Analysis of CCR5 diversity in the South African population

By

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ABSTRACT

Infection with the human immunodeficiency virus (HIV) constitutes a global pandemic, and South Africa forms part of the region known to house over two-thirds of HIV infected individuals worldwide. In the early stages of infection, the C-C chemokine receptor type five (CCR5) is the major HIV-1 co-receptor. The importance of this receptor in HIV infection and disease progression was recognised with the discovery of the CCR5 delta 32 (Δ32) allele. Individuals homozygous for this mutation lack functional CCR5 receptors. Consequently, they are almost completely resistant to HIV infection, while the absence of CCR5 has minimal effects on health. Heterozygous individuals display decreased cell surface CCR5 which slows disease progression. Phenotypic expression of CCR5 is heterogeneous and its relation to genetic mutations in the CCR5 gene is not currently known for the South African population. This together with the effect of CCR5 expression on HIV infection provided the rationale for investigating both the phenotypic and genotypic distribution of CCR5. The aim of this study was therefore 1) to investigate CCR5 phenotypic expression on cluster differentiation four (CD4) T-lymphocytes in a group of South African individuals and 2) to analyse the genetic variation in a South African cohort. Flow cytometric methods were used to measure the phenotypic distribution of CCR5 in 245 individuals by assessing both the percentage of $CD4^{+}CCR5^{+}$ T-cells and CCR5 density. Sixty five individuals, mostly found within the lower CCR5 receptor density range were selected for DNA sequencing. The study found considerable variability in CCR5 expression with South African individuals expressing relatively high $CD4^{+}CCR5^{+}$ T-cell percentages. Ethnicity was established as a significant variable affecting CCR5 expression with Black African individuals displaying higher ($p < 0.05$) $CD4^{+}CCR5^{+}$ T-cell percentages and densities than Caucasians. Genotypic data revealed 70 single nucleotide polymorphisms (SNPs), four insertions and the Δ32 deletion. Results showed that Black African individuals have greater genetic diversity with 39 mutations exclusive to this group. The Δ32 mutation was not detected in the Black African group but was identified in the Caucasian group at a frequency of 18.6 %. Twelve novel mutations were identified in this study with two in the open reading frame (ORF). It is evident from the data that the variability in CCR5 phenotypic expression is difficult to correlate with specific mutations in the gene. This thesis provides information on CCR5 distribution and diversity in the South African population which will be of value to patients, clinicians and health policy officials.

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In the Name of God the Most Gracious the Most Merciful

To my supervisor and co-supervisor, your assistance and guidance throughout my master's programme has ensured that I have developed in both my professional and personal capacity. I sincerely appreciate all the commitment and time you have invested in me.

To my husband, thank you for the sacrifices that you made for me over the past two years. It is through your unwavering support and motivation that I have achieved my goal.

To all my family and friends, your constant motivation has taken me to the end.

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List of Abbreviations and Symbols

%	Percentage
®	Registered sign
°C	Degrees Celsius
µl	Microliter
µM	Micromolar
Δ32	Delta 32
A	Adenine
AIDS	Acquired immune deficiency syndrome
ARV	Anti retroviral therapy/drugs
Bp	Base pairs
C	Cytosine
CCR5	C-C chemokine receptor type five
CD	Cluster differentiation
cDNA	Complementary DNA
CEBPβ	CCAAT/enhancer-binding protein beta
C-terminal	Cytoplasmic/ Carboxyl terminal
CXCR4	C-X-C chemokine receptor type four
DNA	Deoxyribonucleic acid
dNTPs	Deoxribonucleotides
EDTA	Ethylenediaminetetraacetic acid
Env	HIV envelope
ECL	Extracellular loop
FITC	Fluorescein isothiocyanate
FS	Forward scatter
G	Guanine
Gag	Group specific antigen (HIV gene)
gDNA	Genomic DNA
Gp41	Glycoprotein 41 (HIV receptor)
Gp120	Glycoprotein 120 (HIV receptor)
Gp160	Glycoprotein 160
GPCR	G protein coupled receptor
GRKs	G protein coupled receptor kinases

HHA – G	Human haplotype A – G
HIV	Human immunodeficiency virus
HLA	Histocompatibility leukocyte antigen
ICL	Intracellular loop
IL	Interleukin
Indels	Inserts and deletions
INF-γ	Interferon gamma
IRR	Incidence rate ratio
Kb	Kilo bases
Lin	Linear
Log	Logarithm
LPS	Lipopolysaccharide
MCP-2	Monocyte chemotactic protein two
MFI	Mean fluorescence intensity
MIP1-α	Macrophage inflammatory protein one alpha
MIP1-β	Macrophage inflammatory protein one beta
ml	Millilitre
mRNA	Messenger RNA
MTb	*Mycobacterium Tuberculosis*
N-terminal	Amino terminal
Nef	Negative factor (HIV gene)
NFW	Nuclease free water
Oct-1	Octamer one
Oct-2	Octamer two
ORF	Open reading frame
PCR	Polymerase chain reaction
Pd	Downstream promoter
PE	Phycoerythrin
Pol	Polymerase (HIV gene)
PSC	Primary Sclerosing Cholangitis
Pu	Upstream promoter
RANTES	Regulated upon activation, normal T-cell expressed and secreted
RBC	Red blood cell
Rev	Regulator of virion (HIV gene)

RNA	Ribonucleic acid
SIV	Simian immunodeficiency virus
SNPs	Single nucleotide polymorphisms
T	Thymine
Taq	*Thermus Aquaticus*
Tat	Trans-activator of transcription
TB	Tuberculosis
TBE	Tris borate EDTA
TDN	*Trans* dominant negative
Tm	Melting temperature
TMD	Transmembrane domain
UNAIDS	Joint United Nations Programme on HIV/AIDS
UV	Ultraviolet
Vif	Viral infectivity factor (HIV gene)
Vpr	Viral protein r (HIV gene)
Vpu	Viral protein u (HIV gene)
WNV	West Nile virus
Wt	Wild type

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Introduction

South Africa is faced with a major challenge in the prevention and treatment of human immunodeficiency virus (HIV) and the acquired Immunodeficiency Syndrome (AIDS). According to the mid-year population estimates for 2011 approximately 10.6 % of the South African population is infected with HIV. This equates to a total number of 5.38 million individuals (Statistics South Africa, 2011). These statistics illustrate both the magnitude and severity of HIV infection in South Africa.

The human immunodeficiency virus gains entry into host cells via its extracellular glycoprotein 120 receptor (gp120), which binds to host cell receptor cluster differentiation four ($CD4^+$) on T-lymphocytes (McDougal *et al.*, 1986). In order for the virus to enter the host cell, it requires membrane fusion. This occurs via the virus' glycoprotein 41 receptor (gp41), which binds to a host co-receptor either C-C chemokine receptor type five (CCR5) or C-X-C chemokine receptor type four (CXCR4). The CCR5 co-receptor is crucial for HIV infection as demonstrated by the discovery of the CCR5 delta 32 (Δ32) variant (Samson *et al.*, 1996a). The Δ32 mutation is a 32 base pair (bp) deletion in the open reading frame (ORF) of the CCR5 gene, which confers resistance to HIV in homozygotes and retards disease progression in heterozygotes. The mutated protein consists of 215 amino acids in comparison to the wild-type protein, which consists of 352 amino acids (Liu *et al.*, 1996). This mutated protein is unable to insert in to the host cell membrane (Samson *et al.*, 1996a). This renders the cell resistant to HIV, as the virus cannot dock onto the host cell surface and is thereby denied entry into the cell.

The virus can still use the alternative co-receptor, CXCR4, but in individuals homozygous for Δ32, CXCR4 does not seem to play a role in HIV infection. This is possibly attributed to the *trans* dominant negative (TDN) effect that the Δ32 protein has on CXCR4 (Agrawal *et al*, 2004). A study conducted in 2009 by Hutter and co-workers, showed that performing a stem cell transplant on an HIV positive individual using a histocompatibility leukocyte antigen-matched (HLA) HIV negative donor homozygous for the Δ32 mutation, rendered the HIV positive individual free of viral detection 20 months post transplantation. An update in 2010 by the group confirmed that the HIV infected patient remains free of viral replication and has reconstituted his immune system with that of the Δ32 homozygous donor (Allers *et al.*, 2010). The group suggests that the individual is 'cured' of HIV.

The Δ32 mutation is most common in individuals of European descent, but is absent or rare in the Black African population (Martinson *et al.*, 1997). Other mutations, however, have been identified in African populations, such as the C101X mutation (Quillent *et al.*, 1998). Several novel mutations have also been identified in small cohorts in South Africa (Peterson *et al.*, 2001). These include the

D2V mutation shown to have a decreased interaction with HIV and the R225X mutation, which like the Δ32 mutation results in the absence of receptor expression and no interaction with HIV (Folefoc *et al.*, 2010). A more detailed description of CCR5 mutations will be discussed in Chapter 2 of this thesis.

Few studies have been conducted to determine the frequency of individuals in South Africa with spontaneous CCR5-null phenotypes. Williamson and co-workers (2000) investigated the allelic frequencies of 1 247 black individuals and 147 individuals of Caucasian descent but concentrated on only two CCR5 mutations that protect against HIV infection, Δ32 and C101X. Peterson and co-workers (2001) investigated CCR5 mutations in the coding region of 103 HIV positive and 146 HIV negative individuals mostly of Black African origin, which play a role in HIV vulnerability. In 2010, Picton and co-workers analysed the entire 9.2 (kilo base) kb region of CCR5 in 35 Black African- and Caucasian South Africans for single nucleotide polymorphisms (SNP's), indels (inserts and deletions) and haplotypes. In the small cohort of individuals studied, they found 68 SNP's, five indels and seven haplotypes. Research into the effects of these mutants on CCR5 expression, HIV infection and disease progression has not been reported.

The CCR5 protein is a G-protein coupled receptor (GPCR), which is expressed on various cell populations such as macrophages and T-cells of the immune system, epithelium and endothelium and certain cells of the central nervous system (Rottman *et al.*, 1997). The chemokine co-receptor plays a role in the inflammatory immune response. However, studies show that individuals without CCR5 lead normal healthy lives (Liu *et al.*, 1996). Conversely, some studies indicate that CCR5 is required for immune protection in cases of West Nile virus (WNV) infection (Glass *et al.*, 2006), and may increase disease susceptibility and outcome of primary sclerosing cholangitis (PSC) in individuals lacking CCR5 (Eri *et al.*, 2004).

In 1996, it was found that CCR5 was necessary as a co-receptor for HIV entry (Dragic *et al.*, 1996). The impact of CCR5 expression in HIV susceptible individuals was only recognised later. In 1994, Kabat and co-workers demonstrated that CD4 receptor density on the cell surface affects HIV infection; however, the levels of CD4 receptor protein expression were relatively constant on peripheral blood cells (Poncelet *et al.*, 1991). On the contrary, the expression of CCR5 shows a great degree of heterogeneity in both infected and non-infected individuals (Ostrowski *et al.*, 1998).

In HIV infected individuals, the progression of disease is inversely correlated with $CD4^+$ count and directly correlated with viral load. The development of disease in these individuals shows considerable inter-individual differences. The level of CCR5 was illustrated to associate with viral

load (Reynes *et al.* 2000), with higher CCR5 expression demonstrating a higher viral load. In 2001, Reynes and co-workers extended these findings by showing that CCR5 density on the cell surface established both the rate of HIV production and $CD4^+$ cell reduction *in vivo*. The phenotypic expression of CCR5 in a cohort of South African individuals will be discussed in Chapter 4 of this thesis.

The expression of CCR5 is affected by environmental and host factors. A study conducted in 2000 showed that individuals living in Africa had higher levels of immune activation, which was environmentally driven, when compared to African individuals living in other countries (Clerici *et al.* 2000). This immune activation influenced CCR5 expression, with these individuals possessing higher levels of CCR5. This is due to the elevated inflammatory chemokines known to increase CCR5 gene expression (Patterson *et al.*, 1999). Drugs such as statins are also known to affect CCR5 with a concomitant decrease in expression (Nabatov *et al.*, 2007). Statins reduce cholesterol, a component required in the internalisation of CCR5 receptors, discussed in Chapter 2 of this thesis. Thus, host and environmental factors involved in the internalisation and recycling of CCR5 will affect regulatory mechanisms that control CCR5 expression.

The structure of CCR5 also plays an important role in receptor surface density. Mutations in the CCR5 gene, which affect the structural requirements of the protein or at the level of mRNA transcription, will influence cell surface CCR5. In 1998, McDermott and co-workers discovered the adenine/guanine (A/G) polymorphism at position 59029, which slowed disease progression. Individuals homozygous for the wild type CCR5 promoter had increased numbers of $CD4^+$ cells expressing CCR5 (Shieh *et al.*, 2000). Other promoter mutations have been found, and these promoter alleles have been postulated to affect the stringency of specific DNA binding transcription factors (Bream *et al.*, 1999). Mutations in the regulatory regions which are known to affect CCR5 expression and HIV progression are discussed in Chapter 5.

The protein structure of CCR5 consists of seven transmembrane domains (TMD), an extracellular amino terminal (N-terminal), and an intracellular carboxyl or cytoplasmic terminal (C-terminal) (Horuk, 1994), and includes post-translational modifications. The deletion at codon 299 in the ORF of the CCR5 gene reduces the level of CCR5 expression (Shioda *et al.*, 2001). The deletion comprises a termination of translation with the removal of the cytoplasmic tail of CCR5. This region is palmitoylated and this post translational modification is required for efficient trafficking and integration of CCR5 at the cell surface (Blanpain *et al.*, 2001). This illustrates the pivotal role that mutations play in CCR5 expression and hence the ability of HIV to infect cells.

In South Africa, studies have been conducted to describe previously identified mutations (Williamson *et al.*, 2000) as well as novel mutations in the population (Peterson *et al.*, 2001). While studies have been performed to evaluate the *in vitro* effect of novel South African mutations on HIV (Hayes *et al.*, 2002, Folefoc *et al.*, 2010), there are currently no studies describing CCR5 expression levels in the population groups and how these levels are influenced by genetic variability in CCR5. The above studies have also focussed on discovering mutations in exon 3 of the CCR5 gene that houses the ORF. In addition, these studies concentrated on establishing allelic frequencies of Δ32 in population groups in South Africa. Although the paper published by Picton and co-workers (2010) assessed the entire 9.2kb CCR5 gene, the mutations found were not related to phenotypic expression of the CCR5 protein or the influence this has on HIV.

The research problem indicated above, together with the Hutter report (Hutter *et al.*, 2009), provides the rationale for investigating phenotypic expression of CCR5 and analysing genotype in the South African population.

The aims of this study include:

1) To investigate CCR5 phenotypic expression on $CD4^+$ T-lymphocytes in a group of South African individuals

The objectives include:

- Determine the levels of $CD4^+CCR5^+$ T-cell percentage and CCR5 density in the South African population
- Determine if there is a correlation between the $CD4^+CCR5^+$ T-cell percentage and CCR5 density
- Determine if $CD4^+CCR5^+$ T-cell percentage is dependent on age, gender or ethnicity
- Determine if CCR5 density is dependent on age, gender or ethnicity
- Determine the frequency of phenotypically null individuals in the South African population

The following hypotheses were formulated:

- Levels of $CD4^+CCR5^+$ T-cell percentage and CCR5 density are not different to expected values
- There is no correlation between $CD4^+CCR5^+$ T-cell percentage and CCR5 density
- $CD4^+CCR5^+$ T-cell percentage is not affected by age, gender and ethnicity
- CCR5 density is not affected by age, gender or ethnicity
- There are no null individuals in the SA population

2) To analyse the genetic variation in a South African cohort

The objectives include:

- Report on mutations found in the South African population
- Detect novel mutations
- Correlate mutations found with CCR5 cell surface expression found in this study
- Determine the frequency of Δ32 in the South African cohort
- Determine if the phenotypically null individual detected by flow cytometry has a genetic cause of the phenotype

The following hypotheses were formulated:

- Mutations cannot be correlated with the flow data
- The phenotypically null individual has a genetic cause

The study was carried out as follows:

(1) 245 samples of peripheral blood were collected

(2) The cells were screened for the concomitant expression of $CD4^+$ and CCR5 receptors using flow cytometry

(3) The DNA was extracted from the cells

(4) A 9028 bp region of the CCR5 gene was amplified using polymerase chain reaction (PCR)

(5) The CCR5 gene was sequenced in 65 individuals and cloning techniques were used for unreadable regions

From a global perspective, Sub-Saharan Africa is the region which has the highest prevalence of HIV in the world (www.unaids.org). The epidemic is consuming our workforce and hindering the development of our youth. Currently, therapy includes the use of anti retroviral drugs (ARV's) which prolong disease progression and decrease viral load but can lead to serious side effects. Many South Africans have no access to the drugs and therefore do not adhere to a strict regimen when taking these ARV's. This has led to HIV resistant subtypes. Vaccines have many challenges to overcome before a suitable lifelong suppressing vaccine will be produced.

This study aims to further investigate the incidence of CCR5 mutations and establish the phenotypic expression of CCR5, ultimately hoping to provide useful information on HIV infection and disease progression in South Africa.

This thesis concludes with Chapter 6 providing a discussion on the study's findings, limitations of the study and future applications.

Methodology

The Faculty of Health Sciences Ethics Committee of the University of Pretoria approved this study as Protocol 89/2010 and 146/2011 (Appendix A).

3.1 ASSESSMENT OF CCR5 EXPRESSION ON $CD4^+$ CELLS

3.1.1 Sample size and study population

The cohort consisted of 245 South African individuals, of which 95 subjects were Black female, 29 Black male, 73 Caucasian females and 37 Caucasian males. Eleven individuals formed part of the Coloured and Indian population groups. Participants were initially recruited at the University of Pretoria Clinical Research Unit, Prinshof Campus, with the assistance of Mrs. K Depken, Dr. M Zarrabi and other trained phlebotomists. Participants were also recruited at the National Health Laboratory Service core lab facility, located at the Steve Biko Academic Hospital with the assistance of the resident phlebotomist.

The recruitment strategy is illustrated below:

- Each individual was informed of the objectives of the study and the requirements verbally
- Subjects were then asked whether they consent to participating in the study
- Upon verbal acknowledgement of participation, participants were then required to read the patient information leaflet and informed consent documents (Appendix B) and to ask questions.
- The document required the completion of a few unidentifiable details (individual's age, ethnicity, gender and known medication or disease) (Appendix B). The questions pertaining to medication and disease were not strict requirements for the study but were included for the purpose of interest. Ethical clearance was not obtained to test participants for infectious agents (HIV, TB).
- Only participants above the age of 18, with the ability to understand the document in its entirety and consequences thereof, were selected for inclusion in the study. The participants were encouraged to keep the patient information leaflet.
- Blood was drawn by authorised medical personnel (trained phlebotomists) only.

Peripheral blood (5 ml) was drawn by venipucture and collected in sterile evacuated blood tubes (BD Vacutainer®) containing the anti-coagulant K_2EDTA (7.2 mg). The samples were transported to the laboratory, at the Department of Immunology, Prinshof Campus for further analysis.

3.1.2 Sample Handling

Once the samples were received, they were labelled T001 - T007 (for the first seven samples used in generating preliminary data) and F001 - F238 (for the subsequent experimental samples). The first seven samples (T001 - T007) were collected to set up the protocols and optimise the methodology employed in the study, the results of which were also included in the study

Samples were analysed by flow cytometry within six hours of collection as this is optimal for the lysing reagent used and to ensure accuracy of CD4 and CCR5 enumeration. According to Shalekoff and Tiemessen (2001), the level of HIV-1 co-receptors, CCR5 and CXCR4, are affected by the duration of storage of EDTA collected blood. The authors suggest using a six hour cut-off time for flow cytometric analysis of peripheral blood mononuclear cells. Samples were kept between 18-25 °C until processing.

Once processed for phenotypic expression, aliquots of blood were made and stored at -20 °C to inhibit DNase enzymes until further processing.

3.1.3 Sample preparation

From a review of the literature it is evident that HIV uses host cell receptors to enter into target cells. The virus's gp120 receptor binds to host CD4 on the surface of immune cells. This is followed by gp41 HIV receptor binding to CCR5 or CXCR4 co-receptors on the host surface. Considering the importance of CCR5 in HIV infection, the level of expression of this protein was assessed on $CD4^+$ T-lymphocytes.

The IOTest CD4-FITC (fluorescein isothiocyanate) (Beckman Coulter) and CCR5-PE (phycoerythrin) (BioLegend) monoclonal antibodies were used. Each antibody (10 µl) was placed carefully at the bottom of a 5 ml sterile tube and mixed by repeat pipetting. The antibodies were kept away from light to prevent deterioration of the conjugated fluorochromes. Whole blood (50 µl) was then added to the antibody mixture and mixed by repeat pipetting. The tube was later vortexed for 5 sec to ensure thorough distribution of the antibodies in the blood sample. The samples were then incubated for 20 min at room temperature in the dark.

To prepare a 'fix and lyse' solution, the VersaLyse™ lysing solution (Beckman Coulter) (500 µl) was added to 12.5 µl of the IOTest® 3 fixative solution (Beckman Coulter) and vortexed briefly. This was always freshly prepared before being added to the sample. The purpose of the VersaLyse solution is to lyse red blood cells (RBC) from whole blood, as these cells interfere with flow cytometric readings. According to the product specifications, the solution's major active component is an amine. The

latter reacts with RBC carbonic anhydrase and forms a product which lyses the cells. The product exclusively targets RBCs, thereby leaving cells required for experimentation (mononuclear cells) intact.

After incubation, 500 µl 'fix and lyse' solution was added to the antibody labelled blood samples. The tubes were vortexed immediately for 1 sec. The samples were then incubated for 10 min at room temperature in the dark. They were then ready to analyse on the flow cytometer.

3.1.4 Acquisition and analysis protocol setup

Flow cytometry is a technique used to analyse particles or single cells based on multiple parameters including size, density and fluorescence, determined using a combination of fluidics, optics and electronics (Bakke, 2001). Flow cytometry was performed on a Beckman Coulter Cytomics FC500 Flow cytometer, using CXP system software for analysis.

The parameters selected for the acquisition of data included:

- Forward scatter (FS) [linear (lin) and logarithm (log)]
- Side scatter (SS) (lin and log)
- FL1 (fluorescence channel for FITC)
- FL2 (fluorescence channel for PE)

Unstained whole blood was used to define the instrument settings (voltages and gain) and colour compensation values, to ensure the settings were optimised for the study. Regions and gating strategies were defined to generate required data and statistics. A total of six histograms and dot plots were created during the protocol setup (Figure 8).

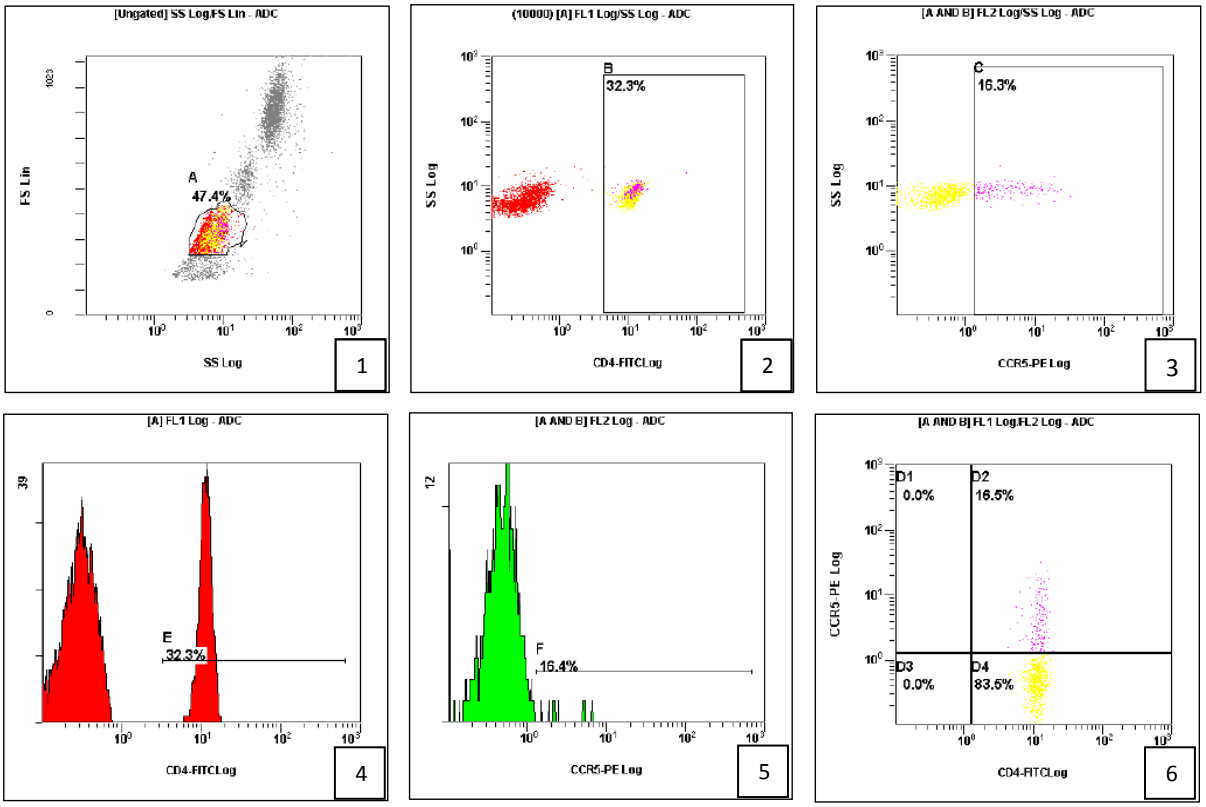


Figure 8: Histograms and Dot plots (1-6) for the flow cytometry protocol used for analysis of CCR5 expression on $CD4^+$ T-lymphocytes

Data was acquired as follows:

- Lymphocytes were identified in dot plot (1), which depicts FS vs. SS by measuring the size and granularity of cells in the peripheral blood. Lymphocytes are the smallest and least complex intracellularly and can be found at the bottom left side of the dot plot. These cells are identified by region A on the dot plot.
- $CD4^+$ T-lymphocytes were identified on dot plot (2), which depicts SS vs. CD4-FITC. The dot plot separates these cells from non-expressing $CD4^+$ cells. The dot plot is gated on region A, so that only lymphocytes appear in dot plot (2) with a region (B) designated for CD4 expressing cells.
- $CD4^+$ T-lymphocytes that express CCR5 on the cell surface were identified on dot plot (3), which depicts SS vs. CCR5-PE. The dot plot is gated on both regions A and B, indicating that only lymphocytes with CD4 expression are included in this dot plot. Region C is designated to depict only the $CD4^+CCR5^+$ T-cells.
- Histograms (4) and (5) are single parameter plots, count vs. CD4-FITC or count vs. CCR5-PE. The former histogram is gated on A indicating all lymphocytes present with a region E encompassing only $CD4^+$ lymphocytes. The latter histogram, however, is gated on A and B

indicating only $CD4^+$ lymphocytes with region F encompassing only $CD4^+CCR5^+$ lymphocytes. The count axis on these two histograms indicates the number of events.

- Dot plot (6) displays both CD4-FITC vs. CCR5-PE on one plot. The dot plot is gated on both A and B, thus only depicting $CD4^+$ lymphocytes. A quadrant was applied to this dot plot with region D2 showing lymphocytes that co-express CD4 and CCR5, while region D4 showed lymphocytes that express CD4 without CCR5.

Data was analysed after acquisition by investigating both percentage positive data (number of cells expressing antigen of interest, that is, $CD4^+$ cells co-expressing CCR5) and the mean fluorescence intensity (MFI) of a particular antigen (a qualitative estimation of the density of CCR5 receptors per $CD4^+$ cell).

A standardised flow check (Beckman Coulter) protocol used in the Department of Immunology was run once a day before sample analysis to ensure the argon laser was properly aligned and the instrument was efficient for use.

3.1.5 Statistical analysis

Statistical analysis was performed in consultation with Prof. Piet Becker (biostatistician) from the Biostatistics unit at the Medical Research Council. The STATA version 11, EViews 5 and MedCalc® version 11.6.1 software packages were used for statistical analysis of data. A p-value of < 0.05 (5%) was used to determine significance.

Pearson's Correlation (r-value) was used to analyse the association (strength or linear relationship) of the two variables, $CD4^+CCR5^+$ T-cell percentage (y) and CCR5 density (MFI) (x).

- Positive r: as x increases, y increases
- Negative r: as x increases, y decreases
- r=0: indicates no relationship between x and y

Values closer to 0 indicate weak relationships

A regression is the study of the dependence of one variable (dependent variable) on one or more other variables (explanatory variable). The dependent variable in this study was $CD4^+CCR5^+$ T-cell percentage and CCR5 density (MFI). The explanatory variables were age, gender and ethnicity. A Poisson regression test (incidence rate ratio/IRR) was used to analyse if age, gender or ethnicity could explain the variation in $CD4^+CCR5^+$ T-lymphocytes percentage and the CCR5 MFI.

A Tukey test was used to check for multiple outlier values in the data sets.

3.2 GENETIC ANALYSIS

3.2.1 Sample selection

Sixty five individuals with variable CCR5 levels (either CD4CCR5% or CCR5 MFI) were selected for genetic analysis. The sample population consisted of 35 Caucasians, 29 Black Africans and one Indian individual.

3.2.2 DNA extraction

Frozen blood was thawed on ice (4 °C) and mixed thoroughly before use. The Maxwell® 16 Blood DNA purification kit (Promega) was used, according to manufacturer's instructions, to extract DNA. According to the product specifications, the kit uses paramagnetic particles in a cartridge based system to extract and elute genomic DNA (gDNA) (Figure 9).

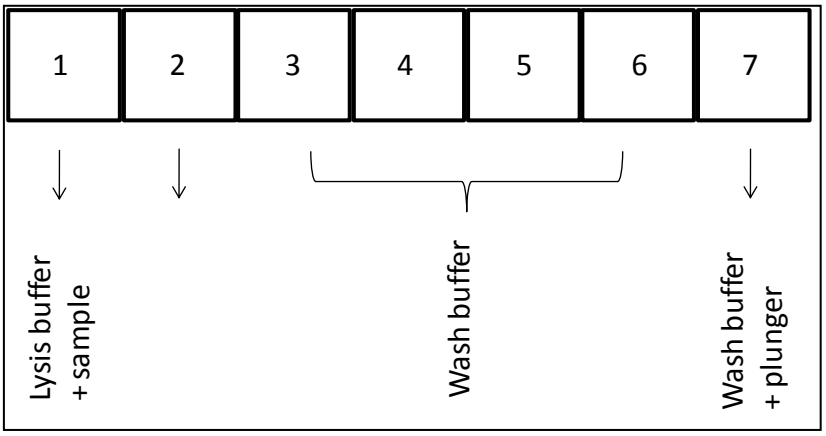


Figure 9: Maxwell cartridge containing sample and reagents (image created by Fatima Barmania)

Whole blood (400 µl) was added to well (1) of the cartridge (Figure 9) which contains lysis buffer. The cartridge was placed in the designated holder and then placed in the Maxwell® 16 MDx Instrument. An elution buffer (500 µl) was positioned in the elution tube slots of the machine. The DNA protocol for blood and cells was then followed.

On completion of extraction, the elution tubes containing the gDNA were placed in the Magnetic Elution tube rack which draws the paramagnetic particles to the sides of the elution tube. The gDNA was later aspirated into a sterile 1.5 ml Eppendorf tube and labelled accordingly.

3.2.3 DNA quantification and storage

Genomic DNA in the elution buffer was quantified using the Nanodrop® ND-1000 version 3.3 spectrophotometer. The concentration of DNA was calculated by the absorbance of ultraviolet (UV) light at 260 nm. Proteins absorb UV light at 280 nm. The ratio of absorbance between DNA and

protein (260/280) was used to assess the purity of the DNA. An aliquot (1.5 µl) of the DNA sample was used to calculate the concentration.

Once quantified, aliquots of the stock solution (20 ul) were made and stored at -20 °C to prevent contamination of the DNA.

3.2.4 Polymerase chain reaction (PCR)

Polymerase chain reaction is a technique used to amplify minute quantities of DNA which can then be used in various applications (Saiki *et al.*, 1985; Mullis and Faloona, 1987). The reaction makes use of temperature and a thermostable bacterial polymerase, *Thermus Aquaticus* (Taq) polymerase. The repetition of three steps, denaturation (conversion of the double stranded DNA into single stranded templates), annealing (binding of short DNA fragments called primers to the template) and elongation (extension of the primers) result in the exponential amplification of the DNA of interest.

3.2.5 Primer design

To amplify a 9028 bp region of the CCR5 gene, four primer pairs (Table 2) were designed using the reference sequence U95626 GenBank accession number (Figure 10). The fragment amplified included the entire coding region as well as flanking regions of the CCR5 gene. Primer design was performed using the CLC Bio Genomic workbench software program. Criteria used to design primers included: low GC content, primer pair melting temperatures (Tm) of similarity with temperatures below 55 °C being excluded. The primers were verified using an Ensembl BLAST to ensure the primers efficiently amplified and was specific for the designated region shown in Figure 10. Primers were synthesised at Inqaba Biotechnical Industries, Pretoria, South Africa.

Table 2: PCR primer sets

PRIMER	SEQUENCE	LENGHT	Tm (°C)	Binding Position
Primer 2 forward	5'CGAAAAGTGCATGGGAAAAG3'	20	58.35	-5471
Primer 2 reverse	5'AGCTTTCTCGTCTGGGTATT3'	20	58.35	-3112
Primer 3 forward	5'ATCATTCCCTCTCCACCACA3'	20	60.40	-3750
Primer 3 reverse	5'CTACCCTCCCTCTCAGTCCT3'	20	64.50	-952
Primer 4 forward	5'TAAGGTGCAGGGAGTTTGAGAC3'	22	62.67	-1311
Primer 4 reverse	5'TTGATGCATGTGAAGGGGAGA3'	21	60.61	+1317
Primer 5 forward	5'ATCAACCCCATCATCTATGCC3'	21	60.61	+874
Primer 5 reverse	5'GCAACCAGCAACCTTACTCT3'	20	60.40	+3557

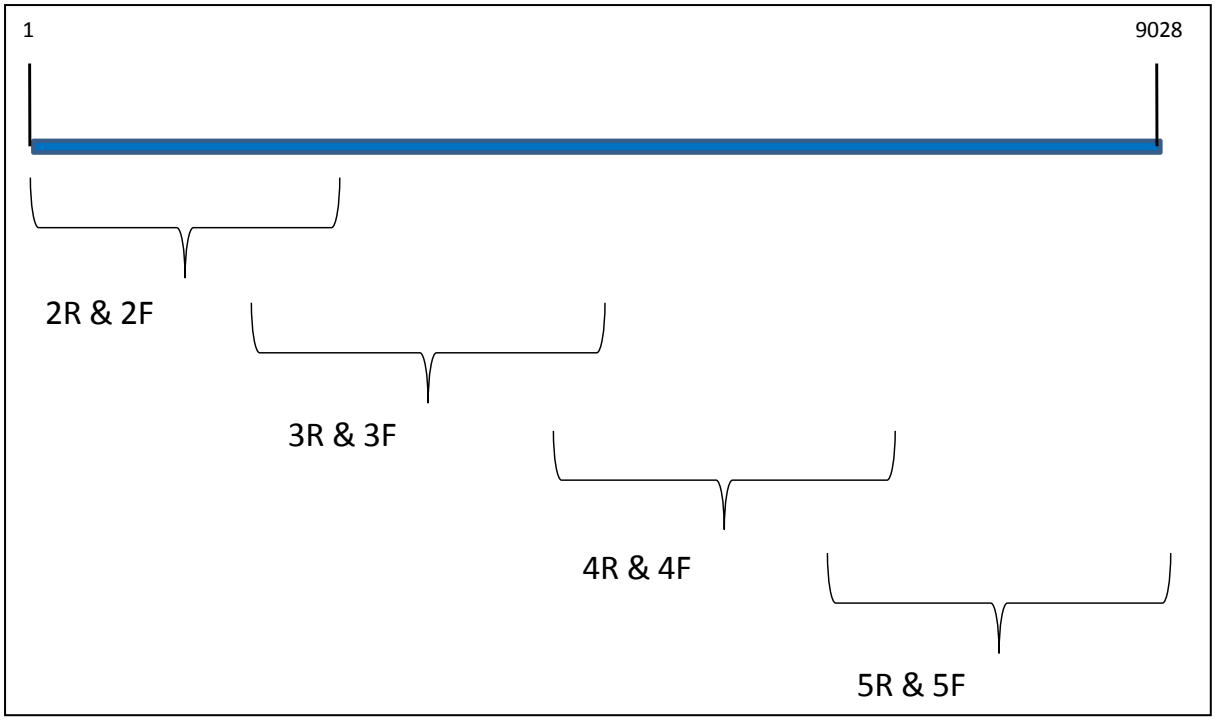


Figure 10: **Primer regions amplified on the 9028 bp CCR5 gene (image created by Fatima Barmania)**

3.2.6 PCR amplification protocol

The PCR reactions were carried out in a 30 µl volume, using the DreamTaq™ Green PCR master mix (Fermentas). The master mix consists of the Taq DNA polymerase, optimised concentrated buffer, deoxribonucleotides (dNTPs) and $MgCl_2$. Aliquots of the mastermix and nuclease free water (nfw) were made upon receiving the reagents. Working dilutions (30 µl) of the primers were made at a concentration of 10 µM (27 µl nfw + 3 µl stock). The reaction mix consisted of the following reagents outlined in Table 3.

Table 3: PCR reaction mixture

Reagent	Stock Concentration	Working dilution	Working concentration	Volume Required (30ul)
Mastermix	2X	-	1X	15
Primer forward	100 uM	(10uM)	10pmol (0.4uM)	1.2
Primer reverse	100 uM	(10uM)	10pmol(0.4uM)	1.2
Nuclease free water	-	-	-	10.6
Template DNA	-	-	-	2

For each primer pair, optimisation of the PCR amplification was performed using a starting temperature of Tm- 5. The final PCR reaction was performed in the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) using the protocols summarised in Figure 11.

Primer 2			
Segment	**Temperature (˚C)**	**Duration**	
Denature DNA	95.0	3 min	
Denature DNA	95.0	30 sec	30 cycles
Anneal Primers	57	30 sec	
Extend DNA	72.0	2:40sec	
Final extension	72.0	7 min	
Store	4.0	∞	

Primer 3			
Segment	**Temperature (˚C)**	**Duration**	
Denature DNA	95.0	3 min	
Denature DNA	95.0	30 sec	30 cycles
Anneal Primers	62	30 sec	
Extend DNA	72.0	2:40sec	
Final extension	72.0	7 min	
Store	4.0	∞	

Primer 4			
Segment	**Temperature (˚C)**	**Duration**	
Denature DNA	94.0	5 min	
Denature DNA	94.0	30 sec	15 cycles
Anneal Primers	62	30 sec	
Extend DNA	72.0	2:40sec	
Denature DNA	94.0	30 sec	15 cycles
Anneal Primers	61	30 sec	
Extend DNA	72.0	2:40sec	
Final extension	72.0	7 min	
Store	4.0	∞	

Primer 5			
Segment	**Temperature (˚C)**	**Duration**	
Denature DNA	95.0	3 min	
Denature DNA	95.0	30 sec	30 cycles
Anneal Primers	58	30 sec	
Extend DNA	72.0	2:40sec	
Final extension	72.0	7 min	
Store	4.0	∞	

Figure 11: PCR protocols used for each primer set

3.2.7 Agarose gel electrophoresis

Electrophoresis is the movement of charged molecules through an electric field (Fairbanks and Andersen, 1999). The velocity of the molecule is dependent on the electric field, charge of the molecule and friction. The latter involves the size and shape of the molecule. The phosphate backbone of DNA provides the molecule with a net negative charge. This means that during electrophoresis, DNA will be repelled from the cathode to the anode (Figure 12) (Fairbanks and Andersen, 1999).

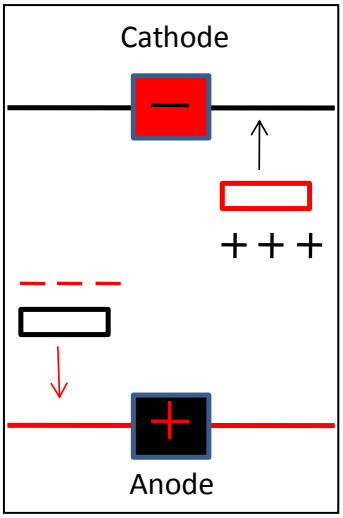


Figure 12: Principle of electrophoresis (image created by Fatima Barmania) (Data source: Fairbanks and Andersen, 1999).

Agarose gel (1%) was used to determine if PCR product was present. The gel was prepared as follows:

- 0.6 g agarose powder (Lonza Seakem® LE Agarose) was weighed in a heat resistant Schott bottle.
- 6 ml Tris borate EDTA (10X TBE) electrophoresis buffer (Fermentas Life Sciences) was added to 54 ml of deionised water to create a 1X electrophoresis buffer solution.
- The above solution was then added to the agarose and heated to dissolve the powder.
- The solution was cooled slightly and 5 µl SYBR® Safe DNA gel stain (Invitrogen) was added.
- Thereafter, the solution was set for a minimum of 30 min in a horizontal gel plate, in which a gel comb was inserted to create wells for loading the sample.

The gel stain used is a nucleic acid stain and when exposed to UV light the DNA fluoresces.

After the gel was set, it was placed in the gel tank which was then filled with 1X TBE buffer to cover the gel. To determine if the product was amplified, 5 µl was added to the wells. To ensure that the correct PCR product was amplified, the Mass Ruler™ DNA Ladder high range (5 µl) (Fermentas Life Sciences) was used as the size standard (Figure 13a). The gel was run at 100V (50 A) for 35 minutes (Power Station 300 plus, Labnet).

The DreamTaq™ Green PCR master mix consists of a density reagent to keep the PCR product in the well while loading. The master mix also consists of two loading dyes to monitor the progress of electrophoresis. The PCR product was visualised using a VersaDoc™ imaging system and the Quantity One program software (BioRad) (Figure 13b).

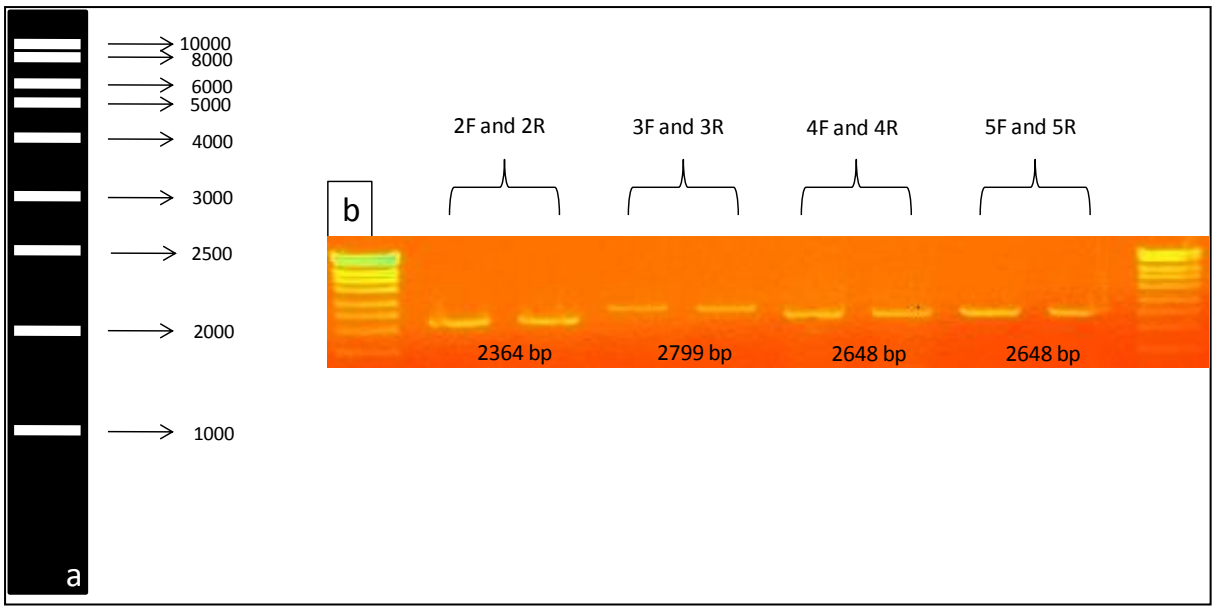


Figure 13: (a) is the molecular weight marker, Mass Ruler™ DNA Ladder high range and (b) illustrates 2364 bp fragments using primers 2F and 2R, 2799 bp fragments using primers 3F and 3R, and two 2648 bp fragments using primers 4F and 4R as well as 5F and 5R respectively.

3.2.8 DNA sequencing

Sequencing DNA generates important information regarding the nucleotide sequence of genes, which can be compared in many different individuals to determine similarities and differences. The CCR5 gene was analysed in this study to determine the prevalence and diversity of SNPs, inserts and deletions present in a selected cohort of South African individuals. All mutations discussed in chapter five display concomitant electropherogram data in Appendix E.

3.2.9 Primer design

With regards to the PCR primer synthesis, sequencing primers (Table 4) were designed using the CLC Bio Genomic workbench software program.

Table 4: List of sequencing primers

Primer 2	Sequence (5'- 3')	Primer 4	Sequence (5'- 3')
CCR5P2a_2FInt	ATGGTGTGAGCAAGGAGA	CCR5P4a_4F	AGAAGAAGAGGCACAGG
CCR5P2a_2RInt	TGGTGGAGAGGGAATGA	CCR5P4a_4R	AGAAGCGTTTGGCAATG
CCR5P2b_2_F	CGAAAAGTGCATGGGAAAAG	CCR5P4b_4F_Seq	GGTGCAGGGAGTTTG
CCR5P2b_2_R	GTTGTTGGGAGGATTCTATGAGG	CCR5P4b_4R_Seq	GATGCATGTGAAGGGG
CCR5P2c_2R	CTCCTTGCTCACACC	CCR5P4c_4InF	GCACCATGCTTGACC
CCR5P2d_2R_Seq	GCTTTCTCGTCTGGGTATT	CCR5P4c_4InR	CCACCACACCAAGCTAA
CCR5P2e_2F	GGGGGTGAGTATGTCT	CCR5P4d_4_F	TAAGGTGCAGGGAGTTTGAGAC
CCR5P2f_2_R_D2	AGCTTTCTCGTCTGGGTATT	CCR5P4d_4_R	TTGATGCATGTGAAGGGGAGA
		CCR5P4e_4Int_Seq	GGCTTGCTCATAGTGC
		CCR5P4f_4R	CCACCACACCAAGCTAA
		CCR5P4g4Rint_rev	TCCTTCTTACTGTCCCCT
		CCR5P4h_4Rint	AGGGGACAGTAAGAAGGA
		CCR5P4i_4F	CCTCCGCTCTACTCACT
Primer 3	**Sequence (5'- 3')**	**Primer 5**	**Sequence (5'- 3')**
CCR5P3a_3_F	ATCATTCCCTCTCCACCACA	CCR5P5a_5_F	ATCAACCCCATCATCTATGCC
CCR5P3a_3_R	CTACCCTCCCTCTCAGTCCT	CCR5P5a_5_R	GCAACCAGCAACCTTACTCT
CCR5P3b_3_F_seq	ATCATTCCCTCTCCACC	CCR5P5b_5F_Seq	ATCAACCCCATCATCTATGC
CCR5P3b_3_R_seq	CCTCCCTCTCAGTCCT	CCR5P5b_5R_Seq	GCAACCAGCAACCTTAC
CCR5P3c_3_Int-F-seq	TTGCTGTTTGGGGTCT	CCR5P5c_5F	TCCAGGCTGTCTTTCAC
CCR5P3c_3Int-R-seq	GGCTTCCCTCTTGTCTG	CCR5P5c_5R	CCAACTCAAATTCCTTCTC
CCR5P3d_3F	AAGATTTGCAGAGAGATGAG	CCR5P5d_5FInt	TGGGGAGAAAAGACATGAAT
CCR5P3d_3R	TTCTCTGCTCATCCCACT	CCR5P5d_5RInt	AGCCATCCCCCAAAT
CCR5P3e_3R	ACTCATCTCTCTGCAAATC	CCR5P5e_5F	AGAAGGAGGAGGAGGAG
CCR5P3f_3R	GGGGAGAGTGGAGAAAAAG	CCR5P5f_5F	AAAGGAGGGTCAGGAAG
CCR5P3g_3F	ACATCCGTTCCCCTAC		
CCR5P3h_3F	GAAGGGCAACAAAATAGTGA		

Footnote: CCR5P2, CCR5P3, CCR5P4, CCR5P5 primers representing those binding to regions amplified by PCR primers 2F and 2R, 3F and 3R, 4F and 4R, 5F and 5R respectively.

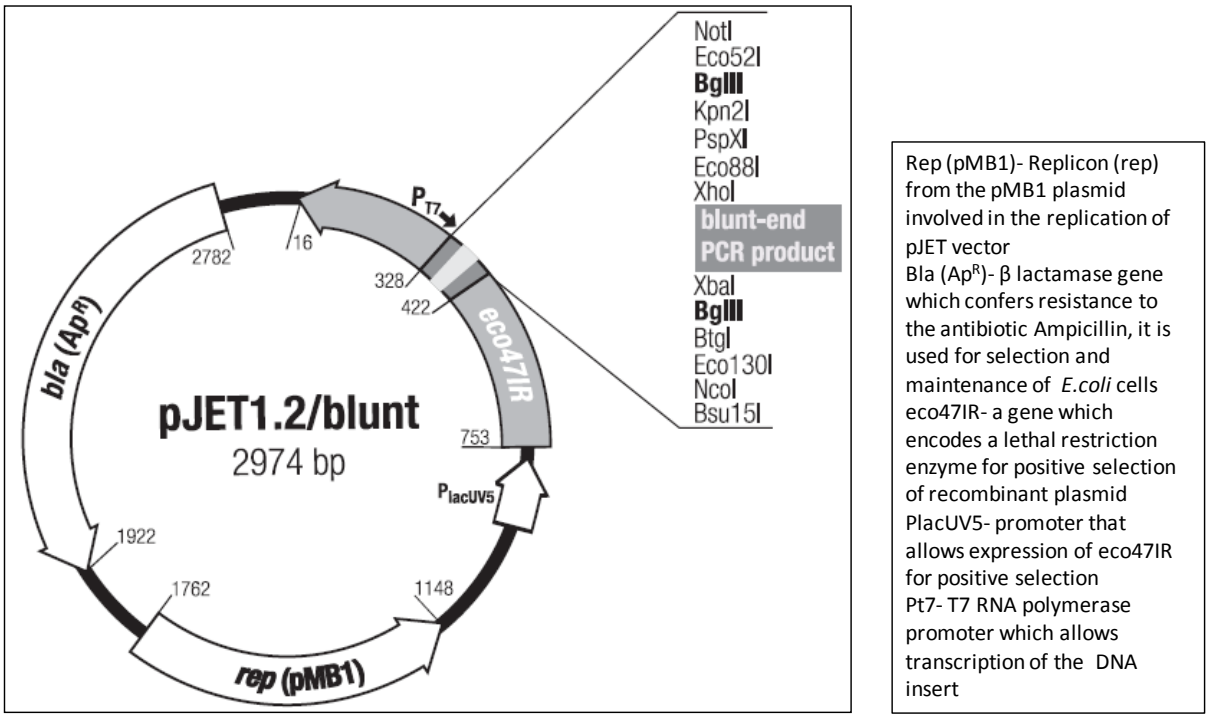
3.2.10 Sequencing protocol

Sequencing was performed at Inqaba Biotec utilizing the ABI version 3.1 Bigdye kit and ABI3500xl Genetic analyzer according to the manufacturer's instructions. The PCR product was purified before sequencing, using the Exo/SAP Amplicon purification kit. This is an enzymatic method which removes excess nucleotides, primers and salts from the PCR reaction which can interfere with the sequencing reaction. The DNA clean and concentration kit (Zymo Research) was used to purify the sequencing product obtained, to optimise the quality of the sequence data.

3.2.11 Cloning

Eleven individuals displayed an 18-20 nucleotide region which could not be sequenced using the above protocol. The sequence was found to be in the ORF of the CCR5 gene and can therefore be expected to have a significant impact on the CCR5 protein. To overcome this problem, cloning and haplotype analysis of the region containing this fragment were performed.

A plasmid is constructed by combining DNA from many sources. The plasmid consists of an origin of replication, selectable markers and several restriction sites. Once the plasmid is inserted into a bacterial cell, it uses the cell's machinery to replicate. The selectable markers ensure that cells which do not contain the plasmid are excluded. The restriction sites, when cut with the appropriate restriction enzyme, allow a DNA fragment cut with the same enzyme to recombine with the plasmid. Once the DNA fragment is inserted it will cause one of the selectable marker genes to lose its function, ensuring that only cells with the plasmid containing the DNA insert are selected. Bacterial cells transformed with the plasmid are allowed to grow for a sufficient period of time, to ensure that many copies of the plasmids in each cell are made. The DNA fragment is then recovered and purified (Fairbanks and Andersen, 1999). In the present study, cloning was performed using the CloneJET™ PCR cloning kit (Fermentas) (Figure 14) and transformation used Z- Competent™ *E. Coli* cells (Zymo Research) according to manufacturer's instructions.



Rep (pMB1)- Replicon (rep) from the pMB1 plasmid involved in the replication of pJET vector
Bla (Ap^R)- β lactamase gene which confers resistance to the antibiotic Ampicillin, it is used for selection and maintenance of *E.coli* cells
eco47IR- a gene which encodes a lethal restriction enzyme for positive selection of recombinant plasmid
PlacUV5- promoter that allows expression of eco47IR for positive selection
Pt7- T7 RNA polymerase promoter which allows transcription of the DNA insert

Figure 14: pJET1.2 vector used in the CloneJET™ PCR cloning kit (Fermentas) and explanation of the components of the vector (product insert).

The following is a summary of the procedure used:

- The PCR product was run on a gel to ensure the correct product was extracted
- The cloning kit was based on a positive selection cloning vector, pJET1.2 (Figure 14). The vector consisted of a gene, Eco47IR, a lethal restriction enzyme that would aid in selecting cells containing plasmid with the DNA insert.
- The gene fragment of interest was blunt ended using the blunting reaction reagents and then inserted into the vector using the ligation reaction mixture.
- The ligation mixture was used to transform the Z- Competent™ cells. These are chemically competent cells that are efficient in taking up the vector without heat shock. Thus the ligation mixture is added directly to the cells.
- The mixture was then spread onto Ampicillin containing culture plates and the colonies were incubated.
- For each individual, eight colonies were picked and used as templates for performing colony PCR using the pJET reaction reagents according to manufacturer's instructions. Colony PCR was performed to analyse the colonies for the correct DNA insert
- Resulting products were run on agarose gels and three of the products for each individual were chosen for sequencing.
- Sequencing was performed as described above.

3.2.12 Statistical analysis

Allele (portion of a particular allele among all alleles in a population) and genotype (distribution of alleles among genotypes) frequencies were calculated using STATA version 11 software.

Chi-Square test was used to compare observed frequencies with expected values. The Fisher exact test was used to determine if there is a significant difference in two data sets, between observed and expected values.

Literature review

2.1 ORIGIN AND HISTORY OF HUMAN IMMUNODEFICIENCY VIRUS (HIV)

The human immunodeficiency virus (HIV) is part of a family of retroviruses, which are ribonucleic acid (RNA) viruses. These viruses undergo replication using a deoxyribonucleic acid (DNA) intermediate (Weiss *et al.*, 1982). The virus forms part of the lentivirus sub class. This sub class is characterised by: 1) long periods of asymptomatic infection (Haase, 1986); 2) constant viremia; 3) specificity for the host; 4) ability to affect the haematopoietic and nervous systems and cause immune suppression; and 5) dysregulation (Letvin, 1990). Infection with HIV eventually causes the disease acquired immune deficiency syndrome (AIDS). The virus causes depletion of the immune system by $CD4^+$ T-cell destruction (Gottlieb *et al.*, 1981) but also an abnormal activation of soluble components of the immune system such as cytokines (Kinter *et al.*, 2000). This renders the infected individual susceptible to rare and opportunistic infections characteristic of AIDS.

The initial discovery of HIV in humans occurred in the United States in 1981. Previously healthy homosexual men began displaying similar conditions to one another, including rare diseases such as Kaposi's sarcoma and rare opportunistic infections such as *Pneumocystis carinii* pneumonia. These diseases were resistant to treatment (MMWR, 1981). The individuals also displayed swollen lymph nodes and insufficient cell mediated immunity characterised by diminished $CD4^+$ T-cell counts. The disease was initially referred to as 'gay compromise syndrome', but when similar cases started appearing in many different groups of individuals the name was changed to AIDS (Brennan and Durack, 1981).

At first, the cause of AIDS was uncertain and HIV was disregarded as the causative agent. This was due to long lag times until development of disease, a characteristic not common to viral like agents. Additionally, various other infectious agents were present in the individuals at the time of disease, providing more than one possibility for the cause. It was only in 1984 that HIV was finally accepted as the cause of AIDS, when evidence of repeated isolation of the virus was shown in AIDS patients (Gallo and Montagnier, 2003).

There are two HIV types, HIV-1 that is the predominant type found worldwide and HIV-2 found mainly in West Africa, with the latter being less pathogenic (Kanki *et al.*, 1994). It is generally accepted that HIV originated by zoonotic transmission (transfer to humans via vertebrate animals (Marx, 2005)) from the simian immunodeficiency virus (SIV) that affects many primates. This is possibly due to the close phylogenetic relationship between HIV-1 and SIVcpz strains found in *Pan Troglodyte* troglodyte chimpanzees and HIV-2 with SIVsm strains in the sooty mangabey monkey

(Gao *et al.*, 1999). Moreover, the viruses originated in the same regional locations as both HIV types, further supporting zoonosis.

There are many theories that explain the mechanism of viral transfer to humans. In Africa, hunting and the consumption of bush meat was common practice while it was also common to keep primates as pets (Peeters *et al.*, 2002). It is possible that individuals were infected during these practices.

The oral polio vaccine theory initiated by Edward Hooper indicated that between the periods of 1957-1960, the polio vaccine was given to millions of African individuals (Hooper, 1999). This vaccine was produced in chimpanzee cell cultures, infected with SIV, facilitating the transfer and subsequent spread of HIV in Africa during this period. However, many authors (Weiss, 2001) have refuted this theory. Other theories include the contaminated needle theory in which non-sterile needles were used in mass vaccinations in Africa. This could have facilitated the spread of the virus from already infected individuals.

The oldest known case of HIV-1 infection dated back to 1959, when HIV was detected in the plasma sample of an adult male living in Kinshasa (Nahmias *et al.*, 1986). In 1998, Zhu and co-workers analysed the genetic sequence of the plasma sample with the results indicating that HIV appeared in humans earlier than the 1940s. A study by Worobey *et al.*, (2008) compared the initial genetic sequence found in 1959 with a sequence discovered in 1960 in the same region. Using phylogenetic methods, they determined that there was a substantial difference in the genetics of the two sequences. They concluded that viral diversification in humans had occurred long before the AIDS pandemic. It was further suggested that the origin of HIV dated between the periods of 1884 and 1924.

2.2 MECHANISM OF HIV INFECTION

Viral transmission occurs via the exchange of bodily fluids that contain the HIV virion or via $CD4^+$ T-cells that have been infected with the virus. These bodily fluids can be in the form of blood, blood products, serum, breast milk, amniotic fluids, seminal fluid and vaginal secretions (Staprans and Feinberg, 1997). The most common mechanism of transmission is through unprotected sexual activity. Other forms of transmission include injection of blood products during transfusions or drug abuse and, mother to child transmissions that occur via placental transfer or during birth. Many factors facilitate the transfer of HIV. Persons exposed to secretions from an individual with a high viral load have an increased chance of infection. Furthermore, if the site of infection has no

protective barrier, such as in an open wound or if the site is infiltrated by immune cells such as $CD4^+$ cells, it will increase the risk of viral transmission (Brennan and Porche, 1997).

Replication of HIV requires entry into a host cell where it uses the host cell machinery to propagate. The virus structure consists of a lipid bilayer that contains two glycoprotein receptors, gp120 and gp41 (designated gp160), which are used to gain entry into the host cell (Greene, 1997; Phillips, 1996) (Figure 1). The viral core contains the p17 matrix protein, p24 capsid protein and the p6 and p7 nucleocapsid proteins. The matrix protein is found beneath the lipid bilayer where it maintains the structural integrity of the virion (Wu *et al.*, 2004). The capsid protein forms a protective case around the genomic material. The nucleocapsid protein, p7, is involved in forming and stabilising the RNA as well as in nucleocapsid assembly (Goel *et al.*, 2002). The p6 nucleocapsid protein is involved in viral assembly and budding of the virus from the host cell (Sandefur *et al.*, 2000). The core also consists of enzymes required in viral replication such as reverse transcriptase, integrase and protease (Geleziunas and Greene, 1999). The viral genome is made up of nine genes: *gag* (group specific antigen), *pol* (polymerase), *env* (envelope), *tat* (trans-activation of transcription), *rev* (regulator of virion), *nef* (negative factor), *vpr* (viral protein r), *vif* (viral infectivity factor), and *vpu* (viral protein u). The *gag* gene codes for proteins that make up the core, while the *env*- and *pol* gene codes for gp160 and enzymes, respectively. The remaining (*tat*, *rev*, *nef*, *vpr*, *vif*, *vpu*) genes are involved in accessory functions that aid viral production and spread (Hirsch and Curran, 1990; Montagnier and Klavel, 1994).

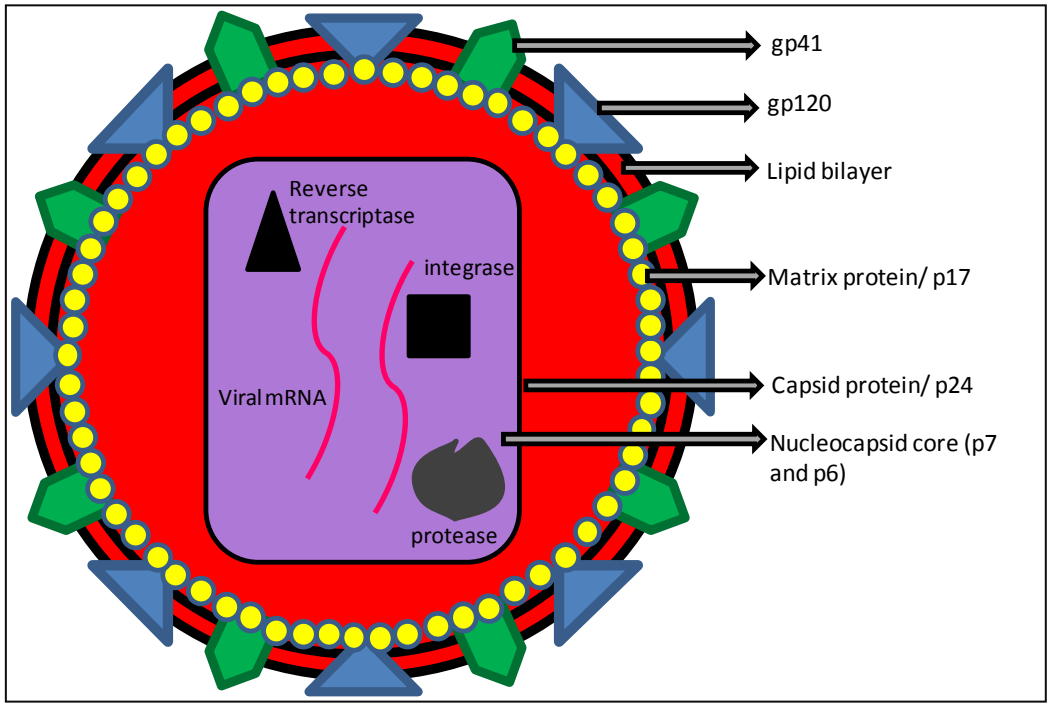


Figure 1: **Structure of a mature HIV virion (image created by Fatima Barmania)**

Viral replication begins with attachment of the virus's gp120 surface protein to the host cell CD4 receptor (McDougal *et al.*, 1986) (Figure 2). This triggers a conformational change in the virus's structure, which reveals the gp41 surface protein (Rizzuto *et al.*, 1998). The latter will bind to a chemokine co-receptor on the host cell being either CCR5 (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996; Doranz *et al.*, 1996) or CXCR4 (Feng *et al.*, 1996). Viruses that bind to CCR5 and CXCR4 are designated R5 and X4, respectively. While viruses that bind to both CCR5 and CXCR4 are R5X4. Co-receptor binding brings the virus in close contact with the host cell membrane as gp41 forms pores in the membrane (Srinivas *et al.*, 1992; Miller *et al.*, 1993). This anchors gp120, and facilitates fusion and entry (Helseth *et al.*, 1991).

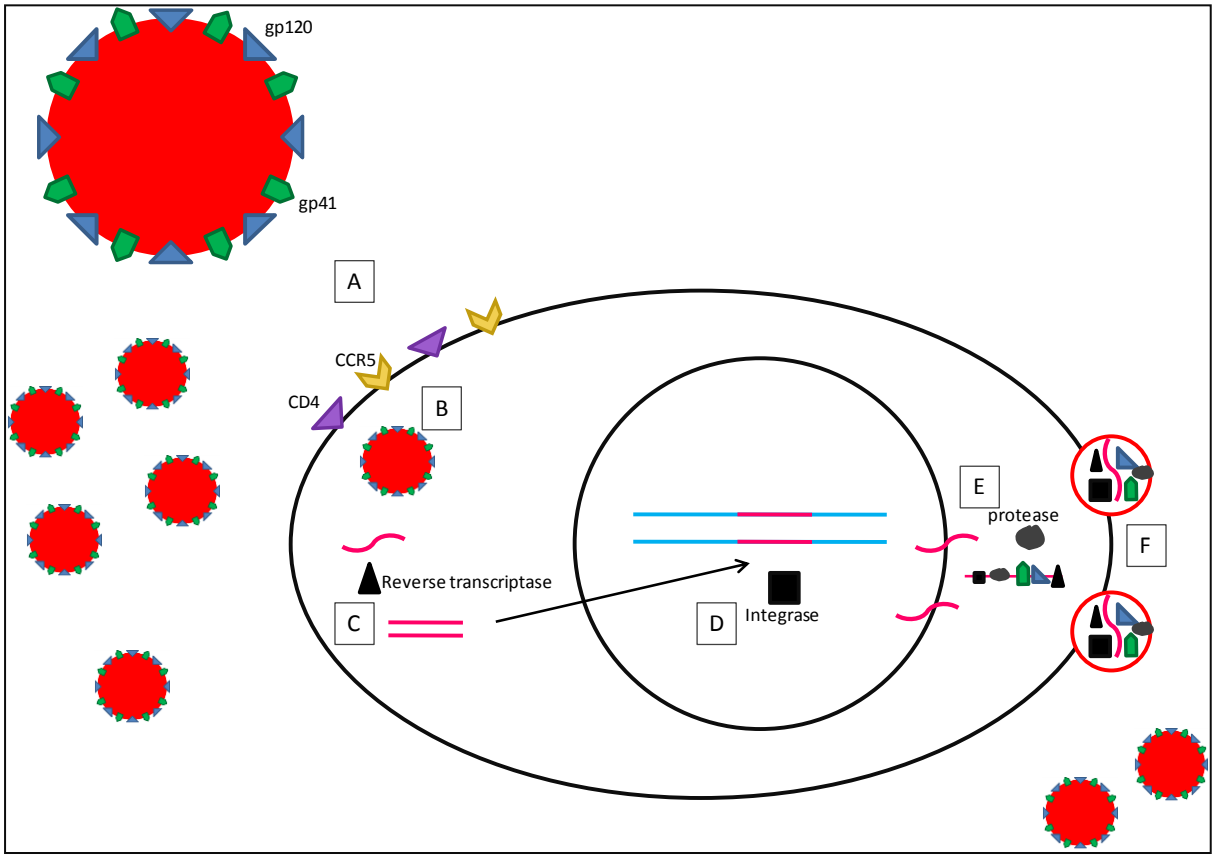


Figure 2: Diagram illustrating HIV replication. (A) The virus glycoprotein receptors bind to host cell CD4 and a co-receptor CCR5. (B) Fusion of the virus with the host cell membrane results in viral uncoating and the release of the viral nucleocapsid into the cytoplasm. (C) The enzyme reverse transcriptase converts the single stranded RNA into double stranded DNA. (D) The viral DNA is transported to the host nucleus where it is integrated into the hosts DNA. (E) Viral DNA is transcribed and translated using host cell machinery and then cleaved by viral protease into functional viral proteins. (F) Viral RNA and proteins assemble at the cell surface and bud of the cellular membrane. (Image created by Fatima Barmania).

Cellular factors as well as p17, Vif and Nef from the virus are involved in viral uncoating which unveils the RNA (Hirsch and Curran, 1990; Harrich and Hooker, 2002). The RNA is transcribed into double stranded complementary DNA (cDNA) by the enzyme reverse transcriptase (Varmas, 1988).

The doubled stranded DNA is then incorporated into a pre-integration complex (Farnet and Haseltine, 1991), which is transported across the nucleopore into the nucleus by the Vpr protein (Popov *et al.*, 1998). The integrase enzyme inserts the DNA into the host chromosome (Varmas, 1988). After integration, transcription is stimulated by host cellular RNA polymerase to produce Nef, Rev and Tat. The latter is involved in promoting the transcription of further HIV products by binding to promoter regions of the gene and additional host factors. The Rev product binds to transporter regions of the gene transcripts and facilitates the movement of these messenger RNA (mRNA) products to the cytoplasm for translation into protein (Pollard and Malim, 1998). The Env protein product, gp160, undergoes posttranslational modification in the endoplasmic reticulum and then inserts into the cell membrane. The Gag and Pol polyprotein products assemble with the envelope proteins where budding occurs. The process of budding triggers the catalytic activity of the protease enzyme. The enzyme cleaves the Gag and Pol polyproteins into its constituent structural and functional proteins, which form the virion (reviewed in Sierra *et al.*, 2005).

2.3 PATHOGENESIS OF HIV

Nadler and Montero, 2005, reviewed the clinical course of HIV disease progression. The following is a summary of their findings. Once HIV infection occurs, the clinical presentation of disease varies from one individual to another and depends on both host and viral factors. The first phase of disease, which proceeds immediately after HIV enters the individual, is known as 'primary infection' or 'acute retroviral syndrome'. This phase is characterised by symptoms such as fever, rash, fatigue, lymphadenopathy, sore throat, muscle pain and headache and is usually present 2-6 weeks subsequent to infection. The symptoms are different to viral or influenza infections as there is an absence of nasal leakage and congestion associated with these conditions. Effects of the first phase are due to viral dissemination, which initiates an immune response.

The second phase of disease is termed the asymptomatic stage as many individuals experience no obvious symptoms but this depends on viral factors and the host's ability to mount a strong, sufficient immune response. During this phase, the virus continues to replicate in various tissues, such as the mucosal-associated lymphoid tissue, causing destruction and decrease of $CD4^+$ T-cells. The immune system's response to HIV during infection will initially cause a decrease in plasma viremia and thereafter a viral load set point is achieved. The viral load (number of viral copies in the blood) is correlated with the progression of HIV. As such, viral load is used as a predictor of the length of this stage of the disease.

Destruction of cells of the immune system by HIV results in depletion of immune system function. This eventually causes the individual to acquire opportunistic infections, autoimmune diseases and neoplasms. Initially, mild infections and symptoms such as lymphadenopathy and fever manifest with the $CD4^+$ T-cell count between 350 and 500 cells/mm^3. When the $CD4^+$ T-cell count drops to between 200-350 cells/mm^3, more severe life-threatening infections, such as tuberculosis (TB) and bacterial pneumonia, persist. Once the $CD4^+$ T-cell counts drops below 200 cells/mm^3, the individual is diagnosed with AIDS and can no longer contain infections. If this condition is left untreated, it is likely to lead to death.

Depletion of immune system cells can occur directly by the virus or indirectly via the immune system. Mechanisms mediated by the virus include direct cell killing and formation of syncytia (Staprans and Feinberg, 1997). The $CD4^+$ cells are infected by many HIV virions with accumulation of HIV in the cell potentially disrupting normal cellular processes and structure, leading to cell death. This is due to the build up of viral DNA in the cell and continuous budding of HIV from the cell surface (Figure 3). Infected cells also display HIV envelope proteins on the cell surface, which bind to other infected $CD4^+$ and uninfected cells. The formation of these large multinucleated syncytia (or cell masses) enhances spread and replication of HIV in uninfected cells. It also prevents the $CD4^+$ cells from performing their immune system function, which contributes to the immunopathogenesis of the disease (Staprans and Feinberg, 1997).

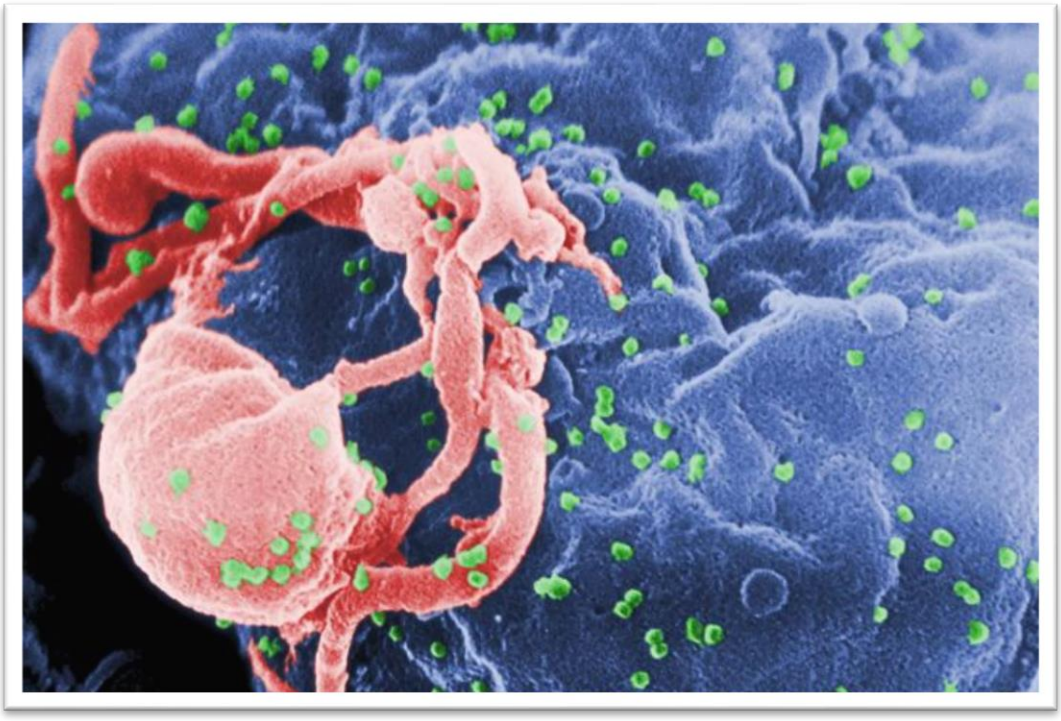


Figure 3: **Scanning electron micrograph of HIV-1 (green spherical molecules) budding from the surface of cultured lymphocyte (red cell exuding pseudopodia) (Original unedited image- www.cdc.gov)**

Dendritic cells and macrophages are also targets of HIV infection and may carry HIV to lymphatic and other tissue. The lymph nodes are known to be reservoirs of HIV, which facilitates the infection of passing $CD4^+$ cells. Continuous HIV replication in the lymph nodes eventually leads to tissue damage, which is harmful to the immune system as these areas are important in pathogen filtering and recognition (Kaplan, 1994; Staprans and Feinberg, 1997). Additionally, macrophages and dendritic cells have a longer life span, which assists in the creation of a long-term reservoir of HIV. Viral replication within these cells disrupts key immune functions such as antigen presentation, phagocytosis and cytokine signalling (Gendelman *et al.*, 1989; Kedzierska *et al.*, 2001).

Immune mechanisms include those carried out by $CD8^+$ cells, which destroy HIV infected $CD4^+$ cells as part of their normal immune function (Staprans and Feinberg, 1997). Furthermore, antibodies produced against viral envelope protein gp120 induce antibody dependent cell mediated cytotoxicity. This occurs in infected cells and uninfected cells that have gp120 proteins attached to the CD4 antigen on the surface (Forthal *et al.*, 2001). Other forms of immune activation that contribute to cell depletion include cytokine release. These cytokines can signal both infected and uninfected surrounding cells to undergo apoptosis. Therefore, cells are killed due to a bystander effect (Kedzierska *et al.*, 2003; Bangs *et al.*, 2006).

In addition, the virus itself and many of its proteins are involved in disease pathogenesis through interference with, and by confounding the immune system. The viral proteins gp160, Nef and Vpu can prevent the expression of the CD4 receptor, leading to cell dysfunction (Staprans and Feinberg, 1997). Binding of gp120 to CD4 also promotes a phenomenon called anergy whereby the $CD4^+$ cell becomes immunologically unresponsive. Binding can also cause aberrant activation of the cell that triggers the cell to undergo apoptosis. This is possibly due to the virus's ability to promote cytokine release. Cytokines such as interferon gamma (INF-γ) can increase the expression of death ligands on the surface of $CD4^+$ cells (Herbeuval *et al.*, 2007). Other cells such as B-lymphocytes can be activated to produce abnormally large amounts of antibody. The antibody can lead to autoimmune diseases and targeting of $CD4^+$ cells infected with HIV (Staprans and Feinberg, 1997).

The outcome of HIV infection is exhaustion of the immune system due to chronic stimulation and activation. Viral proteins Tat and Nef can increase the activity of the immune system while gp120 initiates signalling cascades (Simmons *et al.*, 2001; Misse *et al.*, 2005; Schindler *et al.*, 2006). Furthermore, the envelope protein binds to other receptors such as CD209 (also known as DC-SIGN) and can cause aberrant activation and immune effects (Nobile *et al.*, 2003). The depletion of specific $CD4^+$ regulatory cells can increase immune activation, as these cells are responsible for immunosuppression (Kinter *et al.*, 2007).

The virus also displays molecular mimicry with certain regions of the gp120 protein being similar to host proteins. Individuals with certain HLA (histocompatibility leukocyte antigen) types have structural similarity to the HIV envelope. This structural resemblance allows soluble forms of gp120 to interact with naive $CD4^+$ cells in a manner similar to HLA, causing activation of aberrant clones of T-cells (Susal *et al.*, 1993; Via *et al.*, 1990). Studies in mice show that recurrent stimulation of the immune system diminishes the naive cell pools, increases the risk for opportunistic infections, and decreases the immune systems' overall ability to respond to the infection (Tesselaar *et al.*, 2003).

2.4 THE HIV PANDEMIC

Since the initial discovery of HIV in 1981, 60 million people have been infected with HIV while over 25 million individuals worldwide have already died of AIDS. Published statistics from UNAIDS (Joint United Nations Programme on HIV/AIDS) indicated that there were between 31.4-35.3 million people globally living with HIV/AIDS at the end of 2009. In 2008, 2.4-3.0 million new infections were documented worldwide with approximately 1.7-2.4 million AIDS associated deaths. In developing countries, 9.5 million individuals required antiretroviral (ARV) therapy with only 42% actually receiving therapy. The global trend also illustrated a steady incline in HIV positive individuals from 8 million individuals in 1990 to the estimated 33.4 million in 2008 (www.unaids.org). This is a growth rate of the pandemic of around 320%.

Around two-thirds of HIV infected individuals are found in Sub-Saharan Africa (Figure 4), with 91% of new infections found in children (www.unaids.org). The epidemic has also resulted in 14 million orphans in the region. South Africa is faced with a major challenge in preventing and treating HIV. According to the 2011 mid-year population estimates, there are an estimated 5.38 million individuals living with HIV. This equates to 10.6% of the total population. The total number of new infections in 2011 was recorded at 316 900, of which 63 600 were children. Other indicators for the same year, estimated the number of AIDS orphans to be 2.01 million while the number of adults and children in need of ARV's is approximately 1.2 million and 377 097, respectively (Statistics South Africa, 2011).

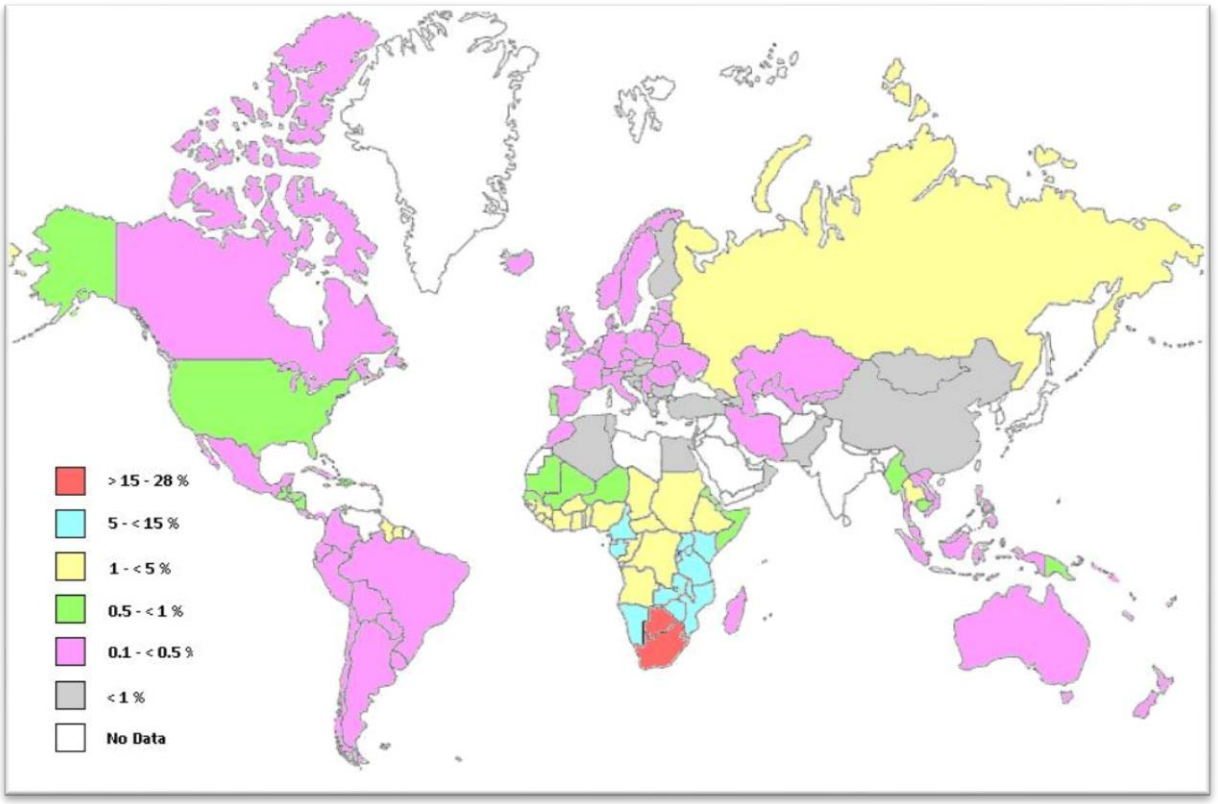


Figure 4: **Prevalence of HIV infected individuals globally in 2009 (image created by Fatima Barmania) (Data Source: www.unaids.org)**

An estimated 16.6% of adults aged between 15-49 years are infected in South Africa (Statistics South Africa, 2011). According to the World Economic Forum (2010-2011), South Africa ranks 54th of the 139 countries surveyed in terms of overall competitiveness. The country, however, was ranked 136th out of 139 countries when the impact of HIV prevalence was estimated. In addition, the health sector is heavily burdened as the increase in HIV infected individuals increases the demand on the health care system. This disease has significant bearing on the productivity and overall efficiency of our domestic labour force, which has both direct and indirect consequence on economic growth.

2.5 HIV: EVASION OF THE IMMUNE SYSTEM

During HIV infection, the virus' RNA is converted into double stranded DNA by the enzyme, reverse transcriptase. This retroviral enzyme is error prone and lacks the ability to proofread the DNA, creating a mis-incorporation at a rate of one nucleotide for every replication cycle (Nowak *et al.*, 1991). This creates what is known as a 'quasispecies', in which viral populations have distinct genetics but are closely related (Nowak *et al.*, 1990). These quasispecies differ in replication rates, HIV infectivity and other viral properties due to the antigenic variability. These nuances lead to viral populations with increased viral fitness that can override the host immune response.

Additionally, being a lentivirus allows HIV to mutate antigens on the viral surface that are involved in eliciting an immune response, thus facilitating immune escape (Nowak *et al.*, 1991). The *env* gene, which codes for the envelope proteins, mutates at a much higher rate than other genes and consists of five hypervariable regions, V1-V5 (Nowak *et al.*, 1990). The V3 region codes for gp120, the dominant surface antigen eliciting an immune response. The high degree of variability in this region prevents immune detection. Thus, antigenic variation is one of the mechanisms used by HIV to evade the immune system.

In 2003, Wei and co-workers demonstrated that neutralising antibodies were produced against HIV in infected individuals. These antibodies were shown to decrease viral entry with an efficiency of 99.8%, but were made ineffective by the virus. With most viruses, mutations in the genome enable changes in the epitope that elicit a neutralising antibody. In the case of HIV, a different mechanism is used (Wei *et al.*, 2003). According to the latter study, analysis of the *env* gene found mutations that did not correspond to the epitopes eliciting neutralising antibodies. However, it did produce changes involving glycosylation (addition of a carbohydrate side chain to an amino acid). The authors theorise that HIV uses glycosylation as a mechanism of masking the epitopes from neutralising antibodies while still allowing receptor binding to the host cell.

Structural analysis of the viruses binding sites for CD4 and the host co-receptor illustrated that two main accessible immune hot spots are found overlapping these regions (Kwong *et al.*, 1998 and 2002). However, the virus uses mechanisms of conformational changes to shield these regions from immune surveillance. The hypervariable regions in the gene code for structural loops, which mask the hotspots. Furthermore, the binding pockets for the CD4 receptor are depressed and thus occluded from neutralising antibodies. Another mechanism seems to be the uneven nature of the viral surface that makes it difficult for neutralising antibody attack. In this way, the virus prevents attack from the immune system while still maintaining HIV replication.

Down-regulation of host cell proteins, required for normal immune function, is another mechanism used by HIV to evade the immune system. Cytotoxic T-cells kill viral infected cells by recognising and binding to viral peptides (Cohen *et al.*, 1999). The viral peptides are displayed in conjunction with HLA class I molecules on the surface of infected cells. The HLA class I types A and B are required for cytotoxic cell killing whereas types C and E are inhibitor molecules for natural killer cell activity. A decrease in HLA class I inhibits cytotoxic cell killing but stimulates natural killer cell activity. According to Cohen and co-workers (1999), HIV uses the Nef protein to decrease the expression of HLA I types A and B. This prevents the cytotoxic T-cells killing of the infected cells. Alternatively, HLA I types C and D are not down regulated, thus natural killer cell activity is not stimulated. In this way,

HIV protects itself from both mechanisms of immunity. In 2003, Schindler and co-workers demonstrated that Nef decreases T-cell antigen interactions as well as $CD4^+$ T-cell function *in vitro*. For antigen presentation and activation of $CD4^+$ T helper cells, there is a requirement for interaction between the T-cell receptor and the antigen, in conjunction with HLA II presented by antigen presenting cells. It also requires accessory molecules CD28 and expression of CD4 and CD3 on the surface of the $CD4^+$ cell. Thus, HIV decreases these required molecules as well as HLA II from the cell surface. The virus also increases the expression of the HLA invariant chain, a molecule known to prevent antigenic peptide presentation. Impairment in antigen presentation and subsequent activation of $CD4^+$ T-cells diminishes the stimulation of B-cell and cytotoxic T-cell responses, allowing HIV to persevere in the infected individual (Schindler *et al.*, 2003).

2.6 HIV TREATMENT

Antiretroviral drugs (ARVs) are the current choice of treatment for HIV and are aimed at inhibiting the various stages of HIV replication. These drugs reduce viral load but do not eradicate the virus and are associated with many adverse effects. The drugs can lead to toxicity with some eliciting mild symptoms in the form of rash and lymphadenopathy, while others cause more severe symptoms such as neuropathy and pancreatitis (Lipsky, 1996). In South Africa, many infected individuals either have no access to ARVs or would rather rely on traditional medicine. Lack of access to treatment, as well as illnesses due to the drug toxicity can prevent infected individuals from complying with a strict regimen of taking their medication. Studies show that compliance is key in the development of HIV resistance to ARVs (Marfatia and Smita, 2005). Lastly, the ability of HIV to evolve at such rapid rates increases the development of resistance to ARVs that can render them ineffective. Irrespective of the current challenges, ARVs are the only treatment for HIV that causes viral suppression and slower disease progression for infected individuals.

When HIV was initially discovered as the causative agent of AIDS, many expected to find a vaccine within a few years. Unfortunately, the road to finding a vaccine has proven long and unsuccessful thus far. It has been approximately 30 years and a suitable vaccine is still not in effect. In 2007, a phase IIb HIV vaccine trial was conducted in various countries including South Africa using a recombinant adenovirus serotype five vector. The study aimed at increasing a cellular immune response towards HIV in uninfected individuals. Results from the study showed that the vaccine did not protect against HIV infection, neither did it inhibit the progress of HIV once individuals were infected. Alarmingly, the vaccine may have increased the susceptibility of participants to HIV infection although the mechanism remains unknown (Sekaly *et al.*, 2008).

Production of an HIV vaccine is fraught with many challenges including the inability to use live or dead virus due to the risk of infecting participants. However, there is no suitable model to investigate the effects of the vaccine; humanised mouse models may alleviate this problem. In addition, there are many HIV variants due to the rapid mutation rate, which complicates the production of a broadly effective vaccine (Gallo, 2010). The viral properties of HIV also contribute to the hurdles faced. Being a retrovirus allows HIV to integrate into the host cell genome where it can remain latent for many years. The virus also damages the immune system and changes the dynamics of the immune environment. This can affect the vaccine's response and diminish its sustainability (Esparza *et al.*, 2010). A suitable vaccine will therefore, need to be one that prevents initial HIV infection (Esparza *et al.*, 2010). Some promise was evident from the Thai phase III HIV vaccine trial, RV144, which studied a two-combination vaccine approach using ALVAC® and AIDSVAX® (Rerks-Ngarm *et al.*, 2009). Results from the trial showed that the vaccine was safe and reduced HIV infection in the participants by 31.2%. However, once the participants were infected, the vaccine did not reduce viral load or $CD4^+$ cell count.

In 2009, Hutter and co-workers reported on a bone marrow transplant performed on an HIV positive individual using stem cells that were derived from an HIV negative donor who was homozygous for a mutation in the CCR5 gene known as CCR5 delta-32 (Δ32). The HIV positive individual became HIV negative and remained free of viral detection 20 months after transplantation despite halting ARV treatment (Hutter *et al.*, 2009). Although a small proportion of CCR5 expressing macrophages were present in the intestinal mucosa, viral DNA was not present. It was found that the patient had a small subset of X4 HIV in the plasma when deep sequencing was performed before the transplant. However, the X4 HIV did not rebound after transplant. An update in 2010 showed that the patient remains free of viral detection three and a half years since the transplant. The recipient's macrophages displayed CCR5 six months after transplant but have subsequently disappeared and the individuals CCR5 alleles are now Δ32 homozygous. The $CD4^+$ T-cell levels have normalised and the authors have suggested that the individual is 'cured' of HIV (Allers *et al.*, 2010).

Considering the complex nature of HIV vaccine development and the issues surrounding ARVs, many researchers are looking at alternative mechanisms of therapy. Current interest is focussed on targeting the co-receptor CCR5, for HIV entry as well as enzymes involved in HIV replication. Other approaches aim at using RNA interference combined with lentivectors as well as zinc finger nucleases. Many of these techniques are associated with safety concerns and regulations.

2.7 HOST FACTORS IN HIV INFECTION AND PROGRESSION

Individuals exposed to HIV can follow different outcomes as the acquisition, progression and control of HIV is governed by viral, environmental and host factors. The majority of HIV infected individuals are typical progressors who develop clinical latency for six to ten years before onset of AIDS. Other subgroups include rapid progressors, who develop AIDS very quickly, and long term non-progressors (Pantaleo and Fauci, 1996). Long-term non-progressors have extended periods of clinical latency usually for more than ten years and have constant $CD4^+$ T-cell levels and low viral loads. Elite controllers form another subgroup of infected individuals and usually consist of less than 1 % of HIV infected individuals. These individuals control HIV replication and keep the levels of virus to less than 50 copies/ml (Walker *et al.*, 2007). Finally, there is a subset of individuals that even with multiple high-risk exposures to HIV are not infected and are termed exposed seronegative (O'Brien and Nelson, 2004).

Host genetic factors that affect HIV fall into three categories: (i) genes involved in innate and adaptive immunity, (ii) genes that can restrict HIV replication or infection and (iii) genes that are required during HIV infection and replication (An and Winkler, 2010). Viral infection begins with attachment of the virus to the host cell surface. This occurs via the virus's gp120 envelope glycoprotein, which binds to CD4 receptors on host cells. This is followed by the unveiling of the second glycoprotein receptor, gp41, which binds to a co-receptor on the host cell being either CCR5 or CXCR4. The significance of CCR5 in HIV infection was discovered when it became apparent that individuals with variations in the CCR5 gene were resistant to HIV despite previous exposure (Dean *et al.*, 1996; Liu *et al.*, 1996; Samson *et al.*, 1996). The mutation resulting in this resistance is known as the Δ32 mutation. Individuals homozygous for this mutation produce a truncated version of the CCR5 protein, which is unable to be expressed on the cell surface, and therefore not available for HIV entry. Individuals heterozygous for the mutation have partial protection as they can still express some normal protein, which allows infection of HIV; however, the progression towards AIDS is much slower.

2.8 INTRODUCTION TO CCR5

The first chemokine was discovered in 1977 (Walz *et al.*, 1977) and since then a large super family of chemokines has been identified. Chemokines are small proteins with a variety of functions including immune surveillance and immune cell recruitment. The effects of these chemokines are mediated by their receptors, which are known to be GPCR's. Chemokines are classified as C, CC, CXC and CX3C

depending on the structure and number of cysteine residues. Moreover, the receptors are designated with an addition of 'R' to indicate receptor (Murphy *et al.*, 2000).

Chemokine co-receptors are GPCR, which consist of seven transmembrane domains, as well as an amino and carboxyl terminal (Horuk, 1994). The CC receptors often share more than 48% homology, with CCR5 and CCR2 sharing 75% homology (Combadiere *et al.*, 1996). Despite the close structure of these receptors, they bind to different ligands and can result in various effects.

The chemokine receptor, CCR5, is expressed on various cell population groups such as macrophages, dendritic cells and memory T cells in the immune system; endothelium, epithelium, vascular smooth muscle and fibroblasts; and microglia, neurons, and astrocytes in the central nervous system (Rottman *et al.*, 1997). Natural ligands for the receptor include macrophage inflammatory protein one alpha (MIP1-α) (Nibbs *et al.*, 1999), macrophage inflammatory protein one beta (MIP1-β), regulated upon activation, normal T-cell expressed, and secreted (RANTES) and monocyte chemotactic protein two (MCP-2) (Combadiere *et al.*, 1996; Raport *et al.*, 1996; Samson *et al.*, 1996b; Gong *et al.*, 1998). The receptor plays a role in the inflammatory response by directing cells to sites of inflammation. Other chemokine co-receptors work together with CCR5 to stimulate T-cell functions (Contento *et al.*, 2008). The receptors enhance T-cell co-stimulation and cytokine release from $CD4^+$ T-cells. Furthermore, ligands for CCR5 augment the activation of T-cell responses and enhance the production of antigen specific T-cells (Taub *et al.*, 1996). During inflammation, the level of CCR5 expression is up-regulated in $CD8^+$ cells, which allows the cells to move towards sites of $CD4^+$ T-cell and dendritic interactions (Castellino *et al.*, 2006). This increases the chance of $CD8^+$ cells encountering antigen specific cells. Thus, CCR5 enhances the specific immune response.

In 1996, it was discovered that CCR5 was necessary as a co-receptor for HIV entry (Dragic *et al.*, 1996) for the macrophage tropic virus strains (Deng *et al.*, 1996). The former group demonstrated that the ligands for CCR5 inhibit viral entry and envelope mediated fusion. The virus uses CCR5 especially during initial infection, whereas the alternative co-receptor CXCR4 is used much later in HIV infection when the infected individual is progressing towards AIDS.

2.9 CCR5 PROTEIN STRUCTURE

The CCR5 protein consists of 352 amino acids with a molecular weight of 40.6 KDa (Samson *et al.*, 1996b). The structure of the protein as found in Figure 5 contains an amino terminal (N-terminal), seven trans-membrane domains (TMD) made up of hydrophobic residues, three extracellular loops (ECL), three intracellular loops (ICL) and a cytoplasmic or carboxyl tail (C-terminal tail). The protein is

composed of conserved residues, specific motifs of charged or hydrophobic regions and posttranslational modifications. These regions are important for chemokine ligand binding, functional response of the receptor, and HIV co-receptor activity.

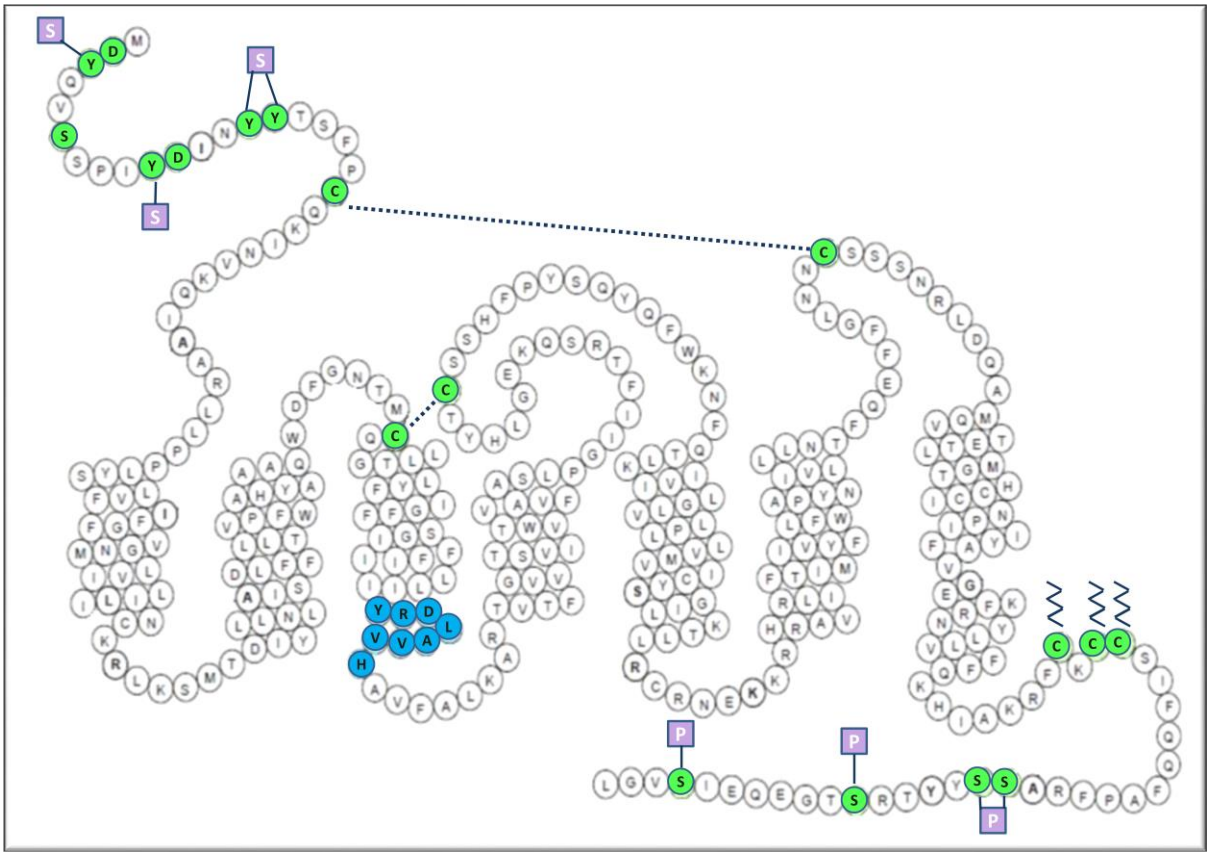


Figure 5: Protein structure of CCR5 indicating regions of importance with the dotted line showing disulfide linkage, the boxed S and P indicating sulphate and phosphate moieties and the three zig zag lines showing palmitoylation of C moieties. The DRYLAVVH sequence is highlighted in blue. Image adapted from Blanpain and Parmentier, 2000.

The N-terminal is rich in tyrosine and acidic amino acids that play an important role in both R5 HIV interaction and chemokine binding (Dragic *et al.*, 1998; Rabut *et al.*, 1998; Farzan *et al.*, 1999). Blanpain and co-workers (1999a) using deletion and mutagenesis studies in this region discovered the role of these amino acids in the N-terminal. Mutants lacking amino acids 2-13 responded weakly to elevated concentrations of ligands. In addition, co-receptor function was reduced by 80 %. Using alanine-scanning mutagenesis, five amino acid positions (2, 3, 10, 11 and 18) were deemed important for ligand and HIV envelope binding affinity.

Similar to other chemokine receptors, cysteine residues are found in all three ECLs and the N-terminal (Blanpain *et al.*, 1999b). The second ICL has a conserved sequence motif, DRYLAVVA, and a short third ICL containing charged amino acids (Opperman *et al.*, 2004). A motif designated TxP, with x referring to any amino acid, was found in the second TMD creating a structural constraint

important for receptor function (Govaerts *et al.*, 2001). The proline and threonine residues in this region provide flexibility to the receptor backbone. Mutation in this area reduces ligand binding and severely affects the functional response of the receptor.

Most GPCRs have a conserved disulfide bond between the first and second ECLs (Fraser *et al.*, 1989; Dohlman *et al.*, 1990; Savarese *et al.*, 1992; Perlman *et al.*, 1995). Chemokine receptors contain an additional disulfide bond between the N-terminal and third ECL (Baggiolini *et al.*, 1997). These disulfide bonds are important for ligand binding and stable receptor conformation (Perlman *et al.*, 1995). Naturally occurring mutations were found at these positions in long-term non progressors (Carrington *et al.*, 1999) which led to the study conducted by Blanpain and co-workers (1999b) on these cysteine residues. Substitution of alanine at any individual cysteine reduced cell surface expression of CCR5 between 40-70%. The cysteine mutants did not respond to CCR5 agonists, but allowed R5 HIV infection albeit at decreased levels.

As mentioned previously, the tyrosine and acidic residues in the N-terminal are important in HIV infection and chemokine binding. The tyrosines are post translationally modified by sulfation, which increases the net negative charge of the N-terminal region, facilitating interaction with ligands and HIV (Farzan *et al.*, 1999). Viral gp120 has been shown to bind sulphated moieties on the cell surface (Mondor *et al.*, 1998; Rodriquez *et al.*, 1995) and sulphated compounds can inhibit *in vitro* HIV infection by binding to gp120 (Yang *et al.*, 1996). Farzan and co-workers (1999) showed that prevention of tyrosine sulfation reduced ligand and gp120 binding affinity but did not affect CCR5 expression. The serine residue at position six in the N-terminal also contains an O-linked glycosylation modification that affects CCR5 chemokine binding (Bannert *et al.*, 2001).

The C-terminal region of CCR5 consists of various modifications and residues, which affect expression and function of CCR5. Blanpain and co-workers (2001) demonstrated using green fluorescent fusion proteins and immunofluorescence that CCR5 is palmitoylated at the C-terminal. The cysteine residues (positions 321, 323 and 324) that are acylated, serve as an anchor between the C-terminal and the cell membrane. This facilitates receptor transport to the cell surface, affects interaction between the receptor and signalling pathways and is involved in receptor-mediated endocytosis (Blanpain *et al.*, 2001; Percherancier *et al.*, 2001; Kraft *et al.*, 2001). Eliminating palmitoylation reduced surface expression by intracellular trapping of the receptor in organelles and subsequent degradation. This reduces the amount of receptor utilised by HIV for entry. Escape mutants that reach the cell surface have impaired signalling but still maintain intact co-receptor function (Blanpain *et al.*, 2001). The C-terminal region is also enriched in serines and threonines that provide phosphorylation sites for G-protein coupled receptor kinases (Opperman *et al.*, 1999).

Accordingly, amino acid modifications can have consequences on HIV infection and ligand binding affinity but also on downstream CCR5 capabilities.

2.10 CCR5 EXPRESSION AND HIV

The density of CD4 receptors on the cell surface is an important factor for HIV infection; peripheral blood $CD4^+$ cells have however been reported to have a relatively consistent density on the cell membrane (Kabat *et al.*, 1994; Poncelet *et al.*, 1991). Considering the consistency of CD4 receptor expression, Moore and co-workers (1997) found that CCR5 expression consists of large inter-individual variability. This variation in expression was shown to affect HIV infectability *in vitro* in cell lines (Platt *et al.*, 1998), macrophages (Tuttle *et al.*, 1998) and lymphocytes (Wu *et al.*, 1997).

In individuals infected with HIV, the percentage of $CD4^+CCR5^+$ T-cells is higher (13.2 %) in comparison to uninfected individuals (6.2 %) (Ostrowski *et al.*, 1998). The highest percentage of expression was found in an individual with acute HIV syndrome, recorded at around 30-40 %. The variation in CCR5 percentages in HIV infected individuals did not correlate with genotype as three individuals with heterozygosity for Δ32 had different levels of expression (2.7 %, 13.1 % and 17 %). In contrast, the activation state of the $CD4^+$ cells as measured by HLA-DR positively correlated with CCR5 expression.

In 1999, a study conducted by de Roda Husman and co-workers showed CCR5 expression in terms of CCR5 genotype and HIV infection and progression. Individuals with wild-type CCR5 receptors had higher levels of $CD4^+CCR5^+$ T-cells than those with heterozygous Δ32 genotypes, in both HIV infected (wild-type- 28 %; Δ32 heterozygote- 21 %) and uninfected individuals (wild-type- 15 %; Δ32 heterozygote- 10 %). Furthermore, infected individuals in end stage HIV progression had higher percentages than individuals that had not progressed. The study postulated that the $CD4^+CCR5^+$ T-cell percentage is directly correlated to the HIV disease progression due to the constant immune activation associated with HIV. The presence of the CCR5 receptor on memory effector T-cells (Mo *et al.*, 1998) or mature activated T-cells supports the latter finding.

Reynes and co-workers postulated that CCR5 expression affects virus production and viral load (Reynes *et al.*, 2000). The study found a strong correlation between CCR5 density and viral load, but a weak correlation between $CD4^+CCR5^+$ T-cell percentage and viral load. In addition, cell activation did not affect CCR5 density and there was no correlation between cell activation state and viral load. Infection with HIV did not up-regulate CCR5 density, as there was little difference between density in infected and uninfected individuals. Furthermore, treatment of infected individuals with ARVs reduced viral load but not CCR5 density. As such, the strong correlation between viral load and CCR5 density was independent of activation state or up-regulation of CCR5 by HIV. Platt and co-workers

(1998) elucidated a threshold of 10000 CCR5 molecules per $CD4^+$ cell as a requirement for HIV infection. Levels below this threshold value showed extremely severe drops in cell infectability. Most individuals with a low viral load have CCR5 densities below this value (Reynes *et al.*, 2000). Thus, the *in vivo* importance of CCR5 density in determining viral load was established.

2.11 REGULATION OF CCR5 EXPRESSION

Regulation of CCR5 occurs at three levels: (1) genetic factors; (2) factors involved in activation, signalling and trafficking of the receptor including desensitisation (when a receptor stops responding to stimulus), internalisation (receptors are removed from the cell surface after stimulus and move to intracellular organelles) and recycling (receptors return to the cell surface) and; (3) environmental or other triggers. This section will deal with the regulation of CCR5 via levels (2) and (3) while regulation at the level of the gene will be discussed later in this chapter.

Chemokine receptor CCR5 is part of the GPCR family, which upon binding by the relevant ligand results in the release of the αi and βγ G-protein subunits. This in turn mediates an effector response (Aramori *et al.*, 1997; Zhao *et al.*, 1998). These responses include the release of adenylyl cyclase and phospholipase Cβ, which release intracellular calcium and forms inositol triphosphate. These products activate phosphorylation of the receptor via protein kinase C and G-protein coupled receptor kinases (GRK) (Opperman *et al.*, 1999). Phosphorylation via these kinases occurs at the serine and C-terminal residues of CCR5. Binding of β-arrestin 1 and 2, which are regulatory proteins, occurs at the activated serines (Kraft *et al.*, 2001) and the conserved DRY motif (Figure 5) in the ICL (Gosling *et al.*, 1997). The β-arrestin proteins desensitise the receptor to further stimulation but also participate in receptor-mediated endocytosis (Huttenrauch *et al.*, 2002).

The level of CCR5 expression is determined by the rates of endocytosis and recycling. The β-arrestin protein facilitates the former process by participating in the binding process between the phosphorylated receptor and clathrin coated pits (Goodman *et al.*, 1996; Laporte *et al.*, 1999). An alternative pathway of endocytosis can occur by cholesterol rich caveolae dependent pathways (Mueller *et al.*, 2002). Once endocytosis is complete, the phosphorylated receptor collects in perinucleur endosomes. These endosomes then return the receptor to the cell surface in a dephosphorylated form (Signoret *et al.*, 1998; Pollok-Kopp *et al.*, 2003). According to Signoret and co-workers (1998), the receptor is not subject to degradation pathways, as it does not accumulate in late endosomes. Infection and entry of HIV into cells does not require CCR5 internalisation or signalling (Gosling *et al.*, 1997), but the chemokine induced endocytosis decreases available receptor

for HIV use (Amara *et al.*, 1997; Mack *et al.*, 1998). This is the mechanism of chemokine mediated anti-HIV activity.

Environmental factors, affecting CCR5 expression, include infectious agents such as *mycobacterium tuberculosis* (MTb) and HIV. Santucci and co-workers (2004) showed that MTb infection up-regulates CCR5 expression facilitating HIV cell infectability. Endotoxins such as lipopolysaccahride (LPS) can also increase CCR5 expression (Juffermans *et al.*, 2001). The latter study injected HIV negative males with LPS to induce endotoxemia; this resulted in a 2-4 fold increase of CCR5 on $CD4^+$ T-cells. Studies *in vitro* yielded similar results with LPS, MTb and *Staphylococcus Aureus*, demonstrating that infections can aid HIV viral entry by up-regulating CCR5. Shalekoff and co-workers (2001) analysed the effect of TB and HIV infection in leukocytes populations *in vivo*. The study found that CCR5 expression was significantly increased in all leukocyte subsets during TB, HIV and dual infection. However, the level of CCR5 expression on $CD4^+$ cells was not increased.

Conversely, Ostrowski and co-workers (1998) showed that HIV affects the level of expression of CCR5, as there is a direct correlation with HIV disease progression (Ostrowski *et al.*, 1998). Individuals with end stage HIV were shown to have the highest percentages of CCR5 expressing $CD4^+$ cells.

Clerici and co-workers (2000) found that immune activation in Africa is environmentally induced and not due to genetic determinants. This results in an up-regulation of CCR5 mRNA and concomitantly CCR5 cell surface expression, increasing R5 HIV infection.

Other factors affecting expression include drugs such as statins that have been shown to affect CCR5 expression by reducing the level of CCR5 mRNA and increasing CCR5 ligand expression (Nabatov *et al.*, 2007). These drugs decreased both R5 and X4 HIV infection *in vitro*. Perney and co-workers (2005) studied the effect of chronic alcohol consumption on CCR5, since the receptor is involved in inflammatory reactions. The study showed that in comparison to control subjects, alcoholic individuals had lower CCR5 densities on $CD4^+$ T-cells.

The levels of cytokine secretion are also known to affect CCR5. Cytokines affect CCR5 expression with pro-inflammatory cytokines such as interleukin 2 (IL-2) (Wu *et al.*, 1997; Bleul *et al.*, 1997), IL-12, tumor necrosis factor α and INF-γ increasing CCR5 expression on peripheral blood mononuclear cells (Hariharan *et al.*, 1999; Patterson *et al.*, 1999). The anti-inflammatory cytokine, IL-10, also increased CCR5 density on monocytes by prolonging the half-life of CCR5 mRNA (Sozzani *et al.*, 1998).

2.12 CCR5 GENE STRUCTURE

On March 19 1996, Samson and co-workers (1996b) published the first report indicating the molecular cloning, expression and ligand binding properties of CCR5. Four months later, two separate reports confirming these findings were published (Combadiere *et al.*, 1996; Raport *et al.*, 1996). Samson and co-workers (1996b) cloned and expressed the fifth chemokine receptor, designated Chem13. Using a murine clone they examined the human genomic DNA library, isolated, and purified a positive clone, which was physically linked with the already discovered CCR2 gene. The ORF of these two genes were separated by 17.5 kb. The similarity between CCR2B and CCR5 was found to be 71 % identical in amino acid residues and 55 %, 49 % and 48 % identical in residues to CCR1, CCR3 and CCR4, respectively (Raport *et al.*, 1996). Combadiere and co-workers (1996) isolated a human CCR5 clone, identical to the clone identified in the Samson paper (1996b), which differed at position 90 of the protein with leucine substituting for alanine. This finding illustrated that these two sequences are different alleles of the CCR5 gene with a novel variant having been discovered. Additionally, Combadiere and co-workers (1996) demonstrated that the signal transduction pathway is petussis toxin sensitive, confirming its nature as a GPCR.

The CCR5 gene was restricted to chromosome 3p21 by radiation hybrid mapping (Liu *et al.*, 1996) and was found within a cluster of genes encoding for chemokine receptors which included CCR1, CCR2, CCR3, XCR1 and CCBP2 (Samson *et al.*, 1996b; Maho *et al.*,1999). The CCR5 gene is composed of three exons, two introns and two promoters (Figure 6) (Mummidi *et al.*, 1997). The upstream promoter, designated Pu or PR2, consists of a 1.9 kb region preceding exon 1 that is 57 bp in length. Exon 1, the start of the coding region, is followed by the first intron that is 501 bp in length. Exon 2 is intronless and can be found as exon 2a (235 bp) and exon 2b (54 bp). The second promoter, designated Pd or PR1, encompasses the intron 1 and exon 2 regions (Mummidi *et al.*, 2007). A 1.9kb intron is located between exon 2 and exon 3. Exon 3 is intronless and contains the entire ORF of the CCR5 gene, 11bp of the 5' UTR and the complete 3' UTR (Mummidi *et al.*, 1997).

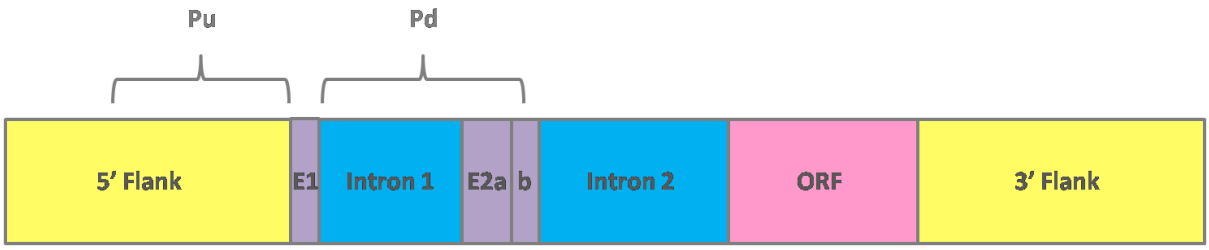


Figure 6: Structure of the CCR5 gene (image created by Fatima Barmania)

The CCR5 gene has several ATG transcription sites, prior to the start codon of exon 3, leading to different CCR5 transcripts, which vary in their 5' UTR regions (Mummidi *et al.*, 1997). These transcription start sites are usually negated by stop codons preceding exon 3, with the longest resulting cistrons being nine and 15 amino acids long. The formation of multiple transcripts upstream of exon 3 is a potential regulator of CCR5, through reduction of protein expression. The CCR5 protein containing transcripts are referred to as CCR5A and CCR5B. These two transcripts code for the same CCR5 protein with CCR5B lacking the 235 bp exon 2b fragment.

The two promoters for CCR5, Pu and Pd, contain sequences with high proportions of pyrimidines, which is unusual for promoters and lack the canonical TATA and CCAAT sequences (Mummidi *et al.*, 1997). However, the Pd promoter consists of a non-consensus TATA box. Furthermore, the upstream or Pu promoter was found to be weaker than the downstream or Pd promoter with the latter having up to five fold greater activity (Liu *et al.*, 1998). Liu and co-workers (1998) characterised the transcriptional activity of the Pd promoter, which contained two TATA motifs as well as a myriad of transcription factor binding sites. Negative regulatory factors were found in intron 2 and the region upstream of the Pd promoter. The use of two promoters results in different CCR5 transcripts with transcription occurring at multiple start sites found in either exon 1 or exon 2 (Mummidi *et al.*, 1997; Mummidi *et al.*, 2007).

Transcription of the CCR5 gene driven by the Pu promoter contains exon 1, and results in CCR5A or B (Mummidi *et al.*, 2007). Alternatively, transcription driven by the Pd promoter results in truncated isoforms, which are not individually named. Contrary to previously established data that suggested the Pu promoter was less transcriptionally active and therefore not an important determinant in CCR5 gene expression, Mummidi and co-workers showed that this was an error (Mummidi *et al.*, 2007). Using RT-PCR based methods, the group identified Pd as the promoter used in unstimulated primary T-cells, whereas Pu was initially used in stimulated T-cells. The erroneous data was a result of transformed T-cells being used, thereby affecting the level of CCR5 protein expression via the Pu promoter.

The promoter regions include various sites for transcription factor binding, which according to Bream and co-workers (1999) can produce various CCR5 promoter alleles that affect CCR5 expression. Mummidi and co-workers (1997) found binding sites for activator protein one, octamer one (Oct-1) and GATA transcription factors on the CCR5 gene. In addition, Moriuchi and co-workers (1997) found 11 designated areas deemed to be protected from DNase digestion. The areas consisted of sequences resembling transcription factor binding sites. In 1999, the latter group discovered that one of the DNase protected areas contained a third GATA binding site (Moriuch *et*

al., 1999). Furthermore, the group found that one of the binding sites for GATA, designated GATA-1, up-regulated CCR5 promoter activity and that mutation of the binding site significantly reduced transcriptional activity. In the Pd promoter, Liu and co-workers found a myriad of transcription factor binding sites, with nuclear factor-kappa-beta being an effective stimulator of the CCR5 promoter (Liu *et al.*, 1998).

The transcription factor Oct-1 was found to negatively regulate the Pu promoter. Octamer two (Oct-2), however, was found to actively stimulate the promoter (Mummidi *et al.*, 2007). The Oct transcription factors have been shown to up-regulate CCR5 protein expression and increase fusion with R5 HIV (Moriuchi and Moriuchi, 2001). Rosati and co-workers (2001) identified multiple binding sites for CCAAT/enhancer-binding protein beta (CEBPβ) within the CCR5 gene, mainly in the intron and promoter. Moreover, they found that an increase in this transcription factor increased the activity of the promoter. In lymphoid and myeloid cell lineages, CEBPβ binds to sites in the intron. Meanwhile, promoter-binding sites were found in specific cell types in the myeloid lineage. The importance of CEBPβ was indicated by the high levels of the factor in HIV positive individual blood cells. Furthermore, these individuals had higher levels of CCR5 positive lymphocytes. Rosati and co-workers (2001) further supported the study conducted by Liu and co-workers (1998), in which a negatively regulating sequence was found in the intronic region, emphasising the importance of intronic regions in CCR5 expression.

The regulation of CCR5 and its effect on protein expression at the level of the cell membrane is complex. The introns as well as sequences in the 5' and 3' UTR have been found to affect CCR5 gene regulation (Mummidi *et al.*, 1997). Thus, mutations in these regions should be considered important in regulation.

The CCR5 gene has been numbered according to the following systems:

- The Genbank accession number U95626
- Delineating position 1 as the start of the coding region with 1 describing the first position in exon 1 (Mummidi *et al.*, 1997)
- Delineating position 1 as the start of translation (CCR5 AIDS Symposium held at the NCI-FCRDC, Frederick, MD, April 30 1999).

For the purpose of the present study, the latter numbering system was used.

2.13 THE CCR5Δ32 MUTATION

The CCR5Δ32 mutation was initially discovered in 1996 (Dean *et al.*, 1996; Liu *et al.*, 1996; Samson *et al.*, 1996a) as a genetic mutation that confers protection to cells from infection by HIV. Genetic analysis of the ORF of the gene by Liu and co-workers (1996) revealed a deletion of 32 basepairs consisting of nucleotides 794 to 825. The deletion involves a frameshift mutation with the inclusion of seven novel amino acids following amino acid 174 and a stop codon at amino acid 182 (Figure 7). The mutant allele contains 215 amino acids in comparison to the full-length 352 amino acid wild type CCR5. Samson and co-workers (1996a) found that the region affected in the protein involved the second extracellular loop. The subsequent protein lacked the last three transmembrane domains as well as regions important in G-protein interaction and signal transduction. Both groups discovered that $CD4^+$ cells with the mutated CCR5 prevented HIV envelope fusion.

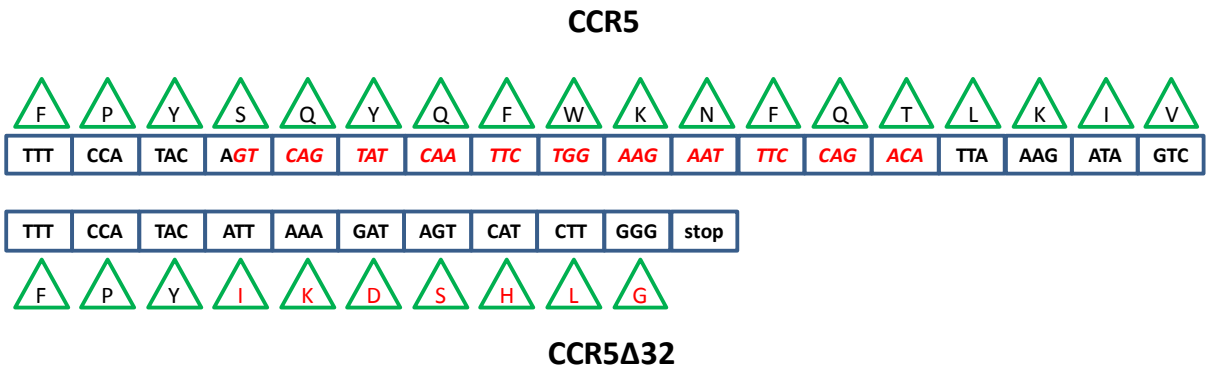


Figure 7: Illustration of the region involving the Δ32 mutation with the upper section showing the translation of the wild type CCR5 protein while the lower section demonstrates the translation of the mutant protein. The red highlighted region in the wild type sequence refers to the region deleted in Δ32. The red highlighted region in the mutant protein sequence refers to the novel amino acids inserted followed by the stop codon (image created by Fatima Barmania).

The Δ32 mutant allele is confined mostly to individuals of European descent, at gene frequencies of approximately 10 %, and has a north to south latitude decline in frequency (Martinson *et al.*, 1997). Martinson and co-workers (1997) analysed the distribution of the Δ32 mutation in more than 3000 individuals from various countries and found a 2-5 % gene frequency in Europe, Middle East and some parts of the Indian subcontinent. Isolated incidences of Δ32 found in other regions were attributed to European gene flows into these areas. The highest frequency of the mutation was discovered in the Ashkenazi Jewish population at frequencies of 20.93 %. In 2005, Novembre and co-workers confirmed these results when they assessed the Δ32 frequency in various population groups worldwide. The mutant allele is absent in Black populations excluding the African American group which may have acquired the mutation through admixture (Dean *et al.*, 1996; Liu *et al.*, 1996; Samson *et al.*, 1996a).

The origin of the Δ32 mutant allele was dated back to between 275 - 1875 years ago, as a unique mutation that increased over the years due to a selective pressure (Stephans *et al.*, 1998). Stephans and co-workers used haplotype analysis on the chromosomes of 192 Caucasian individuals and estimated the origin using a coalescence theory. They hypothesised that the Black plague was the strong selective pressure that caused the mutant allele boom.

On the contrary, historical data suggests that the Black plague is not the selective force. The distribution of Δ32 from a north to south gradient does not correspond with casualties of the plague. In fact, the distribution follows a south to north gradient (Cohn and Weaver, 2006). Moreover, the Black plague showed the greatest casualties and effects in areas with the lowest allele frequencies of Δ32, such as the Mediterranean region and China. In 2004, Mecsas and co-workers infected CCR5 deficient mice with the bacterial pathogen known to cause the Black plague. The experimental data demonstrated no differences in bacteria growth or survival of the deficient mice in comparison to CCR5 containing mice. The finding of ancient DNA in skeletal remains dating back 2900 years presents further evidence. The Δ32 allele frequency found in these remains was similar to that found in individuals ridden by the plague in the same region (Hummel *et al.*, 2005).

Smallpox was another pandemic deemed culpable for the Δ32 mutant allele increase (Galvani and Slatkin, 2003). The pandemic had severe casualties that exceeded that of the plague. Smallpox is a virus similar to HIV, as poxviruses are known to infect lymphocytes using chemokine receptors (Galvani and Novembre, 2005). Conversely, historical evidence refutes this theory as smallpox started outside of Europe and did not affect the country more significantly than any other (Cohn and Weaver, 2006). The discovery of ancient DNA with similar allele frequencies of Δ32 indicated that historic pandemics such as the plague and smallpox did not result in the allele increase.

In correspondence, Faulds and Horuk (1997) suggested that Δ32 arose without a selective event. Tandem repeats found in the coding region of the CCR5 gene could cause unequal homologous recombination, which results in the Δ32 allele. However, the origins of the Δ32 mutation remain an enigma.

The hype surrounding the Δ32 mutation stems from its ability to protect homozygous individuals from HIV. However, in 1997, studies showed that some individuals with homozygous Δ32 were infected with HIV (Biti *et al.*, 1997; O'Brien *et al.*, 1997; Theodorou *et al.*, 1997). Analysis of the HIV strains in these individuals revealed the presence of X4 utilising HIV, accompanied by very rapid $CD4^+$ T-cell decline (Michael *et al.*, 1998). This indicates that the mutation does not protect Δ32 homozygous individuals from viral strains utilising alternative receptors.

The protective effect of the Δ32 mutation towards HIV is a consequence of an inhibition of CCR5 protein expression on the cell surface. This prevents HIV from utilising the receptor for viral entry. In addition, the Δ32 protein, localised to the endoplasmic reticulum, exerts a TDN effect on the wild type CCR5 protein, inhibiting its transport to the cell surface (Benkirane *et al.*, 1997; Chelli *et al.*, 2001). Further *in vitro* investigation by Agrawal and co-workers (2004) showed that the mutant protein reduces the surface expression of wild type CCR5 and CXCR4 by dimerisation. This conferred a broad-spectrum inhibition to R5, X4 and R5X4 HIV infection. Homozygous Δ32 individuals with mutant protein expression in $CD4^+$ cells were shown *in vivo* to have down-regulated CXCR4 surface protein expression and decreased susceptibility of the cells to X4 infection. Thus, the Δ32 protein product is of great importance in providing resistance to HIV infection. This was further supported by evidence suggesting that Δ32 homozygous individuals with HIV infection do not stably express the Δ32 protein, and are devoid of the molecular mechanism of protection (Agrawal *et al.*, 2004; Agrawal *et al.*, 2007). Stable expression of the mutant protein can be affected by polymorphisms in the promoter region of the gene (Jin *et al.*, 2008). Individuals homozygous for Δ32 with the 59537-A/A variant had reduced expression of mutated protein in comparison to homozygous individuals with the wild type 59537-G/G promoter variant.

2.14 CCR5 MUTATIONS

Mutations in the coding and promoter regions of the CCR5 gene have been well documented in relation to HIV infection and progression. Coding region mutations affect the CCR5 protein structure, which can affect production, transport, chemokine binding, signalling and expression of the CCR5 receptor. Mutations in the promoter region can affect the DNA transcription factor binding or regulatory sites leading to aberrations in CCR5 mRNA production.

2.14.1 Coding region mutations

Six of the variants in Table 1 (I12T, C20S, I42F, L55Q, A73V, and C101X) have been previously identified in individuals with Δ32 (Carrington *et al.*, 1997; Quillent *et al.*, 1998).

Table 1: List of previously identified mutations in the protein-coding region of the CCR5 gene

Variant	Nucleic Acid Substitution	Protein Region
I12T[a,b]	A25C	N- terminal
C20S[b]	T58A	N- terminal
A29S[a,b]	G85T	N- terminal
I42F[b]	A124T	TMD 1
L55Q[b,c]	T164A	TMD 1
R60S[b]	G180T	ICL 1
A73V[b]	C218T	TMD 2
C101X[d]	T303A	ECL 1
G106R[b]	G316A	TMD3
C178R[e]	T532C	ECL 2
S185R[b]	A553C	ECL 2
L215S[a,c]	A187T	TMD 5
I254T[a,b]	T758C	TMD 6
R223Q[b,c]	G668A	ICL 3
228delK[b]	680del3	ICL 3
C269F[b]	G806T	ECL 3
G301V[b]	G902T	TMD 7
FS299[c]	893delC	TMD 7
A335V[b,c]	C1004T	C-terminal
Y339F[a,b,c]	A1016T	C-terminal

Footnote: All variants (except 228delK and FS299) are named with the first letter indicating wild-type amino acid. The number between the wild type amino acid and the last letter indicates the position on the CCR5 protein, and the last letter indicates the mutant amino acid. 228delK is a deletion of a three-nucleotide codon, which codes for lysine at position 228 while FS299 is a frameshift caused by single basepair deletion. [a] Represents variants causing conservative amino acid changes. [b] represents variants discovered in Carrington *et al.*, 1997 and [c] represents variants discovered by Ansari-Lari *et al.*, 1997, [d] represents variants discovered by Quillent *et al.*, 1998, [e] represents variants discovered by Magierowska *et al.*, 1999.

The C101X (Quillent *et al.*, 1998) and FS299 (Ansari-Lari *et al.*, 1997) variants result in premature termination of translation. The C101X variant discovered in an HIV negative homosexual male individual, frequently exposed to HIV, results in substitution of cysteine at position 101 of the CCR5 protein with a stop codon and has a high allele frequency in central Africa (Blanpain *et al.*, 2000). Consequently, this premature termination of translation forms a protein that contains only two transmembrane domains. Studies performed *in vitro* indicate that this protein is not expressed on

the cell surface, does not function as an HIV co-receptor and is misfolded. The consequence of these factors is that the protein is retained intracellularly (Blanpain *et al.*, 2000). The FS299 variant, found at 3-4 % allele frequency in Asia (Blanpain *et al.*, 2000), is a frameshift mutation causing absence of the latter portion of the seventh TMD and complete absence of the C-terminal. Protein expression on the cell surface is reduced because of intracellular retention. Furthermore, it does not bind or respond to chemokines but still has the ability to bind to HIV, albeit with reduced efficiency (Blanpain *et al.*, 2000).

The I12T, C20S and A29S variants are all located in the N-terminal. According to Carrington and co-workers (1999), the variants markedly reduce cell surface expression and ligand binding with the former two not functioning as HIV co-receptors. Conversely, Blanpain and co-workers (2000) found the I12T variant mediated HIV entry. The C20S variant prevents disulfide bond formation between the N-terminal and ECL 3. Considering the importance of this bond in chemokine binding (Blanpain *et al.*, 1999b), the variant is unable to function or respond to chemokines *in vitro* (Blanpain *et al.*, 2000). The variant also reduces cell surface expression but does not prevent HIV co-receptor function. Blanpain and co-workers (2000) also refute the findings of the A29S variant, as expression on the cell surface was found to be within the normal wild-type range. However, the variant does not respond to MIP-1α and MIP-1β but does respond to MCP-2 and can function as an HIV co-receptor (Blanpain *et al.*, 2000).

The I42F, L55Q, and A73V variants are found in the first and second TMDs, and according to Carrington and co-workers (1999), these variants support HIV infection and have a 4 - 8 fold higher affinity for ligands. Howard and co-workers (1999) demonstrated that both I42F and A73V have reduced surface expression in comparison to wild-type receptors, with the former showing increased binding to RANTES and the latter having decreased HIV co-receptor activity. The L55Q receptor is a variant affecting a highly conserved residue, which is important in mediating receptor activation but not chemokine binding affinity (Blanpain *et al.*, 2000).

The C178R variant, initially discovered in the Vietnamese population (Magierowska *et al.*, 1999), affects a highly conserved cysteine involved in disulfide bonding between ECL-1 and ECL-2, which is important for CCR5 structure and in HIV binding (Wu *et al.*, 1997). Studies show the variant causes a dramatic reduction in cell surface expression with intracellular retention of the misfolded receptor (Blanpain *et al.*, 2000). The mutant receptor does not bind or respond to chemokines but can still bind HIV.

The R223Q variant is also known to affect a conserved residue. Carrington and co-workers (1999) observed a decrease in variant co-receptor function while still maintaining the ability to bind to gp120. Analysis by Capoulade-Metay and co-workers (2004) found no change in CCR5 expression or chemokine binding while Zhao and co-workers (2005) found no effect on HIV infection *in vitro* or the CCR5 mRNA level.

The G106R variant changes the residue hydrophobicity in the third TMD, resulting in reduced surface expression (Capoulade-Metay *et al.*, 2004) without affecting levels of mRNA production (Zhao *et al.*, 2005). The variant also decreases binding to chemokines and HIV.

The S185R, I254T and C269F variants are found in the South East Asian population group (Capoulade-Metay *et al.*, 2004). The former two variants may alter residue charge and hydrophobicity respectively. The receptor function and HIV co-receptor activity, however, remain similar to the wild-type receptor. In contrast, the C269F variant disrupts the conserved cysteine involved in disulphide linkage of ECL-3 to the N-terminal. This results in reduced cell surface expression, decreased binding to MIP-1β and RANTES and weak HIV co-receptor binding.

In South Africa, the Δ32 mutation is found heterozygously in the Caucasian population at an allelic frequency of 9.4% and is virtually absent from the Black African population. The C101X variant was not detected in the South African Black population but was found at an allelic frequency of 0.7% in Caucasians (Williamson *et al.*, 2000).

In 2001, Peterson and co-workers identified seven novel variants (D2V, P35, Y89, L107F, P162, R225X and R225Q) exclusive to the Black African and Coloured population groups as well as six previously discovered variants (L55Q, S75, R223Q, A335V and Y339F). Further investigation by Hayes and co-workers (2002) showed that the novel P35 variant was not significantly different in slow versus fast progressors. The A335V variant, found homozygously in a Black African female and heterozygously in a coloured male and female, may have significantly contributed to the long-term non-progression seen in these individuals.

In 2010, mutant receptor constructs from four novel variants (L107F, R225Q, D2V, and R225X) identified in the Peterson paper were analysed *in vitro* for there effects on expression, chemokine binding and response, and HIV co-receptor properties (Folefoc *et al.*, 2010). The D2V variant found in the N-terminal showed decreased CCR5 expression, reduced chemokine binding and response, and lowered HIV infection. The R225X mutation, on the other hand, resulted in premature termination of translation in the third cytoplasmic loop, showed no surface expression, chemokine binding and

response, and could not support HIV infection. The latter mutation was found heterozygously and could partially protect against HIV in these individuals.

Studies in South Africa have focussed mostly on determining Δ32 frequency and mutations in the ORF. However, in 2010 a 9.2 kb region encompassing the entire CCR5 gene was analysed for SNPs, indels and haplotypes in 35 Black and 35 Caucasian individuals in South Africa (Picton *et al.*, 2010). The study revealed 68 SNPS, four indels, the Δ32 mutation as well as seven complex haplotypes, while illustrating that the diverse variation in the CCR5 gene in the South African population may explain differences in response to HIV.

2.14.2 Promoter region mutations

A plethora of studies surrounding mutations and effects has been completed on the ORF of the CCR5 gene. Although these mutations significantly influence CCR5 and HIV infection, the mutations within the intronic, promoter and untranslated exons should be included.

Martin and co-workers (1998) performed denaturing high pressure liquid chromatography and single strand confirmation polymorphism techniques on HIV positive patients. They identified 10 SNPs that in different combinations created four common haplotypes (CCR5 P1-4) and six infrequent haplotypes (CCR5 P5-10). These haplotypes affect the progression of HIV at varying rates.

A CCR2 variant, known as CCR2-64I and located in the first TMD of the CCR2 protein, slows the progression of HIV irrespective of Δ32 status (Smith *et al.*, 1997; Kostrikis *et al.*, 1998). Due to the close proximity of CCR2 and CCR5, linkage disequilibrium between variants in the two genes may cause the slow progression as the CCR2 receptor is rarely used for HIV infection (Deng *et al.*, 1996). A cytosine/thymine (C/T) polymorphism at position 59653 (according to U95626) in intron 2 of the CCR5 gene was shown to be in 100 % linkage disequilibrium with the CCR2-64I polymorphism (Kostrikis *et al.*, 1998); however, the protection provided via linkage of the variants could not be established (Martin *et al.*, 1998).

An A/G polymorphism at position 59029 (according to U95626) in the promoter region of CCR5 has been shown to affect HIV progression (McDermott *et al.*, 1998). Individuals with an A/A genotype progress rapidly towards AIDS in comparison to individuals with the G/G genotype which progress 3.8 years more slowly. The latter genotype was associated with 45 % lower promoter activity (McDermott *et al.*, 1998). The A/A allele is found in the CCR5 P1 haplotype and has potentially greater efficient promoter activity, even though it is not found in any transcription factor binding regions. Shieh and co-workers (2000) found that individuals homozygous for the A/A genotype had

higher levels of $CD4^+$ cells expressing CCR5. This polymorphism was shown to determine CCR5 expression and HIV infection *in vitro* (Salkowitz *et al.*, 2003).

A study performed by de Souza and co-workers (2006) on the effect of four promoter polymorphisms on HIV perinatal transmission in Brazillian children, revealed two genotypes affecting HIV transmission. The 59353 T/T genotype was associated with an increase in HIV transmission, while the 59402 A/A genotype has a protective effect. Strong linkage disequilibrium between the 59029 A/A and 59353 T/T genotypes has been identified. Individuals lacking these genotypes progress more slowly towards AIDS (Clegg *et al.*, 2000). Promoter variants can also affect transcription factor binding. This was illustrated by Bream and co-workers (1999) who showed that certain variants bind more efficiently than other variants do.

2.15 ROLE OF CCR5 IN DISEASE

Individuals homozygous for the Δ32 mutation, which abolishes CCR5 receptor expression, are healthy and at no apparent disadvantage (Biti *et al.*, 1997; Theodorou *et al.*, 1997). Other than its protective effect in HIV infection, the mutations role in providing a protective or negative impact on other diseases remains controversial.

In rheumatoid arthritis, $CCR5^+$ monocytes are found in the synovial fluid (Kohem *et al.*, 2007) as the receptor plays a role in the inflammatory process. A meta-analysis showed that the Δ32 mutation provides protection in the latter disease (Prahalad *et al.*, 2006), as the severity of the disease is reduced (Scheibel *et al.*, 2008). In renal allograft outcome, CCR5 deficient mice had a significant decrease in chronic renal transplantation rejection (Dehmel *et al.*, 2010). This favourable outcome is due to a decrease in inflammatory reactions in the acute phase while in the chronic phase the alternative macrophage pathway is activated. During the classic macrophage pathway a strong pro-inflammatory response ensues, but in the alternative pathway a wound healing or immune-suppressive response is characterised (Martinez *et al.*, 2009).

The Δ32 allele has also been associated with protection in coronary artery disease and myocardial infarction (Szalai *et al.*, 2001; Gonzalez *et al.*, 2001; Pai *et al.*, 2006). The mutant allele was associated with lower levels of triglycerides and higher HDL plasma levels, both beneficial in reducing the risk of cardiovascular disease (Hyde *et al.*, 2010). The use of the CCR5 inhibitor, maraviroc, was also associated with better lipid profiles (DeJesus *et al.*, 2008). In 2006, Henckaerts and co-workers postulated that a deficiency of CCR5 could be protective in primary sclerosing cholangitis (PSC). Conversely, the Δ32 allele frequency was significantly higher in PSC patients

compared to controls and furthermore the former individuals had more severe liver disease (Eri *et al.*, 2004).

Studies in mice show that CCR5 deficiency increases the severity of brain injury in stroke (Sorce *et al.*, 2010). Deficiency in CCR5 is also associated with impaired osteoclast differentiation and osteoblast maturation leading to defective bone repair in mice (Hoshino *et al.*, 2009). Glass and co-workers (2005) showed that in mice, CCR5 is essential for survival against West Nile virus (WNV) infection. A further study in humans homozygous for Δ32 illustrated a significant correlation between the mutation and fatality in WNV infection (Glass *et al.*, 2006).

High levels of CCR5 are significantly associated with non-metastatic colorectal cancer, whereas weak or deficient CCR5 expression is significantly associated with an advanced form of the disease (Zimmerman *et al.*, 2010). In bone marrow transplants, CCR5 presence on $CD4^+$ T-regulatory cells is important in prolonging graft survival (Wysocki *et al.*, 2005). This was corroborated by a study, which showed that CCR5 genotypes with higher levels of expression have a significant survival outcome (McDermott *et al.*, 2010). However, contrary to these findings, Bogunia-Kubik and co-workers (2006) demonstrated that Δ32 individuals have a reduced likelihood of developing graft vs. host disease.

Phenotypic expression of CCR5 on CD4$^+$ T-lymphocytes

4.1 INTRODUCTION

Chemokine co-receptor 5 (CCR5) can be measured phenotypically on CD4$^+$ T-cells in two ways:

- the number of CD4$^+$ cells co-expressing CCR5 (CD4$^+$CCR5$^+$ T-cell percentage)
- the number of CCR5 receptors per CD4$^+$ cell (CCR5 density)

The phenotypic expression of CCR5 is an important factor for HIV infection and progression. In Δ32 homozygous individuals, the absence of CCR5 phenotypically, promotes resistance to HIV (Dean *et al.*, 1996; Liu *et al.*, 1996; Samson *et al.*, 1996). Heterozygotes can still be infected with HIV, but have the advantage of slower disease progression. In comparison to individuals with wild-type CCR5, heterozygotes tend to live longer with HIV, have lower viral loads and slower diminishing CD4$^+$ cell counts (Dean *et al.*, 1996; Huang *et al.*, 1996; Zimmerman *et al.*, 1997). This is attributed to decreased infectability by macrophage tropic viral strains (Liu *et al.*, 1996) and reduced cell surface CCR5. Wu and co-workers (1997) showed a positive correlation between CCR5 expression and *in vitro* HIV infection. In addition, CCR5 expression is a key determinant in predicting viral load (Reynes *et al.*, 2000; Reynes *et al.*, 2001). Thus, the aim of this study (Chapter 4) was to analyse CCR5 expression in a selected cohort of the South African population using the two measurements indicated above.

Reynes and co-workers (2000) found that there was no correlation between these two measurements. Some individuals express low CD4$^+$CCR5$^+$ T-cell percentages with high density, while other individuals have the opposite pattern. This lead to the null hypothesis investigated in this study:

H_0: There is no correlation between CD4$^+$CCR5$^+$ T-cell percentage and CCR5 density.

Ethnicity, gender and age were the three independent variables selected to explain the variation in the CD4$^+$CCR5$^+$ T-cell percentages and CCR5 density. The null hypotheses includes: (1) There is no relationship between CD4$^+$CCR5$^+$ T-cell percentages and age, gender or ethnicity and (2) there is no relationship between CCR5 density and age, gender or ethnicity.

The level of transcription, and subsequently protein expression, has been shown to decrease with age (Heydari *et al.*, 1993; George *et al.*, 1995). However, it has previously been shown that CCR expression, including CCR1 - CCR5, increases with age on the surface of T-cells in both humans (Yung *et al.*, 2003) and mice (Mo *et al.*, 2003). In a meta-analysis of HIV data known as the Cascade study

(2000), results from thousands of individuals were pooled. The results demonstrated that the incidence of AIDS and associated death, increased significantly with age. The cause of this incidence was not established, however, the present study will attempt to determine if there is a link between CCR5 expression and age.

Many studies have indicated gender specific variations in HIV infection and progression (Gandhi *et al.*, 2002). Mo and co-workers (2005) showed that T-cells express estrogen receptors which play an important role in the cell function. The group illustrated that five different strains of female mice over-express CCR1-CCR5, with the up-regulation of gene expression increasing CCR expression phenotypically.

Compared to Caucasians, the Δ32 mutation is rarely found in Black populations (Williamson *et al.*, 2000). These variances in CCR5 genetics affect phenotypic differences in CCR5 amongst ethnic groups. Gonzalez and co-workers (1999) found that there are altered rates of HIV disease progression in Caucasians and African Americans depending on the CCR5 haplotype. The CCR5 human haplotype C (HHC) is associated with delaying HIV disease progression in Caucasians, while the same haplotype may accelerate progression in African Americans (Gonzalez *et al.*, 1999). Conversely, the HHE haplotype is associated with disease acceleration in homozygous Caucasians while in African Americans it is not associated with disease modifying effects. A study by Kalinkovich and co-workers (2001) compared the surface expression of CCR5 in Ethiopian immigrants living in Israel to native Caucasian Israelis. The study found that in Ethiopian immigrants, activated and resting $CD4^+$ T-cells express higher levels of both $CD4^+CCR5^+$ T-cell percentage and CCR5 density than the native Caucasian Israelis. They inferred that this high level of CCR5, independent of environmental factors, may account for the high rates of HIV infection in Africa.

Although studies have demonstrated that infectious agents such as HIV and TB can affect CCR5 expression (Juffermans *et al.*, 2001; Shalekoff *et al.*, 2001) the present study did not test participants for these infections.

Considering the low frequency of individuals that are phenotypically null and the rarity of Δ32 homozygotes in South Africans (Williamson *et al.*, 2000; Peterson *et al.*, 2001; Picton *et al.*, 2010), this study will also establish phenotypic CCR5 null prevalence in the South African population.

4.2 RESULTS AND DISCUSSION

4.2.1 Distribution of CCR5 in the South African population

The results indicate that the percentage of $CD4^+CCR5^+$ T-lymphocytes relative to the total $CD4^+$ cell population is in the range between 0-90 % (Figure 15). The total sample consisted of 245 individuals, with 188 (77 %) of these individuals found in the 10.1-30 % range. The largest number of individuals (57/245 or 23 %) was found in the 15.1-20 % cell expression bracket, followed by individuals in the 10.1-15 % bracket. This amounts to a total of 108 individuals (44 %) expressing $CD4^+CCR5^+$ cells between 10.1-20 %. A total of 80 individuals (33 %) were also found to express $CD4^+CCR4^+$ cells in the 20.1-30 % bracket. The average $CD4^+CCR5^+$ cell percentage was 21 (95 % confidence interval 19.5-22.3 %).

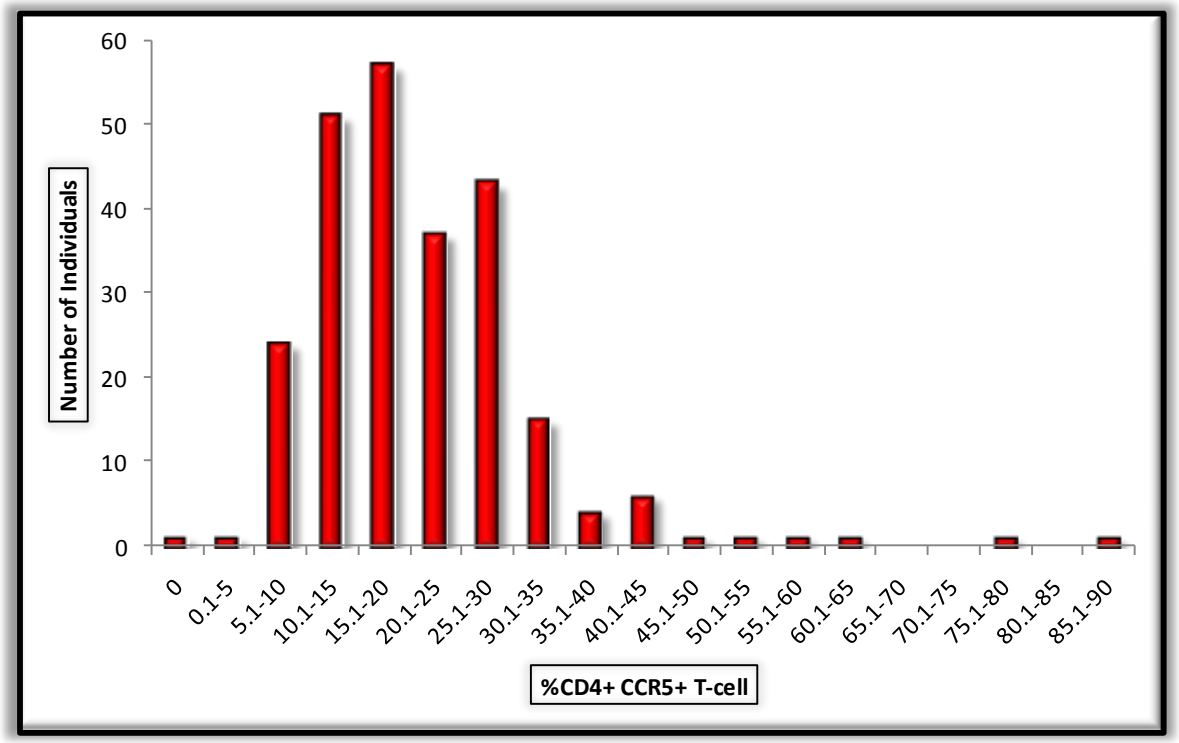


Figure 15: **Histogram illustrating the percentage of $CD4^+CCR5^+$ T-lymphocytes in a sample of the South African population (Raw data in Appendix C).**

A total of 24 (9.8 %) and 15 individuals (6.1 %) express $CD4^+CCR5^+$ cells at between 5.1-10 % and 30.1-35 %, respectively. Very few individuals, however, were found to exceed 35 % with only single individuals expressing extremely high $CD4^+CCR5^+$ cell percentages. The highest cell percentage (86.9 %) was found in a coloured individual. Absent or very low $CD4^+CCR5^+$ cell percentages are rare with only a solitary individual found in each of the 0 and 0.1-5 % brackets.

Previous studies in South Africa using healthy uninfected adult individuals found $CD4^+CCR5^+$ cell percentages of approximately 18-38 % with an average of 23 % (Shalekoff *et al.*, 2004). A study in 2001 showed a mean percentage of 30 for normal individuals and 70 for HIV infected individuals (Shalekoff and Tiemessen, 2001).

A number of different studies indicate that peripheral blood $CD4^+$-T lymphocytes express CCR5 between 5-10 % (Bleul *et al.*, 1997). Zaunders and co-workers (2001) corroborated these measurements, with individuals in their study reporting CCR5 levels between 2.5-7.5 %. Other studies have also shown mean percentages of $CD4^+CCR5^+$ cells of 6.5 (Ostrowski *et al.*, 1998) and 15 (de Roda Husman *et al.*, 1999), respectively.

In comparison to these studies, the levels of $CD4^+CCR5^+$ cells are higher in the South African population. According to Clerici and co-workers (2000), the levels of CCR5 expression in Africa are higher due to environmental factors which increase immune activation. Infection with HIV (Tuttle *et al.*, 2004) or TB (Juffermans *et al.*, 2001) is reported to increase CCR5 on $CD4^+$ T-lymphocytes. Both HIV and TB are rampant in South Africa, which could account for the high levels of $CD4^+CCR5^+$ cells found in this study. Results reported in this study (Figure 15) for individuals with very high percentages (exceeding 50 %) may indicate co-infection with HIV and TB, as studies have shown that co-infection can cause expression exceeding 65 % (Juffermans *et al.*, 2001). The HIV and TB status of the individuals in the current study was not recorded.

Individuals with very low CCR5 expression (0.1-5 %) or the absence thereof as illustrated in Figure 15 may indicate the presence of genetic factors affecting CCR5 expression. However, as discussed previously (Chapter 2) many factors affect CCR5 expression.

The density of CCR5 was measured qualitatively using MFI units (Figure 16). The results indicate that most individuals (167/245 or 68 %) had CCR5 densities between 3.6-6 arbitrary units. The greatest number of individuals was found at a density of between 4.1- 4.5 arbitrary units followed by density between 5.1-5.5 arbitrary units. The average CCR5 density in the population studied was found at 4.5 arbitrary units (95 % confidence interval 4.3-4.7). A single male Caucasian individual expressed no CCR5 at the cell surface while few individuals (6/245 or 2.5 %) exceeded 7.6 arbitrary units.

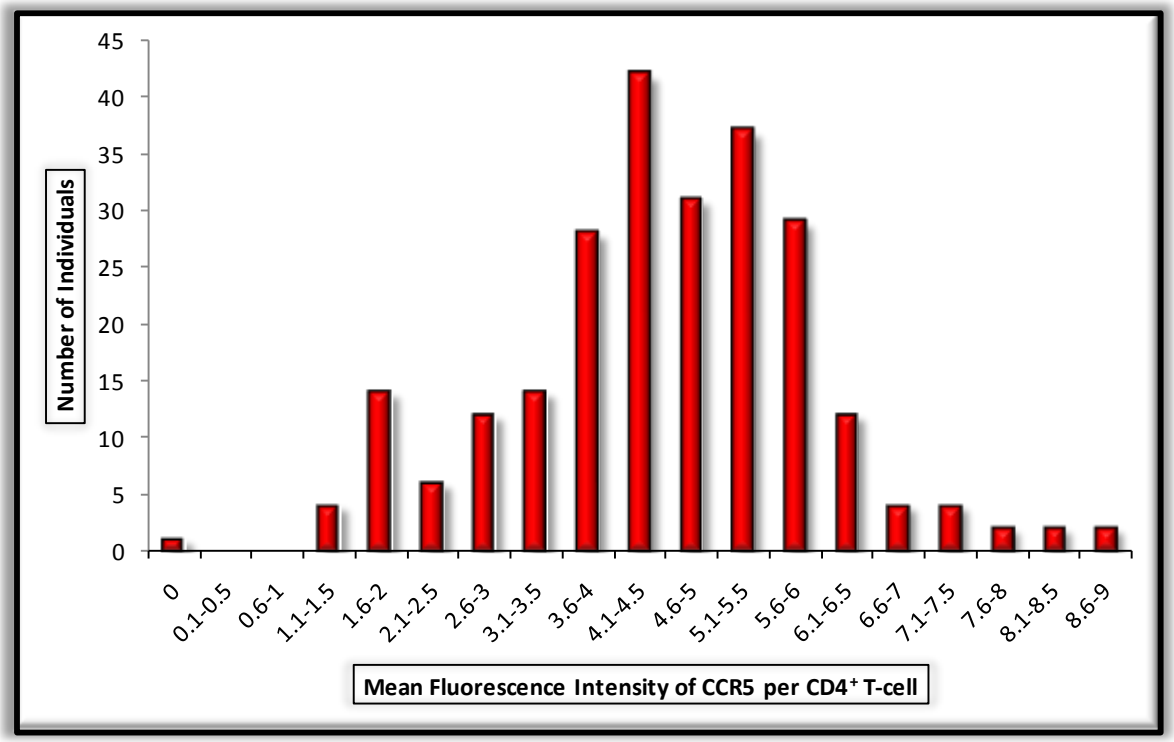


Figure 16: Histogram illustrating the qualitative CCR5 density per $CD4^+$ cell as indicated by mean fluorescence intensity of CCR5 in a sample of the South African population.

The CCR5 density as reviewed in Chapter two is an important measurement for HIV as it predicts both viral load and HIV disease progression (Reynes *et al.*, 2000; Reynes *et al.*, 2001). High levels of CCR5 density indicate a greater number of CCR5 receptors per cell. This allows a greater proportion of virions to enter the host cell. Reynes and co-workers (2001) showed that infected individuals who progress towards AIDS quickly, have higher densities than those who progress slowly. These individuals also display a rapid loss of $CD4^+$ T-cells. The density of CCR5 on $CD4^+$ cells is consistent over time and is not influenced by HIV infection (Reynes *et al.*, 2001). As a result, individuals with a higher MFI (Figure 16) will have higher viral loads and increased disease progression if infected with HIV and are possibly fast progressors. Conversely, individuals with lower CCR5 density will have lower viral loads and progress slowly.

4.2.2 Correlation between $CD4^+CCR5^+$ T-cell percentage and CCR5 MFI

The data on correlation indicates a very weak relationship between $CD4^+CCR5^+$ T-cells and CCR5 density. Pearson's correlation coefficient was used to analyse the relationship between the variables and was found at 0.2467 (95 % confidence interval 0.1253-0.3609). The scatter plot (Figure 17) further illustrates this weak correlation between the variables as individuals at 20 % $CD4^+CCR5^+$ T-cells have different CCR5 densities. The density in these individuals varies from 1.8-8.6 arbitrary units.

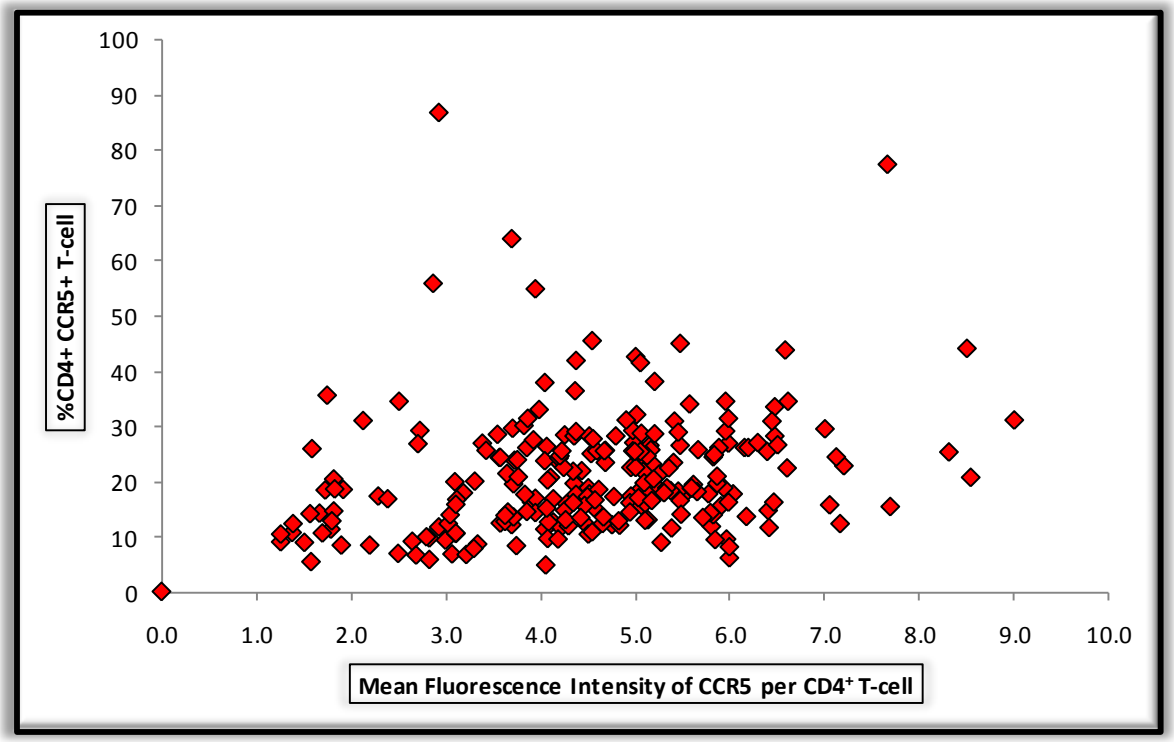


Figure 17: **Scatter plot illustrating the relationship between the percentage of $CCR5^+CD4^+$ T-cells and CCR5 density as measured by mean fluorescence intensity.**

Reynes and co-workers (2000) determined the effect of CCR5 expression, both density and percentage expression, on HIV viral load. In the control subjects, healthy and uninfected with HIV, the CCR5 density and percentage expression of CCR5 were not related. One individual expressed a $CD4^+CCR5^+$ T-cell percentage of 16 with a CCR5 density of 19 147 molecules/cell. The other individual, meanwhile, had a $CD4^+CCR5^+$ T-cell percentage of 35 but with a density of 9 132 molecules/ cell. Furthermore, HIV infected individuals with similar densities had similar viral loads irrespective of the percentage of $CD4^+CCR5^+$ T-cells.

As reviewed in Chapter two many factors affect CCR5 expression. These factors, however, affect the density and percentage expression differently, accounting for the weak correlation between these

variables. The $CD4^+CCR5^+$ T-cell percentage is induced by cell activation whereas CCR5 density is unaffected (Ostrowski *et al.*, 1998). The number of activated $CD4^+CCR5^+$ T-cells in HIV infected individuals was higher (18.5 and 39.3 %) than in uninfected individuals (3.9 and 8.9 %). These cells had higher levels of activation markers and a significant correlation was found between $CD4^+CCR5^+$ T-cell percentages and disease progression. This means that as the disease progresses, the percentage of $CD4^+CCR5^+$ T-cells increases. De Roda Husman and co-workers (1999) suggested that the increased percentages in HIV progression are due to continual immune activation via HIV.

Conversely, the CCR5 density is not affected as individuals with or without HIV display similar densities (Reynes *et al.*, 2000). In addition, the density of CCR5 before using ARVs is the same or similar to the density after treatment. Thus the two variables measured in this study are regulated at different levels which results in the very weak correlation found in this study.

4.2.3 Effect of age, gender and ethnicity on CCR5 expression

The present study recorded a total number of 58, 44 and 140 individuals between the ages 21-30, 31-40 and above 40 years, respectively. Of these age sub-categories, 57.7 % of the total sample was above 40 years of age. Figures 18 and 19 illustrate the distribution of $CD4^+CCR5^+$ T-cell percentages and CCR5 MFI respectively, in the various age categories.

The mean $CD4^+CCR5^+$ T-cell percentages were found to be 18.8, 21.3 and 21.8 for each age category, respectively. Percentages were found between 6.1 and 37.9 for the age 21-30 years (Figure 18). For 31-40 years, the distribution was found within 9.3 and 34.5 % with an outlier value of 77.5 %. For >40 years of age, the distribution was found within 0 and 45.5 %. The latter category consisted of four outlier values at 54.9 %, 55.9 %, 64 % and 86.9 %.

The data indicates that individuals in the age category 21-30 may have lower $CD4^+CCR5^+$ T-cell percentages. The incidence rate ratio (IRR) analysed in the Poisson regression test was used to explain whether the variation in $CD4^+CCR5^+$ T-cell percentage was influenced by the independent variable, in this case age. The regression results showed an IRR of 1.12 (p= 0.01) for age group 31-40 years and 1.15 (p= 0.00) for age group >40 years. This indicates that, *ceteris paribus,* age groups 31-40 and above 40 had a 1.12 and 1.15 greater rate of $CD4^+$ $CCR5^+$ T-cell percentages in comparison to age group 21-30. Therefore, as age increases the percentage of CCR5 expression on $CD4^+$ T-cells increases.

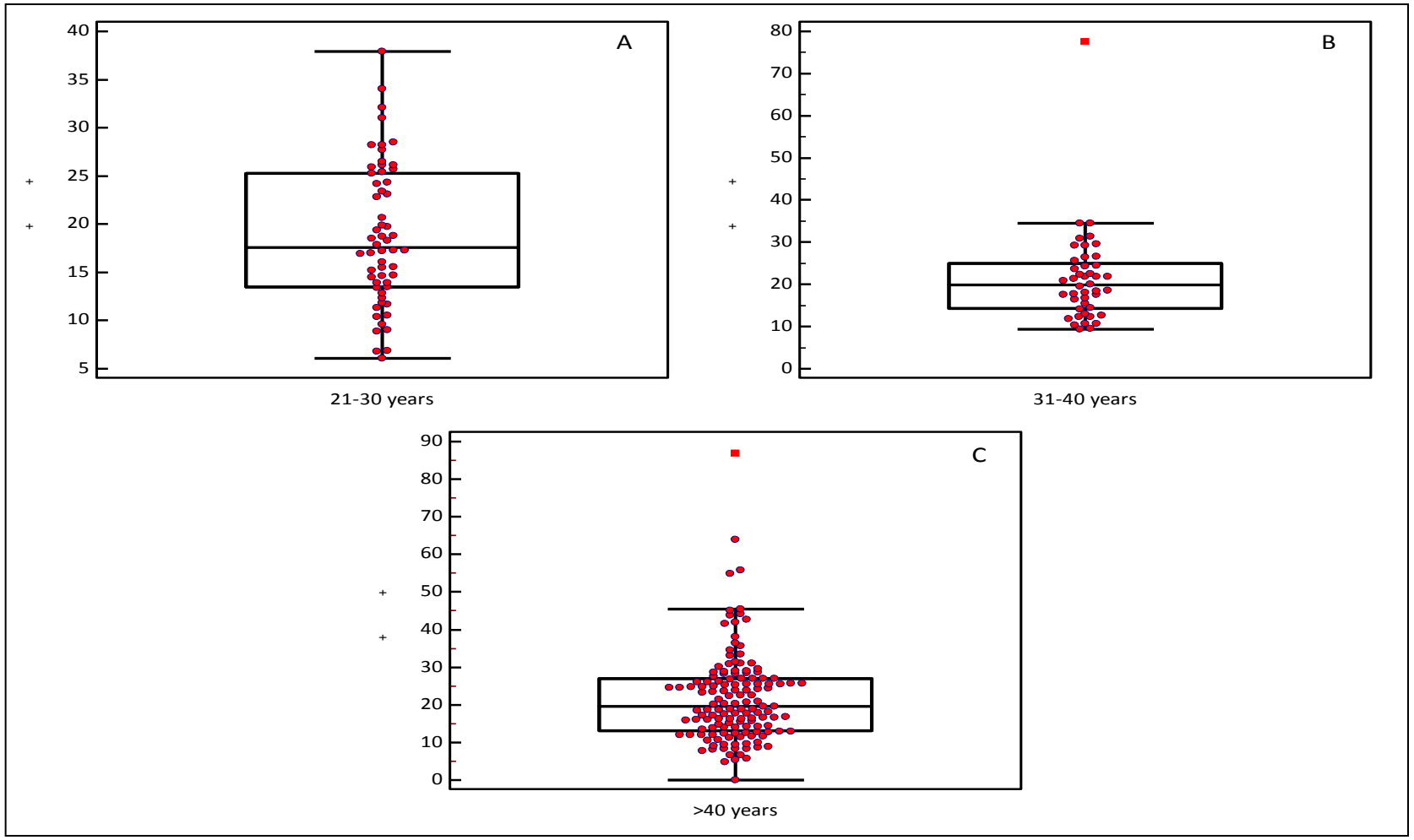


Figure 18: Distribution of $CD4^{+}CCR5^{+}$ T-cells based on ages with plot (A) representing ages 21-30 years (B) 31-40 years and (C) > 40 years. The boxes represent the middle 50 % of data values. The horizontal line within the boxes represents the median data value. The error bars indicate the 10^{th} and 90^{th} percentile values of the data set. Red squares (Figure B and C) indicate outlier values.

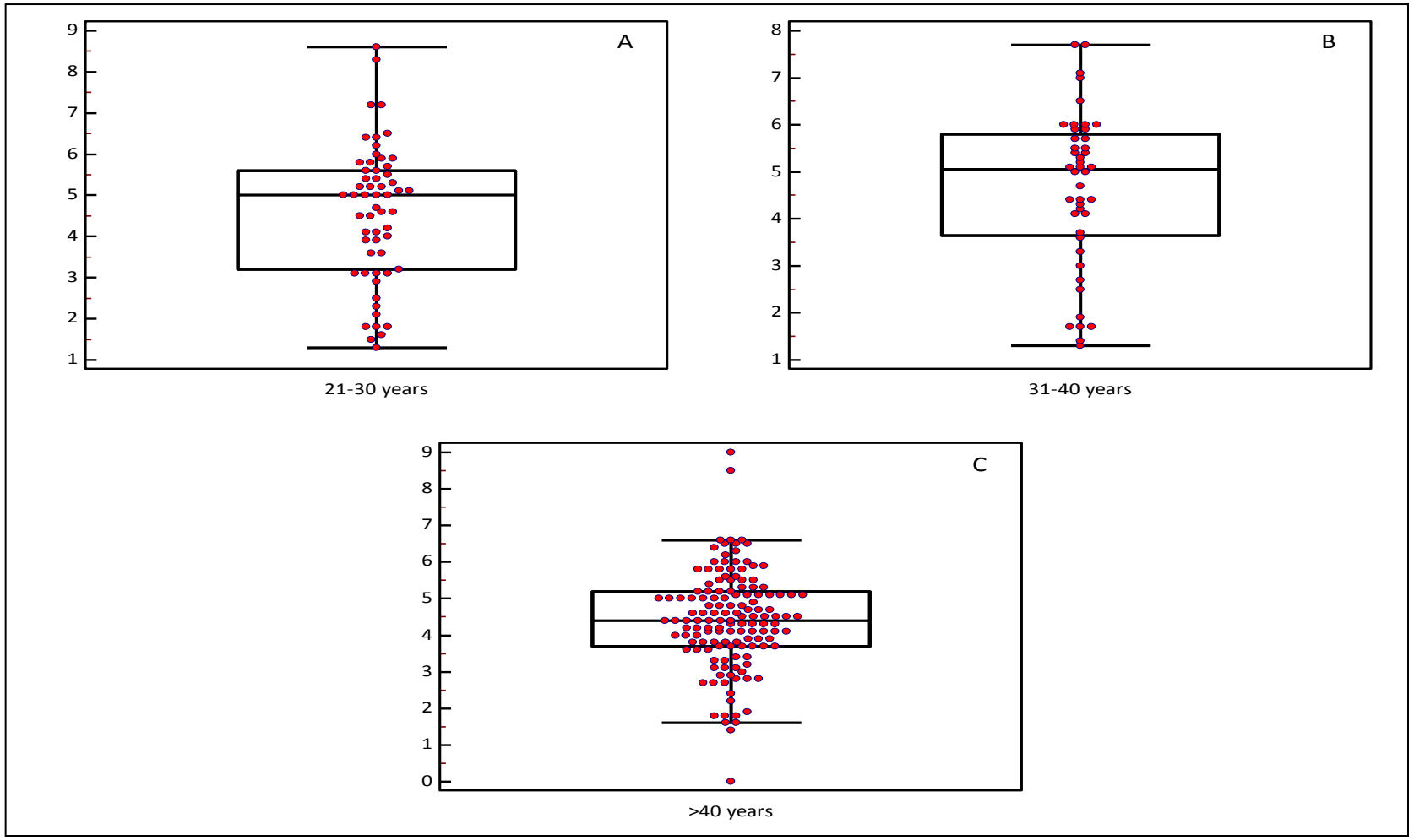


Figure 19: **Distribution of CCR5 density (MFI) based on ages with plot (A) representing ages 21-30 years (B) 31-40 years and (C) > 40 years. The boxes represent the middle 50 % of data values. The horizontal line within the boxes represents the median data value. The error bars indicate the 10th and 90th percentile values of the data set.**

The mean CCR5 density, as measured by MFI, is 4.6, 4.5 and 4.4 for each age category, respectively. The MFI (Figure 19) was found between 1.3 and 8.6 for the age 21-30 years. For 31-40 years, the MFI was found within 1.3 and 7.7.

For individuals >40 years of age, the MFI was found between 0 and 6.6 with outlier values of 8.9 and 9 arbitrary units. The mean values were not significantly different in the three age categories indicating that age does not play a role in CCR5 density. The IRR of the 31-40 and > 40 age groups were both 0.98 ($p>0.05$), which illustrates that age does not play a role in the number of CCR5 receptors per cell.

In 2003, Chen and co-workers studied the variation in chemokine expression in young and old mice. The study found increased expression of IFN-γ, MIP-1α, MIP-1β, and RANTES in the older mice. The latter three are known ligands of CCR5 whereas IFN-γ as reviewed in Chapter two of this thesis is known to increase CCR5 expression (Hariharan *et al.*, 1999; Patterson *et al.*, 1999). Further studies showed that $CD4^+$ T-cells display higher levels of chemokine receptors including CCR5 in older mice in comparison to young mice. Yung and Mo (2003) corroborated these studies in humans, showing that aging is associated with increased CCR5 expression on $CD4^+$ T-cells.

Age is associated with a decrease in naive $CD4^+$ T-cells and an increase in the memory T-cell subset (Lerner *et al.*, 1989; Ernst *et al.*, 1990). The CCR5 receptor is known to reside mostly on the memory cell phenotype (Wu *et al.*, 1997). This may explain the higher levels of $CD4^+CCR5^+$ T-cell percentages found in the older age groups (Figure 18) in the present study. Older individuals usually have quicker progression to AIDS and increased co-morbidity (Chiao *et al.*, 1999), which could be attributed to the age associated increases in CCR5. Results from this study show that age has an impact on $CD4^+CCR5^+$ T-cell percentages but not on CCR5 density. However, given the limited number of individuals in each age group in the present study, sample bias may have had an influence on the data.

The present study recorded a total number of 174 females (71 %) and 71 males.

Figure 20, illustrates the distribution of $CD4^+CCR5^+$ T-cell percentages in the two genders. Mean percentages were found at 20.8 and 21.2 for females and males, respectively. For females, the distribution was found within 4.8 % and 45.5 % with outlier values of 64 %, 77.5 % and 86.0 %. For males, the distribution was found within 0 and 45 % with outlier values of 54.9 % and 55.9 %.

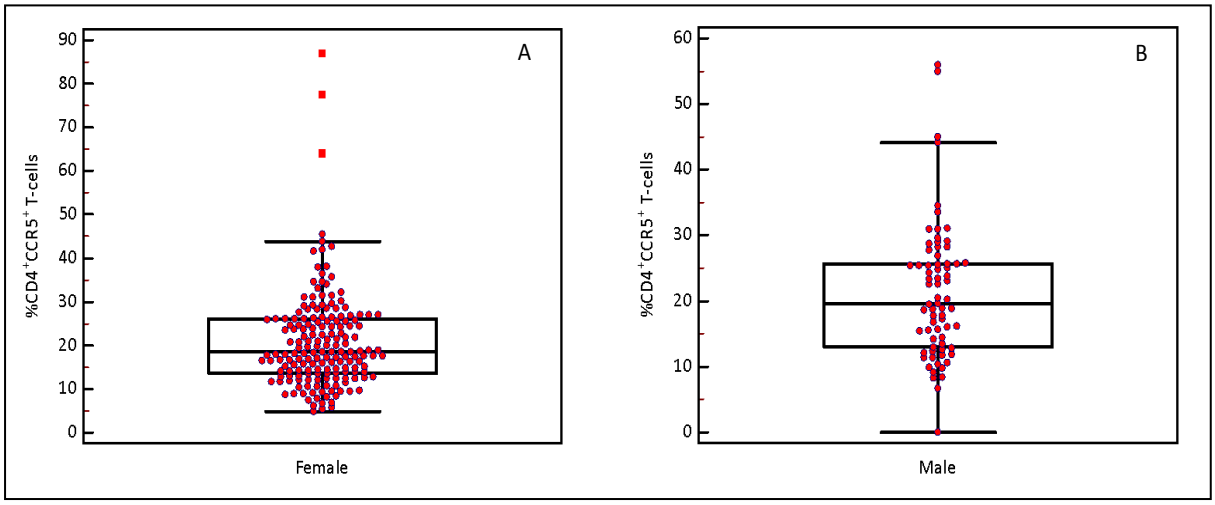


Figure 20: Distribution of $CD4^+CCR5^+$ T-cells based on gender with plot (A) representing females and (B) representing males. The boxes represent the middle 50 % of data values. The horizontal line within the boxes represents the median data value. The error bars indicate the 10^{th} and 90^{th} percentile values of the data set. Red squares (Figure A) indicate outlier values.

The data indicates that female individuals may have a lower $CD4^+CCR5^+$ T-cell percentage than males. The IRR analysed in the Poisson regression test was used to explain if the variation in $CD4^+CCR5^+$ T-cell percentage was influenced by the independent variable, in this case gender. Regression results showed an IRR of 0.93 (p= 0.03) for females. This indicates that in comparison to males, *ceteris paribus*, females have a 0.93 lower rate of $CD4^+$ $CCR5^+$ T-cell percentage. Although the statistics show this is significant, the difference in mean percentages between males and females is very small. Therefore, gender may have a possible effect on $CD4^+CCR5^+$ T-cell percentage.

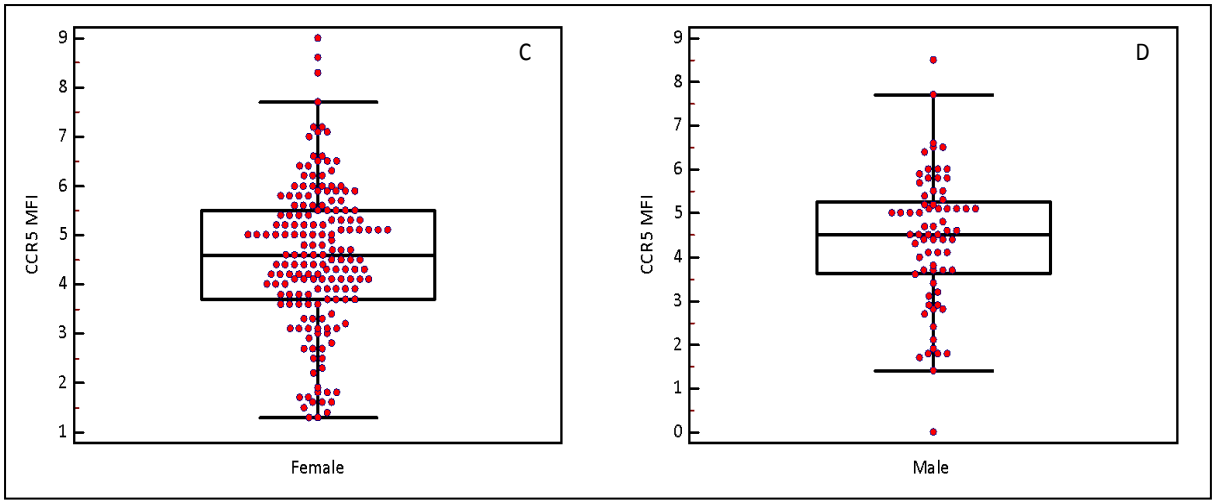


Figure 21: Distribution of CCR5 density (MFI) based on gender with (A) representing females and (B) representing males. The boxes represent the middle 50 % of data values. The horizontal line within the boxes represents the median data value. The error bars indicate the 10th and 90th percentile values of the data set.

Figure 21 illustrates the distribution of CCR5 density in females and males. Mean CCR5 density was found at 4.6 and 4.4 for females and males, respectively. For females, the distribution was found within 1.3 and 7.7 with outlier values of 8.3, 8.6 and 9. For males, the distribution was found within 1.4 and 7.7 with outlier values of 0 and 8.5 arbitrary units.

Regression results for the independent variable, gender, reported an IRR of 1.01 (p= 0.87) for females. This indicates that in comparison to males, *ceteris paribus*, females have no difference to males. Therefore, gender does not affect CCR5 density.

Differences in response to HIV have been recorded between females and males. The Δ32 heterozygous mutation does not protect against transmission in males but has been shown to protect or convey partial protection in females (Philpott *et al.*, 2003). Furthermore, a meta-analysis of 13 studies found that females display HIV RNA levels between 2-6 fold lower than males (Gandhi *et al.*, 2002). Considering the dependence of viral load on CCR5, the decreased HIV RNA in women may be due to decreased CCR5 expression. This was verified by Portales and co-workers (2001) in a study which showed that women have lower CCR5 densities compared to men.

Conversely, studies in female mice showed that estrogen increases the levels of $CD4^+CCR5^+$ T-cell percentages (Mo *et al.*, 2005). Oral contraceptives have also been demonstrated to increase both the percentage of $CD4^+CCR5^+$ T-cells and CCR5 density (Prakash *et al.*, 2002). Results from the present study show slightly lower $CD4^+CCR5^+$ T-cell percentages in females while no differences were found in CCR5 density between males and females. However, considering the number of females assessed in this study, sample bias may have had some influence in the reported results.

The present study recorded a total number of 124 (52 %) Black Africans and 110 (48 %) Caucasian individuals. The mean $CD4^+CCR5^+$ T-cell percentages were 23.4 and 17.5 for Black African and Caucasians, respectively. Figure 22 illustrates the distribution of $CD4^+CCR5^+$ T-cell percentages in the two ethnicities. Percentages were found between 4.8 and 45.5 for the Black African group with outliers of 54.9, 55.9, 64 and 77.5. For the Caucasian group, the distribution was found between 0 and 37.9 % with outlier values of 41.5 % and 45 %.

The data indicates that Black African individuals may have higher $CD4^+CCR5^+$ T-cell percentages. The regression results for the independent variable, ethnicity, showed an IRR of 1.34 ($p< 0.05$) for the Black African group. This indicates that in comparison to the Caucasian group, *ceteris paribus*, the Black African group had a 1.34 greater rate of $CD4^+$ $CCR5^+$ T-cell percentages. Therefore, ethnicity affects $CD4^+$ $CCR5^+$ T-cell percentages.

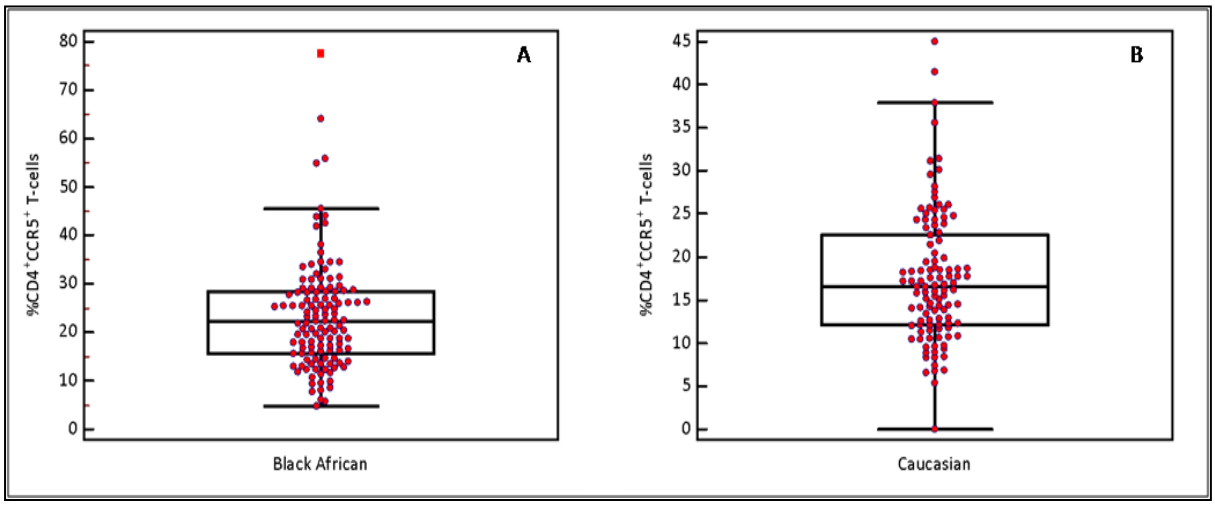


Figure 22: Distribution of $CD4^+CCR5^+$ T-cells based on ethnicity with plot (A) representing the Black African group and (B) representing the Caucasian group. The boxes represent the middle 50 % of data values. The horizontal line within the boxes represents the median data value. The error bars indicate the 10^{th} and 90^{th} percentile values of the data set. Red squares (Figure A) indicate outlier values.

The mean CCR5 densities measured by MFI were 4.8 and 4.1 for Black African and Caucasian groups, respectively. Figure 23, illustrates the distribution of CCR5 density in various ethnicities. The MFI is found between 1.4 and 8.6 for the Black African group. For the Caucasian group, the distribution is found within 1.3 and 7.7 with outlier values of 0 and 9.

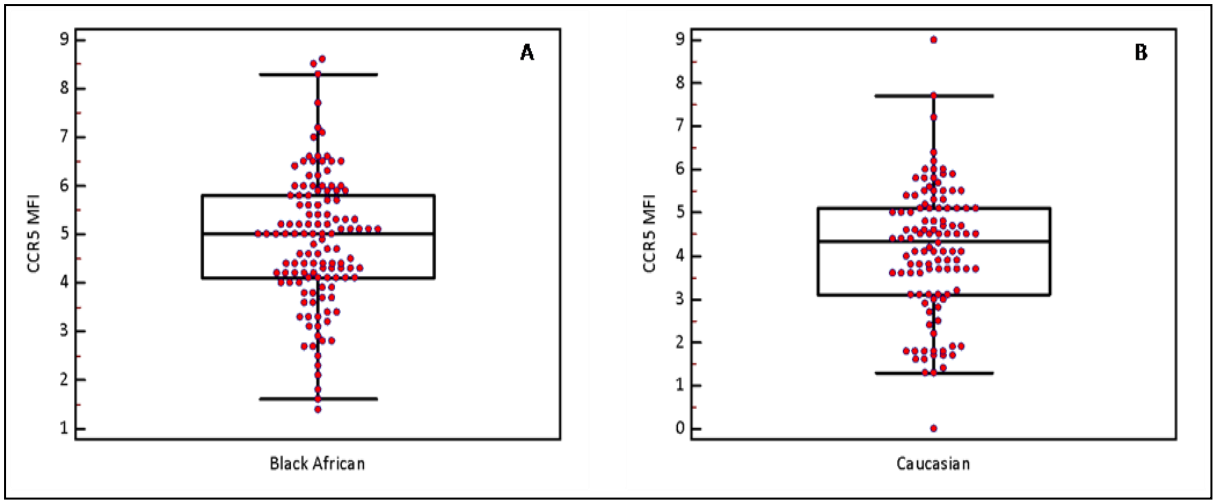


Figure 23: Distribution of CCR5 density (MFI) based on ethnicity (A) representing the Black African group and (B) representing the Caucasian group. The boxes represent the middle 50 % of data values. The horizontal line within the boxes represents the median data value. The error bars indicate the 10th and 90th percentile values of the data set.

The results indicate that the Black African group has a higher CCR5 density than the Caucasian group. The IRR of the Black African group was 1.16 (p= 0.02) which indicates that this group had 1.16 the rate of CCR5 density compared to the Caucasian group. Therefore ethnicity affects CCR5 density as indicated by MFI.

Genetic differences in CCR5 have been shown in different ethnicities with the Δ32 mutation being very rare in the Black African population (Martinson *et al.*, 1997; Williamson *et al.*, 2000). Black homosexual men also have a greater risk of HIV infection (Millett *et al.*, 2006), with genetic factors in CCR5 being deemed a possible cause. This present study indicates that Black African individuals have both higher levels of $CD4^{+}CCR5^{+}$ T-cells and CCR5 density compared to the Caucasian group.

4.3 CONCLUDING REMARKS

The results from this study support previous studies performed in South Africa. The mean $CD4^+CCR5^+$ T-cell percentages have previously been reported to be at 23 % for healthy individuals (Shalekoff, 2004). A similar $CD4^+CCR5^+$ T-cell percentage of 21% was measured in the present study although unlike the previous report, the health status of the individuals was unknown. However, the results reject the hypothesis which states that $CD4^+CCR5^+$ T-cell percentages are not different to expected values, as the *a priori* expectations as indicated by other studies was not met. Levels of $CD4^+CCR5^+$ T-cells found in this study were higher than values found in other studies which find mean percentages between 5-10% (Bleul *et al.*, 1997; Ostrowski *et al.*, 1998). The density measured in this study was qualitative, as it used MFI as a measure of CCR5 receptors per $CD4^+$ T-cell. A shortfall of this approach, however, is the inability to make comparisons with data from studies which used a quantitative measure of CCR5 density.

The increased $CD4^+CCR5^+$ T-cell percentages found in the present study is supported by Clerici and co-workers (2000) who found that CCR5 is up-regulated in Africa due to environmental factors which increase immune activation. In addition, genetic factors may affect expression as African individuals have low frequencies of the Δ32 mutation (Martinson *et al.*, 1997), which is associated with decreased CCR5 expression. Infectious diseases such as TB and HIV are found predominantly in sub-Saharan regions, and both are associated with increased CCR5 expression (Juffermans *et al.*, 2001; Shalekoff *et al.*, 2001). This occurrence can be examined by evaluating CCR5 expression in HIV and TB infected individuals and comparing the results to uninfected individuals.

Results from this study found a very weak correlation (r= 0.2456) between percentage $CD4^+CCR5^+$ expression and CCR5 density, which supports the null hypothesis of no correlation between these measurements. This also supports previous data which indicated that high $CD4^+CCR5^+$ T-cell percentages can have either low or high CCR5 densities (Reynes *et al.*, 2000). Additional studies using quantitative measures of CCR5 density will further aid in supporting this observation.

Results from this study show that age affects the levels of $CD4^+CCR5^+$ T-cells, as increasing age increases the percentage of cells. However, age was not found to affect CCR5 density. Previous data showed that older individuals progress to AIDS more rapidly and have higher levels of memory T-cell subsets (Lerner *et al.*, 1989; Ernst *et al.*, 1990; Chiao *et al.*, 1999). Rapid progression (Reynes *et al.*, 2001) and memory T-cells (Wu *et al.*, 1997) are associated with high levels of CCR5. The role of sample bias may have influenced the results as a substantially higher number of individuals in the >40 year age category were analysed.

In terms of the influence of gender on CCR5 expression, females were found to have lower $CD4^+CCR5^+$ T-cell percentages than males. However, gender did not play a role in CCR5 density. Although the density of CCR5 has previously been shown to be lower in females (Portales *et al.*, 2001); no significant difference could be found in the present study. The role of sample bias may influence these results as just over 70% of the individuals assessed in this study were female.

Ethnicity was found to significantly affect both $CD4^+CCR5^+$ T-cell percentage and CCR5 density. Black African individuals had higher levels of both measurements when compared to Caucasian individuals. Genetic factors may provide a possible explanation as Kalinkovich and co-workers (2001) demonstrated that in comparison to Caucasian Israelis, Black Ethiopians living in Israel displayed higher levels of CCR5 expression on $CD4^+$ T-cells. Analysis of genotypic differences between individuals in these two ethnic groups will aid in establishing the reason for this difference in phenotype.

The null hypotheses stated that age, gender and ethnicity do not affect either $CD4^+CCR5^+$ T-cell percentage or CCR5 density. However, results from this study reject these null hypotheses.

Lastly, one of the objectives of this study was to determine the frequency of CCR5 null individuals in the South African population. A single Caucasian male individual (sample number F092) was found in the cohort of 245 individuals who expressed no CCR5, neither in terms of percentage of $CD4^+$ T-cells nor in terms of CCR5 density. The individual's blood sample was analysed twice by flow cytometry to confirm the findings. The null hypothesis is therefore rejected as CCR5 null individuals, although rare, are found in the South African population.

Text Box 1: Summary of Findings

- High degree of variability for phenotypic CCR5 expression
- South African individuals express higher levels of $CD4^+CCR5^+$ T-cells
- No correlation between $CD4^+CCR5^+$ T-cell percentage and CCR5 density
- An age associated increase was found for $CD4^+CCR5^+$ T-cell percentages, but not for density
- Females may display lower $CD4^+CCR5^+$ T-cell percentage but gender does not affect CCR5 density
- Ethnicity significantly affects both $CD4^+CCR5^+$ T-cell percentages and CCR5 density

Individuals homozygous for the Δ32 mutation do not express CCR5 at the cell surface (Liu *et al.*, 1996; Samson *et al.*, 1996). The following chapter (Chapter five) will aim to establish if the CCR5 null individual found in the present study has a genetic cause to the phenotype.

The flow data discussed in this chapter emphasises the heterogeneity in CCR5 expression in the South African population. Although many factors affect CCR5, genetic factors will be assessed in a select group of individuals in the following chapter. The distribution of CCR5 (Figures 15 and 16) was used to select individuals with varying expression for sequence analysis.

Genetic analysis of the CCR5 gene in a selected cohort of South Africans

5.1 INTRODUCTION

Mutations in the CCR5 gene have been associated with HIV infectivity or lack thereof (Liu *et al.*, 1996; Samson *et al.*, 1996a). Although emphasis has been placed on the ORF, mutations in the non protein coding regions have been found to affect HIV disease progression (Martin *et al.*, 1998).

Studies conducted in South Africa have largely focussed on mutations affecting CCR5 protein structure. Williamson and co-workers (2000) concentrated on establishing the frequency of the Δ32 and the C101X mutations. The Δ32 mutation was found at 9.8 % allelic frequency in the Caucasian group and at a 0.1 % allelic frequency in the African group. A mutation detection assay was used by Peterson and co-workers (2001), the results of which found six previously identified mutations and seven novel mutations in the South African population. All the novel mutations were found exclusively in the African population including the identification of a nonsense mutation known as R225X, discussed in Chapter two. The Δ32 mutation was not detected in the Black African population. Although generally confined to the Caucasian population, the Δ32 mutation was found in the Coloured population, at an allelic frequency of 0.03 in five HIV negative and one HIV positive individual.

In 2010, Folefoc and co-workers determined the effect of four novel mutations identified in the Peterson study using mutant receptor constructs. The study assessed the effect of the mutations on the ability of CCR5 to function as a receptor and as an HIV co-receptor. Only the D2V and R225X mutants were found to affect HIV infection. The D2V mutant reduced cell surface expression thereby reducing HIV infectability. The R225X mutant abolished cell surface CCR5 which prevented HIV infection of the cells.

Picton and co-workers (2010) analysed a 9.2 kb CCR5 gene fragment in the Black African and Caucasian groups and detected 68 SNPs, four indels and the Δ32 mutation. Twenty four of the mutations identified were novel. The Δ32 mutation was found at an allelic frequency of 0.071 in the Caucasian group only.

The aim of the present study is to analyse the entire CCR5 gene in 65 individuals and to report on the mutations detected. In addition, the frequency of the Δ32 mutation will be determined. The phenotypic data for these individuals was determined using flow cytometry (Chapter 3); therefore

the objective of this chapter was to use the phenotypic data as a screening procedure for the identification of low expressors on whom genotype will be determined.

5.2 RESULTS AND DISCUSSION

The present study genotyped a region of 9028 bp which included the 5' flanking region, three exons, two introns, the ORF and the 3' flanking region.

5.2.1 Selection of individuals sequenced

A total of 65 South African individuals were genotyped. This included 29 Black Africans, 35 Caucasians and one Indian individual. Flow cytometry as discussed in chapter four was used to determine $CD4^+CCR5^+$ T-lymphocyte percentage and CCR5 density and on the basis of the data obtained, the individuals to be sequenced were selected (Table 5). The percentage of $CD4^+CCR5^+$ T-cells is dependent on various factors such as cell activation state or infectious agents (Mo *et al.*, 1998; Juffermans *et al.*, 2001). The number of CCR5 receptors per cell (CCR5 density) is dependent on genetic factors as observed in Δ32 individuals. In addition, as reviewed in Chapter two, mutations which affect important regions in the CCR5 gene can affect CCR5 expression at the cell surface (Blanpain *et al.*, 2000). This supported the use of CCR5 density as measured by MFI in the present study as a tool for selection of the 65 individuals for sequencing (Figure 24).

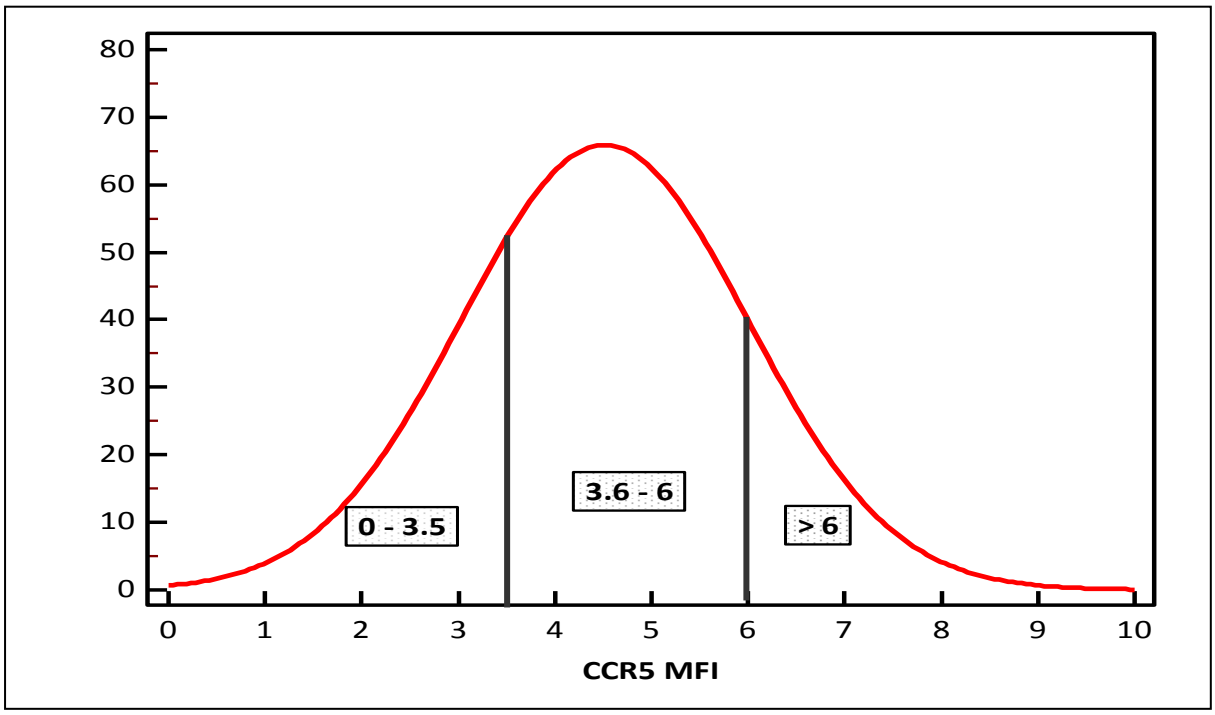


Figure 24: Histogram illustrating the distribution of CCR5 density (MFI) in the South African population. Most individuals were found within 3.6 – 6 arbitrary units.

A total of 45 individuals sequenced, were found within the lower range of CCR5 density (0-3.5) (Table 5). This was followed by 19 individuals within the normal range of CCR5 density (3.6-6) and one individual that exceeded a density of 6 arbitrary units.

Table 5: List of 65 individuals sequenced with concomitant CCR5 flow data

CCR5 MFI Range	Black African			Caucasian		
	Sample ID	MFI	$CD4^+CCR5^+$ %	Sample ID	MFI	$CD4^+CCR5^+$ %
0 – 3.5	F051	1.4	12.3	F092[a]	0.0	0
	F039	1.6	25.9	F054[a]	1.3	10.4
	F037[c]	1.8	11.3	F042[a]	1.4	10.7
	F047	2.1	31	F044	1.6	14.1
	F048	2.3	17.3	F043	1.6	5.4
	F114	2.5	34.5	F050	1.7	10.7
	F158	2.7	26.8	F040	1.7	18.4
	F142	2.7	29.2	F052	1.8	35.6
	F099	2.8	9.9	F055[c]	1.8	12.8
	F172	2.8	5.8	F046[c]	1.8	14.6
	F086	2.9	55.9	F049	1.8	20.4
	F059	3.1	13.9	F056	1.8	18.6
	F135	3.1	10.6	F053	1.9	8.4
	F009	3.2	17.9	F045	1.9	18.5
	F216	3.3	7.8	F087[a]	2.4	16.8
	F084	3.3	20	T001[a]	2.5	6.9
	F173	3.3	8.6	F157[a]	2.7	6.6
	F081	3.4	26.9	F024[a]	2.8	9.7
	F184	3.4	25.6	F014	2.9	8.35
				F002[a]	2.9	11.7
				F033[a]	3.0	12.3
				F029	3.1	6.8
				F030	3.1	19.9
				F154[a]	3.1	15.8
				F104[a]	3.1	16.7
				F103	3.2	6.7
3.6 - 6	F115	3.7	13.5	F070	3.7	12.1
	F203	4.1	4.8	F197[a]	3.8	8.3
	F102	4.1	12.6	F105	4.1	9.6
	F007	5.0	17.3	F195	4.2	9.5
	F119	5.0	42.6	F093[c]	4.7	23.4
	F021[c]	5.0	32.1	F121	5.1	41.5
	F101	5.9	9.4	F062	5.3	8.9
	F068	6.0	9.5	T002[c]	5.5	17.2
	F200[c]	6.0	6.1			
	F228	6.0	8.1			
> 6				F010	7.2	22.8
	F032[b]	5.2	26.5			

Footnote: [a] indicates individuals found with the Δ32 mutation [b] F032 indicates the individual of Indian descent, the flow data falls within the commonly found range in the present study (Chapter 4) (3.6-6) and [c] indicates individuals found homozygous for the P1 promoter haplotype.

From the total number of individuals sequenced, 19 Black Africans and 26 Caucasians were found within the low CCR5 density range (0-3.5). Ten Black Africans and eight Caucasians were found in the normal range (3.6-6) while one Caucasian was found to exceed 6 arbitrary units.

5.2.2 Report of previously identified CCR5 mutations detected in the present study

The present study recorded 63 previously identified mutations in the 65 South African individuals genotyped (Table 6 and 7) (Appendix D). The mutations found included 58 SNPs, four inserts and one deletion. Of the total number of mutations found 53 occurred in the Black African population while 31 mutations were identified in the Caucasian group. Therefore, 32 mutations were detected exclusively in the Black African population and 10 only in the Caucasian group.

Table 6: Mutations detected in the Black African and Caucasian cohort of the present study which have previously been identified

Gene Region	Position	Base Change wt/m	Accession number[a]	Population Frequency	
				Black African[b]	Caucasian[c]
5' Flanking region (2715 bp)	**-5272**[d]	G/A	rs3136535	1 (0.017)	16 (0.229)
	-5270	G/A	rs6776227	1 (0.017)	1 (0.014)
	-5218	T/C	Picton *et al.*, 2010	2 (0.034)	0
	-5084	T/A	rs41429449	1 (0.017)	0
	-4749	C/T	rs3136536	5 (0.086)	0
	-4349[d]	A/G	rs7637813	3 (0.052)	22 (0.314)
	-4248[d]	A/C	rs41490645	0	14 (0.200)
	-4079	T/A	rs41499550	9 (0.155)	0
	-3891[d]	C/A	rs72622924/rs2856757	44 (0.759)	46 (0.657)
	-3886	T/C	rs41395049	9 (0.155)	0
	-3879[e]	-/C/TTAT	rs145381188/ rs41412948	0	2
	-3875[d, e]	-/CTAT	rs10577983	18	13
	-3827	C/T	Picton *et al.*, 2010	4 (0.069)	0
	-3453[d]	G/T	rs2734225	45 (0.776)	49 (0.700)
	-3256	G/A	rs41475349	9 (0.155)	0
	-2847[d]	A/G	rs2227010	17 (0.293)	36 (0.514)
Exon 1 (57 bp)	**-2728**[d]	A/G	rs2856758	2 (0.034)	17 (0.243)
Intron 1 (501 bp)	**-2572**	T/G	Picton *et al.*, 2010	1 (0.017)	0
	-2549[d]	T/G	rs2734648	37 (0.638)	49 (0.700)
	-2454[d]	G/A	rs1799987	29 (0.500)	43 (0.614)
	-2449	G/A	Picton et al., 2010	1 (0.017)	0
Exon 2a (235 bp)	**-2130**[d]	T/C	rs1799988	27 (0.466)	45 (0.643)
	-2127	C/T	rs41469351	5 (0.086)	0
	-2081[d]	G/A	rs1800023	46 (0.793)	47 (0.671)
	-2043	C/G	rs41355345	1 (0.017)	0
Intron 2 (1909 bp)	**-1830**	C/T	rs1800024	11 (0.190)	6 (0.086)
	-1681	A/C	rs9282632	10 (0.172)	0

	Position	Base Change wt/m	Accession number[a]	Population Frequency	
				Black African[b]	Caucasian[c]
Intron 2 (1909 bp)	**-1459**	A/G	rs3181037	9 (0.155)	0
	-1188	C/T	Picton *et al.*, 2010	2 (0.034)	0
	-1126[d, e]	GA/-	rs3054375	18	15
	-1057	C/T	rs2856762	0	3 (0.043)
	-973[d]	C/T	rs2254089	44 (0.759)	49 (0.700)
	-972	G/A	rs41395249	4 (0.069)	0
	-648[d]	C/T	rs2856764	46 (0.793)	48 (0.686)
	-441[d]	A/G	rs2856765/rs35046662	46 (0.793)	49 (0.700)
	-359[d]	G/A	rs41515644	46 (0.793)	49 (0.700)
	-353	CAA/-	Mummidi *et al.*, 1997	18	15
	-113	G/T	rs3176763	9 (0.155)	0
	-112	G/A	rs41352147	1 (0.017)	0
3' Flanking region (2498 bp)	**1253**	A/G	Picton et al., 2010	1 (0.017)	0
	1752	G/A	rs41495153	12 (0.207)	0
	1823	C/T	rs17765882	0	3 (0.043)
	1843	G/A	rs41418945	5 (0.086)	0
	1846	G/A	rs41466044	4 (0.069)	0
	2066	G/A	Picton *et al.*, 2010	3 (0.052)	0
	2077[d]	T/G	rs1800874	46 (0.793)	47 (0.671)
	2225	T/C	rs41535253	3 (0.052)	0
	2381	A/G	Picton *et al.*, 2010	2 (0.034)	0
	2435	T/A	Picton *et al.*, 2010	3 (0.052)	0
	2458	A/C	rs3188094	4 (0.069)	0
	2676	C/A	rs41442546	0	4 (0.057)
	2838	C/G	rs41512547	2 (0.034)	0
	2919[d]	T/G	rs746492	28 (0.483)	46 (0.657)
	3132	T/G	Picton *et al.*, 2010	0	1 (0.014)

Footnote: [a] Accession numbers as found in the SNP database (dbSNP) or reference to article which discovered mutation. [b] Frequency of mutation in the Black African population calculated using the total number of alleles (58). [c] Frequency of mutation in the Caucasian population calculated using the total number of alleles (70). [d] mutations found in a single Indian individual. [e] Refers to the number of individuals found with insertions (not frequency). The insertion at position -3879 is a combination of two reference sequences. For base change wt refers to wild-type allele and m refers to mutant allele.

As illustrated in Figure 25 there were no mutations detected in the 54 bp region of exon 2b. This was also observed by Picton and co-workers (2010). The regions with the highest number of mutations were the 3' flanking region and intron 2. Ten mutations are exclusive to the South African population and were previously discovered by Picton and co-workers (2010). Mutations with the highest frequency in the Caucasian group were found at positions -3453, -2549, -973, -441 and -359. In the Black African population mutations with the highest frequency were found at positions -2081, -648, -441, -359 and +2077.

Table 7: Open reading frame mutations detected in the Black African and Caucasian cohort of the present study which have been previously identified

DNA Position	Amino acid position	Base Change wt/m	Accession number[a]	Population Frequency	
				Black African[b]	Caucasian[c]
105	P35	G/A	Peterson *et al.*, 2001	0.017	0.000
164	L55Q	A/T	rs1799863	0.000	0.043
180	R60S	G/T	rs1800940	0.017	0.000
303	C101X	T/A	rs1800560	0.000	0.014
554	Δ32	D32	rs333	0.000	0.186
582	Q194H	G/T	rs62625034	0.000	0.157
673	R225X	C/T	Petersen *et al.*, 2001	0.034	0.000
1004	A335V	C/T	rs1800944	0.121	0.000
1016	Y339F	A/T	rs1800945	0.017	0.000

Footnote: [a] Accession numbers as found in the SNP database (dbSNP) or reference to article which discovered mutation. [b] Frequency of mutation in the Black African population calculated using the total number of alleles (58). [c] Frequency of mutation in the Caucasian population calculated using the total number of alleles (70). For base change wt refers to wild-type allele and m refers to mutant allele.

The ORF contained nine previously found mutations. Five of these mutations were found exclusively in the Black African individuals while four were found in the Caucasian group only. There were no mutations in the ORF that coincided in both population groups. The Δ32 mutation was found in 12 Caucasian individuals at a frequency of 0.186 but was not detected in the Black African group. All individuals who displayed the Δ32 mutation were heterozygous, except a single male who was homozygous.

Mutations in the coding region of the CCR5 gene are known to affect CCR5 expression (Chapter 2) (Carrington *et al.*, 1999) and thus HIV susceptibility and disease progression (Reynes *et al.*, 2001). However, these mutations were found in isolated groups of individuals and are usually population specific (Carrington *et al.*, 1999; Blanpain *et al.*, 2000). Individuals exposed to HIV show considerable variation in HIV susceptibility and once infected, display differences in disease progression. Considering the low frequency of CCR5 coding mutations, Bamshad and co-workers (2002), analysed the sequence variation in the CCR5 5' *cis*-regulatory region (between -2867 and -1745), a region known to affect HIV susceptibility and disease progression (Martin *et al.*, 1998). The group demonstrated a high concentration of SNPs in this region that outweighs SNP density found normally in regulatory and non-coding regions. The variation in this region did not follow a normal pattern of genetic drift but may have been a target of natural selection. The discussion below will concentrate on the mutations in this region as well as mutations found in the ORF of the present study.

The study by Martin and co-workers (1998) discovered 10 SNP variants in the downstream promoter region of the CCR5 gene which includes intron 1, exon 2a and part of exon 2b. These promoter SNPs constitute 10 CCR5 promoter haplotype alleles. Additional promoter alleles have been defined (Mummidi *et al.*, 1997; McDermott *et al.*, 1998) and together the SNPs represent CCR5 HHA-HHG (Gonzalez *et al.*, 1999). Seven promoter SNPs defined in the literature have been found in the present study. These include SNPs found at positions -2728 (58755), -2549 (58934), -2454 (59029), -2130 (59353), -2127 (59356), -2081 (59402) and -1830 (59653). The positions in brackets refer to the numbering system based on the Genbank accession number U95626.

The 59029 G/A mutation is a common polymorphism that affects HIV disease progression (McDermott *et al.*, 1998) and this was substantiated in the present study which found frequencies of 0.5 and 0.614 for the Black African and Caucasian groups respectively. McDermott and co-workers (1998) demonstrated that the 59029 G/G promoter has 45% lower activity than the 59029 A/A promoter. Individuals with the homozygous G wild-type sequence had a median survival time of 10.4 years to AIDS. Individuals with the homozygous A SNP had a median survival of 6.6 years. Individuals heterozygous for the mutation were found at intermediate survival curves with a median of 8 years. Due to the effect on promoter activity, the protection of the homozygous G sequence may arise from reduced mRNA production. The present study identified 17 Black Africans who were heterozygous (59029 A/G) and six who were homozygous (59029 A/A) for the mutation. In the Caucasian group 21 individuals were heterozygous and 11 were homozygous for the mutation.

The 59353 T/C mutation is found in strong linkage disequilibrium with the 59029 G/A mutation, with both mutations contributing to greater promoter activity in the homozygous C and A states respectively (McDermott *et al.*, 1998). In assessing the effect of promoter mutations in perinatal HIV-1 transmission, de Souza and co-workers (2006) found that the 59353 T/T genotype increased the risk of HIV vertical transmission. In addition, the allelic frequency of the 59353 C/C genotype is much higher in individuals with AIDS when compared to long-term non progressors (Jang *et al.*, 2008). Both the 59029 G and 59353 T genotypes were also found to protect against perinatal HIV transmission in a Malawian cohort analysed by Pedersen and co-workers (2007). The protection was associated with decreased CCR5 receptor expression. The present study identified 17 Black Africans who were heterozygous (59353 T/C) and five who were homozygous (59353 C/C) for the mutation. In the Caucasian group 21 individuals were heterozygous and 12 were homozygous for the mutation.

The 59402 A/A mutation has been shown to protect against perinatal HIV transmission by de Souza and co-workers (2006) whereas Kostrikis and co-workers (1999) showed the G/G genotype conferred protection. The latter genotype was found at a lower frequency in African Americans in comparison

to Caucasians and Hispanics (Kostrikis *et al.*, 1999). Kaur and co-workers (2007) confirmed that the 59402 A allele increases HIV susceptibility and progression in the Asian Indian population. The 59029 A/A, 59353 C/C and the 59402 A/A genotypes are associated with accelerated disease progression (Mummidi *et al.*, 1998). The present study identified 10 Black Africans who were heterozygous (59402 G/A) and 18 who were homozygous (59402 G/G) for the mutation. In the Caucasian group 21 individuals were heterozygous and 12 were homozygous for the mutation.

The 59356 T/T mutation was previously found to be common among African Americans but rare in Caucasians and Hispanics (Kostrikis *et al.*, 1999). This was substantiated in the present study as the mutation was exclusive to the Black African population being found in five individuals who were heterozygotes. The latter mutation is known to increase the risk of perinatal HIV transmission. The 59653 C/T mutation is usually in linkage disequilibrium with the protective CCR2- 64I mutation, the latter which is known to delay HIV disease progression (Kostrikis *et al.*, 1998). In the present study, the mutation was found to occur in 11 Black Africans who were heterozygotes. In the Caucasian group it was found in six individuals who were heterozygotes.

According to McDermott and co-workers (1998) the Δ32 mutation is genetically linked to the 59029 A allele. The study found that all Δ32 homozygotes were also homozygous for the 59029 A allele and in addition, no Δ32 heterozygotes were homozygous for the 59029 G allele. In the present study, 12 individuals were found to possess the Δ32 mutation (Table 8). The Δ32 homozygous individual (F092) contained the 59029 A/A genotype, substantiating the findings of McDermott and co-workers (1998). However, in the current study 10 of the 12 Δ32 heterozygous individuals were genetically linked to the 59029 A allele with one individual being homozygous for the G allele (F002). According to Hladik and co-workers (2005) only Δ32 heterozygotes with the A/G genotype and not the A/A genotype, have protection against HIV infection as these individuals have lower CCR5 densities. As found in the current study, Δ32 heterozygotes with the A/A genotype (n= 5) had a mean density of 2.58 while those with A/G genotype (n= 5) had a mean density of 2.56. The similarity in the density between the two genotypes does not concord with the findings in the Hladik paper. This might be due to sample size. Interestingly, the individual with G/G genotype had a higher density of 2.93. To date the present study is the first to report on a Δ32 individual without a 59029 A allele association.

Table 8: Distribution of five mutations found in the present study in the regulatory region of the CCR5 gene known to affect HIV progression with 0 indicating wild-type allele, 1 indicating heterozygous mutation and 2 indicating homozygous mutation.

Ethnicity	Sample ID	MFI	%	59029 G/A	59353 T/C	59356 C/T	59402 G/A	59653 C/T
Caucasian	T001[a]	2.5	6.9	1	1	0	0	0
	T002	5.5	17.2	2	2	0	2	0
	F002[a]	2.9	11.7	0	2	0	2	0
	F010	7.2	22.8	1	1	0	1	1
	F014	2.9	8.35	1	1	0	1	0
	F024[a]	2.8	9.7	1	1	0	2	0
	F029	3.1	6.8	1	1	0	1	0
	F030	3.1	19.9	1	1	0	1	1
	F033[a]	3.0	12.3	2	1	0	2	0
	F040	1.7	18.4	1	1	0	1	0
	F042[a]	1.4	10.7	2	2	0	2	1
	F043	1.6	5.4	1	1	0	1	1
	F044	1.6	14.1	1	1	0	1	0
	F045	1.9	18.5	2	2	0	2	1
	F046	1.8	14.6	2	2	0	2	0
	F049	1.8	20.4	0	0	0	0	0
	F050	1.7	10.7	1	1	0	1	0
	F052	1.8	35.6	1	1	0	1	0
	F053	1.9	8.4	0	0	0	1	0
	F054[a]	1.3	10.4	1	1	0	1	0
	F055	1.8	12.8	2	2	0	2	0
	F056	1.8	18.6	1	1	0	1	0
	F062	5.3	8.9	1	1	0	1	0
	F070	3.7	12.1	1	1	0	1	0
	F087[a]	2.4	16.8	2	2	0	2	0
	F092[a, b]	0	0	2	2	0	2	0
	F093	4.7	23.4	2	2	0	2	0
	F103	3.2	6.7	1	2	0	2	0
	F104[a]	3.1	16.7	1	1	0	1	0
	F105	4.1	9.6	1	1	0	1	1
	F121	5.1	41.5	1	1	0	1	0
	F154[a]	3.1	15.8	1	1	0	1	0
	F157[a]	2.7	6.6	2	2	0	2	0
	F195	4.2	9.5	1	1	0	1	0
	F197[a]	3.8	8.3	2	2	0	2	0
Black African	F007	5.0	17.3	1	1	1	2	0
	F009	3.2	17.9	2	2	0	2	1
	F021	5.0	32.1	2	2	0	2	0
	F037	1.8	11.3	2	2	0	2	0
	F039	1.6	25.9	0	0	0	1	0
	F047	2.1	31	0	0	0	2	0
	F048	2.3	17.3	0	0	1	2	0
	F051	1.4	12.3	1	1	1	2	1
	F059	3.1	13.9	0	0	0	1	0

	Sample ID	MFI	%	59029 G/A	59353 T/C	59356 C/T	59402 G/A	59653 C/T
Black African	**F068**	6.0	9.5	1	1	0	1	1
	F081	3.4	26.9	1	1	0	2	1
	F084	3.3	20	1	1	0	2	1
	F086	2.9	55.9	1	1	0	2	0
	F099	2.8	9.9	1	1	0	1	0
	F101	5.9	9.4	1	0	0	2	0
	F102	4.1	12.6	1	1	0	2	0
	F114	2.5	34.5	0	0	1	1	0
	F115	3.7	13.5	1	1	0	1	1
	F119	5.0	42.6	1	1	0	1	0
	F135	3.1	10.6	1	1	0	2	1
	F142	2.7	29.2	1	1	0	1	0
	F158	2.7	26.8	0	0	0	0	0
	F172	2.8	5.8	1	1	0	1	1
	F173	3.3	8.6	1	1	0	1	1
	F184	3.4	25.6	2	2	0	2	1
	F200	6.0	6.1	2	2	0	2	0
	F203	4.1	4.8	2	1	0	2	0
	F216	3.3	7.8	1	1	0	2	0
	F228	6.0	8.1	1	1	1	2	1

Footnote: [a] illustrating individuals with the Δ32 mutation, [b] representing the Δ32 homozygous individual.

The 59029 G/A allele is known to determine CCR5 expression (Salkowitz *et al.*, 2003) with individuals homozygous for the A genotype displaying increased numbers of $CD4^+CCR5^+$ T-cells, excluding individuals with Δ32 (Shieh *et al.*, 2000). However in the present study, mean $CD4^+CCR5^+$ T-cell percentages in the South African population were found to be 16.75, 16.8 and 22.8 % for the 59029 A/A, A/G and GG genotypes, respectively.

McDermott and co-workers (1998) also illustrated a strong linkage between the 59029 A and 59353 C alleles. Individuals homozygous for 59029 A are also homozygous for 59353 C and similarly individuals homozygous for 59029 G are also homozygous for 59353 T. Results from the present study confirm these findings as all except for five individuals follow this linkage pattern (F002, F033, F101, F103 and F203).

As stated above, the 59029 A/A, 59353 C/C and the 59402 A/A genotypes are associated with accelerated disease progression (Mummidi *et al.*, 1998). To assess if the latter genotypes are associated with increased CCR5 expression, the density was correlated with the genotypes. Seven individuals were found with these genotypes. Four of these individuals were found with densities of 6, 5.5, 5 and 4.7 while three of the individuals were found with lower CCR5 densities of 1.8. Therefore, the genotypes 59029 A/A, 59353 C/C and 59402 A/A associated with accelerated HIV progression was not correlated with higher CCR5 density.

Individuals with lower CCR5 densities could not be associated with specific promoter genotype mutations as these individuals varied in genotype constitution.

Thus far, the discussion has been based on mutations found in the Pd promoter. However, the Pu promoter was also found to be an important determinant of CCR5 expression and may provide a target for HIV therapy (Mummidi *et al.*, 2007). Mummidi and co-workers (2007) discovered that the Pu promoter is responsible for forming exon 1 containing transcripts especially in T-cell receptor stimulated cells and is tightly regulated by the Oct transcription factors. In addition, the previous assumption of Pu being transcriptionally weaker than Pd is a result of using transformed T-cell lines. The data from the present study does not show a link between -2847 and CCR5 density (Appendix D) but the role of this mutation in CCR5 expression merits further investigation.

In the present study, a polymorphism at amino acid number 35 (Table 7) was found to occur in a Black African female heterozygote (F216) but was not detected in the Caucasian group. The CCR5 density was measured at 3.3 arbitrary units, which is in the lower range, with a concomitant $CD4^{+}CCR5^{+}$ T-cell percentage of 7.8 (Table 5). The mutation has previously been discovered exclusively in the South African population (Peterson *et al.*, 2001). The study found the mutation at high frequencies in both HIV positive and negative Black African and Coloured individuals. The mutation was heterozygous in all except one HIV positive Coloured female in which the mutation was found in a homozygous state. The mutation results in a G/A change at position 105 in the ORF causing a silent mutation. Formerly, silent mutations were considered ineffective in causing changes to the protein function as there is no change in amino acid. Many codons can code for an amino acid however, the use of a preferential codon is known as 'codon usage bias' and this optimises translational machinery and growth (Fairbanks and Anderson, 1999). Studies in *Drosophila* have shown there is a strong correlation between gene expression and codon usage bias (Powell and Moriyama, 1997). Duan and co-workers (2003) discovered that two silent mutations affect the mRNA stability and protein expression of a GPCR. Thus, the silent mutation present at P35 may affect CCR5 protein expression, resulting in CCR5 density at the lower level as was found in the present study.

In the present study a T/A SNP was found at position 164 (Table 7) of the nucleotide sequence. The mutation at this position is known as L55Q (Ansari-Lari *et al.*, 1997; Carrington *et al.*, 1997). The mutation was present in three Caucasian individuals (F014, F052 and F103) at CCR5 densities of 2.9, 1.8 and 3.2 arbitrary units (Table 5). In a previous South African study the mutation was also absent in the Black and Coloured groups, but found to be present in an HIV positive Caucasian female (Peterson *et al.*, 2001). The mutation has been previously found at high frequency in the Caucasian

group and is suggested to be of Caucasian ancestry. Although the mutation has been found in African Americans this was suggested to be due to admixture of the genetic pool (Carrington *et al.*, 1997). The L55Q mutant was inferred to be of European origin (Boldt *et al.*, 2009). The mutation is found in HIV positive individuals and thus does not prevent HIV infection (Carrington *et al.*, 1997). However, the mutation is located in a region which is highly conserved for β-chemokine receptors. The mutation results in the replacement of leucine, a neutral amino acid, with glutamine a polar amino acid. The mutation is also found in the first TMD and thus the change of hydrophobicity caused by the mutation may affect the CCR5 receptor. Previous studies have demonstrated that the L55Q mutant receptor can bind to β-chemokines such as MIP-1β but has a reduced functional response (Blanpain *et al.*, 2000). Considering the role of CCR5 ligands in hindering HIV infection, the mutation has been suggested to increase HIV infection and progression (Howard *et al.*, 1999). However, in the present study, the CCR5 densities are found in the lower range of expression for individuals with this mutation.

The mutation found at position 180 (Table 7) in the DNA sequence of the present study corresponds to the previously identified R60S mutation (Ansari-Lari *et al.*, 1997; Carrington *et al.*, 1997). In the present study the mutation was found to occur in a single Black African female heterozygote (F172) with a CCR5 density of 2.83 and $CD4^{+}CCR5^{+}$ T-cell percentage of 5.8 %. The mutation was not found in other South African studies (Peterson *et al.*, 2001; Picton *et al.*, 2010) although Boldt and co-workers (2009) inferred that the mutation may have originated in Africa. The mutation is found in the first ICL of the CCR5 protein and results in an amino acid change from arginine, a basic residue, to the polar serine. The mutant receptor was shown to be poorly expressed at the cell surface and is considered to be a candidate for protection against HIV infection and progression (Tamasauskas *et al.*, 2001). In further studies, Blanpain and co-workers (2000) showed that the mutant receptor decreased HIV gp120 binding as a result of poor expression at the cell surface. In the present study the mutation, although heterozygous, was found in an individual with CCR5 density and percentage expression in the lower range.

The C101X mutation at nucleotide position 303 was found in the present study in a Caucasian male individual (F103) who also presented with the L55Q mutation. The mutation has previously been discovered in the South African population in a heterozygous state and restricted to the Caucasian group at an allelic frequency of 0.7 %. The mutation was originally discovered in a male homozygous individual who remained uninfected despite continuous exposure from an infected partner over 10 years (Quillent *et al.*, 1998). Analysis of the mutant receptor showed lack of expression on the cell surface, rendering the individual resistant to HIV infection. The mutation affects an important

cysteine residue involved in disulfide linkage, discussed in Chapter two, by replacing the residue with a stop codon. This results in premature termination of translation with intracellular retention of the unfolded receptor (Blanpain *et al.*, 2000). According to Blanpain and co-workers (2000) the mutation is frequent in Central Africa.

The homozygous Δ32 mutation found in the present study was detected in a Caucasian male individual who did not express CCR5, as measured by flow cytometry (Chapter 4). The cause of the null expression is therefore due to genetic determinants as the Δ32 mutation in a homozygous state is known for absence of expression (Samson *et al.*, 1996a). This individual will likely be resistant to HIV if infected with the R5 strain. Additionally, DNA sequencing in the present study identified 11 Caucasian individuals (T001, F002, F024, F033, F042, F054, F087, F104, F154, F157 and F187) who contained a sequence within the ORF that could not yield sequencing results. Haplotype analysis using blunt end cloning was utilised to verify the nature of the unidentified sequences. Analysis revealed that all 11 individuals were heterozygous for the Δ32 mutation. The frequency of the Δ32 mutation was found at 0.186 and is significantly different ($p < 0.05$) to that found in other South African studies (0.071) (Picton *et al.*, 2010). Ten of the heterozygous individuals display CCR5 densities in the low range between 1.38 and 3.11 arbitrary units with one individual (F197) having a density of 3.75. The $CD4^+CCR5^+$ T-cell percentages for these individuals are all found below the mean percentage ranging between 6.9 and 16.8 %.

An unusual SNP was found at nucleotide position 582, known as Q194H, in all individuals found to be heterozygous for Δ32. The effect of this mutation on CCR5 expression requires further investigation.

A mutation exclusive to the South African Black African and Coloured populations originally discovered by Peterson and co-workers (2001), was found in the present study. The mutation at nucleotide position 673, known as R225X, was found in two Black African individuals in a heterozygous state (F135 and F203). The mutation is found in the third ICL with arginine being replaced with a stop codon resulting in premature termination of translation. Folefoc and co-workers (2010) created a mutant receptor construct containing the mutation and found complete absence of CCR5 expression at the cell surface. Furthermore, this prevented HIV binding and cells containing the mutant receptor were resistant to R5 HIV infection. Thus, the mutation in a homozygous state may provide resistance to HIV. The two individuals, F135 and F203, displayed CCR5 densities of 3.11 and 4.06 with $CD4^+CCR5^+$ T-cell percentages of 10.6 and 4.8, respectively.

The A335V mutation, at nucleotide position 1004, was found exclusively in the Black African population in the present study. The mutation was found in one homozygous individual (F021) and

in five heterozygous individuals (F037, F086, F119, F142 and F200). The mutation has previously been detected in the Black African and Coloured populations in both HIV positive and negative individuals (Peterson *et al.*, 2001). In addition, it was detected in the Black African population and the Caucasian group although at a lower frequency in the latter group (Picton *et al.*, 2010). The mutation is found in the C-terminal tail of the CCR5 protein with an amino acid change from alanine to valine, both of which are non-polar amino acids. Carrington and co-workers (1997) suggested that the A335V mutation did not have an effect on HIV progression but was found in a long-term non progressor. Additional studies have shown that the mutation may significantly aid in the occurrence of long-term non progression as the mutation was present in individuals living with HIV for many years (Hayes *et al.*, 2002). However, the study mentioned that the mutation does not contribute solely to the effect on HIV progression but may be linked to another genetic marker. Interestingly, the mutation was found in most of the individuals with higher CCR5 densities and $CD4^{+}CCR5^{+}$ T-cell percentages (Table 5) in the present study. Further investigation found that these individuals were also heterozygous or homozygous for the P1 promoter haplotype known to accelerate disease progression (Table 8).

Another mutation in the C-terminal tail was found in the present study at nucleotide position 1016 known as Y339F (Ansari-Lari *et al.*, 1997; Carrington *et al.*, 1997). The mutation was found in a Black African homozygous individual. Previously, the mutation was detected in an HIV negative Black African individual (Peterson *et al.*, 2001). The mutation causes an amino acid change of the polar tyrosine residue to non-polar phenylalanine. The importance of the C-terminal tail in receptor trafficking to the cell surface and in CCR5 functional response was discussed in Chapter two. Therefore, the mutant receptor may affect these functions.

5.2.3 Report of novel CCR5 mutations discovered in the present study

A total of 12 novel SNPs were identified in the present study including two in the ORF (Table 9). Seven mutations were exclusive to the Black African group while three were exclusive to the Caucasian group.

Table 9: Novel mutations detected in the Black African and Caucasian groups in the present study

Region	Position	Base Change wt/m	Black Africans[a]	Caucasians[b]
5' Upstream	**-5240**	C/T	0.000	1 (0.014)
Intron 1	**-2273**	A/G	2 (0.034)	0.000
Intron 2	**-1498**	C/T	8 (0.138)	4 (0.057)
	-783	G/A	2 (0.034)	0.000
	-508	G/A	0.000	1 (0.014)
	-10	C/G	1 (0.017)	0.000
ORF/Exon 3	**667**	C/T	2 (0.034)	2 (0.029)
	817	A/C	1 (0.017)	0.000
3' Flanking region	**1134**	G/A	0.000	1 (0.014)
	1197	C/A	1 (0.017)	0.000
	1349	A/G	1 (0.017)	0.000
	1477	C/T	1 (0.017)	0.000

Footnote: [a] Frequency of mutation in the Black African population calculated using the total number of alleles (58). [b] Frequency of mutation in the Caucasian population calculated using the total number of alleles (70). For base change, wt refers to wild-type allele and m refers to mutant allele.

A novel SNP in the 5' region upstream of exon 1 was found at position -5420. This C/T mutation was in a heterozygous Caucasian individual (F197). Many mutations have been found within this region; however their clinical significance is unknown.

A novel SNP was detected in the intron 1 region of the CCR5 gene at position -2273. The A/G SNP was found in two Black African heterozygous individuals (F086 and F119). Studies have shown that the region encompassing intron 1 is crucial to CCR5 gene regulation and forms part of the Pd promoter (Moriuchi *et al.*, 1997; Guignard *et al.*, 1998). The region consists of several protected areas containing important *cis* acting elements which are the reason why promoter mutations in the area, as discussed previously, affect CCR5 expression and HIV progression. The novel mutation detected in the present study affects an ATG codon. According to Mummidi and co-workers (1997), these upstream ATG codons play important roles in regulating CCR5 expression as the upstream minicistrons created regulate CCR5 protein output. Thus it is possible that the -2273 mutation affects CCR5 expression as it is found in this important area.

A novel mutation in the ORF of the CCR5 gene was found at nucleotide position 667. The mutation is a C/T SNP found in two heterozygous Black Africans (F037 and F048) and in two Caucasian individuals (F044 and F055). The mutation affects the third ICL of the CCR5 protein where it results in replacement of the basic arginine residue to the non-polar tryptophan, with the arginine residue being highly conserved (Blanpain *et al.*, 2000). A mutation known as R223Q has previously been defined in this position and is known to significantly affect HIV co-receptor function, although it can still bind gp120 (Blanpain *et al,*, 2000). The novel mutation found in the present study, R223W, may

have a similar effect. Individuals containing the latter mutation in the present study displayed CCR5 densities in the lower range.

A mutation at nucleotide position 817 corresponding to position 273 in the protein was discovered in a single Black African heterozygous individual (F048). The individual also presented with the mutation R223W discussed above. The A/C SNP results in an amino acid change of the polar residue asparagine to the basic residue histidine (N273H). The mutation is located in the third ECL however the effect of this mutation still needs to be investigated.

The 5'UTR, introns and 3'UTR are important in CCR5 gene regulation (Mummidi *et al.*, 1997). Therefore, novel mutations found within intron 2 may play a significant role in CCR5 expression. In particular, the C/G SNP (-10) found in a single Black African heterozygous individual (F172), may be of considerable importance. The SNP is found adjacent to the translation start site in a region known to be important in initiating translation (Fairbanks and Anderson, 1999).

Four novel mutations were detected within the 3' flanking region, all occurring in heterozygous individuals. The effect of mutations in this region is not well understood and thus further investigation into these mutations should be completed.

5.3 CONCLUDING REMARKS

The present study analysed the sequence variation in two population groups in South Africa, Black African and Caucasian. The study discovered 63 previously identified mutations (Figure 25), 32 of which were found exclusively in the Black African population. A total of 12 novel mutations were found (Figure 25), seven of which are exclusive to the Black African population and three in the Caucasian group. According to Bamshad and co-workers (2002), genetic diversity is substantially greater in the African population which is confirmed by the higher proportion of CCR5 mutations detected in this group. Furthermore, the present study confirmed 11 mutations that have been previously discovered in the South African population (Peterson *et al.*, 2001; Picton *et al.*, 2010).

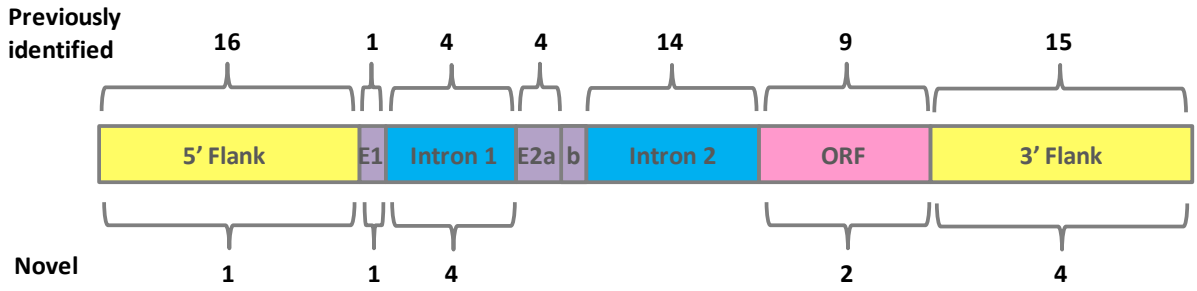


Figure 25: Illustration indicating the number of previously identified and novel mutations detected in the present study (Image created by Fatima Barmania)

Mutations in the Pd promoter have been shown to affect HIV disease progression (Martin *et al.*, 1998). These mutations were found in both the population groups analysed in this study. Correlation between CCR5 expression, described by flow cytometry, and promoter genotypes was insufficient to explain the effect on HIV disease progression. Studies have shown that these mutations are in strong linkage disequilibrium (Clegg *et al.*, 2000) and thus analysis of single site mutations may result in an erroneous interpretation. In retrospect, HIV status, viral load and the number of years an individual has been infected may have been useful in predicting the role of CCR5 mutations.

Shieh and co-workers (2000) illustrated that individuals with the 59029 A/A genotype have higher $CD4^{+}CCR5^{+}$ T-cell percentages. However, the present study could not confirm these findings as some individuals expressed higher percentages with the 59029 G/G genotype. This suggests the presence of other mutations which confound the results. Additionally, environmental factors can affect CCR5 expression, with individuals in Africa being found to express higher levels of CCR5 (Clerici *et al.*, 2000).

Although the present study has concentrated the discussion on mutations in the Pd promoter, the role of mutations in other regions is worth investigation. Mummidi and co-workers (2007) have shown the importance of the region upstream of exon 1, designated Pu, in CCR5 gene regulation.

An objective of the present study was to determine if the individual (F092) found to be CCR5 phenotypically null (Chapter 4) had a genetic cause. Genetic analysis confirmed that the phenotype was due to the individual being homozygous for the Δ32 mutation. Thus, the hypothesis which stated that the phenotypic absence of expression was due to a genetic cause is not rejected. Resistance to HIV in these individuals depends on the expression of the mutated protein which provides protection via a TDN effect (discussed in Chapter 2). Jin and co-workers (2008) have shown that homozygous individuals can be infected with HIV if they contain a promoter mutation at position 59537 which abolishes mutant protein expression completely. The homozygous individual

found in the present study was not found to display the latter mutation and is likely to be resistant to R5 HIV infection.

The frequency of Δ32 in the present study was found to be significantly different to that found in other South African studies. According to the null hypothesis, there should be no difference between the frequencies of Δ32 in this study in comparison to others. Thus, the null hypothesis regarding similarity in frequency of Δ32 in South African studies is rejected.

Concluding Discussion

The devastating effect of HIV is tacit and statistics (Chapter 2) have shown how the virus has infected millions of individuals around the world. The uncanny nature of the virus has made it relatively difficult to create a vaccine or cure, with reliance on ARVs, which are relatively toxic, being the best choice of treatment. The virus uses the host's receptors for entry, with the co-receptor CCR5 being critical for this purpose

The importance of the CCR5 receptor was discovered in individuals with genotypes that abolished the receptors expression from the cell surface (Dean *et al.*, 1996; Liu *et al.*, 1996; Samson *et al.*, 1996). These individuals, homozygous for a mutation known as Δ32, were resistant to HIV infection. The well publicised research by Hutter and co-workers (2009), in which they performed a bone marrow transplant on an HIV positive individual using a Δ32 homozygous donor has inspired many research groups. The HIV positive individual from the Hutter study has since been free of viral detection. Currently, many groups have invested research into mimicking the role of Δ32 using recombinant DNA technology to target CCR5 expression.

Studies have shown that CCR5 expression is highly heterogenous amongst individuals (Wu *et al.*, 1997). Considering the role of CCR5 in HIV infection, the present study aimed to determine the distribution of CCR5 in the South African population. In addition, a selected cohort of individuals was genotypically analysed to assess CCR5 mutations in the South African population.

Chapter four of this thesis provides data for the phenotypic distribution of CCR5 on $CD4^+$ T-cells in 245 individuals living in South Africa (Section 4.2.1). Expression of CCR5 was measured in two ways by flow cytometry: (a) as the percentage of $CD4^+CCR5^+$ T-cells, and (b) CCR5 density on the cell surface, with the latter being measured qualitatively. Analysis of the correlation between these two measurements was also determined (Section 4.2.2). Finally, the chapter discussed the effect of three variables, age, gender and ethnicity on CCR5 phenotypic expression (Section 4.2.3).

The percentage of $CD4^+CCR5^+$ T-cells was found to have a weak correlation with viral load, whereas CCR5 density is strongly correlated with the latter (Reynes *et al.*, 2000). The amount of CCR5 on the surface of $CD4^+$ T-cells can determine *in vivo* HIV production and the rate of $CD4^+$ T-cell decline (Reynes *et al.*, 2001). Previous studies have demonstrated mean $CD4^+CCR5^+$ T-cell percentages in healthy individuals to be between 5-15 % (Ostrowski *et al.*, 1998; de Roda Husman *et al.*, 1999; Zaunders *et al.*, 2001). In HIV infected individuals this percentage was between 10-28 % (Ostrowski *et al.*, 1998; de Roda Husman *et al.*, 1999).

In the present study, mean $CD4^+CCR5^+$ T-cell percentage was found at 21 % (standard deviation ± 10.9). Outliers were found at 45, 45.5, 54.9 and 55.9 with far outliers at 64, 77.5, and 86.9. Previously published South African studies (Shalekoff *et al.*, 2004) extrapolated mean percentages of 23 %, which is consistent with the findings of this thesis.

In contrast to data published in other studies, the results from this study suggest that South African individuals express higher $CD4^+CCR5^+$ T-cell percentages. The data for CCR5 density in the present study was measured qualitatively, making it relatively more difficult to make comparisons with published data which used quantitative methods.

However, the null hypothesis was rejected as data in the present study for phenotypic expression was different to previously found percentages. This unexpected finding may be due to the high rate of HIV and TB in South Africa. Cell activation is known to increase CCR5 expression; however, the effect on CCR5 density is moderate in comparison to the effect on $CD4^+CCR5^+$ T-cell percentages (Reynes *et al.*, 2000). In HIV infected individuals, the percentage of activated cells is much higher than in uninfected individuals (Ostrowski *et al.*, 1998). The $CD4^+CCR5^+$ T-cell percentage is known to increase with HIV disease progression, possibly due to HIV associated immune activation (de Roda Husman *et al.*, 1999). Furthermore, TB has been shown to increase CCR5 expression on $CD4^+$ T-cells (Juffermans *et al.*, 2001).

The findings in the present study is supported by Clerici and co-workers (2000) in a study which found that individuals living in Africa possess higher levels of CCR5 expression due to environmentally determined immune activation. Moreover, Kalinkovich and co-workers (2001) showed that $CD4^+$ T-cells from African immigrants living in Israel had increased CCR5, both in $CD4^+CCR5^+$ T-cell percentages and CCR5 density when compared to Caucasian Israelis. The study theorised that African individuals may be genetically predisposed to higher CCR5 levels.

Sub-Saharan Africa has the highest number of HIV and TB infected individuals, which may account for the higher percentages illustrated in this study (www.unaids.org). These possible explanations can be tested by including knowledge of the HIV and TB status of these individuals, in order to perform a comparative analysis on CCR5 expression in HIV infected and uninfected individuals with or without TB.

Chapter four (Section 4.2.2) discusses the correlation analysis between $CD4^+CCR5^+$ T-cell expression and CCR5 density. Previous studies have shown discrepancies between these two measurements (Reynes *et al.*, 2000). Reynes and co-workers (2000) showed individuals expressing low $CD4^+CCR5^+$ T-cell percentages but high density. Consistent with the aforementioned study, the present study

showed a very weak correlation ($r = 0.25$, $p < 0.05$) between these measurements. As such, the null hypothesis of no correlation between these measurements could not be rejected. As discussed in Chapter two, different factors govern CCR5 density and $CD4^+CCR5^+$ T-cell percentages. Immune activation and HIV infection may increase $CD4^+CCR5^+$ T-cell percentages but does not have a sufficient effect on CCR5 density (Reynes *et al.*, 2000). Thus, one may deduce that the correlation between these measurements is weak.

Based on the high levels of $CD4^+CCR5^+$ T-cells found in this study, a question arises as to the impact of HIV on individuals with high levels at the time of infection in comparison to those with low levels. The CCR5 density per cell might be considered low. However, if the number of cells expressing CCR5 is high, would this balance out the effect of lower density on viral load? If there are a greater number of cells prone to infection it may indicate higher levels of HIV virions produced. Conversely, high levels of $CD4^+CCR5^+$ T-cells indicate immune activation with subsequent CCR5 ligand release. The ligands for CCR5 are known to inhibit HIV infection by reducing CCR5 density as a consequence of ligand induced receptor endocytosis (Amara *et al.*, 1997; Mack *et al.*, 1998). The effect of ligand increase on CCR5 density may nullify the effect of high $CD4^+CCR5^+$ T-cell percentages on viral load.

Section 4.2.3 of Chapter four discussed the effect of age, gender and ethnicity on CCR5 expression, with the hypothesis stating that these variables do not affect CCR5 expression. The results from the present study illustrate that the age categories, 31-40 and > 40 years had higher levels of $CD4^+CCR5^+$ T-cell percentages than age 21-30 ($p < 0.05$). The former two age groups expressed outlier values between 54.9 % to 86.9 % which was not present in the 21-30 age category. In contrast, CCR5 density was not significantly different in the various age categories.

The effect of older age in $CD4^+CCR5^+$ T-cell percentages is supported by Yung and Mo (2003) in a study which showed that older individuals express increased chemokine receptors such as CCR5. In addition, studies demonstrate an age associated increase in the memory T-cell subset (Lerner *et al.*, 1989; Ernst *et al.*, 1990) which is known to express CCR5 (Wu *et al.*, 1997). Older individuals are known to have quicker progression to AIDS with data from the present study suggesting this could be due to an age associated increase in CCR5.

Although density was not significantly associated with age, both the $CD4^+CCR5^+$ T-cell percentage and density are regulated by different factors discussed above which can account for this result. Mention should also be made of the disproportionate number of individuals in each age category, with the > 40 age group constituting 57.7 % of the cohort studied. Sample bias can be a variable which affects the results.

Age-related diseases have been associated with increased CCR5 expression (Balistreri *et al.*, 2007). In the present study, outlier values or extremely high $CD4^+CCR5^+$ T-cell percentages were found in the 31-40 and > 40 age groups. Therefore, these individuals may be afflicted with age related illnesses leading to this high expression of $CD4^+CCR5^+$ T-cells.

Infection with HIV shows gender specific differences, with females having lower viral loads than men (Gandhi *et al.*, 2002). This is due to the lower densities of CCR5 found on $CD4^+$ T-cells (Portales *et al.*, 2001). In the present study, mean $CD4^+CCR5^+$ T-cell percentages in females were slightly lower (20.8 %) when compared to males (21.8 %). Regression analysis showed that females had a significantly lower percentage expression than males ($p < 0.05$), however, the difference is very small. Conversely higher outlier values were found in females, although this could be due to age related illnesses or HIV infection. In addition, the regression results illustrated no difference in gender for CCR5 density. Females accounted for 71 % of the sample size, and thus sample bias may have influenced the results.

A well known example of ethnic differences in CCR5 expression is the Δ32 mutation. Gonzalez and co-workers (1999) have also demonstrated that race specific differences occur in CCR5 haplotype expression. Results from the present study are consistent with previous reports, as Black African individuals showed higher $CD4^+CCR5^+$ T-cell percentages and CCR5 density. The mean $CD4^+CCR5^+$ T-cell percentage was significantly different in Black African individuals (23.4 %) when compared to Caucasian individuals (17.5 %). The distribution of $CD4^+CCR5^+$ T-cells was found to be between 4.8 and 45.4 % for Black Africans and between 0 and 37.9 % for Caucasians. The CCR5 density was also significantly higher. The cause of this difference is unknown, but may be influenced by genetic factors.

Therefore, the null hypothesis which states that the $CD4^+CCR5^+$ T-cell percentage is not affected by age, gender and ethnicity is rejected as these variables were indeed found to affect $CD4^+CCR5^+$ T-cells. Additionally, the null hypothesis stated that the variables age, gender and ethnicity do not affect CCR5 density. The latter was also rejected, although, only ethnicity was found to affect CCR5 density significantly.

The prevalence of individuals with CCR5 null phenotypes has not been established in the South African population. As a result of this, an objective of this study was to assess the CCR5 null frequency as these individuals are seemingly resistant to R5 HIV infection. The findings from the present study provide evidence that very few individuals fall within the low ranges of CCR5 expression. One of these individuals (0.4 % of cohort) was found to be phenotypically null for CCR5.

Therefore, the hypothesis which states that there are no null individuals in the South African population was rejected. Although rare, CCR5 null individuals are found in the South African population presumably limited to the Caucasian group.

Chapter five of this thesis begins with a discussion of the selection criteria used to determine individuals for genetic analysis (Section 5.2.1). The entire CCR5 gene (9028 bp) of 65 individuals was selected for sequencing. The cohort selected included 29 Black African, 35 Caucasians and one Indian individual. The chapter also reported on the mutations detected in the present study, which have previously been identified (Section 5.2.2). This was followed by a discussion of the novel mutations discovered in the present study (Section 5.2.3). Analysis of data revealed 11 individuals with a fragment of ORF that yielded unreadable sequencing results. The cloning and analysis of this region was discussed in Chapter five.

The CCR5 density as evident from the results in Chapter four was used to select the individuals sequenced. Considering that $CD4^{+}CCR5^{+}$ T-cell percentages are affected by diverse environmental factors (Chapter 2) as well as variables such as age and gender (Chapter 4), CCR5 density was a more reliable choice for correlation with constitutive factors. In addition, mutations in the CCR5 gene are known to affect CCR5 density (Liu *et al.*, 1996), but not necessarily $CD4^{+}CCR5^{+}$ T-cell percentages (Reynes *et al*., 2000). Most of the individuals sequenced (45) were found in the 0-3.6 CCR5 density category detected in the present study. Only 20 individuals above this CCR5 density were sequenced. The rationale behind this stems from the inhibiting effect of low CCR5 density with HIV infection and disease progression. Platt and co-workers (1998) found a 10 000 molecules/per cell density threshold for CCR5. Below this threshold, infection of cells with HIV was reduced considerably.

South African studies to date have largely focused on determining frequencies of CCR5 ORF mutations (Williamson *et al.*, 2000; Peterson *et al.*, 2001), as well as the functional effects of novel South African mutations in the ORF (Hayes *et al.,* 2002; Folefoc *et al.*, 2010). A study published in 2010 by Picton and co-workers, was the first paper to analyse the genetic variation in the entire CCR5 gene in both Black Africans and Caucasians. The study characterised the CCR5 genotype in 35 Black Africans and 35 Caucasian individuals. The results revealed 68 SNP positions, four indels and the Δ32 mutation.

In the present study, sequence analysis detected a total of 75 mutations in the study cohort. This included 63 previously known and 12 newly identified mutations. The mutations comprised 70 SNPs, four insertions and the Δ32 mutation. A total of 39 mutations were exclusive to the Black African

group, while 13 were exclusive to the Caucasian group. The data suggests that the Black African individuals have a greater genetic diversity. This finding is supported by previous data which shows that African populations have greater diversity and lower levels of linkage disequilibrium when compared to European populations (Tishkoff *et al.*, 2009). This was further corroborated by Picton and co-workers (2010), who also found a significantly higher number of mutations in the Black African group in comparison to the Caucasian group.

Of the four insertions detected, two were found in the 5' flanking region (-3879 and -3875) and two in intron 2 (-1126 and -353) (Table 6). The insert at position -3879 was found as a combination of two previously described polymorphisms with accession number rs145381188 reporting a CTAT insertion and accession number rs41412948 reporting a C/T SNP at the same location. The mutation was found in two Caucasian individuals. Cloning of this region will need to be performed in further studies to confirm this insertion. The insertion found at position -3875 in the present study was present in 18 Black Africans and 13 Caucasian individuals. The mutation was more common in the Black African group, although Picton and co-workers found this mutation higher in the Caucasian group. The insertion at position -1126 in the present study was also detected at higher levels in the Black African group unlike the study by Picton and co-workers (2010), which found the mutation to be more frequent in the Caucasian group. In the present study, all inserts were not cloned thus the allelic frequency could not be determined. As indicated in Table 6 (Chapter 5), the insert frequency was calculated by the number of individuals identified. Future research will need to use cloning to ensure the presence of the insert on both alleles. The last insert detected in the present study was located at position -353. Although the mutation was detected as an ACAA insert in the study by Picton and co-workers (2010), in the present study, it was found as a CAA insert. In the characterisation of the CCR5 gene by Mummidi and co-workers (1997), the mutation was also identified as a CAA insert. However, dbSNP reports two different mutations at this position, an A/G SNP or a guanine nucleotide insertion (rs41515644 and rs71619644). An interesting observation is the occurrence of the insertions at -3875, -1126 and -353, concurrently in individuals present with these insertions. According to Picton and co-workers (2010) these mutations were found in strong linkage disequilibrium in all three insertions in both populations.

The largest number of mutations in the present study was found in the introns and UTR regions, similar to that of Picton and co-workers (2010). The 5' UTR is known to be highly polymorphic (Bamshad *et al.*, 2002), which may account for the large number of mutations found in this region. Furthermore, both the present study and the Picton study found no mutations in the exon 2b region.

Mutations in the promoter region of the CCR5 gene have been well studied for their effects on HIV infection and disease progression. Particular mention must be made of the 59029 SNP (-2454), in which the wild-type homozygous G sequence is known to confer protection against HIV as it has reduced promoter activity in comparison to the homozygous mutant A sequence (McDermott *et al.* 1998). The -2454 SNP was found at relatively high frequencies in both the Black African (0.64) and the Caucasian groups in the present study. Consistent with previous reports, the 59029 A SNP was found in linkage with the Δ32 mutation. However, a single male individual heterozygous for Δ32 was not found with the 59029 mutation. The discovery of an individual with Δ32 and homozygous G wild type sequence was surprising as this has not been reported in other studies. The present study also found a linkage between the 59029 (-2454) and 59353 (-2130) SNPs as reported previously (McDermott *et al.*, 1998; Clegg *et al.*, 2000). However, unlike the previous reports, not all individuals with a heterozygous or homozygous 59029 genotype were found with similar allelic constituency for the 59353 genotype.

An objective of the present study was to correlate the phenotypic data (Chapter 4) with the genotypic data found in Chapter five. Individuals with the P1 promoter haplotype are associated with accelerated disease progression due to increased CCR5 expression. Individuals in the present study with this genotype had varying levels of CCR5 expression ranging from low to high (Table 5). An explanation for this is the presence of other polymorphisms which also affect CCR5 expression in different degrees.

Individuals with heterozygous Δ32 also displayed varying levels of CCR5 expression (Table 5). This was consistent with previous reports which found that heterozygosity for the Δ32 mutation is not necessarily a singular determinant affecting CCR5 expression (Ostrowski *et al.*, 1998). In addition, Reynes and co-workers (2000) found that the individual with the lowest CCR5 density had a wild-type ORF, whereas the highest density was present in a Δ32 heterozygote. It is difficult to correlate a set of mutations with CCR5 expression as it appears to rely on many factors or mutations that may not be associated directly with the CCR5 genotype. As such, the null hypothesis which stated that mutations can not be correlated with the flow data was not rejected.

Nine previously found mutations were detected in the ORF of the current study. The C101X mutation was found in a single Caucasian individual. The mutation has been found at a frequency of 0.9 % in the Caucasian group of the South African population (Williamson *et al.*, 2000). A mutation exclusive to the South African Black and Coloured groups, initially discovered by Peterson and co-workers (2001), was detected in two Black African heterozygous individuals. Folefoc and co-workers (2010)

have shown that this mutation resulted in premature termination of translation and was subsequently resistant to HIV *in vitro*.

The Δ32 mutation was detected in the Caucasian group at a frequency of 0.186, but was not found in the Black African group. The frequency differed significantly from those found in previous South African studies (Williamson *et al.*, 2000; Peterson *et al.*, 2001; Picton *et al.*, 2010). However, although the same numbers of Caucasian individuals were assessed in this study in comparison to Picton and co-workers, one can not exclude sample bias from the present study. The individuals sequenced in the previous South African studies consisted of randomly chosen individuals. In the present study, individuals were chosen based on CCR5 density with most of the individuals falling within the lower CCR5 density category. Hence, it is difficult to make a direct comparison with other South African publications.

A further objective of the present study was to assess if the phenotypically null individual, F092, identified by flow cytometric analysis (Chapter 4) had a genetic cause for this particular phenotype. Genetic analysis confirmed that the null phenotype displayed, was indeed due to a genetic cause with the individual being homozygous for Δ32. Therefore, the hypothesis which states that there is a genetic cause for the phenotype was not rejected.

Lastly, the current study found 12 novel mutations, two of which were in the ORF. The effect of these mutations is unknown, but in theory the SNP at position 667 may have a possible effect on HIV co-receptor function as a previously identified mutation in the same location has been found to affect HIV infection (Blanpain *et al.*, 2000). Further investigation into these novel mutations will need to be performed as the mutations' effect on CCR5 expression cannot solely be determined by the flow data.

In conclusion the main findings of this study include:

- South African individuals show considerable heterogeneity in CCR5 expression
- In comparison to previously published data, South African individuals express higher $CD4^+CCR5^+$ T-cell percentages which may result from environmental factors
- There was no correlation found between $CD4^+CCR5^+$ T-cell percentage and CCR5 density consistent with previously published data
- An age associated increase in $CD4^+CCR5^+$ T-cell percentage was noted, but not found for CCR5 density, confirming that the same factors may not necessarily affect both measurements of CCR5 expression

- Gender is weakly associated with $CD4^+CCR5^+$ T-cell percentage, with females showing slightly lower mean percentages. Gender was not found to associate with CCR5 density
- Black African individuals were found to have significantly higher $CD4^+CCR5^+$ T-cell percentages and CCR5 densities in comparison to Caucasian individuals, emphasising the role of ethnic differences in CCR5 expression
- A total of 75 mutations were detected in the current study, with 12 mutations to the best of the authors knowledge, being novel
- Greater genetic diversity is present in Black Africans, with 39 mutations being exclusive to this group
- Although certain mutations could be correlated with the phenotype (CCR5 null individual), overall, the genotype phenotype correlation was insufficient as individuals with similar mutations had varied CCR5 expression
- The Δ32 mutation and C101X mutants were detected in the Caucasian group
- The Δ32 mutation was found to be significantly higher than those found in previous South African studies, due to the selection of low density expressors for sequencing in this thesis

Limitations of the present study:

- CCR5 density was not measured quantitatively which resulted in the inability to compare to previous data
- Both the HIV and TB statuses of the individuals assessed were unknown and both infectious agents are known to affect CCR5 expression. Results from the present study may therefore, have been confounded by these unknown variables
- Selection of individuals should have been random with an equal number being chosen from each density category in order to conduct a comparative analysis
- Inability to confirm the effect of novel mutations on CCR5 expression

Future studies implemented should address the following:

- Taking into consideration the importance of both HIV and TB in CCR5 expression, it will be fundamental for future studies to include the status of individuals when assessing expression
- Research should focus on confirming if South African individuals express higher CCR5 levels and correlate this with high rates of HIV infection
- Although inter-individual variability has been shown in the present study, research should be conducted on the intra-individual variability

- Establishing a quantitative methodology for assessing CCR5 density in the South African population such as that described by Reynes *et al.*, 2000 or Zhang and co-workers (2010)
- Analysing the mutations in a larger cohort of South African individuals in order to confirm the frequency of mutations
- Cloning inserts and deletions to confirm the presence on both alleles
- Comparison with an African gene reference sequence (recently published Southern African genes by Schuster *et al.*, 2010), since reference sequences on current databases are developed from Caucasian or European individuals

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Appendix A

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

* **FWA** 00002567, Approved dd 22 May 2002 and Expires 13 Jan 2012.
* **IRB** 0000 2235 IORG0001762 Approved dd Jan 2006 and Expires 13 Aug 2011.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee
Fakulteit Gesondheidswetenskappe Navorsingsetiekkomitee

DATE: 11/10/2010

PROTOCOL NO.	**89/2010**
PROTOCOL TITLE	Rendering the Immune System Resistant to HIV
INVESTIGATOR	**Principal Investigator:** Prof. Michael S. Pepper
SUBINVESTIGATOR	None
SUPERVISOR	None
DEPARTMENT	**Dept:** Immunology **Phone:** 012-420-5317 **Mobile:** 072-209-6324 **E-Mail:** michael.pepper@up.ac.za
STUDY DEGREE	Grant
SPONSOR	None
MEETING DATE	26/05/2010

The Protocol and Informed Consent Document were approved on 29/09/2010 by a properly constituted meeting of the Ethics Committee subject to the following conditions:

1. The approval is valid until the end of December 2012, and
2. The approval is conditional on the receipt of 6 monthly written Progress Reports, and
3. The approval is conditional on the research being conducted as stipulated by the details of the documents submitted to and approved by the Committee. In the event that a need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Members of the Research Ethics Committee:

Prof M J Bester	(female)BSc (Chemistry and Biochemistry); BSc (Hons)(Biochemistry); MSc(Biochemistry); PhD (Medical Biochemistry)
Prof R Delport	(female)BA et Scien, B Curationis (Hons) (Intensive care Nursing), M Sc (Physiology), PhD (Medicine), M Ed Computer Assisted Education
Prof VOL Karusseit	MBChB; MFGP(SA); MMed(Chir); FCS(SA) - Surgeon
Prof JA Ker	MBChB; MMed(Int); MD – Vice-Dean (ex officio)
Dr NK Likibi	MBBCh – Representing Gauteng Department of Health)
Prof TS Marcus	(female) BSc(LSE), PhD (University of Lodz, Poland) – Social scientist
Dr MP Mathebula	(female)Deputy CEO: Steve Biko Academic Hospital
Prof A Nienaber	(female) BA(Hons)(Wits); LLB; LLM(UP); PhD; Dipl.Datametrics(UNISA) – Legal advisor
Mrs MC Nzeku	(female) BSc(NUL); MSc(Biochem)(UCL, UK) – Community representative
Prof L M Ntlhe	MBChB(Natal); FCS(SA)
Snr Sr J Phatoli	(female) BCur(Eet.A); BTec(Oncology Nursing Science) – Nursing representative
Dr R Reynders	MBChB (Prêt), FCPaed (CMSA) MRCPCH (Lon) Cert Med. Onc (CMSA)
Dr T Rossouw	(female) M.B.,Ch.B. (cum laude); M.Phil (Applied Ethics) (cum laude), MPH (Biostatistics and Epidemiology (cum laude), D.Phil
Dr L Schoeman	(female) B.Pharm, BA(Hons)(Psych), PhD – Chairperson: Subcommittee for students' research
Mr Y Sikweyiya	MPH; SARETI Fellowship in Research Ethics; SARETI ERCTP; BSc(Health Promotion) Postgraduate Dip (Health Promotion) – Community representative
Dr R Sommers	(female) MBChB; MMed(Int); MPharmMed – **Deputy Chairperson**
Prof TJP Swart	BChD, MSc (Odont), MChD (Oral Path), PGCHE – School of Dentistry representative
Prof C W van Staden	MBChB; MMed (Psych); MD; FCPsych; FTCL; UPLM - **Chairperson**

DR R SOMMERS; MBChB; MMed(Int); MPharmMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

* **FWA** 00002567, Approved dd 22 May 2002 and Expires 13 Jan 2012.
* **IRB** 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee
Fakulteit Gesondheidswetenskappe Navorsingsetiekkomitee

DATE: 1/09/2011

PROTOCOL	**146/2011** (previously accepted as 89/2010)
OLD TITLE	~~Analysis of CCR5 expressing CD4 cells and mutations in the CCR5~~ gene of African and Caucasian South African population groups
NEW TITLE	Analysis of CCR5 diversity in the South African population
INVESTIGATOR	**Principal Investigator:** Prof MS Pepper
SUBINVESTIGATOR	Ms F Barmania
SUPERVISOR	Prof MS Pepper **E-Mail:** Michael.Pepper@up.ac.za
DEPARTMENT	**Dept:** Immunology; Steve Biko Academic Hospital **Phone:** +27 (0)12 420 3845 **Fax:** +27 (0)12 420 3953 **E-Mail:** Michael.Pepper@up.ac.za **Cell:** +27 (0)72 209 6324
STUDY DEGREE	MSc Immunology
SPONSOR	Not Applicable
CONTACT DETAILS	**Representative:** Ms F Barmania **Phone:** 012 319 26 46 **E-Mail:** barmaniaf@gmail.com
VAT NO.	Not Applicable
SPONSORS POSTAL ADDRESS	Not Applicable
ORDER / CONTRACT NUMBER	Not Applicable
MEETING DATE	31/08/2011

The Protocol was approved on 31/08/2011 by a properly constituted meeting of the Ethics Committee subject to the following conditions:

1. The approval is valid for 2 years period [till the end of December 2013], and
2. The approval is conditional on the receipt of 6 monthly written Progress Reports, and
3. The approval is conditional on the research being conducted as stipulated by the details of the documents submitted to and approved by the Committee. In the event that a need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Members of the Research Ethics Committee:

Prof M J Bester	(female)BSc (Chemistry and Biochemistry); BSc (Hons)(Biochemistry); MSc(Biochemistry); PhD (Medical Biochemistry)
Prof R Delport	(female)BA et Scien, B Curationis (Hons) (Intensive care Nursing), M Sc (Physiology), PhD (Medicine), M Ed Computer Assisted Education
Prof JA Ker	MBChB; MMed(Int); MD – Vice-Dean (ex officio)
Dr NK Likibi	MBB HM – Representing Gauteng Department of Health) MPH
Dr MP Mathebula	(female)Deputy CEO: Steve Biko Academic Hospital; MBCHB, PDM, HM
Prof A Nienaber	(female) BA(Hons)(Wits); LLB; LLM; LLD(UP); PhD; Dipl.Datametrics(UNISA) – Legal advisor
Mrs MC Nzeku	(female) BSc(NUL); MSc(Biochem)(UCL, UK) – Community representative
Prof L M Ntlhe	MbChB (Natal) FCS (SA)
Snr Sr J Phatoli	(female) BCur(Eet.A); BTec(Oncology Nursing Science) – Nursing representative
Dr R Reynders	MBChB (Prêt), FCPaed (CMSA) MRCPCH (Lon) Cert Med. Onc (CMSA)
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Dr L Schoeman	(female) B.Pharm, BA(Hons)(Psych), PhD – Chairperson: Subcommittee for students' research
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Dr R Sommers	(female) MBChB; MMed(Int); MPharmMed – **Deputy Chairperson**
Prof TJP Swart	BChD, MSc (Odont), MChD (Oral Path), PGCHE – School of Dentistry representative
Prof C W van Staden	MBChB; MMed (Psych); MD; FCPsych; FTCL; UPLM - **Chairperson**

DR R SOMMERS; MBChB; MMed(Int); MPharmMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

Appendix B

PATIENT INFORMATION LEAFLET AND INFORMED CONSENT

(Each patient must receive, read and understand this document before the start of the study)

STUDY TITLE

Analysis of CCR5 diversity in the South African Population

INTRODUCTION

You are invited to participate in a laboratory-based research study conducted by the Department of Immunology from the University of Pretoria. This information leaflet is to help you to decide whether you would like to participate in a research project. Before agreeing to partake in this study, please familiarise yourself with all procedures involved and do not hesitate to ask the investigator about anything that you might be uncertain about. You should not agree to take part unless you are completely satisfied about all procedures involved.

WHAT IS THE PURPOSE OF THIS STUDY?

Researchers would like to perform analysis on cells in the blood of South African Individuals. For the purposes of this study, cells will be studied in a laboratory to look at a receptor (CCR5) on the surface of blood cells. The collection of these cells does not make use of any unethical procedures.

HOW ARE THE SAMPLES COLLECTED?

Collection will be based on a voluntary basis. A small amount of blood will be taken using routine procedures. You might experience discomfort/pain during the donation process, and there might be bruising or bleeding at the site from which the blood is taken.

WHAT WILL BE EXPECTED OF ME?

You will only need to give the researchers the permission to use the collected blood and provide some unidentifiable details such as age, gender, ethnicity and known medical conditions.
All your personal information will be regarded as confidential during this study. Each participant will be assigned a specific code and this code will be the only information that the researchers will have and the researchers will not be able to trace the identity of the blood.

HAS THE STUDY RECEIVED ETHICAL APPROVAL?

A study protocol for this study was submitted to the Research Ethics Committee and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki, which deals with the recommendations guiding doctors in biomedical research involving humans. A copy of this may be obtained from the investigator should you wish to review it.

WHAT ARE MY RIGHTS AS A PARTICIPANT IN THIS STUDY?

Your participation in this study is entirely voluntary and you can refuse to participate or withdraw consent at any time without stating any reason.

MAY ANY OF THESE STUDY PROCEDURES RESULT IN DISCOMFORT OR INCONVENIENCE

Venipunctures (i.e. drawing blood) are normally done as part of routine medical care and may present a slight risk of discomfort. Drawing blood may result in a bruise at the puncture site, or less commonly fainting or swelling of the vein, infection or bleeding from the site.

IS THERE FINANCIAL GAIN / LOSS FOR MY ACCOUNT IN THIS STUDY?

There will be no gain or loss should you participate in the study. This research could potentially lead to future profitable medical technologies, however you will not have access to these profits because of participation. There will be no financial costs for you to participate in the study.

SOURCE OF ADDITIONAL INFORMATION

If at any time you have any questions about the study, please do not hesitate to contact Prof. Michael Pepper at Tel: 012 420 3845 (Secretary) or Tel: 012 420 5317 (Direct) and Fatima Barmania (0826651888).

CONFIDENTIALITY

All information obtained during the course of this study is strictly confidential. Data that may be reported in scientific journals will not include any information which identifies you.

INFORMED CONSENT

I hereby confirm that I have been informed by the investigators, Prof. Michael Pepper and Fatima Barmania about the nature, conduct, benefits and risks of this study. I have also received, read and understood the above written information (Patient Information Leaflet and Informed Consent) regarding the study.

I am aware that the results of the study, including anonymous personal details regarding my ethnicity, gender, age, and other unidentifiable details will be processed into a study report.

I may, at any stage, without prejudice, withdraw my consent and participation in the study. I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

Patient's name _____
Patient's signature _____ Date _____

I, Prof Michael Pepper herewith confirm that the above patient has been informed fully about the nature, conduct and risks of the above study.

Investigator's name _____
Investigator's signature _____ Date _____

Patient Information Sheet							
Reference Number:	Date:			Collected By:			
Gender: M F		Race:	Black African	Caucasian	Coloured	Indian	Other
Age Group:	10-20 years	21-30 years	31-40 years	>40 years			
Known Conditions:	Diabetes	Cardiovascular	Other				
Medication:							

Appendix C

Raw Data for Flow cytometric results

CODE	AGE	GENDER	ETHNICITY	MFI	%$CD4^+CCR5^+$
T001	21-30 years	F	Caucasian	2.5	6.9
T002	21-30 years	F	Caucasian	5.5	17.2
T003	21-30 years	F	Caucasian	6.2	26.1
T004	>40 years	M	Caucasian	4.5	18.8
T005	>40 years	F	Caucasian	4.8	12.1
T006	31-40 years	F	Caucasian	3.0	9.3
T007	>40 years	M	Caucasian	6.0	26.9
F001	21-30 years	F	Caucasian	1.3	9.0
F002	21-30 years	M	Caucasian	2.9	11.7
F003	21-30 years	M	Caucasian	5.9	15.5
F004	21-30 years	F	Caucasian	5.3	18.5
F005	21-30 years	M	Caucasian	5.1	18.7
F006	21-30 years	F	Caucasian	5.5	18.2
F007	21-30 years	F	Black African	5.0	17.3
F008	21-30 years	F	Caucasian	3.1	10.5
F009	21-30 years	F	Black African	3.2	17.9
F010	21-30 years	F	Caucasian	7.2	22.8
F011	21-30 years	M	Coloured	6.4	25.4
F012	31-40 years	F	Indian	7.1	24.4
F013	31-40 years	M	Caucasian	6.0	17.7
F014	>40 years	F	Caucasian	2.9	8.4
F015	>40 years	F	Caucasian	4.5	17.7
F016	>40 years	F	Caucasian	4.8	12.0
F017	21-30 years	F	Caucasian	4.6	15.2
F018	21-30 years	M	Indian	5.8	11.8
F019	>40 years	F	Caucasian	3.9	16.2
F020	21-30 years	F	Black African	4.0	16.9
F021	21-30 years	F	Black African	5.0	32.1
F022	>40 years	M	Caucasian	3.7	19.5
F023	>40 years	F	Caucasian	4.5	12.1
F024	>40 years	M	Caucasian	2.8	9.7
F025	21-30 years	F	Caucasian	5.6	19.4
F026	31-40 years	M	Caucasian	5.7	25.7
F027	21-30 years	F	Caucasian	5.8	13.9
F028	21-30 years	M	Caucasian	4.5	10.4
F029	21-30 years	F	Caucasian	3.1	6.8
F030	21-30 years	F	Caucasian	3.1	19.9
F031	>40 years	F	Caucasian	3.8	17.6
F032	21-30 years	F	Indian	5.2	26.5
F033	>40 years	F	Caucasian	3.0	12.3
F034	21-30 years	F	Black African	5.9	18.8
F035	31-40 years	F	Black African	5.2	17.5
F036	31-40 years	F	Caucasian	4.7	12.3
F037	21-30 years	M	Black African	1.8	11.3
F038	21-30 years	F	Indian	1.5	8.9

CODE	AGE	GENDER	ETHNICITY	MFI	%CD4$^+$CCR5$^+$
T001	21-30 years	F	Caucasian	2.5	6.9
T002	21-30 years	F	Caucasian	5.5	17.2
T003	21-30 years	F	Caucasian	6.2	26.1
T004	>40 years	M	Caucasian	4.5	18.8
T005	>40 years	F	Caucasian	4.8	12.1
T006	31-40 years	F	Caucasian	3.0	9.3
T007	>40 years	M	Caucasian	6.0	26.9
F039	21-30 years	F	Black African	1.6	25.9
F040	31-40 years	F	Caucasian	1.7	18.4
F041	31-40 years	M	Caucasian	1.7	14.2
F042	31-40 years	F	Caucasian	1.4	10.7
F043	>40 years	F	Caucasian	1.6	5.4
F044	>40 years	F	Caucasian	1.6	14.1
F045	31-40 years	F	Caucasian	1.9	18.5
F046	21-30 years	F	Caucasian	1.8	14.6
F047	21-30 years	M	Black African	2.1	31.0
F048	21-30 years	F	Black African	2.3	17.3
F049	>40 years	F	Caucasian	1.8	20.4
F050	31-40 years	F	Caucasian	1.7	10.7
F051	>40 years	M	Black African	1.4	12.3
F052	>40 years	F	Caucasian	1.8	35.6
F053	>40 years	M	Caucasian	1.9	8.4
F054	31-40 years	F	Caucasian	1.3	10.4
F055	21-30 years	M	Caucasian	1.8	12.8
F056	>40 years	M	Caucasian	1.8	18.6
F057	21-30 years	F	Black African	5.2	25.7
F058	>40 years	F	Coloured	5.0	27.0
F059	21-30 years	F	Black African	3.1	13.9
F060	21-30 years	F	Caucasian	5.0	16.1
F061	>40 years	F	Black African	5.8	24.4
F062	>40 years	F	Caucasian	5.3	8.9
F063	>40 years	M	Coloured	2.7	9.1
F064	>40 years	M	Caucasian	4.5	25.0
F065	>40 years	M	Caucasian	3.7	29.6
F066	>40 years	M	Black African	5.1	15.6
F067	>40 years	M	Coloured	5.0	23.3
F068	31-40 years	F	Black African	6.0	9.5
F069	>40 years	F	Caucasian	4.1	12.6
F070	>40 years	M	Caucasian	3.7	12.1
F071	>40 years	F	Caucasian	4.3	15.9
F072	31-40 years	F	Caucasian	4.4	21.9
F073	>40 years	F	Caucasian	3.7	21.4
F074	>40 years	M	Caucasian	3.7	23.8
F075	>40 years	M	Caucasian	4.7	25.6
F076	>40 years	F	Caucasian	5.4	11.5
F077	>40 years	M	Black African	4.4	19.6
F078	>40 years	F	Black African	4.3	14.7
F079	>40 years	F	Caucasian	4.0	14.3
F080	21-30 years	F	Black African	6.5	28.2

CODE	AGE	GENDER	ETHNICITY	MFI	%CD4$^+$CCR5$^+$
T001	21-30 years	F	Caucasian	2.5	6.9
T002	21-30 years	F	Caucasian	5.5	17.2
T003	21-30 years	F	Caucasian	6.2	26.1
T004	>40 years	M	Caucasian	4.5	18.8
T005	>40 years	F	Caucasian	4.8	12.1
T006	31-40 years	F	Caucasian	3.0	9.3
T007	>40 years	M	Caucasian	6.0	26.9
F081	>40 years	F	Black African	3.4	26.9
F082	>40 years	F	Black African	4.2	11.6
F083	>40 years	F	Black African	3.8	20.8
F084	31-40 years	F	Black African	3.3	20.0
F085	31-40 years	F	Black African	3.6	12.4
F086	>40 years	M	Black African	2.9	55.9
F087	>40 years	M	Caucasian	2.4	16.8
F088	31-40 years	F	Black African	4.3	11.9
F089	>40 years	M	Caucasian	5.1	16.0
F090	31-40 years	F	Black African	5.9	19.6
F091	21-30 years	F	Caucasian	5.0	17.0
F092	>40 years	M	Caucasian	NULL	NULL
F093	>40 years	M	Caucasian	4.7	23.4
F094	31-40 years	F	Black African	4.2	23.7
F095	21-30 years	M	Black African	5.2	23.1
F096	>40 years	F	Caucasian	3.6	12.7
F097	>40 years	F	Black African	4.1	26.3
F098	>40 years	M	Caucasian	4.1	11.3
F099	>40 years	M	Black African	2.8	9.9
F100	31-40 years	F	Black African	5.0	29.2
F101	>40 years	F	Black African	5.9	9.4
F102	31-40 years	M	Black African	4.1	12.6
F103	>40 years	M	Caucasian	3.2	6.7
F104	>40 years	F	Caucasian	3.1	16.7
F105	21-30 years	F	Caucasian	4.1	9.6
F106	>40 years	F	Black African	4.3	28.4
F107	>40 years	F	Black African	4.6	45.5
F108	31-40 years	F	Black African	5.3	21.3
F109	31-40 years	F	Black African	5.0	22.5
F110	31-40 years	F	Black African	4.1	16.8
F111	>40 years	M	Black African	4.4	28.2
F112	31-40 years	F	Caucasian	4.4	17.6
F113	21-30 years	F	Black African	3.9	26.1
F114	31-40 years	F	Black African	2.5	34.5
F115	21-30 years	F	Black African	3.7	13.5
F116	>40 years	F	Caucasian	9.0	31.1
F117	>40 years	F	Black African	6.6	43.8
F118	21-30 years	F	Caucasian	5.4	18.3
F119	>40 years	F	Black African	5.0	42.6
F120	21-30 years	F	Black African	5.6	34.0
F121	>40 years	F	Caucasian	5.1	41.5
F122	31-40 years	F	Coloured	5.5	26.5

CODE	AGE	GENDER	ETHNICITY	MFI	%CD4$^+$CCR5$^+$
T001	21-30 years	F	Caucasian	2.5	6.9
T002	21-30 years	F	Caucasian	5.5	17.2
T003	21-30 years	F	Caucasian	6.2	26.1
T004	>40 years	M	Caucasian	4.5	18.8
T005	>40 years	F	Caucasian	4.8	12.1
T006	31-40 years	F	Caucasian	3.0	9.3
T007	>40 years	M	Caucasian	6.0	26.9
F123	31-40 years	F	Caucasian	3.7	14.4
F124	31-40 years	F	Caucasian	5.5	16.5
F125	>40 years	M	Black African	6.0	29.1
F126	>40 years	F	Black African	4.1	20.7
F127	>40 years	F	Caucasian	6.4	11.6
F128	>40 years	F	Caucasian	4.1	23.7
F129	>40 years	F	Caucasian	4.6	10.8
F130	>40 years	M	Caucasian	4.8	17.2
F131	>40 years	F	Black African	4.0	33.0
F132	>40 years	F	Black African	4.3	13.0
F133	>40 years	F	Caucasian	3.9	27.5
F134	>40 years	F	Black African	3.7	64.0
F135	>40 years	M	Black African	3.1	10.6
F136	31-40 years	F	Black African	5.1	13.0
F137	21-30 years	F	Black African	4.2	24.2
F138	31-40 years	F	Black African	6.0	34.5
F139	31-40 years	F	Caucasian	5.1	24.3
F140	31-40 years	F	Caucasian	6.0	31.4
F141	31-40 years	F	Black African	7.7	77.5
F142	31-40 years	F	Black African	2.7	29.2
F143	>40 years	M	Black African	8.5	44.1
F144	>40 years	M	Caucasian	4.6	25.5
F145	21-30 years	F	Black African		28.2
F146	>40 years	F	Black African	4.9	31.1
F147	31-40 years	F	Black African	5.9	20.9
F148	>40 years	F	Caucasian	4.5	17.2
F149	>40 years	M	Caucasian	5.8	17.7
F150	>40 years	F	Caucasian	5.1	24.6
F151	>40 years	F	Black African	4.8	12.8
F152	>40 years	M	Caucasian	5.5	45.0
F153	>40 years	F	Black African	5.6	18.7
F154	>40 years	F	Caucasian	3.1	15.8
F155	>40 years	M	Caucasian	5.1	25.4
F156	21-30 years	F	Caucasian	3.9	14.5
F157	>40 years	F	Caucasian	2.7	6.6
F158	>40 years	F	Black African	2.7	26.8
F159	21-30 years	F	Caucasian	4.1	37.9
F160	>40 years	F	Caucasian	4.6	18.5
F161	>40 years	F	Caucasian	3.6	13.8
F162	>40 years	F	Black African	4.4	36.4
F163	>40 years	F	Black African	3.8	23.9
F164	>40 years	F	Black African	4.4	41.9

CODE	AGE	GENDER	ETHNICITY	MFI	%CD4$^+$CCR5$^+$
T001	21-30 years	F	Caucasian	2.5	6.9
T002	21-30 years	F	Caucasian	5.5	17.2
T003	21-30 years	F	Caucasian	6.2	26.1
T004	>40 years	M	Caucasian	4.5	18.8
T005	>40 years	F	Caucasian	4.8	12.1
T006	31-40 years	F	Caucasian	3.0	9.3
T007	>40 years	M	Caucasian	6.0	26.9
F165	>40 years	M	Black African	5.1	28.7
F166	31-40 years	F	Black African	5.7	18.1
F167	21-30 years	F	Black African	6.4	14.7
F168	31-40 years	M	Black African	5.4	30.9
F169	>40 years	F	Caucasian	4.1	15.1
F170	21-30 years	F	Black African	4.5	15.6
F171	31-40 years	F	Black African	7.0	29.5
F172	>40 years	F	Black African	2.8	5.8
F173	>40 years	F	Black African	3.3	8.6
F174	>40 years	F	Black African	5.0	25.5
F175	>40 years	M	Black African	5.0	14.4
F176	31-40 years	F	Black African	4.4	21.8
F177	>40 years	M	Black African	6.5	33.5
F178	21-30 years	F	Black African	6.2	13.6
F179	>40 years	M	Black African	4.3	22.5
F180	31-40 years	F	Black African	5.1	21.8
F181	>40 years	M	Black African	5.8	24.8
F182	>40 years	M	Black African	6.5	30.9
F183	>40 years	M	Caucasian	3.6	24.3
F184	>40 years	M	Black African	3.4	25.6
F185	21-30 years	F	Black African	5.4	23.4
F186	>40 years	M	Caucasian	4.4	13.4
F187	>40 years	F	Black African	5.8	14.3
F188	>40 years	F	Caucasian	5.5	14.0
F189	>40 years	M	Caucasian	5.0	22.5
F190	>40 years	F	Caucasian	5.9	26.1
F191	>40 years	F	Caucasian	5.2	16.5
F192	21-30 years	F	Caucasian	3.6	24.3
F193	>40 years	F	Caucasian	5.8	24.8
F194	>40 years	M	Black African	4.0	54.9
F195	>40 years	F	Caucasian	4.2	9.5
F196	>40 years	M	Caucasian	4.5	28.2
F197	>40 years	M	Caucasian	3.8	8.3
F198	>40 years	M	Coloured	5.5	28.9
F199	>40 years	F	Caucasian	3.8	30.1
F200	21-30 years	F	Black African	6.0	6.1
F201	>40 years	F	Black African	6.2	26.1
F202	>40 years	F	Black African	3.9	31.4
F203	>40 years	F	Black African	4.1	4.8
F204	>40 years	M	Caucasian	5.1	12.9
F205	>40 years	F	Black African	6.5	16.2
F206	31-40 years	M	Caucasian	7.7	15.4

CODE	AGE	GENDER	ETHNICITY	MFI	%$CD4^+CCR5^+$
T001	21-30 years	F	Caucasian	2.5	6.9
T002	21-30 years	F	Caucasian	5.5	17.2
T003	21-30 years	F	Caucasian	6.2	26.1
T004	>40 years	M	Caucasian	4.5	18.8
T005	>40 years	F	Caucasian	4.8	12.1
T006	31-40 years	F	Caucasian	3.0	9.3
T007	>40 years	M	Caucasian	6.0	26.9
F207	>40 years	F	Black African	6.0	16.2
F208	>40 years	M	Black African	5.3	18.8
F209	>40 years	F	Black African	6.3	27.0
F210	21-30 years	F	Black African	4.7	13.5
F211	21-30 years	F	Black African	7.2	12.3
F212	21-30 years	F	Black African	5.1	19.7
F213	>40 years	F	Black African	4.2	24.6
F214	>40 years	F	Black African	4.2	25.5
F215	21-30 years	M	Black African	4.6	27.7
F216	>40 years	F	Black African	3.3	7.8
F217	21-30 years	F	Black African	3.6	28.5
F218	>40 years	F	Black African	5.3	17.9
F219	>40 years	M	Black African	6.6	34.5
F220	>40 years	F	Black African	4.4	29.0
F221	>40 years	M	Black African	5.2	20.4
F222	>40 years	F	Coloured	2.9	86.9
F223	>40 years	F	Black African	5.6	18.8
F224	>40 years	F	Black African	5.2	28.6
F225	>40 years	F	Black African	5.2	38.1
F226	>40 years	M	Black African	4.1	20.2
F227	21-30 years	F	Black African	5.7	13.4
F228	>40 years	F	Black African	6.0	8.1
F229	>40 years	F	Black African	4.6	16.6
F230	21-30 years	F	Black African	7.1	15.7
F231	>40 years	F	Black African	4.7	25.5
F232	>40 years	M	Black African	5.0	25.4
F233	21-30 years	F	Black African	8.6	20.7
F234	>40 years	M	Black African	4.4	16.1
F235	31-40 years	F	Black African	5.4	22.4
F236	>40 years	F	Black African	6.6	22.4
F237	31-40 years	F	Black African	6.5	26.6
F238	21-30 years	F	Black African	8.3	25.3

Appendix D

Raw Data for Sequencing results (0 = wild type; 1 = heterozygous mutation; 2 = homozygous mutation)

	-5272	-5270	-5240	-5218	-5084	-4749	-4349	-4248	-4079
T001	1	0	0	0	0	0	0	1	0
T002	0	0	0	0	0	0	2	0	0
F002	2	1	0	0	0	0	0	1	0
F010	0	0	0	0	0	0	0	0	0
F014	0	0	0	0	0	0	1	0	0
F024	1	0	0	0	0	0	0	1	0
F029	0	0	0	0	0	0	1	0	0
F030	0	0	0	0	0	0	0	0	0
F032	1	0	0	0	0	0	1	1	0
F033	1	0	0	0	0	0	1	0	0
F040	0	0	0	0	0	0	1	0	0
F042	1	0	0	0	0	0	0	1	0
F043	0	0	0	0	0	0	0	0	0
F044	1	0	0	0	0	0	0	1	0
F045	0	0	0	0	0	0	1	0	0
F046	1	0	0	0	0	0	1	1	0
F049	0	0	0	0	0	0	0	0	0
F050	0	0	0	0	0	0	1	0	0
F052	0	0	0	0	0	0	1	0	0
F053	0	0	0	0	0	0	0	0	0
F054	1	0	0	0	0	0	0	1	0
F055	0	0	0	0	0	0	2	0	0
F056	0	0	0	0	0	0	0	0	0
F062	0	0	0	0	0	0	0	0	0
F070	0	0	0	0	0	0	1	0	0
F087	1	0	0	0	0	0	1	1	0
F092	2	0	0	0	0	0	0	2	0
F093	0	0	0	0	0	0	2	0	0
F103	0	0	0	0	0	0	2	0	0
F104	1	0	0	0	0	0	0	1	0
F105	0	0	0	0	0	0	0	0	0
F121	0	0	0	0	0	0	1	0	0
F154	1	0	0	0	0	0	0	1	0
F157	1	0	0	0	0	0	1	1	0
F195	0	0	0	0	0	0	1	0	0
F197	1	0	1	0	0	0	1	1	0
F007	0	0	0	0	0	0	1	0	1
F009	0	0	0	0	0	0	1	0	0
F021	0	0	0	0	0	0	0	0	0
F037	1	1	0	0	0	0	0	0	0
F039	0	0	0	0	0	1	0	0	0
F047	0	0	0	0	0	1	0	0	1
F048	0	0	0	0	0	0	0	0	1
F051	0	0	0	0	0	0	0	0	1

F059	0	0	0	1	0	1	0	0	0
F068	0	0	0	0	0	0	0	0	0
F081	0	0	0	0	1	0	0	0	1
F084	0	0	0	0	0	1	0	0	0
F086	0	0	0	0	0	0	0	0	1
F099	0	0	0	0	0	0	0	0	0
F101	0	0	0	0	0	0	0	0	0
F102	0	0	0	0	0	1	0	0	0
F114	0	0	0	0	0	0	0	0	1
F115	0	0	0	1	0	0	0	0	0
F119	0	0	0	0	0	0	0	0	0
F135	0	0	0	0	0	0	0	0	0
F142	0	0	0	0	0	0	0	0	0
F158	0	0	0	0	0	0	0	0	0
F172	0	0	0	0	0	0	0	0	0
F173	0	0	0	0	0	0	0	0	0
F184	0	0	0	0	0	0	0	0	0
F200	0	0	0	0	0	0	1	0	0
F203	0	0	0	0	0	0	0	0	0
F216	0	0	0	0	0	0	0	0	1
F228	0	0	0	0	0	0	0	0	1
	-3891	**-3886**	**-3879**	**-3875**	**-3827**	**-3453**	**-3256**	**-2847**	**-2728**
T001	1	0	0	0	0	1	0	1	1
T002	2	0	0	IN	0	2	0	2	0
F002	2	0	0	IN	0	2	0	2	2
F010	0	0	0	0	0	1	0	0	0
F014	1	0	0	0	0	1	0	1	0
F024	2	0	IN	0	0	2	0	1	1
F029	1	0	0	0	0	1	0	1	0
F030	2	0	0	0	0	1	0	0	0
F032	2	0	0	IN	0	2	0	2	1
F033	2	0	0	IN	0	2	0	2	1
F040	1	0	0	0	0	1	0	1	0
F042	2	0	0	IN	0	2	0	1	1
F043	1	0	0	0	0	1	0	0	0
F044	1	0	0	0	0	1	0	1	1
F045	2	0	0	IN	0	2	0	1	0
F046	2	0	0	IN	0	2	0	1	1
F049	0	0	0	0	0	0	0	0	0
F050	1	0	0	0	0	1	0	1	0
F052	1	0	0	0	0	1	0	1	0
F053	0	0	0	0	0	1	0	0	0
F054	1	0	0	0	0	1	0	1	1
F055	2	0	0	IN	0	2	0	1	0
F056	0	0	0	0	0	1	0	1	0
F062	0	0	0	0	0	1	0	1	0
F070	1	0	0	0	0	1	0	1	0
F087	2	0	0	IN	0	2	0	2	1
F092	2	0	0	IN	0	2	0	2	2
F093	2	0	0	IN	0	2	0	2	1

F103	2	0	0	IN	0	2	0	1	0
F104	2	0	IN	0	0	2	0	1	1
F105	1	0	0	0	0	1	0	0	0
F121	1	0	0	0	0	1	0	1	0
F154	1	0	0	0	0	1	0	1	1
F157	2	0	0	IN	0	2	0	2	1
F195	1	0	0	0	0	1	0	1	0
F197	2	0	0	IN	0	2	0	1	1
F007	2	1	0	IN	0	2	1	1	0
F009	2	0	0	IN	0	2	0	1	0
F021	2	0	0	IN	0	2	0	2	0
F037	2	0	0	IN	0	2	0	2	1
F039	0	0	0	0	0	1	0	0	0
F047	2	1	0	IN	1	2	1	0	0
F048	1	1	0	0	0	1	1	0	0
F051	2	1	0	IN	0	1	1	0	0
F059	1	0	0	0	0	1	0	0	0
F068	1	0	0	0	0	1	0	0	0
F081	2	1	0	IN	1	2	1	0	0
F084	2	0	0	IN	0	2	0	0	0
F086	2	1	0	IN	1	2	1	1	0
F099	1	0	0	0	0	1	0	1	0
F101	2	0	0	IN	0	2	0	1	0
F102	2	0	0	IN	0	2	0	1	0
F114	2	1	0	IN	0	2	1	0	0
F115	1	0	0	0	0	1	0	0	0
F119	1	0	0	0	0	1	0	1	0
F135	2	0	0	IN	0	2	0	0	0
F142	0	0	0	0	0	1	0	1	0
F158	0	0	0	0	0	0	0	0	0
F172	1	0	0	0	0	1	0	0	0
F173	1	0	0	0	0	1	0	0	0
F184	2	0	0	IN	0	2	0	1	1
F200	2	0	0	IN	0	2	0	2	0
F203	2	0	0	IN	0	2	0	1	0
F216	2	1	0	IN	1	2	1	1	0
F228	2	1	0	IN	0	2	1	0	0
	-2572	**-2549**	**-2454**	**-2449**	**-2273**	**-2130**	**-2127**	**-2081**	**-2043**
T001	0	1	1	0	0	1	0	0	0
T002	0	2	2	0	0	2	0	2	0
F002	0	2	0	0	0	2	0	2	0
F010	0	1	1	0	0	1	0	1	0
F014	0	1	1	0	0	1	0	1	0
F024	0	2	1	0	0	1	0	2	0
F029	0	1	1	0	0	1	0	1	0
F030	0	1	1	0	0	1	0	1	0
F032	0	2	2	0	0	2	0	2	0
F033	0	2	2	0	0	1	0	2	0
F040	0	1	1	0	0	1	0	1	0
F042	0	2	2	0	0	2	0	2	0

F043	0	1	1	0	0	1	0	1	0
F044	0	1	1	0	0	1	0	1	0
F045	0	2	2	0	0	2	0	2	0
F046	0	2	2	0	0	2	0	2	0
F049	0	0	0	0	0	0	0	0	0
F050	0	1	1	0	0	1	0	1	0
F052	0	1	1	0	0	1	0	1	0
F053	0	1	0	0	0	0	0	1	0
F054	0	1	1	0	0	1	0	1	0
F055	0	2	2	0	0	2	0	2	0
F056	0	1	1	0	0	1	0	1	0
F062	0	1	1	0	0	1	0	1	0
F070	0	1	1	0	0	1	0	1	0
F087	0	2	2	0	0	2	0	2	0
F092	0	2	2	0	0	2	0	2	0
F093	0	2	2	0	0	2	0	2	0
F103	0	2	1	0	0	2	0	2	0
F104	0	2	1	0	0	1	0	1	0
F105	0	1	1	0	0	1	0	1	0
F121	0	1	1	0	0	1	0	1	0
F154	0	1	1	0	0	1	0	1	0
F157	0	2	2	0	0	2	0	2	0
F195	0	1	1	0	0	1	0	1	0
F197	0	2	2	0	0	2	0	2	0
F007	0	1	1	0	0	1	1	2	0
F009	0	2	2	0	0	2	0	2	0
F021	0	2	2	0	0	2	0	2	0
F037	0	2	2	0	0	2	0	2	0
F039	0	1	0	0	0	0	0	1	0
F047	0	1	0	0	0	0	0	2	0
F048	0	0	0	0	0	0	1	2	0
F051	0	1	1	0	0	1	1	2	0
F059	0	1	0	0	0	0	0	1	0
F068	0	1	1	0	0	1	0	1	0
F081	0	1	1	0	0	1	0	2	0
F084	0	2	1	0	0	1	0	2	0
F086	0	1	1	0	1	1	0	2	0
F099	0	1	1	0	0	1	0	1	0
F101	0	2	1	0	0	0	0	2	0
F102	1	2	1	0	0	1	0	2	0
F114	0	1	0	0	0	0	1	1	0
F115	0	1	1	0	0	1	0	1	0
F119	0	1	1	0	1	1	0	1	0
F135	0	2	1	0	0	1	0	2	0
F142	0	1	1	0	0	1	0	1	0
F158	0	0	0	0	0	0	0	0	0
F172	0	1	1	0	0	1	0	1	1
F173	0	1	1	0	0	1	0	1	0
F184	0	2	2	0	0	2	0	2	0
F200	0	2	2	0	0	2	0	2	0

F203	0	2	2	0	0	1	0	2	0
F216	0	1	1	1	0	1	0	2	0
F228	0	1	1	0	0	1	1	2	0
	-1830	**-1681**	**-1498**	**-1459**	**-1188**	**-1126**	**-1057**	**-973**	**-972**
T001	0	0	0	0	0	0	0	1	0
T002	0	0	0	0	0	IN	0	2	0
F002	0	0	0	0	0	IN	0	2	0
F010	1	0	0	0	0	0	0	1	0
F014	0	0	0	0	0	0	0	1	0
F024	0	0	0	0	0	IN	1	2	0
F029	0	0	0	0	0	0	0	1	0
F030	1	0	0	0	0	0	0	1	0
F032	0	0	0	0	0	IN	0	2	0
F033	0	0	0	0	0	IN	0	2	0
F040	0	0	0	0	0	0	0	1	0
F042	1	0	0	0	0	IN	0	2	0
F043	1	0	0	0	0	0	0	1	0
F044	0	0	0	0	0	0	0	1	0
F045	1	0	0	0	0	IN	0	2	0
F046	0	0	0	0	0	IN	0	2	0
F049	0	0	0	0	0	0	0	0	0
F050	0	0	0	0	0	0	0	1	0
F052	0	0	0	0	0	0	0	1	0
F053	0	0	0	0	0	0	1	1	0
F054	0	0	0	0	0	0	0	1	0
F055	0	0	0	0	0	IN	0	2	0
F056	0	0	0	0	0	0	0	1	0
F062	0	0	0	0	0	0	0	1	0
F070	0	0	0	0	0	0	0	1	0
F087	0	0	0	0	0	IN	0	2	0
F092	0	0	0	0	0	IN	0	2	0
F093	0	0	0	0	0	IN	0	2	0
F103	0	0	0	0	0	IN	0	2	0
F104	0	0	0	0	0	IN	1	2	0
F105	1	0	1	0	0	0	0	1	0
F121	0	0	0	0	0	0	0	1	0
F154	0	0	1	0	0	0	0	1	0
F157	0	0	1	0	0	IN	0	2	0
F195	0	0	1	0	0	0	0	1	0
F197	0	0	0	0	0	IN	0	2	0
F007	0	0	0	0	0	IN	0	2	0
F009	1	0	0	0	0	IN	0	2	0
F021	0	0	0	0	0	IN	0	2	0
F037	0	0	0	0	0	IN	0	2	0
F039	0	1	0	1	0	0	0	1	1
F047	0	1	0	1	0	IN	0	2	1
F048	0	0	0	0	0	0	0	1	0
F051	1	0	0	0	0	IN	0	2	0
F059	0	1	0	1	1	0	0	1	1
F068	1	0	0	0	0	0	0	1	0

F081	1	0	0	0	0	IN	0	2	0
F084	1	1	0	1	0	IN	0	0	1
F086	0	0	0	0	0	IN	0	2	0
F099	0	0	0	0	0	0	0	1	0
F101	0	1	0	1	0	IN	0	2	0
F102	0	1	0	1	0	IN	0	2	0
F114	0	1	0	1	0	IN	0	2	0
F115	1	0	0	0	1	0	0	1	0
F119	0	0	1	0	0	0	0	1	0
F135	1	1	0	1	0	IN	0	2	0
F142	0	1	1	0	0	0	0	1	0
F158	0	0	0	0	0	0	0	0	0
F172	1	0	1	0	0	0	0	1	0
F173	1	0	1	0	0	0	0	1	0
F184	1	0	1	0	0	IN	0	2	0
F200	0	0	1	0	0	IN	0	2	0
F203	0	1	1	1	0	IN	0	2	0
F216	0	0	0	0	0	IN	0	2	0
F228	1	0	1	0	0	IN	0	2	0
	-783	**-648**	**-508**	**-441**	**-359**	**-353**	**-113**	**-112**	**-10**
T001	0	1	0	1	1	0	0	0	0
T002	0	2	0	2	2	IN	0	0	0
F002	0	2	0	2	2	IN	0	0	0
F010	0	1	0	1	1	0	0	0	0
F014	0	1	0	1	1	0	0	0	0
F024	0	2	0	2	2	IN	0	0	0
F029	0	1	0	1	1	0	0	0	0
F030	0	1	0	1	1	0	0	0	0
F032	0	2	0	2	2	IN	0	0	0
F033	0	2	0	2	2	IN	0	0	0
F040	0	1	0	1	1	0	0	0	0
F042	0	2	0	2	2	IN	0	0	0
F043	0	1	0	1	1	0	0	0	0
F044	0	1	0	1	1	0	0	0	0
F045	0	2	0	2	2	IN	0	0	0
F046	0	2	0	2	2	IN	0	0	0
F049	0	0	0	0	0	0	0	0	0
F050	0	1	0	1	1	0	0	0	0
F052	0	1	0	1	1	0	0	0	0
F053	0	1	0	1	1	0	0	0	0
F054	0	1	0	1	1	0	0	0	0
F055	0	2	0	2	2	IN	0	0	0
F056	0	1	0	1	1	0	0	0	0
F062	0	1	0	1	1	0	0	0	0
F070	0	1	0	1	1	0	0	0	0
F087	0	1	1	2	2	IN	0	0	0
F092	0	2	0	2	2	IN	0	0	0
F093	0	2	0	2	2	IN	0	0	0
F103	0	2	0	2	2	IN	0	0	0
F104	0	2	0	2	2	IN	0	0	0

F105	0	1	0	1	1	0	0	0	0
F121	0	1	0	1	1	0	0	0	0
F154	0	1	0	1	1	0	0	0	0
F157	0	2	0	2	2	IN	0	0	0
F195	0	1	0	1	1	0	0	0	0
F197	0	2	0	2	2	IN	0	0	0
F007	0	2	0	2	2	IN	0	0	0
F009	0	2	0	2	2	IN	0	0	0
F021	0	2	0	2	2	IN	0	0	0
F037	0	2	0	2	2	IN	0	0	0
F039	0	1	0	1	1	0	1	0	0
F047	0	2	0	2	2	IN	1	0	0
F048	0	1	0	1	1	0	0	0	0
F051	0	2	0	2	2	IN	0	0	0
F059	0	1	0	1	1	0	1	0	0
F068	0	1	0	1	1	0	0	0	0
F081	1	2	0	2	2	IN	0	0	0
F084	0	2	0	2	2	IN	1	0	0
F086	1	2	0	2	2	IN	0	0	0
F099	0	1	0	1	1	0	0	0	0
F101	0	2	0	2	2	IN	1	0	0
F102	0	2	0	2	2	IN	1	0	0
F114	0	2	0	2	2	IN	1	0	0
F115	0	1	0	1	1	0	0	0	0
F119	0	1	0	1	1	0	0	0	0
F135	0	2	0	2	2	IN	1	0	0
F142	0	1	0	1	1	0	0	0	0
F158	0	0	0	0	0	0	0	0	0
F172	0	1	0	1	1	0	0	0	1
F173	0	1	0	1	1	0	0	0	0
F184	0	2	0	2	2	IN	0	0	0
F200	0	2	0	2	2	IN	0	0	0
F203	0	2	0	2	2	IN	1	0	0
F216	0	2	0	2	2	IN	0	0	0
F228	0	2	0	2	2	IN	0	1	0
	105	**164**	**180**	**303**	**554**	**582**	**667**	**673**	**817**
T001	0	0	0	0	1	1	0	0	0
T002	0	0	0	0	0	0	0	0	0
F002	0	0	0	0	1	1	0	0	0
F010	0	0	0	0	0	0	0	0	0
F014	0	1	0	0	0	0	0	0	0
F024	0	0	0	0	1	1	0	0	0
F029	0	0	0	0	0	0	0	0	0
F030	0	0	0	0	0	0	0	0	0
F032	0	0	0	0	0	0	0	0	0
F033	0	0	0	0	1	1	0	0	0
F040	0	0	0	0	0	0	0	0	0
F042	0	0	0	0	1	1	0	0	0
F043	0	0	0	0	0	0	0	0	0
F044	0	0	0	0	0	0	1	0	0

F045	0	0	0	0	0	0	0	0	0
F046	0	0	0	0	0	0	0	0	0
F049	0	0	0	0	0	0	0	0	0
F050	0	0	0	0	0	0	0	0	0
F052	0	1	0	0	0	0	0	0	0
F053	0	0	0	0	0	0	0	0	0
F054	0	0	0	0	1	1	0	0	0
F055	0	0	0	0	0	0	1	0	0
F056	0	0	0	0	0	0	0	0	0
F062	0	0	0	0	0	0	0	0	0
F070	0	0	0	0	0	0	0	0	0
F087	0	0	0	0	1	1	0	0	0
F092	0	0	0	0	2	0	0	0	0
F093	0	0	0	0	0	0	0	0	0
F103	0	1	0	1	0	0	0	0	0
F104	0	0	0	0	1	1	0	0	0
F105	0	0	0	0	0	0	0	0	0
F121	0	0	0	0	0	0	0	0	0
F154	0	0	0	0	1	1	0	0	0
F157	0	0	0	0	1	1	0	0	0
F195	0	0	0	0	0	0	0	0	0
F197	0	0	0	0	1	1	0	0	0
F007	0	0	0	0	0	0	0	0	0
F009	0	0	0	0	0	0	0	0	0
F021	0	0	0	0	0	0	0	0	0
F037	0	0	0	0	0	0	1	0	0
F039	0	0	0	0	0	0	0	0	0
F047	0	0	0	0	0	0	0	0	0
F048	0	0	0	0	0	0	1	0	1
F051	0	0	0	0	0	0	0	0	0
F059	0	0	0	0	0	0	0	0	0
F068	0	0	0	0	0	0	0	0	0
F081	0	0	0	0	0	0	0	0	0
F084	0	0	0	0	0	0	0	0	0
F086	0	0	0	0	0	0	0	0	0
F099	0	0	0	0	0	0	0	0	0
F101	0	0	0	0	0	0	0	0	0
F102	0	0	0	0	0	0	0	0	0
F114	0	0	0	0	0	0	0	0	0
F115	0	0	0	0	0	0	0	0	0
F119	0	0	0	0	0	0	0	0	0
F135	0	0	0	0	0	0	0	1	0
F142	0	0	0	0	0	0	0	0	0
F158	0	0	0	0	0	0	0	0	0
F172	0	0	1	0	0	0	0	0	0
F173	0	0	0	0	0	0	0	0	0
F184	0	0	0	0	0	0	0	0	0
F200	0	0	0	0	0	0	0	0	0
F203	0	0	0	0	0	0	0	1	0
F216	1	0	0	0	0	0	0	0	0

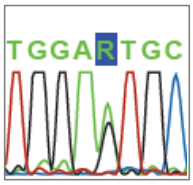
F228	0	0	0	0	0	0	0	0	0
	1004	**1016**	**1134**	**1197**	**1253**	**1349**	**1477**	**1752**	**1823**
T001	0	0	0	0	0	0	0	0	0
T002	0	0	0	0	0	0	0	0	0
F002	0	0	0	0	0	0	0	0	0
F010	0	0	0	0	0	0	0	0	0
F014	0	0	0	0	0	0	0	0	0
F024	0	0	0	0	0	0	0	0	1
F029	0	0	0	0	0	0	0	0	0
F030	0	0	0	0	0	0	0	0	0
F032	0	0	0	0	0	0	0	0	0
F033	0	0	0	0	0	0	0	0	0
F040	0	0	1	0	0	0	0	0	0
F042	0	0	0	0	0	0	0	0	0
F043	0	0	0	0	0	0	0	0	0
F044	0	0	0	0	0	0	0	0	0
F045	0	0	0	0	0	0	0	0	0
F046	0	0	0	0	0	0	0	0	0
F049	0	0	0	0	0	0	0	0	0
F050	0	0	0	0	0	0	0	0	0
F052	0	0	0	0	0	0	0	0	0
F053	0	0	0	0	0	0	0	0	1
F054	0	0	0	0	0	0	0	0	0
F055	0	0	0	0	0	0	0	0	0
F056	0	0	0	0	0	0	0	0	0
F062	0	0	0	0	0	0	0	0	0
F070	0	0	0	0	0	0	0	0	0
F087	0	0	0	0	0	0	0	0	0
F092	0	0	0	0	0	0	0	0	0
F093	0	0	0	0	0	0	0	0	0
F103	0	0	0	0	0	0	0	0	0
F104	0	0	0	0	0	0	0	0	1
F105	0	0	0	0	0	0	0	0	0
F121	0	0	0	0	0	0	0	0	0
F154	0	0	0	0	0	0	0	0	0
F157	0	0	0	0	0	0	0	0	0
F195	0	0	0	0	0	0	0	0	0
F197	0	0	0	0	0	0	0	0	0
F007	0	0	0	0	0	0	0	0	0
F009	0	0	0	0	0	0	0	0	0
F021	2	0	0	0	0	0	0	0	0
F037	1	0	0	0	0	0	0	0	0
F039	0	0	0	0	0	0	0	1	0
F047	0	0	0	0	1	0	0	1	0
F048	0	0	0	0	0	0	0	0	0
F051	0	0	0	0	0	0	0	0	0
F059	0	0	0	0	0	0	0	2	0
F068	0	0	0	0	0	0	0	0	0
F081	0	0	0	0	0	0	0	0	0
F084	0	0	0	0	0	0	0	1	0

F086	1	0	0	0	0	0	0	0	0
F099	0	1	0	0	0	0	0	0	0
F101	0	0	0	0	0	0	0	1	0
F102	0	0	0	0	0	0	0	2	0
F114	0	0	0	0	0	0	0	1	0
F115	0	0	0	1	0	0	0	1	0
F119	1	0	0	0	0	0	0	0	0
F135	0	0	0	0	0	0	0	1	0
F142	1	0	0	0	0	0	0	0	0
F158	0	0	0	0	0	0	0	0	0
F172	0	0	0	0	0	0	0	0	0
F173	0	0	0	0	0	1	0	0	0
F184	0	0	0	0	0	0	0	0	0
F200	1	0	0	0	0	0	1	0	0
F203	0	0	0	0	0	0	0	1	0
F216	0	0	0	0	0	0	0	0	0
F228	0	0	0	0	0	0	0	0	0
	1843	**1846**	**2066**	**2077**	**2225**	**2381**	**2435**	**2458**	**2676**
T001	0	0	0	1	0	0	0	0	1
T002	0	0	0	2	0	0	0	0	0
F002	0	0	0	2	0	0	0	0	0
F010	0	0	0	1	0	0	0	0	0
F014	0	0	0	1	0	0	0	0	0
F024	0	0	0	0	0	0	0	0	0
F029	0	0	0	1	0	0	0	0	0
F030	0	0	0	1	0	0	0	0	0
F032	0	0	0	2	0	0	0	0	0
F033	0	0	0	2	0	0	0	0	0
F040	0	0	0	1	0	0	0	0	0
F042	0	0	0	2	0	0	0	0	0
F043	0	0	0	1	0	0	0	0	0
F044	0	0	0	1	0	0	0	0	0
F045	0	0	0	2	0	0	0	0	0
F046	0	0	0	2	0	0	0	0	0
F049	0	0	0	0	0	0	0	0	0
F050	0	0	0	1	0	0	0	0	0
F052	0	0	0	1	0	0	0	0	0
F053	0	0	0	1	0	0	0	0	0
F054	0	0	0	1	0	0	0	0	1
F055	0	0	0	2	0	0	0	0	0
F056	0	0	0	1	0	0	0	0	0
F062	0	0	0	1	0	0	0	0	1
F070	0	0	0	1	0	0	0	0	0
F087	0	0	0	2	0	0	0	0	0
F092	0	0	0	2	0	0	0	0	0
F093	0	0	0	2	0	0	0	0	0
F103	0	0	0	2	0	0	0	0	0
F104	0	0	0	2	0	0	0	0	0
F105	0	0	0	1	0	0	0	0	0
F121	0	0	0	1	0	0	0	0	1

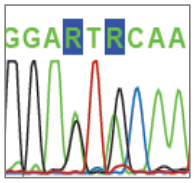
F154	0	0	0	1	0	0	0	0	0
F157	0	0	0	2	0	0	0	0	0
F195	0	0	0	1	0	0	0	0	0
F197	0	0	0	2	0	0	0	0	0
F007	0	0	0	2	0	0	0	0	0
F009	0	0	0	2	0	0	0	0	0
F021	0	0	0	2	0	0	0	0	0
F037	0	0	0	2	0	0	0	0	0
F039	0	0	1	1	0	0	0	0	0
F047	0	0	0	2	0	0	1	0	0
F048	0	0	0	1	0	0	0	1	0
F051	0	0	0	2	0	0	0	0	0
F059	0	0	0	2	0	1	0	0	0
F068	0	0	0	1	0	0	0	0	0
F081	0	0	0	2	0	0	1	0	0
F084	0	0	0	2	0	0	0	0	0
F086	0	0	0	2	0	0	1	0	0
F099	0	0	0	1	0	0	0	1	0
F101	1	1	0	1	1	0	0	0	0
F102	1	1	0	2	1	0	0	0	0
F114	1	0	0	2	1	0	0	0	0
F115	0	0	0	2	0	1	0	0	0
F119	0	0	0	1	0	0	0	1	0
F135	1	1	0	2	0	0	0	0	0
F142	0	0	1	1	0	0	0	0	0
F158	0	0	1	1	0	0	0	0	0
F172	0	0	0	1	0	0	0	0	0
F173	0	0	0	1	0	0	0	1	0
F184	0	0	0	2	0	0	0	0	0
F200	0	0	0	2	0	0	0	0	0
F203	1	1	0	2	0	0	0	0	0
F216	0	0	0	2	0	0	0	0	0
F228	0	0	0	0	0	0	0	0	0
	2838	**2919**	**3132**						
T001	0	1	0						
T002	0	2	0						
F002	0	2	0						
F010	0	1	0						
F014	0	1	0						
F024	0	1	0						
F029	0	0	0						
F030	0	1	1						
F032	0	2	0						
F033	0	2	0						
F040	0	1	0						
F042	0	2	0						
F043	0	1	0						
F044	0	1	0						
F045	0	2	0						
F046	0	2	0						

F049	0	0	0
F050	0	1	0
F052	0	1	0
F053	0	0	0
F054	0	1	0
F055	0	2	0
F056	0	1	0
F062	0	1	0
F070	0	1	0
F087	0	2	0
F092	0	2	0
F093	0	2	0
F103	0	2	0
F104	0	2	0
F105	0	1	0
F121	0	1	0
F154	0	1	0
F157	0	2	0
F195	0	1	0
F197	0	2	0
F007	0	1	0
F009	0	2	0
F021	0	2	0
F037	0	2	0
F039	0	0	0
F047	0	0	0
F048	0	0	0
F051	0	1	0
F059	0	0	0
F068	0	1	0
F081	0	1	0
F084	0	1	0
F086	0	1	0
F099	0	1	0
F101	1	1	0
F102	1	0	0
F114	0	0	0
F115	0	1	0
F119	0	1	0
F135	0	1	0
F142	0	1	0
F158	0	1	0
F172	0	1	0
F173	0	1	0
F184	0	2	0
F200	0	2	0
F203	0	1	0
F216	0	1	0
F228	0	1	0

Appendix E



-5272



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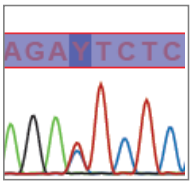

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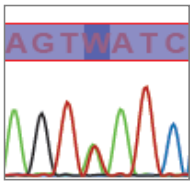

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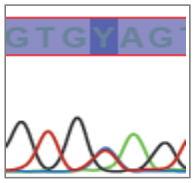

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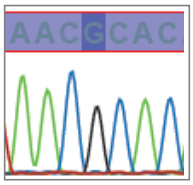

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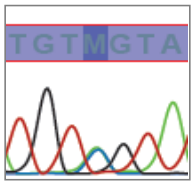

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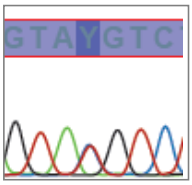

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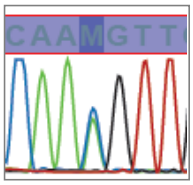

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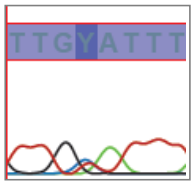

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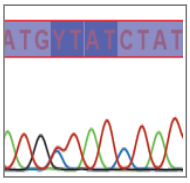

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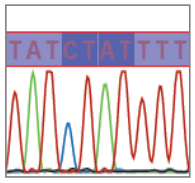

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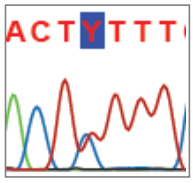

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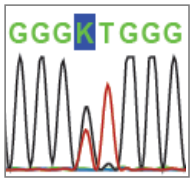

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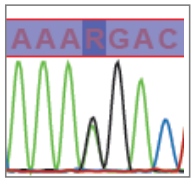

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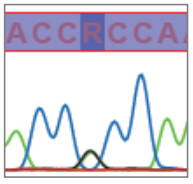

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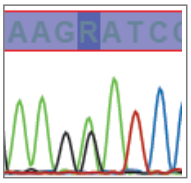

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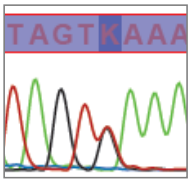

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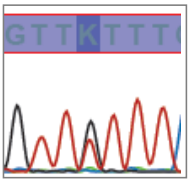

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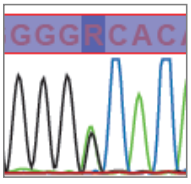

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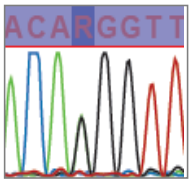

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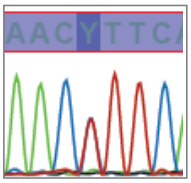

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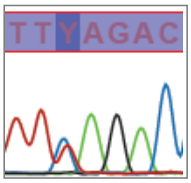

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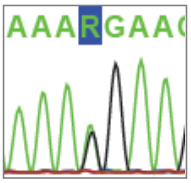

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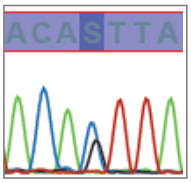

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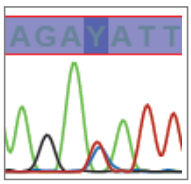

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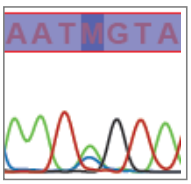

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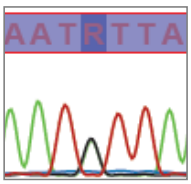

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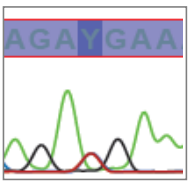

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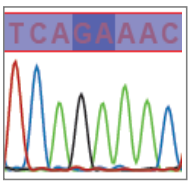

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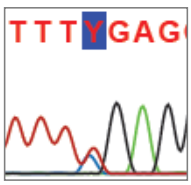

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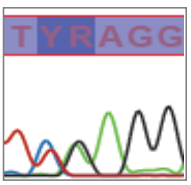

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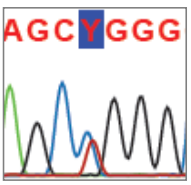

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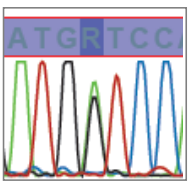

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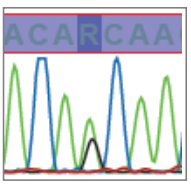

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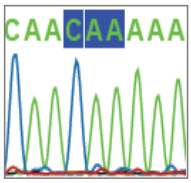
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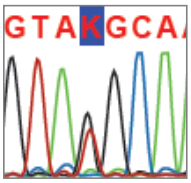
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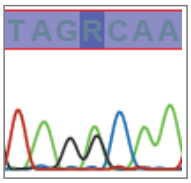
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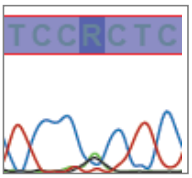
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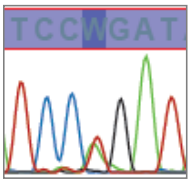
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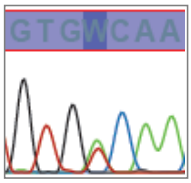
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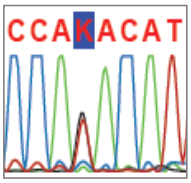
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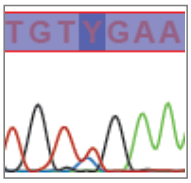
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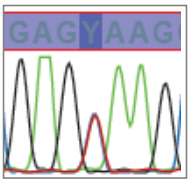
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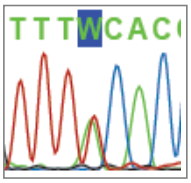
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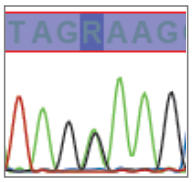
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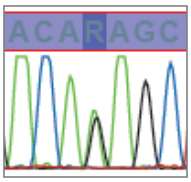
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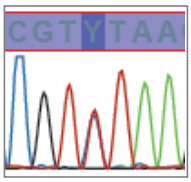
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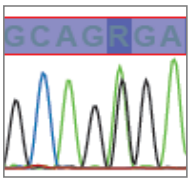
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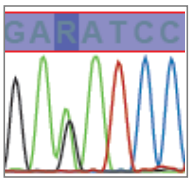
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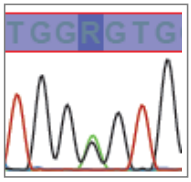
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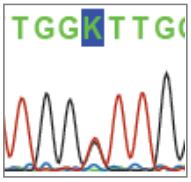
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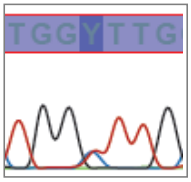
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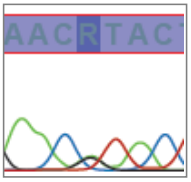
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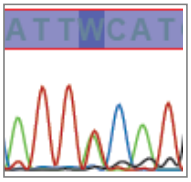
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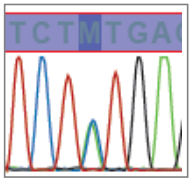
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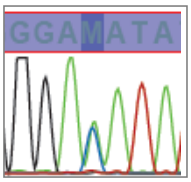
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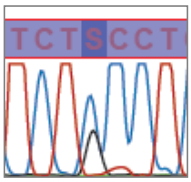
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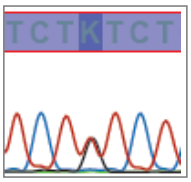
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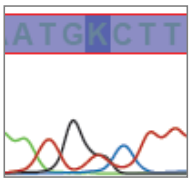
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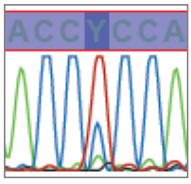
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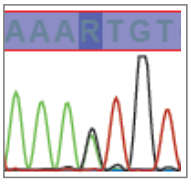
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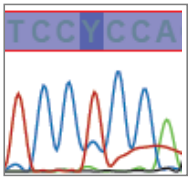
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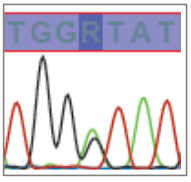
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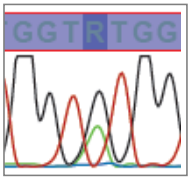
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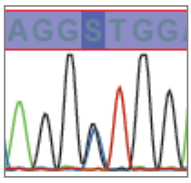
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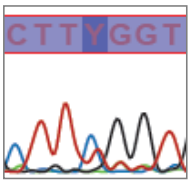
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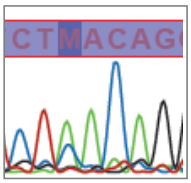
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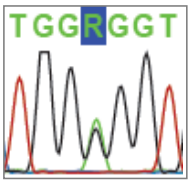
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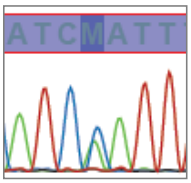
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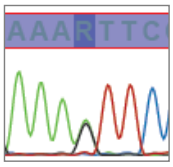

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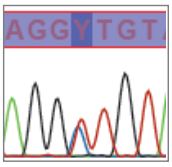

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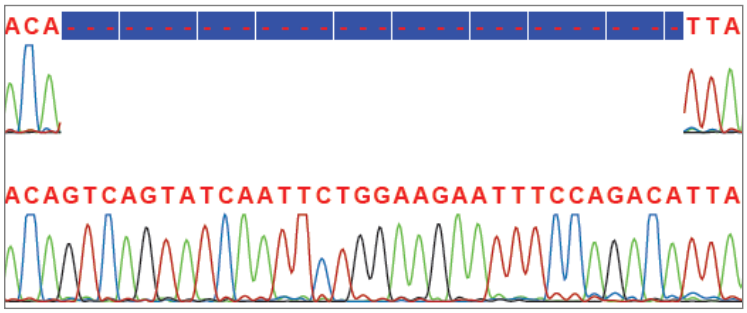

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