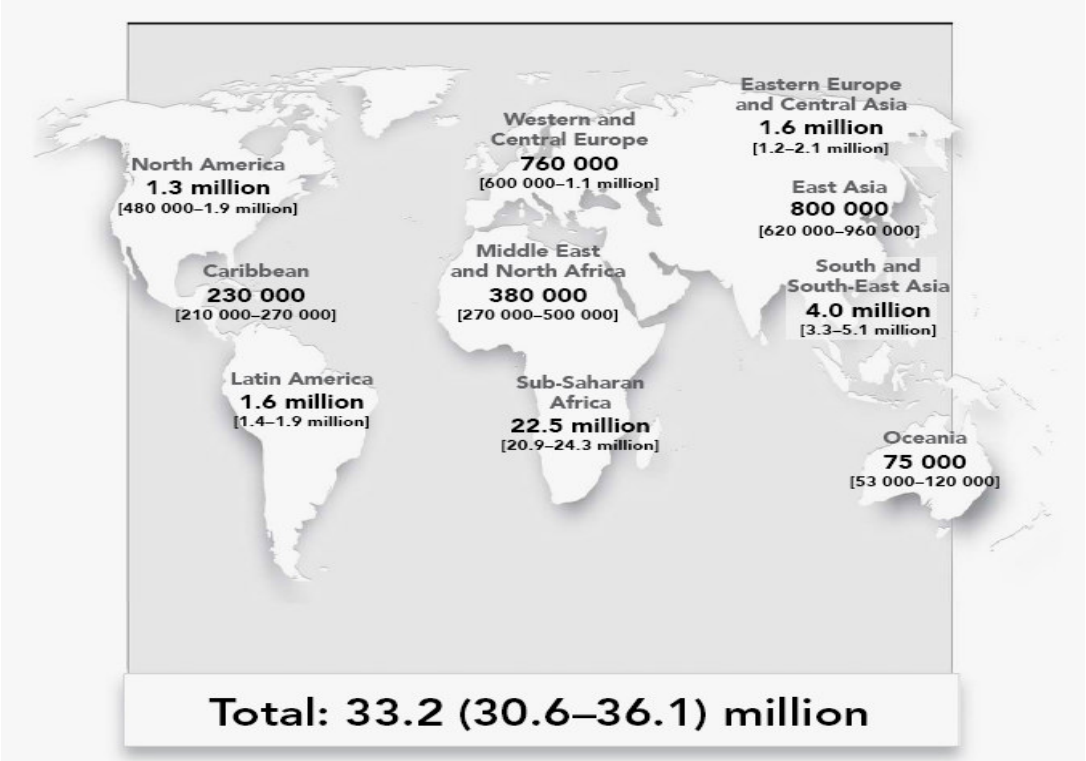


Figure 1.1. A Global view of HIV infection (taken from UNAIDS, 2007). An overview of the geographical distribution of HIV prevalence in adults and children. This distribution shows that almost two-thirds of HIV-infected individuals are from sub-Saharan Africa.

Many different factors may contribute to the uneven distribution of HIV-infected individuals. For example, socio-economic factors such as poor nutrition and poverty can make populations vulnerable to HIV infection and spread. It has been suggested that the circulating virus strain or subtype is also a factor which could add to this uneven distribution. Another important factor which affects HIV infection is host biological factors. The genetic background of the population plays a major role in HIV susceptibility as well as HIV pathogenesis (O'Brien and Nelson, 2004; Montoya *et al.,* 2006; Burgner *et al*., 2006).

1.2. HIV

1.2.1. The Structure of HIV-1

As demonstrated in Figure 1.2, the human immunodeficiency virus is spherical in shape. The outer protein coat is known as the envelope, and the shape is determined by proteins that make up the outer protein coat. Glycoproteins are embedded throughout the lipid membrane. The envelope glycoprotein of HIV is produced as precursor polypeptide which is proteolytically processed to produce two subunits, a 41 kilodalton (kDa) transmembrane region, i.e. gp41; and a 120 kDa surface unit, i.e. gp120. HIV contains ribonucleic acid (RNA) as its genetic material (Turner & Summers, 1999; Freed & Martin, 1995). Two copies of the RNA are contained within the protein capsid. Also found within the protein capsid is reverse transcriptase, which is an enzyme that catalyses the synthesis of DNA from the RNA, subsequent to infection of the host cell.

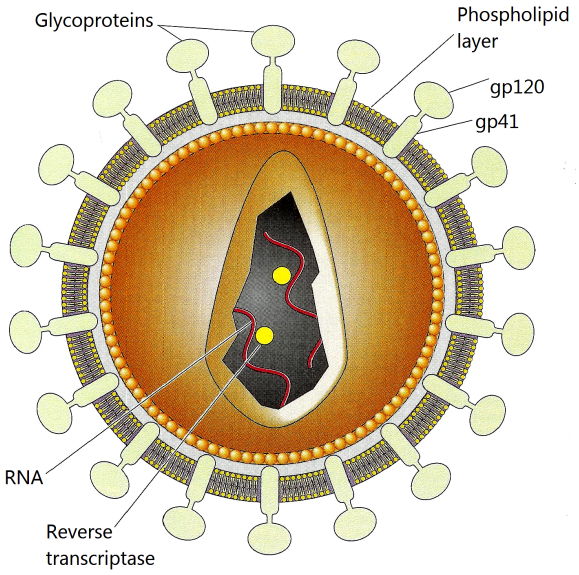


Figure 1.2. The Human Immunodeficiency Virus (taken from Wallace *et al.,* 1996). The virus consists of an outer envelope (phospholipids layer) which contains glycoproteins. The glycoproteins are known as gp120 and gp41 depending on the size of the protein. The RNA material and accessory enzymes are contained within the core of the virus.

1.2.2. The Life Cycle of HIV-1

The replicative part of the HIV life cycle occurs within a host cell, as the virus utilises host enzymes to perform vital steps for producing the components that will be assembled into new virions. HIV infection of the host cell commences with the interaction of the gp120 glycoprotein and the CD4 receptor (Haseltine, 1988; Fan *et al*., 2007). Interaction with the CD4 molecule leads to a conformational change of the envelope glycoprotein, which then results in the envelope making contact with a coreceptor found on the cell surface of the T-helper immune cell and other cells of the immune system (Moore *et al*., 1993), as shown in Figure 1.3. The binding of the HIV envelope to the coreceptor is followed by fusion of the virus with the host cell membrane. Fusion is mediated partially by the gp41 glycoprotein, and the virus then enters the host cell.

Once the virus has entered the cell, the surrounding layers of the virus are removed and the uncoated RNA is now exposed within the host cell. The viral RNA is then reverse transcribed into viral DNA by the viral enzyme reverse transcriptase. A complementary strand of the HIV DNA is formed from the viral RNA. During HIV reverse transcription many mutations arise, as there is a lack of a proof-reading function from the RNA polymerase enzyme (Preston *et al*., 1988; Quiñones-Mateu & Arts, 1999). The viral DNA is then transported into the nucleus in a preintegration complex (Levy, 1998) and integrates into the host genome with the aid of the viral integrase enzyme.

Once the viral DNA has integrated into the host genome, it is transcribed with the host genome and replication of the virus occurs. The host cell's RNA polymerase transcribes the HIV DNA, forming HIV messenger RNA (mRNA), which is then translated into the viral proteins; as well as the viral genomic RNA, which will be packaged into the new virions. This results in several copies of the viral RNA being formed. Along with the copies of viral RNA, other components of the virus are produced as well as the necessary viral proteins, which will be assembled into new virions. The final assembly of new virions occurs at the host cell membrane, and new virions bud from the surface of the infected cell. The released virions continue to infect other surrounding cells within the host.

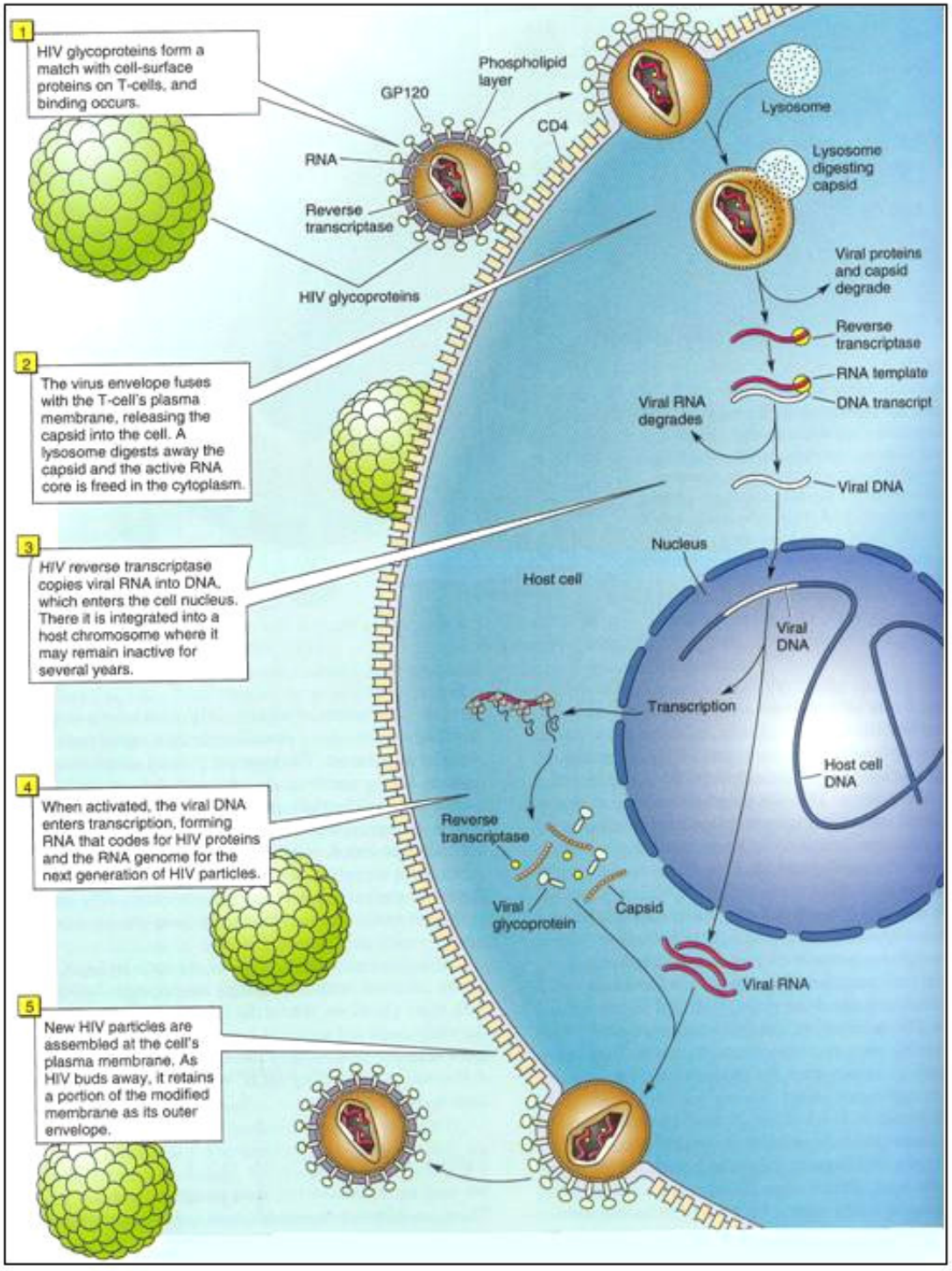


Figure 1.3. The Life Cycle of HIV (taken from Wallace *et al*., 1996) The HIV virus fuses to the host cell membrane and enters the host cell. When the virus enters the cell the viral RNA is transcribed into viral DNA, which then gets integrated into the host genome. The viral DNA is then transcribed, and viral proteins are produced by the host cell. The RNA and viral proteins are assembled into new virions, which then bud from the surface of the host cell. The virions are therefore released from the host cell, and then continue on to infect other cells.

1.2.3. The Genomic Organisation of HIV-1

The genes that are found within the genome of all retroviruses are *gag*, *pol* and *env*, which encode viral structural proteins and are essential for viral replication (see Figure 1.4). However, the HIV genome is more complex, and therefore contains several other genes, i.e. *tat*, *rev*, *nef*, *vif*, *vpr* and *vpu* (Levy, 1998; Emerman & Malim, 1998). These genes have regulatory or accessory functions in viral replication. The different genes have different functions and these functions are displayed in Table 1.1 below.

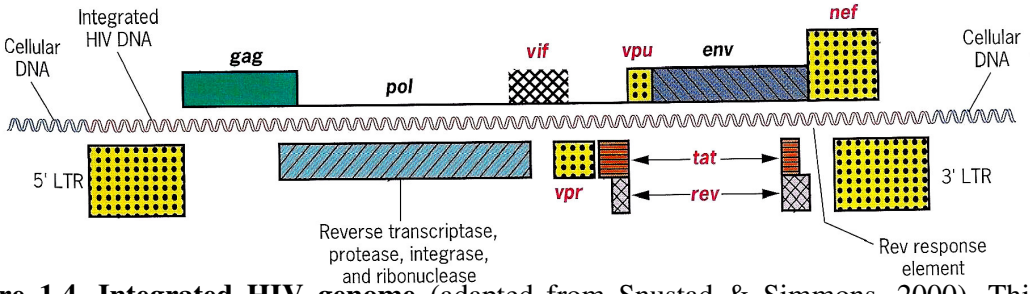


Figure 1.4. Integrated HIV genome (adapted from Snustad & Simmons, 2000). This shows the integrated HIV genome within the host genome (provirus). The diagram shows the organisation of the 9 HIV genes as they are arranged within the HIV genome.

Table 1.1. Genes of the HIV genome. The following table summarises the genes of the HIV genome and their corresponding function.

Gene	Function
gag	• Ecodes nulcear capsid core protein • Interacts with vpr • Involved with virion binding
pol	• Encodes viral enzymes (protease, reverse transcriptase & integrase
env	• Encodes viral coat proteins • Mediates CD4 binding • Mediates membrane fusion
vif	• Promotes infectivity of virus
vpr	• Weak transcriptional activator • Promotes infection of macrophages
vpu	• Involved in virion budding
tat	• Potent transcriptional activator
rev	• Regulator of structural gene expression
nef	• Enhances virion infectivity
LTR	• Binding sites for host transcription factors • Contains regulatory elements which control gene expression

Genes that are essential for viral replication

Genes that have regulatory functions

1.2.4. HIV Subtypes

During reverse transcription, mutations arise as there is a lack of proof-reading function by viral RNA polymerase. HIV has a very high replication rate, and therefore mutations rapidly accumulate resulting in the development of viral quasi-species *in vivo*. In addition to the error-prone nature of the viral reverse transcriptase, genetic variability and molecular evolution is also driven by the high rate of recombination within these viruses (Wei *et al*., 1995; Coffin, 1996; Moore et al., 2002). Due to the high mutation rate and recombination, the virus has diversified significantly. HIV can be divided into two types, i.e. HIV type 1 (HIV-1) and HIV type 2 (HIV-2). The HIV-1 virus is responsible for the majority of cases world-wide and can be further phylogenetically divided into three groups. The three HIV-1 groups are M (major/main), O (outlying) and N (non-M, non-O). Groups M and O appear to be genetically equidistant from group N (Quiñones-Mateu & Arts, 1999). A genetic distance of 35 – 49% separates group M from group O. The genetic sequence diversity found in the HIV genome is so significant that there is further distinct subclassification into nine major (M) clades or subtypes. These clades are designated A-D, F-H, J and K, as shown in Figure 1.5 below. Several circulating recombinant forms (CRFs) of the HIV virus also occur.

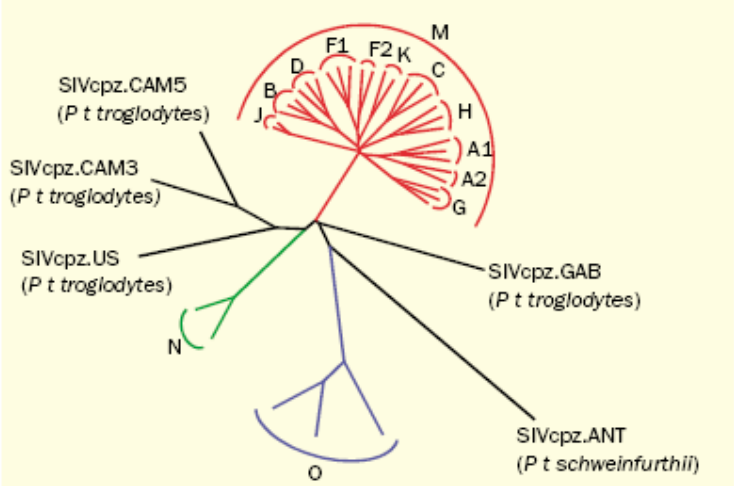


Figure 1.5. Phylogenetic tree showing HIV-1 groups and subtypes (taken from Thompson *et al*., 2002). This phylogenetic tree shows the three HIV-1 groups, i.e. group M, group N and group O. A genetic distance of 35 – 49% separates group M from group O. Simian immunodeficiency virus (SIV) isolates are also incorporated in this tree.

Due to the genomic diversity resulting from high recombination rates, as well as mutations, there is also a diversity of viral subtypes in accordance with different geographical regions. In sub-Saharan Africa, where the prevalence of HIV infected individuals makes up almost two-thirds of the world's infected population, the viral diversity is the greatest. In other words, there are several different subtypes found in sub-Saharan Africa. Figure 1.6 below shows the geographical distribution of HIV-1 subtypes globally. Clade C is the predominant HIV-1 subtype of the HIV pandemic, see Figure 1.7 (Hemelaar *et al.*, 2006). HIV-1C has caused the increase in HIV-1 infection in southern and eastern Africa (Janssens *et al.*, 1997; Spira *et al.*, 2003), as well as India and Nepal, and in countries such as Botswana, Zimbabwe, Malawi, Zambia, Namibia, Lesotho, South Africa, and China (Wainberg, 2004).

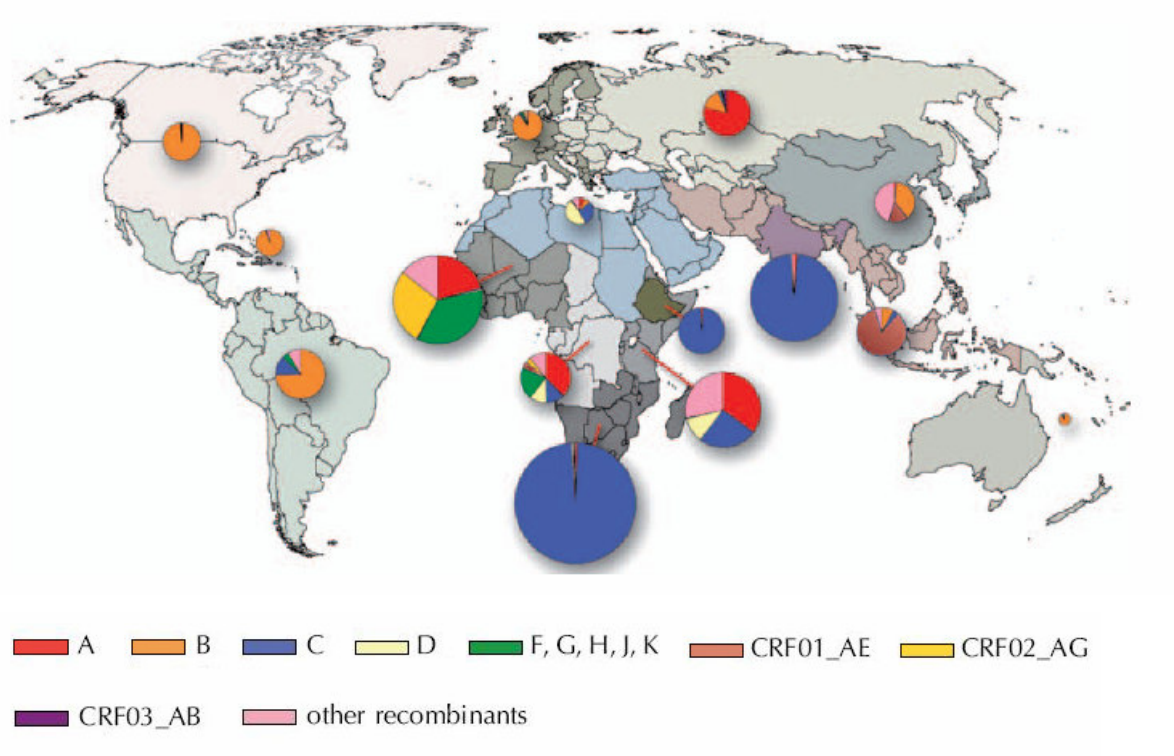


Figure 1.6. The global distribution of HIV-1 subtypes (taken from Hemelaar *et al.*, 2006). This world map shows the geographical distribution of the different HIV-1 subtypes. Countries which form a region are shaded in the same colour, and different regions are shaded accordingly, for example, the countries which form the sub-Saharan African region are shaded in the same colour. The designated pie at the different regions indicates the predominant HIV-1 clade circulating in that region.

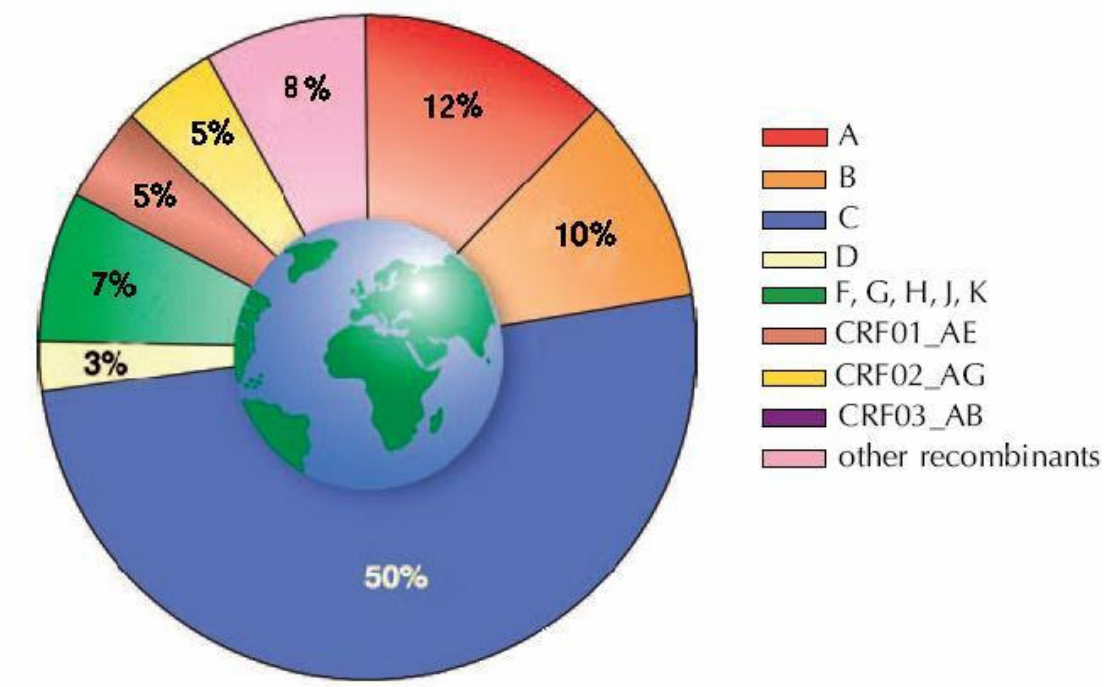


Figure 1.7. The occurrence of different HIV-1 subtypes and recombinants in 2004 (adapted from Hemelaar *et al*., 2006)**.** The percentages represent the proportion of individuals globally living with a specific HIV-1 subtype or recombinant. The colours representing different HIV-1 subtypes or recombinants are represented in the key.

1.2.5. The Phases of HIV-1 Infection

There are different phases during the natural course of HIV-1 infection. Immediately after infection, most individuals experience influenza-like symptoms (Snustad & Simmons, 2000). This early, short and symptomatic phase of HIV-1 infection is known as the acute phase of infection. During the acute phase, HIV-1 undergoes robust replication in infected individuals and the viral load rapidly rises to very high levels. Subsequently, the viral load declines to a steady state known as the viral set point, but it is not eliminated (Janeway *et al*., 1999), and it therefore continues to rapidly replicate within the host, and infection of new cells continues, accompanied by a rapid T-cell turnover. During infection the T-cells are the primary target for HIV replication and the depletion of $CD4^+$ helper T cells begin (demonstrated in Figure 1.8.). The $CD4^+$ T helper cell plays a key role in the general coordination of the immune response (Sherwood, 2001). The virus also attacks other cells

of the immune system, such as macrophages, which leads to further destruction of the immune system.

Following the acute phase of infection the person then enters an asymptomatic phase, which lasts from 1 year to as long as 15-20 years. A gradual decline in T-cells occurs, but the immune system continues to fight the infection and $CD4^+$ cells are rapidly replenished, therefore the individual appears to be asymptomatic following the acute phase of infection. However, persistent viral replication continues (Koup *et al*., 1994; Bailey *et al*., 2006), and the T cells gradually diminish. The degree of viral control determines disease progression (van den Bosch *et al*., 2006, Arnaout *et al*., 1999). A high viral set point following acute infection is a prognostic indicator of rapid disease progression and a low viral set point is a predictive marker of slow disease progression (Schacker *et al*., 1998; Mellors *et al*., 1996; O'Brien *et al*., 1996; Lyles *et al*., 2000). The viral load set point can therefore be used as a surrogate indicator of slow or rapid disease progression to study the factors that determine the rate of disease progression, in cases of primary infection cohorts with short longitudinal follow-up periods. On the other hand, several studies indicate that viral load and $CD4^+$ T lymphocytes counts are inversely proportional (Arnaout *et al*., 1999). Therefore, viral load and $CD4^+$ T cell measurements can be used as surrogate markers of disease progression within HIV-1 infected individuals, particularly following the resolution of acute viremia.

Following the asymptomatic phase, the T cells continue to diminish and eventually symptoms of generalized immunodeficiency begin to appear. The immune system is suppressed and as a result opportunistic infections occur. This continues until the infected individual develops an AIDS-defining condition, during which the T-cell count drops to less than 200 cells/µl of blood.

Figure 1.8 demonstrates the typical course of HIV infection; however, this natural course of infection can vary widely between individuals. Most HIV-infected individuals will follow this course and continue to develop AIDS, and eventually this leads to death due to opportunistic infections. However, some individuals, known as long-term non-progressors, are able to control the virus to relatively low levels of viremia, while other individuals, known as rapid-progressors show an atypical fast disease progression course (Bacchetti & Moss, 1989; Cao *et al*., 1995). It has been reported that HIV specific $CD4^+$ T-cell function

is inversely related to viral load (Jansen *et al.,* 2006), but other factors may also play a role in differential outcome of HIV-1 infection. It has also been demonstrated that certain individuals remain seronegative despite high levels of exposure to HIV-1, and numerous factors including host genetic factors are likely to play a role in this phenomenon.

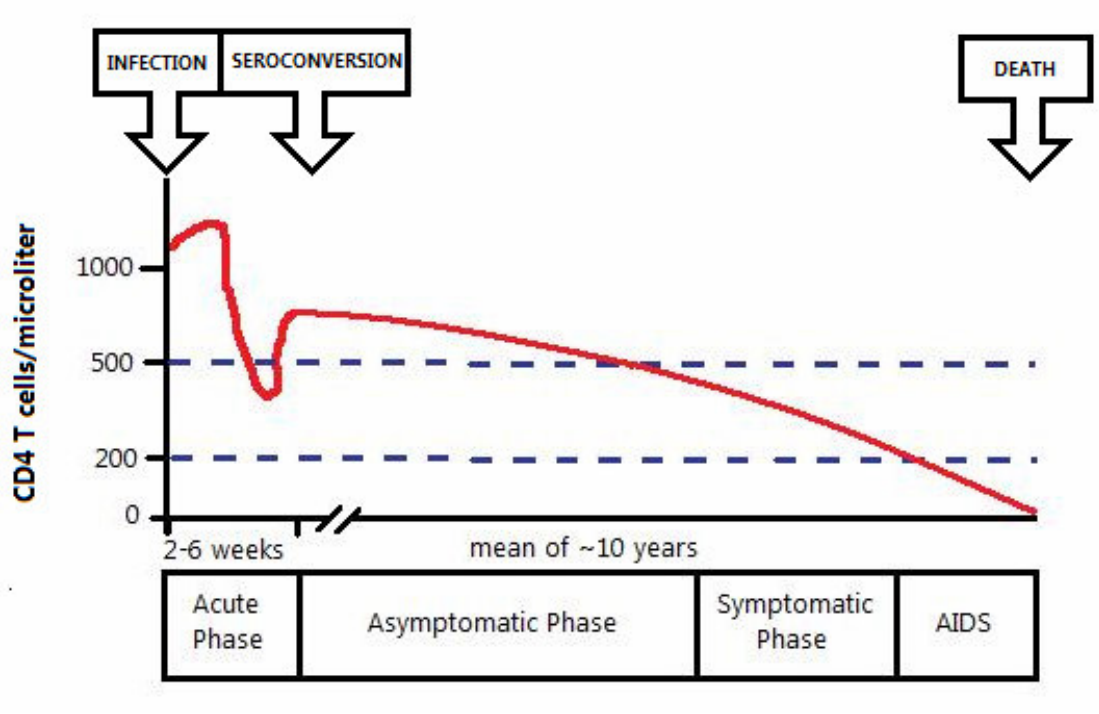


Figure 1.8. The typical course of HIV infection (adapted from Janeway *et al.*, 1999). This demonstrates the depletion of $CD4^+$ T cells during HIV infection. After infection, the $CD4^+$ cell count drops drastically as a result of rapid replication of the virus. Following this acute phase, the body is able to control viral replication to a certain point, leading to restoration of levels of $CD4^+$ T cells. The body is, however, unable to eliminate the virus.

1.2.6. Host Factors which limit HIV-1

Allelic variants in the human genome most likely regulate susceptibility or resistance to HIV infection and disease progression, as well as the environment and the variability of the pathogen (Carrington and O'Brien, 2003; Shrestha *et al*., 2006). Host factors known as AIDS restricting genes (ARGs) modulate disease resistance or disease progression, mainly by affecting HIV cell entry, immune recognition and antigen presentation (Winkler *et al*., 2004). According to O'Brien and Nelson (2004), ARGs are defined as human genes with polymorphic variants that influence the outcome of HIV-1 exposure or infection. Differences in controlling viral replication are somewhat determined by allelic variants in genes that are thought to play a role in viral replication as well as immunity (O'Brien and Nelson, 2004). Different ARGs are involved at different stages of HIV entry, HIV life cycle or HIV infection, as well as the host immune response.

There are two cytokine genes, interleukin 10 (IL-10) and interferon gamma (IFN-γ) that were identified as ARGs (O'Brien and Nelson, 2004; Winkler *et al*., 2004). These ARGs are thought to influence HIV pathogenesis either by a direct effect on viral replication or through modulation of the immune response, which then affects replication of the HIV-1 virus. IL-10 promoter polymorphic variants limit HIV-1 infection and affect disease progression, while IFN-γ allelic variants have been shown to affect disease progression. The polymorphisms in these genes that limit HIV-1 infection or influence disease progression are mainly single nucleotide polymorphisms (SNPs). SNPs are single base mutations in DNA, and about 90% of sequence variations in humans are due to SNPs (Collins *et al*., 1998).

1.3. The Human Immune System

The immune system aims to protect the body from potential harmful foreign molecules or abnormal cells. There are two types of immunity that come into play during the immune response, i.e. the innate immunity and adaptive immunity, shown in Figure 1.9. The innate

immune response mediates defence against microbes by early reactions, whereas the later responses are mediated by the adaptive immune response (Levy, 2001).

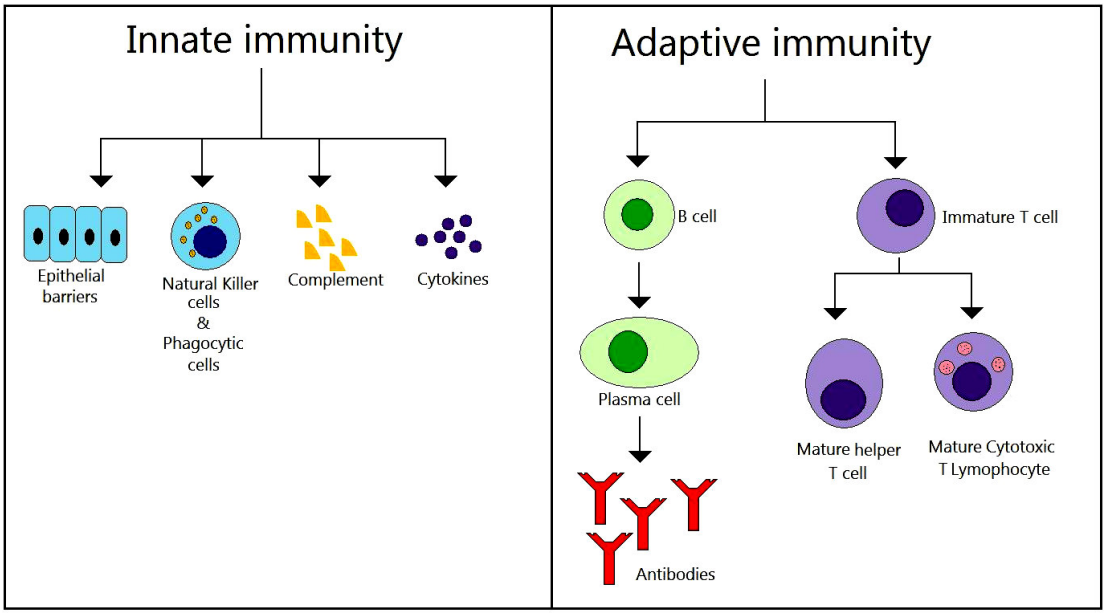


Figure 1.9. Overview of the human immune system. The innate immunity response reacts over a short period following pathogen invasion. The immune system is made up of epithelial barriers, natural killer cells & phagocytic cells, complement, and cytokines. The adaptive immunity response reacts over a longer period. The adaptive immunity is composed of humoral and cellular immunity, whose principal players are thought to be B and T cells respectively.

The innate immune response mechanisms present the initial defence against infection. Innate immunity consists of physical (epithelial) and chemical (antimicrobial) barriers; phagocytic cells such as neutrophils and macrophages, and natural killer (NK) cells; the complement system, which are plasma proteins that attack the plasma membrane of foreign cells; and cytokines, which are a group of proteins that regulate and directs the activities of the cells of innate immunity.

The adaptive immune response has “memory” of previous infections and can therefore respond more robustly to repeated exposures of the same microbe. Adaptive immune responses could be mediated by antibodies secreted by B lymphocyte derived cells; or by T lymphocytes which directly engage pathogens or secrete chemical messengers that mediate

the immune response. B cells are lymphocytes which have matured and differentiated in the bone marrow. T lymphocytes are thought to be the key players in cell-mediated immunity (Schindler, 1991). T cells are also derived from lymphocytes that originated in the bone marrow; however they mature and differentiate within the thymus (a gland situated within the chest cavity). Cell-mediated immunity is coordinated by T helper cells (Asadullah et al., 2003; Roitt & Delves, 2001) and these cells are divided into two subsets of cells, depending on their cytokine production pattern. T helper cell subsets have been identified as type 1 T helper cells, known as Th1 cells; and type 2 T helper cells, known as Th2 cells. Th1 cells are responsible for promotion of cell-mediated effector responses (Bidwell *et al*., 1999), and Th2 cells are involved in promoting B cell mediated humoral responses.

1.4. CYTOKINES

1.4.1 Properties of Cytokines

Cytokines are small secreted proteins that mediate and regulate immunity and inflammation (Oppenheim *et al*., 1994). Cytokines are involved in regulating the intensity and duration of the immune response, and many significant interactions between cells of the immune system are controlled by cytokines, see Figure 1.10. These intercellular communicators are synthesised, released and recognised by cells of the immune system (Nacy *et al*., 1990). Their molecular weights range from 8 – 70kDa.

Cytokines are produced by a diverse range of cell types including cells of the immune system (Bidwell *et al*., 1999). The broad classification of cytokines produced by cells of the monocyte lineage is known as monokines, and cytokines produced by lymphocytes are known as lymphokines. Cytokines that are released by T helper (Th) cells are named interleukins (IL). T helper 1 (Th1) cell populations produce a different set of cytokines to T helper 2 (Th2) cell populations. Examples of some cytokines produced by Th1 cells are: interferon-gamma (IFN-γ), interleukin 2 (IL-2), and tumor necrosis factor-α (TNF-α). Some cytokines that are produced by Th2 cells are: IL-4, IL-5, and IL-10. The Th1/Th2

balance between the secreted cytokines has the ability to influence the phenotype and the outcome of several diseases (Tsiligianni *et al*., 2005).

Cytokines which are released as a result of infection can directly up-regulate or down-regulate viral replication, depending on the type of cytokines released and the role they play. The initiation of cytokine binding to specific membrane receptors, results in a specific cytokine action.

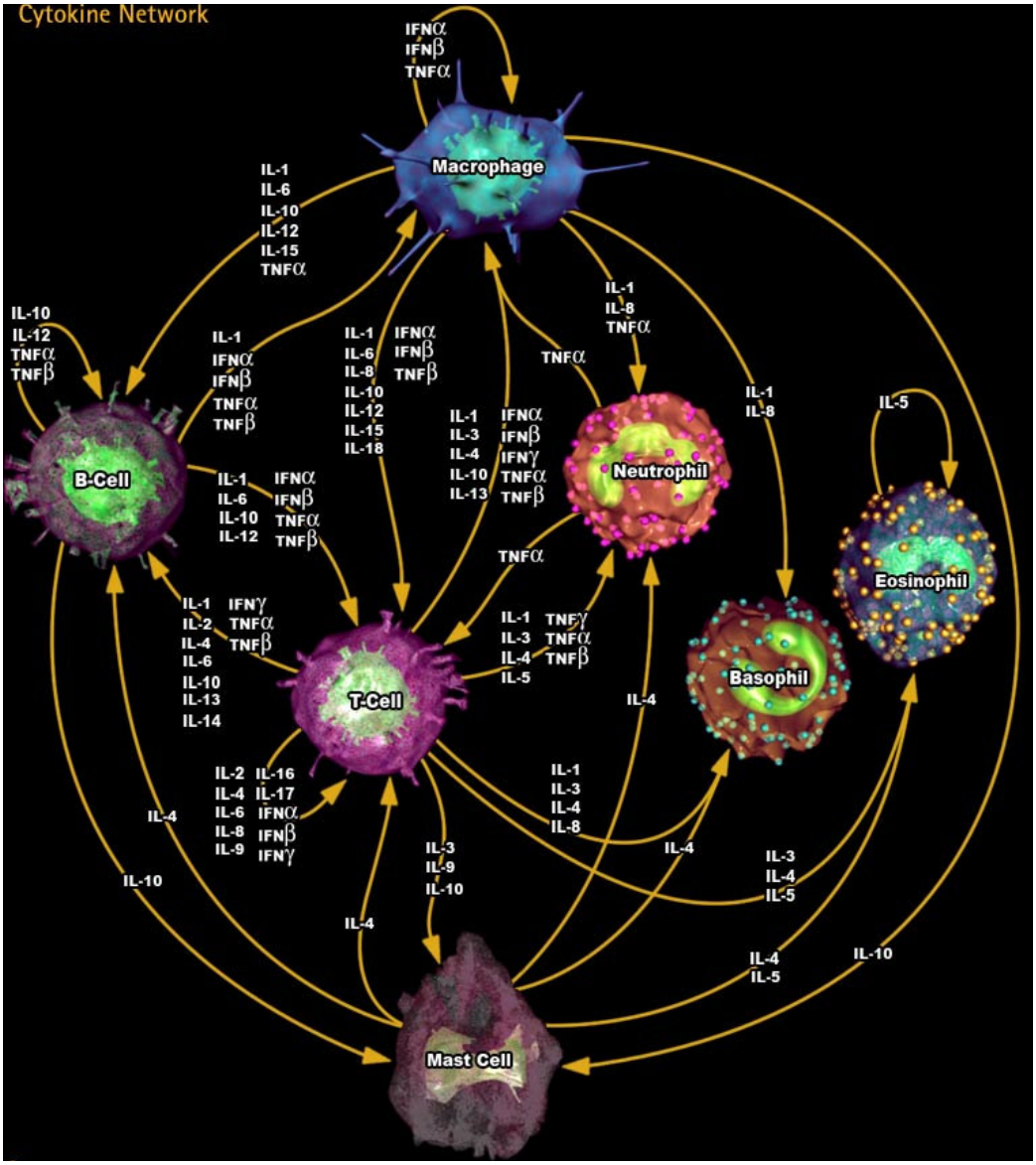


Figure 1.10. The Cytokine Network (taken from www.emdbiosciences.com). These intercellular signalling proteins, cytokines, operate in a highly complex but coordinated network. They are able to inhibit or stimulate their own synthesis, along with other cytokines and cytokine receptors.

1.4.2. Interleukin-10

Interleukins are a group of cytokines which stimulate immune cell proliferation and differentiation. Interleukin-10 is one of two cytokines that have been identified as AIDS restriction genes (O'Brien and Nelson, 2004). The IL-10 gene is located on human chromosome 1, between 1q31 and 1q32 (Lazarus *et al*., 2002). The gene spans about 5 kilobases (kb) and consists of five exons, and the resulting 36 kDa protein has a structure which is made up of a four α-helical globular domain structure (Janeway, *et al*., 1999). IL-10 is a pleiotropic cytokine that is involved with immunoregulation and down regulation of inflammation (Moore *et al*., 2001; Lech-Maranda *et al*., 2004). IL-10 was originally referred to as cytokine synthesis-inhibitory factor (CSIF) (Takeshita *et al*., 1995). IL-10 enhances B cell survival, proliferation and antibody production (Hunt *et al*., 2000; Croxford *et al*., 1998). IL-10 is a powerful cytokine that is produced by Th2 cells, that down-regulates the major histocompatibility class (MHC) class II antigens and expression of numerous proinflammatory Th1 cytokines such as IL-1, IL-6, IL-8, IL-12 and TNF-α from activated macrophages; and Th1 cytokines in turn down-regulate the expression of IL-10 (Tedgui & Mallat, 2006; Ness *et al*., 2004).

IL-10 interacts with specific cell surface receptors to mediate a specific biologic effect on target cells. IL-10 is an anti-inflammatory cytokine that controls its own expression, and prevents unnecessary inflammation (Abbas *et al*., 2007; O'Farrell *et al*., 1998). IL-10 is an example of a negative feedback regulator, as it is produced by macrophages, and acts to inhibit activated macrophages. The importance of IL-10 as an anti-inflammatory cytokine is demonstrated in knockout mice, which exhibit dysregulated inflammatory responses (Rennick *et al*., 1995).

Cytokine production is under genetic control (Hutchinson *et al*., 1998). Cytokine gene polymorphisms located in the upstream regulatory regions (promoter) can affect gene expression as well as disease pathogenesis. The IL-10 promoter region (Figure 1.11) contains three biallelic polymorphisms at positions (relative to the transcription start site) -1082 (A to G transition), -819 (C to T transition) and -592 (C to A transversion), which are related to levels of IL-10 production (Temple *et al*., 2003; Haukim *et al*., 2002; Kamali-Sarvestani *et al*., 2006; Lan *et al*., 2006). The T substitution at position -819 is in

complete linkage disequilibrium with the A substitution at position -592 (Stanford *et al.*, 2005), and therefore the alleles are inherited together, i.e. if the mutation occurs at the one position, then the mutation will always occur at the other position. The mutation from A to G at position -1082 has been associated with higher IL-10 production *in vitro* (Turner *et al.*, 1997). Previous reports demonstrate that the -1082A allele is associated with low IL-10 production, and the -592A allele is associated with low IL-10 production (Hutchinson, *et al.*, 1998; Turner *et al.*, 1997). Previous studies have also proven that IL-10 production varies between different IL-10 haplotypes (Eksdale *et al.*, 1998). The GCC/GCC haplotype is associated with high IL-10 production; the GCC/ATA or GCC/ACC haplotypes are associated with intermediate IL-10 production; the ATA/ATA, ATA/ACC or ACC/ACC haplotypes are associated with low IL-10 production (Edwards-Smith *et al.*, 1999).

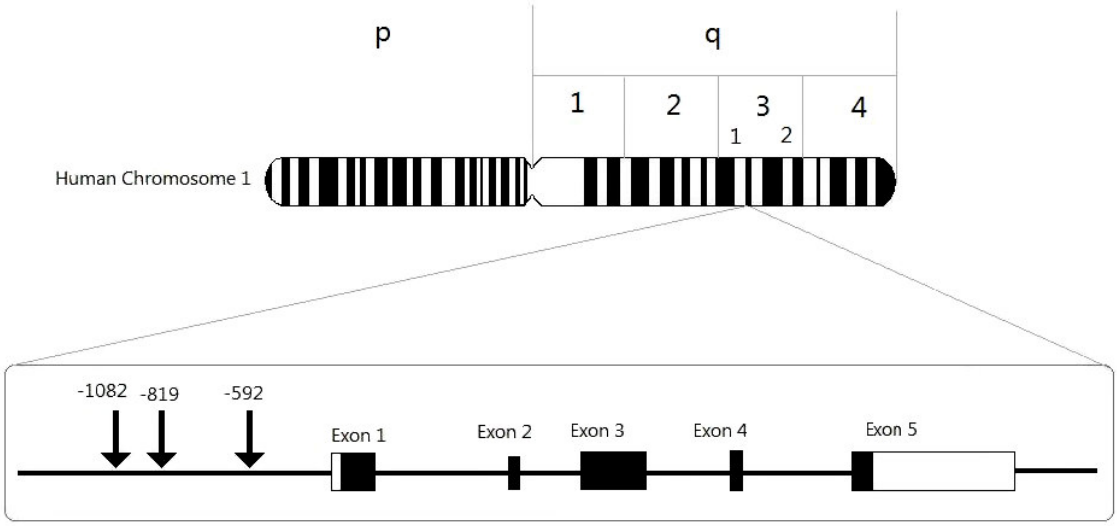


Figure 1.11. The location of the IL-10 gene on human chromosome 1 (adapted from Shin *et al.*, 2000 & http://ghr.nlm.nih.gov)**.** The IL-10 gene is located on the q arm of human chromosome 1, i.e. between 1q31 and 1q32. The IL-10 gene consists of 5 exons, and the polymorphic regions are located within the promoter region upstream from the transcription start site.

1.4.3 Interleukin-10 and Human Disease

IL-10 plays a significant role in the regulation of immune responses as well as inflammatory responses. Therefore IL-10 promoter polymorphisms, which affect the expression of the gene, may affect disease risk, pathogenesis or protection, and therefore these polymorphisms are of importance in several human diseases. Since IL-10 plays a key role in immunoregulation, it has been associated with a range of human diseases and this extensive list includes the following: atherosclerosis, pulmonary sarcoidosis, sudden infant death syndrome (SIDS), breast cancer, prostate cancer, asthma, rheumatoid arthritis, systemic lupus erythematosus (SLE), among others (Tedgui & Mallat, 2006; Tsiligianni *et al*., 2005; Opdal *et al*., 2003, Palmeri *et al*., 2005; McCarron *et al*., 2002). IL-10 has also been shown to play a role in transplantation due to its anti-inflammatory properties (McDaniel *et al.*, 2004).

Several studies have shown an association between IL-10 promoter polymorphisms and the pathogenesis of certain viral infections (Sabouri *et al*., 2004). Some viral infections that have been associated with IL-10 include hepatitis C virus (HCV) infection, hepatitis B virus (HBV) infection, Epstein-Barr virus (EBV) infection, lymphocytic choriomeningitis virus (LCMV) and human immunodeficiency virus (HIV) infection. Untreated chronic HCV infection has been associated with increased IL-10 levels. Edwards-Smith *et al.* (1999) showed that different polymorphisms within the IL-10 promoter region were associated with the initial response of chronic HCV infection to IFN-γ therapy. Hyodo *et al.* (2003) showed that increased levels of IL-10 were possibly associated with HBV viral persistence and the suppression of antiviral immune responses. The EBV encodes a viral homolog of IL-10, which appears to suppress early immunoregulatory cytokines, leading to ineffective immune responses (Moore *et al.*, 1990).

IL-10 production was found to be higher in asymptomatic individuals with severely compromised T cell functions (Clerici *et al*., 1994). Recent evidence has emerged that IL-10 may play a crucial role in the establishment and persistence of chronic viral infections (Jones, 2006). It was demonstrated in the lymphocytic choriomeningitis virus (LCMV) mouse model that IL-10 receptor blockade with a monoclonal antibody results in clearance of the infection (Ejrnaes *et al*., 2006). This resolution of persistent viral infection was

accompanied by reduced IL-10 production and enhanced production of interferon-gamma (IFN-γ) by anti-viral $CD8^+$ T cells. In another study, genetic removal of IL-10 resulted in induction of robust T cell immune responses, the rapid elimination of virus and the development of antiviral memory T cell responses, resulting in the complete clearance of the LCMV virus infection (Brooks *et al.*, 2006). These studies suggest that IL-10 primarily favours viral persistence by inducing immunosuppression, and that high levels of IL-10 lead to impaired T cell function with resultant establishment of viral persistence. Targeting IL-10 in therapeutic interventions could have a profound public health benefit, given the widespread impact of persistent viral infections on human health.

1.4.4 Role of Interleukin-10 with HIV-1 susceptibility and pathogenesis

The cytokine pattern during HIV infection may play a role in HIV pathogenesis (Clerici *et al*., 1994; Crabb Breen *et al*., 2000), and the regulation of HIV replication by the intricate cytokine network is multifaceted (Clerici *et al*., 1994; Kinter *et al*., 1996, Unutmaz *et al*., 1999; Tomiyama *et al*., 2002). IL-10 has been associated with a number of immune-mediated diseases, including HIV infection and pathogenesis. IL-10 is a Th2 cytokine that down-regulates the production of Th1 cytokines, therefore it has been suggested that decreased production of Th1 cytokines and the inhibition of cell-mediated responses as well as the inhibition of antigen-presenting cell function have adverse effects on HIV-infected individuals (Ji *et al*., 2005). It has been suggested that viral infection results in a reduction of IL-2 expression and IFN-γ production, and a decrease in IL-4 and IL-10 (Meira *et al*., 2004), in accordance with the hypothesis of the Th1 to Th2 shift during HIV progression and a possible role in the pathogenesis of AIDS (Trivedi *et al*., 2001; Fiore *et al*., 2006).

HIV replication occurs within monocytes/macrophages (Amirayan-Chevillard *et al*., 2000), and is thought to play a role in viral spread and persistence. It has been shown that HIV-1 has the ability to induce IL-10 production in monocytes, and the HIV-1 Tat protein facilitates the induction of IL-10 production (Badou *et al*., 2000). IL-10 has been shown to inhibit HIV replication in monocytes and macrophages (Wang & Rice, 2006; Porcheray *et al*., 2006). Infection in monocytes and macrophages appears to be less productive than in

$CD4^+$ T lymphocytes, but replication within monocytes and macrophages is thought to have significant consequences for HIV transmission and pathogenesis especially during the late stages of AIDS, when the $CD4^+$ T lymphocytes have been depleted (Orenstein *et al*., 1997).

IL-10 has been shown to down-regulate the expression of cyclin T1, which is a major component of the positive transcription elongation factor b (P-TEFb), a transcription elongation complex found within human monocyte derived macrophages (MDMs). Figure 1.12 shows the mechanism of transcriptional elongation within MDMs. The HIV Tat protein is required for efficient transcriptional elongation of the HIV-1 proviral genome by RNA polymerase (Wang and Rice, 2006). A cellular kinase complex which is made up of Cdk9 and cyclin T1 is recruited to the TAR RNA stem-loop structure by Tat. The Cdk9 and cyclin T1 complex is known as P-TEFb. Tat binds to cyclin T1 and is therefore able to recruit the P-TEFb complex to the TAR RNA stem-loop. Elongation occurs after the P-TEFb complex joins to the Tat and the TAR RNA stem-loop. IL-10 plays a role in the inhibition of HIV-1 replication within monocytes/macrophages by down-regulating cyclin T1.

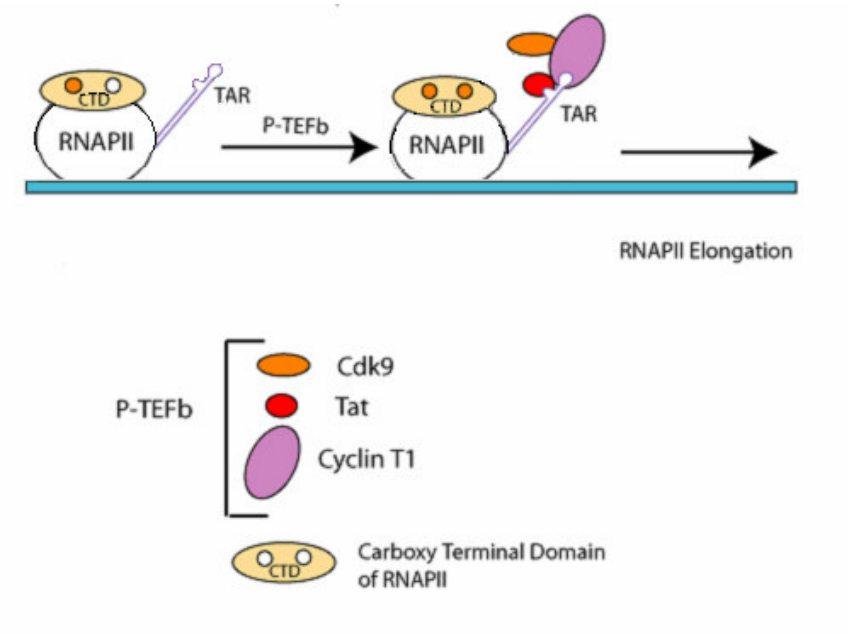


Figure 1.12. The transcriptional elongation of the proviral genome in monocytes/macrophages (adapted from Barboric & Peterlin, 2005). Elongation of the proviral genome by RNA polymerase in monocytes/macrophages requires the recruitment of the P-TEFb complex to the TAR stem-loop structure. The P-TEFb complex is composed of cyclin T1 and Cdk9. Tat binds directly to cyclin T1 and recruits it to the TAR structure.

IL-10 production is dependent on IL-10 promoter polymorphisms and the resulting genotype and haplotype. Different haplotypes and genotypes have been associated with different levels of IL-10 gene expression, as these polymorphisms occur within the promoter region, which is found upstream from the transcription starting site and therefore controls expression of the gene.

Shin *et al.* (2000) demonstrated that IL-10 promoter polymorphisms play a role in HIV-1 susceptibility as well as HIV disease pathogenesis. The mutation at the -592 position (nucleotide C to A mutation), which is associated with diminished IL-10 production, was present in all four ethnic groups included in the study, i.e. Caucasians, African Americans, Hispanics and Asians. Results from this study suggested that the -592 mutation was associated with increased risk for HIV-1 infection. Also there was a significant association between disease progression to AIDS in HIV-1-infected Caucasians carrying the mutation. Analyses demonstrated that the AIDS-accelerating effect of the -592 mutation in carriers was more pronounced starting approximately 5 years after HIV-1 exposure, also the -592 A allele (i.e. the mutation) had no significant effect on AIDS progression in the initial 5 years after infection. It was suggested that decreased IL-10 could be consistent with more pronounced AIDS acceleration effects in later stages of infection, i.e. more than 5 years after infection.

The AIDS-delaying influence of the -1082G SNP was shown when considering a recessive model when comparing individuals homozygous for the mutation, compared to other genotypes. The AIDS-accelerating effect of the -592A SNP was shown when considering a dominant model by comparing carriers of the mutation compared to individuals homozygous for the wild-type allele.

1.5 PROJECT AIM

There are host genetic factors, which modulate resistance to infection with HIV-1 or the rate of disease progression among those already infected. These genes are collectively known as AIDS restriction genes (ARGs) (O'Brien and Nelson, 2004). The interleukin-10 (IL-10) gene was previously identified as an ARG (O'Brien and Nelson, 2004; Winkler *et al.*, 2004). Polymorphisms within the IL-10 gene have been shown to have an effect on susceptibility to HIV infection and disease progression (Shin *et al.,* 2000). Studies on IL-10 gene polymorphisms have been conducted in developed countries where the burden of disease is low, particularly among European Americans. However, it is not entirely clear what the frequencies of these polymorphisms are in African populations and whether they have a similar HIV/AIDS restriction effect in sub-Saharan African populations. This is particularly so because the genetic subtypes of HIV-1 found in Africa differ from the predominant subtype found in Europe and North America, where most previous studies on the role of genetic factors in HIV-1 infection have been done. It is also unclear whether IL-10 promoter genetic variants have differential effects depending on the stage of disease of the infected person since the earlier study focused on chronic infections.

The first objective of the study was to determine the frequency of three IL-10 promoter single nucleotide polymorphisms, namely positions -1082, -819 and -592, within the CAPRISA Acute Infection cohort, which is a predominantly African population. The second objective was to determine if polymorphic variants at these positions are associated with differential likelihood of HIV-1 acquisition among HIV-1 negative women at high risk for HIV-1 infection. The third objective was to determine whether these polymorphisms have an effect on disease progression using viral load and $CD4^+$ T lymphocyte counts as surrogate markers during primary infection. We also assessed if there was an association of these IL-10 polymorphisms and plasma IL-10 expression levels.

Therefore the aim of this research was to determine whether genetic variation in the proximal promoter region of the IL-10 gene plays a role in susceptibility to HIV infection and in disease pathogenesis, particularly in early infection among HIV-1C infected Africans. This project focused on determining the frequency of the specific polymorphisms in a South African population, where the burden of HIV is high, in contrast to studies that

have been performed in populations that are not as heavily burdened. We hypothesized that the allele frequency of single nucleotide polymorphisms in the proximal region of the IL-10 promoter might be significantly different in an African population compared to well characterised populations in the West. Specifically, 228 women, who were part of the CAPRISA Acute Infection study, were genotyped for single nucleotide polymorphisms at positions -1082 and -592 of the proximal promoter region. Polymorphisms in the -819 region were inferred since this region is in complete linkage disequilibrium with -592. This study investigated whether these IL-10 promoter polymorphisms are associated with a higher likelihood of HIV-1 acquisition in a longitudinal cohort of high risk seronegative women from KwaZulu Natal, South Africa. Furthermore, the aim of this research was to identify whether IL-10 promoter polymorphisms play a role in infection pathogenesis during early HIV-1 infection, using viral load and $CD4^+$ T cell counts as surrogate indicators. This study also assessed whether these IL-10 promoter polymorphisms had an affect on IL-10 production to determine if polymorphisms in these regions affected plasma expression levels of IL-10.

1.6 PROJECT STRATEGY

Association studies aim to identify immunogenetic markers for specific diseases. An outline of the project strategy is shown in Figure 1.13. Association analysis between genetic polymorphisms within the IL-10 gene and clinical outcomes were performed by analysing individual IL-10 genotypes at three different regions within the promoter of the IL-10 gene, i.e. the -1082 position, -819 position and the -592 position relative to the transcription start site. Positions -819 and -592 are in complete linkage disequilibrium, and therefore genotype analysis was performed at positions -1082 and -592 (tracking position -819).

As viral load and $CD4^+$ T lymphocyte cell count can be used as prognostic markers for disease progression, in this study we aimed to use these diagnostic clinical data to determine association between viral load and IL-10 genotype at both positions, as well as $CD4^+$ T lymphocyte cell count and IL-10 genotype. In this study we wanted to utilise genotype data generated from genotyping, and perform association analysis between IL-10 expression and IL-10 promoter genotype.

If we can identify that IL-10 promoter polymorphisms play a role in HIV infection and disease progression within South African populations, then further studies can be undertaken to investigate the underlying mechanisms so that appropriate intervention strategies can be devised. This study was done among a predominantly African population, which eliminates the complexities associated with admixtures inherent in previous studies from North America. Data from this study therefore complements previous published studies from individuals with other ethnic backgrounds.

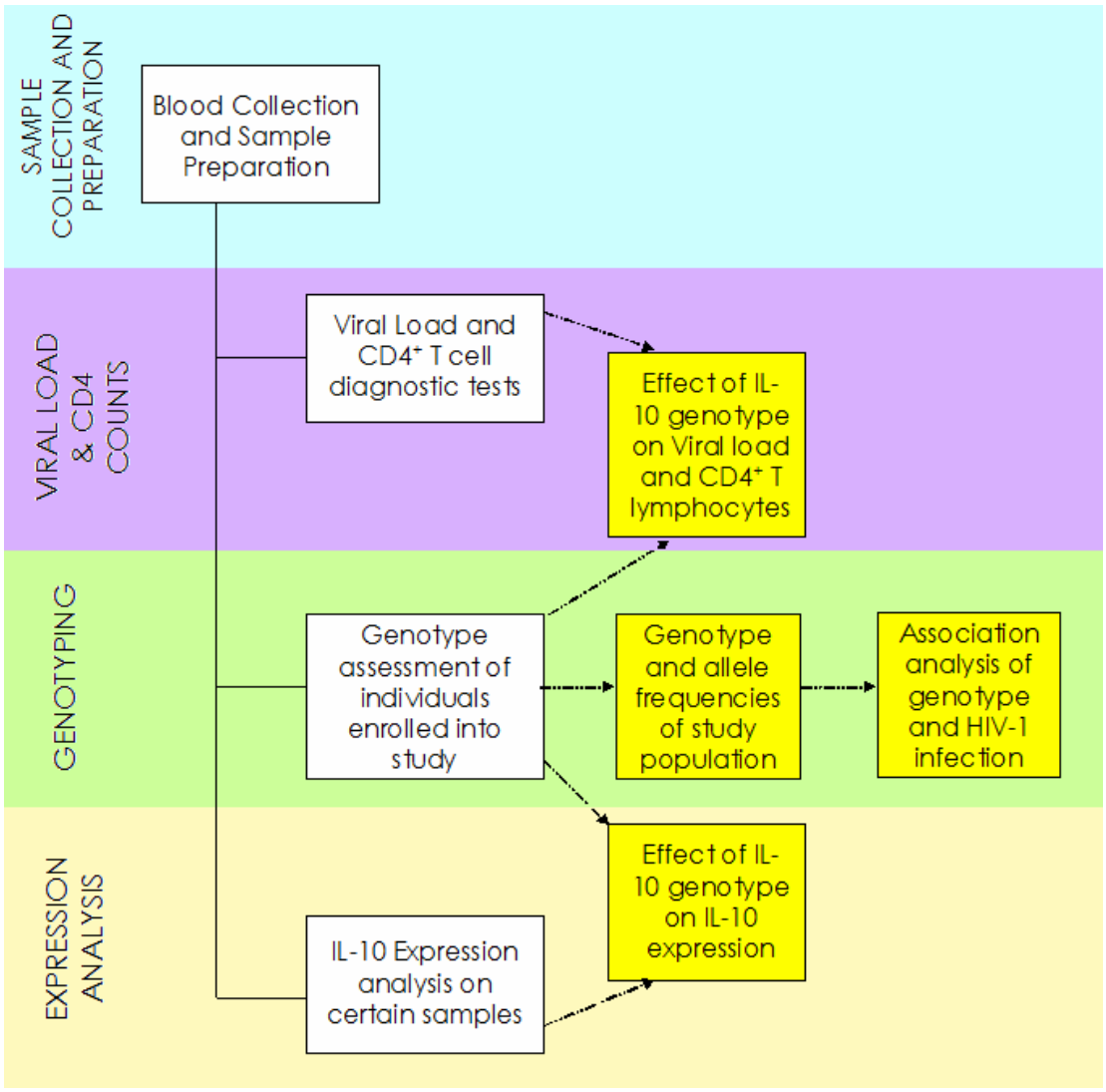


Figure 1.13. Outline of Project Strategy

Chapter 2

Genotype Analysis of IL-10 Promoter Polymorphisms, and Association with HIV-1 Infection

Chapter 2

Genotype Analysis of IL-10 Promoter Polymorphisms, and Association with HIV-1 Infection

Page

2.1. INTRODUCTION

The HIV/AIDS epidemic is characterised by uneven global distribution (UNAIDS, 2007). This uneven distribution of the epidemic is possibly due to multiple factors, including socio-economic factors and biological factors. Among the biological factors shown to play a role in differential susceptibility and in the observed different rates of disease progression are host genetic factors. Host genetic factors can affect the HIV-1 life cycle because host proteins are required for the successful completion of the life cycle (cofactors), or because they are involved in antiviral immune responses. Genetic variation and differential expression of these cofactors and antiviral proteins can in turn affect susceptibility to infection or the rate of disease progression. Most studies that have focused on the influence of host genetics on susceptibility to HIV and on HIV pathogenesis have been done in North America and Europe. In addition, few studies have investigated the role of genetic factors in primary HIV-1 pathogenesis, despite recent findings that pathogenic events in early infection profoundly affect the rate of progression and eventual outcome of disease (Hecht *et al*., 2002; Kassutto & Rosenberg, 2002). Furthermore, it has recently been demonstrated that the influence of AIDS restriction genes can differ according to the interval of pathogenesis (Gao *et al.*, 2005).

Differences in distribution and allelic frequencies of ARGs can be attributed to the age of the population and varied mutational forces that shape human evolution (Donfack *et al*., 2006). Systematic and random evolutionary forces shape the process of evolution (Snustad and Simmons, 2000). Some forces which drive these genetic variations are natural selection, random genetic drift, migration, and mutation. Geographical and ecological factors are possibly correlated with genetic differences. Therefore it is possible that there is a difference in the allelic frequency of ARGs from one geographical region to another, and that the frequency of a specific allele in one geographical region might not translate into another region, and therefore ethnic diversity might possibly play a role in the distribution of ARGs.

HIV-1 requires the use of two surface receptors to enter a cell. The first is the CD4 receptor, and the second is the chemokine binding co-receptor (Kostrikis *et al*., 1998). CCR5 is the chemokine co-receptor most commonly used by HIV-1 (Marmor *et al*., 2006).

An example of the difference in geographical distribution of alleles, is shown by the difference in the geographical distribution of the allele frequency of the ARG, *CCR5*. A specific mutation of the *CCR5* gene results in the deletion of a 32-bp region and is known as the *CCR5*-△32 mutation. If an individual is homozygous for this mutation, then the resulting CCR5 co-receptor is non-functional, and this phenotype is strongly associated with protection against HIV-1 infection by R5 HIV-1 variants (Kostrikis *et al*., 1999). The distribution of the CCR5-△32 mutation differs geographically, forming a frequency gradient from north to south Europe (Winkler *et al*., 2004). It was demonstrated that the frequency of the CCR5-△32 allele is highest in the Swedish population, and decreased as we move geographically north to south, in the British, German, French, Italian, Greek and Turkish populations (Stephens *et al*., 1998). This mutation is very rare among Africans.

Numerous studies have shown that not all individuals that are exposed to HIV-1 become infected. Therefore there are underlying host mechanisms which may predispose to HIV-1 susceptibility or resistance. The CCR5-△32 allele has been shown to be associated with resistance to HIV-1 infection (Marmor *et al*., 2006). The human leukocyte antigen (HLA) genes form part of the major histocompatibility complex (MHC). These HLA genes have been identified as important determinants resistance or susceptibility to HIV-1 infection. HLA class I molecules function to present pathogen-derived peptides on the cell surface of infected cells, for recognition by $CD8^+$ T lymphocytes (Carrington & O'Brien, 2003). Due to the difference between the expression of the different HLA class I alleles, these molecules will affect the difference in immune response and exposure outcome or disease progression. In addition to chemokine receptors, HLA alleles may also affect HIV-1 resistance or susceptibility as has been suggested for HLA-DRB1*01 (MacDonald *et al*., 2000 and Ndung'u *et al*., 2005).

A previous study by Shin *et al*. (2000) investigated the frequencies of the different IL-10 -592 and -1082 promoter alleles in varied North American cohorts. The cohorts studied were: AIDS Link to the Intravenous Experience, Hemophilia growth and Development Study, Multicenter Hemophiliac Cohort Study, Multicenter AIDS Cohort Study, and San Francisco City Clinic Study. Shin *et al.* (2000) studied IL-10 promoter polymorphisms in these five cohorts which, overall, consisted of 848 seroconverters, 1 863 seroprevalents and 627 seronegatives (total 3 337). Of the total study population, 2 208 were Caucasian, 937 African American, 153 Hispanic, and 47 Asian. The study demonstrated that

individuals carrying the IL-10 -592A allele, i.e. the mutant allele, were possibly at increased risk for HIV-1 infection and those HIV-infected individuals carrying this promoter polymorphism progressed to AIDS more rapidly than individuals who were homozygous for the wild type occurring allele. Within the long-term nonprogressors studied, between 25-30% of the individuals were homozygous wild type at the -592 position. It was suggested that the mutation at the -592 position (C to A mutation) possibly results in the down regulation of IL-10 production which facilitates HIV-1 replication, resulting in acceleration to AIDS, particularly in late stages of HIV infection.

The primary aim of this chapter was to determine, in a South African cohort, the allele and genotype frequencies of the two IL-10 promoter polymorphisms previously shown to influence HIV-1. The previous study was done in diverse North American cohorts. We hypothesized that the allele and genotype frequencies of the IL-10 promoter polymorphisms may differ significantly in the South African cohort compared to the North American cohorts. Further, this study investigated whether these polymorphisms are associated with increased likelihood of HIV-1 acquisition in this high risk cohort. Therefore, the focus of this study was to investigate the role of a previously identified ARG in mediating differential susceptibility to HIV-1 infection in a high risk cohort of African women. Understanding the protective mechanisms against HIV-1 infection could lead to the design and development of therapeutic interventions or possible vaccine strategies.

2.2. MATERIALS AND METHODS

2.2.1. STUDY POPULATION

The study population consisted of individuals that were enrolled into the CAPRISA (Centre for the AIDS Program of Research in South Africa) Acute Infection study. This is a longitudinal cohort study on viral set point and clinical progression in HIV-1 subtype C infection. The study aims to define the role of immunological, viral and genetic factors during acute and early infection. Individuals were enrolled in the study if they met the following criteria: they must be willing to participate in the follow-up phase, must be willing to receive the HIV test result, be able to provide documentation of informed consent. Individuals were excluded from the study if they were pregnant on screening for Phase I, and would be away from area of recruitment for more than 3 months of the total 24 months. The maximum duration of participation for those who become HIV-1 infected is 66 months (5.5 years). HIV-negative individuals enrolled into the cohort are followed up until seroconversion or a maximum of 24 months.

In 2004, 245 high risk HIV negative women were enrolled into a sex worker cohort in Durban, South Africa (see Figure 2.2.1). Monthly screens for HIV-infection were performed based on two HIV-1 rapid antibody tests. Antibody negative samples were subjected to pooled PCR testing for HIV-1 RNA. All samples that were tested positive for the pooled testing were confirmed using a quantitative RNA and HIV enzyme immunoassay (EIA) test. Women from this HIV-negative cohort, as well as from other seroincidence cohorts were enrolled into phase II of the study if they became HIV-positive during follow-up. Plasma viral loads and $CD4^+$ T cell counts were performed at various study time points post-infection.

At the time of assessment the total number of individuals included in the CAPRISA Acute infection study was 273. Of this total 40 individuals were lost to follow-up. Also 5 samples were unavailable at time of assessment. Therefore the total number of individuals genotyped for this study was 228, 48 of which had acquired HIV infection. After they were enrolled in the study, an intense follow-up which included virologic studies as well as $CD4^+$ and viral load counts were performed. An overview of the study is shown in Figure

2.2.2. HIV negative individuals enter into Phase I of the study. They are followed up longitudinally in this phase for 24 months or until seroconversion. Individuals enter Phase II of the cohort when they become HIV-infected. The time of infection was taken as the midpoint between the last antibody-negative and the first antibody-positive test, or 14 days prior to PCR positive and antibody negative.

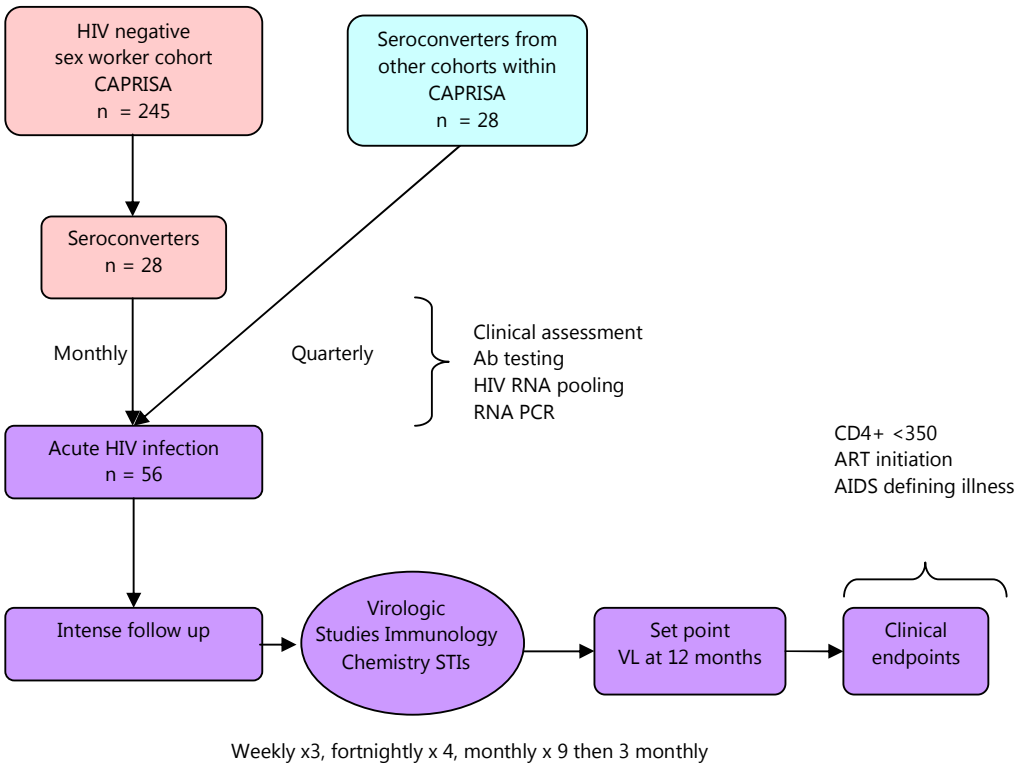


Figure 2.2.1. An overview of the Acute Infection cohort. 245 HIV negative women were enrolled into Phase I of the study, of which 28 seroconverted and went to Phase II. A further 28 women were recruited from other cohorts within CAPRISA. A total of 56 HIV positive participants were followed up.

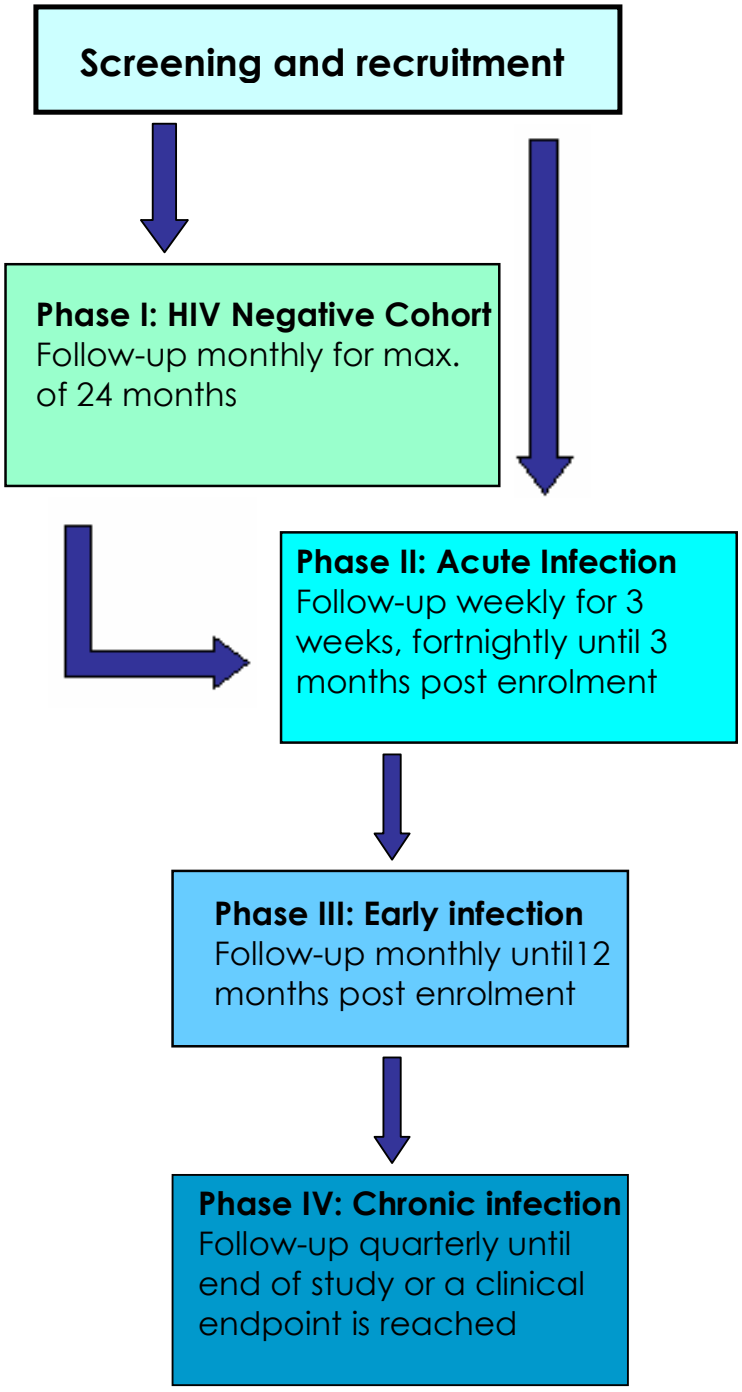


Figure 2.2.2. Overview of the Phases of the CAPRISA Acute Infection cohort. This diagram demonstrates the different phases into which CAPRISA Acute Infection study participants are categorized. Individuals are recruited and placed into different phases accordingly. Evaluation schedule is dependent upon the phase of infection that each participant is in.

2.2.2. SAMPLE PREPARATION

Patients enrolled in the study provided blood samples at scheduled visits. At each visit blood samples were collected by venipuncture into EDTA tubes. To separate cellular components from plasma, whole blood was centrifuged at 2500xg for 10 min at room temperature, using the Jouan MR23i centrifuge. The centrifuged whole blood separates into 3 layers (see Figure 2.2.3). Red blood cells (erythrocytes) settle at the bottom of the tube, a thin intermediate layer of white blood cells (leukocytes) and platelets (thrombocytes) settles on top of the red cells, and there is an upper layer consisting of clear yellow plasma. The intermediate layer of cells is known as the buffy coat.

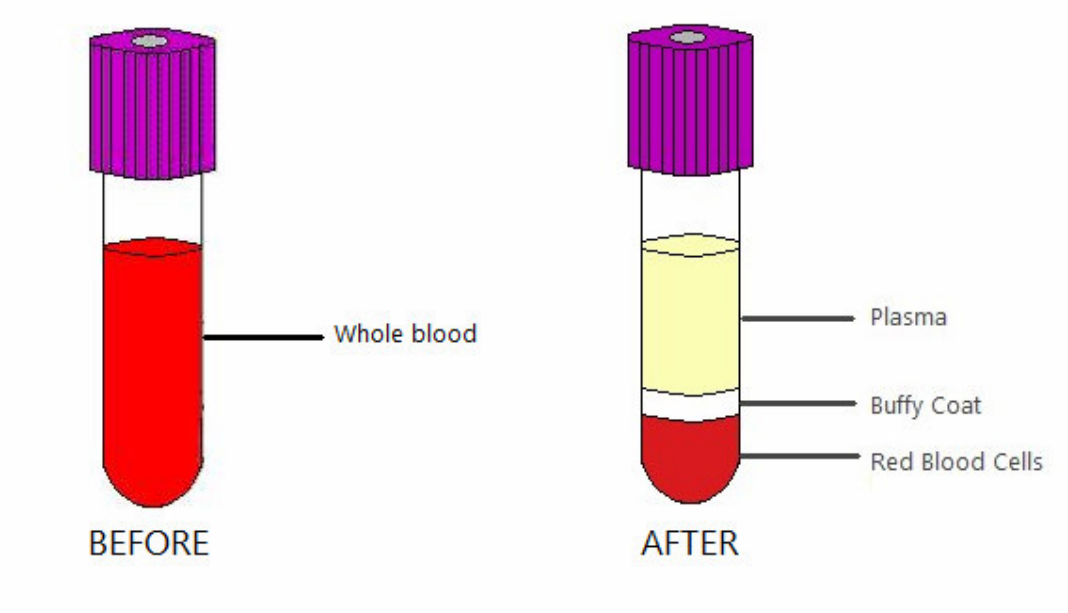


Figure 2.2.3. Centrifugation of whole blood. This diagram demonstrates the separation of the different blood layers by density separation. Before centrifugation the whole blood has no layers. After centrifugation the blood separates into three different layers based on their density.

Buffy coats, which are enriched with lymphocytes, were processed for DNA extraction. The buffy coats were carefully removed using fine tip plastic Pasteur pipettes (Copan) and placed into clean 1.5 ml tubes (Axygen). The buffy coats were then stored at -80^{o}C until used.

During the process of buffy coat removal from the centrifugation tube, some red blood cells contaminate the buffy coat layer. These red blood cells (RBCs) need to be lysed or removed from the sample to avoid downstream inhibition of PCR reactions. To remove excess red blood cells, the samples were treated with red blood cell lysis solution prior to DNA extraction. Red blood cell lysis solution was prepared and made up to 10x concentrate solution. 10x concentrate of red blood cell lysis solution (also known as ammonium chloride (NH_4Cl) lysis solution) was prepared as follows: 3.7g of EDTA (Ethylene diamine tetracetic acid) disodium salt; 80.2g ammonium chloride (NH_4Cl); 8.4g sodium bicarbonate ($NaHCO_3$); and distilled water was added to make up to one litre. The pH of the solution was adjusted to 7.0 using the laboratory pH meter CG842 (Schott Instruments). This solution can then be stored at 4^{o}C for up to 6 months.

The buffy coat samples were then treated with the red blood cell lysis solution to remove excess red blood cells from the sample. 1ml of 1x RBC lysis solution was added to the samples; the samples were then left at room temperature for ten minutes with periodic inversion of the sample, to allow for lysis of the red blood cells; and the samples were then centrifuged at 4000rpm for ten minutes at room temperature, using the Jouan A14 microcentrifuge. The buffy coat then formed a pellet at the bottom of the tube. The supernatant, consisting of lysed red blood cells, was removed leaving behind about 200µl of the supernatant to allow for the resuspension of the pellet as a starting point for DNA extraction.

2.2.3. DNA EXTRACTION

DNA extraction was performed on the samples using the QIAamp DNA Blood Mini Spin kit (QIAGEN). The manufacturer's protocol for DNA extraction from blood and body fluid spin protocol was followed. An overview of the technique is shown in Figure 2.2.4. 200µl of buffy coat was used for DNA extraction. Protease was added to the sample to lyse the cells and expose the contents of the cell. Buffers were added to the sample which allowed the DNA to bind to the column membrane during centrifugation. The sample was then added to a spin column, which the DNA binds to, and during centrifugation, the protein cellular debris will flow out of the column and are discarded. Protein and other contaminants will not bind to the membrane, due to the salt and pH conditions of the lysate buffer, and will therefore prevent downstream inhibition of PCR. Two wash steps followed, which allowed for the removal of residual contaminants. The last step involved the elution of the purified DNA into Buffer AE (provided with the kit), which allows for long term storage, as compared to elution with water.

Following extraction, the DNA concentration was quantified using the GeneQuant pro (Amersham Biosciences). Glass capillaries were used to quantify DNA using the GeneQuant pro. 5µl of eluted DNA was taken up using a capillary, which was then placed into the cuvette, and a reading was then taken.

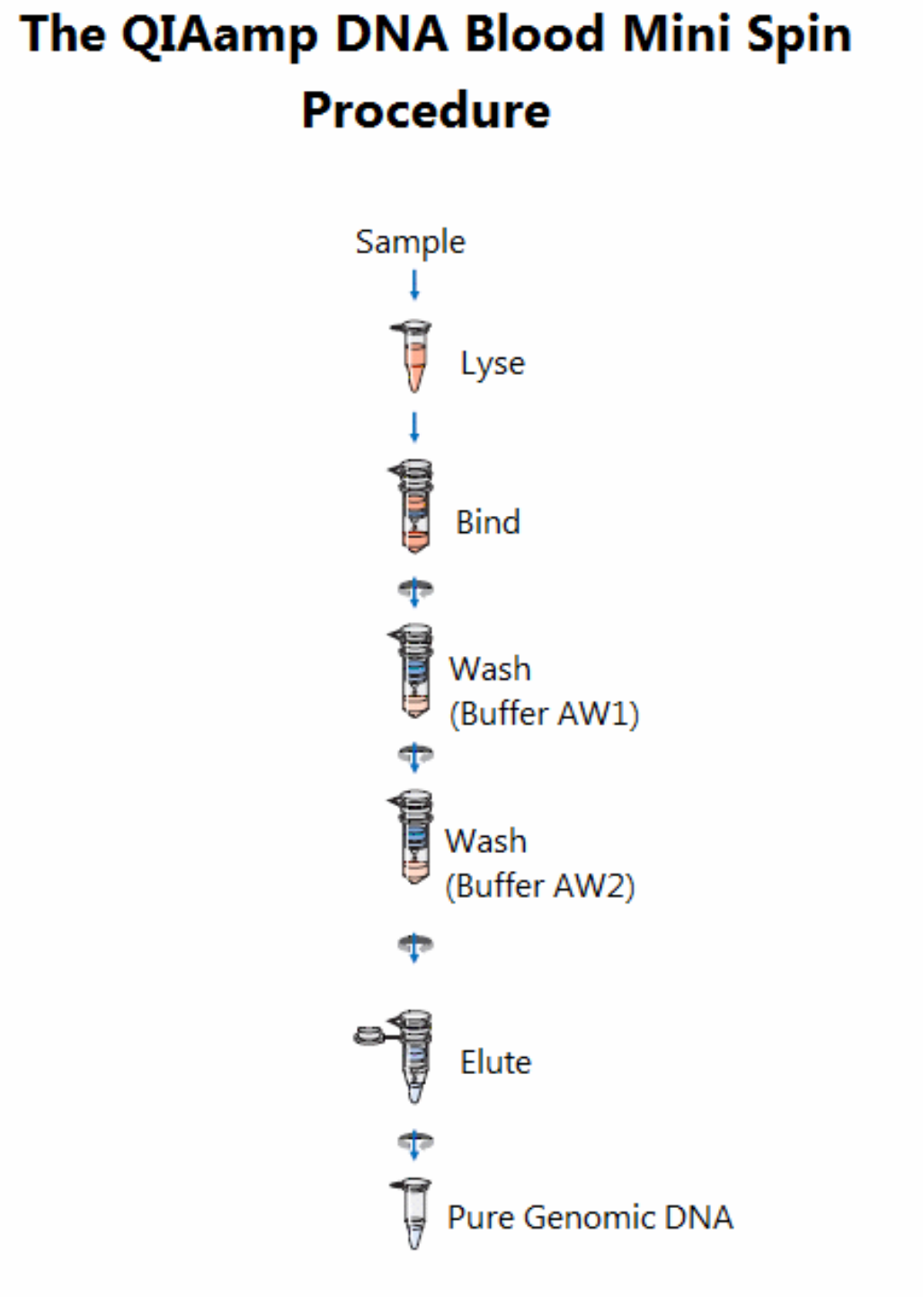


Figure 2.2.4. An overview of the QIAGEN QIAamp DNA Blood Mini Kit Spin Procedure for DNA Extraction (adapted from QIAGEN, 2003). This shows the steps involved in DNA extraction from buffy coat samples.

2.2.4. GENOTYPE ASSESSMENT

In this study positions -1082 and -592 (tracking -819) were genotyped. Genotype assessment was done by Amplification Refractory Mutation System (ARMS-PCR). In this system the primers are designed in such a way that single nucleotide polymorphisms can be detected on an electrophoresis gel after PCR amplification (Newton *et al*., 1989; Ye *et al*., 2001). An internal control primer was used to determine if the PCR was successful or unsuccessful. ARMS-PCR works so that one pair of primers amplifies the wild type allele and another pair of primers amplifies the mutated allele. Therefore two reactions are required for each sample. Figure 2.2.5 demonstrates how ARMS-PCR works. The primers used can distinguish single nucleotide polymorphisms within the IL-10 promoter region between samples (Schneider *et al*., 2004; Smith *et al*., 2004; McCarron *et al*., 2002; Girndt *et al*., 2001, 2002; Crilly *et al*., 2003; Palmeri *et al*., 2005). The primers used for genotype assessment of samples were previously described by Opdal *et al.* (2003). The primer sequences used for PCR amplification are shown in Table 2.2.1.

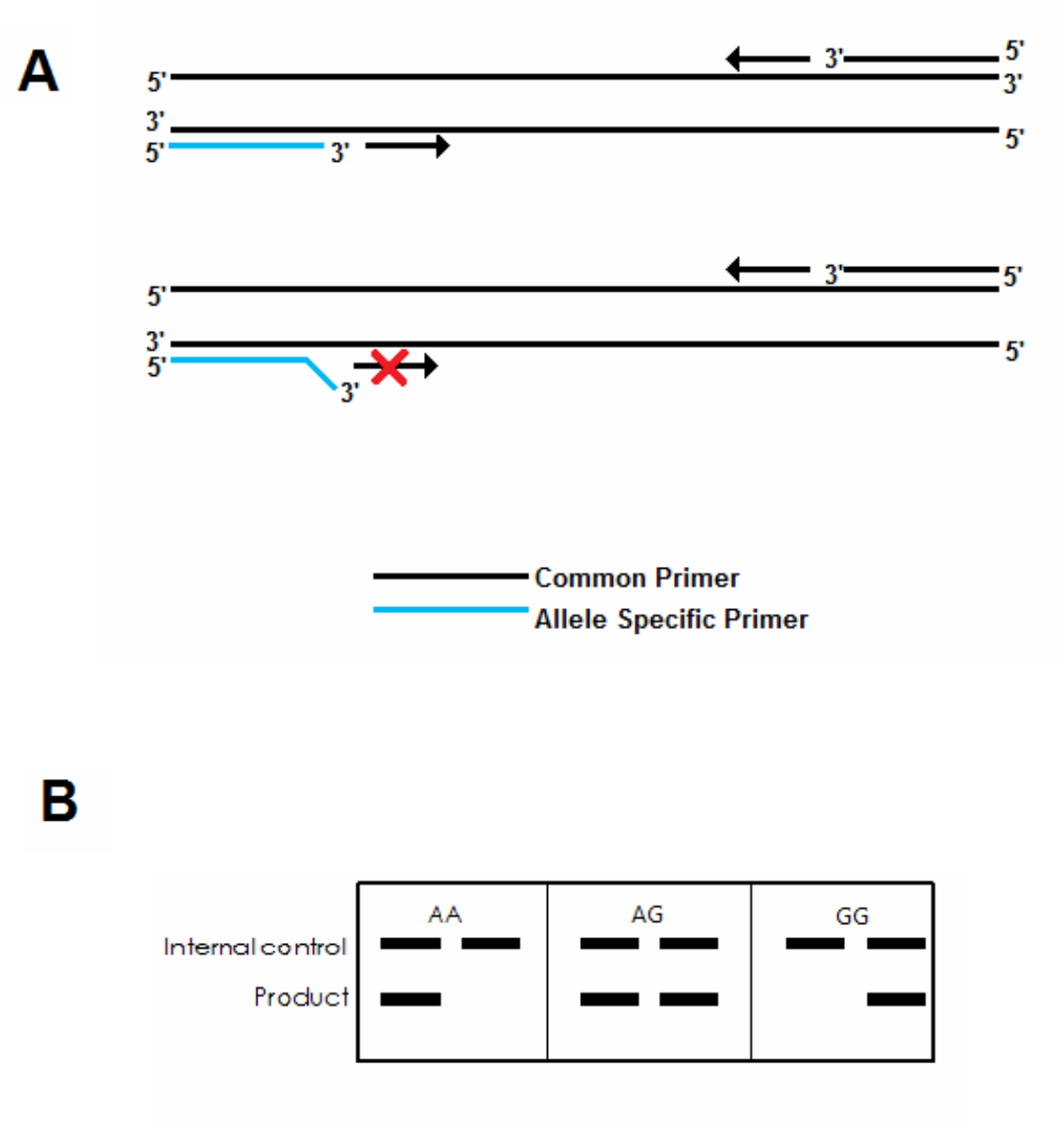


Figure 2.2.5. Demonstration of the ARMS-PCR Methodology. (A) Generally, the allele specific primer differs by a single nucleotide base at the 3' end. If the primer is complementary to the template sequence, then elongation occurs, however if the primer is not complementary to the template sequence, then elongation does not occur. (B) Using this method, we can therefore differentiate between different genotypes based on the banding pattern after running the PCR products on an agarose gel.

Table 2.2.1. Primers sequences used and PCR conditions for genotype assessment (adapted from Opdal *et al.*, 2003)

Primer name	Area	Sequence	Product length
A	SNP-1082	5'-tgtaagcttctgtggctgga	159 bp
B	SNP-1082	5'-ctactaaggcttctttgggag	
C	SNP-1082	5'-actactaaggcttctttgggaa	
D	SNP-592	5'-ggcacatgtttccacctctt	188 bp
E	SNP-592	5'-cccttgtacaggtgatgtaac	
F	SNP-592	5'-acccttgtacaggtgatgtaat	
IK1	Human growth hormone	5'-gccttcccaaccattccctta	429 bp
IK2	Human growth hormone	5'-tcacggatttctgttgtgtttc	

PCR was performed on the DNA samples using AmpliTaq Gold PCR Master Mix (Applied Biosystems). This master mix reagent is premixed with all the components used during amplification of DNA. The AmpliTaq Gold PCR Master Mix contains AmpliTaq Gold DNA Polymerase, 250 U (0.05 U/µl); GeneAmp PCR Gold Buffer, 30 mM Tris/HCl, pH 8.05, 100 mM KCl; dNTP, 400 µM each; $MgCl_2$, 5 mM; and stabilisers. The PCR conditions used are described in Table 2.2.2. The samples were run on the GeneAmp PCR system 9700 (Applied Biosystems). The positive control used was the internal control, i.e. the IK1 and IK2 primers which amplified the human growth hormone gene. This product should amplify in every positive sample in the run, except the no template negative control. If the IK1 and IK2 primers do not amplify a product, then the PCR is unsuccessful. If the internal control did not show up, then DNA was re-extracted and subjected to a second round of amplification. The negative control used was water, which was included in every PCR run, to control for contamination problems. The negative control contained the internal control primers only. If the negative control amplified the human growth hormone, then there was contamination of the PCR, and the PCR was deemed unreliable.

Table 2.2.2. PCR conditions used for ARMS-PCR.

Step	AmpliTaq Gold Enzyme Activation	PCR Cycle (30 cycles)			PCR (Final step)
		Denature	Anneal	Extend	
Temp.	95°C	95°C	63°C	72°C	72°C
Time	5min	30s	30s	30s	7min

2.2.5. AGAROSE GEL ELECTROPHORESIS

The genotyping results are interpreted by running the products from the ARMS-PCR on an agarose gel. Figure 2.2.6 demonstrates the overall technique, as well as how the genotyping results are interpreted. The two different reactions per sample, are run on two consecutive lanes on an agarose gel. The internal control primers IK1 and IK2, which amplify the human growth hormone gene, are used in every reaction, therefore the amplification of the human growth hormone will determine whether the PCR was successful or not. With every ARMS-PCR run, water was used as a negative control, therefore this reaction should not produce any products, and therefore no bands should appear in this lane.

After samples are subjected to ARMS-PCR amplification, they were then analysed on a 2% agarose gel stained with ethidium bromide (final concentration 0.5mg/ml), within the gel, to visualize the PCR products. The 2% agarose gel was prepared using 8g Agarose Low Electroendosmosis (Roche); dissolved in 400ml of 1x Tris-Borate EDTA buffer (TBE), (Sigma®). The samples were run in a Hoefer electrophoresis unit, using the electrophoresis power supply EPS 301 (Amersham Biosciences) at 120V for 2 hours. The bands were then visualised using ethidium bromide (Sigma®) under ultraviolet light, and the gel was documented using the gel documentation and analysis system G: Box HR (SYNGENE).

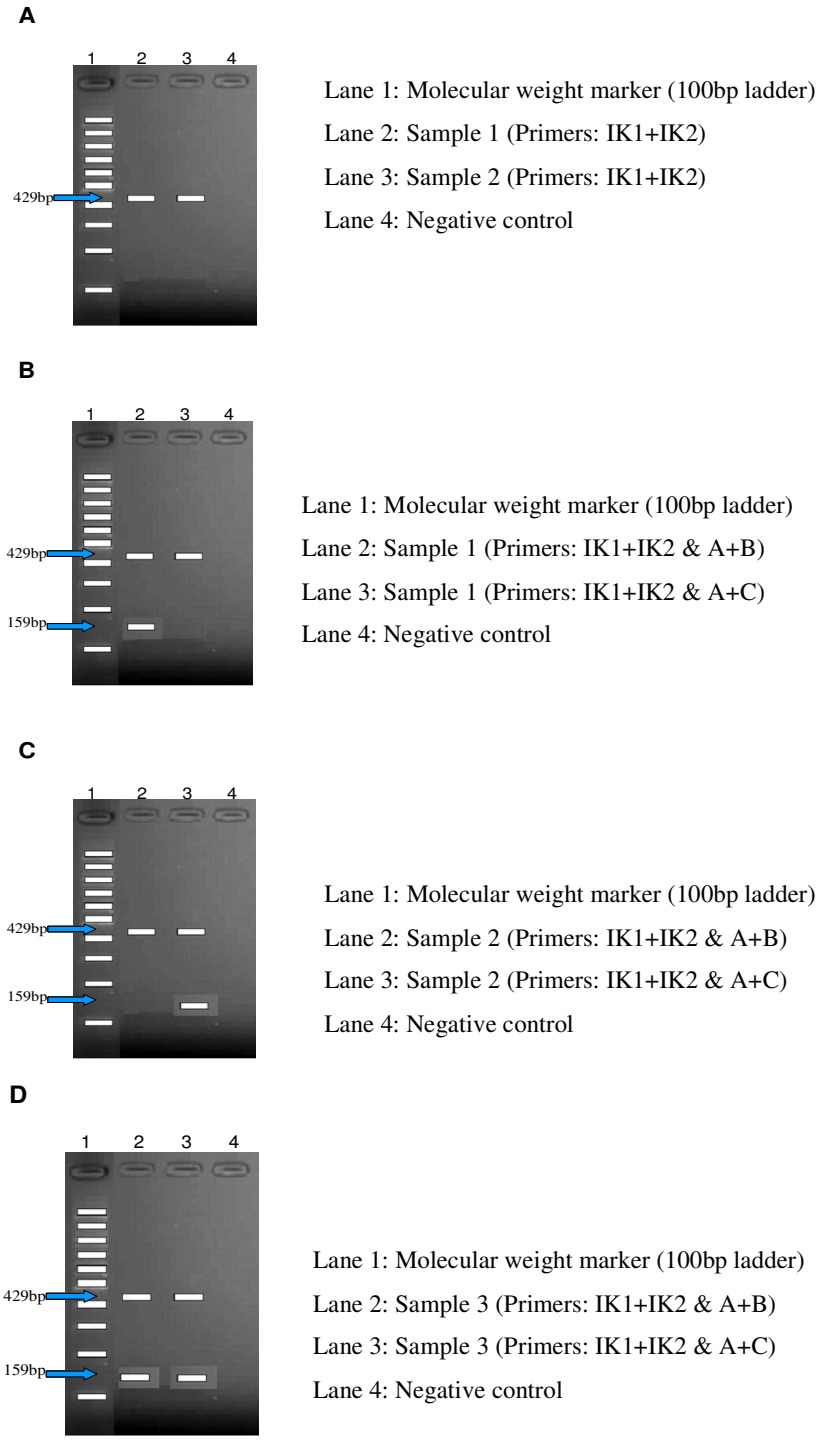


Figure 2.2.6. Interpretation of genotyping results for position -1082 on an agarose gel. The human growth hormone product is expected to be present in every lane with a sample if the PCR is successful. (A) The human growth hormone gene product (size 429 bp) can be visualized. (B) If an individual is homozygous for the mutation, a band should be visible at 159 bp in lane 2 and not lane 3. (C) If an individual is homozygous for the wild-type then a band is present in lane 3 and not lane 2. (D) If an individual is heterozygous at this position then a band will be visualized in both lane 2 and lane 3.

2.2.6. STATISTICAL ANALYSIS

Hardy-Weinberg equilibrium of the alleles and genotypes was used to determine whether the genotype distributions observed was in agreement with expected values calculated from allele frequencies. Chi-square test compared the allelic frequencies to confirm their fit to the Hardy-Weinberg equilibrium test. To test the association between HIV status and the genotype at the different positions, we used a Fisher's exact test. Kaplan-Meier graphs, which determine the time between entry into a study and a subsequent outcome/end-point event, were prepared (Swinscow & Campbell, 2002). Kaplan-Meier graphs give a graphical representation of the survival functions, or the time until HIV infection occurrence in these participants. These survival curves are modelled for each different allele in order to determine whether participants with a certain allele, or combination of alleles, were more susceptible to becoming HIV infected.

2.3. RESULTS

2.3.1. Clinical details of participants included in the study

Participants enrolled in the CAPRISA Acute Infection cohort are screened monthly for HIV-1 infection. HIV-negative individuals are in Phase I of the cohort and were followed up for a maximum of 24 months, or until seroconversion. One hundred and eighty HIV-negative women and forty eight HIV-infected women were included in this study. Table 2.3.1 illustrates the demographic characteristics of the HIV-positive individuals as well as the HIV-negative individuals included in this study. The genotypic data of all the individuals included in this study are in Appendix 1.

Table 2.3.1. Overall demographics of HIV-positive vs. HIV-negative individuals. Of the 228 individuals included in this study, 180 were HIV-negative and 48 were HIV-positive.

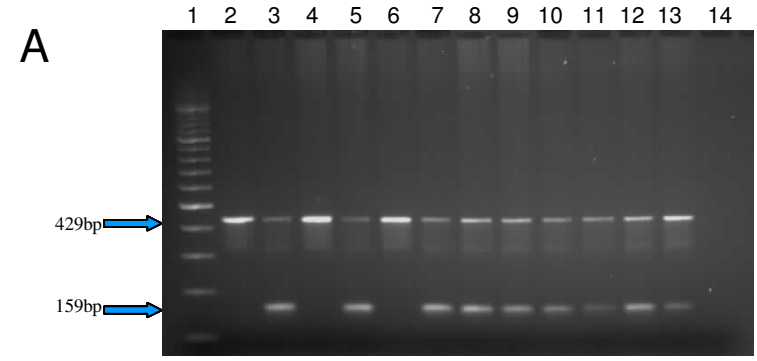
HIV Status	Number of Individuals	Median Age	Mean Age $\pm$ SD
Positive	48	24	27.02 $\pm$ 9.005
Negative	180	37.5	35.88 $\pm$ 9.787

2.3.2. DNA Extraction and Quantification

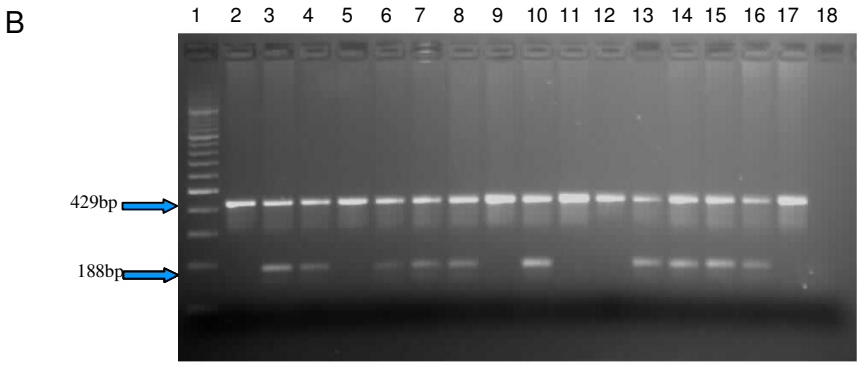
DNA was extracted for the one hundred and eighty HIV-negative women and forty eight HIV-positive women included in this study. According to Opdal *et al.* (2003), 150ng of DNA is required for PCR amplification using the ARMS-PCR methodology. The concentrations of the DNA samples ranged between 40-120ng/µl, with a purity $\pm$ 1.8 (A260/A280 ratio). This concentration of DNA was adequate for PCR amplification.

2.3.3. Agarose Gel Electrophoresis

ARMS-PCR methodology was used to genotype the samples for IL-10 promoter polymorphisms. This system utilises a PCR primer which is designed in such a way that it is able to discriminate among templates that differ by a single nucleotide residue. The 3' end of the allele specific primer contains the single nucleotide base difference. The advantage of the ARMS-PCR is that the amplification and diagnostic steps are combined, and results are interpreted by running the products on an agarose electrophoresis gel. To investigate the reproducibility of the assigned genotypes, ten samples were chosen at random and their genotypes were confirmed by repeating the genotyping using ARMS-PCR. Figure 2.3.1 is a typical result of samples run on an agarose gel. From the resulting banding pattern, the genotype can be assigned to each sample.



Lane 1: 100bp Ladder Molecular Weight Marker
Lane 2: Sample 1 (Primers A+B)
Lane 3: Sample 1 (Primers A+C)
Lane 4: Sample 2 (Primers A+B)
Lane 5: Sample 2 (Primers A+C)
Lane 6: Sample 3 (Primers A+B)
Lane 7: Sample 3 (Primers A+C)
Lane 8: Sample 4 (Primers (A+B)
Lane 9: Sample 4 (Primers A+C)
Lane 10: Sample 5 (Primers A+B)
Lane 11: Sample 5 (Primers (A+C)
Lane 12: Sample 6 (Primers (A+B)
Lane 13: Sample 6 (Primers (A+C)
Lane 14: Negative control



Lane 1: 100bp Ladder Molecular Weight Marker
Lane 2: Sample 1 (Primers D+E)
Lane 3: Sample 1 (Primers D+F)
Lane 4: Sample 2 (Primers D+E)
Lane 5: Sample 2 (Primers D+F)
Lane 6: Sample 3 (Primers D+E)
Lane 7: Sample 3 (Primers D+F)
Lane 8: Sample 4 (Primers D+E)
Lane 9: Sample 4 (Primers D+F)
Lane 10: Sample 5 (Primers D+E)
Lane 11: Sample 5 (Primers D+F)
Lane 12: Sample 6 (Primers D+E)
Lane 13: Sample 6 (Primers D+F)
Lane 14: Sample 7 (Primers D+E)
Lane 15: Sample 7 (Primers D+F)
Lane 16: Sample 8 (Primers D+E)
Lane 17: Sample 8 (Primers (D+F)
Lane 18: Negative control

Figure 2.3.1. Sample agarose gel after running products from ARMS-PCR. (**A**) This is an example of a gel obtained from products of the -1082 Region. The band at the 429bp position is the internal control (human growth hormone) and shows up in every lane (excluding the negative control). From the gel we can determine that Sample 1 is Hom-WT; Sample 2: Hom-WT; Sample 3: Hom-WT; Sample 4: Het; Sample 5: Het; Sample 6: Het.

(**B**) This is an example of an agarose gel showing the products of ARMS-PCR at the -592 region. This shows that Sample 1 is Hom-P; Sample 2: Hom-WT; Sample 3: Het; Sample 4: Hom-WT; Sample 5: Hom-WT; Sample 6: Hom-P; Sample 7: Het; Sample 8: Hom-WT

2.3.4. Overall Genotype Frequencies

The overall allele frequencies at both the -1082 and the -592 positions are shown in table 2.3.2. The allele frequency was obtained by calculating it from the genotype frequency obtained after genotype assessment of the 228 study individuals. Table 2.3.3 shows the allele frequency obtained at the -592 position, compared with the allele frequency obtained from the Shin *et al* (2000) study.

Table 2.3.2. Allele frequency of the Wild-type and the Mutation at both regions analysed. This table shows the allele frequency obtained after genotype assessment of the 228 individuals included in this study.

Region	Mutation	Wild-type
-1082	0.3202	0.6798
-819/-592	0.3333	0.6667

Table 2.3.3. Allele frequency for the -592 mutation and the wild-type, from this study and the Shin *et al* study (2000).

Ethnic Group	Mutation (592A)	Wild-type (592C)
Caucasians		
(n=2208)	0.236	0.746
African Americans		
(n=937)	0.4	0.6
Hispanics		
(n=153)	0.327	0.673
Asians		
(n=47)	0.6	0.4
CAPRISA AIC		
(n=228)	0.333	0.667

2.3.5. Genotype Frequencies and Distribution at the -1082 Position

Genotype data for two hundred and twenty eight individuals was obtained using ARMS-PCR methodology to determine IL-10 promoter genotypes. Hardy-Weinberg equilibrium was determined using the Chi-square test, to confirm that observed frequencies were not significantly different from expected frequencies. Figure 2.3.2 shows the distribution of the genotypes of all individuals in this study. The genotype frequencies were in agreement with Hardy-Weinberg equilibrium.

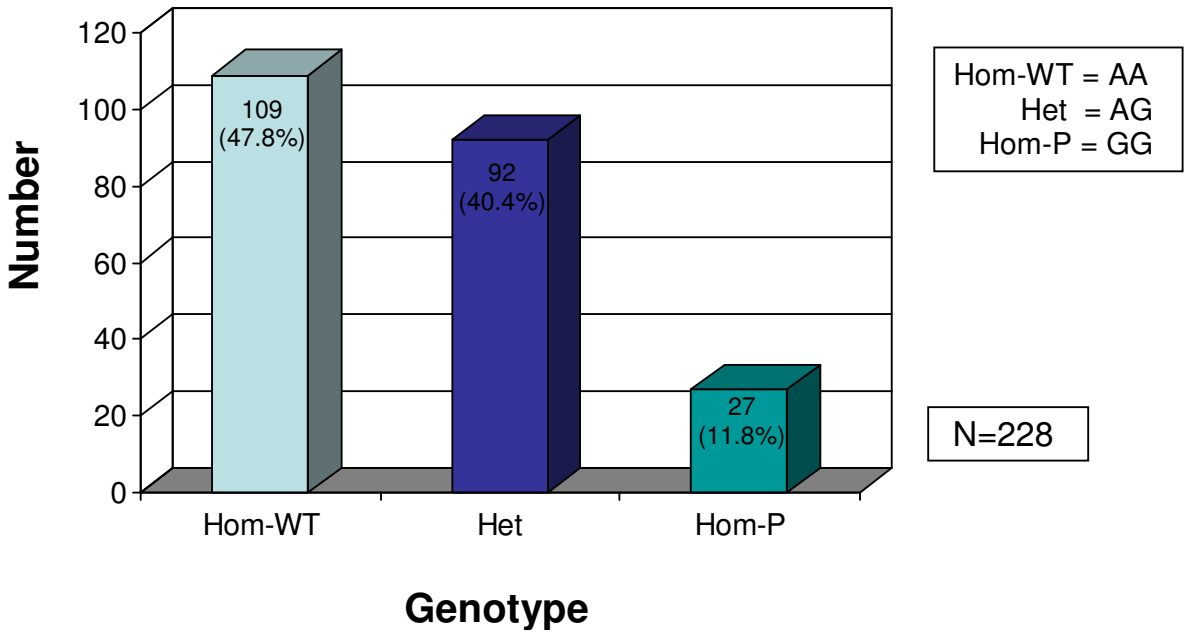


Figure 2.3.2. Genotype distribution of whole study population, at the IL-10 -1082 Position. The distribution of the genotype frequencies between all three groups were in Hardy-Weinberg equilibrium.

The IL-10 genotype data was analysed for association with HIV status. The results obtained for the -1082 position are shown in Figure 2.3.3. The Fisher's exact test was used to determine if there was an association between IL-10 genotype and HIV status. There was no significant association between HIV status and the IL-10 genotype (p= 0.2800). Therefore there is no significant difference in the distribution of IL-10 genotypes between the HIV-negative and the HIV-positive individuals.

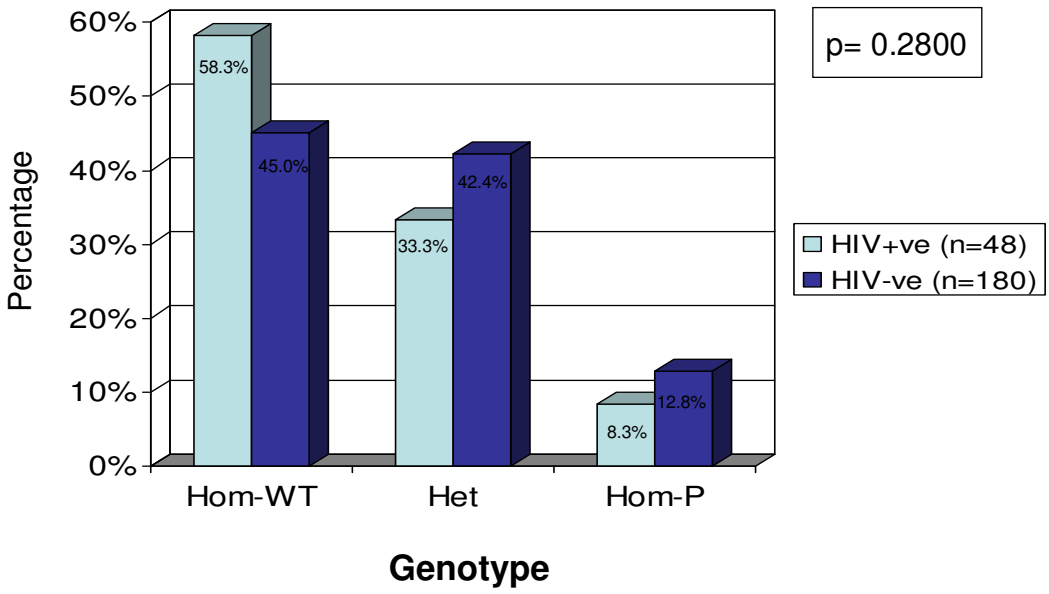


Figure 2.3.3. Genotype distribution of IL-10 genotype at the -1082 position based on HIV status. There is no significant association between IL-10 genotypes and HIV-1 status.

2.3.6. Association of Genotype with Survival/Time to HIV Infection for the - 1082 Polymorphism

The Kaplan-Meier graphs give a graphical representation of survival functions, or the time until these individuals become HIV infected. These survival curves utilised the genotype data to determine whether participants with a certain allele or combination of alleles were more likely to become HIV infected over the follow-up period. Analysis of IL-10 genotype at the -1082 position is shown in Figure 2.3.4. This graph indicates that those individuals who were homozygous wild type (Hom-WT) were more likely to become HIV infected as compared to heterozygous (Het) individuals or individuals homozygous for the polymorphism (Hom-P), however this was not statistically significant (Cox regression, p=0.1211).

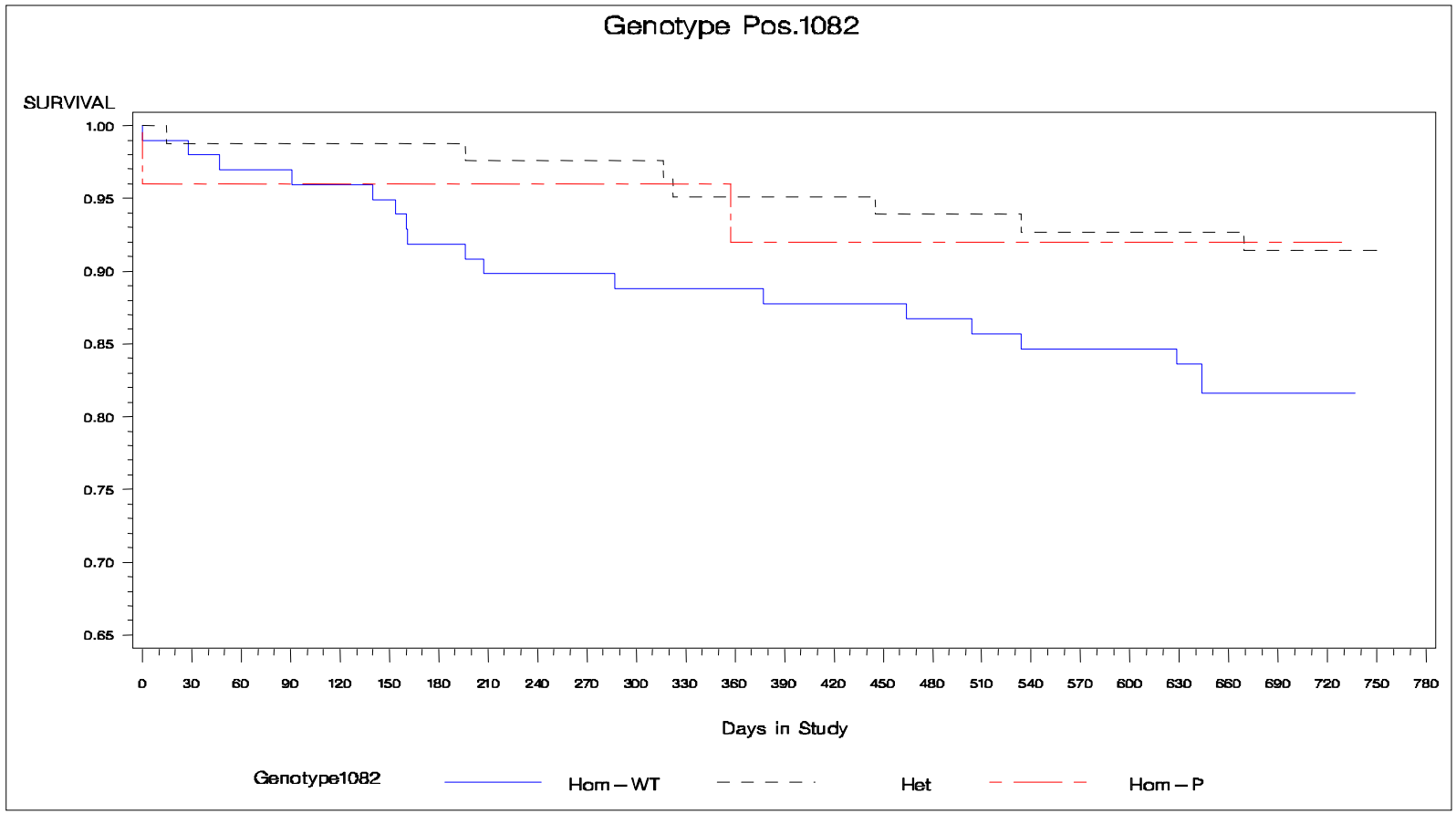


Figure 2.3.4. Kaplan-Meier graph showing the IL-10 -1082 genotype associated with survival to HIV infection. This graph shows that those individuals displaying the homozygous wild type genotype were more likely to become infected, compared to heterozygotes or individuals homozygous for the polymorphism, however, this difference was not statistically significant (Cox regression, p=0.1211)

2.3.7. Genotype Frequencies and Distribution at the -592 Position

Genotype assessment was also performed at the -592 position. The C to A substitution at position -592 is in complete linkage disequilibrium with the C to T substitution at the -819 position. Genotype assessment was performed on all two hundred and twenty eight individuals included in this study. To determine if the distribution of genotype frequencies was in Hardy-Weinberg equilibrium, the chi-square test was performed. The distribution of IL-10 -592 genotypes overall, is shown in Figure 2.3.5. The distribution of IL-10 -592 genotypes was in Hardy-Weinberg equilibrium, which shows that the observed frequencies were not significantly different from what was expected, using allele frequencies to determine this.

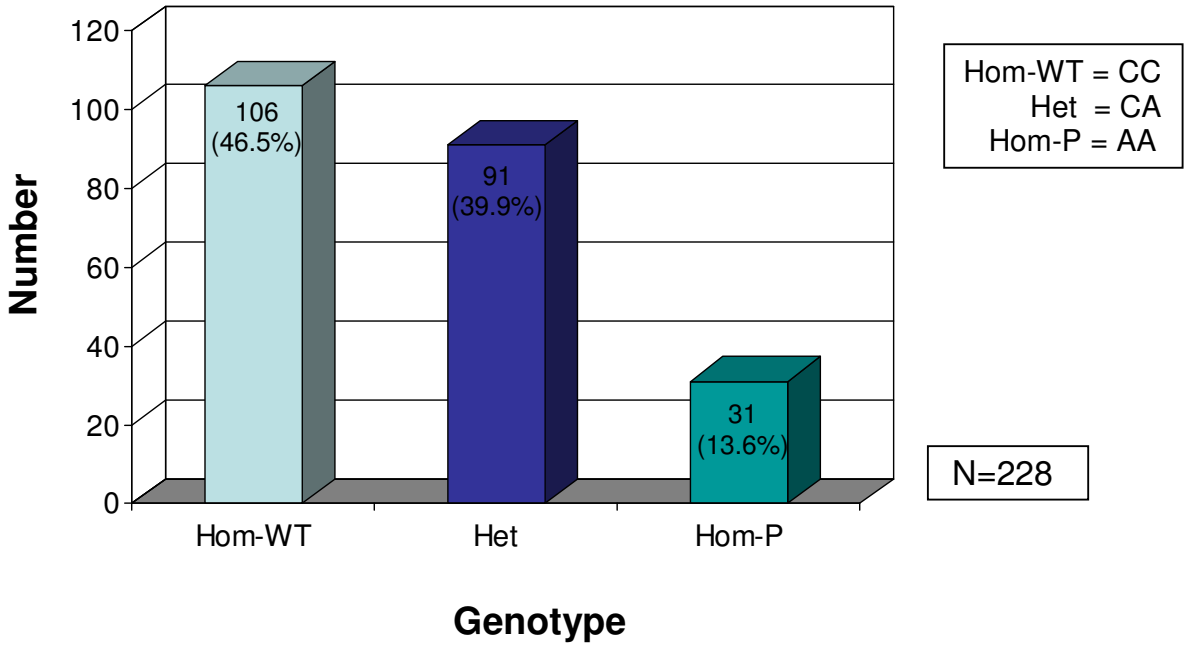


Figure 2.3.5. Overall genotype distribution at the -592 Position. This distribution is also representative of the genotype frequencies at the -819 position, as substitutions in these two positions are in complete linkage disequilibrium. The frequencies are in agreement with the Hardy-Weinberg equilibrium.

The distribution of the -592 genotypes among HIV-negative individuals and HIV-positive individuals was also examined, to determine if there was an association between HIV

status and IL-10 -592 genotype. The data is represented in Figure 2.3.6. The Fisher's exact test indicated that there is a significant association between HIV status and the IL-10 -592 genotype, with a p value of 0.0136 (p=0.0136). Thus, there is a significant difference in the distribution of IL-10 -592 genotypes between the HIV-negative and HIV-positive individuals. This significant difference in the distribution of genotypes is mainly due to the large difference in the Hom-P individuals between HIV- and HIV+ with a 9.5% and 28.6% prevalence, respectively.

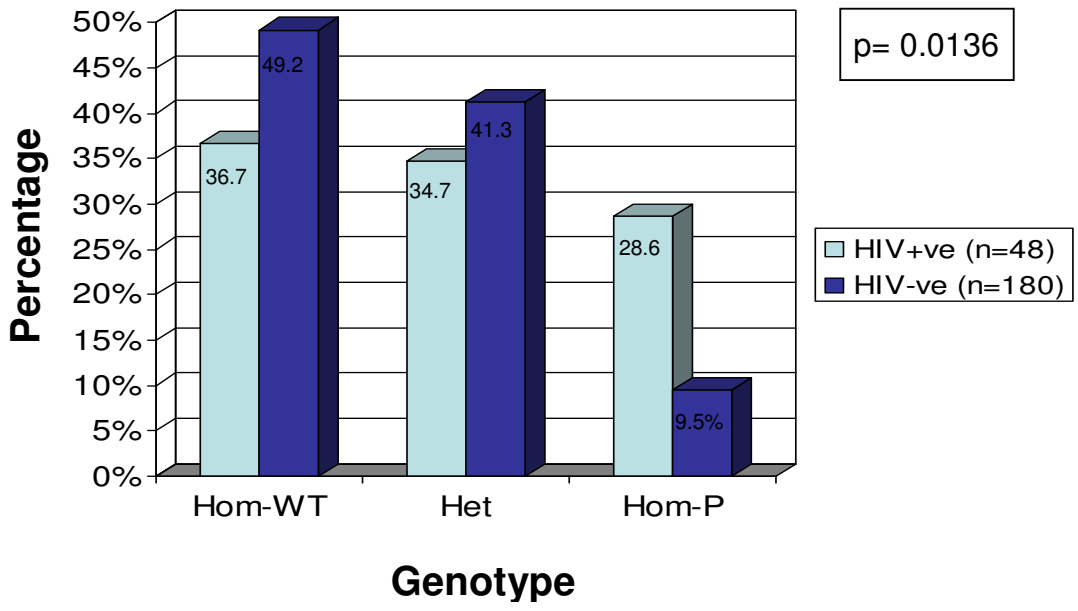


Figure 2.3.6. Distribution of IL-10 -592 genotype vs. HIV status. There is a significant association between IL-10 genotype and HIV status. This mainly is due to the difference between individuals that are HIV-negative and individuals that are HIV-positive among those homozygous for the mutation.

2.3.8. Association of Genotype with Survival versus Time to HIV Infection at the -592 Position

The Kaplan-Meier graph for survival or time to HIV-1 infection based on IL-10 -592 genotype is represented in Figure 2.3.7. This graph determines whether there is an association with IL-10 genotype at the -592 position and HIV susceptibility. This graph indicates that individuals that were Hom-P were more likely to become HIV infected as compared to individuals who were Het or Hom-WT, this was statistically significant with a p value of 0.0335 (Cox regression, p=0.0335). Using the Cox proportional hazards model, if an individual was Hom-P at the -592 position, they were at increased risk of becoming HIV infected with a hazard ratio of 2.78 (95%CI: 1.10-7.07), as compared to individuals who were Hom-WT in the same region and these two groups were significantly different (p=0.0315). If an individual was Het, they had a lower risk of becoming HIV infected with a hazard ratio of 0.78 (95%CI: 0.32-1.92), compared to individuals who were Hom-WT in the same position, however these groups were not significantly different (p=0.5920).

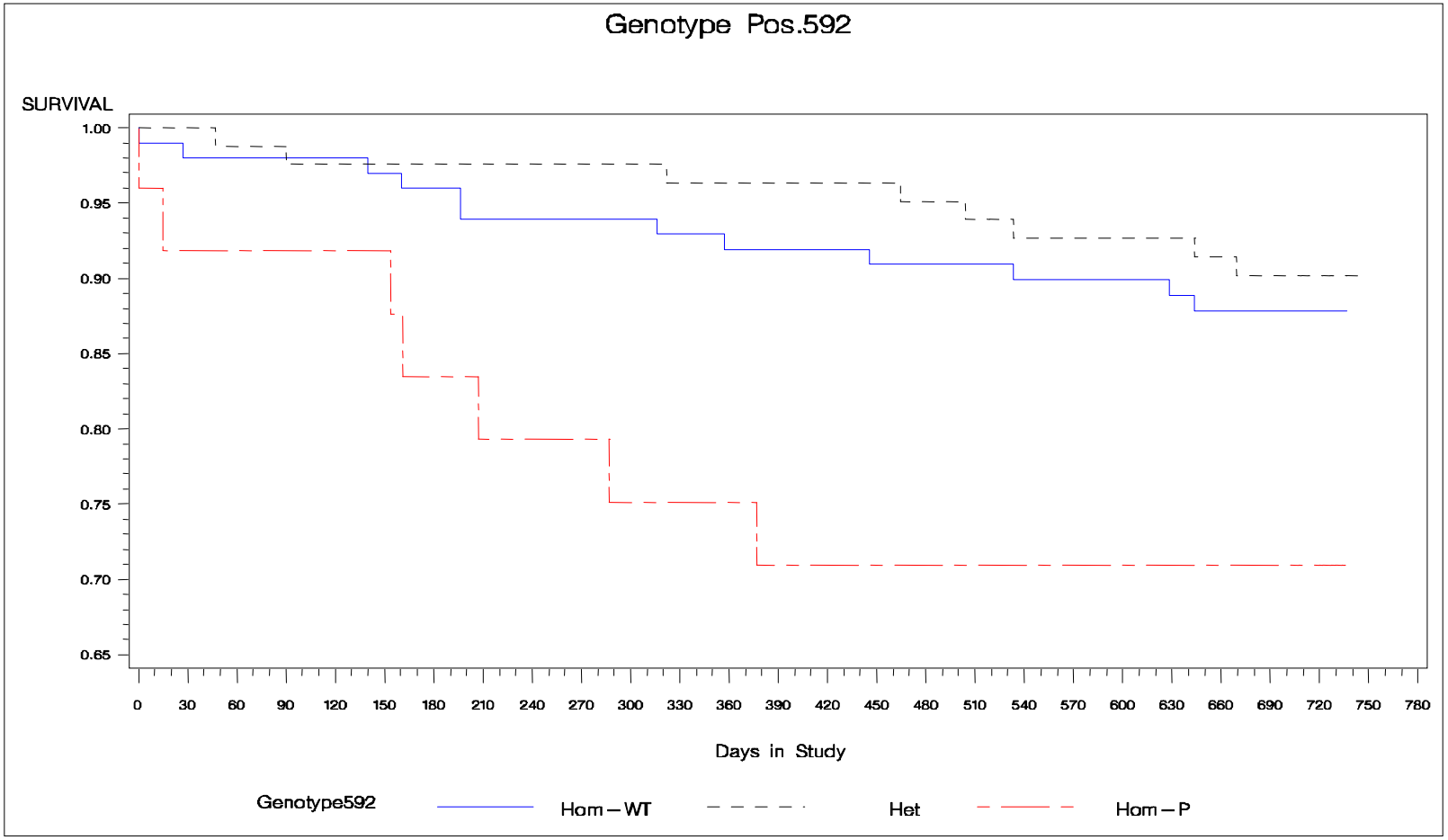


Figure 2.3.7. Kaplan-Meier graph showing IL-10 genotype at -592 position and time to HIV infection. This graph indicates that those individuals that are Hom-P are more likely to become HIV infected with a p value of 0.0335. They were also 2.78 times at increased risk of becoming HIV infected as compared to Hom-WT.

2.4. DISCUSSION

In this study, we investigated the allele and genotype frequencies of two hundred and twenty eight women from the CAPRISA Acute Infection study. HIV-1 seronegative participants at high risk for HIV infection were enrolled into the CAPRISA Acute Infection cohort and followed longitudinally. Routine HIV diagnostic tests were done at monthly intervals. Women who tested positive by rapid HIV tests were enrolled as acutely infected (Phase II of the study). Women who tested negative by rapid testing were subjected to plasma RNA testing, and those found to be positive were then recruited into phase 2 (acute phase) of the study. At the time of this study, there were 228 evaluable women, 180 remained HIV-negative while 27 had become HIV-positive over the follow-up period and 21 acutely infected women had been recruited from other cohorts.

The genotype distribution of the promoter polymorphisms at the -1082 position, as well as the -592 position, are in agreement with Hardy-Weinberg equilibrium, which shows that observed frequencies of the genotypes was not significantly different from the expected frequencies calculated using allele frequency. If the population is in Hardy-Weinberg equilibrium, then the genotype frequencies, as well as the allele frequencies will be preserved generation after generation (Snustad & Simmons, 2000). The allele frequency at the -592 position is similar to what was previously observed among in the Shin *et al.* (2000) study.

We found no association between the genotype at position -1082 and HIV status, as the distribution of HIV-positive and HIV-negative individuals was not significantly different between the groups (*p*-value = 0.2800). Kaplan-Meier graphs revealed that the polymorphism at position -1082 did not affect the risk of HIV acquisition (Cox regression, p=0.1211). This result is in agreement with Shin *et al.* (2000) which investigated the effect of this polymorphism in several American cohorts. A recent study undertaken in Zimbabwe is also in agreement with this observation (Erikstrup *et al.*, 2007). However, at the -592 position there was a significant difference between -592 genotype and HIV status, and this was due to the large difference between HIV-positive individuals homozygous for the mutation and HIV-negative individuals homozygous for the mutation. 28.6% of HIV-positive individuals were homozygous for the -592 mutation, while only 9.5% of HIV-

negative individuals were homozygous for this mutation. Therefore individuals who were homozygous for the mutation at the -592 position are more likely to become HIV-1 infected compared to those with other genotypes.

The Kaplan-Meier graphs were modelled to determine the association of genotype with survival or to the outcome of HIV infection. At the IL-10 -1082 position, the Kaplan-Meier graph shows that individuals who were homozygous for the wild-type allele, i.e. AA, were more likely to become HIV infected, as compared to heterozygous individuals or individuals homozygous for the mutation, however this was not statistically significant (Cox regression, p=0.1211). According to Shin *et al.* (2000) this mutation was also shown to be a recessive mutation, therefore individual's homozygous wild-type or heterozygous at the -1082 position would seem to be phenotypically similar. Our results suggest a trend towards increased likelihood of HIV acquisition for those who were homozygous wildtype at the -1082 position, but it is impossible to tell whether this trend would disappear or become statistically significant with an increased number of study participants in our setting.

We examined whether the -592 polymorphism has an effect with respect to likelihood of HIV acquisition within our study cohort. Our analysis indicates that individuals who were homozygous for the mutation were more likely to become HIV infected compared to individuals who were heterozygous or homozygous for the wild-type allele. This difference was statistically significant (Cox regression, p=0.0335). This was in agreement with data from the Shin *et al.* (2000) study, where individuals that were carriers of the mutation were at increased risk of HIV-infection. According to the Cox proportional hazards model, if a participant was homozygous for the polymorphism at the -592 position then they were at 2.78 times increased risk of becoming HIV-infected, as compared to individuals homozygous for the wild-type allele. These two groups were also significantly different, with a *p*-value of 0.0315. There was no statistically different risk of acquiring HIV infection between individuals who were heterozygous at this position, compared to individuals who were homozygous for the wild-type allele (p=0.5920). The C to A mutation was previously shown to be dominant (Shin *et al.*, 2000) therefore one would expect heterozygotes and individuals homozygous for the mutation to be phenotypically similar, however we did not see this in our study population, again possibly due to small population size.

These results suggest that the mutations at the -1082 and -592 positions might possibly play a protective role in HIV susceptibility. The possible effect of these mutations on HIV susceptibility is demonstrated in Figure 2.4 below. Since IL-10 is an anti-inflammatory cytokine, high expression of IL-10 will lead to general inactivation of T lymphocytes, rendering them less susceptible to HIV-1 infection. On the other hand, low IL-10 production is likely to result in increased T cell activation, thereby making the HIV target cells more susceptible to HIV infection. Individuals who are genetically predisposed to low IL-10 production will therefore be more likely to have activated T cells particularly when they become infected with other infectious pathogens such as sexually transmitted infections. Overall then, the effect of mutations associated with reduced IL-10 production should be to predispose to HIV-1 infection.

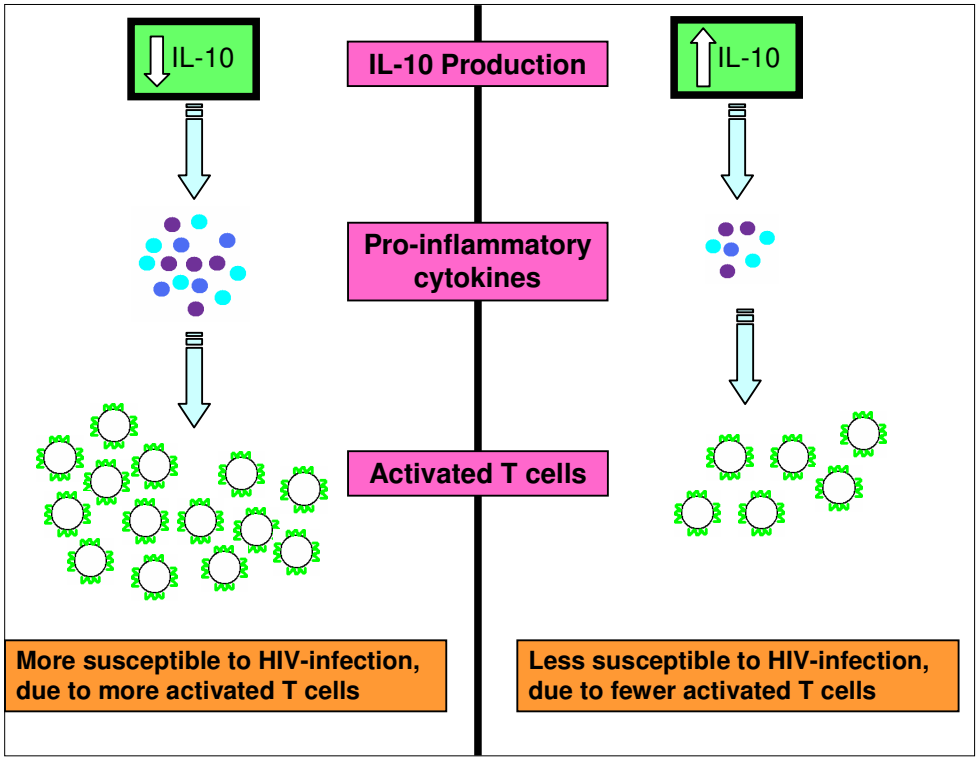


Figure 2.4. The possible role of IL-10 levels on HIV susceptibility. With lower levels of IL-10, there are higher levels of pro-inflammatory cytokines, which results in more activated T cells being present, rendering them more susceptible to HIV infection. However with higher levels of IL-10, there is less production of pro-inflammatory cytokines, and therefore fewer activated T cells, rendering them less susceptible to HIV infection.

However, if the above scenario is correct, how come we do not see the same effect with the -1082 wildtype genotype, which is also associated with lower IL-10 production? First, it is important to note that we observed a trend towards increased HIV-1 infection for carriers of the wildtype allele although this did not reach statistical significance. It is possible that this scenario could have been different if the study sample size was larger. However, we consider this unlikely given the fact that the larger previous study (Shin *et al*., 2000) also found no association with HIV-1 infection for the -1082 polymorphism. Instead, it is possible that the -592 polymorphism has a much stronger effect on IL-10 production and T cell activation in mucosal tissues or in target cells that are more likely to come into contact with HIV-1 during the initial transmission events. This study was not designed to address this question but this should constitute an important focus of future work in order to better understand the mechanisms by which IL-10 promoter polymorphisms may affect susceptibility to HIV-1 infection.

Chapter 3

Effects of IL-10 Promoter Polymorphisms on Primary HIV-1 Pathogenesis

Chapter 3

Effects of IL-10 Promoter Polymorphisms on Primary HIV-1 Pathogenesis

Page

3.1. INTRODUCTION

It has been suggested that both anti-inflammatory as well as pro-inflammatory cytokines play a role in HIV disease progression to AIDS. AIDS Restriction Genes (ARGs) are host genes with polymorphic variants that have been shown to play a role in HIV pathogenesis (O'Brien & Nelson, 2004). These previously identified ARGs have been associated, at different stages of HIV infection, i.e. with prevention of infection, progression to AIDS or limiting infection. Gao *et al.* (2005) demonstrated that the protective effect of certain HLA allotypes changes at different intervals of HIV-1 pathogenesis.

ARGs have been shown to influence the progression of HIV-infection, because not all individuals that become HIV-infected progress to AIDS within the same time frame. Certain HLA Class I alleles have been associated with an AIDS-delaying effect. Killer immunoglobulin-like receptors (KIR) are found on NK cells. The specific HLA ligand must be present on the KIR receptor to regulate NK cell activity (Qi *et al.*, 2006). The specific combination of the receptor *KIR3DS1* with the HLA allele *HLA-Bw4*, has been reported to predispose the protective effect against AIDS progression. Gao *et al.* (2005) showed that *HLA-B*27* and *HLA-B*57* show an AIDS-delaying effect, and the *B*57*-mediated protection is associated with delayed $CD4^+$ T cell decline, and protection occurs early after infection. *HLA-B35*Px* shows an association with acceleration to AIDS (Gao *et al*., 2001).

Different levels of gene expression affect HIV pathogenesis. The up-regulation or down-regulation of IL-10 expression could possibly affect HIV viral load, as well as $CD4^+$ T cell count. It is well documented that a high viral load following the acute phase of infection (a high viral load set point) is a predictor of rapid progression to AIDS, and a low viral load following the acute phase of infection predicts slow progression, therefore viral load can be utilised as a surrogate marker for HIV disease progression, and $CD4^+$ T cell counts are inversely proportional to viral load in HIV-infected individuals.

IL-10, which is an anti-inflammatory cytokine, has been identified as an ARG. Previous studies have demonstrated that polymorphisms found upstream of the transcription start site are associated with differing levels of IL-10 production (Turner *et al*., 1997). The three biallelic polymorphic regions previously identified to be associated with IL-10 production

are -1082, -819 and -592 (nucleotide substitutions at -819 and -592 are in complete linkage disequilibrium). The resulting genotypes and haplotypes have been associated with different effects on IL-10 production. The IL-10 –592A allele (i.e. the mutation) has been associated with acceleration to AIDS, while the IL-10 –1082G allele (i.e. the mutation) has been associated with an AIDS-delaying effect (Shin *et al.*, 2000). A recent study looked at the effect of the IL-10 promoter polymorphisms in a Zimbabwean cohort of HIV-1 infected adults over a three-year follow-up period (Erikstrup *et al*., 2007). This study showed that the IL-10 –1082 mutation was associated with lower viral load and also the decline in $CD4^+$ T cell count after a three-year period was attenuated in individuals that were carriers of the mutation at the -1082 position. It was also shown that the IL-10 -1082G allele was associated with higher levels of IL-10.

IL-10 has the ability to induce the loss of T cell activity and this in turn promotes viral persistence. Brooks *et al.* (2006) documented the effect of IL-10 levels on viral persistence in the LCMV/mouse model. It was shown that viral infection rapidly results in upregulation of IL-10 which then leads to impaired T cell responses. Furthermore, the genetic removal of the IL-10 gene resulted in the maintenance of effector T cell responses, and subsequent viral clearance. In yet another study in the LCMV model, the blockade of the IL-10 receptor with a monoclonal antibody resulted in restoration of T cell function during chronic infection with resultant viral clearance (Ejrnaes *et al*., 2006). These two studies in mice suggest that increased IL-10 production is associated with a reversible loss of effector antiviral immune responses, which then leads to enhanced viral replication. This is in contrast with the earlier data from Shin *et al*. (2000) and more recent data from Erikstrup *et al*. (2007) in which polymorphisms associated with increased IL-10 production appear to be protective against $CD4^+$ T cell loss and progression to AIDS during chronic HIV-1 infection. Thus we hypothesized that IL-10 genetic polymorphisms that affect expression (and by implication IL-10 levels) may differ regarding their effect on HIV pathogenesis depending on the stage of infection studied.

In addition to influencing antiviral immune responses, IL-10 may also directly affect viral replication. It has previously been demonstrated that IL-10 inhibits HIV-1 replication in monocytes and macrophages (Naif *et al*., 1996 and Ancuta *et al*., 2001). Macrophages are thought to play an important role in AIDS pathogenesis, particularly in the late stage of AIDS when $CD4^+$ lymphocytes have been depleted (Orenstein, *et al*., 1997; Swingler *et al.*,

1999; Verani *et al.*, 2005). Although viral replication appears to be more productive in $CD4^+$ T cells as compared to monocytes or macrophages (Wang and Rice, 2006), high levels of IL-10 may be protective against HIV replication within cells of the monocyte/macrophage lineage, thus leading to less rapid immune depletion in stages of infection or in compartments where the macrophages and monocytes are the primary drivers of HIV-1 pathogenesis. It is therefore possible that IL-10 plays significant but divergent roles in HIV replication within monocytes or macrophages especially in the later stages of HIV progression, compared to the earlier stages of infection when $CD4^+$ T lymphocytes are abundant and thought to be more crucial for HIV-1 pathogenesis.

This study was therefore undertaken to describe the effect of the proximal IL-10 gene promoter polymorphisms on viral load and $CD4^+$ T cell counts during the early phase of HIV-1 infection. We reasoned that there were several different possible scenarios with regard to the role of IL-10 promoter polymorphisms that needed to be addressed. First, IL-10 promoter mutations that lead to increased IL-10 production may be protective against high viral load and $CD4^+$ T cell loss during primary HIV-1 infection, as has been demonstrated for chronic (particularly late stage AIDS) phase. This scenario is in line with the hypothesis that the level of viremia and $CD4^+$ T cell count during primary infection is generally predictive of the rate of progression during HIV infection, and therefore we should expect to see similar effects for IL-10 irrespective of disease stage. Second, polymorphisms associated with high levels of IL-10 production may be associated with high viremia and low $CD4^+$ T cell counts, due to the adverse effects of IL-10 on antiviral immune responses during the stage when the virus establishes itself. Third, if HIV replication in macrophages and monocytes is an important source of the virus during early HIV-1 pathogenesis, polymorphisms associated with high IL-10 production might lead to reduced viral replication and thus lower viral load compared to those associated with low IL-10 production, in line with the observation that IL-10 inhibits HIV-1 replication in macrophages (Wang and Rice, 2006). This study may lead to a better understanding of primary HIV-1 pathogenesis and may be informative on strategies for immunotherapeutic interventions during the different phases of HIV-1 infection.

3.2. MATERIALS AND METHODS

3.2.1. STUDY POPULATION

The study population consisted of the HIV-1 infected subset of study participants enrolled in the CAPRISA Acute Infection study. 27 Study participants were enrolled from the longitudinal high risk women cohort, while 21 were enrolled from other CAPRISA studies. A total of 48 HIV-positive women were included in this part of the study. The demographic data for the HIV-positive individuals included in this part of the study is shown in Table 3.2.1. Of the 48 HIV-1 infected individuals included in this part of the study, 43 had IL-10 expression data available. As part of routine diagnostic testing viral load measurements as well as $CD4^+$ T cell counts were carried out by CAPRISA laboratory staff at the Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal. IL-10 expression analysis was performed by Lisa Bebell and Jo-Ann Passmore at the Institute of Infectious Diseases and Molecular Medicine, University of Cape Town.

Table 3.2.1. HIV-positive demographic data. This table shows the demographics of the positive individuals included in this part of the study.

Number of individuals	Mean WPI $\pm$ SD	Mean Age $\pm$ SD
48	72.56 $\pm$ 30.24	27.02 $\pm$ 9.005

WPI – Weeks of follow-up post-infection

3.2.2. VIRAL LOAD ANALYSIS

Participants enrolled in the Acute Infection cohort are screened monthly for HIV infection. HIV-infection was determined using two HIV-1 rapid antibody tests- Determine (Abbott Laboratories) and Capillus (Trinity Biotech). Viral load analysis was performed on all HIV-infected participants enrolled in the CAPRISA Acute Infection cohort. All antibody

negative samples were subjected to pooled PCR testing using the COBAS AmpliScreen™ HIV-1 Test (version 1.5), which is a qualitative *in vitro* test, used to detect HIV-1 RNA in human plasma (Roche Molecular Systems). After the pooling assay was performed, positive samples were then confirmed using a quantitative RNA and an HIV enzyme immunoassay (EIA) test. The COBAS AmpliPrep/COBAS Amplicor HIV-1 Monitor Test (version 1.5) is an *in vitro* diagnostic nucleic acid test for quantification of HIV-1 RNA from human plasma using the COBAS AmpliPrep instrument for RNA extraction, and COBAS AMPLICOR Analyzer for PCR amplification and detection (Roche Molecular Systems, 2006).

3.2.3. $CD4^+$ CELL COUNT ASSESSMENT

$CD4^+$ cell counts were performed on all HIV-infected participants enrolled in the CAPRISA Acute Infection cohort. $CD4^+$ cell counts were determined using the FACS® MultiSET™ System (Becton Dickinson) by the CAPRISA laboratory staff. The CAPRISA laboratory used the BD MultiTEST™. The standard for four colour cytometric assay was used to enumerate $CD4^+$ T lymphocyte counts (Hilerio & Neisler, 1998). The samples were analysed on the BD FACSCalibur™ flow cytometer (BD Biosciences, 2002).

3.2.4. INTERLEUKIN-10 EXPRESSION ANALYSIS

IL-10 expression analysis was performed on 43 out of 48 individuals included in this part of the study. The Becton Dickenson Human Inflammation Cytometric Bead Array® (CBA) system (from BD Biosciences) and FACS analysis were used to measure IL-10 (and other cytokines) in cervicovaginal lavage. Analysis of cytokine production profiles was performed according to manufacturer's instructions. Further details are provided in the study by Bebell *et al*. (2007).

3.2.5. STATISTICAL ANALYSIS

The GraphPad Prism 5 software was used to produce the dot plots. The signed- rank test is used to measure statistical significance of two correlated samples (Wilcoxon, 1945; Paul, V. W., 1999). The Wilcoxon signed-rank test was used to compare viral load measurements; and $CD4^+$ T lymphocytes; as well as IL-10 expression, between the different genotype groups at the different polymorphic IL-10 promoter positions.

3.3. RESULTS

3.3.1. IL-10 Promoter Polymorphisms and the Effect on Viral Load

Viral load analysis was performed on HIV-positive individuals included in this study at different time points during the longitudinal Acute Infection Study. Viral load measurements over all time points, between the three groups Hom-WT, Het and Hom-P, were performed. When the viral load measurements are grouped overall, this allowed us to determine if there was a difference between viral loads in general.

Viral load measurements over all time points were compared between the genotypes at the -1082 position, as shown in Figure 3.3.1. The Hom-P group had the highest median viral load as compared to Hom-WT (p=0.0016) and Het (p=0.0020). The median viral load for the Het and Hom-WT groups were not significantly different.

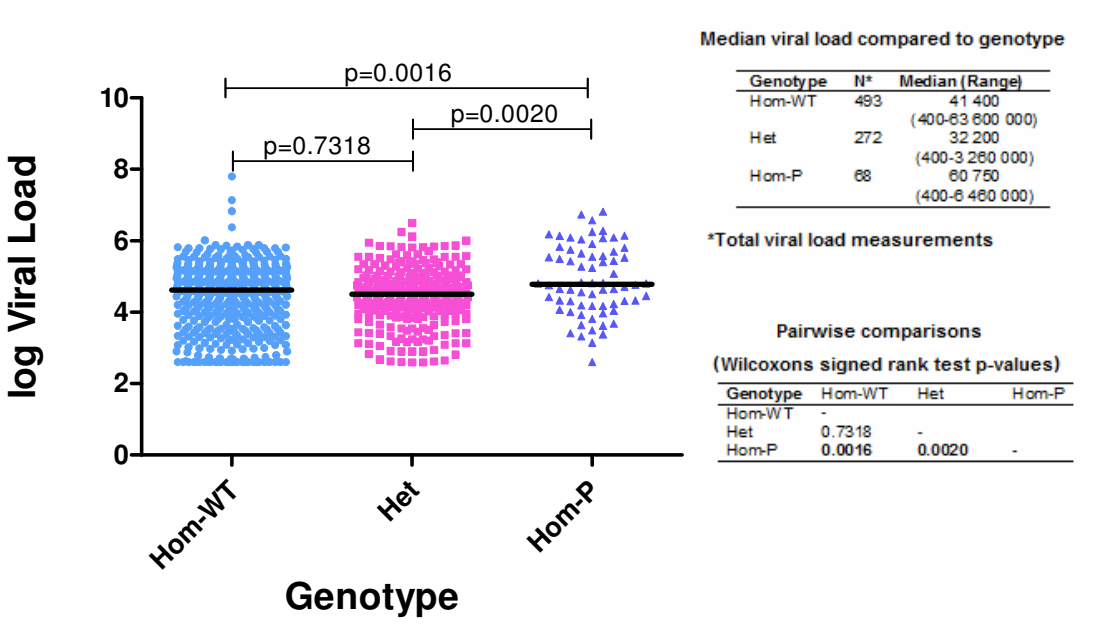


Figure 3.3.1. IL-10 -1082 genotype vs. viral load measurements over all time points. The Hom-P group was significantly different from both the Hom-WT and the Het groups.

Figure 3.3.2 shows the viral load measurements over all time points compared to -592 genotypes. The Hom-WT group had the highest median viral load which was significantly different to the Het group (p=0.0002) but not significantly different to the Hom-P group (p=0.0703). The Het group had the lowest median viral load compared to the other two groups for the -592 position.

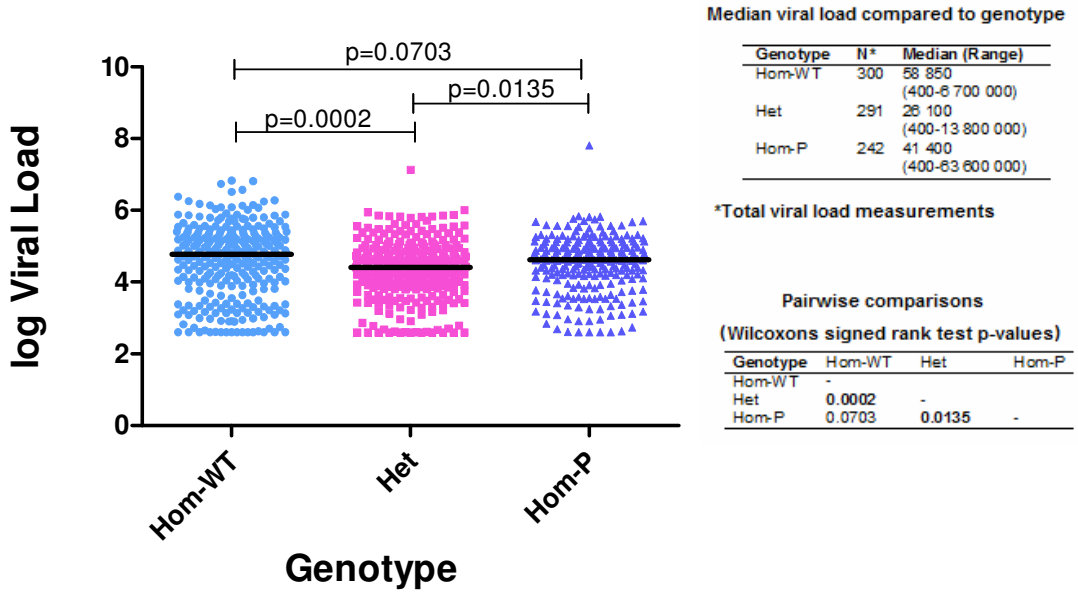
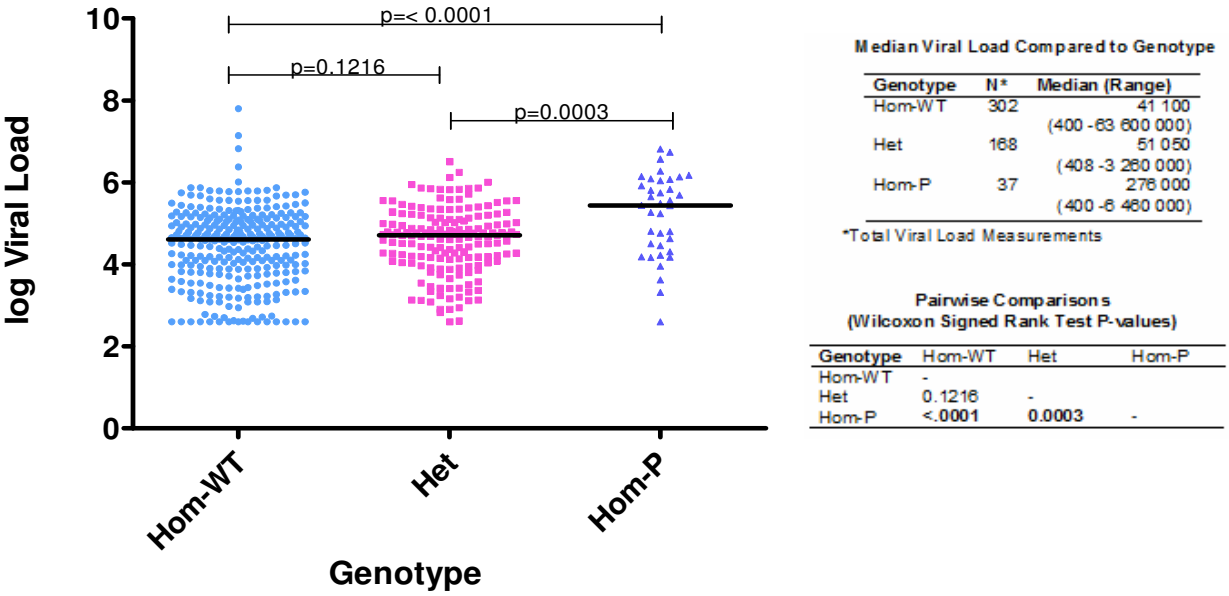


Figure 3.3.2. Viral load over all time points vs. -592 genotype. The median viral load for the Het group was significantly different from the Hom-WT and the Hom-P group.

If one considers viral load to be indicative of disease progression, this data appears to be in contrast to what Shin *et al.* (2000) showed for the mutation at the -1082 position, i.e. this mutation has an AIDS-delaying effect in the late stages of AIDS; and the mutation at the -592 position was shown to have an AIDS-accelerating effect. To determine if there was a change in median viral load between the three genotypic groups for both positions over the early post-infection period, we categorized the viral load data into three different time points, i.e. zero to three months post-infection, three to six months post-infection and six to twelve months post-infection.

3.3.1.1. -1082 Genotype and viral load measurements during early HIV-1 infection

The analysis of viral load at 0-3 months post-infection and -1082 genotype is shown in Figure 3.3.3. This figure shows the log viral load vs. -1082 genotype at 0-3 months post-infection. The individuals that were homozygous for the polymorphism had significantly higher median viral loads at this time point as compared to individuals homozygous for the wild-type allele (p=<0.0001) and heterozygotes (p=0.0003). Hom-WT individuals and Het individuals were not significantly different with a p value of 0.1216.



Median Viral Load Compared to Genotype

Genotype	N*	Median (Range)
Hom-WT	302	41 100 (400 -63 600 000)
Het	168	51 050 (408 -3 260 000)
Hom-P	37	276 000 (400 -6 460 000)

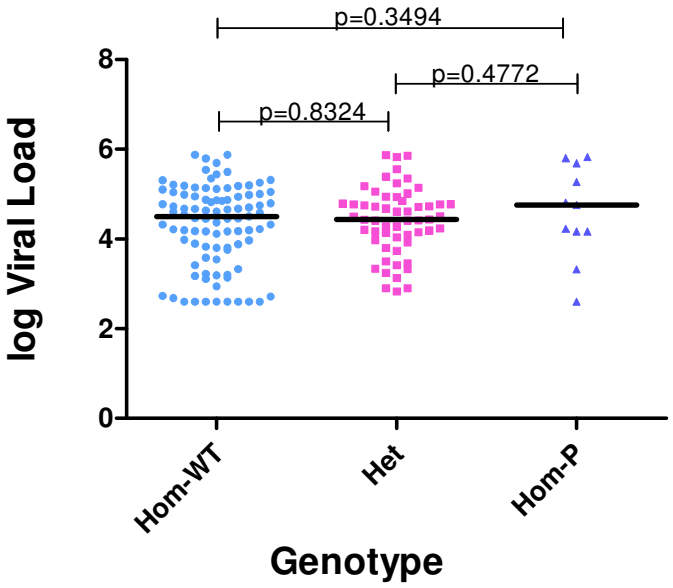
*Total Viral Load Measurements

Pairwise Comparisons (Wilcoxon Signed Rank Test P-values)

Genotype	Hom-WT	Het	Hom-P
Hom-WT	-		
Het	0.1216	-	
Hom-P	<.0001	0.0003	-

Figure 3.3.3. The effect of IL-10 -1082 genotype on viral load at 0–3 months post-infection. Hom-P individuals had the highest median viral load. Hom-WT had the lowest median viral load, however Hom-WT and Het are not significantly different.

The viral load vs. -1082 genotype at 3-6 months post-infection is shown in Figure 3.3.4. Although the Hom-P group seems to have a higher median viral load (Median = 57 300) than the Hom-WT or Het groups, it was not a significant difference (p=0.8324 & p= 0.3494 respectively). Also Hom-WT and Het were not statistically different (p=0.8324).



Median Viral Load Compared to Genotype

Genotype	N*	Median (Range)
Hom-WT	93	32 100 (400 - 751 000)
Het	56	27 400 (685 - 750 000)
Hom-P	11	57 300 (400 - 673 000)

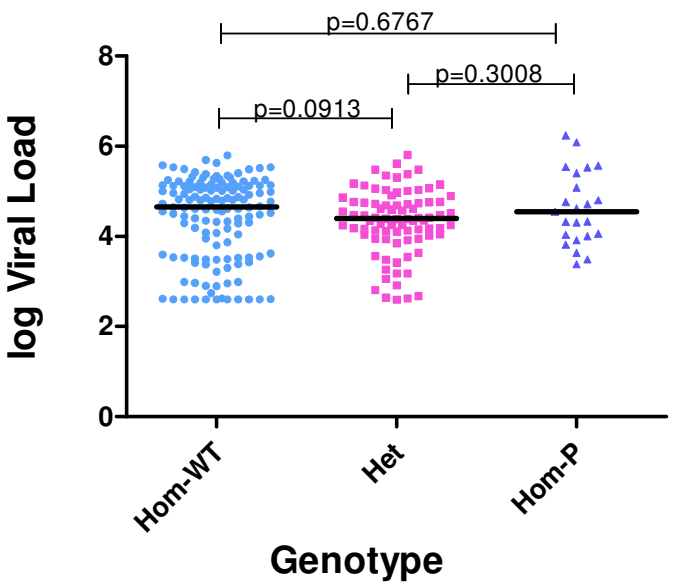
*Total Viral Load Measurements

Pairwise Comparisons (Wilcoxon Signed Rank Test P-values)

Genotype	Hom-WT	Het	Hom-P
Hom-WT	-		
Het	0.8324	-	
Hom-P	0.3494	0.4772	-

Figure 3.3.4. IL-10 -1082 genotype vs. viral load at 3-6 months post-infection. Although the Hom-P group has a higher median viral load than both the Hom-WT and Het groups, there was no significant difference between the groups.

We next analyzed the viral loads at 6-12 months post-infection and genotype at the -1082 position, to determine if there was an association between -1082 genotype and viral load at 6-12 months post-infection. The results are shown in Figure 3.3.5. The results show that Hom-P group has a lower median viral load than the Hom-WT, however this was not significantly different (p= 0.6767). Hom-P group had a higher median viral load than Het, however this was not significantly different (p=0.3008) and Hom-WT group had a higher median viral load than the Het group but this was not significantly different (p=0.0913).



Median Viral Load Compared to Genotype

Genotype	N*	Median (Range)
Hom-WT	134	45 250 (400 - 626 000)
Het	83	25 000 (400 - 659 000)
Hom-P	23	35 500 (2 410 - 1 750 000)

*Total Viral Load Measurements

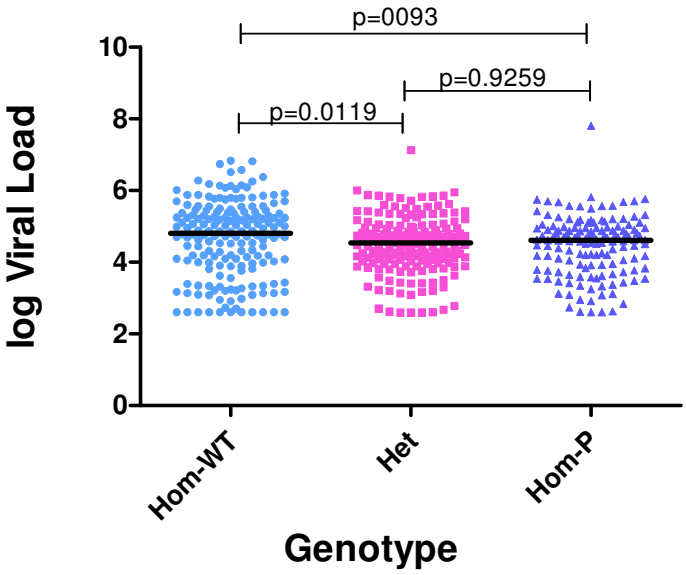
Pairwise Comparisons (Wilcoxon Signed Rank Test P-values)

Genotype	Hom-WT	Het	Hom-P
Hom-WT	-		
Het	0.0913	-	
Hom-P	0.6767	0.3008	-

Figure 3.3.5. IL-10 -1082 genotype vs. viral load at 6-12 months post-infection. There was no statistically significant difference between the groups even though Hom-WT displayed the highest median viral load as compared to Hom-P, which displayed intermediate median viral load, while Het displayed the lowest median viral load at 6-12 months post-infection.

3.3.1.2. -592 Genotype and viral load measurements during early HIV-1 infection

Viral load vs. genotype at the -592 position was also analyzed according to the different time points post-infection, i.e. 0-3 months, 3-6 months, and 6-12 months post-infection. For the analysis at the -592 position at 0-3 months, the viral load analysis is shown in Figure 3.3.6. This data shows that the Hom-WT group was significantly different from Het group and Hom-P group with p values of 0.0119 and 0.0093 respectively. The Het and Hom-P groups were not statistically different. Overall the Hom-WT group had the highest median viral load at the 0-3 months post-infection time point.



Median Viral Load Compared to Genotype

Genotype	N*	Median (Range)
Hom-WT	190	63 800 (400 -6 700 000)
Het	175	34 700 (400 - 13 800 000)
Hom-P	142	40 900 (400 - 63 600 000)

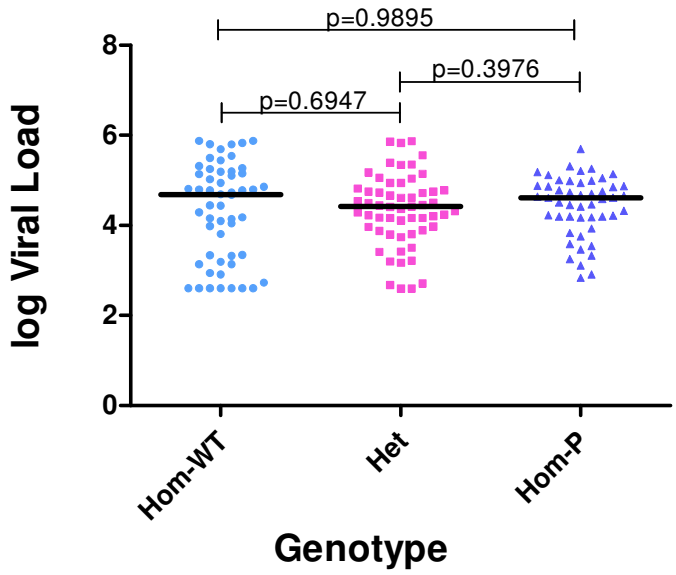
*Total Viral Load Measurements

Pairwise Comparisons (Wilcoxon Signed Rank Test P-values)

Genotype	Hom-WT	Het	Hom-P
Hom-WT	-		
Het	**0.0119**	-	
Hom-P	**0.0093**	0.9259	-

Figure 3.3.6. IL-10 -592 genotype vs. viral load at 0-3 months post-infection. Hom-WT group had significantly higher median viral load than Het or Hom-P group. Het and Hom-P group were not statistically different.

The viral load data at 3-6 months post-infection and was analyzed according to the IL-10 -592 genotype. The analysis is shown in Figure 3.3.7. This analysis shows that Hom-WT and Hom-P groups have higher viral loads than the Het group, however, there was no significant difference between the groups.



Median Viral Load Compared to Genotype

Genotype	N*	Median (Range)
Hom-WT	54	48 250 (400 - 751 000)
Het	57	26 100 (400 - 750 000)
Hom-P	49	40 600 (685 - 496 000)

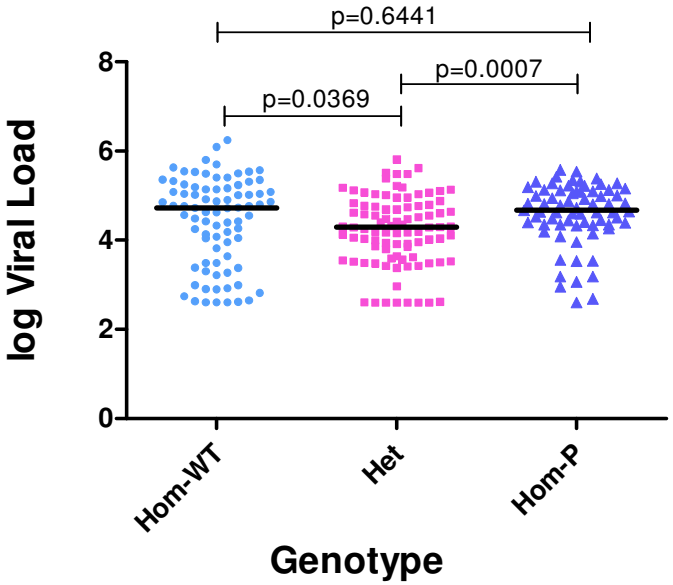
*Total Viral Load Measurements

Pairwise Comparisons (Wilcoxon Signed Rank Test P-values)

Genotype	Hom-WT	Het	Hom-P
Hom-WT	-		
Het	0.6947	-	
Hom-P	0.9895	0.3976	-

Figure 3.3.7. IL-10 -592 genotypes vs. viral load at 3-6 months post-infection. There was no significant difference between the groups even though the Hom-WT and Hom-P groups have a higher median viral load than the Het group.

Analysis of the -592 genotype and viral load at 6-12 months post-infection is shown in Figure 3.3.8. This analysis shows that both Hom-WT and Hom-P groups were statistically different from Het (p= 0.0369 & 0.0007 respectively). The Hom-WT and Hom-P groups were not significantly different.



Median Viral Load Compared to Genotype

Genotype	N*	Median (Range)
Hom-WT	81	52 800 (400 -1 750 000)
Het	90	19 500 (400 -659 000)
Hom-P	69	46 500 (400 -376 000)

*Total Viral Load Measurements

Pairwise Comparisons (Wilcoxon Signed Rank Test P-values)

Genotype	Hom-WT	Het	Hom-P
Hom-WT	-		
Het	**0.0369**	-	
Hom-P	0.6441	**0.0007**	-

Figure 3.3.8. IL-10 -592 genotype vs. viral load at 6-12 months post-infection. The Hom-WT group had the highest median viral load, and was statistically different to the Het group. The Hom-P group had an intermediate median viral load and was statistically different from the Het group, but not the Hom-WT group.

3.3.2. IL-10 Promoter Polymorphisms and the Effect on CD4$^+$ Cell Count

To determine if there was an association between IL-10 promoter variants and CD4$^+$ cell counts in general, CD4$^+$ cell counts over all time points were compared to IL-10 genotypes at positions -1082 and -592. Figure 3.3.9 shows the CD4$^+$ cell counts overall as compared according to the -1082 genotype. This data shows that there was a significant difference between the Hom-P and the Hom-WT group (p=0.0465).

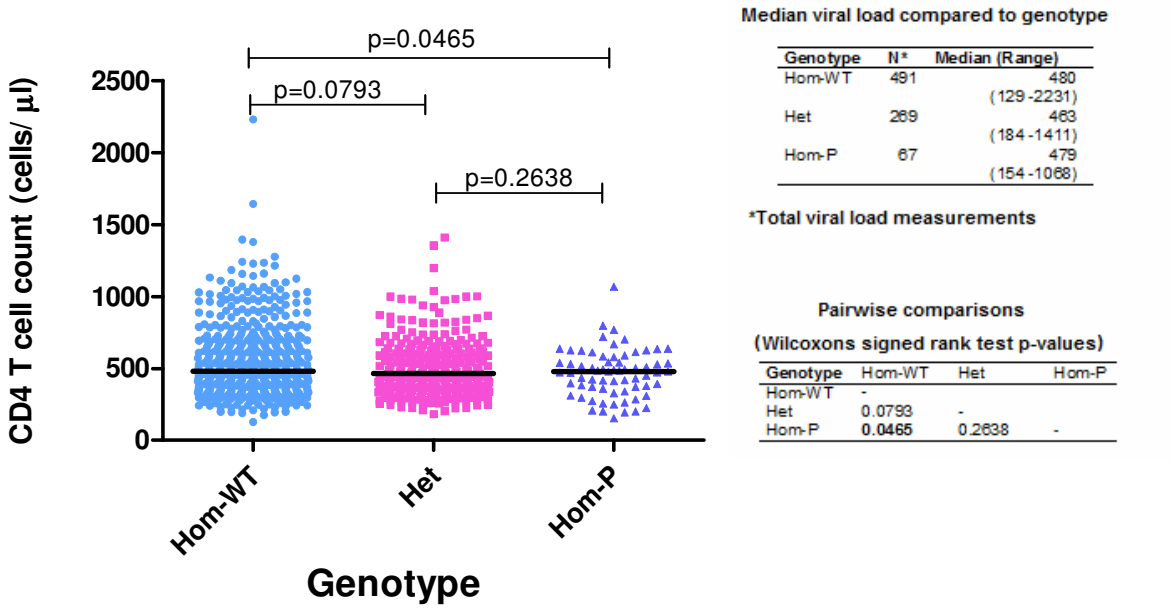


Figure 3.3.9. CD4$^+$ cell count overall vs. -1082 genotype. The Hom-P group had a significantly different median CD4$^+$ cell count compared to the Hom-WT group.

CD4$^+$ cell count over all time points were also compared to genotypes for the 592 position, as shown in Figure 3.3.10. This data shows that the Hom-P group had a higher median CD4$^+$ cell count overall, as compared to the Hom-WT and Het groups with a *p*-value of 0.0484 and 0.0049 respectively.

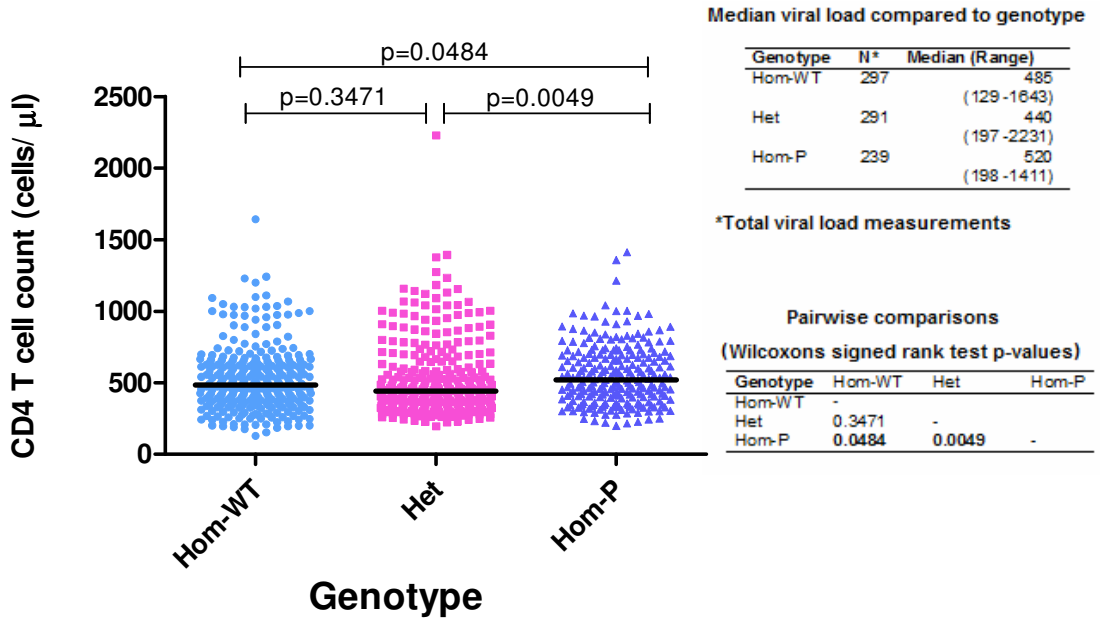
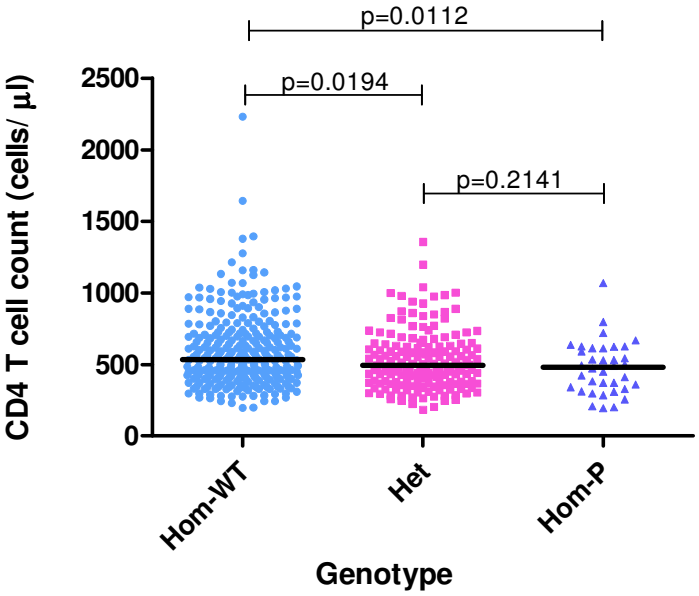


Figure 3.3.10. CD4$^+$ cell count overall vs. -592 genotype. The median CD4$^+$ cell count for the Hom-P group was significantly different to both the Het and Hom-WT groups.

The analysis performed for CD4$^+$ T cell counts and IL-10 genotype at the -592 position appeared to yield results that were in contrast to those of Shin *et al.* (2000) who showed that the mutation at the -592 position has an AIDS-accelerating effect, therefore individuals carrying the mutation should have lower CD4$^+$ cell counts. To determine if there was a change in CD4$^+$ cell count according to time post-infection during the early phase of HIV-1 infection, we categorized post-infection CD4$^+$ cell counts according to different time points post-infection, i.e. 0-3 months post-infection, 3-6 months post-infection and 6-12 months post-infection.

3.3.2.1. -1082 Genotype and CD4$^+$ cell count during early HIV-1 infection

Analysis of IL-10 -1082 genotype and CD4$^+$ cell count at the 0-3 months post-infection time point is shown in Figure 3.3.11. This analysis shows that the median CD4$^+$ cell count for the Hom-WT group was significantly different from the Het group (p= 0.0194) and Hom-P group (p= 0.0112). The Hom-WT group had the highest median CD4$^+$ cell count. The Het and Hom-P groups were not significantly different (p= 0.2141).



Median CD4 Cell Count compared to Genotype

Genotype	N*	Median (Range)
Hom-WT	301	535 (197 - 2231)
Het	166	493 (184 - 1358)
Hom-P	36	480.5 (194 - 1068)

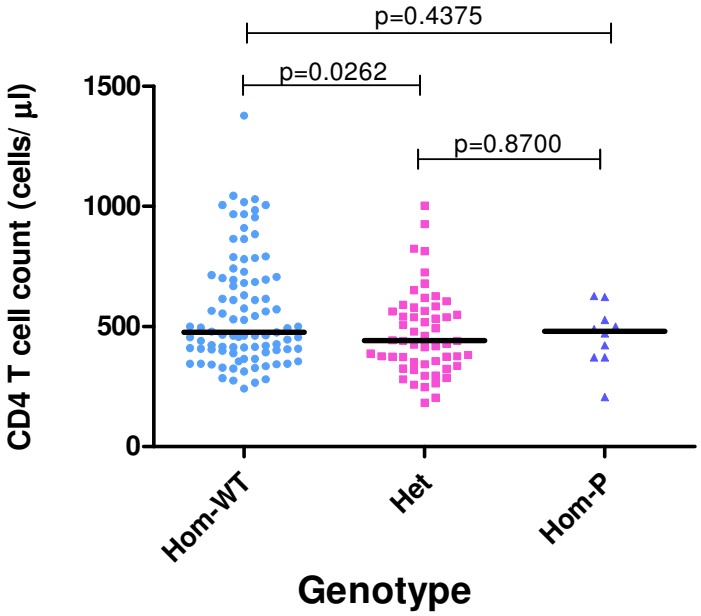
*Total CD4 Count Measurements

Pairwise Comparisons (Wilcoxon Signed Rank Test P-values)

Genotype	Hom-WT	Het	Hom-P
Hom-WT	-		
Het	0.0194	-	
Hom-P	0.0122	0.2141	-

Figure 3.3.11. Genotype at the -1082 position vs. CD4$^+$ cell count at 0-3 months post-infection. The Hom-WT group had the highest median CD4$^+$ cell count which was significantly different from Het and Hom-P groups. Het and Hom-P groups were not significantly different.

The analysis for the -1082 genotype and $CD4^+$ cell count at 3-6 months post-infection is shown in Figure 3.3.12. The Hom-WT group was significantly different from the Het group (p= 0.0262). The Hom-WT group and the Hom-P group were not significantly different.



Median CD4 Cell Count compared to Genotype

Genotype	N*	Median (Range)
Hom-WT	92	477 (242 - 1378)
Het	55	442 (184 - 1004)
Hom-P	10	480.5 (207 - 627)

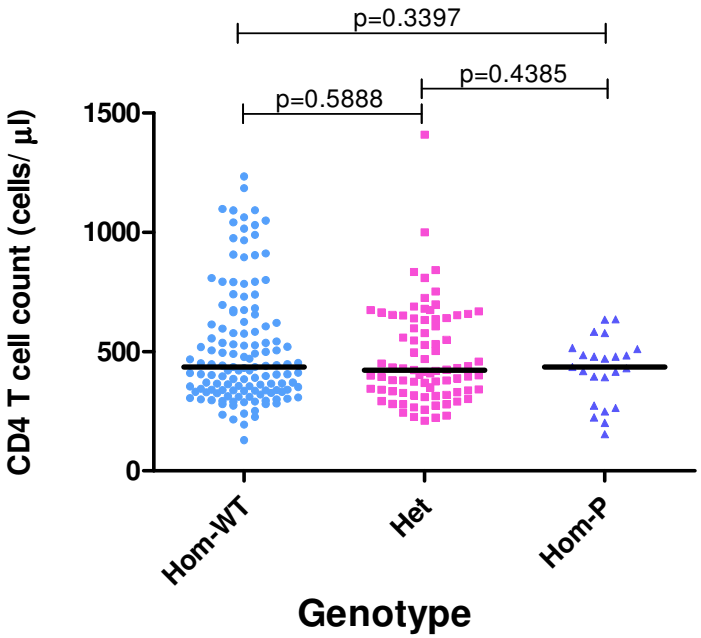
*Total CD4 Count Measurements

Pairwise Comparisons (Wilcoxon Signed Rank Test P-values)

Genotype	Hom-WT	Het	Hom-P
Hom-WT	-		
Het	0.0262	-	
Hom-P	0.4375	0.8700	-

Figure 3.3.12. $CD4^+$ cell count vs. -1082 Genotype at 3-6 months post-infection. The Hom-WT group was significantly different from the Het group. There was no significant difference between the median $CD4^+$ cell count between Hom-WT and Hom-P; and Het and Hom-P.

The analysis between -1082 genotype and $CD4^+$ cell count at 6-12 months post-infection is shown in Figure 3.3.13. The pairwise comparisons between the -1082 genotype and the $CD4^+$ cell count at the 6-12 months post-infection time point, shows that there was no significant difference between the three genotype groups.



Median CD4 Cell Count compared to Genotype

Genotype	N*	Median (Range)
Hom-WT	133	435 (129 - 1234)
Het	82	422 (212 - 1411)
Hom-P	23	435 (154 - 636)

*Total CD4 Count Measurements

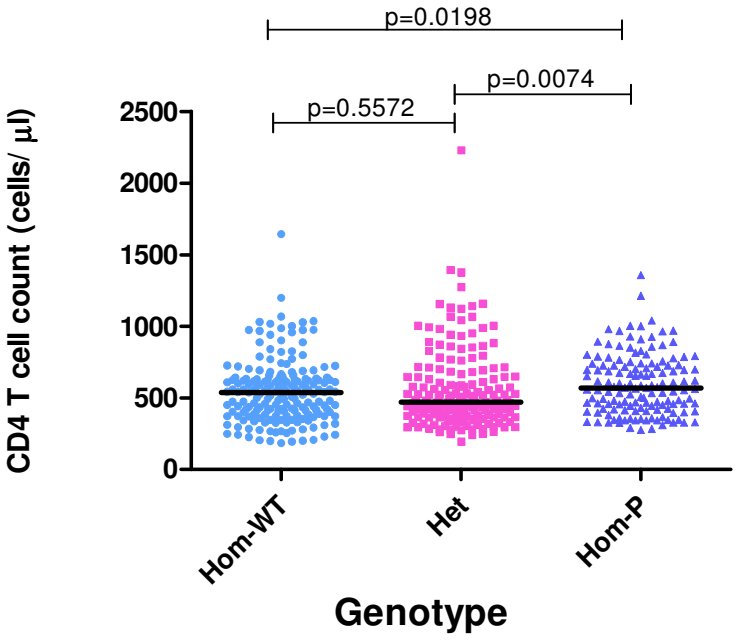
Pairwise Comparisons (Wilcoxon Signed Rank Test P-values)

Genotype	Hom-WT	Het	Hom-P
Hom-WT	-		
Het	0.5888	-	
Hom-P	0.3397	0.4385	-

Figure 3.3.13. CD4$^+$ cell count vs. -1082 genotype at 6-12 months post-infection. The results suggest that there was no significant difference between the median CD4$^+$ cell count between the -1082 genotypes at the 6-12 month post-infection time point.

3.3.2.2. -592 Genotype and CD4$^+$ cell count during early HIV-1 infection

As with the analysis of the IL-10 -1082 genotype and CD4$^+$ cell count, analysis of the -592 genotype and CD4$^+$ cell count was performed at three different time points, i.e. 0-3 months post-infection, 3-6 months post-infection and 6-12 months post-infection. The analysis between -592 genotype and CD4$^+$ cell count at the 0-3 months post-infection time point is shown in Figure 3.3.14. There was a significant difference between the median CD4$^+$ cell count between the Hom-WT group and Hom-P group (p=0.0198), as well as the Het and Hom-P groups (p=0.0074). The Hom-WT and Het groups were not significantly different.



Median CD4 Cell Count compared to Genotype

Genotype	N*	Median (Range)
Hom-WT	187	539 (184 - 1643)
Het	176	469.5 (197 - 2231)
Hom-P	140	567.5 (276 - 1358)

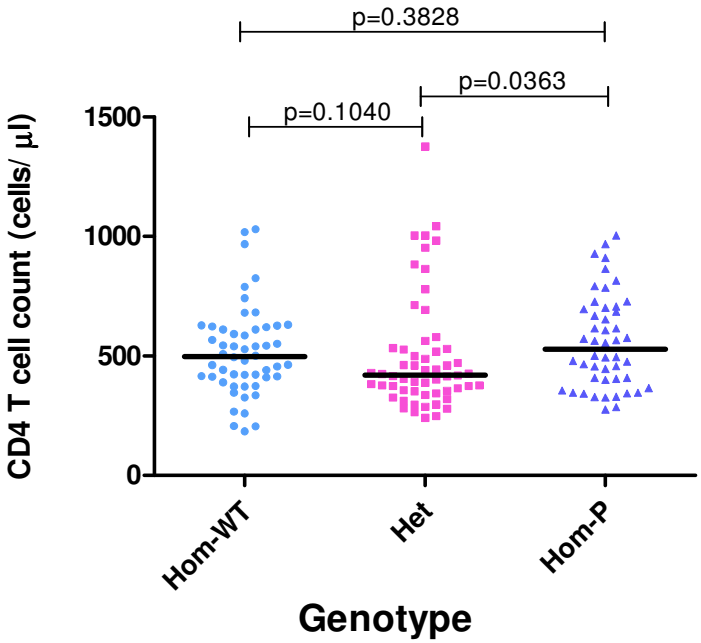
*Total CD4 Count Measurements

Pairwise Comparisons (Wilcoxon Signed Rank Test P-values)

Genotype	Hom-WT	Het	Hom-P
Hom-WT	-		
Het	0.5572	-	
Hom-P	**0.0198**	**0.0074**	-

Figure 3.3.14. CD4$^+$ cell count vs. -592 genotype at 0-3 months post-infection. There was a significant difference between the Hom-WT and Hom-P median CD4$^+$ cell count, and the Het and Hom-P median CD4$^+$ cell count. There was no significant difference between the median CD4$^+$ cell count between the Hom-WT group and the Het group.

The analysis of the -592 genotype and $CD4^+$ cell count at the 3-6 months post-infection time point is shown in Figure 3.3.15. There was a significant difference between the Het and Hom-P groups median $CD4^+$ cell count (p= 0.0363). There was no significant difference between Hom-WT and Het, or Hom-WT and Hom-P groups (p=0.140 and p=0.3828 respectively).



Median CD4 Cell Count compared to Genotype

Genotype	N*	Median (Range)
Hom-WT	52	497 (184 - 1030)
Het	57	419 (242 - 1378)
Hom-P	48	527.5 (276 - 1004)

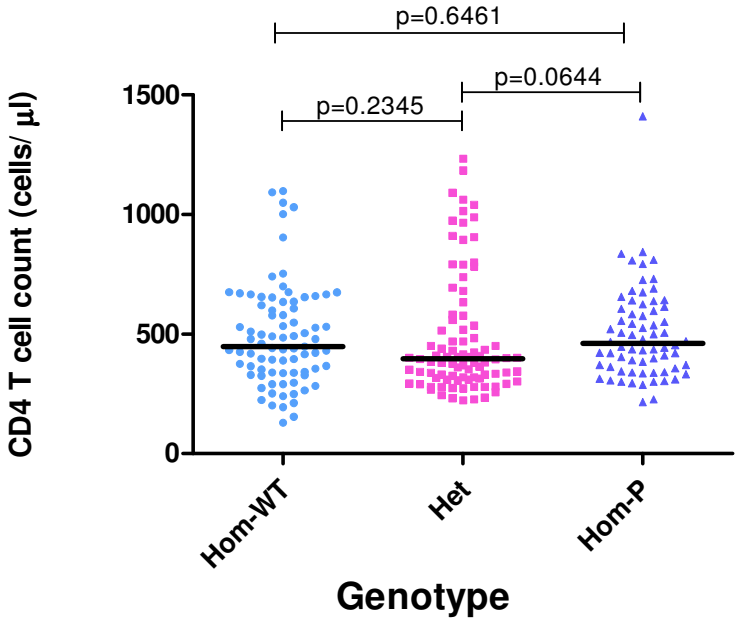
*Total CD4 Count Measurements

Pairwise Comparisons (Wilcoxon Signed Rank Test P-values)

Genotype	Hom-WT	Het	Hom-P
Hom-WT	-		
Het	0.1040	-	
Hom-P	0.3828	0.0363	-

Figure 3.3.15. $CD4^+$ cell count vs. -592 genotype at 3-6 months post-infection. There was a significant difference between the Het and Hom-P median $CD4^+$ cell count. There was no significant difference between the median $CD4^+$ cell count between Hom-WT and Het and Hom-WT and Hom-P groups.

The analysis of the -592 genotype and $CD4^+$ cell count at 6-12 months post-infection is shown in Figure 3.3.16. Although the median $CD4^+$ cell count for the Het group is lower than both the Hom-WT and Hom-P groups, there was no significant difference between the groups for $CD4^+$ cell count and -592 genotype at the 6-12 months post-infection time point.



Median CD4 Cell Count compared to Genotype

Genotype	N*	Median (Range)
Hom-WT	81	448 (129 - 1098)
Het	89	397 (224 - 1234)
Hom-P	68	460.5 (216 - 1411)

*Total CD4 Count Measurements

Pairwise Comparisons (Wilcoxon Signed Rank Test P-values)

Genotype	Hom-WT	Het	Hom-P
Hom-WT	-		
Het	0.2345	-	
Hom-P	0.6461	0.0644	-

Figure 3.3.16. $CD4^+$ cell count vs. -592 genotype at 6-12 months post-infection. There was no significant difference between $CD4^+$ cell count and -592 genotype at the 6-12 months post-infection time point.

3.3.3. IL-10 Promoter Polymorphisms and the Effect on IL-10 Expression

The IL-10 expression data was generated by Lisa Bebell and Jo-Ann Passmore at the Institute of Infectious Diseases and Molecular Medicine, University of Cape Town Medical School. IL-10 levels were measured in cervicovaginal lavage. IL-10 expression data was available from 43 women with acute/recent HIV-1 infection enrolled in the CAPRISA Acute Infection cohort study. The sensitivity of this method was between 1.9 and 7.2 pg/ml. If a sample had levels below the lower detection limit, then the measurement was recorded as zero; and 5000pg/ml for measurements above the upper detection limit of 5000pg/ml. We looked at all available measurements for all time points within a certain genotypic group.

3.3.3.1. -1082 Genotype and IL-10 Expression

43 HIV-positive individuals were included in this analysis, i.e. -1082 genotype and the effect on IL-10 expression. The results are shown in Table 3.3.1. These results show that for the IL-10 -1082 genotype, there was no significant difference between the groups for individuals that expressed IL-10 at all visits, as well as those individuals that had an increase in IL-10 expression after HIV infection. Therefore there was no significant difference between the -1082 genotypic groups and IL-10 expression.

Table 3.3.1. IL-10 expression and association with IL-10 -1082 genotype. (A) Proportion of individuals who had expressed IL-10 at all visits. This data shows that there was no significant difference between the three genotypic groups and IL-10 Expression. (B) The proportion of individuals who had increased IL-10 expression from HIV-negative status. This shows that there was no significant difference between the -1082 genotype and IL-10 expression. (N represents all available time points for n number of individuals)

A

Genotype	N	Proportion who had expressed IL-10 at all visits	Fisher's Exact Test for Association
Hom-WT	54	35.2% (n=19)	
Het	23	52.2% (n=12)	p=0.2518
Hom-P	8	25.0% (n=2)	

B

Genotype	N	Proportion who had increased IL-10 expression from HIV Negative	Fisher's Exact Test for Association
Hom-WT	9	33.3% (n=3)	
Het	1	100.0% (n=1)	p=0.6182
Hom-P	1	0% (n=0)	

3.3.3.2. -592 Genotype and IL-10 Expression

As with the analysis of the -1082 position, the -592 position was analysed for an association between -592 genotype and IL-10 expression. This data is shown in Table 3.3.2. This data suggests that there was no significant difference between the -592 genotype groups and IL-10 expression, for individuals that expressed IL-10 at all visits, as well as the proportion of individuals that showed an increase in IL-10 expression from HIV-negative status. Therefore there was no significant difference between the groups for -592 genotype vs. IL-10 expression.

Table 3.3.2. IL-10 -592 genotype and IL-10 expression. (A) The proportion of individuals expressed IL-10 at all visits. This analysis shows that there was no association between IL-10 -592 genotype and IL-10 expression. (B) The proportion of individuals who had an increase in IL-10 expression after HIV-negative status. There was no significant difference between the -592 genotype and IL-10 expression. (N represents all available time points for n number of individuals)

A

Genotype	N	Proportion who had expressed IL-10 at all visits	Fisher's Exact Test for Association
Hom-WT	31	51.6% (n=16)	
Het	24	33.3% (n=8)	p=0.1971
Hom-P	30	30.0% (n=9)	

B

Genotype	N	Proportion who had increased IL-10 expression from HIV Negative	Fisher's Exact Test for Association
Hom-WT	5	40.0% (n=2)	
Het	1	0% (n=0)	p=1.0000
Hom-P	5	40.0% (n=2)	

3.3.4. Genotype Details of HIV-positive individuals included in study

HIV-negative and HIV-positive individuals were enrolled in the CAPRISA Acute Infection cohort. 228 individuals enrolled in the CAPRISA Acute Infection cohort, were included in this study. Of the 228 individuals included in this study, 48 were HIV-positive and 180 were HIV-negative. Viral load and $CD4^+$ count, is based on HIV-positive individuals only. The IL-10 genotype details of the 48 HIV-positive individuals included in this study are shown in Appendix 1. There were different groups of HIV-positive individuals identified in this study, i.e. Rapid Progressors, individuals with a high viral load (above 100 000 copies/ml) and/or a low $CD4^+$ cell count (less than 350 cells/µl) for at least two consecutive months; the second group was the controllers/slow progressors. The results for IL-10 genotype for the different groups are shown in Table 3.3.3 and Table 3.3.4 respectively.

Table 3.3.3 shows the results for genotype assessment of the Rapid Progressors (according to the Acute Infection cohort). These results show that 12 out of 13 (92.31%) of these individuals display the non-protective allele at the -1082 position, that is, the WT allele - A

at the -1082 position, and eight out of 13 (61.54%) of these individuals display the non-protective allele of G (mutation) at the -592 position.

Table 3.3.3. Genotype details of the rapid progressors. 12/13 individuals display the non-protective allele at the -1082 position and 8/13 individuals display the non-protective allele at the -592 position.

PID	IL-10 -1082 genotype	IL-10 -592 genotype
AI0008	AA	AA
AI0037	AA	CA
AI0065	AA	CC
AI0069	GG	CC
AI0085	AA	CA
AI0174	AA	AA
AI0206	AA	CC
AI0210	AA	AA
AI0256	AA	CC
AI0258	AG	CA
AI0260	AA	AA
AI0264	AG	CA
AI0270	AG	CA

PID – Participant Identification

AI – Acute Infection

*** -1082 AA = Hom-WT; AG=Het; GG=Hom-P**

 # -592 CC=Hom-wt; CA=Het; AA=Hom-P

Table 3.3.4. shows the genotype assessment of the individuals termed as Controllers or Slow Progressors, according to the Acute Infection Cohort. In this group none of the individuals displayed the protective GG genotype at the -1082 position, however four out of the five (80%) of these individuals displayed the protective C allele at the -592 position. This suggests that the -592 mutation might have a stronger effect on HIV pathogenesis than the -1082 mutation.

Table 3.3.4. Genotype details of the controllers (slow progressors). None of these individuals display the protective -1082 allele. At the -592 position 4/5 (80%) of these individuals display the protective C allele.

PID	IL-10 -1082 Genotype	IL-10 -592 Genotype
AI0045	AA	CC
AI0061	AA	CA
AI0222	AG	CC
AI0228	AG	AA
AI0269	AA	CC

PID – Participant Identification

AI – Acute Infection

*** -1082 AA = Hom-WT; AG=Het; GG=Hom-P**

 # -592 CC=Hom-wt; CA=Het; AA=Hom-P

3.4. DISCUSSION

48 HIV-positive individuals enrolled in the CAPRISA Acute Infection cohort were genotyped at the -1082 position and the -592 position of the IL-10 promoter. These genotypes were then compared to viral load, which has been used as a surrogate marker for HIV progression (Arnaout *et al*., 1999; Meira *et al*., 2004); $CD4^+$ T lymphocyte counts; and for 43 of the 48 HIV-positive individuals, IL-10 expression (Brooks *et al*., 2006).

The viral load analysis over all time points for the -1082 position appears to be in conflict with the findings of Shin *et al*. (2000) and Erikstrup *et al*. (2007), if one assumes that viral load during early infection is a good indicator of the rate of disease progression and immune deterioration during chronic and late phases of infection. The viral load analysis was performed at three different time points, i.e. 0-3 months post-infection, 3-6 months post-infection and 6-12 months post-infection, to determine if there was a difference in viral load between the genotypes in early infection. Data from this analysis, based on genotype at the -1082 position, showed that at 0-3 months post-infection there was a significant difference in median viral load between the Hom-WT and Hom-P groups, as well as between the Het and Hom-P groups. Results show that the Hom-P median viral load was highest and the Hom-WT group had the lowest median viral load. This could possibly be due to the mutation at this position resulting in up-regulation of IL-10 production (Turner *et al*., 1997) which subsequently results in decreased antiviral T cell activity (Brooks *et al*., 2006), which leads to more viral replication. However, as the time increased post-infection, we observed a change in the order of median grouping. At 3-6 months post-infection the Hom-P had the highest median viral load, and the Het group had the lowest median viral load, however at this time point the genotype groups were not significantly different. At the 6-12 months post-infection time point we saw that the Hom-WT group now had the highest median viral load, the Hom-P group now had the intermediate median viral load, and the Het group had the lowest median viral load. Also there was no significant difference between -1082 genotype and median viral load at the 6-12 months post-infection time point. Overall it seems as though the Hom-P group might possibly be decreasing in median viral load while the Hom-WT might possibly be increasing in median viral load, when looking at the -1082 genotype, which might indicate

the protective role of the -1082G mutation in chronic and late infection, as shown by Shin *et al.* (2000) and Erikstrup *et al*. (2007).

The mutation at the -592 position has been associated with low IL-10 production (Turner *et al*., 1997) and is associated with acceleration to AIDS (Shin *et al*., 2000). Low IL-10 levels were shown to result in LCMV viral clearance in the mouse model (Brooks *et al*., 2006). The analysis of the effect of the IL-10 -592 genotype on viral load was also performed at three different time points. At the 0-3 months post-infection time point the Hom-WT group was significantly different from both the Het and Hom-P groups, with Hom-WT having the highest median viral load and Hom-P displaying the lowest median viral load. This is possibly due to low IL-10 expression within the Hom-P individuals, which possibly results in inhibition of less viral replication. However at 3-6 months post-infection the Hom-P group had a higher median viral load than the Het group, and the Hom-WT groups still displayed the highest median viral load. At this time point there was no significant difference between the three genotypic groups. At the 6-12 months post-infection time point Hom-WT remained the highest median viral load, Hom-P had the intermediate median viral load and the Het group had the lowest median viral load. There was significant difference between the Hom-WT and Het group, as well as the Hom-P and Het group. As time increased, the Hom-P group seems to have an increase in median viral load, and this might be indicative of the non-protective characteristic of the SNP at the 592 position in chronic infection, as demonstrated by Shin *et al.* (2000).

The IL-10 genotypes were also compared to the $CD4^+$ T cell counts for both the -1082 and the -592 positions, at the three different time points. When we examined the $CD4^+$ cell count vs. -1082 genotype at the 0-3 months post-infection time point, the Hom-WT group had the highest median $CD4^+$ cell count, with the Hom-P group displaying the lowest median $CD4^+$ cell count, with a significant difference between the Hom-WT and Hom-P groups, and the Hom-P and Het groups. Due to the possible role of the IL-10 inducing T cell inactivation and high viral replication, the $CD4^+$ cell count should be low, as viral load and $CD4^+$ cell measurements have been shown to be inversely proportional (Meira *et al*., 2004). At the 3-6 months post-infection time point the Hom-P group now displayed the highest median $CD4^+$ cell count and the Het group displayed the lowest median $CD4^+$ cell count. At the 6-12 months post-infection time point there was no significant difference between the -1082 genotype and $CD4^+$ cell count.

At the -592 position the $CD4^+$ cell count was performed at the three time points and compared to -592 genotype. At the 0-3 months post-infection time point the Hom-P group had the highest median $CD4^+$ cell count and the Het group had the lowest median $CD4^+$ cell count. The Hom-P group was significantly different from the Hom-WT group as well as the Het group. The -592 mutation has been associated with low IL-10 production, which could possibly indicate the higher median $CD4^+$ cell count for the Hom-P group. As time post-infection increased, the order of the $CD4^+$ cell count and genotype groupings remained as Hom-P having the highest median $CD4^+$ cell count, Hom-WT with the intermediate median $CD4^+$ cell count, and the Het group within the lowest median $CD4^+$ cell count.

IL-10 expression has been associated with the three biallelic polymorphic promoter regions, i.e. the -1082, -819 and the -592 regions. The -819 and -592 regions are in complete linkage disequilibrium. These proximal haplotypes together with extended distal haplotypes, consisting of seven polymorphic regions, have also been associated with IL-10 production (Gibson *et al*., 2001). The mutation at the -1082 position has been associated with high IL-10 production, while the mutation at the -592 position has been associated with low IL-10 production. The IL-10 expression analysis was performed on 43 of the 48 HIV-positive individuals included in this study. The analysis between IL-10 genotype, at both the -1082 and -592 positions, showed no significant association with IL-10 production. This is possibly due to the small number of individuals included in this part of the analysis, and therefore the results did not show a significant correlation with IL-10 production. Hel *et al*. (2006) suggested that HIV replication has a more pronounced impact on the mucosal system in initial HIV infection. Massive HIV replication in the gut-associated lymphoid tissue (GALT), as well as the evident loss of $CD4^+$ T cells, is detectable in peripheral blood and lymph nodes. Therefore future IL-10 expression studies could focus on GALT and lymph nodes, where we might observe a difference in IL-10 production based on genotype.

Brooks *et al.* (2006) demonstrated in the mouse model that high levels of IL-10 results in T cell inactivation and viral persistence. It was also shown that the genetic removal, as well as blocking of the IL-10 receptor resulted in viral clearance, due to low IL-10 levels. Shin *et al.* (2000) demonstrated that the -1082G-SNP was associated with an AIDS-delaying influence, while the -592A-SNP was associated with an AIDS-accelerating influence, and

that individuals that were carriers of this mutation were possibly at increased risk of HIV infection.

Results from this study suggest that the data is in agreement with previous studies at different time points for the different polymorphisms. The results for early time points and IL-10 -1082 genotype analysis shows that the mutation at the -1082 position resulted in higher median viral loads. Brooks *et al*. (2006) demonstrated that persistent viremia in the LCMV/mouse model, due to immunosupression via T cell inactivation, is associated with increased IL-10 production. The mutation at the -1082 position has been associated with high IL-10 production, therefore this data suggests that the high viral loads are possibly a result of high IL-10 production. As the time increases the Hom-P median viral load decreases and is possibly demonstrating the AIDS-delaying influence of the mutation at the -1082 position, as demonstrated by Shin *et al*. (2000), in later stages of HIV infection.

At the -592 position the early time points show that the mutation at this region has an association with low viral load, possibly suggesting the impact of robust T cell responses in individuals with low IL-10 production, however as time increases the median viral load of the Hom-P group seems to be increasing, possibly suggesting the association of the mutation with an AIDS-accelerating influence, as demonstrated by Shin *et al*. (2000).

Shin *et al*. (2000) demonstrated that the mutations within the IL-10 promoter regions do not show any significant association with HIV disease progression during the first five years post-infection, however these effect of these mutations are more evident after five years of infection. Therefore it is possible that the effects of these mutations are not very pronounced in this study cohort, as the mean follow up period is 72.56 ± 30.24 weeks post-infection, i.e. approximately 1.4 years post-infection.

The hypothesis we formed to try and explain the different role of IL-10 at different stages of infection is shown in Figure 3.4.1. In early HIV infection IL-10 results in a loss of T cell function. Therefore IL-10 causes immunosuppression via T cell inactivation. This results in the failure to control HIV replication. The infection of $CD4^+$ T lymphocytes is more productive than in macrophages. Therefore in early infection, higher levels of IL-10 results in higher levels of viral replication. In late infection the $CD4^+$ T lymphocytes have been extensively depleted and HIV replication occurs more productively within macrophages.

This results in IL-10 displaying a protective role, due to the function of macrophage inhibition by IL-10. Therefore in the late stages of HIV-infection high levels of IL-10 results in the inhibition of macrophage activation, which in turn results in lower levels of viral replication. Lower levels of IL-10 results in more macrophage activation and this results in higher viral replication rates.

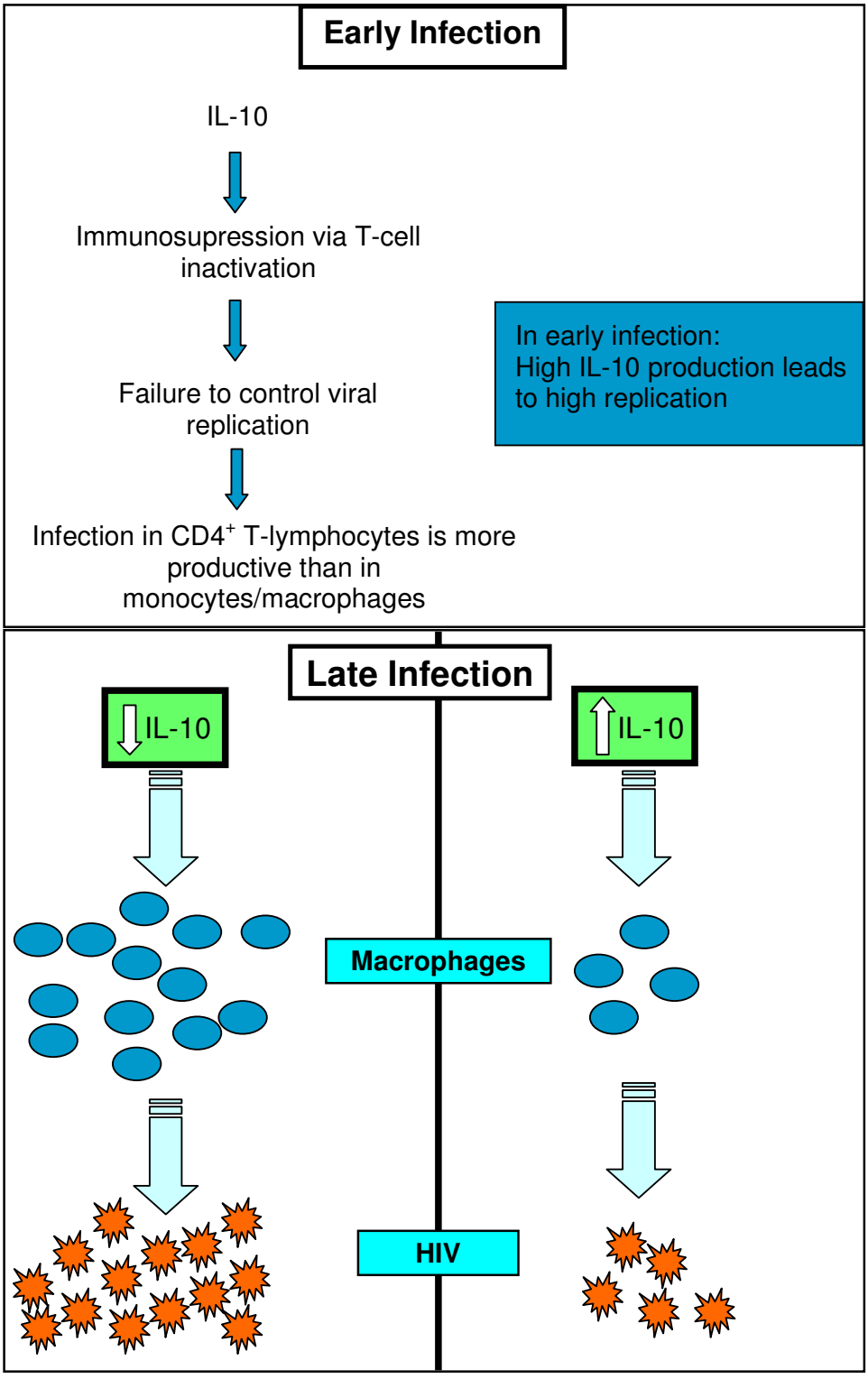


Figure 3.4.1. The possible role of IL-10 during early and late HIV infection. Early infection is aimed at $CD4^+$ T lymphocytes, and in early infection IL-10 enhances HIV replication. However, in late infection, when $CD4^+$ T lymphocytes have been depleted, HIV replication occurs more productively in macrophages. IL-10 has a protective role in late infection due to macrophage inactivation by IL-10.

Chapter 4

DISCUSSION

DISCUSSION

In this study the IL-10 promoter variants were assessed and genotypes were assigned to 228 women that were enrolled in the CAPRISA Acute Infection cohort (Chapter2). The genotypes were then analysed for an association with HIV status and susceptibility. There was no significant association between -1082 genotype and HIV status, however there was a significant difference between the genotype and serostatus at the -592 position and this was due to the large difference between the individuals homozygous for the mutation (Hom-P) and Hom-WT. Shin *et al.* (2000) demonstrated that the mutation at the -1082 position has an AIDS-delaying influence, while the mutation at the -592 position has an AIDS-accelerating influence and individuals that are carriers of the -592 mutation were possibly at higher risk of becoming HIV infected as compared to individuals carrying the wild-type allele. In this study the genotype at the -1082 position as well as the -592 position were analysed, this analysis showed that individuals that were homozygous for the wild-type allele at the -1082 position were at a higher risk of HIV infection, and individuals that were homozygous for the mutation at the -592 position were more likely to become infected, this was in agreement with Shin *et al.* (2000).

The mutations were then analysed for an association with IL-10 production as well as HIV progression (Chapter 3). IL-10 promoter polymorphisms have been associated with differing levels of IL-10 production as compared to the wild-type allele (Turner *et al*., 1997; Eksdale *et al*., 1998; Lazarus *et al*., 2002). The IL-10 expression analysis was performed on 43 of the 48 HIV-positive individuals included in this study, however 228 women were analysed for genotype assessment. The IL-10 expression data did not show any significant difference between the groups, this was likely to be the result of limited sample size used in this part of the analysis. Although sexually transmitted infections (STIs) would have an impact on IL-10 expression, we did not address this within this study. We assumed that the impact of the STIs across the cohort would be equally distributed.

Viral load analysis was compared to the IL-10 genotype to determine if there was an association between IL-10 genotype and progression. Brooks *et al.* (2006) demonstrated in the LCMV/mouse model, that IL-10 results in the inactivation of T cell responses. The

high IL-10 levels resulted in loss of T cell activity and viral persistence, however the genetic removal or IL-10 receptor blocking resulted in viral clearance. In this study the viral load analysis seems to favour what was shown by Brooks *et al.* (2006) at earlier time points, but at later time points these results seem to favour what was demonstrated by Shin *et al.* (2000). Also the $CD4^+$ T cell count is inversely proportional to the viral load (Arnaout *et al*., 1999). At some time points the $CD4^+$ levels change with the inverse change in viral load.

These results suggest that polymorphisms within the IL-10 gene are important for identifying individuals which may be at risk for HIV susceptibility and these genotypes may influence HIV progression. IL-10 genotype is just one of several factors that may be associated with HIV, and therefore other factors also contribute to susceptibility and disease progression in addition to IL-10. Further investigation, such as expression analysis, T cell activation and expanded SNP analysis of the entire IL-10 gene, is required to determine whether other IL-10 promoter polymorphisms play a role in HIV susceptibility and HIV pathogenesis and to determine the mechanisms underlying these effects.

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APPENDIX 1
Genotypic Data of Study Participants

PID	-1082 GENOYPE	-592 GENOTYPE
AI0001	Het	Het
AI0006	Hom-WT	Hom-WT
AI0007	Het	Hom-WT
AI0008	Hom-WT	Hom-P
AI0010	Het	Het
AI0011	Hom-WT	Het
AI0012	Het	Hom-WT
AI0015	Het	Het
AI0016	Hom-WT	Hom-WT
AI0017	Hom-WT	Hom-P
AI0020	Het	Hom-WT
AI0021	Hom-WT	Hom-P
AI0022	Hom-WT	Het
AI0023	Het	Hom-WT
AI0027	Hom-WT	Hom-P
AI0028	Hom-WT	Hom-P
AI0032	Hom-WT	Hom-WT
AI0033	Hom-WT	Hom-P
AI0034	Het	Hom-WT
AI0035	Hom-WT	Hom-WT
AI036	Het	Het
AI0037	Hom-WT	Het
AI0038	Hom-WT	Hom-P
AI0039	Het	Het
AI0040	Hom-WT	Het
AI0041	Hom-P	Hom-WT
AI0042	Het	Hom-WT
AI0044	Het	Hom-WT
AI0045	Hom-WT	Hom-WT
AI0047	Het	Hom-WT
AI0048	Het	Hom-WT
AI0052	Het	Het
AI0053	Hom-WT	Hom-P
AI0054	Het	Hom-WT
AI0055	Hom-WT	Het
AI0056	Het	Het
AI0057	Het	Hom-WT
AI0058	Hom-P	Het
AI0059	Hom-WT	Hom-WT
AI0060	Hom-WT	Hom-P
AI0061	Hom-WT	Het
AI0065	Hom-WT	Hom-WT
AI0066	Hom-P	Hom-WT
AI0067	Hom-WT	Het
AI0069	Hom-P	Hom-WT

PID	-1082 GENOYPE	-592 GENOTYPE
AI0070	Hom-WT	Het
AI0072	Hom-WT	Hom-P
AI0074	Hom-WT	Hom-WT
AI0076	Hom-WT	Het
AI0077	Hom-WT	Het
AI0078	Het	Het
AI0080	Hom-P	Hom-WT
AI0081	Hom-WT	Hom-WT
AI0083	Hom-WT	Hom-P
AI0084	Hom-P	Hom-WT
AI0085	Hom-WT	Het
AI0086	Hom-WT	Het
AI0087	Het	Hom-WT
AI0088	Hom-WT	Hom-P
AI0089	Het	Het
AI0091	Hom-WT	Hom-WT
AI0092	Hom-WT	Het
AI0093	Hom-WT	Het
AI0094	Hom-WT	Het
AI0095	Het	Het
AI0097	Het	Het
AI0098	Hom-WT	Het
AI0099	Het	Het
AI0100	Het	Het
AI0101	Hom-WT	Het
AI0102	Hom-P	Hom-WT
AI0103	Hom-WT	Het
AI0106	Hom-P	Het
AI0107	Hom-WT	Hom-P
AI0108	Hom-WT	Het
AI0109	Hom-WT	Hom-WT
AI0110	Het	Hom-WT
AI0113	Hom-P	Het
AI0114	Hom-P	Hom-WT
AI0115	Hom-P	Hom-WT
AI0116	Het	Hom-WT
AI0117	Hom-P	Hom-WT
AI0119	Hom-WT	Hom-WT
AI0120	Het	Het
AI0121	Hom-WT	Het
AI0122	Hom-WT	Het
AI0123	Hom-WT	Het
AI0124	Het	Hom-WT
AI0125	Het	Hom-WT
AI0126	Hom-WT	Het
AI0128	Hom-WT	Hom-WT
AI0129	Het	Hom-WT
AI0130	Het	Hom-WT
AI0131	Het	Hom-WT

PID	-1082 GENOYPE	-592 GENOTYPE
AI0132	Hom-WT	Het
AI0133	Hom-WT	Hom-WT
AI0134	Hom-WT	Het
AI0135	Het	Het
AI0136	Hom-WT	Het
AI0137	Hom-WT	Hom-P
AI0138	Hom-WT	Het
AI0139	Hom-WT	Het
AI0140	Hom-WT	Het
AI0141	Het	Het
AI0142	Hom-WT	Het
AI0143	Het	Hom-WT
AI0144	Het	Hom-WT
AI0145	Het	Het
AI0146	Het	Hom-WT
AI0147	Hom-WT	Hom-WT
AI0148	Het	Hom-WT
AI0149	Hom-WT	Hom-WT
AI0150	Het	Het
AI0151	Hom-WT	Hom-WT
AI0152	Het	Hom-WT
AI0153	Het	Hom-WT
AI0154	Het	Het
AI0155	Hom-WT	Het
AI0157	Hom-WT	Hom-P
AI0158	Het	Hom-WT
AI0159	Hom-WT	Hom-P
AI0161	Hom-P	Hom-WT
AI0162	Het	Hom-WT
AI0163	Hom-WT	Hom-WT
AI0164	Het	Het
AI0165	Hom-P	Hom-WT
AI0166	Hom-WT	Het
AI0167	Hom-WT	Hom-WT
AI0168	Hom-WT	Hom-P
AI0169	Het	Het
AI0170	Hom-WT	Het
AI0171	Hom-WT	Het
AI0172	Hom-WT	Hom-WT
AI0173	Hom-P	Het
AI0174	Hom-WT	Hom-P
AI0175	Hom-WT	Het
AI0176	Hom-P	Hom-WT
AI0177	Het	Het
AI0178	Hom-P	Hom-WT
AI0179	Hom-P	Hom-P
AI0180	Het	Het
AI0181	Het	Het
AI0182	Het	Hom-WT

PID	-1082 GENOYPE	-592 GENOTYPE
AI0183	Hom-WT	Het
AI0184	Het	Hom-WT
AI0185	Hom-WT	Hom-WT
AI0186	Hom-WT	Het
AI0187	Hom-WT	Het
AI0188	Hom-WT	Het
AI0189	Hom-WT	Hom-WT
AI0190	Het	Het
AI0191	Hom-WT	Hom-WT
AI0192	Het	Hom-WT
AI0193	Hom-WT	Hom-WT
AI0194	Het	Het
AI0195	Het	Het
AI0196	Hom-P	Het
AI0197	Hom-WT	Het
AI0198	Hom-WT	Hom-WT
AI0199	Hom-WT	Het
AI0200	Hom-WT	Hom-WT
AI0201	Hom-WT	Hom-P
AI0202	Hom-WT	Het
AI0203	Het	Hom-WT
AI0205	Het	Het
AI0206	Hom-WT	Hom-WT
AI0207	Het	Hom-WT
AI0208	Het	Het
AI0209	Het	Hom-WT
AI0210	Hom-WT	Hom-P
AI0211	Het	Het
AI0212	Het	Hom-WT
AI0214	Het	Hom-WT
AI0215	Het	Hom-WT
AI0216	Het	Hom-WT
AI0217	Het	Hom-WT
AI0219	Het	Hom-WT
AI0220	Hom-WT	Hom-WT
AI0221	Hom-WT	Hom-P
AI0222	Het	Hom-WT
AI0223	Het	Hom-WT
AI0224	Het	Hom-WT
AI0225	Hom-WT	Hom-P
AI0227	Hom-WT	Hom-WT
AI0228	Het	Hom-P
AI0229	Het	Hom-P
AI0230	Hom-WT	Hom-P
AI0231	Hom-WT	Het
AI0232	Het	Hom-WT
AI0233	Het	Hom-WT
AI0234	Het	Hom-WT
AI0235	Hom-WT	Het

PID	-1082 GENOYPE	-592 GENOTYPE
AI0236	Hom-P	Hom-WT
AI0237	Hom-WT	Hom-WT
AI0238	Het	Het
AI0239	Hom-WT	Het
AI0240	Het	Hom-P
AI0241	Het	Hom-WT
AI0242	Hom-P	Hom-WT
AI0243	Hom-WT	Het
AI0244	Het	Het
AI0245	Het	Hom-WT
AI0246	Hom-P	Hom-WT
AI0247	Hom-P	Hom-WT
AI0248	Hom-WT	Hom-P
AI0249	Het	Hom-WT
AI0250	Het	Hom-WT
AI0251	Hom-WT	Hom-WT
AI0252	Hom-WT	Het
AI0253	Hom-P	Hom-WT
AI0254	Hom-P	Hom-WT
AI0255	Het	Hom-WT
AI0256	Hom-WT	Hom-WT
AI0257	Hom-P	Het
AI0258	Het	Het
AI0260	Hom-WT	Hom-P
AI0261	Hom-P	Hom-WT
AI0262	Het	Het
AI0264	Het	Het
AI0265	Hom-WT	Het
AI0266	Hom-WT	Hom-WT
AI0267	Het	Het
AI0268	Hom-WT	Hom-P
AI0269	Hom-WT	Hom-WT
AI0270	Het	Het
AI0271	Het	Hom-WT
AI0273	Het	Hom-WT
AI0274	Hom-WT	Hom-P