THE ROLE OF APOBEC3G IN ACUTE AND EARLY HIV-1 SUBTYPE C INFECTION

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Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in the School of Laboratory Medicine and Medical Sciences at the HIV Pathogenesis Programme, Nelson R. Mandela School of Medicine, College of Health Sciences, University of KwaZulu-Natal, Durban

DECLARATION

This study represents original work by the author and has not been submitted in any other form to another University. Where use was made of the work of others, it has been duly acknowledged in the text. The experimental work described in this thesis was performed primarily in the Hasso Plattner and HIV Pathogenesis Programme Laboratory in the Doris Medical Research Institute at the Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban under the supervision of Professor Thumbi Ndung'u.

A portion of the laboratory work was also performed at the Laboratory of Genomic Diversity at the National Cancer Institute Frederick, Maryland, USA under the mentorship of Dr. Cheryl Winkler and at the Department of Microbiology, Mount Sinai School of Medicine, New York, USA under the mentorship of Dr. Viviana Simon.

Kavidha Reddy _____ Date: 03/03/2014

Professor Thumbi Ndung'u _____ Date: 03/03/2014

DECLARATION

I, **Kavidha Reddy** declare that:

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- **Kavidha Reddy,** Cheryl A. Winkler, Lise Werner, Koleka Mlisana, Salim S. Abdool Karim, Thumbi Ndung'u, the CAPRISA Acute Infection Study Team, APOBEC3G expression is dysregulated in primary HIV-1 infection and polymorphic variants influence CD4 T-cell counts and plasma viral load. Published in *AIDS*, 2010, 24:195–204

CONFERENCES PRESENTATIONS

- **Kavidha Reddy**, Cheryl Winkler, Lise Werner, Kholeka Mlisana, Salim Abdool Karim, Thumbi Ndung'u on behalf of the CAPRISA Acute Infection Team. The Role of APOBEC3G in Primary HIV-1 Subtype C Infection. XVII International AIDS Conference, August 2008, Mexico City, Mexico.

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The publication, fully referenced below, has been reproduced non-continuously throughout the thesis.

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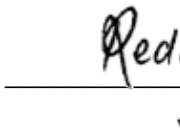
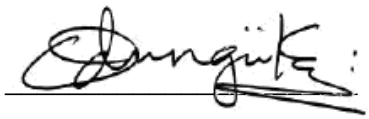
The candidate performed the experiments described in the above research paper, and where others have made contributions it has been duly acknowledged. The candidate drafted the published manuscript in full and it was reviewed by co-authors.

Kavidha Reddy  Date: 03/03/2014

Professor Thumbi Ndung'u  Date: 03/03/2014

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ABSTRACT

Introduction

APOBEC3G and other related cellular cytosine deaminase family members have potent antiviral activity. In the absence of HIV-1 Vif, APOBEC3G mutates the viral DNA during viral reverse transcription. Our knowledge of the Vif-APOBEC3G interaction in human populations infected with subtype C HIV-1 is limited. Investigation of interactions between HIV and its host is crucial as it can ultimately be exploited in vaccine and therapy design. We hypothesised that certain *APOBEC3G* haplotypes and/or their expression in peripheral blood mononuclear cells of seroconverters affect viral setpoint and CD4+ T cell counts. We also hypothesised that certain *APOBEC3G* genetic variants are associated with increased frequency of G to A hypermutations during primary HIV-1 infection and that Vif variability influences disease progression and its ability to neutralise *APOBEC3G* haplotypes.

Methods

Our South African study cohort consisted of females at high risk for HIV-1 infection and women with known recent HIV-1 infection. We used quantitative real-time PCR to measure *APOBEC3G* expression in HIV- and HIV+ samples during primary infection. *APOBEC3G* variants were identified by DNA sequencing and TaqMan Genotyping. The HIV-1*env* gene was sequenced to assess Env diversity and the extent of APOBEC3G induced hypermutations. Vif variability was assessed by plasma derived clonal Vif sequences (n= 10-20 per patient) and Vif function was assessed by APOBEC3G degradation assays and HIV-1 infectivity assays.

Results

We found no correlation between *APOBEC3G* expression levels and plasma viral loads (r=0.053, p=0.596) or CD4+ T cell counts (r=0.030, p=0.762) in 32 seroconverters. However, *APOBEC3G* expression levels were significantly higher in HIV- individuals compared to HIV+ individuals (p<0.0001) including matched pre- and post-infection samples from the same individuals (n=13, p<0.0001). Twenty five single nucleotide polymorphisms (SNPs) were identified within the *APOBEC3G* region. SNP *186R/R* was associated with significantly higher viral loads (p=0.0097) and decreased CD4+ T cell levels (p=0.0081), indicating that *186R/R* has a negative effect on HIV restriction. Overall HIV-1 *env* sequences contained a higher number of APOBEC3F compared to APOBEC3G-induced hypermutations and the number of APOBEC3F-induced hypermutations correlated negatively with viral load (r= -0.6, p=0.006) and positively with CD4 T cell counts (r=0.6, p=0.004). We cloned and sequenced a total of 392 subtype C Vifs, which showed an interpatient diversity of 6.2% to 19.2% at the amino acid level. Interestingly, Vif sequence comparison showed a strong preference for a Lysine or a Serine at position 36 for APOBEC3G *186R/R* and APOBEC3G *186H/H* individuals, respectively. Selected natural subtype C Vif alleles had greater ability to counteract wild type APOBEC3G *186H* as compared to the APOBEC3G *186R* variant as shown by both functional and HIV infectivity assays.

Conclusions

In conclusion, *APOBEC3G* expression in peripheral blood mononuclear cells does not correlate with viral loads or CD4+ T cell counts during primary HIV-1 subtype C infection. However, genetic variants of *APOBEC3G* may affect HIV-1 pathogenesis. Amino acid changes in Vif may influence its anti-APOBEC3 activity. HIV-1 subtype C Vifs may have adapted to counteract the more active wild type APOBEC3G as compared to the less active

APOBEC3G *186R* variant. These studies have improved our understanding of viral-host interactions in African populations and HIV-1 subtype C infections.

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ABBREVIATIONS

°C	degree celcius
µl	microliter
µM	micromolar
A	adenine
aa	amino acid
ABI	Applied Biosystems
AIDS	acquired immune deficiency syndrome
ANOVA	analysis of variance
APOBEC3F	Apolipoprotein B mRNA-editing enzyme 3F
APOBEC3G	Apolipoprotein B mRNA-editing enzyme 3G
ARG	AIDS restriction gene
ARV	AIDS-associated retroviruses
bp	base pair
BST-2	bone marrow stromal cell antigen 2
C-	carboxy
C	cytidine
CA	capsid
CAPRISA	Centre for the AIDS Programme of Research in South Africa
CCR5	C-C chemokine receptor 5
CD	cluster of differentiation
CDAs	cytidine deaminase domains
cDNA	complementary deoxyribonucleic Acid
cPPT	central PPT
CTL	cytotoxic T lymphocytes

CTS	central termination sequence
CXCR4	CXC chemokine receptor 4
dA	deoxyadenosine
dC	deoxycytidine
DC	dendritic cells
DC-SIGN	Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-Integrin
dG	deoxyguanosine
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dU	deoxyuridine
e.g.	example
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
EIAV	equine infectious anaemia virus
ELISA	enzyme linked immunosorbent assays
Env	envelope
ER	endoplasmic reticulum
FBS	fetal bovine serum
G	guanine
Gag	Group specific Antigen
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gDNA	genomic deoxyribonucleic acid
GEE	Generalised Estimating Equation
GFP	green fluorescence protein

gp120	glycoprotein 120
gp41	glycoprotein 41
GPI	glycosyl-phosphatidylinositol
H	histamine
HA	haemagglutinin
HCl	hydrochloric acid
HIV	human immunodeficiency virus
HLA	human leukocyte antigens
HMM	high molecular mass
HTLV	human T-cell lymphotropic virus type
HWE	Hardy Weinberg Equilibrium
I	inosine
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IN	integrase
K	Lysine
kb	kilobase
kDa	kilo Dalton
KIRs	killer cell immunoglobulin-like receptors
LAV	lymphadenopathy-associated virus
LB	Luria Bertani
LINEs	long interspersed nuclear elements
LMM	low molecular mass
LTR	long terminal repeat

MA	matrix shell
mg	milligram
MGB	minor groove binder
MgCl2	magnesium chloride
MHC	major histocompatibility complex
min	minutes
MIP-1	Macrophage Inflammatory Protein 1
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
MSM	men who have sex with men
N-	amino
NaCl	sodium chloride
NC	nucleocapsid
NEB	New England Biolabs
Nef	negative effector
ng	nanogram
NIH	National Institutes of Health
NK	natural killer
nm	nanometre
No.	Number
PAK	p21-activated protein kinase
PAMPs	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cell
PBS	primer binding site

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEI	polyethylenimine
PIC	pre-integration complex
pNL4-3	NL4-3 plasmid
pNL4-3Δvif	vif deleted NL4-3 plasmid
Pol	polymerase
PPT	polypurine tract
PR	protease
PRRs	pathogen recognition receptors
PVDF	polyvinylidene difluoride
R	arginine
RANTES	Regulated on Activation Normal T Cell Expressed and Secreted
Rev	regulator of virion protein
RFLP	restriction fragment length polymorphism
RIG	retinoic acid induced gene
RNA	ribonucleic acid
RRE	Rev response element
RT	reverse transcriptase
S	Serine
SAMHD1	sterile α motif and an HD domain protein
SAP	shrimp alkaline phospatase
SDS	sodium dodecyl sulfate
SINEs	short interspersed nuclear elements
SIV	simian immunodeficiency virus

SNP	single nucleotide polymorphism
ssDNA	strong-stop DNA
SU	surface gp120 molecules
Tat	transcriptional transactivator
TFH	T follicular helper
Th	T helper
TM	transmembrane gp41 molecules
TNF-α	tumour necrosis factor-α
Treg	T regulatory
TRIM5α	tripartite motif protein 5 alpha
rhTRIM5 α	rhesus tripartite motif protein 5 alpha
tRNA	transfer ribonucleic acid
Trp	tryptophan
U	uridine
U	unit
V	volt
VCT	voluntary counselling and testing
VESPA	Viral Epidemiology Signature Pattern Analysis
Vif	virion infectivity factor
Vpr	viral protein R
Vpu	viral protein U
WT	wild type
α	alpha
β	beta
γ	gamma

-ve	negative
+ve	positive

CHAPTER ONE

LITERATURE REVIEW

1.1 The HIV Epidemic

The human immunodeficiency virus (HIV) is the causative agent of the acquired immune deficiency syndrome (AIDS), a disease characterized by progressive failure of the immune system which allows life-threatening opportunistic infections and cancers to thrive.

June 2011 marked the third decade since the documentation and publication of the first AIDS related cases (Gottlieb et al. 1981). Tragically almost 60 million people have been afflicted since the start of the AIDS epidemic but significant progress is being made in our understanding of the causative HIV. According to the 2012 UNAIDS, Global Report, 34 million people were living with HIV worldwide at the end of 2011 and alarmingly sub-Saharan Africa was home to 69% of these infections. Sub-Saharan Africa also accounted for 71% of new infections in 2011. Further, the sub-region of Southern Africa is the most severely affected by this epidemic with South Africa being hardest hit, and harbouring the world's largest population of people living with HIV (5.6 million) (UNAIDS 2012; UNAIDS 2012). Additionally, within South Africa, the province of KwaZulu-Natal is the epicentre of the HIV pandemic with a 39% prevalence rate (Figure 1.1).

Although, the number of new infections has decreased by more than 700 000 globally in 2011 compared to 2001, with Africa having reduced AIDS-related deaths by approximately 30% in the past six years (UNAIDS 2012), the statistics remain disturbing and advocate the urgent need for an effective vaccine against HIV. *S*everal effective therapeutic drugs against HIV have been developed, however, the development of an efficacious vaccine has been

challenging and hindered by HIV's high replicative capacity, extensive tissue dissemination and its extreme degree of variability. In addition, there are many pathways and factors of the human immune system which are not yet well understood or identified, and further knowledge and understanding of such factors and pathways will increase the prospect of finding an effective vaccine and new therapies.

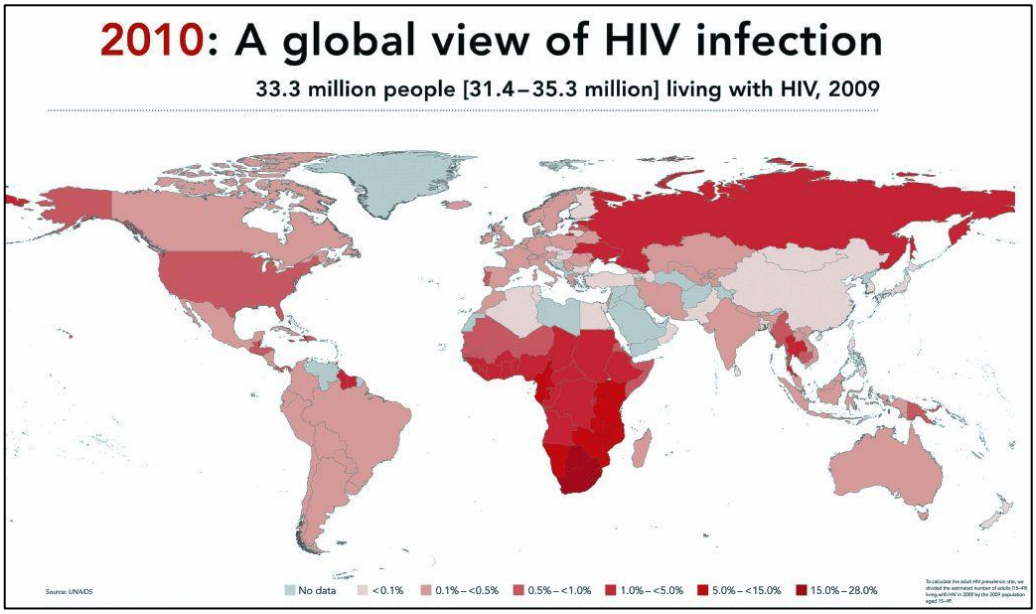


Figure 1.1 Global HIV prevalence map showing that the highest burden of HIV is in southern Africa (UNAIDS, Report on Global AIDS Epidemic, 2010)

Scientists have for some time questioned the heterogeneity of disease progression in HIV infected individuals, where some progress unusually slowly to AIDS despite many years of untreated HIV infection, while in contrast, rapid progressors progress to AIDS within 5 years. And interestingly, there are also individuals who remain uninfected despite repeated exposure to HIV. Studies have shown that individual and population susceptibility to AIDS may be influenced by the possession of genetic variants that modulate immune function and viral replication (An et al. 2004; O'Brien and Nelson 2004; An and Winkler 2010). Identification

and investigation of such genes, that influence resistance and susceptibility to HIV infection may be crucial in understanding the interaction between HIV and its host, which can ultimately be exploited in vaccine and therapy design.

The first evidence that genetic resistance to HIV infection existed, was from studies that discovered and characterised the mutant CCR5Δ32 allelle which has been shown to confer resistance to HIV infection in those individuals who are homozygotic for the mutation (Dean et al. 1996; Liu et al. 1996). The mutation, found predominantly in Caucasians, is a 32 base pair deletion in the single coding exon of the gene that causes a frame shift in the coding sequence and produces a defective CCR5, which is not expressed on the surface of cells. A recent study reported the case of an HIV-infected patient in whom viral replication could no longer be detected after transplantation with CCR5Δ32 /Δ32 stem cells even though antiretroviral therapy was discontinued (Hutter et al. 2009). After a 4 year follow up, the study strongly suggested that a cure for HIV had been achieved in this patient (Allers et al. 2011). This demonstrates that virus-host interaction sites can definitely be utilized in therapy design.

In recent years a number of host innate cellular factors that target HIV-1 and restrict intracellular viral replication have been identified and these include, Apolipoprotein B mRNA-editing enzyme 3G (APOBEC3G) (Sheehy et al. 2002), bone marrow stromal cell antigen 2 (BST-2) also known as Tetherin (Neil et al. 2008), tripartite motif protein 5 alpha (TRIM5α) (Stremlau et al. 2004), and sterile α motif (SAM) and an HD domain protein SAMHD1 (Goldstone et al. 2011; Laguette et al. 2011).

Of particular interest is APOBEC3G, a member of the human cytidine deaminase family, has been shown to have potent antiviral activity (Mangeat et al. 2003; Bishop et al. 2004; Harris and Liddament 2004; Huthoff and Malim 2005), and has been described as a novel candidate AIDS restriction gene (ARG) (Stephen and Nelson 2004). APOBEC3G acts as a post-entry restriction factor where it enters nascent virions and its enzymatic activity induces detrimental mutations in the viral genome. However, the apparently minor HIV accessory protein virion infectivity factor (Vif) counteracts APOBEC3G in its defense against this host immune mechanism. This interaction between virus and host has been a focus of intense study in recent years as it presents a potential novel opportunity for drug intervention and therapy.

Our knowledge of viral-host interactions in human populations infected with subtype C HIV-1 is limited, particularly in southern Africa where the HIV-1 epidemic is severest. Studies have shown that APOBEC3G is an important intrinsic antiretroviral host factor that has significant anti-HIV-1 activity *in vitro*. The purpose of this study was to investigate its antiviral effects *in vivo* which we hypothesized may be more prominent during primary HIV-1 subtype C infection before adaptive immune responses become established. In addition, amino acid changes in Vif may influence its anti-APOBEC3G activity and in this study our goal was also to further understand the complex attack-counterattack interaction between APOBEC3G and Vif in the HIV-1 subtype C context. This may open a window for the design of pharmacological inhibitors that could complement existing AIDS therapies.

1.2 Discovery and Origin of HIV

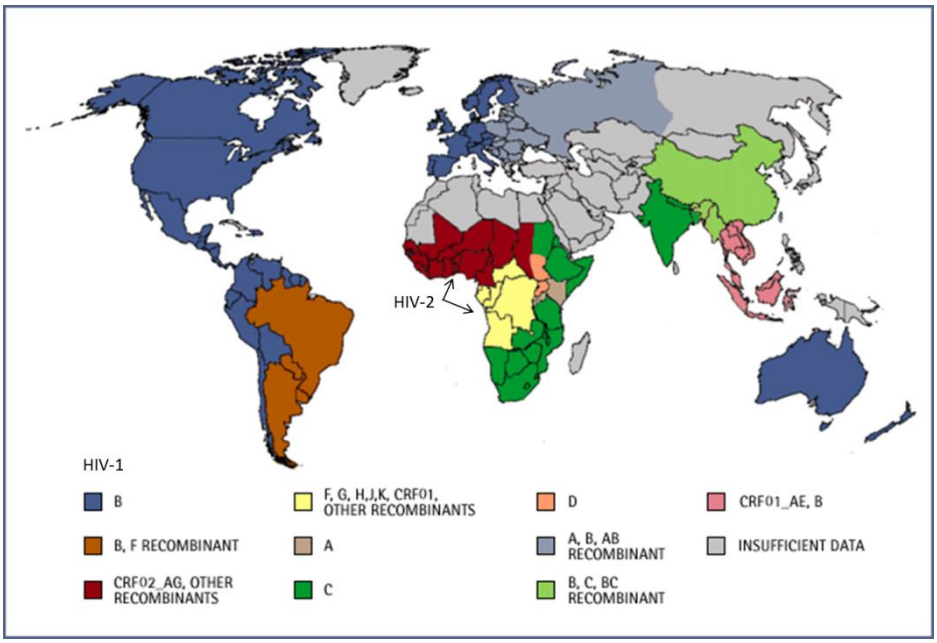
AIDS was first recognised in 1981 in the United States of America after an increase in the incidence of *Pneumocystis carinii* pneumonia, extensive mucosal candidiasis and Kaposi's sarcoma in young homosexual men (Gottlieb et al. 1981). These were shown to be caused by a general immune deficiency and the condition became known early on as AIDS, however, it was only in 1983 that the etiological agent was identified. At the outset several retroviral isolates were implicated as the causative agents of AIDS and these included the lymphadenopathy-associated virus (LAV) (Barre-Sinoussi et al. 1983), human T-cell lymphotropic virus type III (HTLV III) (Gallo et al. 1984) and AIDS-associated retroviruses (ARV) (Levy et al. 1984). However, in 1986, HIV was internationally recognized as the virus that causes AIDS (Coffin et al. 1986). Soon after this discovery another human retrovirus was isolated from patients with AIDS in West Africa (Clavel et al. 1986). This virus was reasonably genetically different from HIV-1 by up to 40%, and was named HIV-2.

Evidence suggests that HIV-1 and HIV-2 entered the human population through several zoonotic infections from simian immunodeficiency virus (SIV) infected primates (Hahn et al. 2000). It is believed that widespread bush meat hunting and trade in West Africa resulted in the transmission of HIV to humans. This most probably occurred through cutaneous or mucous membrane exposure to SIV infected ape blood and/or other body fluids as a result of injury during hunting (Peeters et al. 2002). The simian source of HIV-1 and -2 have been identified based on the phylogenetic relatedness of their sequences to known SIVs.

HIV-1 is most closely related to SIVcpz isolated from the chimpanzee subspecies *Pan troglodytes troglodytes* (Huet et al. 1990; Gao et al. 1999; Hahn et al. 2000). HIV-1 further comprises distinct groups M, N, O, and a rare group P, each of which resulted from an

independent cross-species transmission event, and all of which are distributed across the geographic area consistent to the spread of *P.t. troglodytes* in west equatorial Africa. Of these the M group which is the most diverse, most likely began in Kinshasa in the Democratic Republic of Congo (Vidal et al. 2000) but has now spread throughout the world and is responsible for more than 95% of HIV infections worldwide. Group M has been further divided into clades referred to as HIV-1 subtype A to K. Subtypes A, B and C are the most prevalent genetic forms with subtype C being most predominant and accounting for approximately 50% of all HIV-1 infections globally (Figure 1.2). HIV-1 subtype C is also most prevalent in those regions that carry the world's largest HIV burden, specifically southern Africa and India (Buonaguro et al. 2007) (Figure 1.2). Additionally, the emergence of HIV circulating recombinant forms or CRFs has resulted from co-infection of viruses with different subtypes (Figure 1.2).

A



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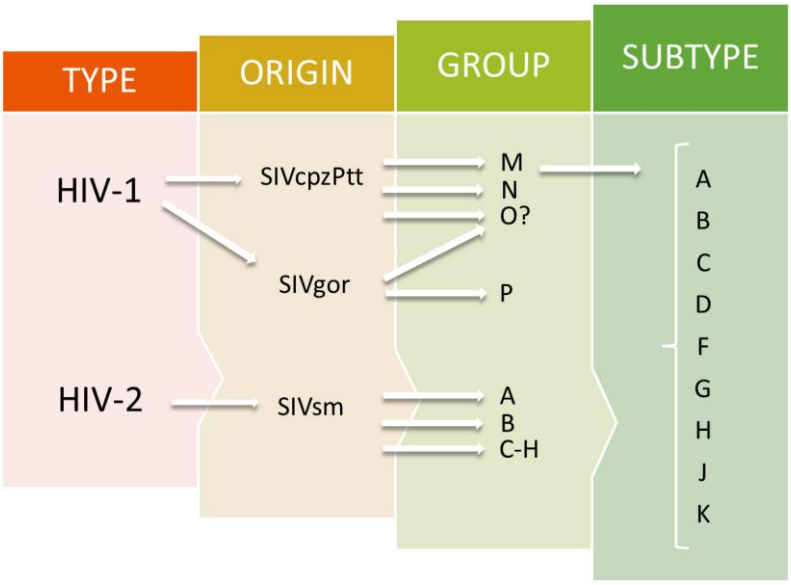


Figure 1.2 [A] Geographical distribution of HIV showing the global spread of HIV-1 subtypes and HIV-2 confined to West Africa. (Adapted from IAVI report, August 2003, http://www.pbs.org/wgbh/pages/frontline/aids/atlas/clade.html) **[B] Classification of HIV subtypes and their origins (self-generated).**

Based on phylogenetic data it is thought that HIV-1 group P may have a gorilla origin via the lineage SIVgor. This lineage clusters with strains from *P. t. troglodytes* apes, and therefore suggests that gorillas acquired SIVgor by cross-species infection from sympatric chimpanzees from a single transmission event over 100 years ago. HIV-1 group P has only been identified in two people from Cameroon (Van Heuverswyn et al. 2006; Sharp and Hahn 2011).

On the other hand, HIV-2 has been shown to be phylogenetically related to SIVsm from sooty mangabeys whose habitat overlaps with the epicenter of the HIV-2 epidemic (Hirsch et al. 1999). HIV-2 also comprises 8 different ancestries, referred to as groups A to H and has been mostly confined to West Africa, with the highest prevalence rates documented in Guinea-Bissau and Senegal (de Silva et al. 2008).

There are more than 40 different SIVs that are known to infect Old World monkeys. However, only three of these (SIVcpz, SIVsm and SIVgor) have been able to make the cross-species jump to humans (Figure 1.3). The ability of an SIV to infect a new primate species depends largely on its ability to counteract different host restriction factors (Sharp and Hahn 2011). These host restriction factors pose a barrier to cross-species infection because they have rapidly evolved as a consequence of their innate immune function to protect against endless attack by various viral pathogens (Malim and Emerman 2008; Neil and Bieniasz 2009). Of note, the three key restriction factors (mentioned on page 3) that have been shown to pose as obstacles to cross-species transmission of SIV include 1) APOBEC3G, which hypermutates the viral genome during reverse transcription (Sheehy et al. 2002), 2) TRIM5α, which induces premature viral uncoating (Stremlau et al. 2004), and 3) Tetherin, which inhibits the release of HIV-1 particles from infected cells (Neil et al. 2008). SAMHD1 is the

most recently discovered restriction factor and is believed to act at the step of reverse transcription (Goldstone 2011). These will be discussed in more detail in subsequent sections.

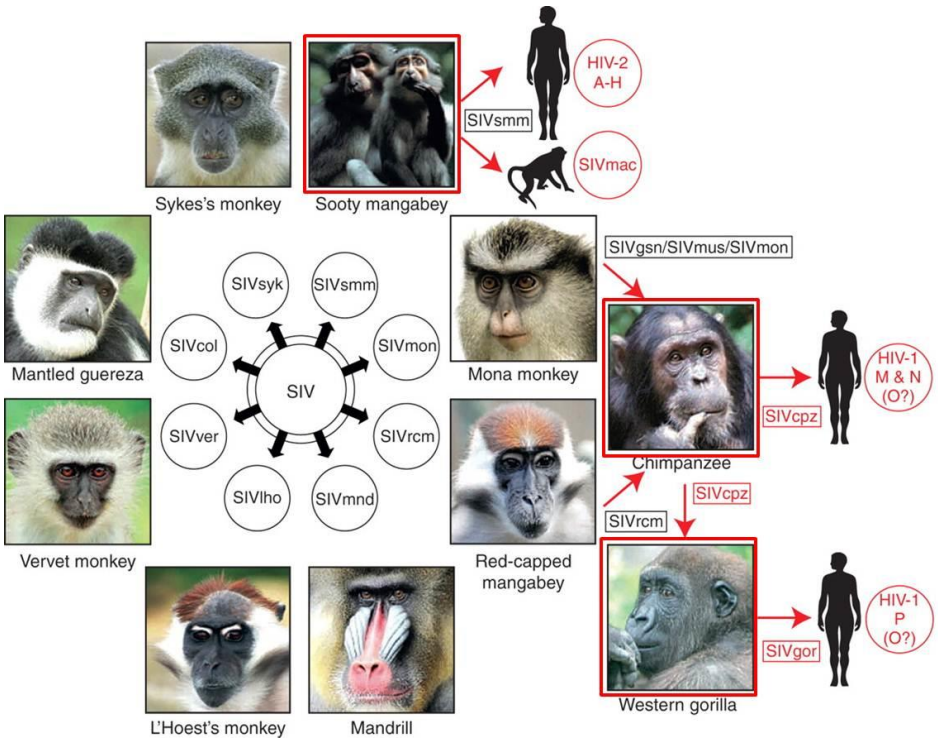


Figure 1.3 Origins of human AIDS viruses. Old World monkeys are naturally infected with more than 40 different lentiviruses, termed simian immunodeficiency viruses (SIVs). Three of these SIVs have crossed the species barrier to humans (highlighted in red). (Source: (Sharp and Hahn 2011) Origins of HIV and the AIDS Pandemic, Cold Spring Harbor Perspectives in Medicine).

1.3 Taxonomic Classification and Morphology of HIV

HIV is a single stranded RNA virus that belongs to the retroviridae family and is a member of the lentivirus genus that causes slow degenerative diseases.

A mature HIV virion has a spherical morphology with a diameter of approximately 100-120nm (Figure 1.4). Like all lentiviruses HIV is enveloped by a lipid bilayer membrane that is derived from the membrane of the infected host cell from which it buds. The lipid bilayer membrane further consists of approximately 72 exposed surface glycoprotein spikes (Hirsch and Curran 1996; Turner and Summers 1999). Each of these spikes are made up of 3 SU glycoprotein 120 (gp120) molecules, which are anchored to the virus via noncovalent interaction with each of 3 transmembrane (TM) glycoprotein 41 (gp41) molecules and held together as a trimer (Weiss et al. 1990). The lipid bilayer also contains host cell membrane proteins such as human leukocyte antigens (HLA), actin and ubiquitin (Arthur et al. 1992).

The viral membrane is lined by a matrix shell (MA) that is made up of approximately 2,000 copies of the MA protein p17 molecules. The capsid (CA) is a shell that surrounds the ribonucleoprotein complex containing the genomic RNA and is made up of approximately 2,000 copies of CA protein p24 molecules (Ganser et al. 1999). The CA and the components it encloses are referred to as the viral core, is located at the center of the virus, and assumes a conical shape.

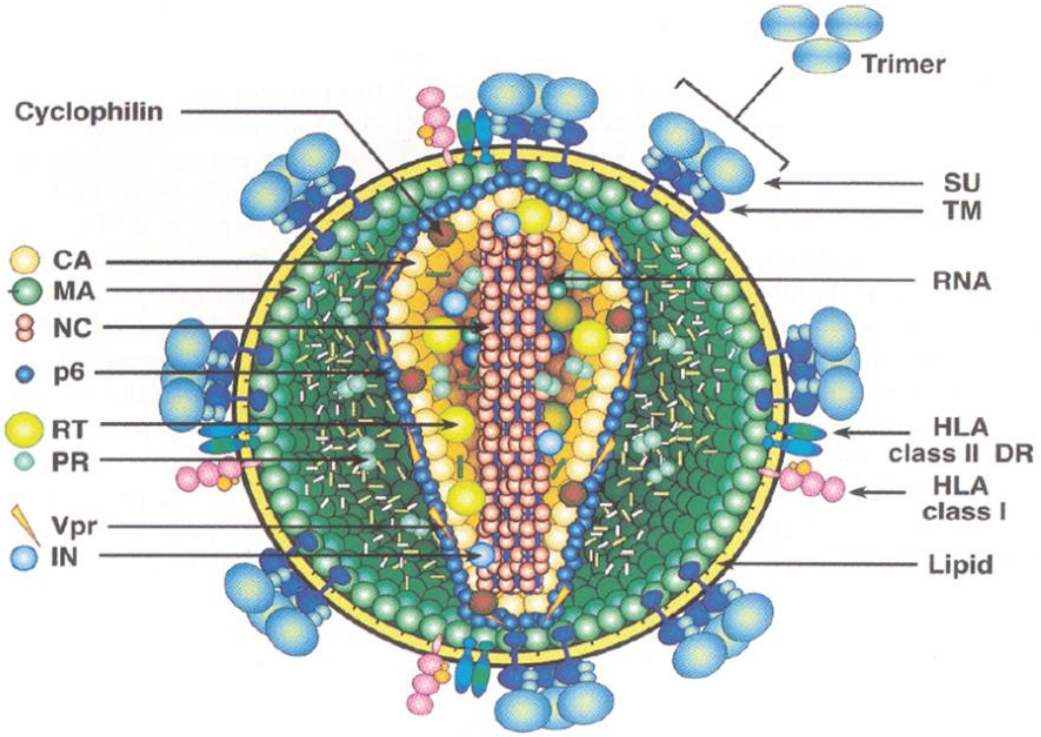


Figure 1.4 Structure of a mature HIV virion (Vogt 1997) .

Within the viral conical CA core exists 2 copies of the single stranded RNA viral genome, which is stabilized as a ribonucleoprotein complex with approximately 2,000 copies of nucleocapsid (NC) protein molecules (p7) (Frankel and Young 1998). The viral core also contains 3 crucial virally encoded enzymes namely reverse transcriptase (RT), integrase (IN) and protease (PR), (located inside and outside the core) (Vogt 1997; Turner and Summers 1999).

1.4 Genomic Organisation of HIV

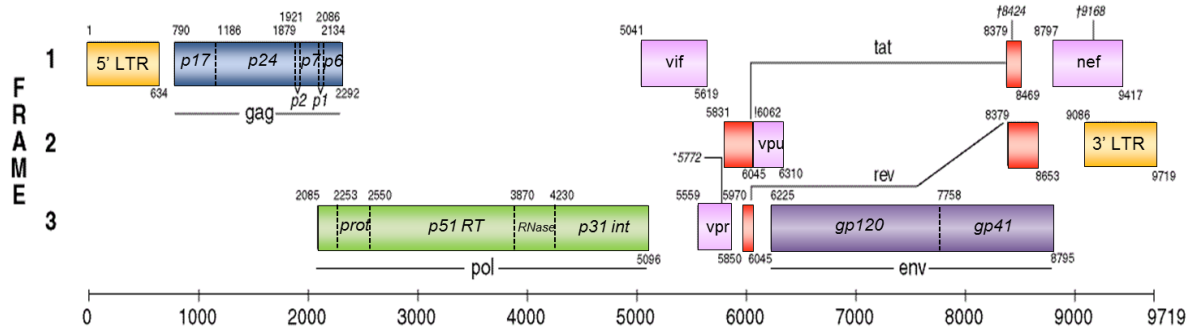


Figure 1.5 The genome organisation of HIV-1 showing the structural and enzymatic genes of HIV, *gag, pol* and *env*; the regulatory *tat* and *rev* genes and four accessory genes *vif, vpr, vpu*, and *nef*. The LTR regions flank the genome on each side. (Adapted from HIV-1 Gene Map, Los Alamos National Library, HIV Database)

The genome of HIV-1 is made up of two identical positive sense single stranded RNA copies, is 9.7 kilobases in length and constitutes nine genes (Figure 1.5) that code for nine primary proteins (Table 1.1). The organisation of the HIV genome is extremely functionally efficient with all 3 reading frames of its genetic sequence being utilised thereby allowing for overlapping of the gene coding regions.

Common to all retroviruses are the three genes *gag*, *pol* and *env* which encode the structural and enzymatic proteins. In addition, the HIV genome has two genes encoding regulatory proteins, the transcriptional transactivator (Tat) and the regulator of virion gene expression (Rev). Also present are four genes that encode accessory proteins: the negative effector (*nef*), viral infectivity factor (*vif*), and the viral proteins "R" (Vpr) and "U" (Vpu). Sixteen proteins are generated in all as a result of cleavage of three of the primary products (Coffin et al. 1997; Frankel and Young 1998).

Flanking the coding region on each end are the long terminal repeat (LTRs) regions. The 5' LTR functions as the promoter for the entire retroviral genome and the 3' LTR provides for nascent viral RNA polyadenylation (Cullen 1991; Jones and Peterlin 1994; Kingsman and Kingsman 1996).

1.5 The Proteins encoded by HIV

Table 1.1 Classification of the nine genes of HIV and their associated proteins

Table 1: Classification of the nine genes of HIV and their associated proteins			
PROTEIN CLASS	**GENE**	**PROTEINS**	
Structural	gag	matrix-MA capsid-CA nucleocapsid-NC	p17 p24 p7 P6
	env	surface-SU transmembrane-TM	gp120 gp41
Catalytic	pol	protease reverse transcriptase-RT integrase Rnase H	p10 p51 p31 p66
Regulatory	tat rev	Tat Rev	p14 p19
Accessory	vpu vif vpr nef	Vpu Vif Vpr Nef	p16 p23 p15 p27

1.5.1 The Structural and Enzymtic Proteins: Gag, Pol and Env

The *gag*, *pol* and *env* genes of HIV encode for the structural and enzymatic proteins that are required for viral replication and assembly of progeny virions.

1.5.1.1 Gag

HIV-1 assembly is driven by the Gag (Group specific Antigen) protein which recruits all the building blocks and components, both viral and cellular, required for the formation of a fully infectious virion. Gag is a 55kDa polyprotein precursor which is cleaved by the viral PR to produce the internal structural proteins of the mature virion namely the MA (p17) proteins, CA (p24) proteins, the NC (p7) proteins (Gottlinger et al. 1991; Freed 1998) as well as the P6 protein that binds Vpr and recruits the cellular machinery required for virus budding (Ganser-Pornillos et al. 2008).

The 132 residue amino acid, 17kDa myristylated MA protein is the N-terminal component of Gag and is important for targeting the Gag polyprotein to the plasma membrane for viral assembly (Adamson and Freed 2007; Bukrinskaya 2007; Ganser-Pornillos et al. 2008; Ono 2009; Chukkapalli and Ono 2011). In mature virus particles the MA protein also lines the inner surface of the virion membrane. Studies also suggest that MA may play a role in facilitating the infection of non-dividing cell types, primarily macrophages (Bukrinsky et al. 1993; Gallay et al. 1995).

The second component of the Gag polyprotein is the 24kDa CA protein that forms the shell of the core of the virus. The capsid contains two domains that have different roles in the development of the virus. The C-terminal domain is vital both for particle assembly and for core formation (Dorfman et al. 1994). The N-terminal domain is primarily responsible for the formation of the mature viral core but is also important for infectivity, by participating in the process of viral coating (Fassati 2012).

The NC is the third fragment of the Gag polyprotein and is found within the viral core where it coats the genomic RNA. The primary function of the NC domain is essentially for the delivery and packaging of two copies of the genomic viral RNA into assembling virions (Berkowitz et al. 1996).

1.5.1.2 Pol

Polymerase is also a polyprotein and is cleaved into the viral enzymes PR, RT, IN and RnaseH which play a vital role in HIV replication.

HIV-1 PR is encoded in the 5' end of the Pol gene. This 99 amino acid protein functions to cleave the Gag polyprotein into its mature components MA, CA and NC which contributes to the structure of the virion and to RNA packaging. PR also cleaves the Pol polyprotein into its enzymatic components PR, RT and IN (Kohl et al. 1988; Peng et al. 1989). In recent years PR has become a key target for drug design with several PR inhibitors currently in use (Flexner 1998).

RT possess two distinct enzymatic activities, a DNA polymerase activity that utilises RNA or DNA as a template, and an RNase H activity that degrades the RNA strand of an RNA/DNA hybrid (Gilboa et al. 1979). RT is a heterodimer with each of its functions being localised in separate protein domains. The first domain is a 560-residue, 66kDa subunit (p66) and the second domain is a 440-residue, 51kDa subunit (p51). The p66 subunit contains the RT domain and the RNase H domain at the C terminal end. The p51 subunit is formed by proteolytic cleavage of p66 and is devoid of RnaseH activity (Freed 2001). RT catalyses the multi-step process of the synthesis of integration competent double-stranded DNA from the positive (+) RNA strand genome of retroviruses. The viral RNase H degrades the RNA from

the DNA/RNA hybrid, so as to free the newly synthesised DNA allowing it to be used as a template for RT to generate the complementary DNA to complete the double stranded DNA copy of the HIV genome (Schultz and Champoux 2008).

The viral IN is a 32 kDa protein that is essential for incorporation of the double stranded viral DNA into host genome after the process of reverse transcription and localization of the viral DNA to the nucleus (Frankel and Young 1998; Turner and Summers 1999).

1.5.1.3 Env

The viral envelope (Env) glycoprotein is the only surface-expressed protein and is a 160kDa polyprotein (gp160), which is exclusively required for binding and entry into host cells. Following translation gp160 is cleaved into two mature envelope glycoproteins; a 120 kDa SU protein (gp120) and a 41 kDa TM protein (gp41). Glycoproteins gp120 and gp41 remain non-covalently linked to form a single subunit of a trimeric spike on the virion surface (Turner and Summers 1999; Arrildt et al. 2012). Gp120 is responsible for interaction with the cell surface receptor CD4 (Dalgleish et al. 1984; Klatzmann et al. 1984) and co-receptor CCR5 (Feng et al. 1996) or CXCR4 (Deng et al. 1996) that are essential for infection (Berger et al. 1999) while gp41 has domains that catalyse the fusion of viral and cellular lipid bilayers during virus entry (Freed and Martin 1995). Gp120 is extensively glycosylated (Leonard et al. 1990) and contains five highly variable regions (V1-V5) (Starcich et al. 1986). It has been established that this surface variability is vital for HIV-1 to escape an effective immune system (Profy et al. 1990).

1.5.2 The Regulatory Proteins, Tat and Rev

Tat and Rev are two crucial viral regulatory factors required for HIV gene expression and replication.

1.5.2.1 Tat

HIV transcription is controlled primarily by the *trans*-activator protein (Tat), a 101 amino acid protein encoded by a two-exon RNA, although there are some isolates that encode a smaller 72 amino acid one-exon Tat (Freed 2001). The HIV-1 promoter is located in the 5' LTR and serves as the site of transcriptional initiation, harbouring several sites for cellular transcription factors that are required for RNA synthesis. However, these transcription complexes initiated at the HIV-1 promoter have low elongation efficiency. Tat functions to enhance transcriptional elongation and RNA synthesis is increased by approximately a 100 fold when Tat is present (Dayton et al. 1986; Fisher et al. 1986). Tat differs from the majority of cellular transcription factors that bind to the viral DNA because it acts by binding to the trans-activating response element, a stem-loop structure at the 5' end of nascent viral RNA. It subsequently recruits Cyclin T1 and CDK9 which phosphorylate RNA polymerase II thereby increasing the efficiency of elongation of the nascent viral mRNA (Berkhout et al. 1989; Reines et al. 1996; Wei et al. 1998).

1.5.2.3 Rev

Rev is 19 kDa phosphoprotein made up of 116 amino acids with two functional domains. The first is an RNA binding and nuclear localisation domain, rich in Arginine while the second is a hydrophobic, Leucine-rich motif that mediates nuclear export (Pollard and Malim 1998).

Viral RNAs generated from HIV-1 LTR transcription exist in three forms: 1) multiply spliced RNAs, which encode Rev, Tat, and Nef proteins, 2) partly spliced RNAs, which function as mRNAs for the Env, Vif, Vpu, and Vpr proteins, and 3) unspliced RNAs, which serve as the mRNAs for the Gag and GagPol polyprotein precursors, as well as genomic RNA which is packaged into virions (Purcell and Martin 1993). It is here that the Rev protein functions by aiding the transport of unspliced and partially spliced viral RNAs out of the nucleus into cytoplasm. Rev binds to the "Rev response element" (RRE) that is found in the *env* gene and also located in all unspliced and partially spliced HIV-1 RNAs. The Rev/RRE complex is able to interact with the cellular nuclear export machinery and transports the unspliced or partially spliced RNAs to the cytoplasm (Hammarskjold et al. 1989; Pollard and Malim 1998).

1.5.3 The Accessory Proteins, Nef, Vpu, Vpr and Vif

In addition to the common primary proteins encoded by other retroviruses specifically Gag, Pol, Env and the regulatory proteins Tat and Rev, a distinctive characteristic of HIV-1 is that it also encodes four accessory proteins: Vpu, Vif, Vpr and Nef. It is believed that these accessory proteins function to alter the milieu within infected cells to ensure viral persistence, replication, dissemination, and transmission (Malim and Emerman 2008).

1.5.3.1 Nef

Nef is a 27kDa, 96 amino acid, myristoylated protein that is mostly cytoplasmic and linked with the plasma membrane via the myristoyl residue. It is the most immunogenic HIV-1 accessory protein and is crucial for efficient viral spread and disease progression. The key functions of Nef are: 1) the downregulation of CD4 and major histocompatibility complex

(MHC) class I, 2) the stimulation of virion infectivity, 3) modulation of the activation state of cells (Goldsmith et al. 1995; Mangasarian and Trono 1997).

High levels of CD4 on the surface of HIV-producing cells inhibit the infectivity of released virions by trapping the viral envelope and may also block virion release (Lama et al. 1999; Ross et al. 1999). Therefore, downregulation of CD4 is required for maintenance of viral infectivity of HIV-1 (Tanaka et al. 2003). Nef steers CD4 from the cell surface and Golgi apparatus to lysosomes resulting in receptor degradation. Nef functions as a bridge between CD4 and cellular endocytic complexes. On one end, Nef binds to CD4 by interacting with dileucine-based signal in the cytoplasmic tail of the receptor (Aiken et al. 1994) while on the other end it interacts with adapter protein complexes in clathrin-coated pits (Mangasarian and Trono 1997).

Nef also downregulates the cell-surface expression of MHC I molecules, however, its mechanism is not completely elucidated. It has been shown that Nef interacts with tyrosine on the cytoplasmic tail of HLA-A and -B. MHC class I is subsequently internalised, directed to the trans Golgi network and eventually degraded (Greenberg et al. 1998; Le Gall et al. 1998). Through this mechanism HIV-infected cells are able to evade detection and eradication by cytotoxic T lymphocytes.

Nef has also been shown to promote virion infectivity in three distinct ways. Firstly, the actin cytoskeleton in the target cell which acts as a barrier to infection can be disrupted and overcome by Nef (Campbell et al. 2004). Secondly, Nef may enhance infectivity by downregulating the protein Dynamin 2, a cellular protein which, in addition to CD4, blocks Env function in the virion (Pizzato et al. 2007). Thirdly, Nef may shield the viral core from

post-entry proteosomal degradation, by facilitating intracytoplasmic transport of the viral core to allow reverse transcription to proceed (Qi and Aiken 2008).

Additionally, it has been demonstrated that Nef forms a complex with p21-activated protein kinase-2 (PAK-2) and induces PAK-2 activation (Renkema et al. 1999; Arora et al. 2000). Besides playing a role in vital cellular functions, the PAKS also play a key role in the entry, replication and spread of many significant pathogenic human viruses including HIV (Van den Broeke et al. 2010).

1.5.3.2 Vpu

Viral protein U (Vpu) is a 16kDa, 81 amino acid membrane phosphoprotein that is expressed late in infection and is unique to HIV-1 and SIVcpz (HIV-1's closest relative) (Cohen et al. 1988; Strebel et al. 1988). The two main functional activities of HIV-1 Vpu are: 1) CD4 degradation in the endoplasmic reticulum (ER) (Levesque et al. 2003) and 2) enhancement of virion release from virus-producer cells (Terwilliger et al. 1989; Klimkait et al. 1990; Bour et al. 1999). Vpu consists of two major domains that control these two biological functions specifically the cytoplasmic domain that is involved in CD4 degradation in ER and the TM domain which plays a role in enhancing virion release (Nomaguchi et al. 2008).

Nascently synthesised Env polyprotein (gp160), are occasionally retained in the ER by interactions with CD4 molecules (Crise et al. 1990). Formation of these Env/CD4 complexes in the ER inhibits the transport and maturation of the Env protein (Bour et al. 1991). Vpu promotes the degradation of CD4, through the host ubiquitin/proteasome pathway, to the release of gp120 from the Env/CD4 complexes in the ER, thereby increasing the amount of Env glycoprotein for transport to the cell surface (Fujita et al. 1997; Schubert et al. 1998).

The second function of Vpu, which is to promote the efficient release of viral particles from the cell surface, has been recently attributed to its ability to inhibit a host protein CD317 (Tetherin). Tetherin has been shown to cause retention of virions to cell surfaces after endocytosis (Neil et al. 2008).

1.5.3.3 Vpr

Viral protein R (Vpr) is a 14kDa protein, composed of 96 amino acids that is packaged into mature virions. Vpr is a multifunctional protein and that plays an essential role in maintaining HIV-infection. Via interactions with nuclear transport machinery, Vpr regulates the transport of nucleoprotein pre-integration complexes into the nucleus of the infected host cell (Heinzinger et al. 1994) and is essential for productive infection in non-dividing cells such as macrophages (Balliet et al. 1994).

Vpr has also been demonstrated to induce cell cycle delay or arrest in proliferating cells in the G2/M phase of the cell cycle (Jowett et al. 1995; Rogel et al. 1995). A study has shown that arrest of cells in the G2 phase of the cell cycle increases transcription from HIV-1 LTR and other promoters (Goh et al. 1998). In addition to its role in nuclear import and cell cycle arrest, Vpr is able to reduce mutation rates during viral DNA synthesis (Mansky 1996), by recruiting Uracil-DNA glycosylase 2, a cellular DNA repair enzyme, into virions (Chen et al. 2002).

The main activities of Vpr are encoded in distinct domains of the protein. The two α-helical domains, in the N-terminal and central regions of Vpr, control cell cycle progression and nuclear translocation respectively, with both domains contributing to incorporation of Vpr into virions (Mahalingam et al. 1997; Subramanian et al. 2011). C-terminal basic domain

functions in both nuclear localisation and cell cycle functions, but is not involved in incorporation of HIV-1 virions (Mahalingam et al. 1995).

1.5.3.4 Vif

Virion infectivity factor (Vif) is encoded by all lentiviruses except equine infectious anaemia virus (EIAV). Vif is a 23kDa, 192 amino acid, cytoplasmic protein that is expressed from a multiply-spliced Rev-dependant mRNA. Vif plays a crucial role in HIV replication and infectivity (Strebel et al. 1987; Goncalves et al. 1994; Karczewski and Strebel 1996).

The Vif protein is essential for the production of infectious particles in peripheral blood mononuclear cells, primary T cells, macrophages and in non-permissive cell lines such as H9 and CEM (Schwartz et al. 1991; Strebel 2007). However, Vif is not required for viral replication in permissive cell lines such as HeLa, Jurkat, SupT1 and 293T cells (Simon et al. 1998; Strebel 2007). This cell type specificity suggested that Vif function may be influenced by the presence of a restrictive endogenous factor in non-permissive cells that inhibits HIV-1 replication in the absence of Vif (Madani and Kabat 1998; Simon et al. 1998). The restrictive factor in non-permissive cells was discovered to be the cytidine deaminase apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G) (Sheehy et al. 2002).

To date, the crystal structure of HIV-1 Vif protein has not been deciphered as Vif is a highly insoluble protein with a strong propensity to aggregate therefore hindering efforts to purify the protein. Vif contains multiple functional domains that might be important for the interaction with viral or cellular proteins. Sequence data reveals that the protein contains several highly conserved motifs, specifically an N-terminal Tryptophan-rich fragment, a conserved HCCH motif and a SOCS-box motif (Oberste and Gonda 1992).

The N-terminal region Tryptophan-rich stretch (residues 1 to 21) mediates Vif recognition and suppression of the target molecules APOBEC3G and APOBEC3F [21]. Amino acids 63–70 and 86–89 are thought to be important for formation of β-strand structures and are critical for maintaining a normal expression level of Vif. A central hydrophilic region, EWRKKR, bears a Glutamic acid and Tryptophan at positions 88 and 89 respectively and is believed to augment steady-state expression of Vif in host cells (Fujita et al. 2004). The HCCH motif (residues 108–139) has two conserved His/Cys pairs flanking an α-helix and was shown to coordinate binding of zinc and Cullin5 (Mehle et al. 2006; Xiao et al. 2006). The HCCH motif also induces a conformational change that promotes Vif multimerisation and aggregation (Paul et al. 2006). The highly conserved SLQYLA motif (Oberste and Gonda 1992) delineates the BC-box motif and is responsible for the binding to Elongin C. This region is often referred to as the SOCS-box as it bears strong similarity to the SOCS-box of the SOCS protein, a suppressor of cytokine signalling (Yu et al. 2004).

Vif is incorporated into HIV-1 virions by interaction with the HIV-1 genomic RNA mediated by the N-terminal region of Vif (Dettenhofer et al. 2000; Zhang et al. 2000), as well as through interaction with the Gag precursor Pr55 gag via its C-terminal region (Bouyac et al. 1997). Several studies have investigated the number of Vif molecules packaged into virions, and estimates have ranged from less than one molecule per virion to as many as 100 molecules of Vif per virion. Generally, studies using virus from chronically infected cells or from stable cell lines stated lower amounts of virus-associated Vif than studies using virus from productively infected cells (Liu et al. 1995; Camaur and Trono 1996; Fouchier et al. 1996; Dettenhofer and Yu 1999).

Studies show that Vif has a tendency to oligomerize and the domain that facilitates Vif's ability to form multimers is found in the conserved PPLP motif (residues 161 to 164) in its C-terminus. The formation of Vif multimers is believed to be essential for Vif function in the HIV-1 life cycle (Yang et al. 2001; Yang et al. 2003). It has been suggested that multimerisation of Vif triggers Vif to form an ordered structure which perhaps enables it to better interact with APOBEC3G and induce its degradation (Auclair et al. 2007; Donahue et al. 2008). Virus-associated Vif is also cleaved by HIV-1 PR between residues 150 and 151 which is a position within the SLQYLA motif necessary for Vif function against APOBEC3 proteins. It is therefore thought that Vif cleavage by PR may be significant for Vif function within non-permissive cell lines (Khan et al. 2002; Barraud et al. 2008).

Vif is found predominantly in the cytoplasm (Simon et al. 1999) but studies also show regulated nuclear localisation of Vif (Simon et al. 1997). Vif may also interact with the cytoskeleton as a result of its association with Pr55gag (Karczewski and Strebel 1996; Bouyac et al. 1997). The association of intracellular Vif with specific subcellular structures may possibly be related to its function of APOBEC3 neutralisation.

1.6 The Replication Cycle of HIV

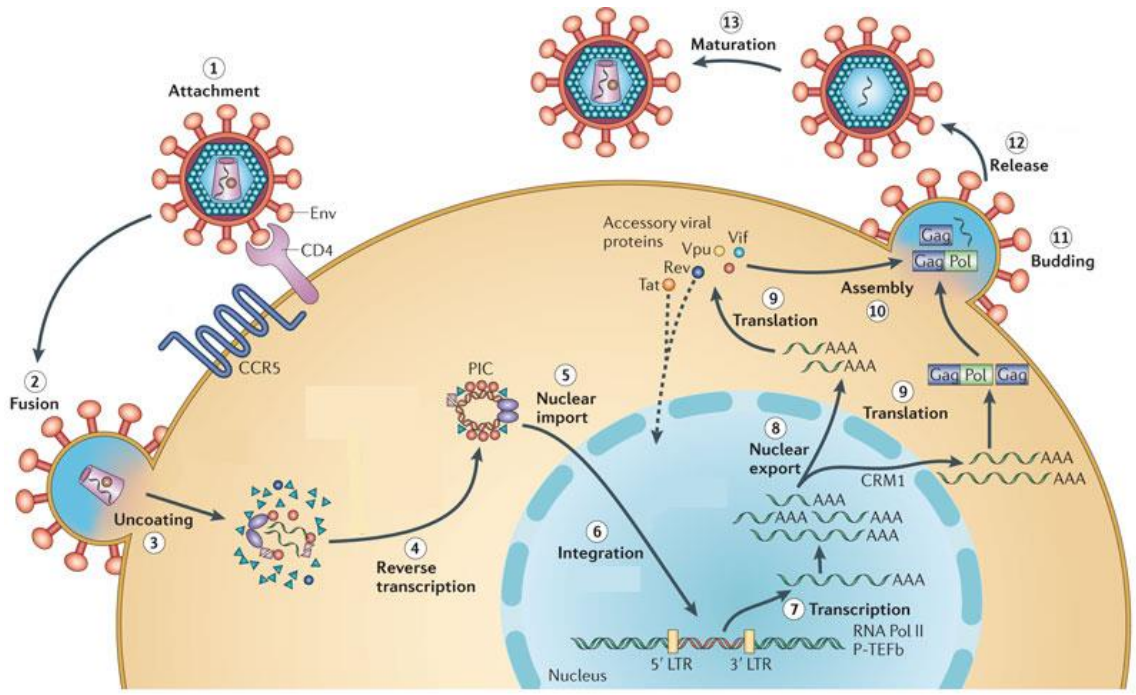


Figure 1.6 The HIV replication cycle consists of a complex train of events that occur in a systematic step-wise fashion. These steps include: (1) attachment of the virus onto the host cell receptors, (2) fusion of virus and host cell membranes (3) uncoating of the viral capsid and (4) reverse transcription of viral genome, (5) nuclear import followed by (6) integration into the host genome, (7) transcription of the provirus and (8) nuclear export of RNA transcripts, then (11) translation into viral proteins (10) viral assembly (11) budding and (12) release of the nascent virion from the host and finally (13) maturation of the virus. (adapted from: (Engelman and Cherepanov 2012) The structural biology of HIV-1: mechanistic and therapeutic insights, Nature Reviews Microbiology)

1.6.1 Binding and Entry

The CD4 molecule on the host cell is the primary cell-surface receptor for HIV entry (Bour et al. 1995) (Figure 1.7). The gp120 region of the HIV viral envelope protein (Env) binds to CD4 via a high affinity binding site on CD4 located in the N-terminal extracellular domain (Kwong et al. 1998) (Figure 1.6). CD4 binding exposes the co-receptor binding site on gp120, formed by the variable regions V1 and V2, conserved region C4 and the variable V3 loop (Sierra et al. 2005). Additionally, CD4 binding promotes the formation of a bridging sheet. The bridging sheet and exposed V3 loop play a vital role in engaging the coreceptors, CCR5 and/or CXCR4, which are essential for viral entry (Choe et al. 1996; Dragic et al. 1996; Wu et al. 1996; Berger et al. 1999) (Figure 1.7). T-cell lines typically express CXCR4 but not CCR5, primary lymphocytes express both CXCR4 and CCR5 and macrophages express CCR5 only. The differential cell-type tropism displayed by viruses is explained by the expression of these co-receptors. T-cell (T) tropic viruses utilise CXCR4 and are termed X4 viruses while macrophage (M) tropic viruses utilise CCR5 and are designated R5 viruses. Dual tropic viruses use both CXCR4 and CCR5 and are named R5X4 viruses (Choe et al. 1996; Dragic et al. 1996; Wu et al. 1996; Berger et al. 1998; Berger et al. 1999; Coakley et al. 2005).

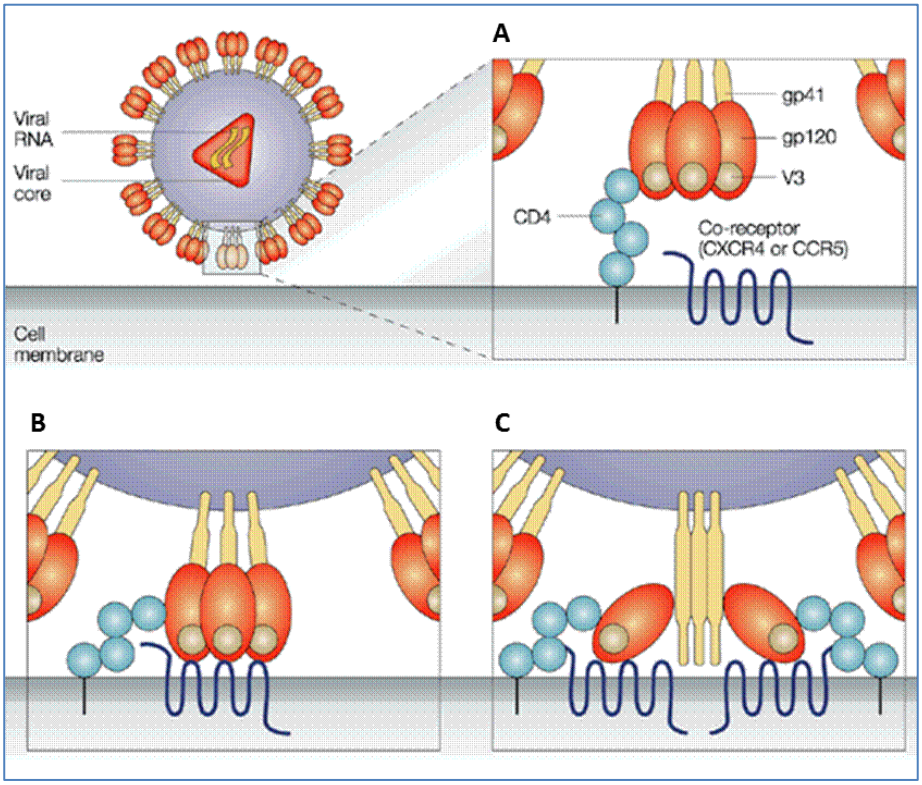


Figure 1.7 During the HIV entry into host cells [A] the viral envelope glycoprotein gp120 interacts with the CD4 receptor at the cell membrane. [B] gp120 interacts with the co-receptors CCR5 and/or CXCR4 [C] the viral glycoprotein gp41 anchors into the cell membrane (Source: (De Clercq 2005), Antiviral drug discovery and development: Where chemistry meets with biomedicine, Antiviral Research).

Co-receptor binding induces further conformational changes in gp41 of HIV-Env which exposes fusion peptides and leads to anchorage of the virus and fusion of viral and cellular membranes (Freed et al. 1990; Doms and Moore 2000). Thereafter during the process of uncoating the viral lipid membrane is stripped away to release the viral core into the cytoplasm of the host cell (Figure 1.6).

1.6.2 Reverse Transcription

A characteristic feature of retroviruses is that they are able to alter their single stranded RNA genomes into double stranded DNA, a process that is catalysed by the RT enzyme (Harrich and Hooker 2002) (Figure 1.8).

Reverse transcription is initiated using the $tRNA_3^{Lys}$ molecule of RT which acts as a primer and base pairs with viral genomic RNA in a region called the primer binding site (PBS) (Figure 1.8). DNA synthesis proceeds in a 3' to 5' direction of the RNA strand and creates a DNA/RNA hybrid (Mak and Kleiman 1997). RNase H subsequently degrades the RNA strand of the hybrid to produce a minus strand strong-stop DNA fragment ([-] ssDNA) (Schatz et al. 1990). The [-] ssDNA is then transferred to the 3' end of the genomic RNA, to allow a continuation of minus-strand DNA synthesis. This step of first-strand transfer is facilitated by the repeat (R) region of the [-] ssDNA that is complementary to the R region of the 3'-end of the RNA template (Peliska and Benkovic 1992).

As minus strand synthesis continues, RNase H cleaves the RNA template except the two purine rich fragments that are highly resistant to degradation and which function as RNA primers for plus strand DNA synthesis. These purine-rich regions of genomic RNA are situated near the 3'-end and in the centre of the viral RNA, and named the polypurine tract (3'PPT) and the central PPT (cPPT), respectively ((Rausch and Le Grice 2004)). Initiation of plus strand synthesis from both the cPPT and PPT results in the formation of two distinct plus-strand DNA segments called the downstream (D+) and upstream (U+) segments (Charneau et al. 1992; Charneau et al. 1994).

The PBS at the 3'-terminus of the [+] ssDNA is regenerated by the tRNA that primed reverse transcription and remained attached to the 5'-terminus of the minus strand DNA. RNase H subsequently removes the tRNA and PPT primers from minus- and plus-strand DNA, respectively. The plus- and minus-strand DNA copies of the PBS facilitates second strand transfer where the PBS sequence of the plus strand DNA anneals to the PBS on the minus strand DNA (Das et al. 1995; Wakefield et al. 1995; Zhang et al. 1998). DNA synthesis proceeds with strand displacement synthesis (Hottiger et al. 1994).

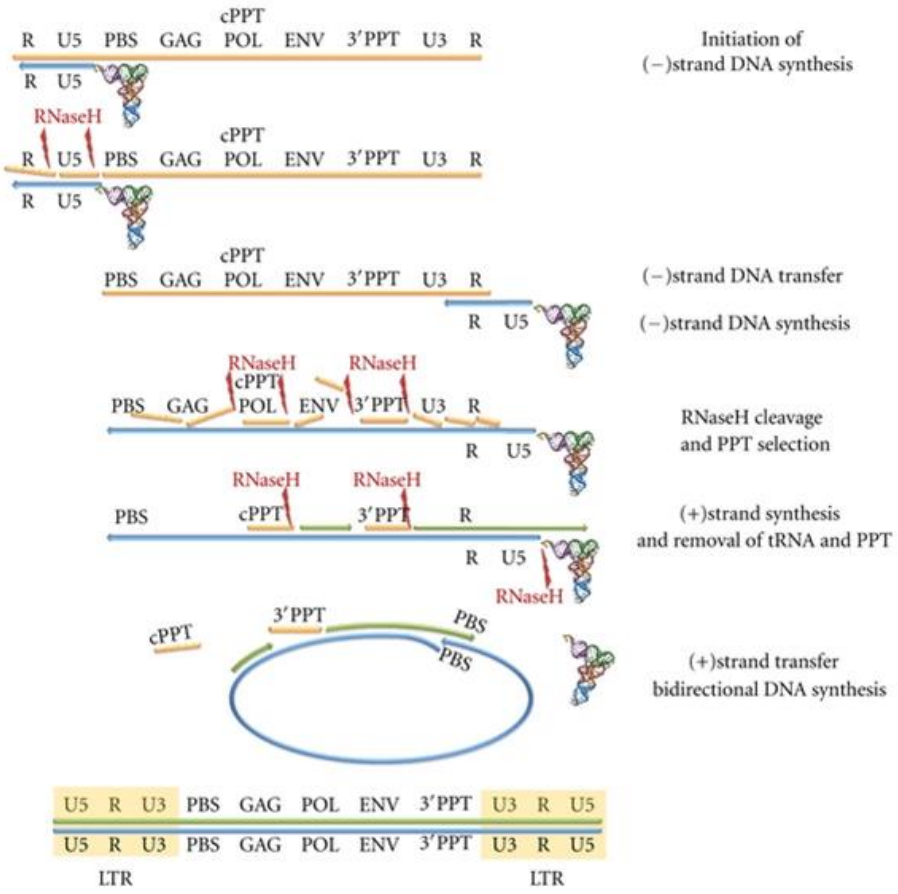


Figure 1.8 The steps of HIV-1 reverse transcription (Source: (Esposito et al. 2012), HIV-1 RT Still Remains a New Drug Target: Structure, Function, Classical Inhibitors, and New Inhibitors with Innovative Mechanisms of Actions, Molecular Biology International)

Upstream plus-strand DNA synthesis terminates when RT encounters the central termination sequence (CTS) located at the centre of the genome. Termination occurs approximately 100 nucleotides downstream of the 5'-end of the downstream segment resulting in the displacement of a fragment of about 100 base pairs. This results is a plus-strand overlap in the full-length double-stranded DNA known as the central DNA flap (Charneau et al. 1994; Hameau et al. 2001).

In addition, the consequence of the two strand transfer reactions is that the provirus acquires a duplicated U3-R-U5 sequence at both of its ends which are known as LTRs.

1.6.3 Nuclear Import, Integration and Transcription

Following the process of reverse transcription, the double stranded DNA forms a complex with host cell and viral proteins IN, Vpr, MA and Gag NC. The complex known as the pre-integration complex (PIC) is actively transported into the nucleus of the cell (Figure 1.6) (Farnet and Haseltine 1991). HIV-Vpr promotes the docking of the PIC into the nuclear membrane (Popov et al. 1998) and enters the membrane through the nuclear pore (Le Rouzic and Benichou 2005). The central DNA flap is also thought to play a role in nuclear import (Zennou et al. 2000).

Once inside the nucleus, the linear double stranded viral DNA in the PIC is inserted into the host cell genome by viral IN protein. During a process called 3'-end processing, IN cleaves off nucleotides from the 3' termini of both strands of viral DNA resulting in double stranded DNA with 3' recessed ends. IN also cleaves the cellular target DNA allowing the 3' recessed ends of the viral DNA to join the ends of the cellular DNA, completing the process of integration (Bushman 2002; Van Maele and Debyser 2005).

Once the proviral DNA is integrated, cellular RNA polymerase II promotes the first round of proviral transcription. Transcription is initiated at the U3/R junction of HIV-1 LTR region. The LTR U3 contains various elements that mediate the binding of RNA polymerase II to the DNA template. The LTR also has binding sites for several host-cell transcription factors, including Sp1, NF-κB, AP-1, and NF-AT which play an important role in regulating the production of RNA transcripts (Garcia et al. 1987; Kawakami et al. 1988; Wu and Marsh 2003). Tat, Rev and Nef are the earliest gene products and are the proteins that alter cellular and viral functions to promote efficient viral replication, but more precisely to control the viral transcription output (Dayton et al. 1986; Hammarskjold et al. 1989; Mangasarian and Trono 1997).

The transcribed viral RNAs are generated as: 1) multiply spliced RNAs, which encode Rev, Tat, and Nef proteins, 2) partly spliced RNAs, which function as mRNAs for the Env, Vif, Vpu, and Vpr proteins, and 3) unspliced RNAs, which serve as the mRNAs for the Gag and GagPol polyprotein precursors, as well as genomic RNA which is packaged into virions. The mixed use of splicing sites results in the production of nearly 40 different transcripts from a single RNA that is generated from the integrated DNA and consequently encodes a total of sixteen different proteins (Purcell and Martin 1993). Transcribed RNAs are exported from the nucleus into the cytoplasm where translation takes place.

1.6.4 Assembly and Release

Once viral proteins have been synthesised they are processed into functional forms and assembled with full-length HIV RNA transcripts into new viral particles. The process of viral assembly and release occurs at the plasma membrane of the infected cell (Figure 1.6) and is driven by the Gag-polyprotein (Pr55 Gag) (Sandefur et al. 2000; Adamson and Freed 2007). Assembly of nascent viral particles at the plasma membrane is essential as it facilitates extracellular virus release and transmission to the next target cells. Viral assembly is a multi-step process involving: (1) the targeting of Gag to the site of assembly and binding of the plasma membrane, mediated by MA, (2) multimerization of Gag that requires CA and NC, (4) encapsidation of the genomic RNA controlled by NC, (5) transport and recruitment of Env into virus particles also mediated by MA (Bieniasz 2009; Ono 2010). The main components of the HIV-1 virion are Gag, which constitutes approximately 50% of the entire virion mass and the viral membrane lipids, which make up 30% of virion mass. An additional 20% is made up by other viral and cellular proteins, while the genomic RNA and other small RNAs contribute 2.5% of virion mass (Sundquist and Krausslich 2012).

The final step is the budding and release of the nascent immature viral particles from the host cell plasma membrane. Sequences that are encoded to facilitate particle release are collectively referred to as “late” or “L” domains to indicate their late role in viral assembly. The L-domain is located in the p6 region of Gag (Gottlinger et al. 1991; Huang et al. 1995). Vpu also facilitates virus release by forming ion conductive pores (Ewart et al. 1996; Schubert et al. 1996). Nef, Env and Vpu are also involved in this process to decrease the number of CD4 molecules present in the plasma membrane to avoid interactions with newly made viruses (Willey et al. 1992; Das and Jameel 2005).

1.6.5 Viral Maturation

Once the virus is released from the plasma membrane, Gag and Gag-Pol are cleaved by viral PR to form mature Gag and Pol proteins which leads to numerous structural rearrangements resulting in viral maturation. The process of viral maturation is essential for viruses to become infectious (Adamson and Freed 2007; Ganser-Pornillos et al. 2008).

1.7. The Immune System

The host immune system provides defence and confers a relative state of resistance to a constant barrage of pathogens and resulting infectious diseases. This defence system is a multifaceted network that constitutes cells and molecules with specialised function in defending against infection (Figure 1.9).

1.7.1 Innate and Adaptive Immunity

The immune system is divided into innate and adaptive components. Innate or natural immunity provides the primary or first line of defence against invading pathogens, and can control many infections and prevent disease. However, the innate response is not always able to eliminate infectious pathogens, and therefore successful immunity may also depend on the second line of defence known as the adaptive or acquired immune response.

Innate immune components play a critical role during early phase of infection and are essential in initiating and guiding the subsequent adaptive immune response. Additionally, the innate immune cells are also involved in removing pathogens and debris that have been targeted by the adaptive immune system.

However, the innate immune system lacks immunologic memory and remains unchanged despite many antigen encounters. On the other hand, the adaptive immune system has adapted to provide a more versatile form of protection and exhibits immunological memory of invading organisms which facilitates a more rapid response on subsequent re-infection. Acting together, the innate and adaptive immune responses provide a highly effective defence system (Janeway et al. 2005).

1.7.2 Components of the Immune System

1.7.2.1 The Major Histocompatibility Complex (MHC)

The MHC also called the HLA system plays a vital role in the immune system where it functions to bind peptide fragments derived from pathogens and display them on the cell surface for recognition by appropriate T cells. There are two classes of MHC molecules, namely, MHC class I and MHC class II which bind peptides fragments from two distinct intracellular compartments. MHC class I molecules bind peptides from intracellular pathogens that multiply in the cytoplasm, MHC class II molecules present peptide antigens from pathogens multiplying in intracellular vesicles and those derived from extracellular bacteria and toxins (Janeway et al. 2005; Neefjes et al. 2011). Studies have shown that the HLA system is associated with susceptibility or resistance to many viruses, and more recently it has been reported that HLA is associated with HIV transmission and disease progression to AIDS (Carrington and O'Brien 2003).

1.7.2.2 The Interferon System

Interferons (IFN) play a crucial role in modulating immune responses and facilitating antiviral and antigrowth responses. The IFN system constitutes cells that produce IFN in response to an external stimulus for example viral infection and cells that react to IFN by establishing an antiviral state (Pestka et al. 1987; Samuel 1991; Stark et al. 1998). The IFN response is an early host defence and is initiated before the onset of the immune response.

The IFNs family constitutes several inducible cytokine genes which are divided into two groups. Type I IFNs are also known as viral IFNs and include IFN-α, IFN-β, and IFN-ω. Type II IFN is also known as immune IFN or IFN-γ. Viral IFNs are induced during viral infection by pathogen recognition receptors (PRRs) such as Toll-like receptors and retinoic

acid induced gene (RIG)-like receptors, whereas type II IFN is initiated by mitogenic or antigenic stimuli (Samuel 1991; Samuel 2001).

IFN-α and –β appear to be synthesised by most types of virally infected cells in cell culture. However IFN-γ is produced only by certain cells of the immune system such as natural killer (NK) cells, CD4 Th1 cells, and CD8 cytotoxic suppressor cells (Young 1996; Bach et al. 1997).

IFN-α is known to downregulate a multitude of viruses by upregulating many different proteins, for instance the protein kinase R, oligoadenylate synthetase, adenosine deaminase and RANTES which are able to inhibit viral replication at the levels of penetration, uncoating, mRNA synthesis, protein synthesis, or assembly (Haller et al. 2007) and most recently discovered host antiviral factors such as, TRIM5α, Tetherin and APOBEC3G (Parmar and Platanias 2003; Asaoka et al. 2005; Tanaka et al. 2006; Pillai et al. 2012).

1.7.2.3 Innate Immune Components

Pathogen recognition and the initial innate immune response is activated by pathogen associated molecular patterns (PAMPs) which are detected by PRRs on the host cell. PAMPs are conserved molecular motifs that are generally expressed on microbes, and include flagellin, lipopolysaccharides, or double-stranded RNA. The PRRs are evolutionary conserved recognition receptors that are confined within the germline of cells. Binding of PRRs with their associated PAMPs induces removal and obliteration of infected cells through a number of mechanisms such as opsonisation, complement activation, acute inflammation, or phagocytosis (Akira 2009; Mogensen 2009).

Immune responses are mediated by leukocytes (white blood cells) that have their origin in the bone marrow stem cells. The innate immune system predominantly involves granulocytes (neutrophils, basophils, eosinophils), dendritic cells (DC), macrophages, mast cells and NK cells which form from myeloid progenitor cells.

Dendritic cells provide the key link between the innate and adaptive immunity. These cells are highly specialised phagocytes and display engulfed antigens, via MHC molecules on their cell surface, for recognition by T-cells, thereby inducing the adaptive immune responses. Activated DCs are also a major producer of cytokines such as type I IFN which activate inflammatory responses (Matzinger 1998; Bell et al. 1999).

Macrophages are the mature form of monocytes and are one of four types of phagocytes in the immune system. Once a macrophage is activated by a pathogen, it engulfs the pathogen and subsequently initiates an inflammatory response. Macrophages possess receptors for carbohydrates like mannose that is expressed by infecting microorganisms but not normally expressed on host cells and are thereby able to recognise self from non-self. Macrophages and neutrophils also have receptors for antibodies and complement and thereby enhance phagocytosis of organisms coated with these (Fraser et al. 1998; Aderem and Underhill 1999). When macrophages take up pathogens they express MHC class II molecules on their surface allowing them to present peptides from degraded pathogens to T lymphocytes, however they are less powerful than DCs at activating naïve T cells.

Mast cells and basophils have similar functional characteristics where both cell types express high-affinity receptors for immunoglobulin E (IgE). These cells are important in initiating allergic responses and are known to secrete inflammatory mediators particularly, histamines,

prostaglandins and leukotrienes. Eosinophils and neutrophils are weakly phagocytic and kill parasites when activated by releasing cationic proteins and reactive oxygen metabolites (Wardlaw et al. 1995; Abraham and Arock 1998).

Lymphoid progenitor cells give rise to NK cells of the innate immune system. NK cells are activated by IFNs and macrophage derived cytokines and plays a significant role in the early immune response to certain infections and malignancies by direct cytolysis of infected or altered cells and by secretion of potent immune mediators. NK cells express inhibitory and activating receptors that interact with MHC class I molecules, MHC class I–like molecules, and molecules unrelated to MHC. Inhibitory receptors enable NK cells to distinguish between infected and uninfected cells thereby ensuring tolerance to self while allowing toxicity toward stressed cells. The MHC class I–specific inhibitory receptors are the killer cell immunoglobulin-like receptors (KIRs). Absence of MHC class I molecules signals an abnormal cell which is subsequently destroyed by NK cells. NK cells also recognise their targets by Fc receptors that bind Immunoglobulin G (IgG) on target cells thereby exerting antibody-dependent cell cytotoxicity (Caligiuri 2008; Vivier et al. 2008; Campbell and Hasegawa 2013).

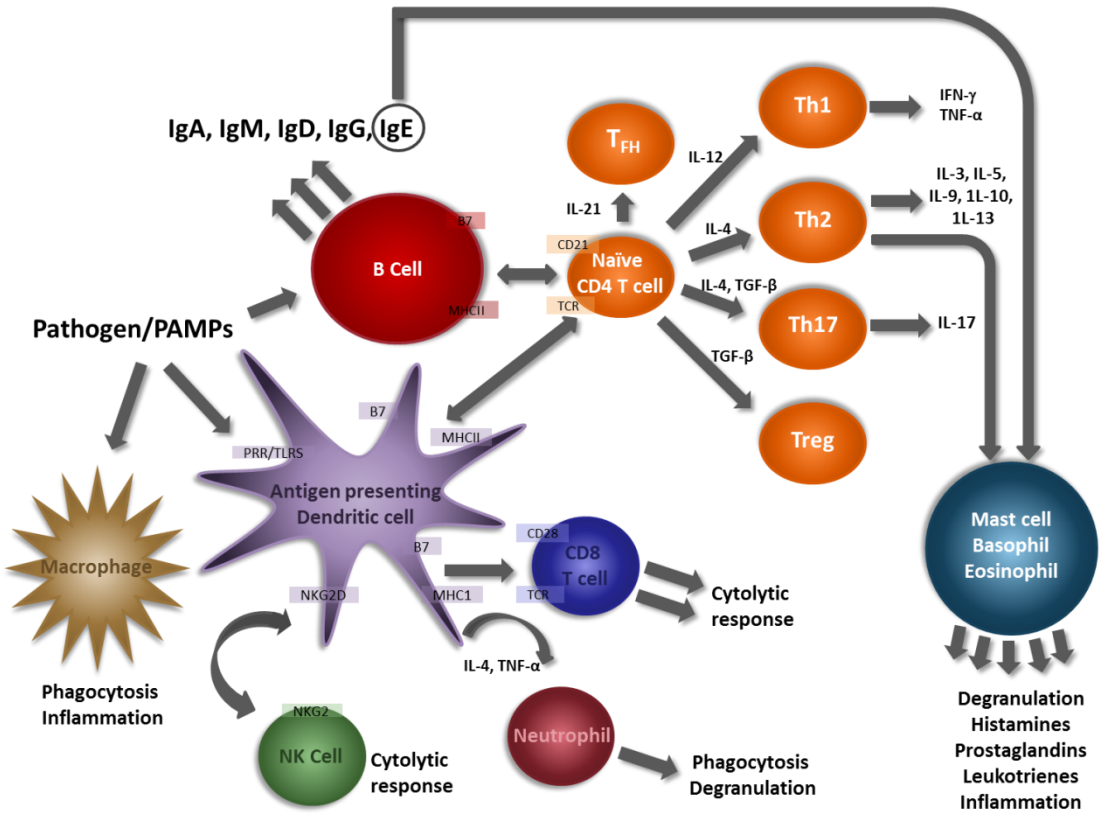


Figure 1.9 Immune system interactions. Cellular interactions lead to different functional responses by different immune cells. Several cell surface receptor–ligand interactions, cytokines, and inflammatory molecules mediate and affect the immune response. Antigen presenting cells, for example dendritic cells, can interact with CD8 T cells to stimulate cytolytic immune responses, or with CD4 T cells to activate humoral antibody responses. CD4 T cells differentiate into an assortment of effector subsets, some of which stimulate B cells to produce antibodies or those that help activate other immune clearance and regulatory processes (Source: (Look et al. 2010). Application of nanotechnologies for improved immune response against infectious diseases in the developing world, Advanced Drug Delivery Reviews).

Innate responses also frequently involve complement, acute phase proteins, and cytokines. Complement is a system of plasma proteins that play a vital role in innate immunity. The complement proteins play a role in immune surveillance; they promote inflammation and eradication of infectious microbes and eliminate cellular debris. The three pathways of complement activation include the classic pathway, activated directly by pathogens or indirectly by antibodies bound to pathogens; the lectin pathway, activated by the interaction of microbial carbohydrates with mannose binding protein in the plasma, and the alternative pathway, activated by microbial cell walls (Rus et al. 2005; Ricklin et al. 2010). Acute phase proteins are secreted during the acute phase response which is a systemic reaction, triggered by cytokines, to local or systemic disturbances in homeostasis caused by infection, tissue injury, inflammation or immunological disorders. Acute phase proteins include C-reactive protein, serum amyloid A protein, proteinase inhibitors and coagulation protein (Gruys et al. 2005). Cytokines are important mediators of immune responses and include a diverse collection of interleukins, IFNs, and growth factors. Cytokines are produced by most cells of the immune system and bind to specific membrane receptors, which then signal the cell via second messengers to alter cellular activity (Mire-Sluis 1993).

1.7.2.4 Adaptive Immune Components

Adaptive immune responses are governed by specialised T and B lymphocytes which arise from lymphoid progenitor cells. The adaptive immune responses are divided into the T-cell responses mediated by T-lymphocytes and humoral immune responses directed by B lymphocytes.

The adaptive immune response is activated when naïve T cells encounter a specific antigen on the surface of an antigen presenting cell. The key antigen presenting cells are DCs that

ingest antigen at sites of infection and migrate to lymphoid tissue where they present the antigen via MHC molecules to T cells. The T cells become activated, and produce interleukin (IL) - 2 which drives them to proliferate and differentiate into effector T cells. Antigens carried by MHC class I molecules are presented to CD8 T cells which differentiate into cytotoxic T lymphocytes (CTL) that kill virally infected cells (Janeway and Medzhitov 2002; Santana and Esquivel-Guadarrama 2006).

On the other hand antigens carried by MHC class II molecules are presented to CD4 T cells which differentiate into T helper cells, specifically Th1, Th2, Th17, and T regulatory (Treg) or T follicular helper (TFH) cells (Janeway et al. 2005). Th1 cells produce IFN-γ and tumour necrosis factor-α (TNF-α) cytokines, which facilitate protection against intracellular pathogens, while Th2 cells stimulate antibody production from B cells and also produce IL-4, IL-5, and IL-13 cytokines, which are important in generating immune responses to extracellular infections. Th17 cells secrete the proinflammatory cytokine IL-17, whereas Treg cells are involved in maintaining tolerance for self-antigen. TFH cells activate B cells in the lymph nodes and spleen and stimulate B cells to produce antibodies (Romagnani 1992; Kaiko et al. 2008; Crotty 2011; Tangye et al. 2013).

T cells produce an array of effector molecules which determine their function. CTLs secrete cytotoxins which are stored in specialised lytic granules and induce apoptosis of target cells. CTLs also produce IFN γ which inhibits viral replication, induces MHC class I molecule expression and macrophage activation. Cytokines and the TNF family of membrane associated effector proteins are produced by CTLs and T helper cells. Th2 cells express effector molecules that activate B cells while Th1 cells express macrophage activating effector molecules (Janeway et al. 2005; Santana and Esquivel-Guadarrama 2006).

B lymphocytes or B cells secrete antigen-specific antibodies against antigens that are presented to the lymphocytes by antigen presenting cells. Th2 helper cells play a role in activation and differentiation of B cells into antibody secreting plasma cells and memory B cells. Antibodies contribute to immunity in different ways: Neutralising antibodies are able to bind to pathogens and prevent their entry into cells. Additionally, antibodies are able to coat the surface of pathogens to enhance phagocytosis in a process call opsonisation. Antibodies coating the pathogen are either recognised by Fc receptors on phagocytic cells or are able to activate the proteins of the complement system (Janeway et al. 2005).

The antibody response begins with the secretion of Immunoglobulin M (IgM) and soon progresses to the production of other isotypes. Antibodies of each isotype have distinct locations and specialised functions. IgM is found predominantly in the blood and is specialised to activate the complement system. IgG antibodies are located in blood and extracellular fluid and function to neutralise toxins, viruses, bacteria, opsonise for phagocytosis and activate the complement system. Immumoglobulin A (IgA) antibodies can also be found in the blood and extracellular fluids as well as in epithelial tissue. They are often transported into the lumen of the gut where they function to neutralise toxins, viruses and bacteria in the intestinal epithelium. IgE is frequently bound to mast cells and triggers local defence reactions (Delves and Roitt 2000; Delves and Roitt 2000; Janeway et al. 2005).

1.8 HIV Pathogenesis and the Host Immune Response

1.8.1 The HIV-1 Transmission Event and the Acute Stage of Infection

HIV infection proceeds by viral transmission; followed by an acute phase of intense viral replication and spread to lymphoid tissues; a chronic phase of continued immune activation and viral replication; and an advanced phase of marked CD4+ T cell loss resulting in acquired immune deficiency syndrome (Moir et al. 2011).

The majority of HIV-1 infections (approximately 70%) occur through exposure of mucosal surfaces to the virus via heterosexual contact. The remaining 30% of infections occur through mucosal transmission in men who have sex with men (MSM), by percutaneous or intravenous drug use, through exposure of blood and blood products via transfusions or by maternal-infant transmission either in-utero, intra-partum or through infected breast milk (Datta et al. 1994; Nduati et al. 2000; 2010). The ratio between these routes of transmission does however differ between different countries.

Once HIV crosses the mucosal barrier, local CD4+ T cells, Langerhans cells and other DCs are the virus's first targets by binding to the high affinity C-type lectin receptor, DC-SIGN. Within a few days the virus begins spreading into lymphoid tissue, with preference for the gut-associated lymphoid tissue, followed by subsequent widespread dissemination (Cohen et al. 2011). The lymphoid tissue becomes the main HIV reservoir and site of persistent viral replication (Fox et al. 1991).

After viral transmission, there is a period of about 10 days, while HIV-1 replicates in the mucosa, submucosa and lymphoid tissues, known as the eclipse phase, where viral RNA is undetectable in the plasma (Figure 1.10 I) (Little et al. 1999). Virus can be detected by the

sensitive, qualitative method of nucleic acid amplification once HIV-1 RNA reaches a concentration of 1-5 copies/ml in plasma. The eclipse phase is followed by the acute phase of HIV-1 infection which is characterised by an exponential increase in viral titres, which reaches a peak (Figure 1.10 I and II) (Clark et al. 1991; Daar et al. 1991) and thereafter spontaneously decreases (Figure 1.10 III - V) to a steady state level or set point (Figure 1.10 VI) that initiates a more chronic phase of the infection and predicts disease progression (Mellors et al. 1995; O'Brien et al. 1996).

Acute or primary HIV infection is defined as the first period of infection from the detection of HIV RNA until the formation of HIV-specific antibodies (seroconversion) 3-4 weeks after infection (Busch and Satten 1997; Mogensen et al. 2010). Early-stage HIV infection usually refers to the interval between seroconversion and the establishment of the viral load set point, which typically occurs 6–12 months after infection.

The stages that delineate acute and early infection (Figure 1.10) are associated with the sequential appearance of viral markers and antibodies in the plasma, specifically HIV-1 viral RNA, the Gag p24 protein antigen, antibodies specific for recombinant HIV proteins (that can be detected by enzyme linked immunosorbent assays (ELISA), and antibodies that bind fixed viral protein, including p31 (detected by Western immunoblot) (Daar et al. 2008).

Additionally, the sharp increase in viral load also coincides with the appearance of acute phase reactants, and a surge of innate immune responses and inflammatory cytokines (Stacey et al. 2009; Kramer et al. 2010). The acute phase of infection is also characterised by a rapid decline in CD4+ T cell levels particularly in the gastrointestinal tract (Brenchley et al. 2004; Mehandru et al. 2004). Additionally, the first CD8 T-cell responses also appear during this

phase, days before the peak of viremia (Lichterfeld et al. 2004). These immune responses are discussed further in section 1.8.2.

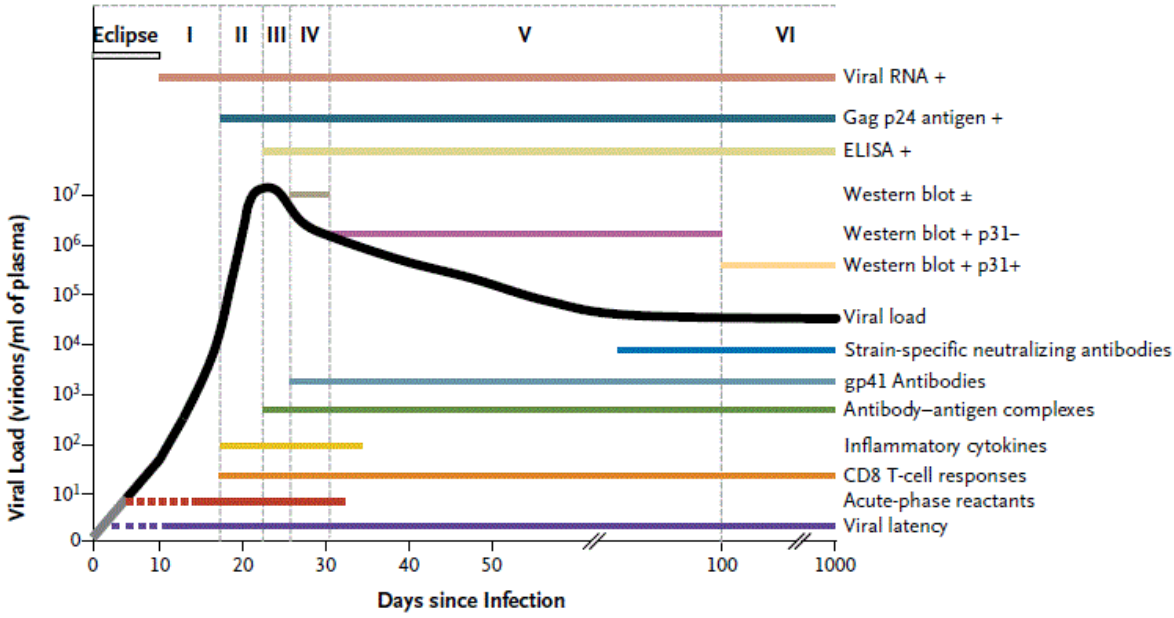


Figure 1.10 Representation of the sequence of events during the acute and early chronic phases of HIV-1 infection (Source: (Cohen et al. 2011), Acute HIV-1 Infection, The New England Journal of Medicine)

1.8.2 Immune Responses to HIV

Even though HIV causes progressive and persistent destruction of the immune system and ultimately extensive immunodeficiency, it also stimulates strong immune responses. During the initial period of rapid viral replication the host immune system attempts to control viral spread and eliminate the virus. Innate responses as well as adaptive cell-mediated immune responses (by HIV-specific CTLs) (Plata et al. 1987; Walker et al. 1987) and HIV-specific humoral responses (from antibodies against HIV proteins) (Gray et al. 2007; Tomaras et al. 2008) have been detected early in the course of infection. However, CTLs have been detected prior to the appearance of neutralising antibodies (Safrit et al. 1994).

The first indication of an immune response during HIV-1 infection is the appearance of acute phase reactants, specifically alpha$_1$ anti-trypsin and serum amyloid, in plasma 3-5 days post-infection (Kramer et al. 2010). Additionally, the rapid increase in viral load overlaps with a surge of inflammatory cytokines directed by interferon-α and interleukin 15 (Stacey et al. 2009). Early cytokines are produced by DCs but later in infection they are also produced by macrophages, NK cells and T cells (Borrow and Bhardwaj 2008)

Acute infection is typically associated with a large T-cell response with substantial oligoclonal expansion in CD8+ T cell numbers, representing approximately 40% of all T cells (Roos et al. 1994). The CTL response emerges during the exponential increase in viral load and although narrowly directed (Radebe et al. 2011), coincides with a decrease of acute viremia (Koup et al. 1994; Borrow and Bhardwaj 2008; Goonetilleke et al. 2009). CTLs peak at approximately 12 months post infection and have been shown to inhibit viral replication by two mechanisms. Firstly, CTLs have the ability to kill infected cells by cell lysis mediated by perforin and granzymes which trigger target-cell death (Shankar et al. 1999). Secondly, CTLs simultaneously secrete soluble antiviral factors, such as cytokines and specifically the chemokines RANTES, MIP-1 alpha, and MIP-1 beta. These chemokines are able to directly inhibit infection by progeny virions, by competition for or downregulation of the cellular coreceptor for the HIV, CCR5 (Cocchi et al. 1995).

CD4+ T cell responses in acute infection are particularly weak. HIV- specific CD4+ T cells are extremely susceptible to attack and destruction by HIV, resulting in rapid and substantial loss of CD4+ T cells in lymphoid tissue, particularly in the gastrointestinal tract, early in infection. Once primary infection resolves CD4 + T cell counts rebound, but rarely return to baseline, however, CD4+ T cell function remains abnormal throughout the course of

infection. In untreated HIV infection CD4+ T cell counts again abruptly decrease late in disease (Gruters et al. 1991; Gupta 1993; Zaunders et al. 2001).

The formation of HIV-specific antibodies is usually detectable by weeks 3-12 of infection. Most early antibodies against HIV are directed against virus debris and non-neutralising epitopes on envelope glycoprotein and therefore are unable to elicit an effective antiviral response. Antibody responses that neutralise transmitted and early infection virus isolates are delayed and arise several months after the transmission event (Tomaras et al. 2008). These neutralising antibodies are targeted particularly against the highly variable V3 loop of the envelope protein involved in virus entry as well as the CD4 binding domain (Burton and Montefiori 1997). Nonetheless, neutralising antibodies against HIV are generally inadequate and lack broad cross-reactivity, and are therefore unable to cross-neutralise newly evolving strains of the virus within a person (Parren et al. 1999). Additionally, by the time the neutralising antibody response develops, it is too late to influence the course of infection.

The production of specific T-helper cells has also been implicated in the HIV cellular immune response and has been shown to assist with maintenance of CTLs in chronic viral infections. T-helper cell responses are mediated by CD4+ cells and are stimulated by the presence of viral peptides bound to the MHC class II binding groove, and become activated to enhance the immune response. However, impairment of CD4+ T cell function early in infection results in loss of T-cell help and ultimate decline in virus-specific CTLs, inevitably resulting in disease progression (Rosenberg et al. 1999; Altfeld and Rosenberg 2000).

Despite the strong initial immune responses which results in reduced viral load and an increase in circulating CD4+ T cell numbers, the host is unable to completely clear the

infection (Grossman et al. 2006). Continued antigen stimulation results in expanded T-cell numbers persisting into chronic infection. Thus, chronic HIV infection is characterised by massive immune activation, high cell turn over, apoptosis and activation induced cell death (Finkel et al. 1995; Grossman et al. 2006; Mogensen et al. 2010). Immune activation ultimately leads to depletion of CD4+ T cell numbers by decreasing the half-life of CD4+ and CD8+ T cells, influencing irregular cell trafficking, causing T cell clonal exhaustion and draining memory T cell pools (Grossman et al. 2002; Douek et al. 2003). This phenomenon of chronic immune activation plays a key part in driving immunopathogenesis and progression to AIDS.

There is also considerable heterogeneity in the strength of innate, humoral and cell-mediated immune responses observed between infected individuals during primary infection and with clinical outcome. Some individuals are able to control HIV infection and progress remarkably slowly to AIDS in the absence of HIV treatment, while others progress rapidly to AIDS. Additionally, a complex interplay of host and viral factors may influence the susceptibility of individuals and populations to HIV infection and disease progression.

In recent years a new component of innate immunity to HIV has been discovered, comprising several host genes encoding innate cellular restriction factors, that demonstrate the ability to inhibit viral replication and influence susceptibility to HIV-1 infection (An et al. 2004; O'Brien and Nelson 2004; An and Winkler 2010). These innate cellular restriction factors and are discussed in subsequent sections.

1.9 Host Restriction Factors that Target HIV Replication

Cellular restriction factors are host proteins that are able to inhibit specific steps in the life cycle of HIV and serve as a potent obstruction to HIV replication (Sheehy et al. 2002; Stremlau et al. 2004; Neil et al. 2008; Goldstone et al. 2011; Laguette et al. 2011). Restriction factors can be identified by four defining traits. Firstly, they must directly and dominantly inhibit viral replication. Secondly, the restriction factor should provoke a strong virus encoded counter-restriction mechanism if they are a significant threat to viral replication. Third, they will carry positive selection signatures that would arise from repeated pathogenic pressure. And fourth, restriction factors should possess interferon responsiveness which strongly associates them with the host's immune system (Harris et al. 2012).

Four key host restriction factors that act at various steps in the HIV-1 replication cycle (Figure 1.11) include APOBEC3G (Sheehy et al. 2002), BST-2 also known as Tetherin (Neil et al. 2008), TRIM5α (Stremlau et al. 2004), and SAMHD1 (Goldstone et al. 2011).

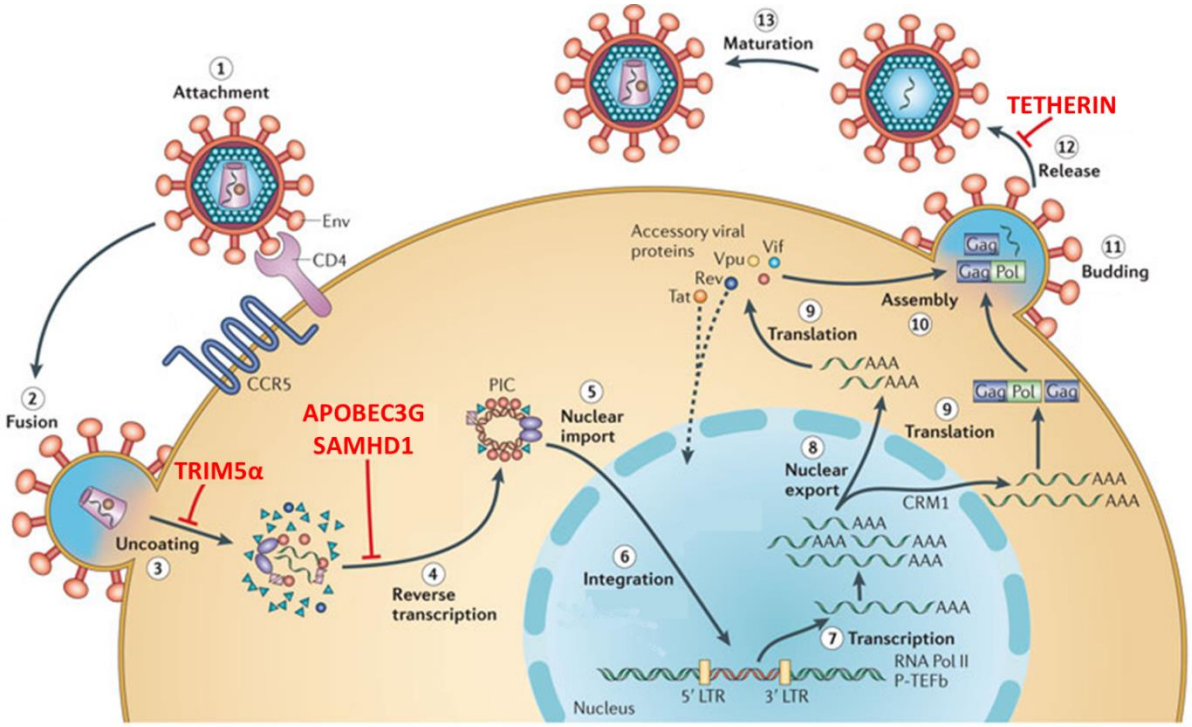


Figure 1.11 Cellular restriction factors acting at specific steps in the life cycle of HIV to inhibit replication of the virus. (Adapted from: (Engelman and Cherepanov 2012), The structural biology of HIV-1: mechanistic and therapeutic insights, Nature Reviews Microbiology)

Type I IFNs, which are activated in the presence of viruses by PPRs, mediate the upregulation of many cellular restriction factors. Type I IFNs induce an autocrine and paracrine-signaling cascade via the Type I IFN receptor and Janus Kinases/Signal Transducers and Activators of Transcription (Jak/Stat) pathway resulting in activation and upregulation of many genes that restrict viral replication thereby initiating an antiviral state (Samuel 2001; Horvath 2004; Baum and Garcia-Sastre 2010).

1.9.1 TRIM5α

TRIM5α was identified as the protein responsible for blocking HIV infection in Old World monkeys (Stremlau et al. 2004). TRIM5 proteins belong to a family of more than 100 tripartite motif genes in the human genome (Han et al. 2011).

Studies thus far indicate that the rhesus orthologue of TRIM5α (rhTRIM5α) is the first restriction factor that acts to inhibit HIV-1 after entry into target cells. rhTRIM5α binds to the viral core and disrupts the normal uncoating process of the core (Chatterji et al. 2006). The B30.2 (SPRY) domain of rhTRIM5α facilitates binding by recognising and interacting with specific sequences on the HIV CA. Human TRIM5α only weakly inhibits HIV-1 replication. However, it is interesting that a single amino acid substitution in B30.2 (SPRY) domain of human TRIM5α enables it to restrict HIV-1 as efficiently as rhTRIM5α (Yap et al. 2005). TRIM5α may additionally block HIV-1 replication at the steps of reverse transcription and nuclear import (Berthoux et al. 2004; Yap et al. 2005). Studies also indicate that HIV-1 CA recognition by rhTRIM5α is facilitated by host cell cyclophilin A (Berthoux et al. 2004; Yap et al. 2005).

Tripartite proteins typically contain a RING finger, B –box and a coiled-coil domain. The B-box is unique to TRIM proteins and is critical for TRIM5 mediated restriction, as deletion of this domain eliminates the ability of TRIM5α to inhibit HIV-1 (Stremlau et al. 2004; Javanbakht et al. 2005; Li and Sodroski 2008). The coiled-coil region plays a role in protein-protein interaction and promotes TRIM5α multimerisation. It has been suggested that multimerisation is important for positioning the B30.2 (SPRY) domain to facilitate CA binding. Additionally mutants in the coiled-coil region fail to hinder HIV-1 (Javanbakht et al. 2006). The TRIM5 RING finger has ubiquitin ligase activity and is not essential for

restriction activity but plays an important role (Yamauchi et al. 2008). Additionally, recent studies showed that high expression of TRIM5 was associated with reduced likelihood of infection in a high risk longitudinal follow-up cohort and that TRIM22 expression levels were associated with lower plasma viral load and higher CD4+ T cell counts during primary HIV-1 infection (Sewram et al. 2009; Ndung'u 2011; Singh et al. 2011).

The mechanism of TRIM5 mediated retroviral restriction remains to be completely elucidated. It is unknown whether its mechanism is as simple as binding to the viral core and initiating premature uncoating or if its mechanism is a more complicated one involving other factors. A recent study highlights a further mechanism of TRIM5 in innate immune signalling where it acts as a pattern recognition receptor specific for the retrovirus CA lattice (Pertel et al. 2011). Additionally, in contrast to the other restriction factors, it has not yet been discovered whether SIV/HIV has a means to counteract TRIM5.

1.9.2 Tetherin

Vpu is required for efficient HIV-1 particle release in certain human cells (Terwilliger et al. 1989; Klimkait et al. 1990). In the absence of Vpu, mature virions are unable to detach from certain types of infected cells and subsequently accumulate on the plasma membrane and within cellular compartments (Geraghty et al. 1994; Varthakavi et al. 2003; Neil et al. 2006).

Studies show that human cells possess an antiviral activity that inhibits the release of retrovirus particles and is antagonized by the HIV-1 accessory protein, Vpu. The protein responsible for this HIVΔVpu antiviral phenotype has been identified as Tetherin (also known as BST-2 or CD317) (Neil et al. 2008). Tetherin is an IFNα -induced protein, as Vpu independent cells can become Vpu dependent by IFN α treatment (Neil et al. 2007).

Tetherin is a 30-36kDa type II transmembrane glycoprotein (Ishikawa et al. 1995), containing an N-terminal cytoplasmic tail, a transmembrane domain, an extracellular coiled coil domain and a C-terminal glycosyl-phosphatidylinositol (GPI) anchor (Kupzig et al. 2003). Tetherin exists as a homodimer formed by disulphide bridges between the coiled coil ectodomain regions of two Tetherin proteins (Andrew et al. 2009).

A few mechanisms of Tetherin-induced inhibition of HIV-1 particle release have been proposed (Perez-Caballero et al. 2009) (Figure 1.12). In the first model, as budding occurs, the GPI anchors of a Tetherin dimer remain embedded in the host-cell membrane and tether budding virus via the transmembrane domains incorporated into the virion envelope. The second model is the reverse of model 1 where transmembrane domains of a Tetherin dimer are incorporated into the host-cell membrane while the GPI anchors remain embedded in the virion envelope. In a third model one disulfide linked Tetherin dimer that has incorporated into the virion envelope interacts with another dimer in the host-cell membrane via coiled-coil-based interactions (Perez-Caballero et al. 2009).

HIV-1 Vpu protein counteracts the antiviral mechanism of Tetherin. Studies suggest that Vpu and Tetherin interact through their respective transmembrane domains and that these domains are essential for Vpu mediated Tetherin degradation (Vigan and Neil 2010; Kobayashi et al. 2011; Skasko et al. 2011). It has been suggested that Vpu degrades tetherin post-translationally, by interaction with β-transducin repeat containing protein (β-TrCP) –E3 ligase complex, thereby inducing the ubiquitination of Tetherin (Guatelli 2009; Mangeat et al. 2009).

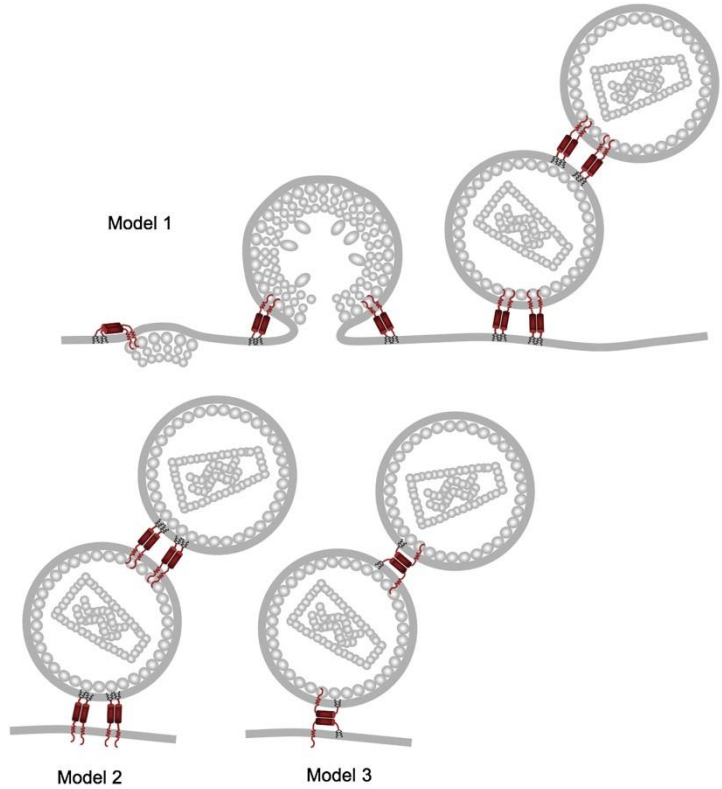


Figure 1.12 Models of interaction of Tetherin with HIV-1 virions (Source: (Perez-Caballero et al. 2009), Tetherin Inhibits HIV-1 Release by Directly Tethering Virions to Cells, Cell)

Ubiquitination of Tetherin results in its endocytosis from the cell membrane and degradation via the proteosomal or lysosomal degradation pathways (Douglas et al. 2009; Mitchell et al. 2009). Additionally, Vpu can alter cell surface expression of Tetherin by sequestering it intracellularly, preventing its trafficking to the cell membrane and subsequently delivering it to late endosomal compartments (Dube et al. 2010; Hauser et al. 2010).

1.9.3 SAMHD1

Cells of the myeloid lineage such as primary monocytes, monocyte-derived DCs and macrophages are more resistant to HIV-1 infection than CD4+ T lymphocytes. SAMHD1 was discovered as the host factor expressed by myeloid cells that restricts HIV-1 by blocking an early step of the HIV-1 replication cycle. SAMHD1 expression levels were found to be high in monocytes and monocyte-derived DCs, low in monocyte-derived macrophages, and lacking from HIV-1-sensitive T cell lines. Interestingly, these expression profiles correlate inversely with the permissiveness of the cell types to HIV-1 infection (Laguette et al. 2011).

SAMHD1 constitutes a putative protein-interacting sterile alpha motif (SAM) and a C-terminal dNTP phosphohydrolase domain (HD domain). The current accepted mechanism for antiviral activity of SAMHD1 is that it causes hydrolysis of cellular deoxynucleoside triphosphates (dNTPs) to the constituent deoxynucleoside and inorganic triphosphate, thereby starving the process of reverse transcription of crucial molecular building blocks required for viral cDNA synthesis (Goldstone et al. 2011).

It has been proposed that IFN production during the innate immune response results in SAMHD1 upregulation. Additionally, SAMHD1 was originally cloned from human DCs as an IFN-γ-inducible gene and has been suggested to act as a negative regulator of the IFN response (Li et al. 2000; Rice et al. 2009).

Studies have shown that viral protein X (Vpx) of HIV-2 and SIV, but absent in HIV-1, is able to enhance the susceptibility of myeloid cells to HIV-1 infection by counteracting SAMHD1 (Hrecka et al. 2011; Laguette et al. 2011). Vpx triggers the degradation of SAMHD1 by facilitating the formation of an E3 ubiquitin ligase complex comprising DCAF1, DDB1 and

CUL4, which facilitates the polyubiquitination and subsequent eradication of SAMHD1 (Ahn et al. 2012; Wei et al. 2012). The C-terminus of SAMHD1 has been reported as the region involved in functional interaction with Vpx (Laguette et al. 2012).

SAMHD1 is the most recently discovered restriction factor and the regulation of SAMHD1 activity remains an important area of study. Additionally, HIV-1 has not yet evolved a counter-attack strategy against SAMHD1 and therefore further understanding of this intrinsic restriction factor may unravel novel methods of combating the virus.

1.9.4 The APOBEC3 Family of Cytidine Deaminases

The mechanism of RNA editing plays an important role in the growth of genetic diversity in many organisms by expanding gene products from a single structural gene without generating new genes. In mammals, there are two types of substitutional RNA editing that occur by deamination of encoded nucleotides. One results in the deamination of adenine (A) to inosine (I) and the other of cytidine (C) to uridine (U) (Blanc and Davidson 2003; Turelli and Trono 2005). Recent evidence shows that RNA editing by cytidine deamination in particular, may play a vital role in host defense against infectious agents, firstly by contributing to antibody diversification and therefore playing an important role in mature B cells for the production of secondary antibody repertoire, a crucial element of adaptive immunity, and secondly, by inhibiting the replication of retroviruses, a possible innate immune strategy (Cascalho 2004; Turelli and Trono 2005).

A recently discovered and well documented RNA editing enzyme, APOBEC3G, a member of the human APOBEC cytidine deaminase family, has been shown to have potent antiviral activity, providing intrinsic immunity to retroviral infection (Bishop et al. 2004; Harris and

Liddament 2004; Mangeat et al. 2004; Huthoff and Malim 2005) and is a novel candidate ARG (O'Brien and Nelson 2004).

The family of cytidine deaminase proteins in humans includes activation induced deaminase (AID) and APOBEC1 (with their gene locus on chromosome 12), APOBEC2 (on chromosome 6), and an array of seven APOBEC3 proteins, named APOBEC3A – 3H, whose genes flank each other on human chromosome 22. Additionally, the APOBEC4 subfamily was recently discovered with genes located on chromosome 1 (Rogozin et al. 2005).

The pattern of APOBEC3 protein expression is tissue specific, with APOBEC3G, -3F, and -3C expressed in spleen, ovary and testes, with little or no APOBEC3B being detectable in these tissues (Jarmuz et al. 2002). Additionally, APOBEC3G and -3F are expressed extensively in hematopoietic cell populations, including T cells, B cells, and in monocytes and DCs, as well as in hepatocytes (Jarmuz et al. 2002; Peng et al. 2007). APOBEC3A is also expressed in monocytes (Peng et al. 2007) and -3B and -3C are expressed in hepatocytes (Bonvin and Greeve 2007).

Along with APOBEC3G, APOBEC3B (Doehle et al. 2005), -3D (Hultquist et al. 2011), -3F (Zheng et al. 2004; Hultquist et al. 2011) and -3H (Dang et al. 2008; Ooms et al. 2010; Hultquist et al. 2011) have also been shown to have antiviral activity.

1.9.4.1 Discovery of APOBEC3G as an Antiviral Factor

Viruses that are *vif* deficient (Δ *vif*) have been shown to be non-infectious. Interestingly, this defective phenotype is cell type dependent, determined by the virus producing cell, and expressed by primary human T cells, the principal cell target for HIV-1 *in vivo*, and a limited number of cell lines, including HUT78 and CEM. These cells have been termed non-permissive. On the other hand, permissive cell lines (eg. HeLa, Jurkat, SupT1, CEM-SS and 293T cells) support the production of fully infectious Δ *vif* viruses (Gabuzda et al. 1992; von Schwedler et al. 1993; Madani and Kabat 1998).

This cell type specificity suggested that Vif function may be influenced by host factors. In cell fusion assays, the heterokaryon formed between non-permissive and permissive cells indicated that the non-permissive phenotype was dominant and suggested that non-permissive cells selectively express an antiviral factor that is suppressed by Vif (Simon et al. 1998). CEM15 (also known as APOBEC3G) was subsequently identified as the cellular gene whose anti-viral action is inhibited by Vif (Sheehy et al. 2002).

1.9.4.2 Structural Characteristics of APOBEC3 Proteins

APOBEC3 proteins have a distinctive domain structure comprising either one (APOBEC3A, -3C, -3H) or two cytidine deaminase domains (CDAs) (APOBEC3B, -3D, -3E, -3F, -3G) (Conticello et al., 2003; Jarmuz et al., 2002). However, generally only the C-terminal CDA is catalytically active while the N- terminal CDA is involved in nucleic acid binding and virus encapsidation (Navarro et. al., 2005) (Figure 1.13). The CDAs are characterized by a conserved zinc-finger binding motif His-X-Glu-X27-28-Pro-Cys-X2-Cys (Conticello et al., 2005; Jarmuzet al., 2002). This site catalyses the hydrolytic deamination of deoxycytidine, to

form a deoxyuridine. During the deamination reaction, the cysteines coordinate a single zinc ion and glutamate is involved in proton shuttling (Betts et al. 1994).

The 3-D structure of the 384 amino acid residue APOBEC3G protein has not yet been resolved by either X-ray or nuclear magnetic resonance. However, a high-resolution crystal structure of the carboxy-terminal deaminase domain of APOBEC3G has been determined. The C-terminal domain, extending from residue 198 – 384, is a well-defined core structure of five alpha helices (α1-α5) and five beta strands (β1-β5), with the zinc-coordinating catalytic domain surrounding α1 and α2 and β3 strands (Chen et al. 2008; Holden et al. 2008). APOBEC3G also has an inherent tendency to multimerise and forms homo-multimers (Jarmuz et al. 2002; Opi et al. 2006). Studies also suggest that APOBEC3G is packaged into viral particles as an oligomer bound to RNA (Burnett and Spearman 2007).

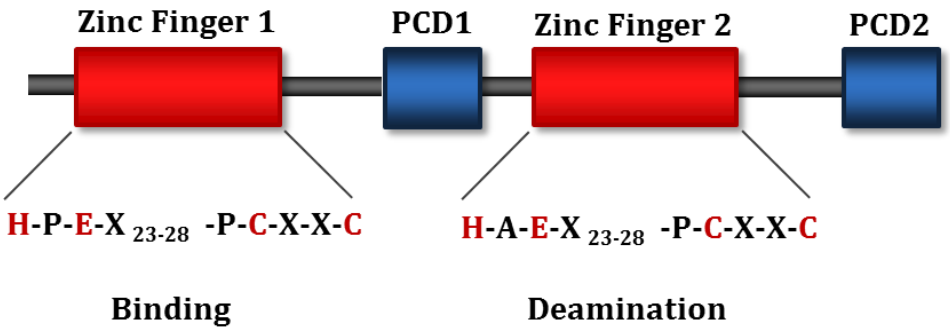


Figure 1.13 Domain Structure of APOBEC3G

Additionally, APOBEC3G is known to exist in two distinct cytoplasmic forms: an enzymatically active low molecular mass form (LMM) and an inactive high molecular mass complex (HMM). In activated cells and T cell lines, APOBEC3G is present as a mixture of both LMM and HMM APOBEC3G. In resting CD4+ T cells APOBEC3G exists

predominantly in the LMM conformation, where it has been shown to inflict strong post-entry restriction on HIV-1 infection (Chiu et al. 2005).

1.9.4.3 Viral Encapsidation of APOBEC3G

Studies have shown that APOBEC3G is incorporated into budding virions via an interaction with the viral Gag precursor protein through its NC component (Alce and Popik 2004; Cen et al. 2004; Schafer et al. 2004; Zennou et al. 2004; Khan et al. 2005). The interaction occurs through the N-terminal deaminase domain of APOBEC3G, which although catalytically inactive, is still crucial for RNA binding and encapsulation into virions (Navarro et al. 2005; Opi et al. 2006). It has also been shown that it is the N-terminal region of HIV-1 NC, vital for binding to RNA and mediating Gag-Gag oligomerisation, which plays a significant part in APOBEC3G binding and virion packaging (Luo et al. 2004).

Further, it has been suggested that 7SL cellular RNA may also facilitate the packaging of APOBEC3G into viruses. 7SL RNA is a component of signal recognition particles but is also an abundant constituent of HIV-1 virions (Onafuwa-Nuga et al. 2006; Khan et al. 2007; Wang et al. 2007). Therefore, APOBEC3G virion incorporation is mediated by APOBEC3G interactions with both viral and nonviral RNAs that are packaged into viral particles.

1.9.4.4 Anti-Viral Mechanism of APOBEC3G

Amongst the APOBEC3 proteins, APOBEC3G appears to have most potent antiviral activity *in vitro*, and most studies to date have focused on the antiviral activity of this isoform as compared to other APOBEC3 proteins.

1.9.4.4.1 Deamination of HIV-1 DNA by APOBEC3G

In the absence of Vif, APOBEC3G is encapsulated into budding virions (as described above) where it primarily directs extensive deamination of deoxycytidine (dC) to deoxyuridine (dU) on viral minus strand DNA, that is formed during the replication step of reverse transcription. These dU rich transcripts serve as templates for plus strand synthesis thus producing extensive deoxyguanosine (dG) to deoxyadenosine (dA) substitutions in plus strand DNA (Sheehy et al. 2002; Mangeat et al. 2003) (Figure 1.14). The consequence of dG to dA hypermutated viral DNA is the alteration of reading frames, introduction of premature stop codons or mutated, non-functional viral proteins (Lecossier et al. 2003; Mangeat et al. 2003; Zhang et al. 2003). Hypermutated viral sequences have been identified in long term non-progressors and associated with increased CD4 counts suggesting an *in vivo* role for cytidine deaminases in disease progression (Huang et al. 1998; Alexander et al. 2000; Land et al. 2008).

Studies show that APOBEC3G preferentially targets sequences in the dinucleotide context 5'-C<u>C</u>, distinguishing it from APOBEC3F which prefers the 5' T<u>C</u> signature dinucleotide (Lecossier et al. 2003; Beale et al. 2004; Liddament et al. 2004; Yu et al. 2004).

In addition, it has been shown that G to A mutations occurs with an increasing 5' to 3' graded frequency over the viral genome (Yu et al. 2004). Furthermore, recent studies actually identified twin gradients of APOBEC3G editing with one peak located just 5' to the cPPT within the *integrase* gene and the second peak 5' to the polypurine tract near the 3' LTR (3'PPT) (Figure 1.15). The region upstream of the primer binding site at the 5' end of the viral genome also appears to be hypersensitive to APOBEC3G editing (Suspene et al. 2006; Wurtzer et al. 2006). It has been suggested that the length of time that each nucleotide

remains single stranded during reverse transcription determines the frequency with which it is mutated (Yu et al. 2004).

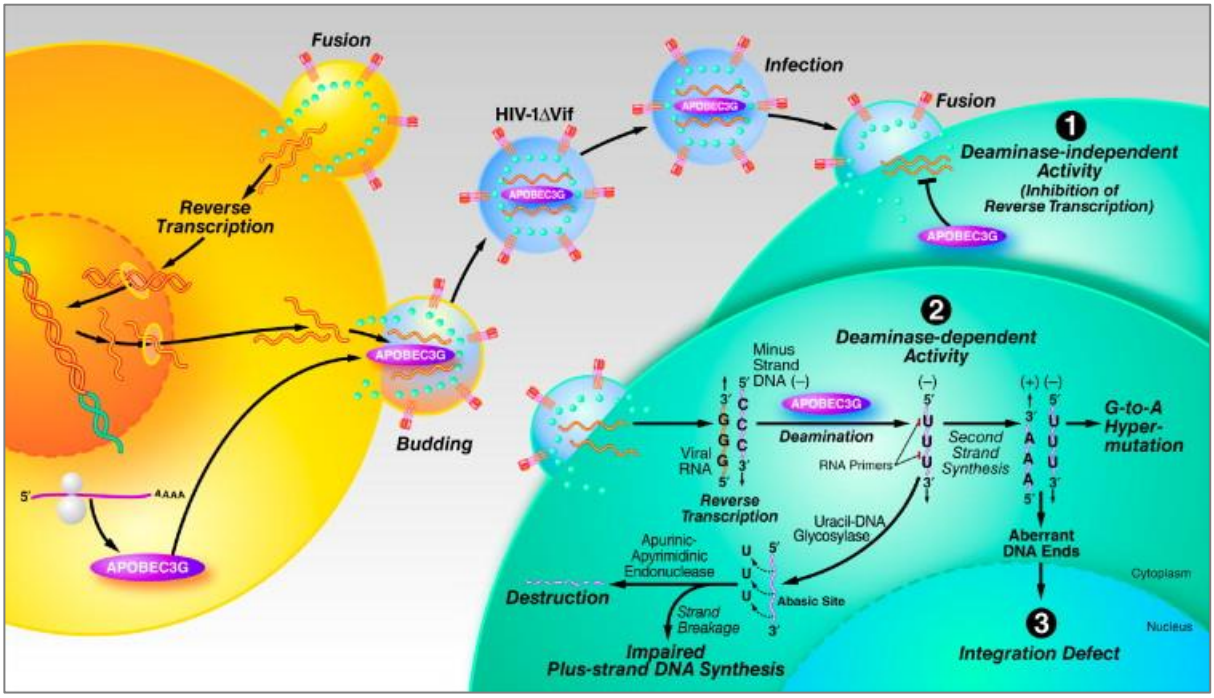


Figure 1.14 Antiviral activity of APOBEC3G. In the absence of Vif, APOBEC3G is effectively packaged into nascent virions where it inhibits viral replication through deaminase dependant and independent mechanisms (Source: (Greene et al. 2008), Novel targets for HIV therapy, Antiviral Research).

During the process of reverse transcription, plus strands of the viral DNA are synthesized as two distinct segments. The downstream segment is initiated at the cPPT and covers the 3' half of the genome, including the entire 3'LTR, while the upstream segment is initiated at the 3'PPT, covers the 5'LTR and the 5' half of the genome (Charneau et al. 1992; Charneau et al. 1994). The PPTs as well as the PBS at which reverse transcription is initiated therefore remain single stranded for the least amount time and are consequently less vulnerable to deamination by APOBEC3G. These sites also offer a degree of protection against

APOBEC3G immediately downstream of their location which decreases gradually further downstream (Wurtzer et al. 2006). Hence, the regions closest to the sites of plus strand initiation such as *gag*, are less prone to editing than *env*, which is located just upstream of the 3' PPT priming site (Yu et al. 2004).

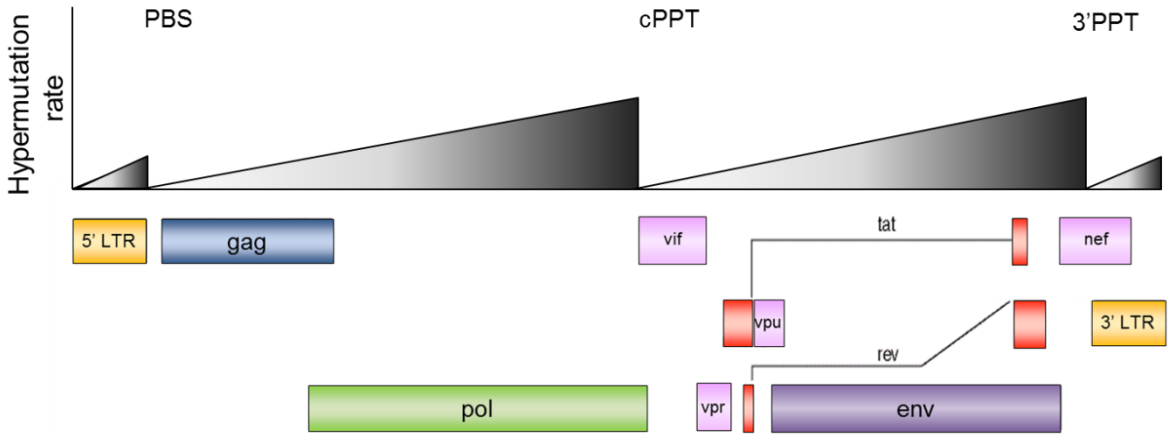


Figure 1.15 Gradients of APOBEC3G mediated hypermutation (Adapted from: (Gandhi et al. 2008), Role of APOBEC3G/F-Mediated Hypermutation in the Control of Human Immunodeficiency Virus Type 1 in Elite Suppressors, Journal of Virology).

In addition to the inhibitory effects of G to A hypermutation, dU's resulting from cytidine deamination, can trigger DNA degradation through the action of DNA repair pathways by recruiting cellular uracil-DNA glycosylases and other endonucleases, thus preventing plus strand viral synthesis (Klarmann et al. 2003; Newman et al. 2005; Yang et al. 2007).

1.9.4.4.2 Deaminase Independent Anti-viral Activity of APOBEC3G

Studies have suggested that APOBEC3G may also be able to inhibit viral propagation through pathways independent of its deaminase activity (Klarmann et al. 2003; Shindo et al. 2003; Li et al. 2004; Newman et al. 2005; Guo et al. 2007; Iwatani et al. 2007; Mbisa et al. 2007; Mbisa et al. 2010).

Expression of the N-terminal domain alone, which is primarily involved in RNA binding and encapsidation, was able to inhibit HIV-1 replication, showing that catalytic activity is not solely required for APOBEC3Gs antiviral function (Li et al. 2004). Additionally, N- and C-terminal deletion mutants of APOBEC3G that were unable to deaminate viral DNA were still able to inhibit viral replication by interfering with reverse transcription priming and extension (Li et al. 2007). APOBEC3G can disrupt priming of HIV-1 reverse transcription by interfering with $tRNA_3^{Lys}$ binding to viral RNA (Guo et al. 2007). In addition, APOBEC3G has been shown to restrict DNA strand transfer (Li et al. 2007; Mbisa et al. 2007), obstruct integration of viral DNA into the host genome (Luo et al. 2007; Mbisa et al. 2007; Mbisa et al. 2010) and inhibit elongation during reverse transcription (Iwatani et al. 2007; Bishop et al. 2008).

There remains uncertainty about whether deaminase activity is essential for the antiviral activity of APOBEC3G. Recent studies indicate that the catalytically inactive mutant form of APOBEC3G maintains very slight antiviral activity compared to APOBEC3G with complete deaminase activity (Newman et al. 2005). It therefore seems possible that both deaminase dependent as well as -independent mechanisms contribute synergistically to the overall antiviral activity of APOBEC3G.

1.9.4.5 Vif - Viral Armour against APOBEC3G

To counteract the potent mutagenic and inhibitory activities of APOBEC3G, HIV-1 has evolved a strategy in the form of the accessory protein Vif, to eradicate this host anti-viral factor. Vif physically interacts with and binds newly synthesised APOBEC3G by hijacking a cellular E3 ligase complex and targets the protein for degradation via the ubiquitin-dependant 26S proteosomal pathway (Conticello et al. 2003; Marin et al. 2003; Sheehy et al. 2003; Stopak et al. 2003; Yu et al. 2003; Mehle et al. 2004).

Studies show that overexpression of Vif results in a striking reduction of APOBEC3G in HIV-1 infected T cells. Additionally, APOBEC3G is a naturally stable protein with a half-life of >8 hrs, calculated in transiently infected HeLa or 293T cells and Vif is able to reduce APOBEC3Gs half-life to between 5 minutes and 4 hours (Conticello et al. 2003; Marin et al. 2003; Stopak et al. 2003; Mehle et al. 2004).

1.9.4.5.1 Sites of Interaction between Vif and APOBEC3G

Several domains within Vif, responsible for the critical interaction with the E3 ligase complex and APOBEC3G have been identified. Studies have shown that there are various regions in the N-terminal domain of Vif that are important for binding to APOBEC3G (Simon et al. 2005; Schrofelbauer et al. 2006; Mehle et al. 2007; Russell and Pathak 2007; Yamashita et al. 2008) (Figure 1.16). Studies have shown that residues in Vif spanning position 40 to 44 (YRHHY) (Schrofelbauer et al. 2006; Mehle et al. 2007; Russell and Pathak 2007; Yamashita et al. 2008) and the region between amino acids 52 to 72 (He et al. 2008) are essential for interaction with APOBEC3G. The amino acids I9, K22, E45 and N48 in Vif have also been shown to be necessary for binding APOBEC3G (Simon et al. 2005; Wichroski

et al. 2005). Additionally, tryptophans (Trp) in the N-terminal region of Vif, specifically Trp 5, 21, 38 and 89, have been found to be important for APOBEC3G binding (Tian et al. 2006). The highly conserved SLQ SOCS-box motif spanning residues 145 to 154 near the C-terminus of Vif was found to mediate binding of Vif to Elongin C, a known component of the E3 ubiquitin ligase complexes (Yu et al. 2003; Mehle et al. 2004; Mehle et al. 2004; Yu et al. 2004). Further, the highly conserved HCCH zinc binding motif, situated upstream of the BC box, mediates interaction with Cullin-5 (Luo et al. 2004; Mehle et al. 2004; Yu et al. 2004).

Both the SLQ and HCCH domains facilitate the recruitment of an ubiquitin-ligase (E3) complex containing elongin –B and –C, cullin-5 and Rbx1 (Yu et al. 2003; Mehle et al. 2004; Yu et al. 2004; Mehle et al. 2006). Thus, Vif forms a bridge between the cullin-5/elonginB/elonginC/Rbx1 complexes and APOBEC3G and accelerates polyubiquitylation of this host defence factor targeting it for destruction by the 26S proteasome (Conticello et al. 2003; Marin et al. 2003; Sheehy et al. 2003; Yu et al. 2003; Yu et al. 2004).

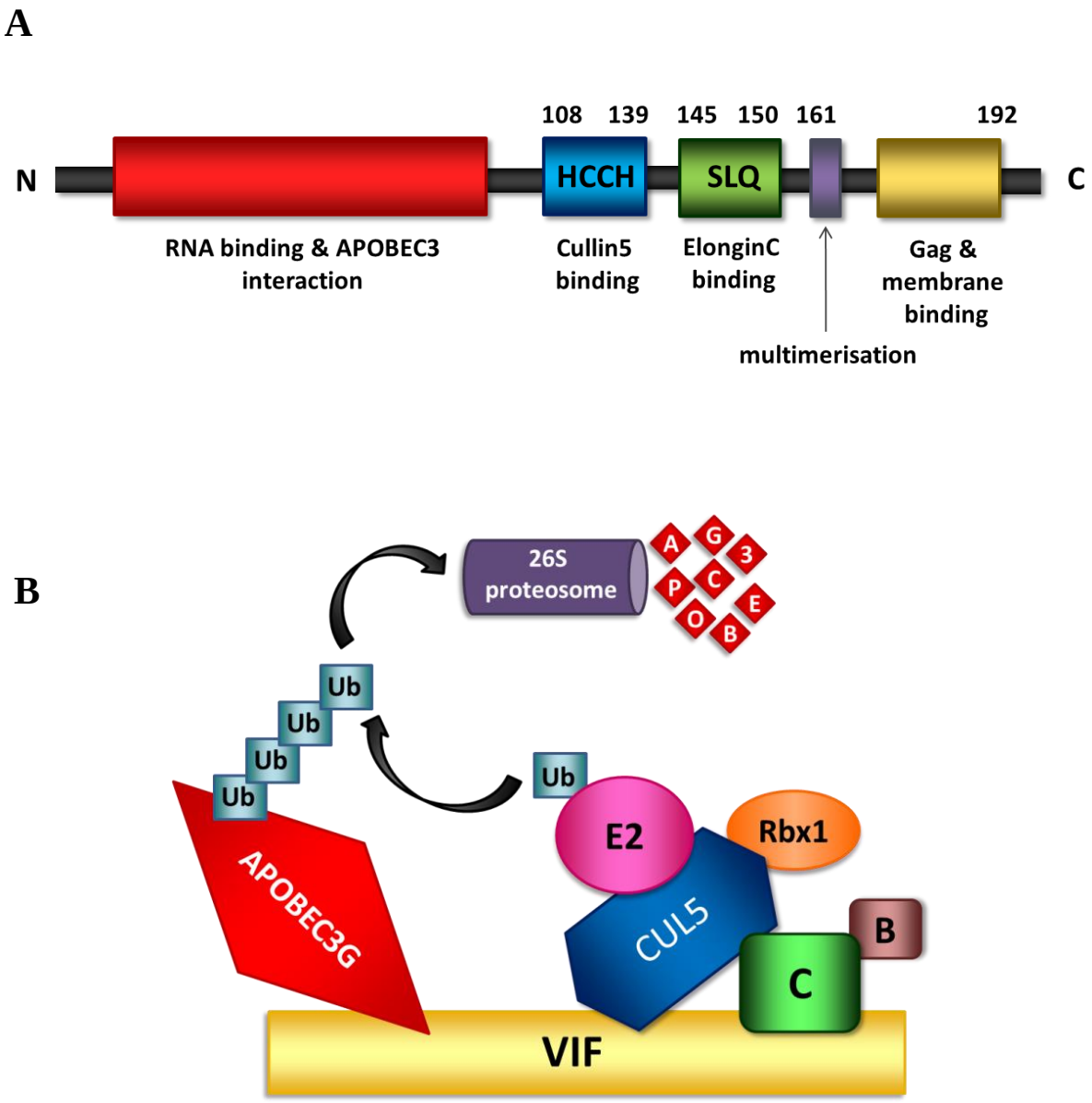


Figure 1.16 **[A] Representation of the functional domains of Vif.** (Adapted from: (Barraud et al. 2008), Advances in the structural understanding of Vif proteins, Current HIV Research) [**B] Vif interaction sites.** VIF targets APOBEC3G for proteosomal degradation by functioning as a bridge that specifically recruits APOBEC3G into an E3 ubiquitin-ligase complex for ubiquitination and subsequent degradation via the 26S proteasome (Adapted from: (Lv et al. 2007), Three-dimensional structure of HIV-1 VIF constructed by comparative modeling and the function characterization analyzed by molecular dynamics simulation, Organic & Biomolecular Chemistry; and (Harris and Liddament 2004), The mechanism of Vif-dependent APOBEC3G degradation, Nature Reviews Immunology).

Studies show that it is also the N-terminal region of APOBEC3G that is involved in Vif binding. Amino acids at positions 128, 129 and 130 and the region spanning residues 54 to 124 in APOBEC3G have been shown to be responsible for its interaction with Vif (Li et al. 2004; Huthoff and Malim 2007).

Additionally, the interaction between Vif and APOBEC3G is species-specific. HIV-1 Vif blocks the inhibitory activity of human APOBEC3G while African Green Monkey (agm) SIV Vif inhibits agmAPOBEC3G. Interestingly, SIV Vif from Rhesus Macaque (rh) can inhibit human, agm and rhAPOBEC3G (Mariani et al. 2003). Residue D128 in the N-terminal region of huAPOBEC3G has been shown to be the determining factor in this species-specific interaction (Bogerd et al. 2004; Mangeat et al. 2004; Schrofelbauer et al. 2004; Xu et al. 2004; Schrofelbauer et al. 2006).

1.9.4.6 APOBEC3F joins APOBEC3G in the fight against HIV-1

Studies have shown that APOBEC3G is joined by APOBEC3F as an anti-viral factor in the fight against HIV-1 infection. It has been shown that APOBEC3F is often co-expressed with APOBEC3G in nonpermissive cells (Liddament et al. 2004; Wiegand et. al. 2004; Zheng et al. 2004). APOBEC3G and APOBEC3F have approximately 49% identity in their amino acid sequences however they have different dinucleotide specificities in editing substrates, where APOBEC3G prefers 5'-C<u>C</u> dinucleotides while APOBEC3F prefers 5'-T<u>C</u> dinucleotides (the underline nucleotide being target for deamination) (Beale et al. 2004; Bishop et al. 2004; Liddament et al. 2004; Wiegand et al. 2004). Most studies to date have shown that APOBEC3G is a significantly more powerful inhibitor of HIV-1 replication and causes a greater frequency of G-to-A mutations than APOBEC3F (Bishop et al. 2004; Liddament et al. 2004; Wiegand et al. 2004; Zennou et al. 2006). However, APOBEC3F

demonstrates a more pronounced deamination-independent effect on HIV-1 replication compared to APOBEC3G (Holmes et al. 2007; Mbisa et al. 2007). Additionally they also differ in their dependency on HIV-1 genomic or non-genomic RNAs for virion incorporation (Simon et al. 2005). Despite these differences HIV-1 degrades both APOBEC3G and APOBEC3F via the same pathway to eliminate its anti-viral activity. HIV-1 Vif is able to bind both proteins and targets them for proteosomal degradation via ubiquitin-dependant 26S proteosomal pathway ((Conticello et al. 2003; Marin et al. 2003; Sheehy et al. 2003; Stopak et al. 2003; Yu et al. 2003; Mehle et al. 2004Liu et al 2007). Even with the evidence of APOBEC3Fs anti-viral activity this host restriction factor has not been as extensively studied as APOBEC3G, particularly in the context of HIV-1 subtype C infection.

1.9.4.7 Other Exogenous and Endogenous APOBEC3 Targets

In addition to providing intrinsic immunity against HIV-1 infection, the spectrum of activity of APOBEC3 proteins extends to other exogenous retroviruses and endogenous mobile retroelements.

Studies have shown that APOBEC3G can block lentiviruses such as SIV, and EIAV, as well as the gammaretrovirus murine leukemia virus. (Harris et al. 2003; Mangeat et al. 2003). It has also been demonstrated that APOBEC3G is able to inhibit replication of hepatitis B virus by extensive editing of its DNA strands (Suspene et al. 2005; Turelli and Trono 2005). APOBEC3G is also able to inhibit human T-lymphotropic virus type I (HTLV-1) via its deaminase independent antiviral activity (Sasada et al. 2005). Editing by APOBEC3 proteins therefore constitutes an obstacle to cross-species transmission of numerous retroviral pathogens and retroelements.

Additionally, APOBEC3G also functions in the inhibition of endogenous retroelements. The human genome contains a large number of mobile genetic elements specifically, DNA transposons and retrotransposons. Retrotransposons account for approximately one-third of the genome and are mobile DNA sequences that are able to integrate into the genome of host cells. In a process called retrotransposition, new copies are generated by coupled transcription and reverse transcription followed by insertion of the new DNA into different sites in the genome. Endogenous retrotransposons include elements that bear LTRs, such as endogenous retroviruses, and non-LTR retroelements, namely long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs). Retrotransposons play an essential role in genome remodelling, evolution and speciation and may facilitate adaptation. Nevertheless, they can also be damaging to the host genome and represent genetic threats, as they are able to disrupt genes by insertional mutagenesis, enhance translocations and other rearrangements by recombination, create aberrant cellular transcripts from their promoter, and exert antisense effects (Prak and Kazazian 2000; Kazazian 2004).

Primates have unusually low levels of retrotransposon activity which interestingly corresponds with the expansion of the APOBEC3 gene cluster from a single gene in mice to seven genes in primates (Jarmuz et al. 2002). APOBEC3 genes have been subjected to strong positive selective pressure throughout the history of primate evolution for more than 30 million years (Sawyer et al. 2004; Zhang and Webb 2004). This selection appears older than modern lentiviruses suggesting that this gene family expanded in order to curtail the genomic instability caused by endogenous retroelements (Sawyer et al. 2004).

1.10 The Tortuous Vif-APOBEC3G Interplay

The interplay between HIV-1 Vif and APOBEC3G appears to be a complex one in the human host. The interaction between the virus and host factor is of particular interest and there is considerable evidence that suggests that the dynamics of this interaction may greatly influence disease progression.

Several studies show a presence of APOBEC3 footprints in HIV-sequences isolated from treated and untreated chronically and early-infected patients (Rose and Korber 2000; Janini et al. 2001; Kieffer et al. 2005; Pace et al. 2006; Wood et al. 2009), infected infants (Koulinska et al. 2003; Amoedo et al. 2011; De Maio et al. 2012), and elite controllers (Wei et al. 2004; Simon et al. 2005). Studies that have attempted to examine the association between the extent of hypermutation in HIV-1 and clinical markers of disease progression, specifically viral loads and CD4 counts, have presented conflicting findings (Gandhi et al. 2008; Land et al. 2008; Ulenga et al. 2008; Piantadosi et al. 2009; Kourteva et al. 2012). Additionally, some studies demonstrate an inverse correlation between APOBEC3G expression (Jin et al. 2005; Ulenga et al. 2008; Vazquez-Perez et al. 2009; Kourteva et al. 2012) and viraemia while others found no such correlation (Cho et al. 2006; Amoedo et al. 2011). It has also been demonstrated *in vitro* that APOBEC3G expression is upregulated upon CD4+ T cell activation (Refsland et al. 2010) and APOBEC3G expression decreases as HIV-1 disease progresses (Vazquez-Perez et al. 2009).

To further investigate the association between APOBEC3G and disease progression, studies have attempted to examine the influence of host and virus variability at the Vif-APOBEC3 system on disease outcome. Several polymorphisms within APOBEC3G have been identified with only a few being associated with increased risk of infection and disease progression or

hypermutation of viral genomes (An et al. 2004; Do et al. 2005; Pace et al. 2006; Valcke et al. 2006; De Maio et al. 2011). The single nucleotide polymorphisms of particular interest are the *H186R* (*rs8177832*) mutation in exon 4 identified in African Americans (An et al. 2004), a 5' extragenic mutation (*rs5757463*) (Bizinoto et al. 2011), and *C40693T* (*rs17496018*) in intron4 (Valcke et al. 2006), all of which were found to be associated with high viral loads and decreased CD4+ T cell counts or accelerated progression to AIDS-defining conditions. Interestingly, APOBEC3G polymorphisms may be able to influence HIV-1 adaptation and evolution of specific Vif variants, as shown by (De Maio et al. 2011) where the Vif variant *E45D* associated with the APOBEC3G *C40693T* minor allele.

Natural variants of Vif have been frequently identified; however, there is uncertainty about how these particular Vif variants may contribute to viral pathogenicity (Zhang et al. 1997; Hassaine et al. 2000; Yamada and Iwamoto 2000; Rangel et al. 2009; Fourati et al. 2010). Vif variants may have an enhanced or diminished capacity to counteract APOBEC3G. (Simon et al. 2005) showed that natural variation in subtype B Vif proteins results in defective or partially active Vif variants. Vif variants that have increased or partial activity against APOBEC3G and may possibly contribute to HIV-1 genome diversification by allowing sub lethal levels of APOBEC3G editing of the viral genome during reverse transcription thus facilitating the emergence of immune escape or drug resistant viral forms. Therefore, an increase or decrease in Vif activity may facilitate accelerated disease progression (Simon et al. 2005; Munk et al. 2012).

Further, degradation of APOBEC3G by Vif may also be regulated by genetic polymorphisms within the components of the E3 ubiquitin ligase complex such as Cullin 5 (An et al. 2007; De Maio et al. 2012).

1.10.1 Harnessing the Vif-APOBEC3G Interaction as a Therapeutic Target

There seems to be a delicate balance between Vif and APOBEC3, which if slightly disturbed may be a key determinant of the disease outcome in retroviral infection. Moreover it might even be possible to take advantage of this balance therapeutically as a potential new target for anti-HIV drugs.

A Vif inhibitor may be able to directly prevent Vif from degrading APOBEC3G allowing the host factor to freely restrict infection. However, as with current anti-HIV-1 therapies, this method might also eventually succumb to viral escape mutants by generating Vif variants that would no longer be bound by the inhibitors. Nevertheless, if used in conjunction with other anti-retroviral drugs, a Vif inhibitor would complement the current pharmaceutical anti-HIV-1 cache. An alternative approach would be to facilitate APOBEC3G mediated viral restriction by using a 'molecular shield' that would shelter APOBEC3G from Vif but at the same time would not hinder its antiretroviral activities. This approach might be more advantageous over Vif inhibitors, as the molecular shield would be directed to a somewhat more stable cellular target (Harris and Liddament 2004). One may even envision a scenario in which APOBEC3G antiviral activity is enhanced, accompanied by its protection from Vif-mediated destruction.

Several groups have already attempted to target the Vif-APOBEC3 interaction with both Vif inhibitors as well as APOBEC3 shields. The first potential Vif inhibitor, the RN 18 molecule was shown to antagonise Vif function and inhibit HIV-1 replication. RN 18 enhanced APOBEC3G incorporation into virions and boosted cytidine deamination of the viral genome (Nathans et al. 2008). The IMB-26 and IMB-35 molecules were shown to bind directly to the APOBEC3G and consequently suppressed Vif-APOBEC3G interaction, therefore protecting

APOBEC3G from Vif-mediated degradation (Cen et al. 2010). The SN-2 compound was also shown to stabilise APOBEC3G in the presence of HIV-1 Vif (Ejima et al. 2011). Additionally, Chim3, an engineered Vif-derived molecule, was shown to preserve the antiviral function of APOBEC3G and impaired the early steps of HIV-1 life cycle (Porcellini et al. 2009; Porcellini et al. 2010).

Additionally, it was recently shown that IFNα potently increases the amount of APOBEC3G mRNA and protein expressed by human primary monocyte-derived macrophages, and this increase correlated with a decrease in viral replication (Stopak et al. 2007). The induction of APOBEC3G expression by IFNα in macrophages possibly tilts the delicate balance between APOBEC3G and Vif in favour of APOBEC3G, and represents a mechanism by which IFNα mediates its anti-HIV effects in macrophages (Peng et al. 2006). This novel tactic by which IFN-α mediates its antiviral activity suggests the possibility of a therapeutic intervention to render the host non-permissive for viral replication.

1.11 Study Objectives and Hypotheses

Restriction factors and viral-host interactions in the HIV-1 subtype C setting are understudied and knowledge is limited, specifically in South Africa which has the highest HIV prevalence in the world. This study was carried out in a South African based cohort with the purpose of investigating the antiviral effects of APOBEC3G in HIV-1 subtype C *in vivo*. We hypothesised that APOBEC3G, an important intrinsic immune factor plays an important role *in vivo*. Specifically, we hypothesised that expression levels of APOBEC3G and genetic polymorphisms may affect susceptibility or disease outcome in early HIV-1 infection. We also hypothesised that the extent of APOBEC induced G-to-A hypermutation within the viral genome may be influenced by expression levels and/or genetic polymorphisms within *APOBEC3G*. We will also test the hypothesis that HIV-1 Vif anti-APOBEC3G activity and evolution is driven by the spectrum of APOBEC3G variants.

1.12 The Aims of the Study were to:

1. To determine the mRNA levels of APOBEC3G in peripheral blood mononuclear cells in HIV-uninfected individuals and during acute HIV-1C infection.

2. To identify single nucleotide polymorphisms within APOBEC3G and to describe the extent of genetic variation within the gene in the acute infection cohort and investigate if the genetic variants of APOBEC3G may affect the course of acute and early HIV-1 infection.

3. To understand the mechanisms of APOBEC3G polymorphisms in driving viral evolution by cloning and sequencing HIV-1 *vif* from patients whose APOBEC3G genotypes are known.

4. To functionally test *vif* alleles for their ability to counteract the action of APOBEC3G variants and to determine whether natural *vif* variants result in altered anti-APOBEC3G activity in the HIV-1 subtype C setting.

5. To study the extent and frequency of APOBEC3G induced G-to-A hypermutation in HIV-1 *env* gene and to determine if there is an association between hypermutations and APOBEC3G variants.

CHAPTER TWO

Materials and Methods

2.1 Study Participants

The CAPRISA 002 Acute Infection Study is an observational natural history study of HIV-1 subtype C infection developed in Durban, KwaZulu-Natal, South Africa in 2004 and was established as a cohort at high risk of HIV infection (van Loggerenberg et al. 2008). The cohort was developed to study the impact of host, immune and viral factors during the phases of acute and early HIV-1 subtype C infection. Seven hundred and seventy five women who were at least 18 years old were screened between August 2004 and May 2005 for participation in the cohort and HIV-1 negative women, considered as high-risk for HIV infection, based on self-identification as sex workers or self-report of more than three sexual partners in the previous 3 months. The process of screening to identify HIV –ve women included voluntary counselling and testing (VCT) and collection of a blood sample for rapid HIV antibody testing (Determine: Abbott Laboratories, Tokyo, Japan) and a urine sample for a pregnancy test. Of the 775 women screened, 313 were HIV negative and 245 of them were eligible for participation in the HIV negative cohort. The HIV negative women who were excluded at screening either had less than three sexual partners in the previous 3 months, or were pregnant at the time of screening or had planned to relocate or were younger than 18 years. Those who were diagnosed as HIV positive, by two positive antibody tests (Determine (Abbott Laboratories, Tokyo, Japan) and Capillus (Trinity Biotech, Jamestown, NY, USA)), were referred for HIV follow-up care.

Thus, 245 females at high risk for HIV infection were enrolled into Phase 1 of the study and underwent baseline evaluation (Figure 2.1). Thereafter, participants were followed monthly

with a series of assessments including a clinical evaluation, two HIV antibody rapid tests, Determine (Abbott Laboratories, Tokyo, Japan) and Capillus (Trinity Biotech, Jamestown, NY, USA), HIV enzyme immunoassay (EIA) test (BEP 2000; Dade Behring, Marburg, Germany) and pooled HIV-1 RNA testing (Ampliscreen v1.5, Roche Diagnostics, Rotkreuz, Switzerland). A urine dipstick was also performed if pregnancy was suspected and STI screening was performed on a 6 monthly basis. Samples that were HIV-1 RNA positive after pooled PCR testing were subsequently confirmed by a quantitative RNA (Amplicor v2.0, Roche Diagnostics) and HIV enzyme immunoassay (EIA) test.

The diagnosis of acute HIV infection was made by either the detection of HIV antibodies within 3 months of a previously negative result or, if HIV-1 antibodies were absent, by sign of HIV-1 viral replication indicated by a HIV RNA PCR positive result. Participants with acute HIV infection were subsequently enrolled into Phase 2 of the study together with seroconverters from the Vulindlela and HIV Trials Prevention Network – 035 (HTPN-035) seroincidence cohorts.

Sixty one participants were recruited into Phase 2 of the study. Their estimated time of seroconversion was determined as the midpoint between the last antibody negative and first antibody positive test or 14 days before the participant was PCR positive and antibody negative. During Phase 2, acutely infected participants were followed weekly for 3 weeks and thereafter fortnightly until 3 months post-infection. Participants subsequently entered Phase 3 of the study where they underwent monthly follow up until 12 months post-infection. In Phase 4 participants were followed quarterly for a maximum of 5.5 years.

2.1.1 Ethics

The present study forms a component of the research protocol for which ethical approval was obtained from the University of KwaZulu-Natal's Biomedical Research Ethics Committee (E013/04). All participants provided written informed consent. All women who were screened for participation received HIV pre- and post-test counseling, risk reduction counseling, condoms, access to clinical care and treatment for sexually transmitted diseases. Additionally, participants who became HIV infected in the duration of the study were referred to the CAPRISA Antiretroviral Treatment Programme, where they were offered continued care and HIV treatment when clinically eligible.

2.2 Sample Collection, measurement of CD4 counts and Plasma Viral Load

Blood samples were collected into acid citrate dextrose tubes (Becton Dickinson Diagnostic Systems, Baltimore, MD, USA) and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Histopaque (Sigma, St Louis, MO) density gradient centrifugation and frozen until use. Blood for viral load quantification and CD4 counting was collected in EDTA anticoagulated vacutainer tubes. Viral load was determined using the automated COBAS AMPLICOR HIV-1 Monitor Test v1.5 (Roche Diagnostics). CD4 cells were enumerated by using the Multitest kit (CD4/CD3/CD8/CD45) on a four-parameter FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA).

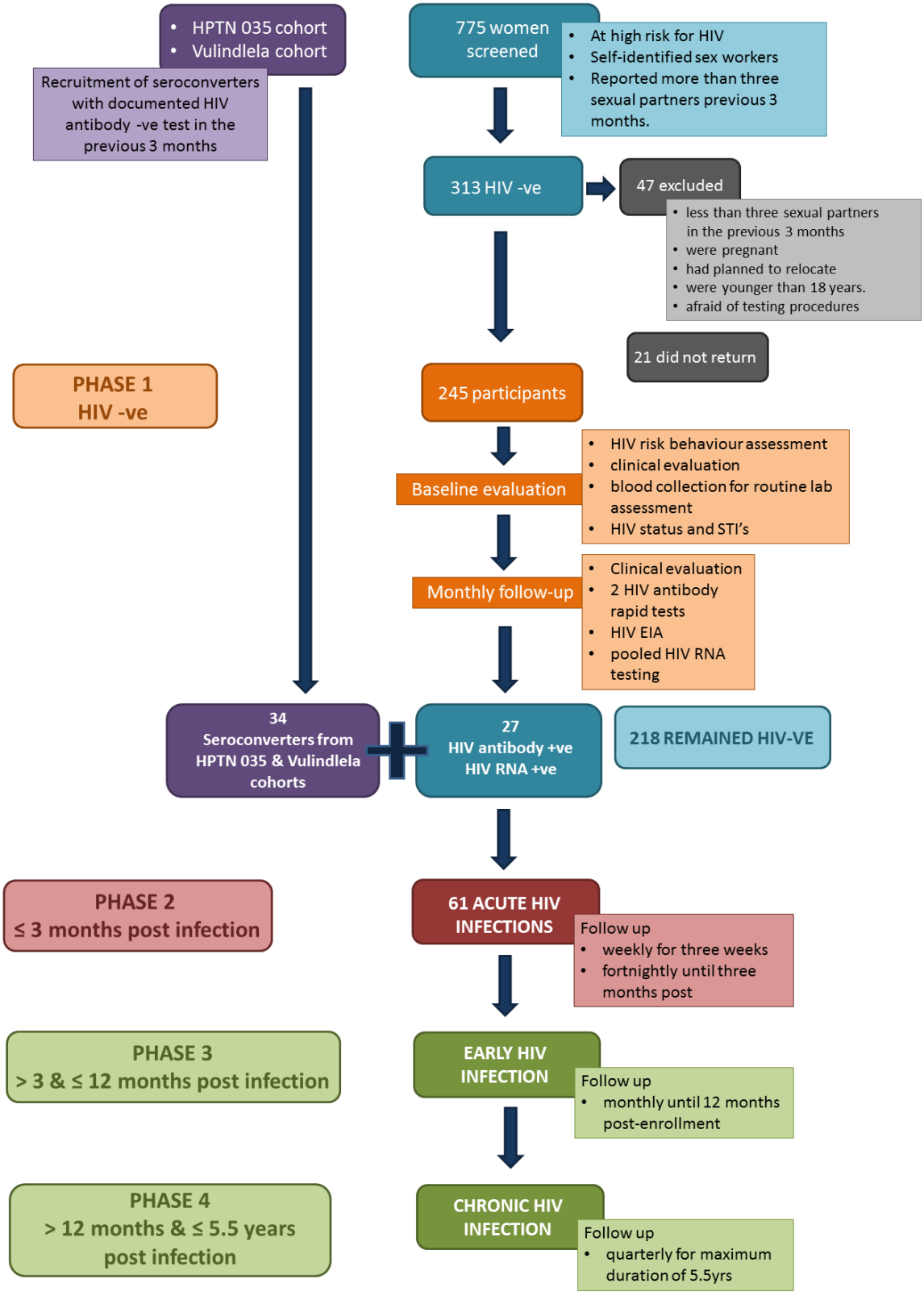


Figure 2.1 Schematic diagram of the CAPRISA Acute Infection Study 002

2.3 Quantification of *APOBEC3G* mRNA Expression by Real Time PCR

2.3.1 RNA Isolation

The accuracy of real time PCR results is heavily dependent on the preparation and isolation of high quality RNA. RNA was isolated from cryopreserved PBMCs using Trizol reagent (Invitrogen, Life Sciences, California, USA) according to the manufacturer's protocol. The quantity and quality of extracted RNA was determined by measuring the optical density at 260 nm and 280 nm. All RNA samples had a 260nm/280nm ratio > 1.8 and < 2.0.

2.3.2 Synthesis of cDNA

Total RNA was reverse transcribed to generate cDNA using the Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions (Table 2.1). The kit consisted of gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase Enzyme, Quantiscript RT Buffer, RT Primer Mix, RNase-Free Water. For a total reaction of 20 µl the following standard volumes were used:

Table 2.1 Components of cDNA Synthesis for *APOBEC3G*

Reagent (µl)	Volume
gDNA Wipeout Buffer	2.0
Template RNA	5.0
Quantiscript Reverse Transcriptase	1.0
Quantiscript RT Buffer	4.0
RT Primer Mix	1.0
RNase-Free Water	7.0
Total Volume	20.0

The reaction was carried out in 200 µl thin walled reaction tubes using a GeneAmp 9700 PCR System (Applied Biosystems (ABI), Lifesciences, California, USA). Template RNA and

gDNA Wipeout Buffer were incubated for 2 min at 42°C and placed immediately on ice. This was then added to the reverse transcription mix and the reaction was incubated at 42^{o}C for 15 minutes and 95^{o}C for 3 minutes. The cDNA was then stored at -20^{o}C until used.

2.3.3 Real Time PCR

APOBEC3G mRNA expression levels were quantified by real-time PCR using SYBR Green chemistry (Roche Applied Science, Mannheim, Germany). Target specific primers were used to amplify *APOBEC3G* and were previously designed and described by (Jin et al. 2005) (Table 2.2).

The method of relative quantification was used where the concentration of the target gene is expressed in relation to the concentration of a reference (housekeeping) gene. The housekeeping gene is used to normalize for variations in cell count or differences in nucleic acid extraction. The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (NM_002046) was used in this study and was amplified with specific primers highlighted in Table 2.2. All primers were synthesized by Roche Applied Sciences.

Table 2.2 Oligonucleotide Sequences for the *APOBEC3G* and *GAPDH* genes

Primer Name	Sequence	Position
APOBEC3G (Fwd)	5’ CGCAGCCTGTGTCAGAAAAG 3’	637 to 657
APOBEC3G (Rev)	5’ CCAACAGTGCTGAAATTCGTCATA 3’	714 to 691
GAPDH (Fwd)	5’ AAGGTCGGAGTCAACGGATT 3’	115 to 134
GAPDH (Rev)	5’ CTCCTGGAAGATGGTGATGG 3’	339 to 320

Primers were optimised and the PCR reactions used for *APOBEC3G* and *GAPDH* amplification was as follows in Table 2.3.

Table 2.3 Components of PCR reaction for amplification of APOBEC3G and GAPDH

Reagent (µl)	***APOBEC3G***	***GAPDH***
$MgCl_2$ (25mM)	1.26	1.47
10X LightCycler FastStart DNA Master SYBR Green I	1.00	1.00
Primer (Forward) (10uM)	0.20	0.50
Primer (Reverse) (10uM)	0.20	0.50
cDNA Template	2.00	2.00
Water	5.34	4.53
Total Volume	10.0	10.0

All reactions were performed on the LightCycler Instrument Version 1.5 (Roche Applied Science). The PCR cycling conditions are described in Table 2.4.

Table 2.4 PCR cycling conditions for *APOBEC3G* and *GAPDH*

	APOBEC3G			*GAPDH*		
Step	**Temperature ^{o}C**	**Time (min)**	**# of cycles**	**Temperature (^{o}C)**	**Time (min)**	**# of cycles**
Initialisation	95	10:00	1	95	10:00	1
Denaturation	95	0.05		95	0.05	
Annealing	55	0:15	40	65	0:15	40
Extension	72	0:05		72	0:05	
Hold	4	∞		4	∞	

Quantification of gene expression by the LightCycler is based on a standard curve for each gene. Standard curves were generated for *APOBEC3G* and *GAPDH* from 10 fold serial dilutions of cDNA of known concentrations. The standard curves were imported into each PCR run and was used by the LightCycler Software to quantify each gene in a sample by extrapolation. All samples and standards were run in duplicate and the average value was used to compute *APOBEC3G* and *GAPDH* copy number. The relative expression levels of *APOBEC3G* to *GAPDH* in each sample were determined by dividing the absolute concentration of the target gene (*APOBEC3G*) by the absolute concentration of the housekeeping gene (*GAPDH*). The resulting target/reference ratio expressed the amount of *APOBEC3G* normalized to the level of *GAPDH* within each sample.

2.3.4 Isolation of CD4+ T cells

As an additional exploratory investigation we also assessed *APOBEC3G* expression in cells primarily targeted by HIV-1 that is CD4+ T cells as *APOBEC3G* expression levels may vary in the different cell types that constitute PBMCs. For this investigation we studied 8 anonymous healthy donors and 8 HIV-1 chronically infected patients from the Sinikithemba Cohort based in Durban, South Africa. Ethical approval for use of study samples was obtained from the University of KwaZulu-Natal's Biomedical Research Ethics Committee (E036/06).

PBMCs were isolated from whole blood by the Histopaque-1077 (Sigma Aldrich, St. Loius, Missouri) density gradient medium. Thereafter, cells were counted by the Guava Viacount assay (EMD Millipore, Merck, Darmstadt, Germany) and 10 million cells were used for subsequent cell separation assays. CD4+ T cells were isolated from total PBMCs using the MACS CD4+ T Cell Isolation Kit II and MS columns (Miltenyi Biotec, California, USA). CD4+ T cells were isolated by the negative selection method where non-CD4+ T cells were depleted. The non-CD4+ T cells were magnetically labeled with a primary labeling cocktail of biotin-conjugated monoclonal antibodies as well as with a secondary labeling reagent of anti-biotin monoclonal antibodies conjugated to microbeads. The magnetically labeled non-CD4+ T cells were depleted by retaining them on a MACS column in a magnetic field while the unlabeled CD4+ T cells pass through the column.

Purity of the isolated CD4+ T cells was evaluated by flow cytometry on the LSR II Flow Cytometer (BD, Biosciences, California, USA). Aliquots of the total PBMC (that is before CD4 isolation) as well as the cell fraction were stained with flourochrome-conjugated antibodies against CD4 (CD4 FITC-A) and against the T cell marker CD3 (CD3 PerCP-A).

Thereafter, RNA was extracted from the isolated CD4+ T cells and processed for quantification of *APOBEC3G* mRNA levels by real time PCR as described in the previous section.

2.4 Detection of *APOBEC3G* polymorphisms

A subset of HIV-ve and HIV+ve samples was selected for resequencing to screen for and identify single nucleotide polymorphisms (SNPs) within *APOBEC3G*.

APOBEC3G amplification and sequencing primers, and protocols that were used have been previously published (An et al. 2004). The primers covered the putative 5' regulatory region, eight exons, exon-intron junctions, intron 1 and the 3' untranslated region of the *APOBEC3G* gene (GenBank sequences AL022318 and AL078641). These regions were amplified separately. The components of each 25µl PCR reaction and PCR cycling conditions are shown in Table 2.5 and Table 2.6 respectively.

Table 2.5 Components of PCR reaction for amplification and sequencing of *APOBEC3G*

Reagent	
$MgCl_2$ (25mM)	1.5 - 2.5mM
10X PCR Buffer	1.50 µl
Taq Gold (ABI)	0.15 µl
dNTPs	2.4 mM
Primer (Forward) (100uM)	5 µM
Primer (Reverse) (100uM)	5 µM
DNA	12.5 ng

Table 2.6 **PCR cycling conditions for amplification of *APOBEC3G***

Step	Temperature (^oC)	Time (min)	#of cycles
Initialisation	95	10:00	1
Denaturation	94	0.30	
Annealing	60	0:30	35
Extension	72	0:45	
Final Extension	72	10:00	1
Hold	4	∞	

For optimal sequencing, PCR products were purified to remove excess dNTPs and primers. The PCR products were purified by addition of Exonuclease and Shrimp Alkaline Phospatase (SAP) (Amersham Biosciences, GE Healthcare, Buckinghamshire, England) and incubation at 37^{o}C for 25 minutes and 72^{o}C for 15 minutes.

The purified PCR products were subsequently sequenced using gene specific overlapping primers (Table 2.7) and a BigDye Terminator v3.1 Cycle Sequencing Kit (ABI) (Table 2.8). Extension products were purified using Sephadex G50 (Amersham, Pharmacia) to remove unincorporated dye terminators in sequencing reactions which would otherwise obscure sequencing data. Sequences were resolved on the 3730xl DNA Analyzer (ABI/Hitachi).

Table 2.7 ***APOBEC3G*** **amplification and sequencing primers, regions amplified and Mg++ concentration used in PCR**

	Amplified Regions	Forward primer	Reverse primer	Mg++ (mM)
CEM15-Ex1	Exon 1, intron 1, exon 2, intron 2	5’ gacggaatttcgctcttgtc 3’	5’ gggagagaaggacacactgg 3’	1.5
CEM15-Ex3	Exon 3, Intron 3, exon 4, intron 4	5’ ggtgagaagtgggaggttca 3’	5’ gacctggtctggaacagagg 3’	1.5
CEM15-Ex4	Intron 4, exon 5	5’ aggtttggaggctctagcaa 3’	5’ cactgaagccgaagtctcc 3’	1.5
CEM15-Ex5	Exon 5, Intron 5, exon 6, intron 6	5’ cctcatggcttgctttcttt 3’	5’ gtcgaccccaaagtcaggt 3’	1.5
CEM15-Ex6	Intron 6, exon 7, intron 7,	5’ gctggaagtggaagcagaac 3’	5’ agtgacaatgatcggagagga 3’	1.5
CEM15-In12	Intron 2	5’ ctgcgtgggtcacgtaca 3’	5’ gcctcgtgtgtgaattctagc 3’	1.5
CEM15-In13	Intron 2, exon 3, intron 3	5’ gctgggaaaacttccaaactc 3’	5’ tttccctccatccccctgt 3’	1.5
CEM15-pm1	5’ region, exon 1	5’ acgcctggccatttactct 3’	5’ aagtgaggcttcatccttgg 3’	2.5
CEM15-pm2	5’ region	5’ ctcctcctgtagcctgttcaa 3’	5’ gacagggagggagagtaa 3’	1.5
CEM15-pm3	5’ region	5’ ggcggtggaaagttacagtc 3’	5’ tttagaagcaggaggggttg 3’	2.5
CEM15-3U	Intron 7, exon 8, 3’ region	5’ cctcctctccgatcattgtc 3’	5’ cctcctctccgatcattgtc 3’	1.5

Table 2.8 Components of *APOBEC3G* Sequencing Reaction

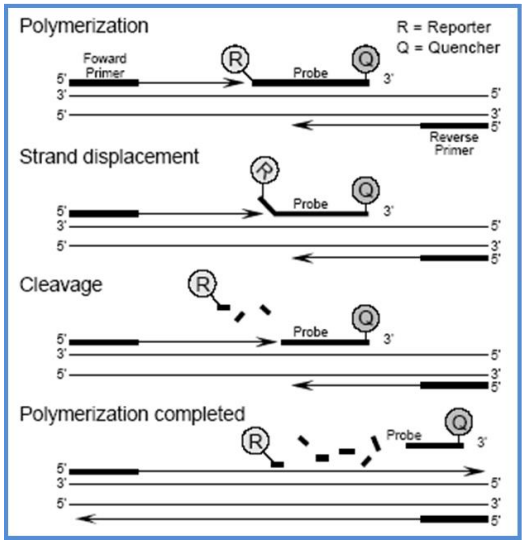
Reagent	Volume (µl)
Big Dye ReadyReaction Mix	0.25
Big Dye Sequencing Buffer (5X)	1.875
Primer (10 µM)	0.32
Water	5.055
DNA	2.5
Total Volume	10.0

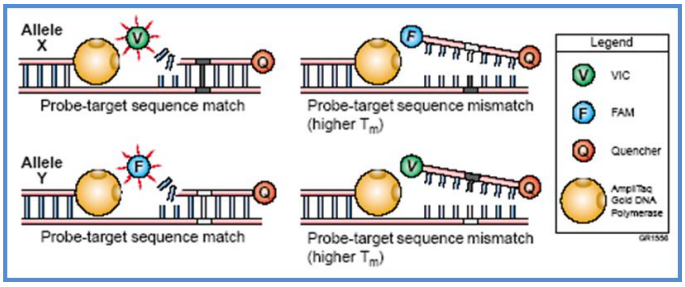
2.5 Genotyping of *APOBEC3G* Variants by TaqMan and RFLP Assays

Following resquencing and screening of *APOBEC3G*, 5 SNPs that were identified were selected to be genotyped in a larger group of samples. The SNPs were selected based on their high minor allele frequencies and previous description in an American cohort (An et al. 2004) . The SNPs were genotyped using pre-designed TaqMan SNP genotyping assays (ABI) (Table 2.9), and where a TaqMan SNP genotyping assay was unavailable, PCR restriction fragment length polymorphism (RFLP) was used. TaqMan assays were carried out according to the manufacturer's protocol (ABI). Details of the reaction mix and cycling conditions used are described in Table 2.10 and 2.11 respectively.

The ABI TaqMan SNP genotyping assay depicted in Figure 2.2 employs 5'-nuclease technology and uses forward and reverse PCR primers, and two differently labelled TaqMan minor groove binder (MGB) probes. The biallelic SNP is located in the middle third of the

probe and each allele-specific MGB probe is labelled differently with either a VIC or FAM fluorescent reporter dye and attached with a fluorescence quencher. The reporter dye is quenched when the MGB probe is intact. During PCR, the 5'-nuclease activity of Taq DNA polymerase cleaves the reporter dye (FAM or VIC) from the MGB probe that has hybridized to the DNA strand. The reporter dye fluoresces when separated from the quencher. An increase in either VIC or FAM dye fluorescence specifies homozygosity for FAM- or VIC specific alleles (X/X or Y/Y), and an increase in the fluorescence of both dyes points to heterozygosity (X/Y) (Shen et al. 2009). The ABI Prism 7900HT sequence detection system was used to distinguish fluorescence and hence genotypes.





A substantial increase in:	Indicates:
VIC™ fluorescence only	Homozygosity for allele X
FAM™ fluorescence only	Homozygosity for allele Y
both fluorescent signals	Heterozygosity

Figure 2.2 TaqMan Genotyping/Allelic Discrimination Chemistry depicting the 5'-nuclease activity of Taq DNA polymerase which cleaves the reporter dye (R) (FAM or VIC) from the probe that has annealed to the DNA strand. The reporter dye (R) fluoresces when separated from the quencher (Q) (Source: ABI PRISM® 7900HT Sequence Detection SystemUser Guide, ABI).

Table 2.9 Details of pre-designed TaqMan SNP genotyping assays

NCBI dbSNP ID	ABI Assay ID
rs8177832	C_2189646_10
rs17496046	C_25649193_10
rs6001417	C_30089175_10
rs3736685	C_27489853_10

Table 2.10 Components of Reaction Mix for TaqMan Genotyping Assays

Reagent	Volume (µl)
TaqMan Universal PCR Master Mix	12.5
Primer and TaqMan Probe (20X)	0.625
Water	5.875
DNA (10ng/µl)	1.0
Total Volume	20.0

Table 2.11 Cycling conditions for TaqMan Genotyping Assays

Step	Temperature (^{o}C)	Time (min)	#of cycles
Activation	95	10:00	1
Denaturation	92	0.15	40
Annealing/Extend	60	1:00	
Hold	4	∞	

PCR primers, PCR conditions and restriction enzymes used for the RFLP assay (NCBI dbSNP ID*: rs5750743)* are described in Tables 2.12, 2.13 and 2.14 respectively. Restriction enzymes and buffers were from New England Biolabs (NEB). The RFLP assay is a commonly used method of genotyping that is based on endonuclease cleavage. A SNP that alters a restriction sequence can be genotyped by an RFLP assay as it produces or eliminates restriction endonuclease recognition sites, thereby affecting quantities and length of DNA fragments that result from restriction endonuclease digestion.

Table 2.12 Components of PCR RFLP Assay

Reagent	Volume (µl)
PCR Buffer (10X) (++Mg)	2.00
dNTPs	0.40
Primer (Fwd) (CEM15-P1-AVAI-S)[a] (100µM)	0.10
Primer (Rev) (CEM15-P1-AVAI-A)[b] (100µM)	0.10
Taq Gold	0.25
Water	12.15

[a] acgcctggccatttactct

[b] gccctccctaaagtgacctc

Table 2.13 Cycling conditions of PCR RFLP Assay

Step	Temperature (^{o}C)	Time (min)	#of cycles
Activation	95	10:00	1
Denaturation	94	0.30	
Annealing	62	0:30	35
Extension	72	0:45	
Final Extension	72	8:00	
Hold	4	∞	

Table 2.14 Components of Restriction Endonuclease Digestion

Reagent	Volume (µl)
Restriction Enzyme (AvaI) (NEB)	2.00
NEB Buffer 3	0.40
DNA (4ng/ µl)	5.00
Water	0.10

The restriction endonuclease digestion was incubated at 37^{o}C for 4 hours and digested products were separated by electrophoresis on a 4% agarose gel. Genotypes were distinguished by the following pattern of digestion:

Undigested:	385 base pairs (bp)
Homozygous (wild type):	316 bp, 69bp
Heterozygous:	316 bp, 180 bp, 136 bp, 69 bp
Homozygous (mutation)	180 bp, 136 bp, 69 bp

2.6 Study of APOBEC3G hypermutation within HIV-1 *env*

A subset of 30 patient samples with known APOBEC3G *H186R* genotypes that was selected for HIV-1 v*if* analysis was also used to assess APOBEC3G hypermutation within HIV-1 *env*. Samples used for this analysis were at the 36 month post infection time point to allow for the accumulation of APOBEC3G induced mutations which are more pronounced in late infection.

2.6.1 Proviral DNA Extraction

Proviral DNA was extracted from cryopreserved PBMCs using a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. The quantity and quality of extracted DNA was determined by measuring the optical density at 260 nm and 280 nm. All DNA samples had a 260nm/280nm ratio > 1.8 and < 2.0.

2.6.2 Amplification of HIV-1 *env*

The 3kb HIV-1 *env* gene was amplified by nested PCR using primers (Singh et al. 2009; Archary et al. 2010) located outside the Env coding region (Table 2.15).

Table 2.15 HIV-1 *env* amplification primer sequences

Primer	Sequence	HXB2 Position
Outer		
OFM19	5' GCACTCAAGGCAAGCTTTATTGAGGCTTA 3'	9604-9632
VIF1	5' GGGTTTATTACAGGGACAGCAGAG 3'	4923-4900
Inner		
Env1A	5' CACCGGCTTAGGCATCTCCTATGGCAGGAAGAA 3'	5955-5982
EnvM	5' TAGCCCTTCCAGTCCCCCCTTTTCTTTTA 3'	9096-9068

Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Life Sciences) was used for the 1st round of PCR amplification while Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes, Finland) was used in the 2nd round of amplification. The nested PCR reactions were prepared as outlined in Tables 2.16 and the PCR cycling conditions that were used are outlined in Table 2.17. All PCR reactions were performed on the GeneAmp PCR System 9700 (ABI).

Table 2.16 Components of nested PCR reactions for amplification of HIV-1 *env*

Reagent	Volume (µl)
1st Round	
Buffer (10X)	2.0
dNTPs (10mM)	0.4
$MgSO_4$	0.8
Primer (Fwd) (OFM19) (20µM)	0.2
Primer (Rev) (VIF1) (20µM)	0.2
Platinum High Fidelity Taq	0.1
Water	12.3
DNA	4.0
Total Volume	20.0
2nd Round	
Phusion Buffer (5X)	10.0
dNTPs (10mM)	1.0
Primer (Fwd) (Env1A) (20µM)	1.0
Primer (Rev) (EnvM) (20µM)	1.0
Taq Polymerase	0.5
Water	31.5
1st round PCR product	3.0
Total Volume	50.0

PCR amplicons were analysed by electrophoresis on a 1% agarose gel. DNA bands were excised from the agarose gel and purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Life Sciences). Purified PCR amplicons were analysed on a 1% agarose gel with a DNA quantification marker to determine the DNA concentration and quality which is crucial for downstream sequencing.

Table 2.17 PCR cycling conditions of nested PCR assay for amplification of HIV-1 *env*

Step	Temperature (^{o}C)	Time (min)	#of cycles
1st Round			
Activation	94	4:00	1
Denaturation	94	0.15	
Annealing	55	0:30	35
Extension	68	4:00	
Final Extension	68	20:00	1
Hold	4	∞	
2nd Round			
Activation	98	0:30	1
Denaturation	98	0.10	
Annealing	62	0:30	35
Extension	72	4:00	
Final Extension	72	10:00	1
Hold	4	∞	

2.6.3 Sequencing of HIV-1 *env*

HIV-1 *env* PCR products were directly sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (ABI) (Table 2.18) and 16 overlapping sequencing primers (Table 2.19) (Rousseau et al. 2006). Purified PCR products were diluted in sterile water in a 1:15 ratio before they were sequenced.

Table 2.18 Components of Sequencing Reaction for HIV-1 *env*

Reagent	Volume (µl)
Big Dye ReadyReaction Mix	0.4
Big Dye Sequencing Buffer (5X)	2.0
Primer (2.5 µM)	2.0
Water	3.6
DNA	2.0
Total Volume	10.0

Sequencing cycling conditions were 96^{o}C for 1 minute, 35 cycles of 96^{o}C for 10 seconds, 50^{o}C for 5 seconds, 60^{o} C for 4 minutes. Sequence extension products were purified using the Ethanol/EDTA/sodium acetate precipitation method to remove unincorporated dye terminators in sequencing reactions which would otherwise obscure sequencing data. Sequences were resolved on the 3730xl DNA Analyzer (ABI/Hitachi). Sequences were edited and analysed with Sequencher DNA Sequence Analysis Software (Genecodes).

Table 2.19 HIV-1 *env* Sequencing Primers

Primer Name	Sequence	Direction	HXB2 Position
SQ4R(2)C	5’ GTTGATCCCTTAGGTATCTTTCTA 3’	Reverse	7972-7995
ED31	5’ CCTCAGCCATTACACAGGCCTGTCCAAAG 3’	Forward	6817-6845
SQ6RC-DESH	5’ ATAATGTATAGGAATTGGGT 3’	Reverse	6856-6875
SQ3RC-DESH	5’ TTTAGTTCCAGACCCCAATA 3’	Reverse	8585-8603
ES7	5’ CTGTTAAATGGCAGTCTAGC 3’	Forward	7002-7021
EnvA	5’ GCTTAGGCATCTCCTATGGCAGGAAGAA 3’	Forward	5955-5982
EnvN	5’ CTGCCAATCAGGGAAGTAGCCTTGTGT 3’	Reverse	9145-9171
SQ11F-DESH	5’ ACTCTATTCTGTGCATCAGA 3’	Forward	6375-6394
BSTQ2+	5’ CCAATTCCTATACATTATTGTGC 3’	Forward	6858-6880
SQ5.5RC	5’ CTAGGAGCTGTTGATCCTTTAGGTAT 3’	Reverse	7979-8004
SQ6RC(2)	5’ GAATTGGGTCAAAAGAGACCTTTGGA 3’	Reverse	6839-6864
SQ3R(2)C	5’ GCTATGGTATCAAGCAGACTAATAGCACTC 3’	Reverse	8651-8680
SQ13F(2)C	5’ TATATAAATATAAAGTGGTAGAAATTAAGC 3’	Forward	7672-7701
SQ14FC	5’ ACTCACGGTCTGGGGCATTA 3’	Forward	7925-7944
EnvM	5’ TAGCCCTTCCAGTCCCCCCTTTTCTTTTA 3’	Reverse	9096-9068
EF00	5’ AAAGAGCAGAAGACAGTGGCAATGA 3’	Forward	6204-6228

2.6.4 HIV-1 *env* Sequence Analysis

HIV-1 *env* sequences were edited and analysed with Sequencher DNA Sequence Analysis Software v5.1 (Genecodes). Sequences were thereafter aligned against an HIV-1 *env* subtype C consensus reference sequence (obtained from the HIV Los Alamos National Laboratory HIV database (http://www.hiv.lanl.gov)) using the Clustal W algorithm in the Molecular Evolutionary Genetics Analysis (MEGA) software v5.05. The aligned nucleotide sequences were translated into corresponding amino acid sequences imported with the Bioedit Sequence Alignment Editor v7.1.3.0 software programme.

Neighbour joining phylogenetic newick tree files were generated by importing the amino acid sequence alignment file into the online Tree Maker tool available in the HIV Los Alamos National Laboratory HIV database. The newick tree files were imported into FigTree v1.3.1 for assembly of phylogenetic trees. Nucleotide and amino acid sequence diversity was calculated using MEGA software v5.05. The online Highlighter tool (HIV Los Alamos National Laboratory HIV database) was used to depict mismatches in aligned HIV-1 *env* sequences as compared to a consensus subtype C HIV-1 *env* reference sequences.

HIV-1 *env* sequences were also assessed for the frequency of APOBEC3G induced hypermutation. The Hypermut 2.0 Tool available on the HIV Sequence Database from the Los Alamos National Laboratory (http://www.hiv.lanl.gov) was used to detect APOBEC-specific hypermutations. Hypermut 2.0 allows for detection of excess APOBEC-induced hypermutation relative to a control reference sequence and employs the Fisher's exact test to detect any increase of mutation for the test sequences compared to the control sequence.

2.7 Cloning and Sequencing of HIV-1 v*if*

A subset of 26 patient samples with known APOBEC3G *H186R* genotypes was selected for HIV-1 v*if* analysis. Samples used for this analysis were chosen at the 36 month post infection time point, as we anticipated that in-patient accumulation of APOBEC immune-driven mutations within Vif would be more evident at this late stage of infection.

2.7.1 Viral RNA Extraction

Viral RNA was extracted from cryopreserved plasma using a Qiagen QIAamp Viral RNA Mini Kit as per manufacturer's instructions. The quantity and quality of extracted RNA was determined by measuring the optical density at 260 nm and 280 nm. All RNA samples had a 260nm/280nm ratio > 1.8 and < 2.0.

2.7.2 Synthesis of cDNA

Viral RNA was then reverse transcribed into a cDNA with the reverse Vif outer primer (Table 2.21) using the Thermoscript RT PCR Sytem (Invitrogen, Life Sciences) (Table 2.20).

T**able 2.20 Components of cDNA synthesis reaction for HIV-1 *vif***

Reagent	**Volume (µl)**
Mix 1	
Primer (SQ8R) (10µM)	2.0
dNTPs (10mM)	4.0
RNA	10.0
Water	8.0
Mix 2	
cDNA Synthesis Buffer (5X)	8.0
DTT	2.0
Rnase out	2.0
Thermoscript Reverse Transcriptase	2.0
Total Volume	40.0

Mix 1 was incubated at 65^{o}C for 5 minutes to denature the RNA sample. Thereafter Mix 2 was added to Mix 1 followed by incubation at 50^{o}C for 60 minutes and 85^{o}C for 5 minutes. Then 1µl of RNase H was added to each reaction and incubated for a further 20 minutes at 37^{o}C, to remove excess mRNA.

2.7.3 Amplification of HIV-1 *vif*

The full length of the HIV-1 *vif* (579 base pairs) was amplified by nested PCR using primers located approximately 225 base pairs on either side of the Vif coding region (Table 2.21, Figure 2.3). Primers sequences were either previously published (Rousseau et. al., 2006) or selected from an HIV primer database supplied by Prof. Carolyn Williamson (HIV Diversity and Pathogenesis Group, University of Cape Town, South Africa).

Table 2.21 Primer sequences used for amplification and sequencing of HIV-1 *vif*

Primer	Sequence	HXB2 Position
Outer		
For9	5' AAAATTAGCAGGAAGATGGCCAGT 3'	4535-4558
SQ8R	5' CTCCGCTTCTTCCTGCCATAGGAGAT 3'	5988-5963
Inner and Sequencing		
P10	5' TACTCTGGAAAGGTGAAGG 3'	4951-4969
7Rev9	5' CTTCCTGCCATAGGAGATGCCTAA 3'	5980-5957
Additional Sequencing		
Rev12	5' AGAGATCCTACCTTGTTATGTCCT 3'	5474-5451

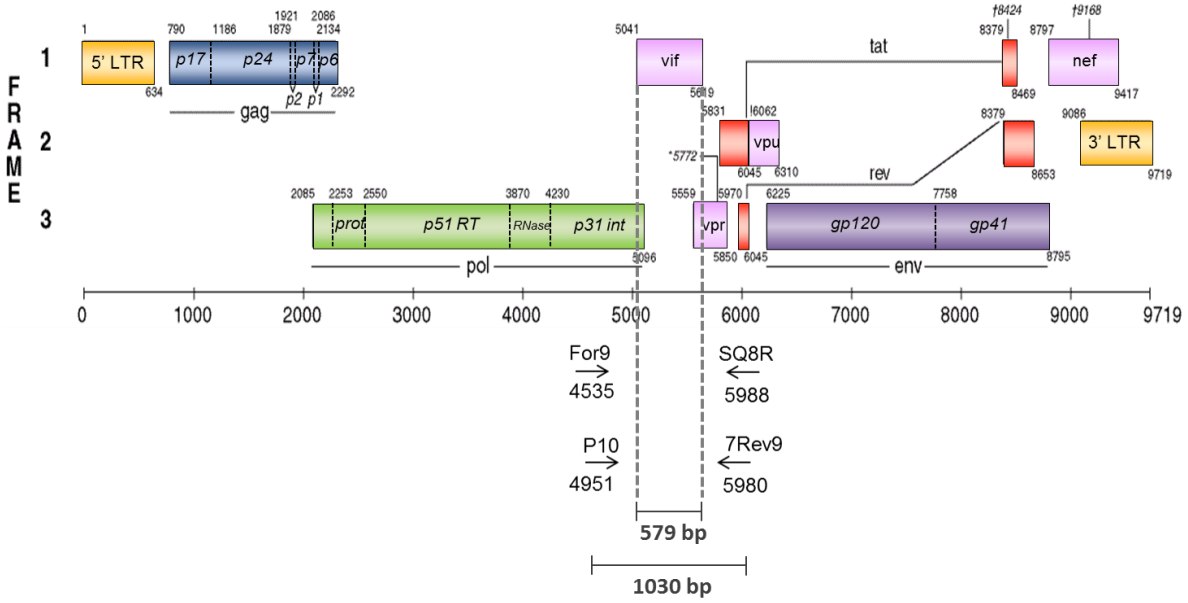


Figure 2.3 Position of primers used to amplify HIV-1 *vif*. (Adapted from HIV-1 Gene Map, Los Alamos National Library, HIV Database).

The Expand High Fidelity PCR system (Roche Diagnostics) was used to amplify HIV-1 *vif*. The nested PCR reactions were prepared as outlined in Tables 2.22 and the PCR cycling conditions that were used are outlined in Tables 2.23. All PCR reactions were performed on the GeneAmp PCR System 9700 (ABI)

Table 2.22 Components of nested PCR assay for amplification of HIV-1 *vif*

Reagent	Volume (µl)
1st Round	
Buffer 1	5.0
dNTPs (10mM)	1.0
Primer (Fwd) (For9) (10µM)	1.0
Primer (Rev) (SQ8R) (10µM)	1.0
Expand High Fidelity Taq	0.75
Water	37.25
cDNA	4.0
Total Volume	50.0
2nd Round	
Buffer 1	5.0
dNTPs (10mM)	1.0
Primer (Fwd) (P10) (10µM)	1.0
Primer (Rev) (7Rev9) (10µM)	1.0
Expand High Fidelity Taq	0.75
Water	38.25
1st round PCR product	3.0
Total Volume	50.0

Table 2.23 PCR cycling conditions of nested PCR assay for amplification of HIV-1 *vif*

Step	Temperature (^{o}C)	Time (min)	#of cycles
1st Round			
Activation	95	2:00	1
Denaturation	95	0.15	
Annealing	52	0:30	10
Extension	72	1:00	
Denaturation	95	0:15	
Annealing	55	0:30	15
Extension	72	1:30	
Final Extension	72	7:00	1
Hold	4	∞	
2nd Round			
Activation	95	2:00	1
Denaturation	95	0.15	
Annealing	55	0:30	35
Extension	72	1:30	
Final Extension	72	7:00	1
Hold	4	∞	

Each sample was amplified in replicates of eight. PCR products were analysed by electrophoresis on a 2% agarose gel. PCR amplification yielded a 1030 base pair amplicon containing the 579 base pair *vif* gene. Five positive amplicons per sample were selected for subsequent cloning assays.

Positive amplicons were excised from the agarose gel and purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare/Life Sciences, Buckinghamshire, United Kingdom). DNA concentration and purity is essential for successful molecular cloning. This was determined by analysing the purified PCR amplicons on 2% agarose gel with the O'Gene Ruler 1kb DNA marker (Fermentas).

2.7.4 TA Cloning of Vif

In order to selectively amplify and generate homogenous HIV-1 *vif* DNA populations for sequencing, the purified HIV-1 *vif* amplicons were cloned by the TOPO TA cloning method (Invitrogen, Life Sciences) (Figure 2.4). The concept behind the TA cloning method utilises the Taq polymerase non template-dependent activity that adds a single deoxyadenosine (A) overhang to the 3´ ends of PCR products. The linearized pCR 2.1vector supplied with the TOPO TA cloning kit has single 3´ deoxythymidine (T) residue overhangs with the topoisomerase enzyme covalently attached at each end. This permits PCR inserts to ligate efficiently with the vector.

Purified HIV-1 *vif* amplicons were ligated into the pCR2.1 vector in a 3:1 ratio as shown in Table 2.24 and incubated at room temperature for 30 minutes. Including salt (200 mM NaCl; 10 mM $MgCl_2$) in the TOPO cloning reaction increases the number of transformants 2- to 3-fold.

Table 2.24 Ligation Mix for HIV-1 *vif* insert and pCR2.1 vector

Reagent	Volume (µl)
HIV-1 *vif* Insert (30ng/ul)	1.0
Salt solution (200 mM NaCl; 10 mM $MgCl_2$)	1.0
Vector (10ng/ul)	1.0
Water	3.0
Total Volume	6.0

The 3µl of insert/vector ligation mix was then chemically transformed into TOP10 One Shot Chemically Competent *E. coli* (Invitrogen, Life Sciences) by heat shock at 42^{o}C for 30 seconds. This was followed by the addition of SOC medium (Sigma-Aldrich) and incubation at 37^{o}C for 1 hour to maximize the transformation efficiency of competent cells. Each transformation was plated onto pre-warmed Xgal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) (Promega, Wisconsin, USA) coated, ampicillin (50 µg/ml) containing, Luria-Bertani (LB) agar plates, and incubated at 37^{o}C overnight.

A blue-white selection method was used to detect the bacterial colonies that contained the HIV-1 *vif* insert. This method is based on the disruption of the *LacZ* gene, which is present in the plasmid vector and codes for the beta-galactosidase enzyme. DNA ligated into the plasmid vector disrupts the *LacZ* gene and therefore inhibits β-galactosidase formation. X-Gal is an inert colorimetric substrate for active beta-galactosidase which hydrolyses X-Gal into colourless galactose and 4-chloro-3-brom-indigo which forms a strong blue precipitate. Therefore, blue colonies indicate the presence of active beta-galactosidase and thus a vector with an uninterrupted *LacZ* gene and therefore no insert. Oppositely, a white

colony, where X-gal is not hydrolysed, confirms the presence of an insert which would have disrupted *LacZ* and hence inhibited the formation of active β-galactosidase.

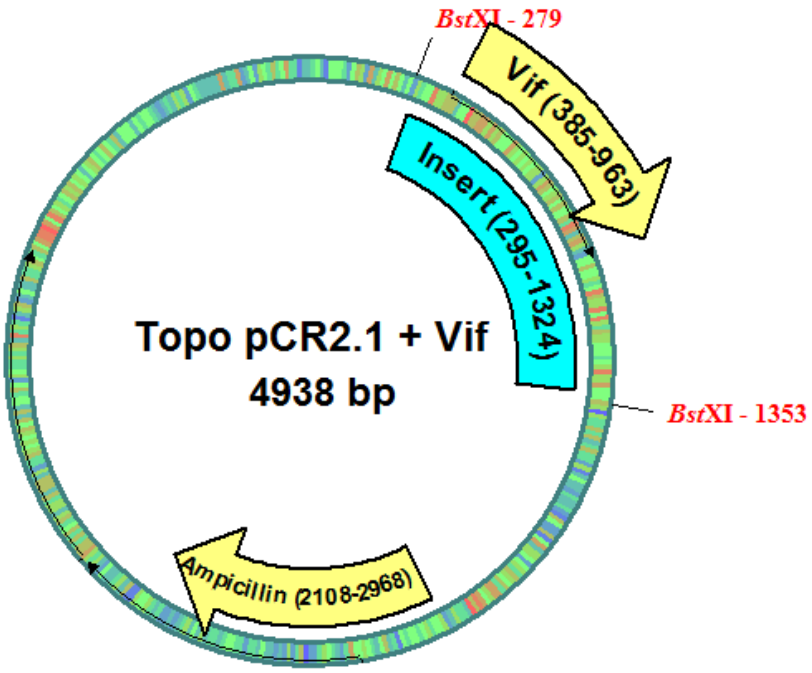


Figure 2.4 Vector Map depicting the location of the 1030 base pair insert containing the 579 base pair HIV-1 *vif* gene within the 3908 base pair pCR2.1TOPO vector, producing a 4938 base pair plasmid vector. Restriction endonuclease digestion at the BstXI restriction sites were used to verify the presence of the insert once the plasmid DNA was isolated. (Generated with pDRAW32 DNA Analysis Software, Acaclone).

Two to four white colonies were picked from each agar plate, resulting in a total of 10 – 20 clones per sample (5 PCRs X (2 to 4 colonies)). It is assumed that each colony is derived from a single bacteria and that it is therefore a single clone. Each colony was placed into 5 ml of LB/ampicillin medium and grown overnight at 37^{o}C.

Plasmid DNA was isolated from the overnight culture using the GeneJET Plasmid Miniprep kit (Fermentas, Thermoscientific, Massachusetts, USA) following the manufacturers protocol. Plasmid DNA isolation yielded concentrations of 150-200 ng/µl with 260 nm/280 nm ratios > 1.8 and < 2.0.

Restriction endonuclease digestion with the BstXI enzyme (New England Biolabs Inc.) was used to confirm the presence of the insert in each plasmid DNA sample (Table 2.25).

Table 2.25 Components of Restriction Endonuclease Digestion by BstXI

Reagent	Volume (µl)
Restriction Enzyme (BstXI) (10U/µl)	1.00
NE Buffer 3 (10X)	2.00
Plasmid DNA	0.50
Water	6.50

Digestion was carried out at 37^{o}C for 1 hour and digested products were separated by electrophoresis on a 2% agarose gel. The following digestion pattern was observed:

Insert present: 2 bands - 3886 bp, 1075 bp,

No insert: 1 band - 3908 bp

Plasmid DNA samples that were positive for the insert was subsequently sequenced.

2.7.5 Sequencing of HIV-1 *vif*

A plasmid DNA concentration of 150 ng or 200 ng, and the primers P10, 7Rev9 and Rev12 was used to sequence HIV-1 *vif*. The BigDye Terminator v3.1 Cycle Sequencing Kit (ABI) was used for sequencing (Table 2.26).

Table 2.26 Components of Sequencing Reaction for HIV-1 *vif*

Reagent	Volume (µl)
Big Dye ReadyReaction Mix	0.4
Big Dye Sequencing Buffer (5X)	2.0
Primer (P10, 7Rev9, R12) (1.6 µM)	2.0
Water	4.6
DNA (150ng/ µl or 200ng/ µl)	1.0
Total Volume	10.0

Sequencing cycling conditions were 96^{o}C for 1 minute, 35 cycles of 96^{o}C for 10 seconds, 50^{o}C for 5 seconds, 60^{o} C for 4 minutes.

Sequence extension products were purified using the Ethanol/EDTA/sodium acetate precipitation method to remove unincorporated dye terminators in sequencing reactions which would otherwise obscure sequencing data. Sequences were resolved on the 3730xl DNA Analyzer (ABI/Hitachi).

2.7.6 HIV-1 *vif* Sequence Analysis

Sequences were edited and analysed with Sequencher DNA Sequence Analysis Software v5.1 (Genecodes). Thereafter the sequences were converted to the FASTA format and aligned against an HIV-1 subtype C consensus reference sequence (obtained from the HIV Los Alamos National Laboratory HIV database (http://www.hiv.lanl.gov) using the Clustal W algorithm in the Molecular Evolutionary Genetics Analysis (MEGA) software v5.05. Thereafter, the aligned nucleotide sequences were imported into the Bioedit Sequence Alignment Editor v7.1.3.0 software programme where they were translated into corresponding amino acid sequences.

The amino acid sequence alignment file was imported into the online Tree Maker tool available in the HIV Los Alamos National Laboratory HIV database where neighbour joining phylogenetic newick tree files were created. The newick tree files were subsequently imported into FigTree v1.3.1 for construction of phylogenetic trees. Nucleotide and amino acid sequence diversity was calculated using MEGA software v5.05.

For the purpose of identifying and comparing regions of conservancy and variability within HIV-1 *vif* sequences, we created consensus sequences from clonal sequences for each of 26 study participants with the online Consensus Maker tool (HIV Los Alamos National Laboratory HIV database). HIV-1 *vif* consensus sequences were aligned against an HIV-1 subtype C consensus reference sequence (from HIV Los Alamos National Laboratory HIV database (http://www.hiv.lanl.gov)) and protein translation inferred using Bioedit Sequence Alignment Editor v7.1.3.0 before analysis of the sequence alignment.

Additionally, signature amino acids were identified by examining HIV-1 *vif* sequences with the Viral Epidemiology Signature Pattern Analysis (VESPA) (Los Alamos National Laboratory HIV database (http://www.hiv.lanl.gov)) to compute the frequency of each amino acid at each position in the HIV-1 Vif sequence alignment. Emphasis was placed on sequences belonging to the *186H/H* (wild type) group and the *186R/R* (mutant) group.

For subsequent functional testing of HIV-1 *vif* alleles, unique HIV-1 *vif* clonal sequences were selected by the Duplicate Sequence Removal (ElimDupes) tool (Los Alamos National Laboratory HIV database). The online tool was used to eliminate clonal sequences that were 90% similar to each other, thereby creating a file of distinctive and diverse HIV-1 *vif* clonal sequences from which *vif* variants were selected for use in functional assays.

2.8 Expression and Functional Analysis HIV-1 Vif

2.8.1 Sample Selection

A subset of HIV-1 *vif* sequences generated from the cloning and sequencing assays were selected for subsequent HIV-1 *vif* functionality assays. HIV-1 *vif* clonal sequences from patients whose *H186R* genotype was wild type (*186H/H*) or homozygous mutant (*186R/R*) were assessed for functionality. A file of unique HIV-1 *vif* sequences was created using the online Duplicate Sequence Removal Tool as described above (in section 2.7.6). The output from a total of 237 HIV-1 *vif* sequences (from 186 -H/H and R/R patients) was 34 unique sequences. Thereafter one HIV-1 *vif* sequence per patient was randomly selected resulting in a subset of 16 HIV-1 *vif* clonal sequences that was used in ensuing functionality assays. We reasoned that selection of the most extreme genotypes would provide good representation of the functional differences that exist between different Vif variants. Of the 16 HIV-1 *vif* clonal

sequences, 11 were from patients with the *186H/H* genotype while 5 were from those carrying the *186R/R* genotype.

2.8.2 Cloning of HIV-1 *vif* into the pCRV1 Expression Vector

Sixteen HIV-1 *vif* clonal sequences that were previously cloned into the pCR2.1 vector were used as templates for PCR amplification with Vif primers designed specifically for the 5' and 3' regions of each variant and containing NotI and EcoRI restriction sites (Tables 2.27). In addition, a second set of reverse primers were designed to introduce a carboxy terminal FLAG-tag (Table 2.28) to each of the sixteen HIV-1 *vif* sequences described above. The peptide sequence of the FLAG-tag from the N-terminus to the C-terminus is DYKDDDDK and was added to the HIV-1 *vif* sequences so that the expressed proteins could also be identified with an antibody against the FLAG sequence.

Further, HIV-1 *vif* sequence analysis to determine signature amino acid patterns had suggested an association between the amino acid at position 36 and the patients *H186R* genotype. Therefore, site-directed mutagenesis was performed on 4 HIV-1 *vif* sequences to mutate the amino acid at position 36 from a serine to a lysine and vice versa. Site directed mutagenesis was carried out using sequence specific overlapping primers that were designed to introduce the mutation at position 36 (Table 2.29). The technique employed for site-directed mutagenesis is described in Figure 2.5. FLAG-tags were also introduced onto the HIV-1 *vif* mutants.

HIV-1 *vif* sequences in pCR2.1 vector were diluted 50X in sterile water and amplified using the specific primers and PCR reaction and cycling conditions described in Tables 2.30 and 2.31.

Table 2.27 Primer sequences used for HIV-1 *vif* amplification designed specifically for each HIV-1 *vif* variant

Primer Name	Sequence	Direction
1770 Vif-C 7G8G EcorI+	5’ AAAGAATTCATGGAAGGCAGATGGCA 3’	Forward
1771 Vif-C 6C EcorI+	5’ AAAGAATTCATGGACAACAGATGGCA 3’	Forward
1772 Vif-C EcorI+	5’ AAAGAATTCATGGAAAACAGATGGCA 3’	Forward
1762 Vif-C1 NOT-	5’ TTTGCGGCCGCCTAATGTCCACTCATT 3’	Reverse
1763 Vif-C2 NOT-	5’ TTTGCGGCCGCCTAGTGTCCATTCATT 3’	Reverse
1764 Vif-C3 NOT-	5’ TTTGCGGCCGCCTAGTGTCCATTCACT 3’	Reverse
1765 Vif-C4 NOT-	5’ TTTGCGGCCGCCTAATGTCCATTCATT 3’	Reverse
1766 Vif-C5 NOT-	5’ TTTGCGGCCGCCTAGTGCCCATTCATT 3’	Reverse
1767 Vif-C6 NOT-	5’ TTTGCGGCCGCCTAGTACCCATTCATT 3’	Reverse
1768 Vif-C7 NOT-	5’ TTTGCGGCCGCCTAATGCCCACTCATT 3’	Reverse
1769 Vif-C8 NOT-	5’ TTTGCGGCCGCCTAATGCCCATTCATT 3’	Reverse
1807 Vif-C1 FLAG-	5’ TCGTCGTCATCCTTGTAATCATGTCCACTCATT 3’	Reverse
1808 Vif-C2 FLAG -	5’ TCGTCGTCATCCTTGTAATCGTGTCCATTCATT 3’	Reverse
1809 Vif-C3 FLAG -	5’ TCGTCGTCATCCTTGTAATCGTGTCCATTCACT 3’	Reverse
1810 Vif-C4 FLAG -	5’ TCGTCGTCATCCTTGTAATCATGTCCATTCATT 3’	Reverse
1811 Vif-C5 FLAG -	5’ TCGTCGTCATCCTTGTAATCGTGCCCATTCATT 3’	Reverse
1812 Vif-C6 FLAG -	5’ TCGTCGTCATCCTTGTAATCGTACCCATTCATT 3’	Reverse
1813 Vif-C7 FLAG -	5’ TCGTCGTCATCCTTGTAATCATGCCCACTCATT 3’	Reverse
1814 Vif-C8 FLAG -	5’ TCGTCGTCATCCTTGTAATCATGCCCATTCATT 3’	Reverse

Table 2.28 Vif specific primers used for amplification of each HIV-1 *vif* variant as well as the reverse primers used to introduce a FLAG-tag to each HIV-1 *vif* variant

HIV-1 *vif* Variant	Forward Primer*	Reverse Primer*	FLAG Reverse Primer
CAP37_P1C1	1772 Vif-C EcorI+	1762 Vif-C1 NOT-	1807 Vif-C1 FLAG-
CAP40_P4C1	1771 Vif-C 6C EcorI+	1764 Vif-C3 NOT-	1809 Vif-C3 FLAG -
CAP45_P1C1	1772 Vif-C EcorI+	1765 Vif-C4 NOT-	1810 Vif-C4 FLAG -
CAP65_P1C1	1772 Vif-C EcorI+	1763 Vif-C2 NOT-	1808 Vif-C2 FLAG -
CAP84_P2C3	1772 Vif-C EcorI+	1763 Vif-C2 NOT-	1808 Vif-C2 FLAG -
CAP137_P1C1	1772 Vif-C EcorI+	1763 Vif-C2 NOT-	1808 Vif-C2 FLAG -
CAP206_P1C3	1772 Vif-C EcorI+	1765 Vif-C4 NOT-	1810 Vif-C4 FLAG -
CAP217_P2C3	1772 Vif-C EcorI+	1766 Vif-C5 NOT-	1811 Vif-C5 FLAG-
CAP221_P3C3	1772 Vif-C EcorI+	1763 Vif-C2 NOT-	1808 Vif-C2 FLAG-
CAP225_P2C2	1772 Vif-C EcorI+	1765 Vif-C4 NOT-	1810 Vif-C4 FLAG-
CAP278_P2C1	1772 Vif-C EcorI+	1768 Vif-C7 NOT-	1813 Vif-C7 FLAG-
CAP174_P3C3	1772 Vif-C EcorI+	1766 Vif-C5 NOT-	1811 Vif-C5 FLAG-
CAP256_P1C1	1772 Vif-C EcorI+	1766 Vif-C5 NOT-	1811 Vif-C5 FLAG-
CAP261_P1C4	1772 Vif-C EcorI+	1765 Vif-C4 NOT-	1810 Vif-C4 FLAG-
CAP262_P1C1	1770 Vif-C 7G8G EcorI+	1768 Vif-C7 NOT-	1813 Vif-C7 FLAG-
CAP280_P2C1	1772 Vif-C EcorI+	1763 Vif-C2 NOT-	1808Vif-C2 FLAG-

*****Primer sequences detailed in Table 2.27

Table 2.29 Primers used for site-directed mutagenesis

Primer Name	Sequence	Direction
1773 37p4c1 S36K+	5’ GTTTCAAGGAGAGCTAaaGGATGGTTTTACAGA 3’	**Forward**
1774 37p4c1 S36K-	5’ TCTGTAAAACCATCCttTAGCTCTCCTTGAAAC 3’	**Reverse**
1775 45p1c1 S36K+	5’ GTTTCCAAGAGAGCTAaaGGATGGTTTTATAGA 3’	**Forward**
1776 45p1c1 S36K-	5’ TCTATAAAACCATCCttTAGCTCTCTTGGAAAC 3’	**Reverse**
1777 256p2c1 K36S+	5’ ATTTCAAGGAGTGCTAgtGGATGGGTTTACAGA 3’	**Forward**
1778 256p2c1 K36S-	5’ TCTGTAAACCCATCCacTAGCACTCCTTGAAAT 3’	**Reverse**
1779 261p1c1 K36S+	5’ GTCTCAAGGAGAGCTAgtGGATGGTTATACAGA 3’	**Forward**
1780 261p1c1 K36S-	5’ TCTGTATAACCATCCacTAGCTCTCCTTGAGAC 3’	**Reverse**

*Mutated nucleotides are highlighted in blue

Table 2.30 PCR reaction for amplification HIV-1 *vif* variants from plasmid DNA

Reagent	Volume (µl)
Buffer (10X)	2.5
dNTPs (10mM)	0.5
Primer (Forward) (10µM)	1.0
Primer (Reverse) (10µM)	1.0
PFU DNA Polymerase (Promega)	0.2
Water	18.8
pDNA	1.0
Total Volume	25.0

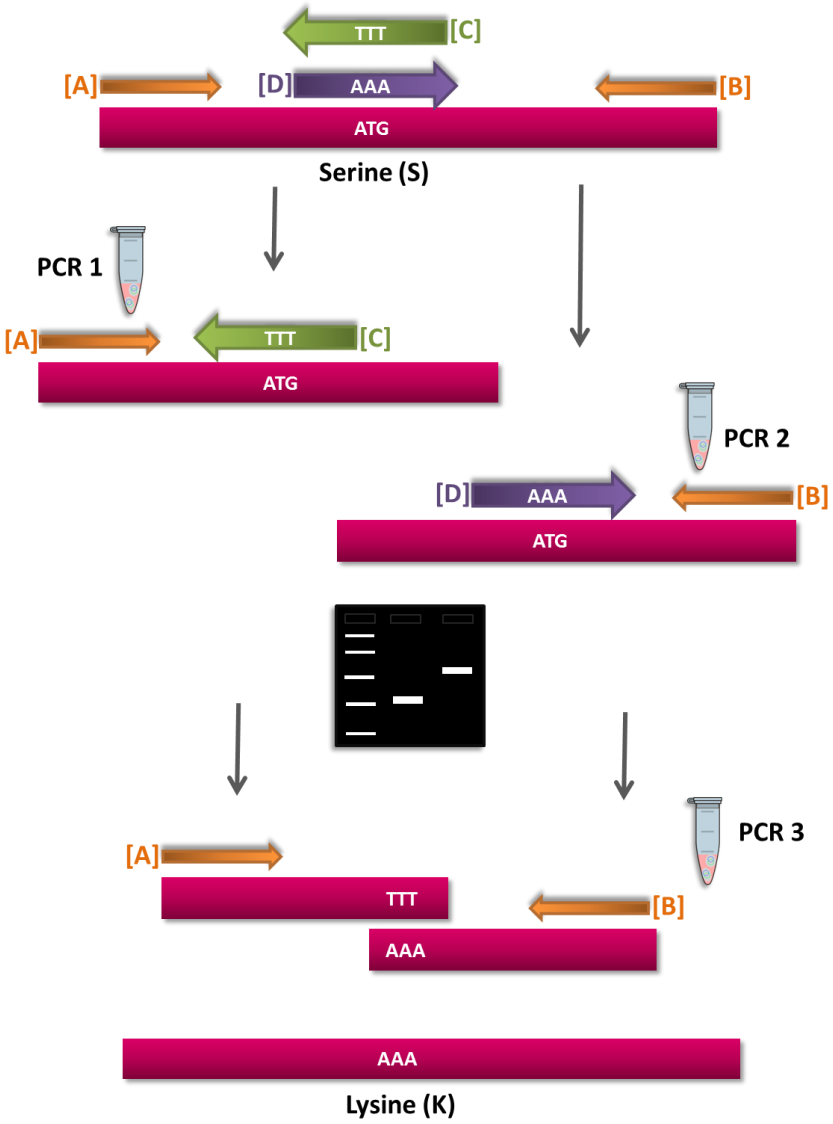


Figure 2.5 **Site-directed mutagenesis by primer extension involves incorporating mutagenic primers in independent, nested PCRs before combining them in the final product. The reaction requires flanking primers (A and B) complementary to the ends of the target sequence, and two internal primers (C and D) that contain the desired mutation and will hybridize to the region to be altered. During PCR 1 and 2 two separate fragments (AC and DB) are created. These products are combined in a 3rd PCR using primers A and B. The complementary ends of the products hybridize in this second PCR to create the final product, AB, which contains the mutated internal sequence.**

Table 2.31 Cycling conditions used for amplification HIV-1 *vif* variants from plasmid DNA

Step	Temperature (^{o}C)	Time (min)	#of cycles
Activation	94	2:00	1
Denaturation	94	0.20	30
Annealing	50	0:20	
Extension	72	0:30	
Final Extension	72	5:00	
Hold	4	∞	

Once amplified, PCR fragments were digested with NotI and EcoRI restriction enzymes (Fermentas, Thermoscientific) (Table 2.32) analysed on a 2% agarose gel and purified with a QIAquick Gel Extraction Kit (Qiagen). T4 DNA Ligase (New England Biolabs Inc.), was used to insert the purified PCR products into a linearized pCRV1 expression vector (Table 2.33) that contains NotI and EcoRI overhangs. pCRV1 provides an HIV-1 Rev response element that is essential for the expression of HIV-1 *vif*. The above cloning procedure is depicted in Figure 2.6.

Table 2.32 Restriction Endonuclease Digestion of PCR products by NotI and EcoRI

Reagent	Volume (µl)
NotI (10U/µl)	2.00
EcoRI (10U/µl)	2.00
Fast Digest Green Buffer (Fermentas)	5.00
Water	16.00
PCR Product	25.00

Table 2.33 Ligation Mix for HIV-1 *vif* insert and pCRV1 vector

Reagent	Volume (µl)
HIV-1 *vif* Insert (30ng/ul)	4.0
Buffer	1.0
pCRV1 Vector	1.0
T4 DNA Ligase	1.0
Water	3.0
Total Volume	10.0

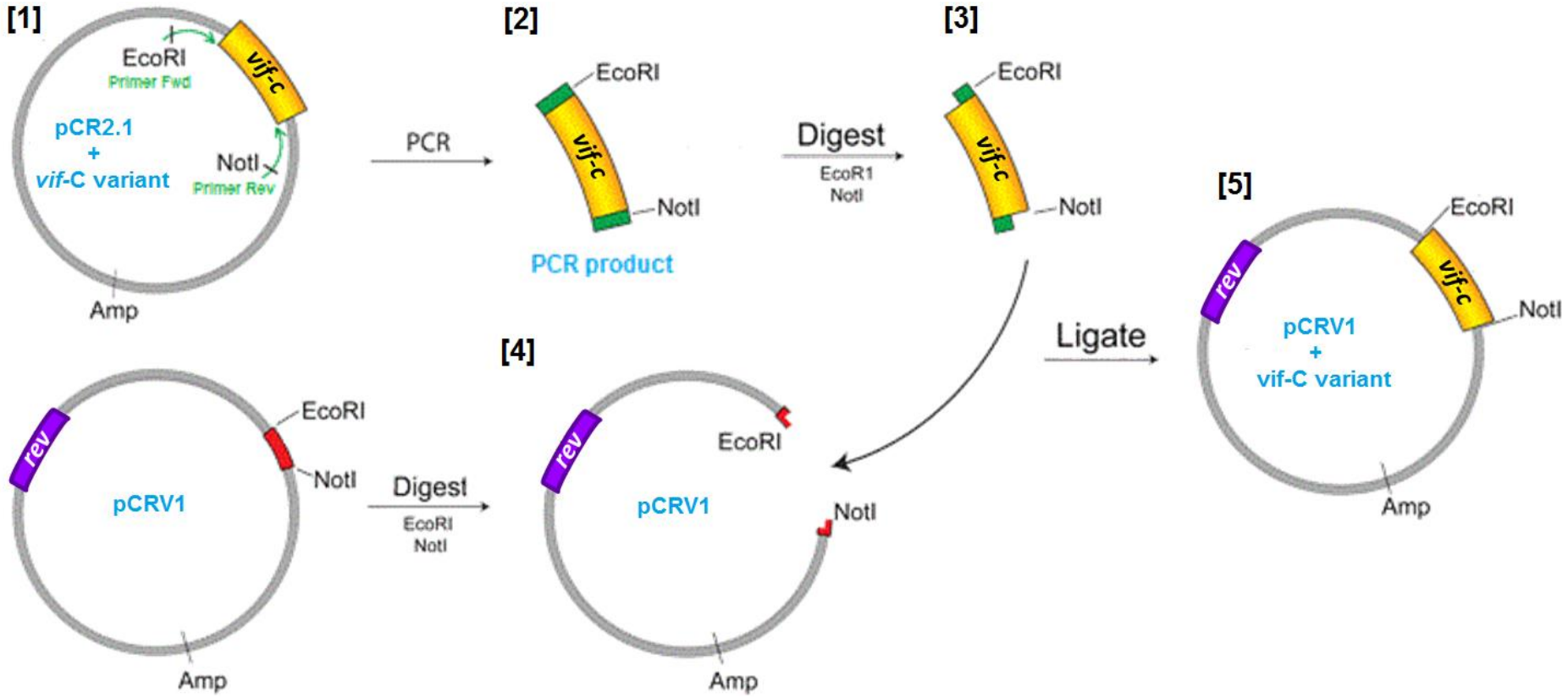


Figure 2.6. Cloning of HIV-1vif variants into the pCRV1 expression vector. [1] HIV-1 *vif* clone in the pCR2.1 vector were used as templates for PCR amplification with primers designed specifically for the 5' and 3' regions of each variant and containing NotI and EcoRI restriction sites. [2] PCR amplicons containing NotI and EcoRI restriction sites were [3] digested with NotI and EcoRI restriction enzymes to create overhangs and [4] inserted into a linearized pCRV1 expression vector containing complementary NotI and EcoRI overhangs to [5] create the virus/vector construct. pCRV1 provides an HIV-1 Rev response element that is essential HIV-1 *vif* expression.

The virus/vector construct was then used to transform ElectroMAX DH10B electrocompetent *E. coli* cells (Invitrogen, Life Sciences) by electroporation at 1.8 volts. This was followed by the addition of SOC medium (Sigma-Aldrich) and incubation at 37^{o}C for 1 hour to maximize the transformation efficiency of competent cells. Each transformation was plated onto pre-warmed ampicillin (50 μg/ml) containing, Luria-Bertani (LB) agar plates, and incubated at 30^{o}C overnight.

Two colonies were picked from each agar plate, placed into 3 ml of LB/ampicillin medium and grown overnight at 30^{o} C. Plasmid DNA was isolated from 1 ml of the mini bacterial culture with the QIAprep Spin Miniprep Kit (Qiagen) as per manufacturer's instruction. Restriction endonuclease digestion by EcoRI and NotI was used to confirm the presence of the insert. DNA preparations that contained the insert were commercially sequenced (Macrogen, USA) with a pCRV1 specific primer (5' GACTGGTGAGTACGCCAAAA 3') to confirm the integrity of the HIV-1 *vif* sequences.

Once the HIV-1 *vif* sequence of the virus/vector constructs was confirmed, the remaining 2 ml of the mini bacterial culture was used to inoculate 100 ml of LB medium containing carbenicillin in preparation of a midi bacterial culture. The midi culture was grown overnight at 30^{o} C. Plasmid DNA was isolated from the midi culture using the High Pure Plasmid Midi Prep Kit (Invitrogen, Life Sciences) according to the manufacturer's protocol. All midi-prepped DNA concentrations were between 1000 – 3000 ng/μl, with 260 nm/280 nm ratios > 1.8 and < 2.0. Plasmid DNA was diluted to a working concentration of 500 ng/ul for subsequent transfection assays.

2.8.3 Vif Expression Assay

Plasmids expressing both untagged and FLAG-tagged HIV-1 *vif* as well as HIV-1 *vif* mutated at position 36 were used in this assay. HEK 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Life Sciences) and supplemented with 10% penicillin/streptomycin and fetal bovine serum (FBS). A total of 3 million 293T cells in a 24 well flat bottom culture plate were transfected with 250 ng of each pCRV1 Vif expression plasmid and 250 ng of PTR600-green fluorescent protein (GFP) plasmid. A total amount of 500 ng of DNA was transfected with 4 mg/ml of the transfection agent polyethylenimine (PEI) (Polysciences, Inc., Pennsylvania, USA). After 48 hours, SDS lysis buffer (1% SDS, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA) was added to the transfected cells, and the clarified lysates were analysed by Western blotting described below.

HIV-1 Vif was detected with a rabbit anti-Vif primary antibody (NIH catalogue number 2221) at a 1:1,000 dilution and with goat anti-rabbit secondary antibody (Sigma) at a 1:5,000 dilution. FLAG-tagged HIV-1 Vif proteins were detected with a mouse anti-FLAG primary antibody (Sigma) at a 1:5,000 dilution.

2.8.4 APOBEC3G Degradation Assay

Plasmids expressing both untagged HIV-1 *vif* as well as HIV-1 *vif* mutated at position 36 were used in this assay. A total of 3 million HEK 293T cells (grown in DMEM (Invitrogen, Life Sciences) and supplemented with 10% penicillin/streptomycin and FBS) in 24-well plates were co-transfected with 10 ng of each pCRV1 Vif expression plasmid, 390 ng of PTR600-GFP and 100 ng of PTR600-A3G (*186H*), or PTR600-A3G (*186R*) expression plasmids, respectively. A total amount of 500ng of DNA was transfected with 4 mg/ml of the transfection agent polyethylenimine (PEI). After 48 hours, SDS lysis buffer (1% SDS, 50

mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA) was added to the transfected cells, and the clarified lysates were analysed by Western blotting. PTR600-A3G (*186H*) and PTR600-A3G (*186R*) expression plasmids were haemagglutimin (HA)-tagged. Therefore APOBEC3G was detected with a rabbit anti-HA primary antibody (Sigma). The PTR600-GFP and PTR600-A3G plasmids were generated at and obtained from the Simon Laboratory, Department of Microbiology at the Mount Sinai School of Medicine, New York.

2.8.5 Western Blotting

Transfected HEK 293T cell lysates were separated on 10% bis-Tris 26-well gels (Invitrogen, Life Sciences) and transferred by semi-dry transfer (BioRad) onto polyvinylidene difluoride (PVDF) membranes (Thermo Scientific). The PVDF membranes were then blocked in 1% low fat milk solution for 1 hour and incubated in primary antibody at 4^{o}C overnight. Membranes were then washed in wash buffer (0.1% Tween in phosphate-buffered saline (PBS)) and incubated in secondary antibody for 2 hours at room temperature. Membranes were subsequently developed with SuperSignal West Pico or Femto substrate (Thermo Scientific) in an LAS-3000 imaging system (FujiFilm).

2.8.6 Infectivity assay

To assess the extent of infectivity rescue that would be achieved by each Vif variant, viral stocks were produced in the presence of APOBEC3G *186H* and *186R* expression plasmids. A total of 3 million HEK 293T cells were co-transfected with 500 ng of HIV pNL4-3Δ*vif*, 25 ng of each pCRV1 Vif expression plasmid, 100 ng of PTR600-A3G (*186H*) or PTR600-A3G (*186R*) expression plasmids and 100 ng of PTR600-GFP respectively, using 4 mg/ml of PEI. Additionally, HIV-1 pNL4-3 as well as pNL4-3 Vif expression plasmids were included as a controls.

HIV-1 infection was quantified using TZM-bl cells, which express a luciferase and β-galactosidase gene under the control of the HIV-1 LTR promoter. After 48 hours post-transfection viral supernatants were used to infect 2 million TZM-bl cells in black 96-well plates. Infections were carried out in triplicate. TZM-bl cells were exposed to virus particles for 48 hours before infectivity was measured using the Galacto-Star System for detecting β-galactosidase activity (ABI). Infectivity was assessed by chemiluminescence on the Wallac 1420 VICTOR (Perkin Elmer).

2.9 Statistical Analysis

APOBEC3G mRNA expression levels was compared between HIV-ve and HIV+ve individuals using the Wilcoxon Signed Rank Test. The association between *APOBEC3G* mRNA levels and viral loads and CD4 counts was determined using rank correlation tests. Fisher's Exact Test was used to test the association between HIV status and *H186R* genotypes. The genetic effect of the *H186R* mutation on viral loads and CD4 counts was determined using a Generalized Estimating Equation model (Liang and Zeger 1986; Zeger and Liang 1986; Ballinger 2004). This analysis takes into account longitudinal (repeated) measures for each participant. Viral loads were log transformed to guarantee normality of these outcomes. Decline in CD4 levels was determined and compared between *H186R* genotype groups. A Kaplan-Meier survival curve was performed to assess the difference between the genotype groups and a Cox Regression was used to acquire Hazard Ratios.

The association between APOBEC3G and -3F hypermutation levels and viral load and CD4 counts, as well as the association between the levels of hypermutation induced by APOBEC3G and -3F was determined by Spearman correlation tests. The Wilcoxon signed rank test was used to compare the number of hypermutations induced by APOBEC3G and -

3F. A one way ANOVA Kruskal-Wallis test was used to compare *APOBEC3G* mRNA expression levels, Vif diversity and APOBEC3G induced hypermutation levels between the three *H186R* genotype groups. The association between Vif diversity and viral loads and CD4 counts was also analysed using Spearman correlation tests.

The SAS statistical package version 9.1 (SAS Institute, Cary, NC, USA.) and GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, California, USA) was used to perform all statistical analyses and all graphs were generated using GraphPad Prism v5.01. *P*-value less than 0.05 were considered significant.

CHAPTER THREE

APOBEC3G Expression is Dysregulated in Primary HIV-1 Infection and a Polymorphic Variant Influences CD4+ T Cell Counts and Plasma Viral Load

3.1 Introduction

Increased expression of *APOBEC3G* mRNA may overcome the effects of Vif by providing a competitive advantage over time and the cumulative G-to-A hypermutations in the HIV genome, induced by APOBEC3G, may eventually incapacitate the virus and suppress viremia (Jin et al. 2005). Additionally, genetic variants of *APOBEC3G* may alter its function or level of expression thereby enhancing or diminishing its anti-HIV activity (Mariani et al. 2003; An et al. 2004; Pace et al. 2006).

Given that APOBEC3G is a key intrinsic antiretroviral host factor that possesses significant anti-HIV-1 activity *in vitro*, we reasoned that its antiviral effects *in vivo* might be particularly pronounced during the primary infection phase before adaptive immune responses become established. We therefore tested the hypothesis that high mRNA levels of *APOBEC3G* in peripheral blood mononuclear cells (PBMCs) of individuals at high risk for HIV-1 infection are associated with decreased likelihood of HIV-1 infection and that in seroconverters, increased *APOBEC3G* mRNA levels are associated with low viral set point and high CD4+ T cell counts during primary HIV-1 subtype C infection. We also investigated the effects of *APOBEC3G* genetic polymorphisms on HIV-1C pathogenesis in a South African cohort, in a population where the HIV-1 epidemic is severest.

3.2 Sample Selection

Figure 3.1 summarizes the study cohort and samples selected for real time PCR assays to study *APOBEC3G* expression and for sequencing and genotyping assays.

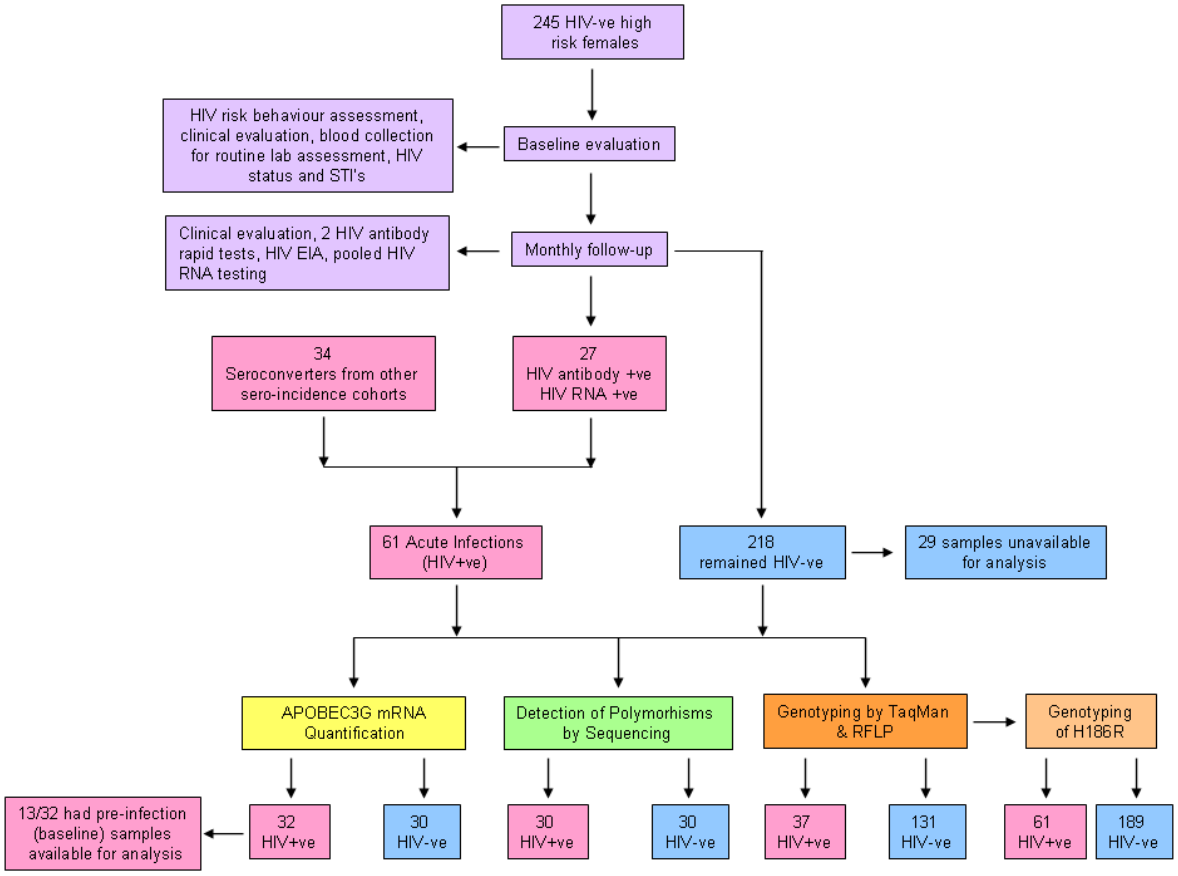


Figure 3.1. Outline of study cohort and experiments. HIV-positive samples are indicated by pink blocks and HIV-negative samples are indicated by blue blocks.

3.3 Quantification of *APOBEC3G* Expression

A significant number of *in vitro* studies suggest that APOBEC3G and other cytidine deaminases may constitute an important mechanism of cellular defense against viruses. However, there is paucity of data regarding the interplay between HIV-1 and *APOBEC3G* expression *in vivo*, particularly during primary infection when rapid viral replication occurs, followed by resolution of viremia and establishment of steady state equilibrium between the virus and the body's immune responses. We therefore investigated whether primary HIV-1C infection is associated with changes of *APOBEC3G* expression in PBMCs compared to HIV -ve samples.

Relative quantification was used to measure *APOBEC3G* mRNA levels in relation to the housekeeping gene *GAPDH*. Quantification of gene expression by the LightCycler is based on a standard curve for each gene. Standard curves were successfully generated for *APOBEC3G* (Figure 3.2) and *GAPDH* (Figure 3.3) from 10 fold serial dilutions of cDNA of known concentrations. A standard curve with a slope of approximately -3.3 indicates optimal PCR efficiency while a low error value of less 0.1 indicates minimal variation between standards and hence accuracy of generated standard curve (Roche Applied Science Technical Note No. LC 10/update 2003). PCR amplicons that are identical melt at the same temperature, therefore melting curves created by the LightCycler can confirm that amplification of each gene product was specific. Additionally, real-time PCR products were analysed on a 2% agarose gel to further confirm amplification specificity of *APOBEC3G* (Figure 3.2D) and *GAPDH* (Figure 3.3D). This also ruled out that a slight shift in the melting peak was due inaccurate amplification.

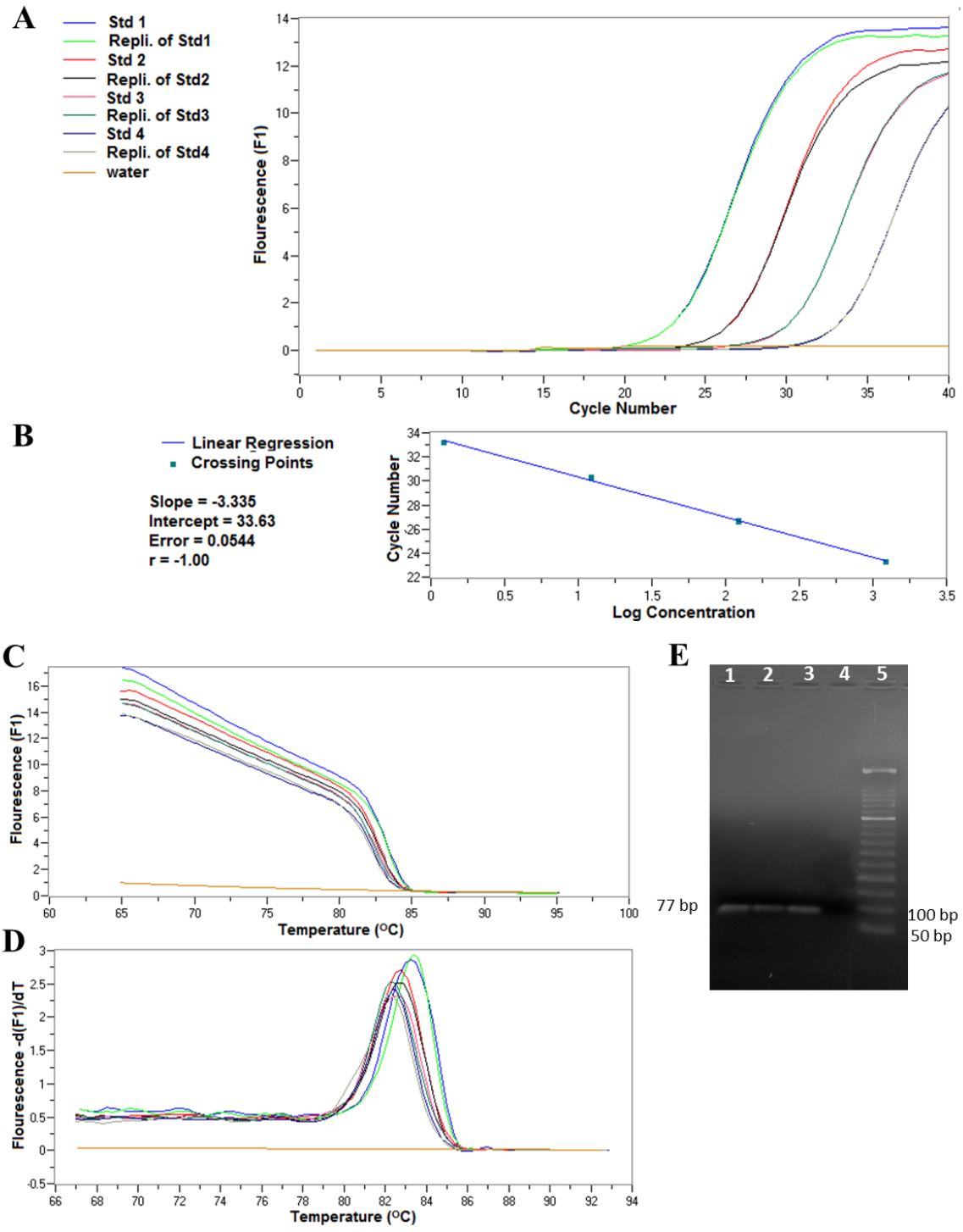


Figure 3.2 ***APOBEC3G*** **real time PCR amplification curves (A), standard curve (B), melting curves (C) melting peaks (D) and 2% agarose gels (E). A slope of -3.3 indicates optimal PCR efficiency while an error value of 0.05 indicates accuracy of the standard curve. Equal melting temperatures of standards confirmed specificity of the PCR. On the agarose gel, lanes 1-3 depict 77 base pair amplicons which further confirmed PCR specificity, lane 4 is the negative control and lane 5 a 50 base pair DNA ladder.**

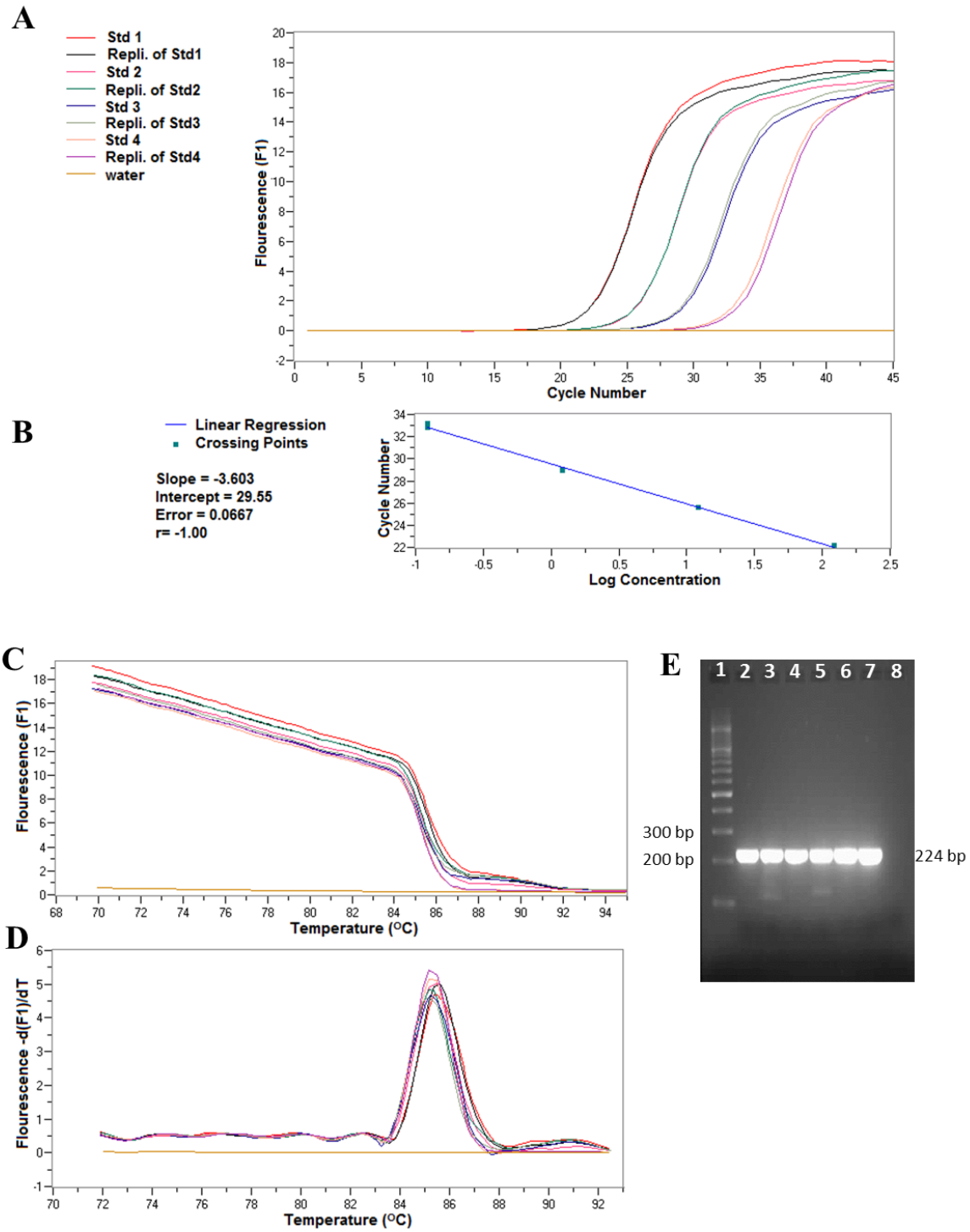


Figure 3.3 ***GAPDH*** **real time PCR amplification curves (A), standard curve (B), melting curves (C) melting peaks (D) and 2% agarose gels (E). A slope of -3.6 indicates optimal PCR efficiency while an error value of 0.06 indicates accuracy of the standard curve. Identical melting temperatures of standards confirmed specificity of the PCR. On the agarose gel, lane 1 is a 100 base pair DNA ladder, lanes 2-7 depict the 224 base pair amplicons which further confirmed PCR specificity, lane 8 is the negative control.**

3.3.1 Analysis of *APOBEC3G* Expression Levels in HIV-ve and HIV+ve Samples

Comparison of *APOBEC3G* mRNA levels between HIV-ve and HIV+ve subjects within 12 months of infection (primary infection) showed that *APOBEC3G* levels were significantly higher in HIV-ve individuals than in HIV+ve individuals ($p<0.0001$) (Figure 3.4A). Additionally, comparison of *APOBEC3G* expression levels in matched pre- and post-infection samples of seroconverters also showed that *APOBEC3G* expression was significantly higher before seroconversion ($p<0.0001$) (Figure 3.4B). Further, there was no significant difference in *APOBEC3G* levels when compared between individuals who are persistently seronegative and pre-infection samples of seroconverters (Figure 3.4C). Comparison of *APOBEC3G* mRNA levels at various time points post-infection (Figure 3.4D) showed no significant change in expression levels over time.

There is conflicting data on the relationship between *APOBEC3G* mRNA levels in PBMCs versus plasma viral load and CD4 cell counts in chronic HIV-1 infection (Jin et al. 2005; Cho et al. 2006; Gandhi et al. 2008). We thus next investigated whether there is a correlation between *APOBEC3G* mRNA levels and HIV-1 plasma viral load (Figure 3.5A).and CD4 cell counts (Figure 3.5B) during primary HIV-1 infection and found no association between these factors.

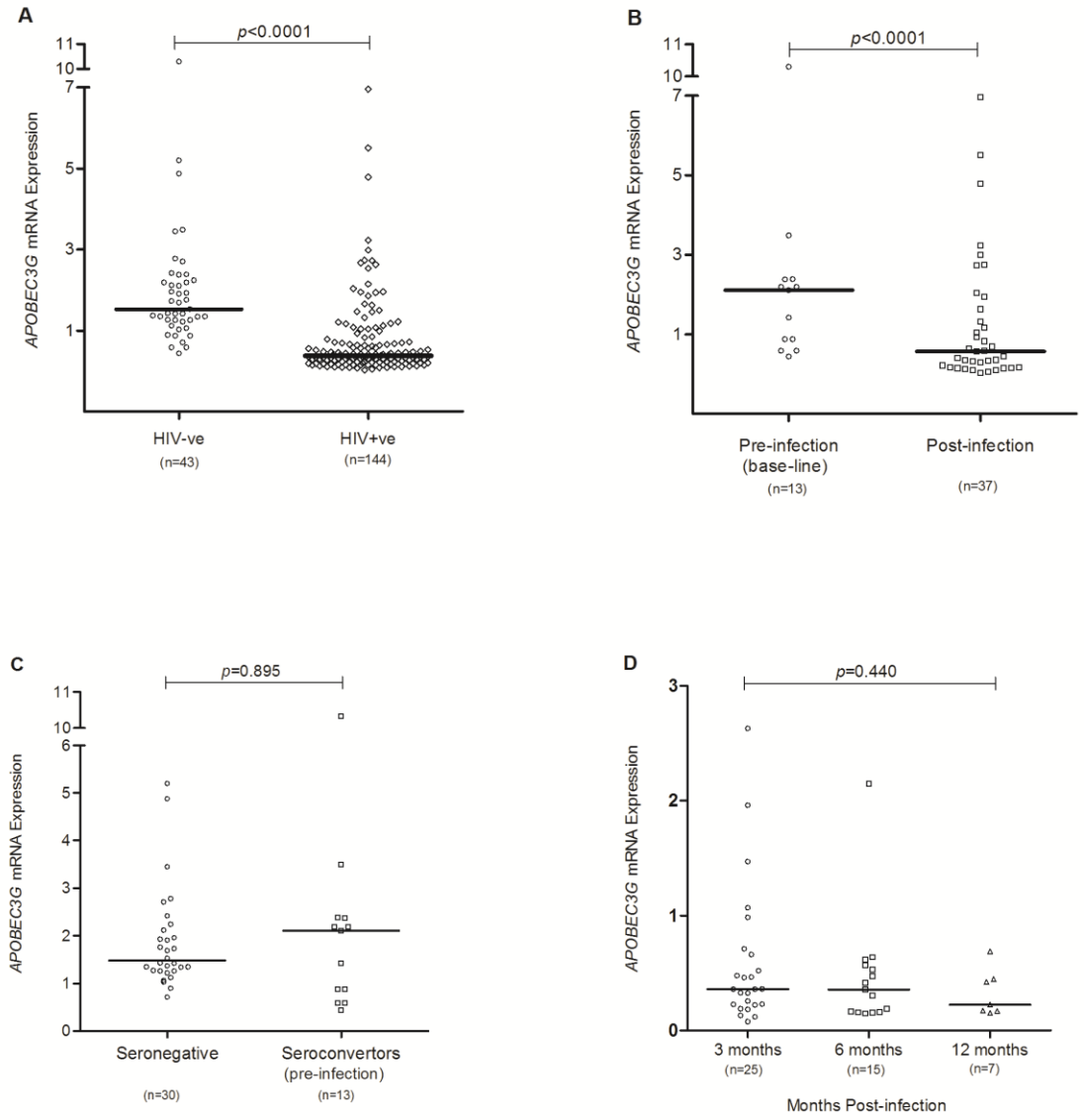


Figure 3.4 Comparison of *APOBEC3G* mRNA levels between [A] HIV-uninfected and HIV-infected participants, [B] pre-infection and post-infection samples of HIV-infected individuals, [C] persistently seronegative individuals and pre-infection samples of seroconverters and [D] longitudinal post-infection samples. In [B], the post-infection column represents post-infection samples from three time points for each individual. These time points are at 3, 6 and 12 months after infection. A generalized estimating equation analysis model was used to analyze these data, as this model takes into account longitudinal (repeated) measures for each participant.

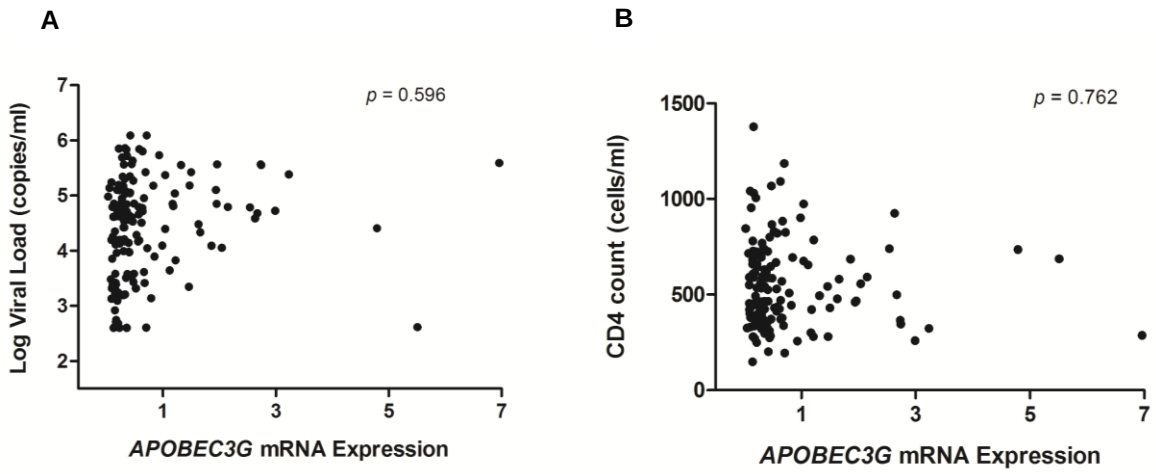


Figure 3.5 Relationship between *APOBEC3G* expression levels and [A] viral load and [B] CD4 counts.

PMBCs constitute a mixture of cells from different lineages and therefore it was important to assess APOBEC3G expression in the different cell types that constitute PBMCs. We were unable to perform extensive experiments to characterize this due to the scarcity of samples as well as low cell counts, of approximately 5 million cells, of the samples which were available. However, we compared the distribution of APOBEC3G in PBMCs and CD4 cells from 8 healthy controls as well as in 8 chronically HIV infected subjects for which samples were available. CD4 cells were isolated by negative selection and purity confirmed by flow cytometry thereafter (Figure 3.6).

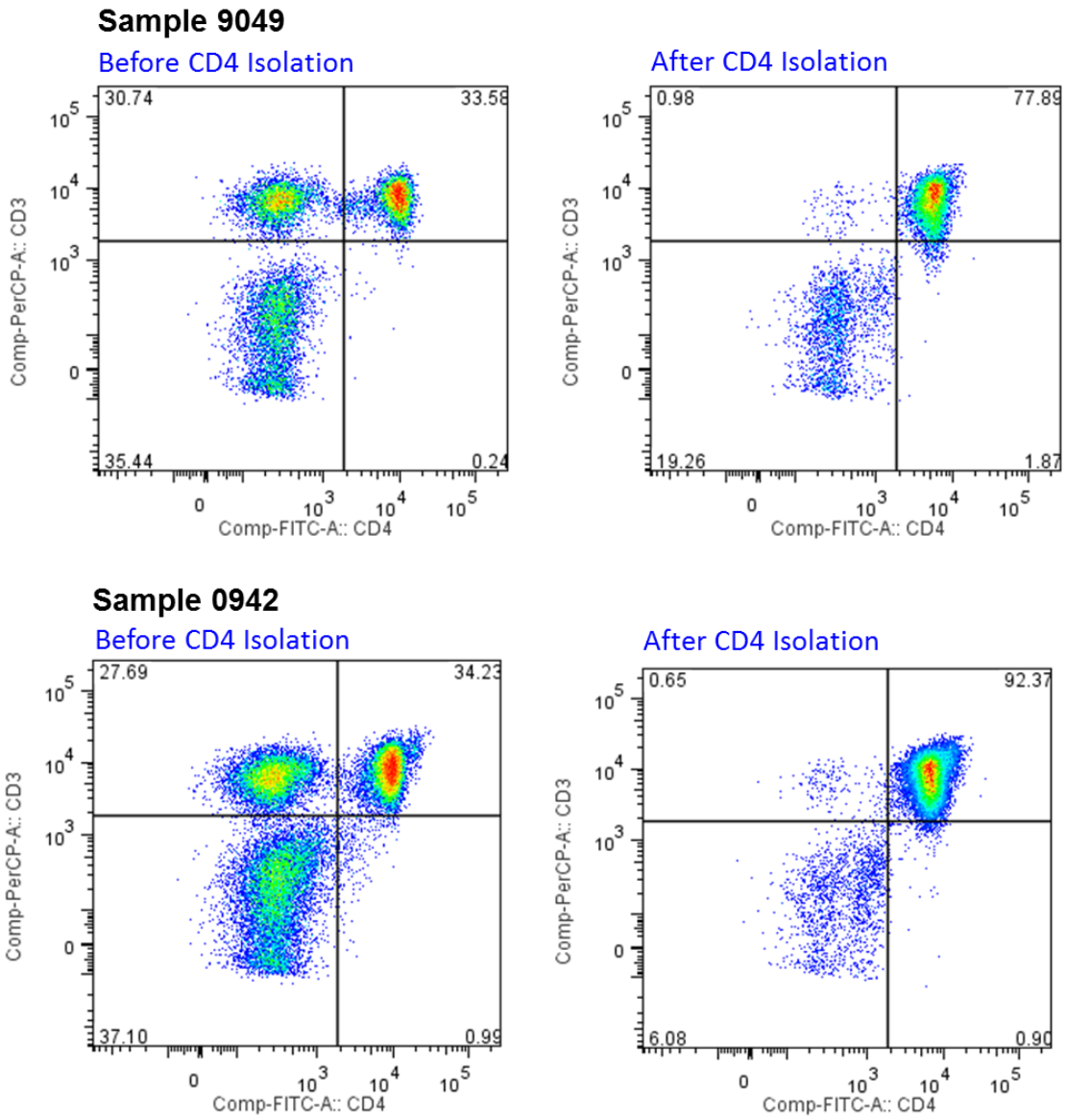


Figure 3.6 Dot plot resulting from a flow cytometry analysis following isolation of CD4+ T cells from PBMCs of two representative samples, using the CD4+ T Cell Isolation Kit II and MS column. Cells were fluorescently stained with CD3 PerCP-A and CD FITC-A. Cell debri and dead cells were excluded based on scatter signals.

Our results suggested that *APOBEC3G* mRNA expression was enriched in the CD4+ T cell subset. *APOBEC3G* mRNA levels were higher in CD4+ T cells as compared to levels in PBMCs in the 8 healthy donors (p=0.08) and in 8 chronically infected subjects (p=0.02) (Figure 3.7). Although sample numbers were too few, this data suggested that *APOBEC3G* levels are augmented in cells primarily targeted by HIV-1 as a crucial factor in the anti-viral immune response. However, further studies are required to investigate *APOBEC3G* expression in a larger sample cohort and in other cell subsets.

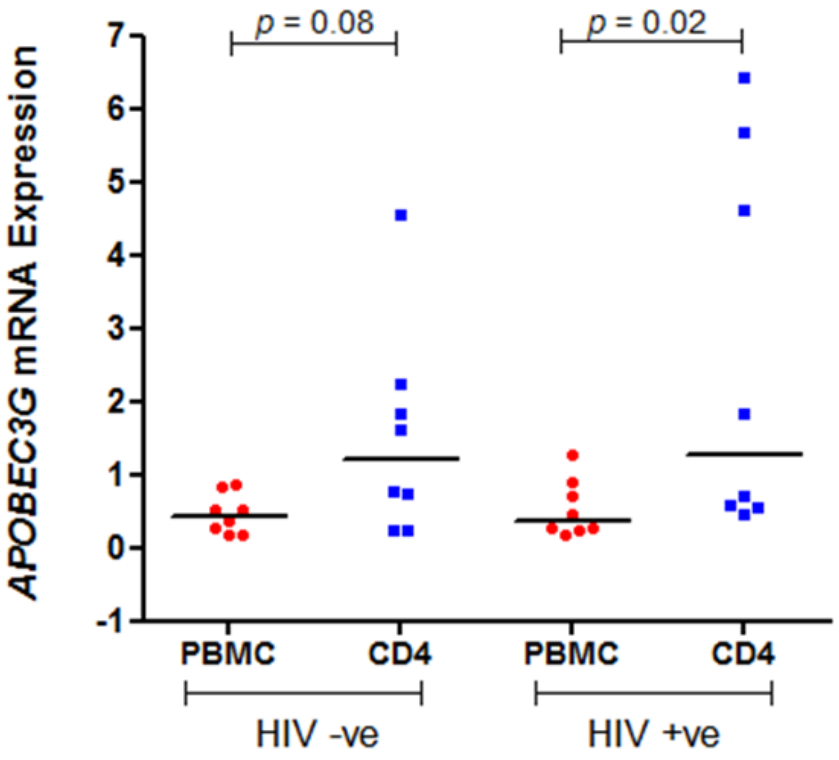


Figure 3.7 Comparison of *APOBEC3G* mRNA levels between total PBMCs and CD4 T cells in HIV-ve and HIV+ve individuals.

3.4 Resequencing of *APOBEC3G*

The *APOBEC3G* gene was amplified and sequenced in separate fragments using several specific amplification primers that covered all regions of the gene. PCR conditions (specifically $MgCl_2$ concentrations and primer annealing temperatures) were optimized for each amplification primer pair before samples were processed for sequencing (Figure 3.8).

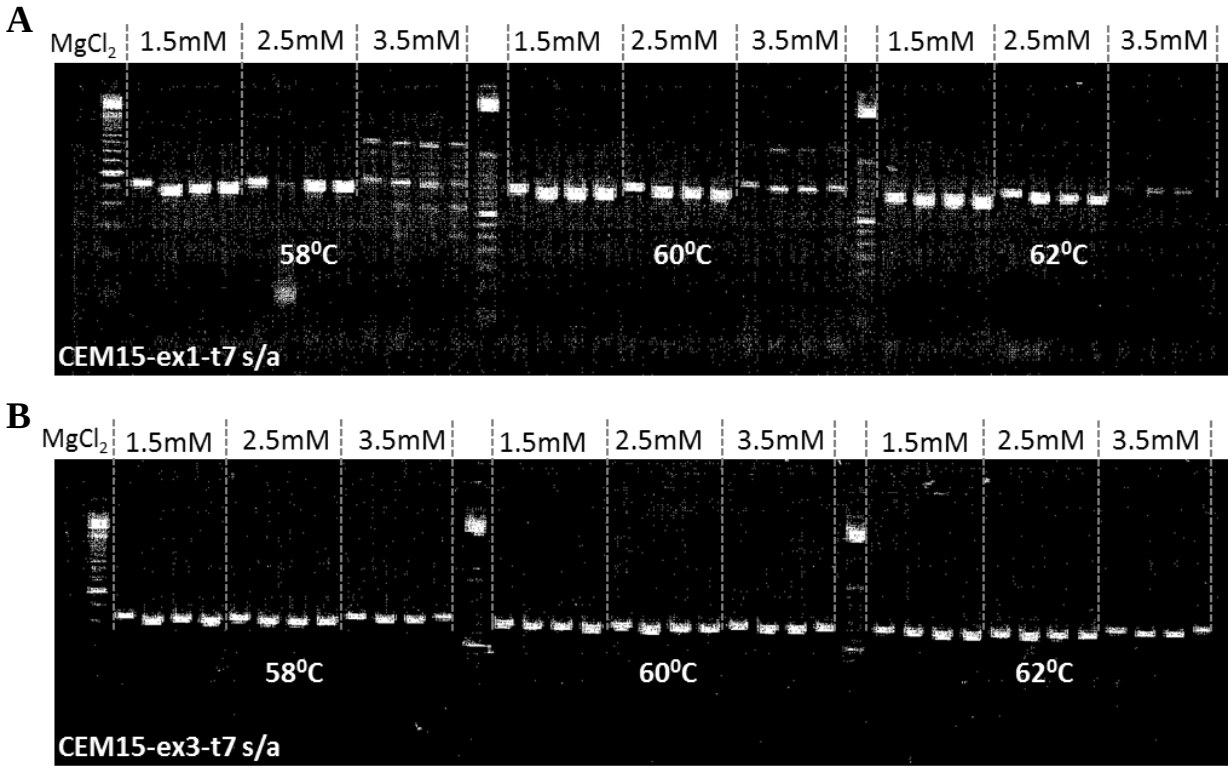


Figure 3.8 Amplification of *APOBEC3G*. Representative agarose gels depicting optimization of PCR conditions for 2 of 11 primers used i.e. [A] cem15-ex1-t7 which amplified a 470 base pair region and [B] cem15-ex3-t7 which amplified a 446 base pair region. $MgCl_2$ concentration of 1.5mM and annealing temperature of 60°C was selected for both primers.

3.4.1 *APOBEC3G* Variants

APOBEC3G genetic variants have not been described in African populations. By re-sequencing a subset of 30 HIV+ve and 30 HIV-ve samples we identified 24 SNPs within *APOBEC3G* in our cohort, which are described in Table 3.1. Sixteen of these SNPs were described previously and 8 were novel. Further, (An et al. 2004) described 7 SNPs within *APOBEC3G* in a United States based study cohort, 4 of which were identified in our study cohort. Frequencies of these SNPs in our cohort were similar to those of the African American group in the United States based cohort (Table 3.1).

Following preliminary analysis of the identified SNPs, 6 were selected to be genotyped in a larger group of samples, based either on their high minor allele frequencies, association with viral load and CD4 counts or previous description in the American study cohort (An et al. 2004). Genotyping was performed by predesigned TaqMan genotyping assays (Figure 3.9) and an RFLP assay (Figure 3.10).

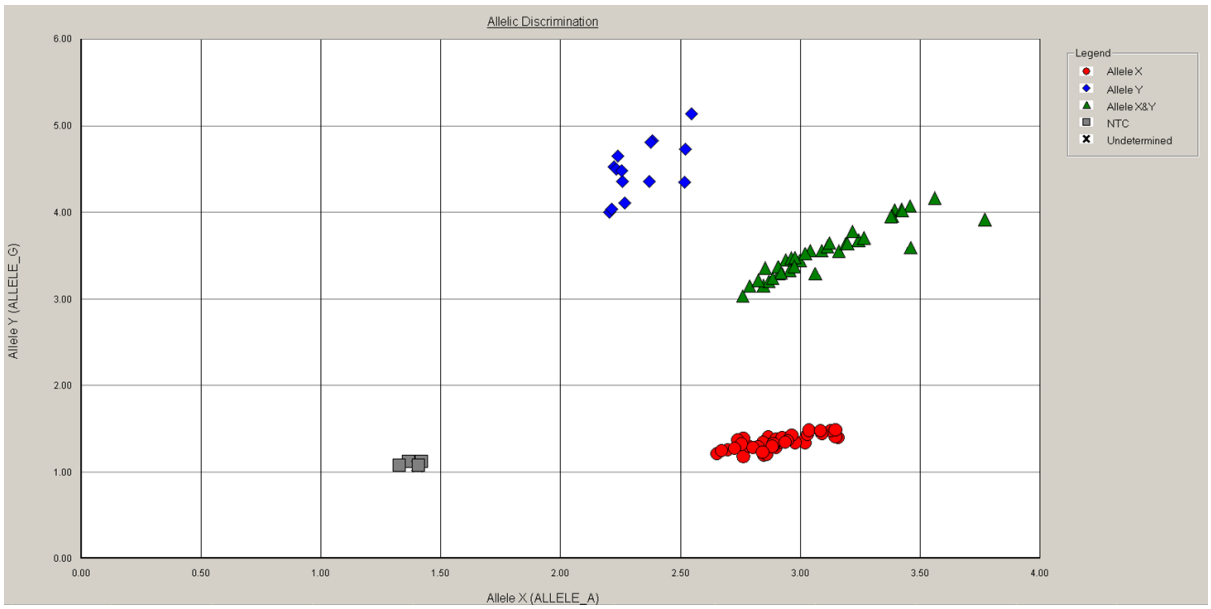


Figure 3.9 A typical allelic discrimination plot obtained from a TaqMan genotyping assay of SNP ID *rs8177832* (*H186R*). Samples of similar genotype cluster together. The clustering of points can vary along the horizontal axis (Allele X), vertical axis (Allele Y), or diagonal (Allele X/Allele Y) due to differences in the extent of reporter dye fluorescent intensity after PCR amplification.

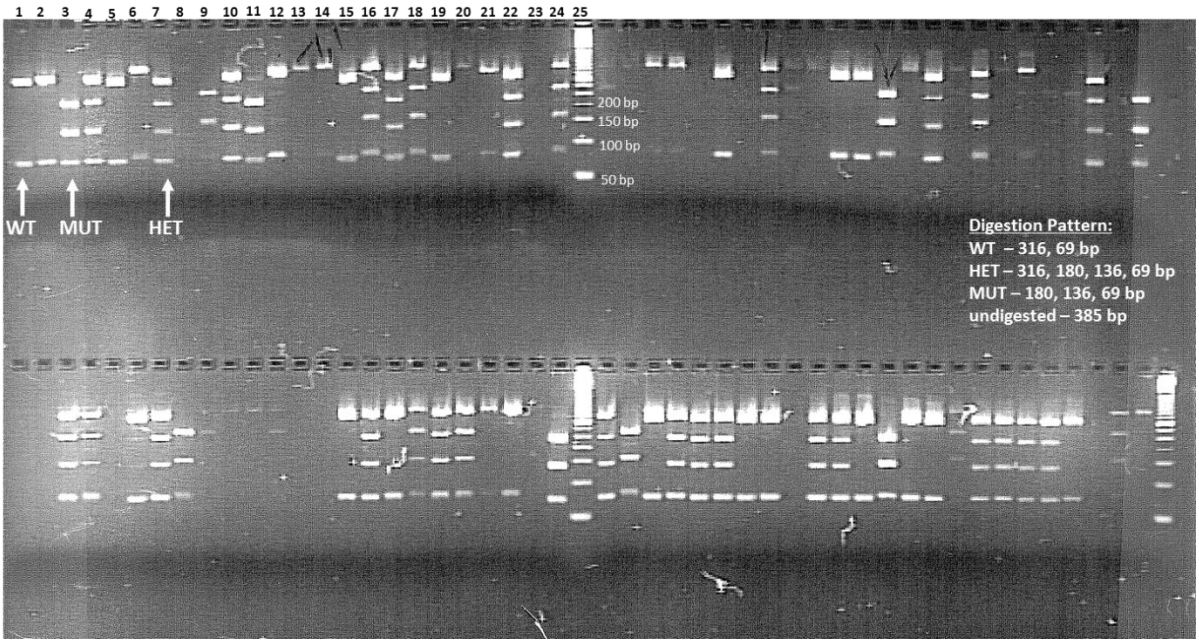


Figure 3.10 Representative agarose gel depicting the RFLP assay and digestion pattern used to identify the genotypes of SNP ID *rs5750743.* Lanes 1, 3 and 7 represent samples whose genotypes for *rs5750743* are wild type (WT), mutant (MUT) and heterozygote (HET) respectively. Lane 25 is a 50 base pair DNA ladder.

3.4.2 Effects of *H186R* mutation on Primary HIV Pathogenesis

The codon changing variant, *H186R* (rs8177832), in exon 4, had a frequency of 0.307, and was analyzed further, as the *186R* allele was previously shown to have AIDS accelerating effects (An et al. 2004).

H186R genotypes were determined for 250 subjects (61 HIV+ve and 189 HIV-ve). We tested the association between HIV status and *H186R* genotypes and found no significant difference in the distribution of *H186R* genotypes between HIV-ve and HIV+ve subjects (p=0.5838) (Figure 3.11).

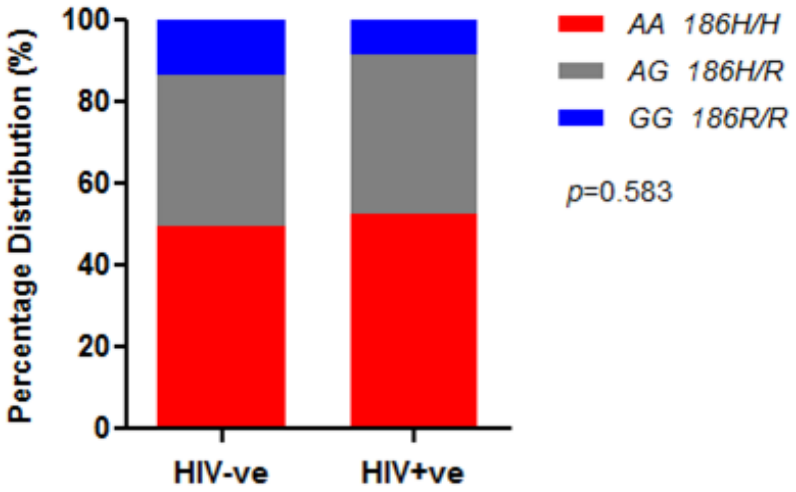


Figure 3.11 Distribution of *H186R* genotypes between HIV-ve and HIV+ve subjects

We also compared viral loads (Figure 3.12A) and CD4+ T cell counts (Figure 3.12B) between genotypes. Viral load and CD4 measurements were classified into 0-3 months post infection and 3-12 months post infection time intervals, to identify possible differences between genotypes during acute infection (0-3 months) and early chronic infection (3-12 months). There was an overall significant difference in viral loads between genotypes (p=0.0097) across both time periods (Figure 3.12A). In addition, during the first 3 months of infection there was a significant difference in viral loads between the wild type reference group (*AA*) and those homozygous for the mutation (*GG*) (p=0.0362), with the *GG* genotype having higher viral loads than the *AA* genotype. There was also an overall significant difference in CD4 counts between the *AA* reference group and the *GG* genotypes (p=0.0081) (Figure. 3.12B). Further, during the first 3 months of infection, there was a significant difference in CD4 counts between *AA* and *GG* genotypes (p=0.0006), with *GG* genotype having lower CD4 counts than the *AA* genotype.

Table 3.1 ***APOBEC3G*** **SNPs and their minor allele frequencies**

NCBI dbSNP ID[a]	Location	Nucleotide Change	Amino Acid Change	Hardy Weinberg	Minor Allele Frequency	Minor Allele Frequency from the work of An. et.al [a]			
						SNP #	AA	EA	CA
rs5757463	5' regulatory region	c/g/t		0.419	0.089	*571G/C*	0.091	0.063	0.116
rs7291971	5' regulatory region	c/g		0.858	0.016				
rs6519166	5' regulatory region	a/c		0.185	0.111				
rs8142124	5' regulatory region	c/t		0.574	0.246				
rs5750743 [c]	5' regulatory region	c/g		0.966	0.327	*90C/G*	0.319	0.340	0.263
rs8177832 [d]	exon 4	a/g	*H186R*	0.040	0.307	*H186R*	0.368	0.029	0.074
rs17496046 [d]	exon 6	c/g	*Q275E*	0.028	0.169				
rs11545130	exon 7	c/t	*L371L*	0.846	0.019				
rs34300092	intron 2	a/g		0.300	0.089				
rs6001417 [d]	intron 3	c/g		0.030	0.303	*197193T/C*[e]	0.368	0.029	0.086
rs3736685 [d]	intron 3	c/t		0.004	0.151				
rs17537581	intron 6	a/g		0.112	0.454				
rs17537574	intron 6	a/g		0.030	0.033				
rs17537588	intron 7	a/g		0.722	0.031				
rs35342554	3' region	c/t		0.155	0.290				
rs35228531 [d]	3' region	c/t		0.519	0.207				
cem15-ex1-snp1-R[f]	intron 1	a/g		0.357	0.078				
cem15-ex3-snp1-R[f]	exon 4	a/g		0.060	0.164				
cem15-ex6-snp1-R[f]	intron 7	a/g		0.846	0.019				
cem15-In13-snp3-R[f]	intron 2	a/g		0.300	0.089				
cem15-In13-snp1-Y[f]	intron 2	c/t		0.401	0.073				
cem15-pm1-snp1-Y[f]	5' regulatory region	c/t		0.344	0.082				
cem15-3u-snp1-R[f]	intron 7	a/g		0.581	0.048				
cem15-3u-snp2-W[f]	intron 7	a/t		0.518	0.056				

NCBI dbSNP ID, National Center for Biotechnology Information SNP database reference number; RFLP=restriction fragment length polymorphism; SNP=single-nucleotide polymorphism. [a]Comparisons with results from a published study, [b]SNPs identified by resequencing, [c]SNP genotyped by RFLP assays, [d]SNPs genotyped by TaqMan assays, [e]T/C denotes complementary alleles, [f]Novel SNPs.

The association with CD4+ T cell count is consistent with observed genotype effects on viral load. At 3-12 months post-infection, the *GG* genotype maintained its association with higher viral loads and lower CD4 counts when compared to the other genotypes. Additionally, the heterozygous *AG* genotype also displays significantly higher viral loads (p=0.0005) and lower CD4 counts (p=0.0078) when compared to the reference *AA* genotype at this later time period.

Kaplan Meier survival analysis (Figure 3.12C) shows that those who have the *GG* genotype have a significantly shorter time to CD4 count < 350 cells/µl. The hazard ratio (HR) for the *GG* group as compared to the *AA* or *AG* group is 3.84 (95% CI 1.43 – 10.35, p=0.0078).

Comparisons to viral load and CD4 counts were also performed for other SNPs highlighted in Table 3.1. *rs6001417* and *rs35228531* demonstrated significant associations with viral load and CD4 counts. *rs6001417* is actually in near complete linkage disequilibrium with *H186R* (D' = 0.95, r^2 = 0.9)1 and therefore displayed similar allele frequencies and associations.

Analysis of the *rs35228531* mutation indicated that individuals who are heterozygous for the mutation (*CT*) had significantly higher viral loads and lower CD4 levels over 12 months of infection as compared to the wild type group (Figure 3.13A and Figure 3.13B). A Kaplan Meier survival analysis (Figure 3.13C) shows that those who have the *CT* genotype progress faster to a CD4 count < 350 cells/µl. The hazard ratio (HR) for the *CT* group as compared to the *CC* group is 2.28 (95% CI 0.85 – 6.07, p=0.1005). Although this was not statistically significant a trend is observable, as only 22.9% of the *CC* group progressed to a CD4 count < 350 cells/µl while 47.1% of individuals with the CT genotype progressed to a CD4 count < 350 cells/µl.

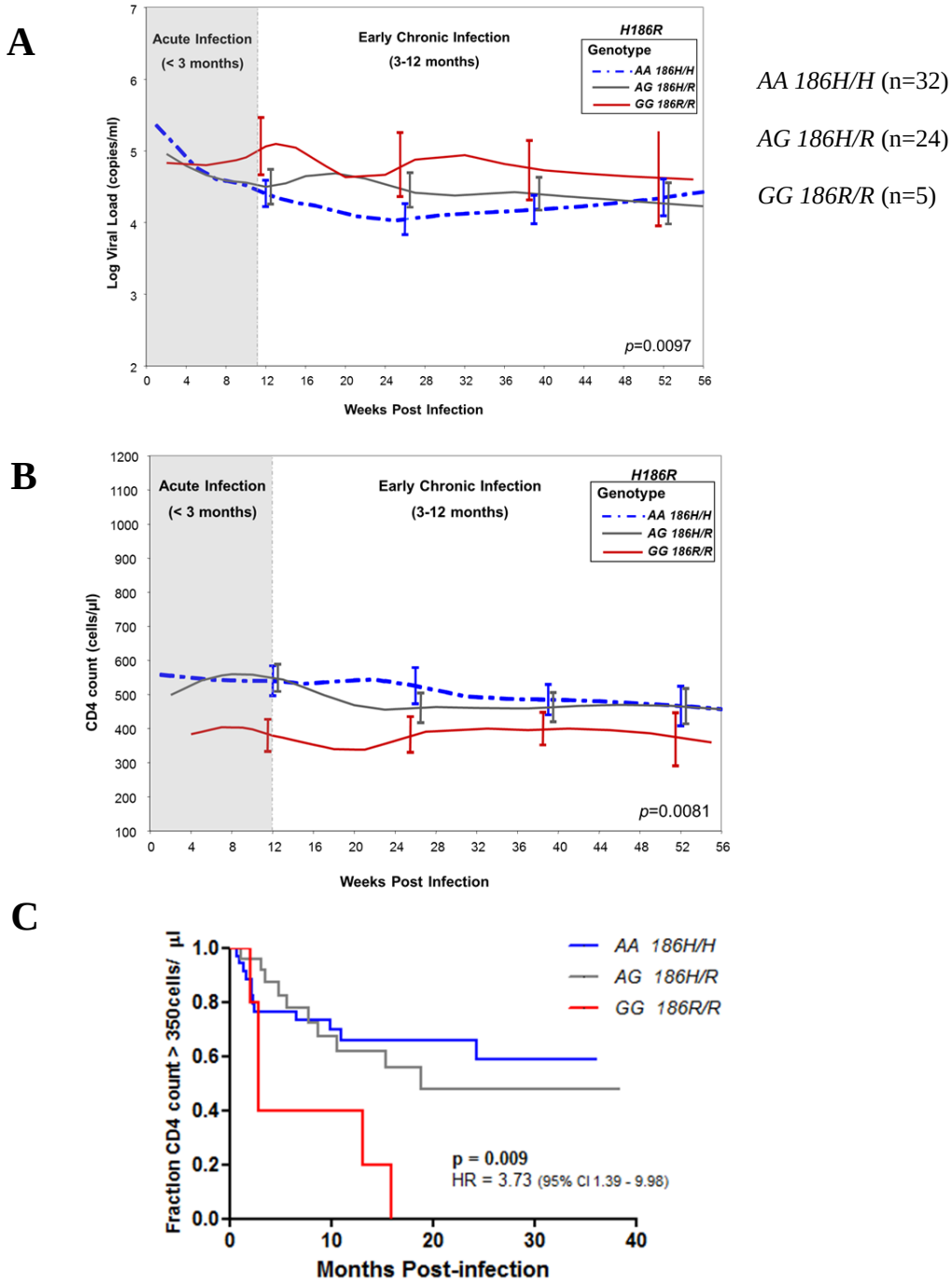
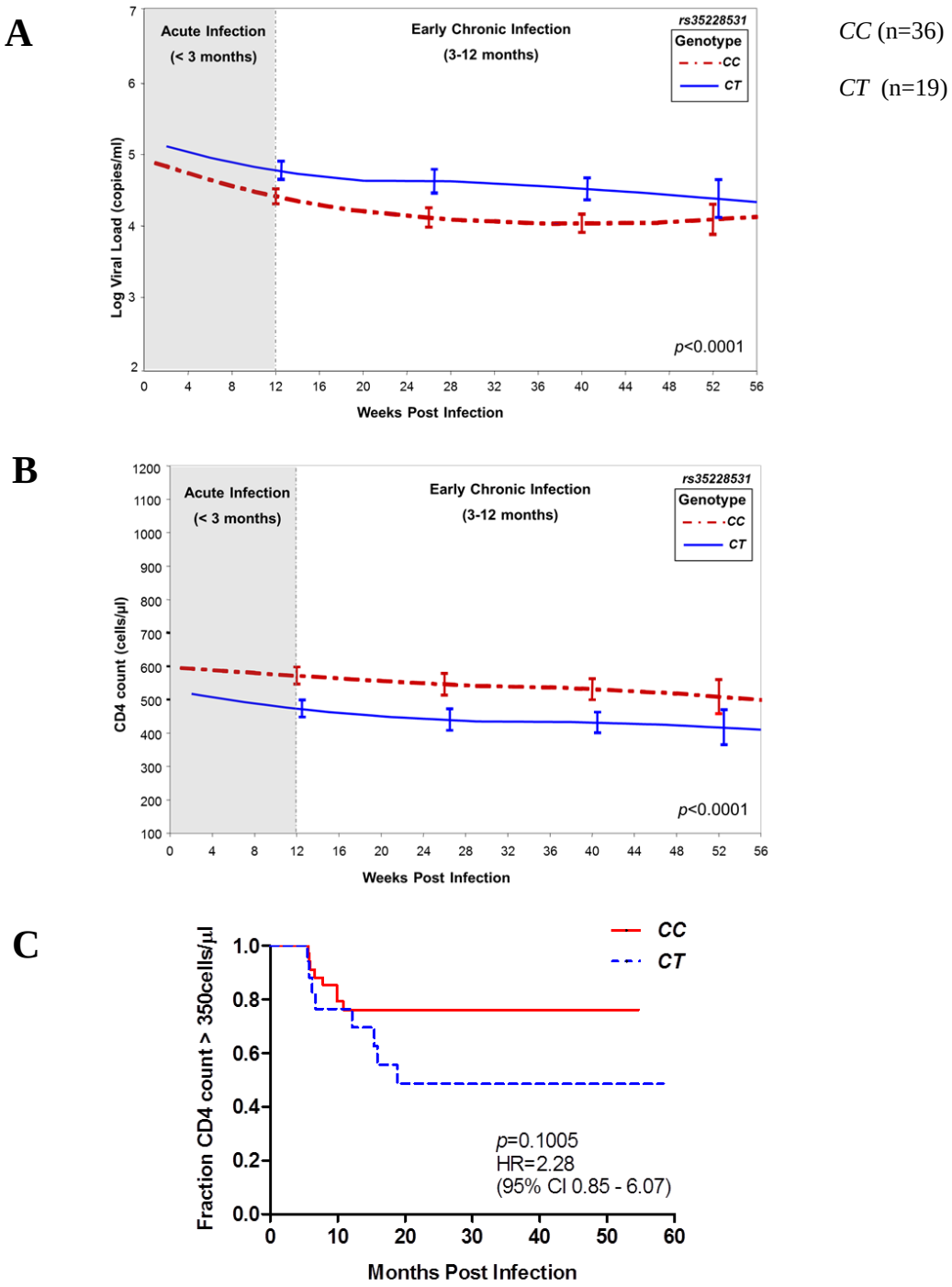


Figure 3.12 Comparison of viral loads and CD4+ T-cell counts between *H186R* genotypes (A and B) over 0–12 months after infection using a locally weighted scatterplot smoothing model. Measurements were classified into 0–3 months after infection and 3–12 months after infection time intervals to identify differences between the genotypes during acute infection and early chronic infection. Confidence intervals are shown by the vertical lines and overall P values are indicated. Kaplan–Meier survival curve (C) were performed to assess the difference between the *H186R* genotype groups in the event of a CD4+ T-cell count less than 350 cells/µl for more than two consecutive visits. Cox regression was used to acquire hazard ratio.



CC (n=36)

CT (n=19)

Figure 3.13 Comparison of viral loads and CD4+ T-cell counts between *rs35228531* genotypes (A and B) over 0–12 months after infection using a locally weighted scatterplot smoothing model. Measurements were classified into 0–3 months after infection and 3–12 months after infection time intervals to identify differences between the genotypes during acute infection and early chronic infection. Confidence intervals are shown by the vertical lines and overall P values are indicated. Kaplan–Meier survival curve (C) were performed to assess the difference between the *rs35228531* genotype groups in the event of a CD4+ T-cell count less than 350 cells/µl for more than two consecutive visits. Cox regression was used to acquire hazard ratio.

We also performed an analysis to determine whether the *H186R* mutation has an influence on the expression levels of APOBEC3G. However, we found no association between *H186R* genotypes and APOBEC3G mRNA expression levels (p=0.695) (Figure 3.14).

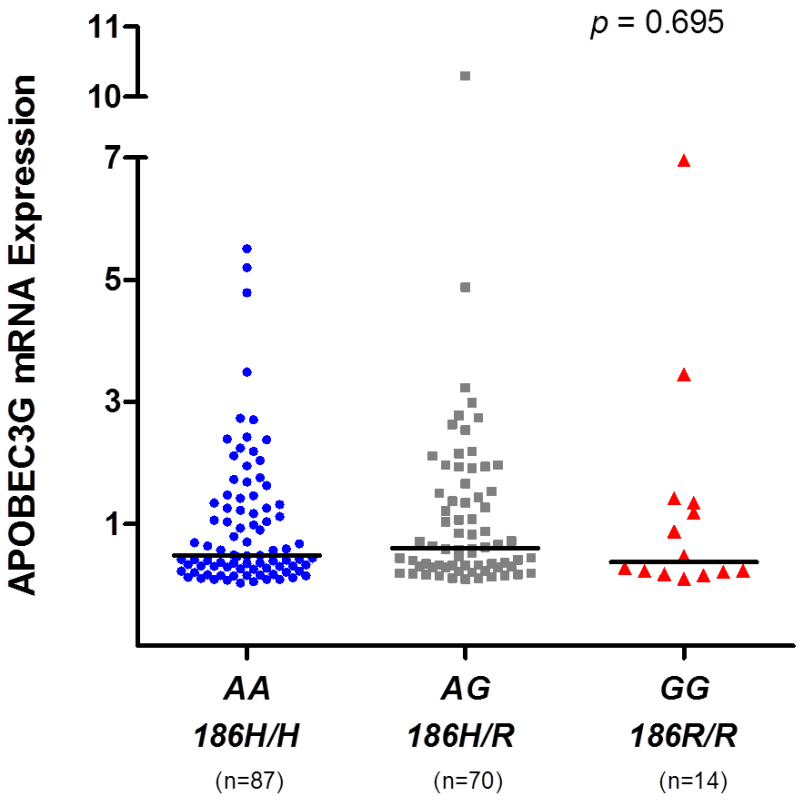


Figure 3.14 Comparison of APOBEC3G levels between *H186R* genotype groups

CHAPTER FOUR

Hypermutation within HIV-1 envelope and its Association with APOBEC3G Genetic Variants

Guanine-to adenine (G-to-A) transitions in retroviruses have an overall expected frequency of 5% of the number of Gs (Koulinska et al. 2003). The phenomenon referred to as G to-A hypermutation occurs when G-to-A substitutions are inordinate and exceed all other mutations in a viral sequence**.** Editing of minus-strand viral DNA by APOBEC3G, as well as APOBEC3F, is the most common mechanism by which G-to-A hypermutation is induced (Lecossier et al. 2003; Mangeat et al. 2003; Zhang et al. 2003; Harris and Liddament 2004). APOBEC3G and -3F differ in their preferred target sequence for mutation. APOBEC3G favours the CC dinucleotide (resulting in GG to AG transition) while APOBEC3F prefers CT dinucleotide (resulting in GA to AA transition) (Mangeat et al. 2003; Zhang et al. 2003; Bishop et al. 2004; Liddament et al. 2004; Yu et al. 2004).

Our previous data suggests that polymorphic variants of APOBEC3G may predispose to accelerated HIV-1 disease progression, however the mechanism remains unknown. It is possible that genetic variation within APOBEC3G may thwart HIV-1 DNA editing *in vivo* and that optimal function of APOBEC3G may further require unhindered conditions, such as defective *vif* activity.

For this reason we chose to assess hypermutation within HIV-1 *env* from patients whose *H186R* genotype were known. This region was selected for its high rate of hypermutation (Suspene et al. 2006). Studies show that regions of the HIV-1 genome at which reverse transcription is initiated i.e. the PPTs as well as the PBS are less vulnerable to deamination by APOBEC3G as they remain single stranded for the least amount time. Additionally, regions

located immediately downstream of the PPTs and PBS remain protected against APOBEC3G however the degree of protection decreases gradually further downstream (Wurtzer et al. 2006). Hence, the regions such as *gag*, are less prone to editing, while *env* is highly susceptible as this region is located immediately upstream of the 3' PPT priming site (Yu et al. 2004) (Figure 1.15).

4.1 Sample Selection

A subset of 22 participants from the Centre for the AIDS Programme of Research in South Africa (CAPRISA) acute infection study cohort (van Loggerenberg et al. 2008) was assessed for hypermutation in HIV-1 *env*. We chose to study proviral DNA isolated from PBMCs at the 36 month post infection time point as APOBEC induced hypermutations are accumulative and may be prominent in archived proviral DNA. The 22 individuals were selected based on their APOBEC3G *H186R* genotypes which we previously characterised. Patient characteristics are described in Table 4.1.

Table 4.1 Characteristics of 22 participants selected for the study of HIV-1 *env*

PID	*H186R*	Viral Load (copies/ml)	CD4 (cells/µl)
CAP40	*AA – 186 H/H*	2,030	685
CAP45	*AA – 186 H/H*	517	1043
CAP65	*AA – 186 H/H*	28,100	214
CAP84	*AA – 186 H/H*	1,710	575
CAP137	*AA – 186 H/H*	24,600	173
CAP188	*AA – 186 H/H*	185,000	352
CAP217	*AA – 186 H/H*	48,900	486
CAP225	*AA – 186 H/H*	22,200	505
CAP278	*AA – 186 H/H*	1,340	635
CAP281	*AA – 186 H/H*	400	809
CAP08	*AG – 186 H/R*	40,700	175
CAP61	*AG – 186 H/R*	400	601
CAP85	*AG – 186 H/R*	242,000	270
CAP211	*AG – 186 H/R*	2,470	424
CAP220	*AG – 186 H/R*	400	546
CAP229	*AG – 186 H/R*	15,400	802
CAP274	*AG – 186 H/R*	125,000	468
CAP282	*AG – 186 H/R*	641	452
CAP174	*GG – 186 R/R*	414,000	131
CAP256	*GG – 186 R/R*	55,000	274
CAP262	*GG – 186 R/R*	1,060	383
CAP280	*GG – 186 R/R*	37,500	355

4.2 Amplification of HIV-1 *env*

Successful amplification of HIV-1 *env* was confirmed by gel electrophoresis of PCR amplicons on a 1% agarose gel. The presence of a 3141 base pair product indicated a specific and successful amplification (Figure 4.1).

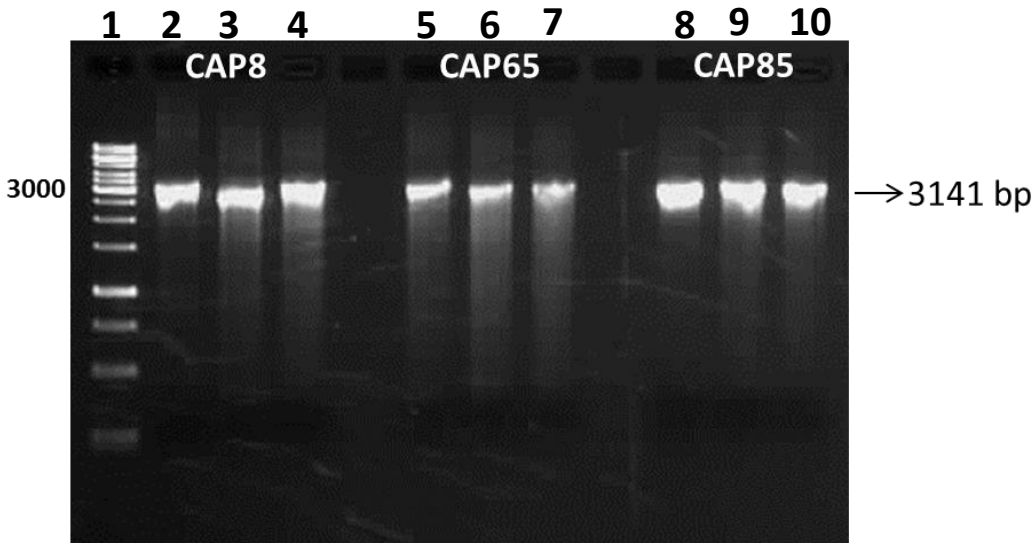


Figure 4.1 Representative agarose gel depicting successful amplification of HIV-1 *env* in patient samples. Lane 1 is a 1 kilo base DNA ladder. The 3141 base pair HIV-1 *env* amplicons are represented in lanes 2-4 from PID CAP8, lanes 5-7 from CAP65 and lanes 8-10 from CAP85.

4.3 Phylogenetic characterisation of subtype C HIV-1 *env*

HIV-1 displays significant diversity, the extent of which evolves over time. The most outstanding changes in diversity take place within the envelope glycoproteins (Env). HIV-1 Env glycoproteins can differ by around 35% across subtypes and HIV-1 Env of subtype C origin has been well studied and characterised (Coetzer et al. 2007; Lynch et al. 2009; Singh et al. 2009; Archary et al. 2010).

In this study we amplified and directly sequenced HIV-1 *env* for the purpose of assessing APOBEC3G induced hypermutation within the gene as described previously (Land et al. 2008). By phylogenetic analysis, we confirmed that the 22 study samples were of subtype C origin. A neighbour joining phylogenetic tree assembled with sequences of the 22 study samples and reference sequences of HIV-1 group M subtypes A to K shows that all study samples clustered with HIV-1 subtype C reference sequences (Figure 4.2).

The mean overall population diversity at the amino acid level was 15.6% but increased to 40% at the nucleotide level. CAP84 displayed the greatest degree of divergence from the consensus subtype C reference (Figure 4.3).

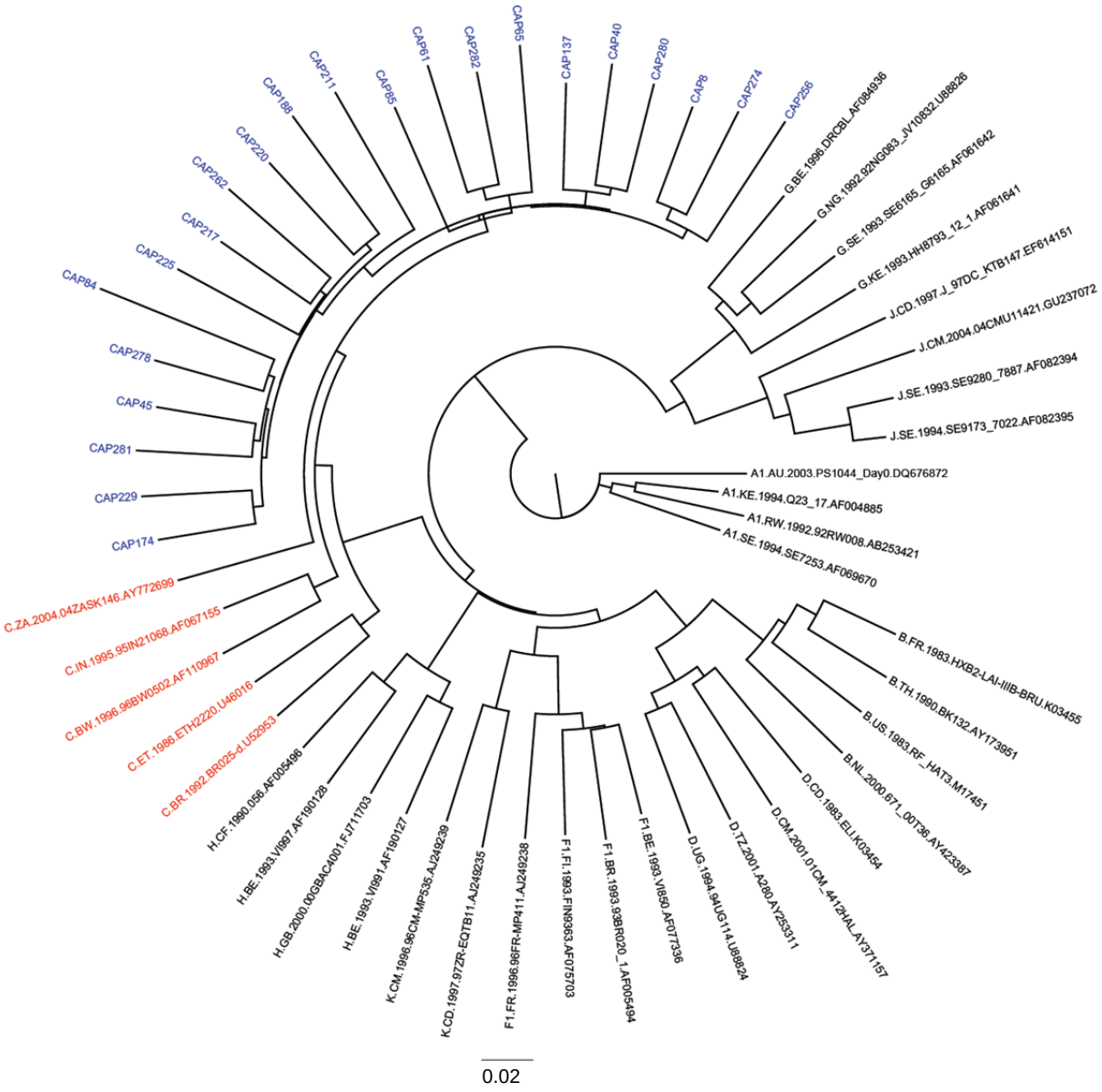


Figure 4.2 Neighbour joining phylogenetic tree of HIV-1 Env sequences. Consensus sequences of 22 samples and reference sequences of HIV-1 group M subtypes A to K (obtained from the Los Alamos National Laboratory HIV database (http://www.hiv.lanl.gov) are included. All samples (blue) cluster with subtype C reference sequences (red). The tree was constructed with Los Alamos National Laboratory HIV database treemaker tool and Fig Tree v1.3.1.

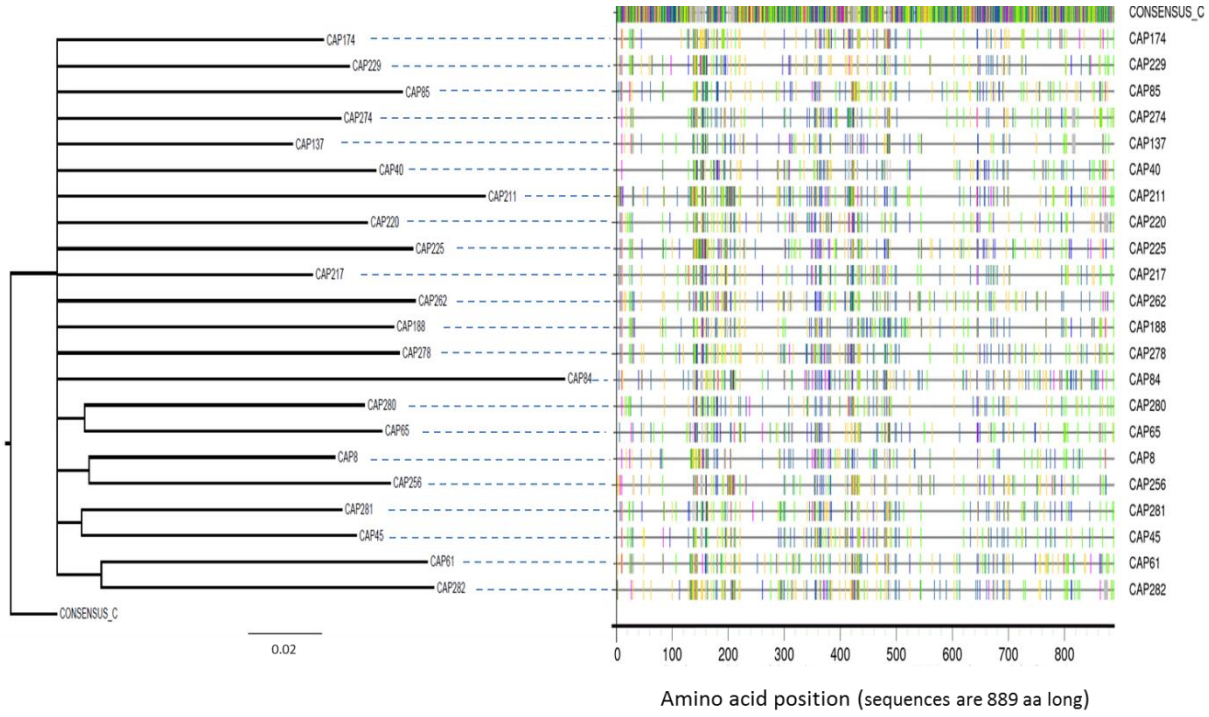


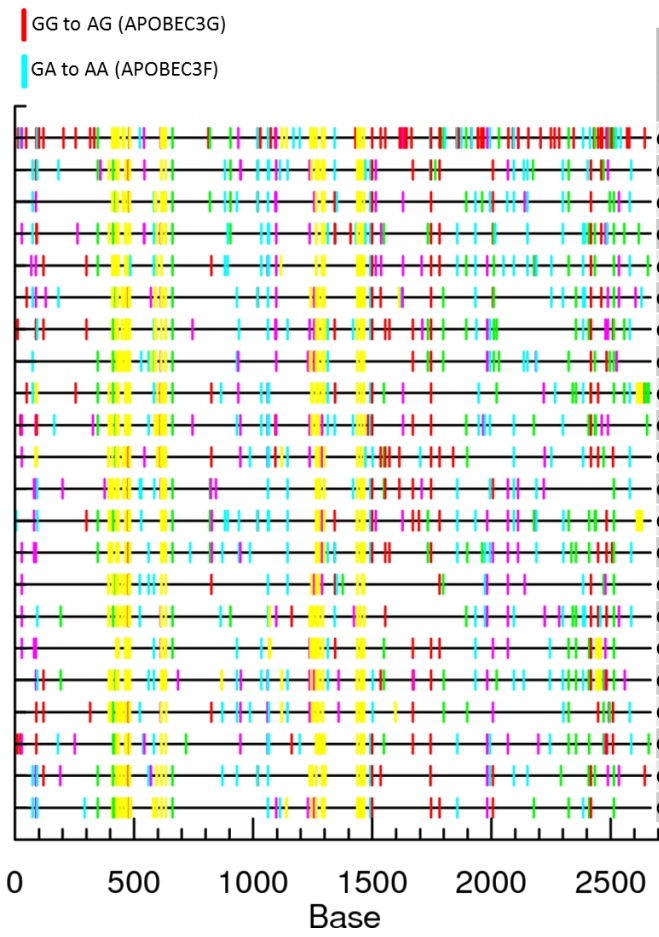
Figure 4.3 Phylogenetic and highlighter analyses depicting amino acid differences in HIV-1 Env sequences compared to a reference consensus C sequence. The neighbour-joining phylogenetic tree and the Highlighter analysis were generated using the HIV Sequence Database from the Los Alamos National Laboratory (http://www.hiv.lanl.gov). In the Highlighter plot, amino acid differences from the consensus C sequence are indicated by vertical bars. Diversity within HIV-1 *env* is extensive with sample CAP84 displaying the greatest divergence.

4.4 Assessment of Hypermutation within HIV-1 *env*

To further understand the mechanism behind the influence of APOBEC3G genetic variants on disease pathogenesis, we assessed the aforementioned HIV-1 *env* sequences for the magnitude of hypermutation induced by APOBEC3G. The Hypermut 2.0 Tool available on the HIV Sequence Database from the Los Alamos National Laboratory (http:// www.hiv .lanl. gov) was employed to detect APOBEC-specific hypermutations as decribed in Materials and Methods section 2.6.4.

The analysis indicated that the HIV-1 *env* sequence of only CAP84 was significantly hypermutated ($p<0.0001$). This sequence contained a total of 56 APOBEC3G induced hypermutations and 19 of the APOBEC3F context (Figure 4.4). Cloning and sequencing for this sample was repeated and sequence contigs were rechecked for errors during editing.

A



B

Sample	Number of APOBEC3G hypermutations (GG)	Number of APOBEC3F hypermutations (GA)	Fishers Exact (p-value)	Viral Load (copies/ml)
CAP84	56	19	<0.0001	1710
CAP278	12	20	0.26	1340
CAP45	7	15	0.79	517
CAP188	12	19	0.62	185000
CAP281	7	17	0.97	400
CAP262	10	13	0.66	1060
CAP217	12	12	0.90	48900
CAP225	4	10	0.91	22200
CAP220	9	13	0.56	400
CAP211	10	15	0.97	2470
CAP256	14	11	0.57	55000
CAP65	7	13	0.37	28100
CAP282	11	16	0.80	641
CAP61	11	15	0.57	400
CAP280	6	9	0.33	37500
CAP40	6	14	0.31	2030
CAP137	5	6	0.90	246000
CAP274	6	15	0.99	125000
CAP8	8	9	0.77	40700
CAP85	11	11	0.57	22000
CAP229	11	12	0.56	15400
CAP174	6	8	0.46	414000

Figure 4.4. [A] Hypermut 2.0 plot of nucleotide changes relative to a reference consensus subtype C sequence. APOBEC3G mutations in the dinucloetide context GG to AG are denoted by red bars while APOBEC3F mutations in the dinucloetide context GA to AA indicated by turqouise bars. [B] Summary of the corresponding hypermutation count for APOBEC3G and -3F. Fisher's exact p-values indicate degree of significance of hypermutation. Hypermutation analysis was performed with the Hypermut 2.0 Tool available on the HIV Sequence Database from the Los Alamos National Laboratory (http://www.hiv.lanl.gov).

Closer examination of the HIV-1 Env amino acid sequence of CAP84 revealed the presence of numerous stop codons across the gene (Figure 4.5). CAP84 carried the wild-type *186H/H* genotype and also maintained a relatively low viral load of 1,710 copies/ml at the time point analysed.

Figure 4.5 HIV-1 Env amino acid sequence of CAP84. Multiple stop codons, represented by red stars, are evident across the gene.

We subsequently assessed whether *H186R* variants had an impact on the editing function of APOBEC3G by comparing the hypermutation count between the *H186R* genotype groups. However, our results showed that there was no significant difference in the number of hypermutations between the genotype groups (p=0.85) (Figure 4.6)

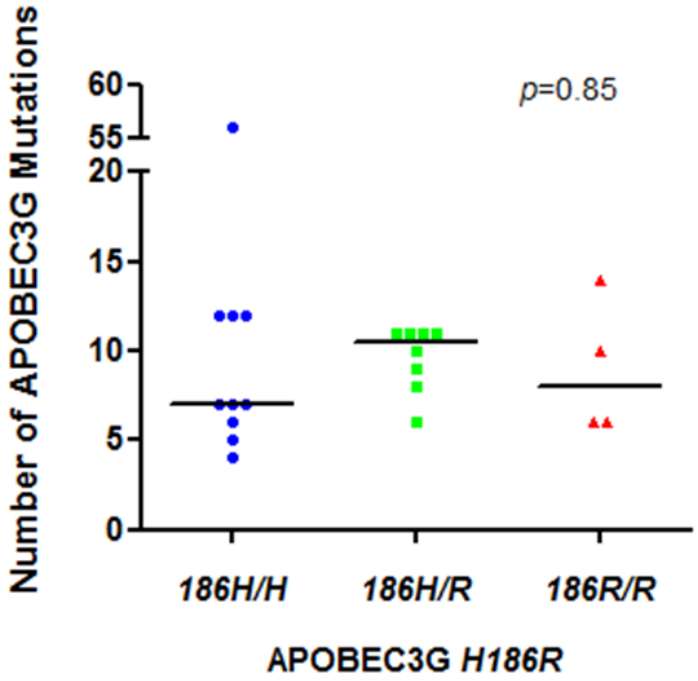


Figure 4.6 Comparison of APOBEC3G hypermutation count between *H186R* genotype groups. Kruskal-Wallis test indicates that there is no significant differences between the groups tested (*p*=0.85).

4.5 Correlation of Hypermutation with Markers of Disease Progression

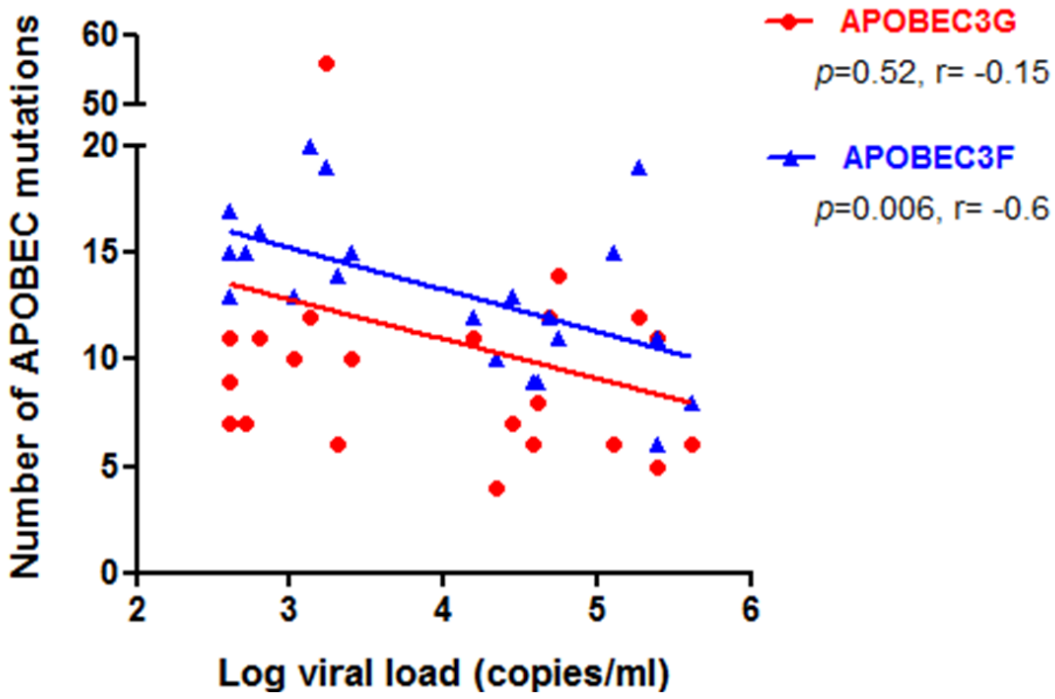
To determine whether the degree of APOBEC3G induced mutations has a clinical influence the next analysis was to the correlate the levels of APOBEC3G hypermutation calculated by Hypermut 2.0 with viral load and CD4 counts. Additionally, out of interest, APOBEC3F hypermutation was included in the analysis as well, as the data was already available.

Data showed that there was no significant correlation between the number of APOBEC3G induced mutations and viral load (p=0.52, r= -0.15) (Figure 4.7A) or CD4 count (p=0.55, r=0.14) (Figure 4.7B). However, there was a weak trend towards a negative correlation with viral load and a positive correlation with CD4 count. Interestingly, there was a strong significant correlation between the number of APOBEC3F induced mutations and viral load

(p=0.006, r= -0.6) (Figure 4.7A) and CD4 count (p=0.004, r=0.6) (Figure 4.7B). The number of APOBEC3F induced mutations associated negatively with viral load and positively with CD4 counts.

This steered us to further assess whether there was association between the number of APOBEC3G and APOBEC3F induced mutations as well as to determine if overall there was a difference in the quantity of APOBEC3G versus APOBEC3F induced mutations. The outcome of this analysis demonstrated that overall there was a greater number of APOBEC3F induced mutations present in the analysed HIV-1 *env* sequences as compared to APOBEC3G induced mutations (p=0.003) (Figure 4.8A). Additionally, there was a positive association between the number of mutations induced by APOBEC3G and APOBEC3F (p=0.027, r=0.5) (Figure 4.8B).

A



B

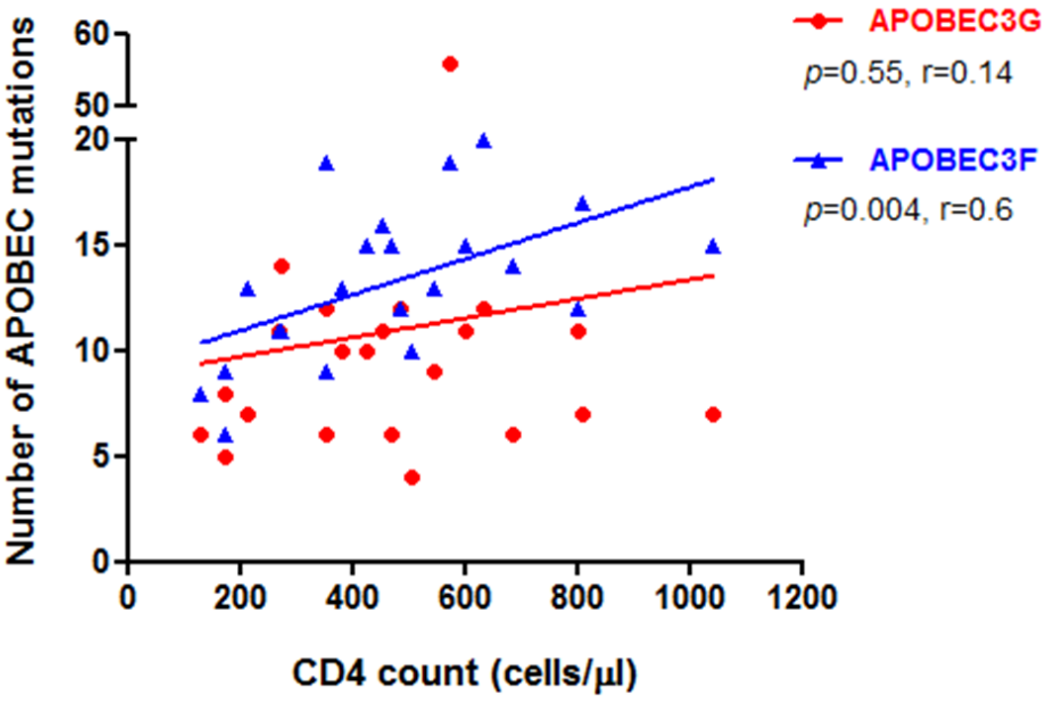


Figure 4.7 Correlation between the number of mutations induced by APOBEC3G (red) and APOBEC3F (blue) and viral load [A] and CD4 counts [B].

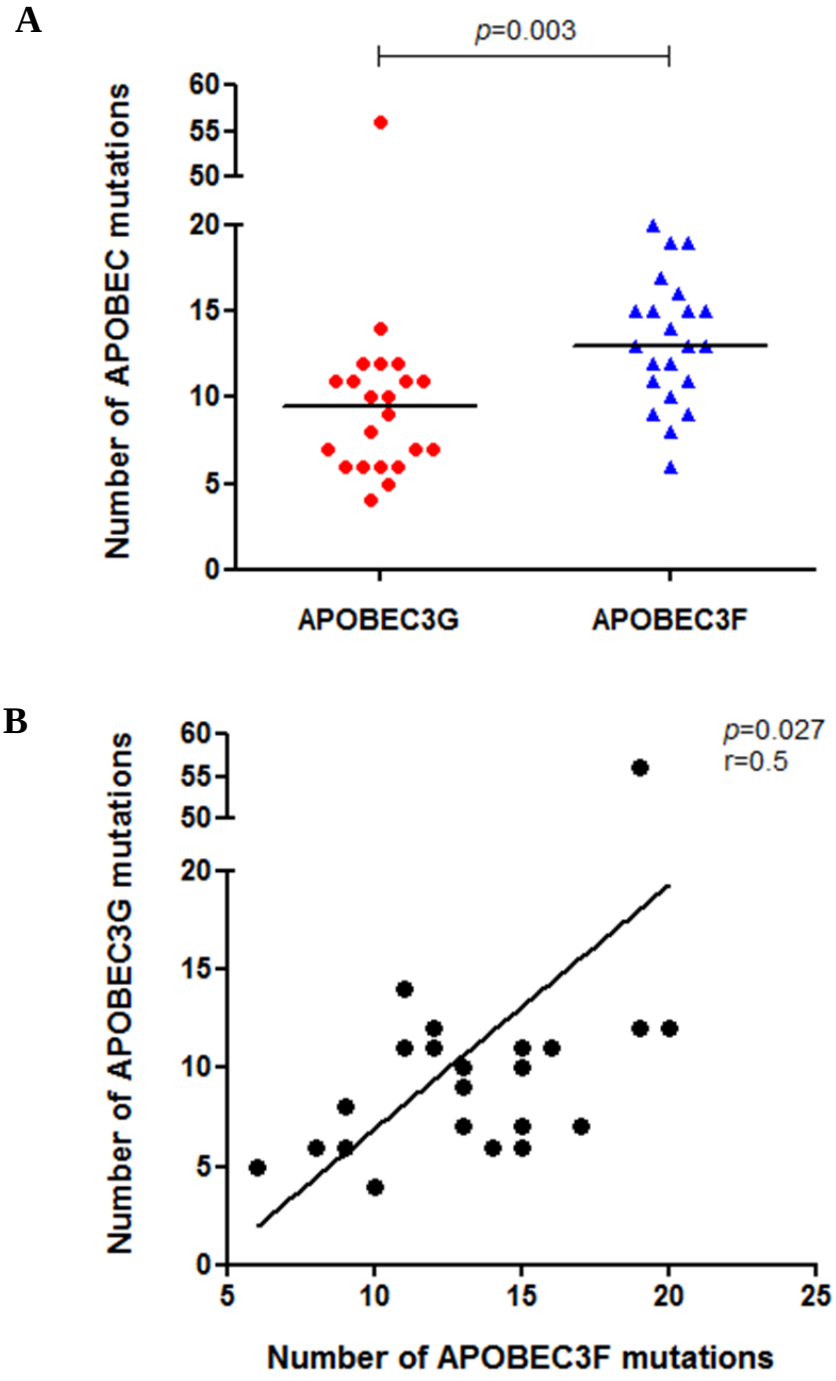


Figure 4.8 APOBEC3G versus APOBEC3F. [A] Overall comparison between the number of APOBEC3G mutations and the number of APOBEC3F mutations present in HIV-1 *env* sequences [B] Association between levels of APOBEC3G vs. APOBEC3F induced mutations.

Although, we did not have corresponding *APOBEC3G* expression data at the time point that hypermutation was analysed, these data was available for seven of the studied samples at an earlier time of infection. We therefore performed an exploratory analysis with these samples to determine if there was correlation between APOBEC3G levels and the number of hypermutations (Figure 4.9). However, our data showed no significant correlation (p=0.23) between these two factors, although there was a weak trend towards a positive correlation.

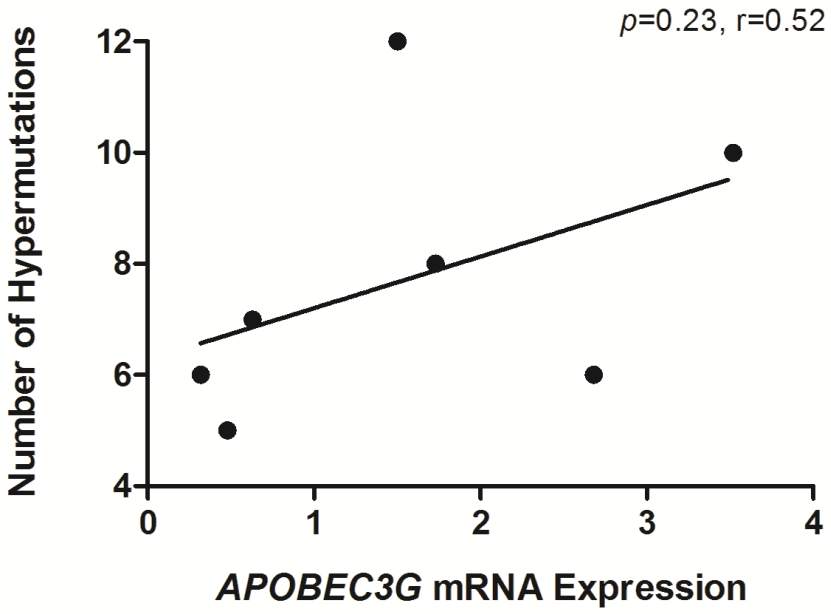


Figure 4.9 Correlation between *APOBEC3G* mRNA expression and the number of hypermutations.

CHAPTER FIVE

Association of APOBEC3G Genetic Variants with HIV-1 *vif* Sequence Variation and Impact on HIV-1 Pathogenesis

5.1 Introduction

The relationship between human immunodeficiency virus-1 (HIV-1) and the infected host is a complex and tumultuous one. Even though HIV-1 depends on its host to replicate it must also escape and shield itself against the host's antiviral restriction factors, which exert strong selective pressure on the virus. An apparent example of this co-evolutionary tussle is the interaction between HIV-1's virion infectivity factor (*vif*) and the cellular restriction factor apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G).

Our data shows that certain *APOBEC3G* genetic variants display differential clinical outcomes following HIV-1 infection. Specifically, the *H186R* mutation was associated with high viral loads and decreased CD4+ T cell counts. These data are consistent with findings from a previous study (An et al. 2004) in which the underlying mechanisms of this association remained unclear. We were therefore interested in extending these observations to investigate the possible underlying mechanisms. Additionally, natural variants in Vif have been frequently identified (Wieland et al. 1994; Adekale et al. 2005; Simon et al. 2005; Jacobs et al. 2008) and have been shown to have variable activity against APOBEC3G resulting in selective and partial neutralization of the host restriction factor (Simon et al. 2005). Studies also demonstrate that the interaction between HIV-1 Vif and APOBEC3G is species-specific, suggesting adaptation of Vif to its host (Mariani et al. 2003; Bogerd et al. 2004; Xu et al. 2004). Interestingly, subtype C Vif proteins have been demonstrated to have

the highest activity against APOBEC3G (Iwabu et al. 2010). However, there remains a gap in knowledge about the effect of subtype C *vif* alleles on different APOBEC3G haplotypes.

The constant conflict and interplay between host and virus may drive and shape the ongoing evolution and adaption of HIV-1 Vif (Compton et al. 2012). The best characterized interplay between HIV-1 and host genetic factors that leads to predictable and reproducible evolution and immune escape for the virus is the HIV-1/HLA class I interaction. HLA molecules are encoded by the MHC genetic loci and are the most polymorphic genes in the human genome as a result of its evolution from the constant barrage of pathogens faced by the human population throughout its existence (Jeffery and Bangham 2000; Klein and Sato 2000). This characteristic of HLA alleles allows them to identify numerous epitopes of pathogens which they present to T cells for activation of suitable immune responses. HLA class I molecules in particular recognize, bind and present viral epitopes to CTLs and have been shown to have a profound impact on the control HIV-1. This host immune response also imposes major selective pressure on the virus and drives the continuous process of immune escape (Phillips et al. 1991; Goulder et al. 2001; Draenert et al. 2004). The HLA protective alleles, HLA-B*57, -B*27 and –B*51, in particular have been shown to drive the formation of specific CTL escape mutants (Carrington and O'Brien 2003; Stephens 2005; Rousseau et al. 2008; Kawashima et al. 2009). The dynamics and patterns of HIV-1 evolution are somewhat predictable based on HLA restriction. For example selection for a T to N mutation at position three of the TW10 epitope in the p24 Gag protein within the first weeks after infection is associated with expression of the HLA-B*57 allele (Leslie et al. 2004). While individuals expressing HLA-B*27, initially select for an L to M change at position 6 in the immunodominant Gag epitope KK10 followed years later by an R to K change at position two (Kelleher et al. 2001).

Additionally, these escape mutations also result in replicative fitness cost in the absence of the selective HLA-allele and CTL pressure, as epitopes are often located in regions of the viral genome that are functionally conserved. Thus, when an escape variant is transmitted between individuals who are HLA-mismatched, rapid reversion of the mutation to wild-type, occurs (Friedrich et al. 2004; Leslie et al. 2004).

For this reason, we wanted to investigate for evidence of HIV adaptation, within Vif in particular, to ongoing *in vivo* pressure by APOBEC3G variants in a similar manner to which HIV adapts to host HLA. The aim of this study was to sequence and analyse subtype C HIV-1 *vif* alleles from patients whose APOBEC3G *H186R* genotypes were previously characterised. We also tested the hypothesis that APOBEC3G genetic variants may differentially influence subtype C Vif protein evolution *in vivo* as the virus adapts to ongoing APOBEC3G immune pressure.

5.2 Sample Selection

A total of 26 participants from the Centre for the AIDS Programme of Research in South Africa (CAPRISA) acute infection study cohort (van Loggerenberg et al. 2008) were selected for this arm of the study. Cryopreserved plasma samples were obtained at 36 months post-HIV-1 infection. The 26 individuals were selected based on their APOBEC3G *H186R* genotypes which were previously characterised. Samples used for this analysis were chosen at the 36 month post infection time point, as we expected that the accumulation of APOBEC immune-driven mutations within Vif would be more apparent at a later stage of infection. Patient characteristics are described in Table 5.1.

Table 5.1 Characteristics of 26 participants selected for the study of HIV-1 *vif*

PID	*H186R*	Viral Load (copies/ml)	CD4 (cells/µl)	# of Vif Clonal Sequences
CAP37	*AA – 186 H/H*	92,300	380	10
CAP40	*AA – 186 H/H*	2,030	685	10
CAP45	*AA – 186 H/H*	517	1043	10
CAP65	*AA – 186 H/H*	28,100	214	10
CAP84	*AA – 186 H/H*	1,710	575	10
CAP137	*AA – 186 H/H*	24,600	173	19
CAP206	*AA – 186 H/H*	431,000	277	19
CAP217	*AA – 186 H/H*	48,900	486	19
CAP221	*AA – 186 H/H*	104,000	264	14
CAP225	*AA – 186 H/H*	22,200	505	17
CAP278	*AA – 186 H/H*	1,340	635	12
CAP8	*AG – 186 H/R*	40,700	175	10
CAP61	*AG – 186 H/R*	400	601	10
CAP85	*AG – 186 H/R*	242,000	270	11
CAP88	*AG – 186 H/R*	142,000	627	17
CAP177	*AG – 186 H/R*	48,300	270	19
CAP200	*AG – 186 H/R*	563,000	183	17
CAP211	*AG – 186 H/R*	2,470	424	20
CAP229	*AG – 186 H/R*	15,400	802	17
CAP274	*AG – 186 H/R*	125,000	468	18
CAP282	*AG – 186 H/R*	641	452	16
CAP174	*GG – 186 R/R*	414,000	131	17
CAP256	*GG – 186 R/R*	55,000	274	16
CAP261	*GG – 186 R/R*	7,490	322	14
CAP262	*GG – 186 R/R*	1,060	383	16
CAP280	*GG – 186 R/R*	37,500	355	17

5.3 Amplification and Cloning of HIV-1 *vif*

A 1030 base pair fragment containing the 579 base pair HIV-1 *vif* gene was successfully amplified as shown by analysis on a 2% agarose gel (Figure 5.1). The amplicons were subsequently cloned into the pCR2.1 vector and successful ligation of the amplicon into the vector was confirmed by a restriction digest performed on plasmid DNA using the BstXI enzyme (Figure 5.2).

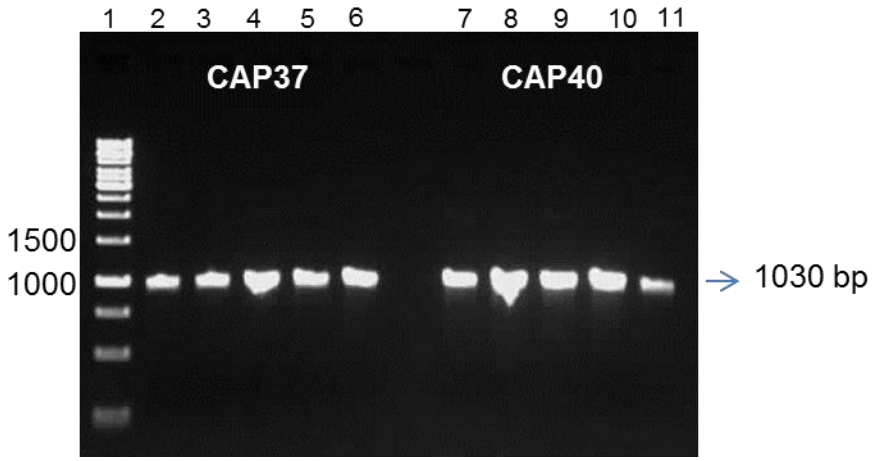


Figure 5.1 Agarose gel depicting successful amplification of the 1030 base pair fragment containing the 579 base pair HIV-1 *vif* gene on a 2% agarose gel. Lane 1 depicts a 1 kilo base DNA ladder. The 1030 base pair amplicons are depicted in lanes 2-6 from sample PID CAP37 and lanes 7-11 from CAP40.

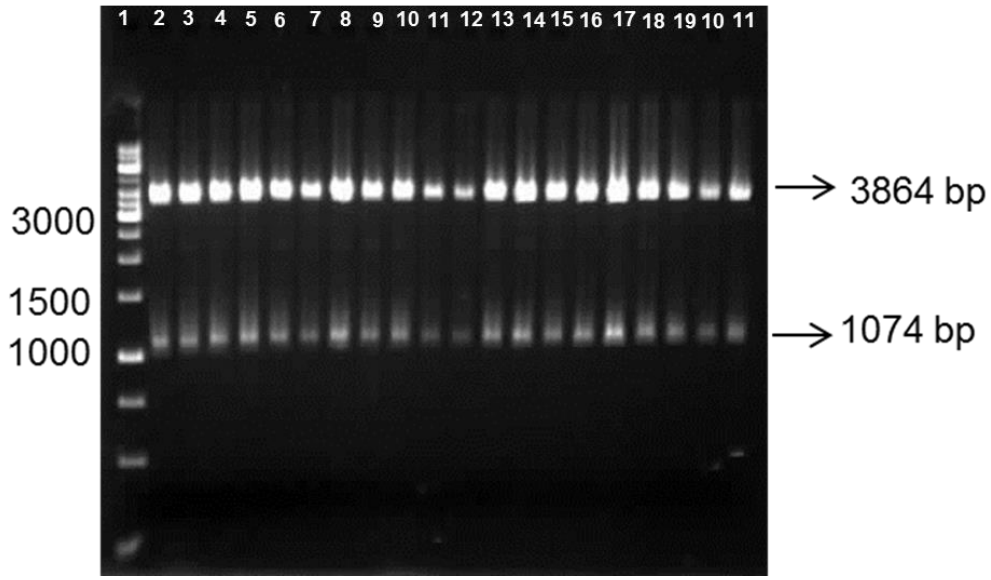


Figure 5.2 Agarose gel depicting plasmid DNA digestion products confirming successful cloning of HIV-1 *vif* amplicons into the pCR2.1 vector. Lane 1 is a 1 kilo base DNA ladder. Lanes 2-11 depicts 10 representative clones.

5.4 Phylogenetic Analysis of subtype C HIV-1 *vif* sequences

There is limited sequence information available for subtype C HIV-1 *vif* of South African origin. In this study we cloned and sequenced HIV-1 *vif* from plasma of 26 participants with recent infection. A total of 392 HIV-1 *vif* clonal sequences were generated. All viral isolates encoded a full length 192 residue amino acid sequence. The phylogenetic relationship between all *vif* sequences is depicted in Figure 5.3 and shows that clonal sequences from each patient sample clustered with each other. This indicated that sequences were free of lab strain contamination or sample mix-ups between patients.

Additionally, a neighbour joining phylogenetic tree constructed with consensus sequences of 26 study samples and reference sequences of HIV-1 group M subtypes A to K. demonstrated that study samples belonged to HIV-1 subtype C (Figure 5.4)

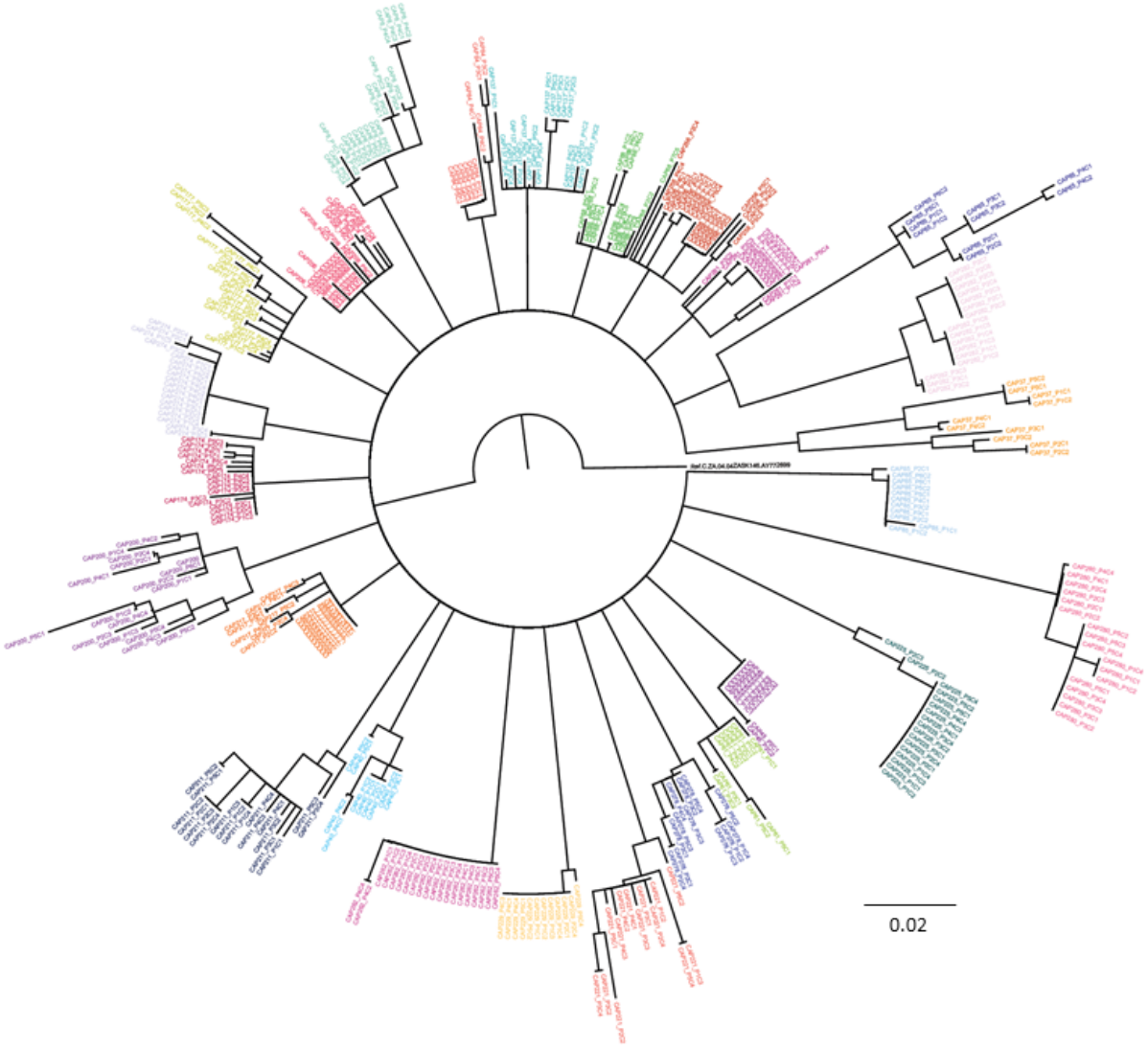


Figure 5.3 Neighbour joining phylogenetic tree of 392 full length HIV-1 *vif* clonal sequences. HIV-1 vif clonal sequences from each of 26 samples (depicted by different colours) cluster together indicating that samples are free of contamination. The tree is rooted with a South African subtype C reference sequence (Ref.C.ZA.04.04Z ASK146.A Y772699) obtained from the Los Alamos National Laboratory HIV database (http://www.hiv.lanl.gov).

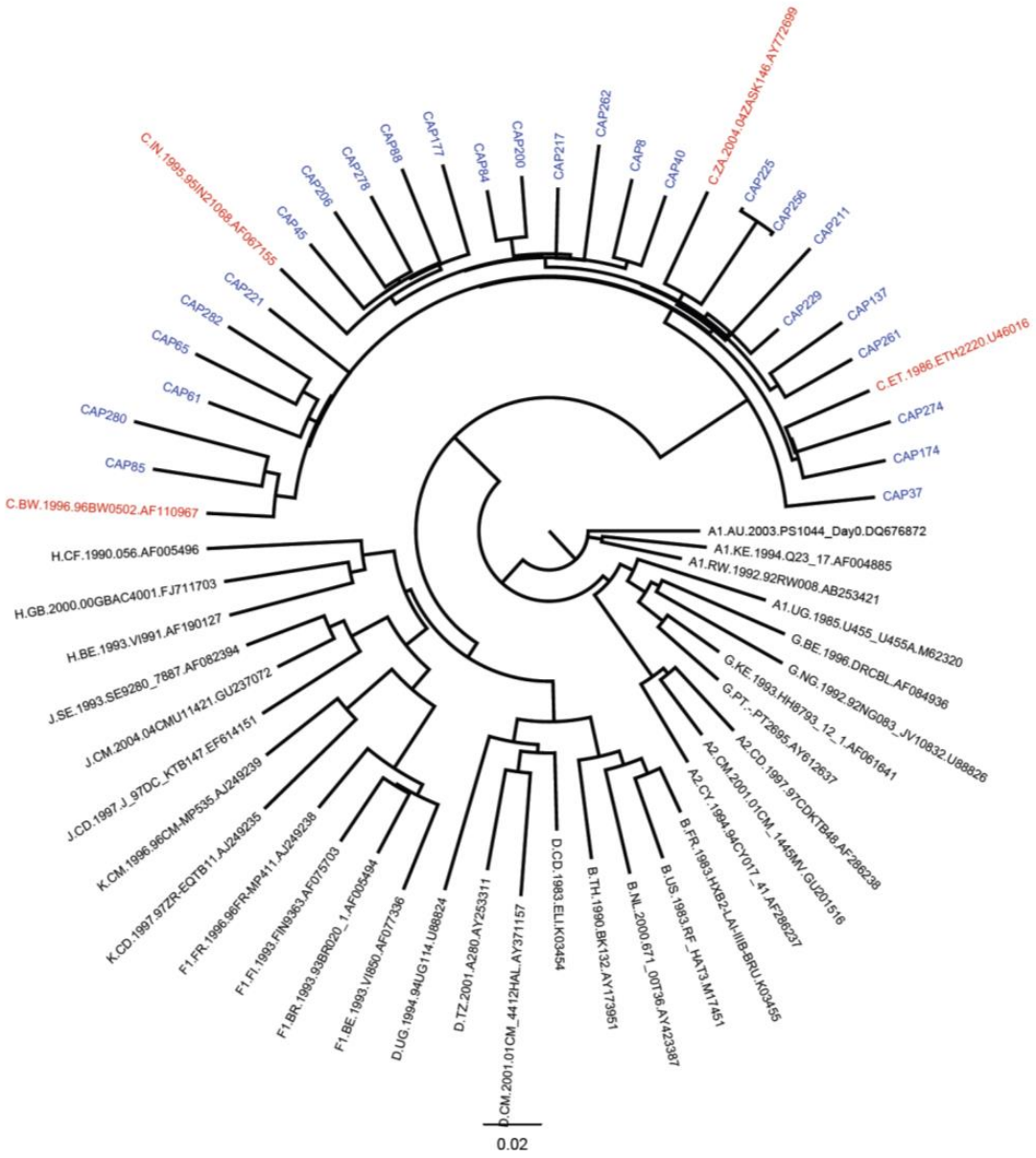


Figure 5.4 Subtype neighbour joining HIV-1 *vif* phylogenetic tree. Consensus sequences of 26 samples and reference sequences of HIV-1 group M subtypes A to K (obtained from the Los Alamos National Laboratory HIV database (http://www.hiv.lanl.gov) are included. All samples (blue) cluster with subtype C reference sequences (red). The tree was constructed with Los Alamos National Laboratory HIV database treemaker tool and Fig Tree v1.3.1.

Intra-individual sequence differences were substantial with a range of 0.1% to 4.9% at the protein level and 0.1% to 2.8% at the DNA level. Inter-individual diversity ranged from 6.2% to 19.2% (protein) and 4.7% to 11.4% (DNA). Assessment of intra-individual sequence diversity for correlation with markers of disease outcome i.e. viral load and CD4 count showed that there was no significant association between these factors (Figure 5.5).

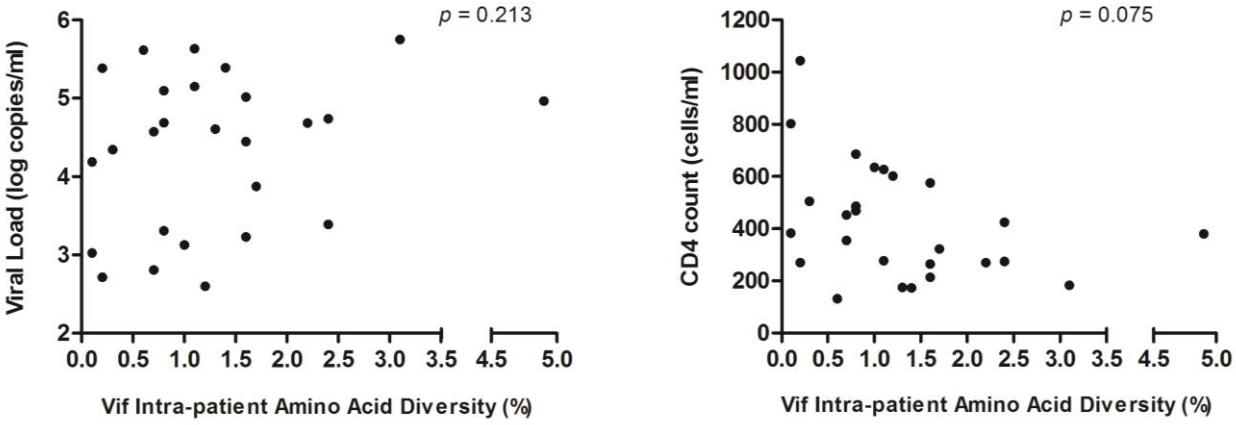


Figure 5.5 Association between Vif intra-patient amino acid diversity and [A] viral load and [B] CD4 count

An alignment with consensus sequences of 26 study samples and a consensus subtype C reference sequence is represented in Figure 5.6. Regions that are crucial for degradation of APOBEC3G are shaded. These sites include amino acids I9, K22, E45 and N48; YRHHY motif (40-44); amino acids 52 to 72 particularly the highlighted VHIPLx_{4-5}LxΦx_2YWGI motif; tryptophan residues Trp 5, 21, 38 and 89; the HCCH motif important for binding to Cullin 5 and the SLQYLA motif important for recruitment of ubiquitin-ligase (E3) complex containing elongin –B and –C, cullin-5 and Rbx. All of these known crucial sites for APOBEC3G/Vif interaction were found to be highly conserved in the studied HIV-1 subtype C sample set.

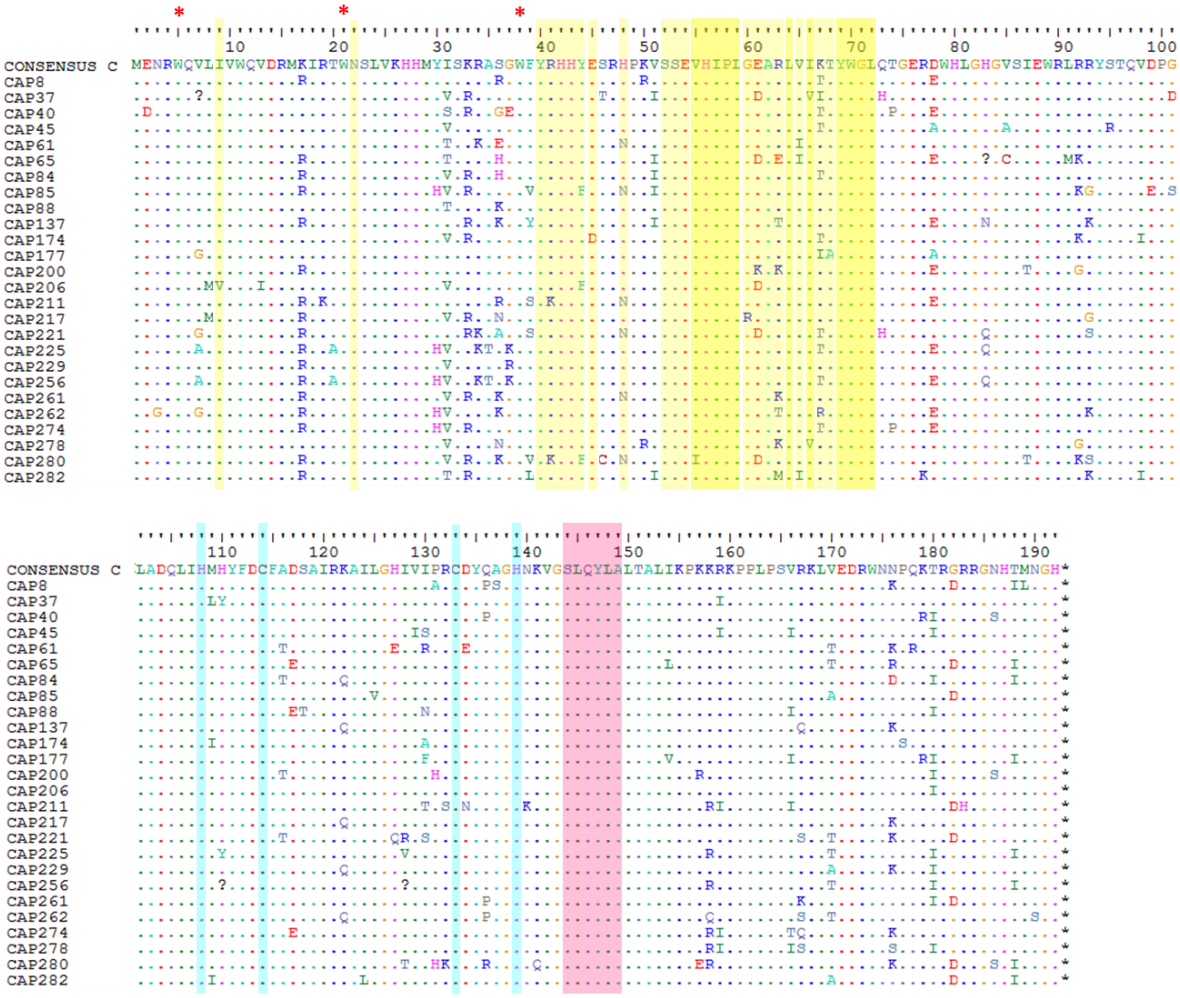


Figure 5.6 Alignment of Vif amino acid consensus sequences of 26 study samples. Nucleotide sequences were aligned and protein translation inferred using Bioedit Sequence alignment editor v7.1.3.0. The sequences are compared to a consensus subtype C reference sequence (obtained from the Los Alamos National Laboratory HIV database (http://www.hiv.lanl.gov). Protein domains involved in degradation of APOBEC3G are shaded or marked as follows: yellow indicates I9, K22, E45 and N48, YRHHY (40-44), amino acids 52 to 72 including the highlighted VHIPLx_{4-5}LxΦx_2YWGI motif which are important for binding to APOBEC3G; * shows tryptophan residues also significant for APOBEC3G binding; blue indicates the HCCH motif important for binding to Cullin 5; pink shows SLQYLA motif important for recruitment of ubiquitin-ligase (E3) complex containing elongin –B and –C, cullin-5 and Rbx.

5.5 *In Vivo* Effect of APOBEC3G *H186R* Genotypes on HIV-1 Vif Sequence Diversity

Evolution of HIV-1 Vif may be influenced by the continuous selective pressure imposed by APOBEC3G. HIV-1 Vif divergence may therefore occur as an adaptive response to the APOBEC3G repertoire present within the host.

For subsequent analysis we stratified the study samples according to their APOBEC3G *H186R* genotypes to determine if there were sequence differences in HIV-1 Vif between the genotype groups. Our data shows that overall there was no significant pattern or difference in HIV-1 Vif diversity between the *H186R* genotypes groups (Figure 5.7).

We next employed the Viral Epidemiology Signature Pattern Analysis (VESPA) (Los Alamos National Laboratory HIV database (http://www.hiv.lanl.gov)) to calculate the frequency of each amino acid at each position in our HIV-1 Vif alignment focusing on sequences belonging to the *186H/H* (wild type) group and the *186R/R* (mutant) group. Positions 36 and 93 were the most distinguishing sites with the most common amino acid in the *186R/R* group differing approximately five fold from that in the *186H/H* group (Figure 5.8A). Interestingly, Vif sequence comparison at position 36, showed a strong preference for Lysine (K) in APOBEC3G *186R/R* individuals and for Serine (S) in *186H/H* individuals (Figure 5.8B). Additionally, at position 93 there was a strong preference for an Arginine (R) or a Serine (S) for APOBEC3G *186H/H* and *186R/R* individuals respectively (Figure 5.8C).

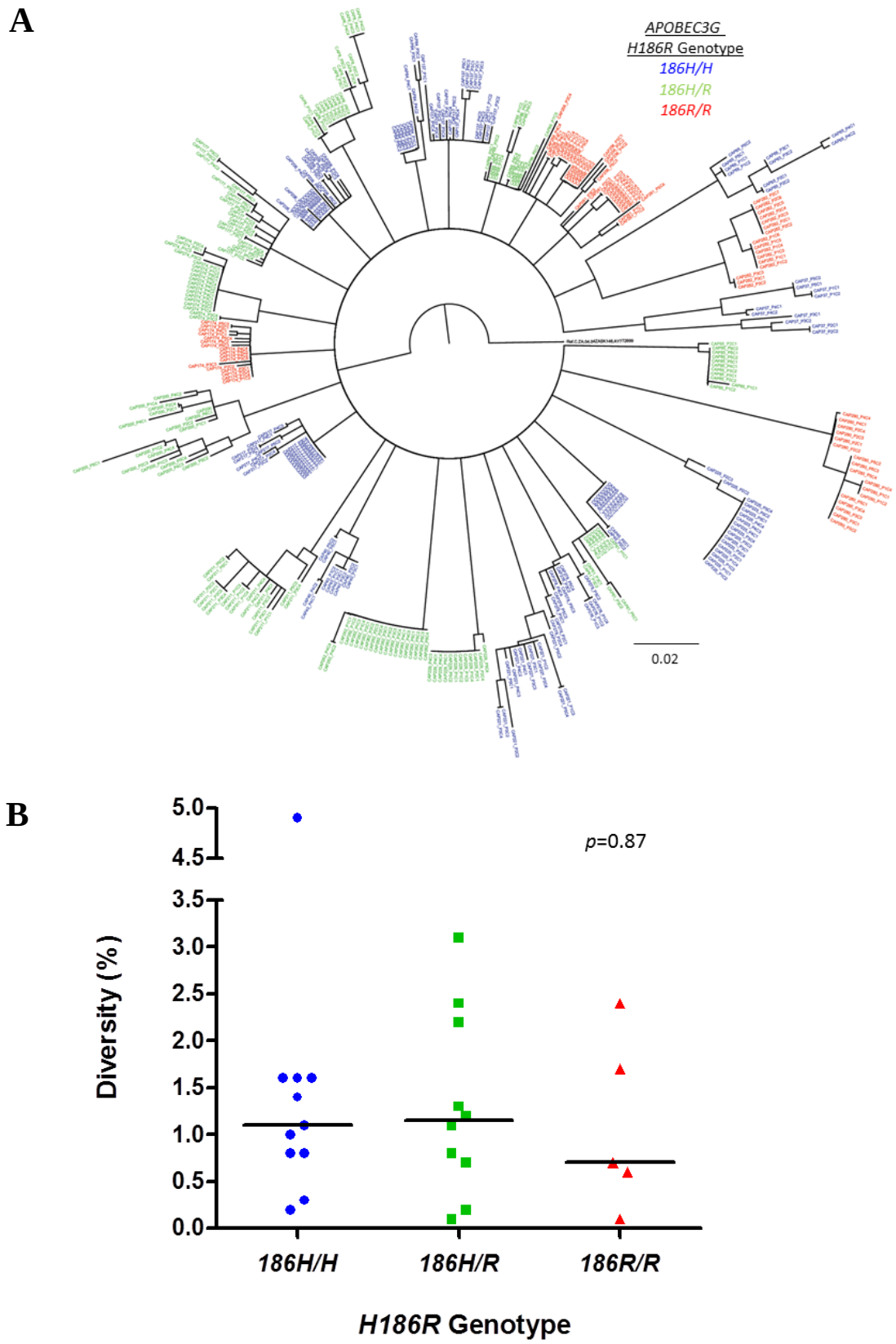


Figure 5.7 Vif Diversity versus *APOBEC3G H186R* genotype. [A] Phylogenetic tree of all Vif clonal sequences stratified by *H186R* genotype. [B] Comparison of Vif diversity between genotypes groups.

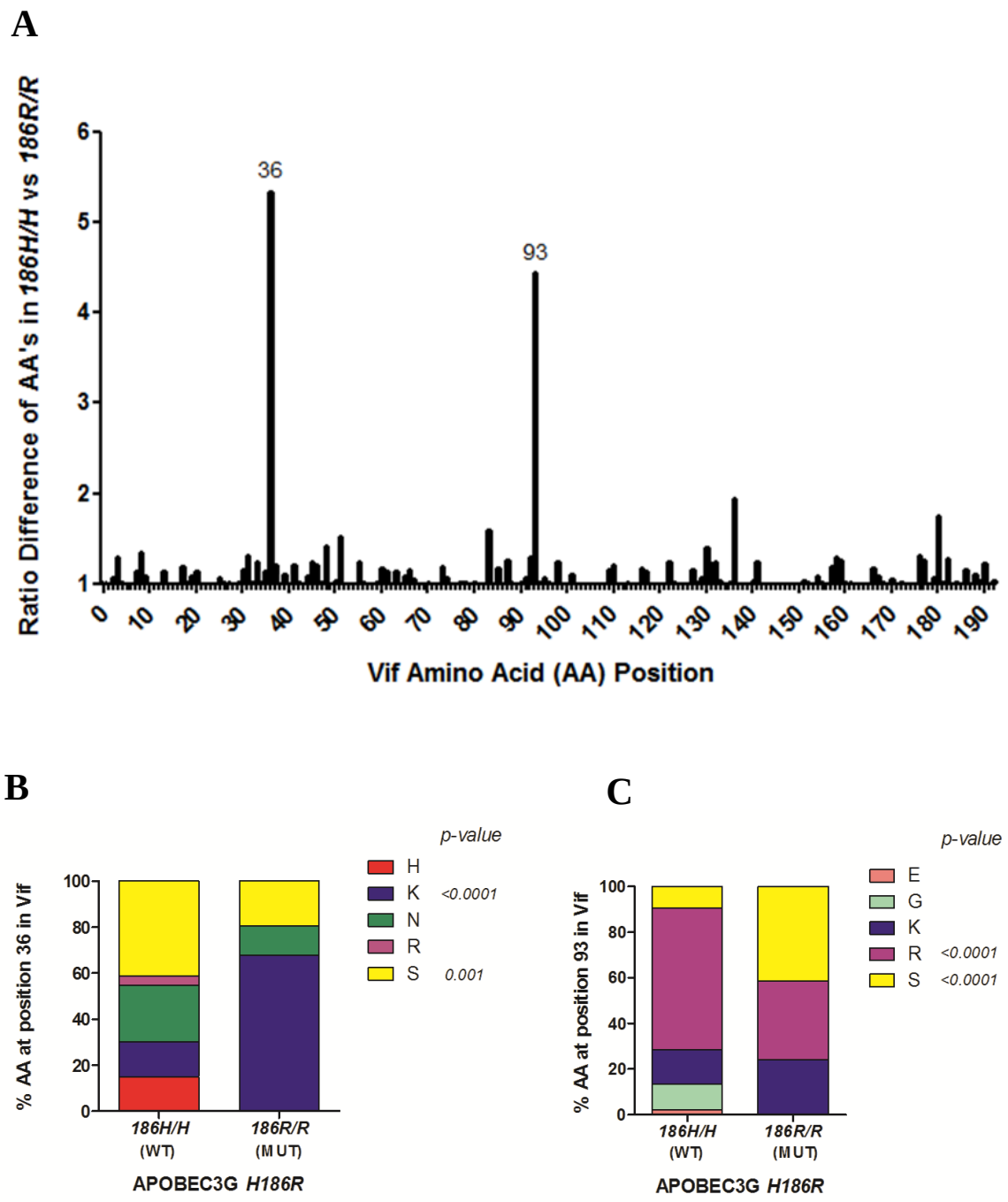


Figure 5.8 Comparison of amino acid frequencies at each position of Vif for APOBEC3G *186H/H* (wild type) group and the *186R/R* (mutant) group. Amino acid frequencies were calculated by VESPA available at Los Alamos National Laboratory HIV database. At Vif position 36 there is a strong preference for Lysine in *186R/R* individuals and for Serine in *186H/H* individuals [A,B] while at position 93 there is preference for an Arginine (R) or a Serine (S) for *186H/H* and *186R/R* individuals [A,C].

5.6 Amino acid frequencies differ at Vif position 36 and 93 between 186H/H and 186R/R individuals.

Inspection of Vif positions 36 and 93 in other HIV-1 subtypes (Figure 5.9A and 5.9B respectively) revealed that the amino acid frequency and distribution at these loci in our Vif sequences conforms to other subtype C Vif sequences. Interestingly, for subtype C the predominant amino acid expressed at position 36 is a Serine (S), while all other subtypes typically express a Lysine (K) at this site. At position 93, Arginine (R) is the predominant amino acid expressed in all subtypes.

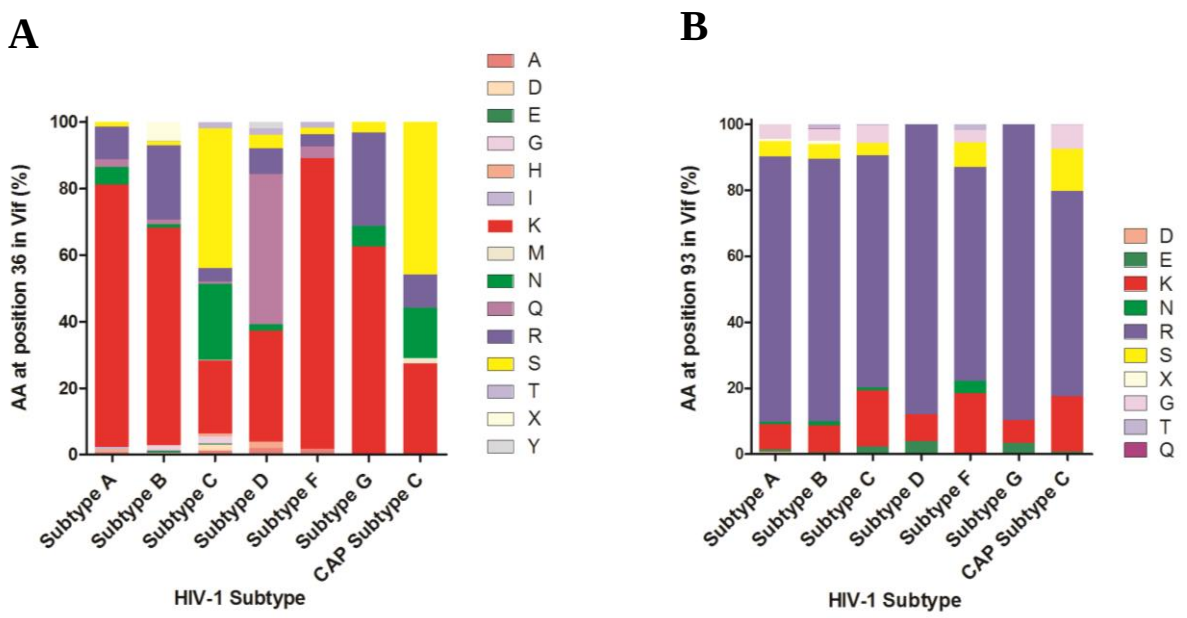


Figure 5.9 Comparison of amino acid frequencies at Vif positions 36 [A] and 93 [B] between HIV-1 subtypes. Amino acid frequency at these sites in our study samples (CAP subtype C) conform to other subtype C Vif alleles. Subtype A (n=135), subtype B (n=771), subtype C (n=414), subtype D (n=51), subtype F (n=55), subtype G (n=32), CAP subtype C (n=332).

5.7 Amplification and Cloning of Subtype C HIV-1 *vif* into the pCRV1 Expression

HIV-1 *vif* clones within the pCR2.1 vector was successfully amplified with specific primers containing EcoRI and NotI restriction sites and digested with the same enzymes to create suitable overhangs for further cloning into the pCRV1 expression as depicted on a 2% agarose gel (Figure 5.10).

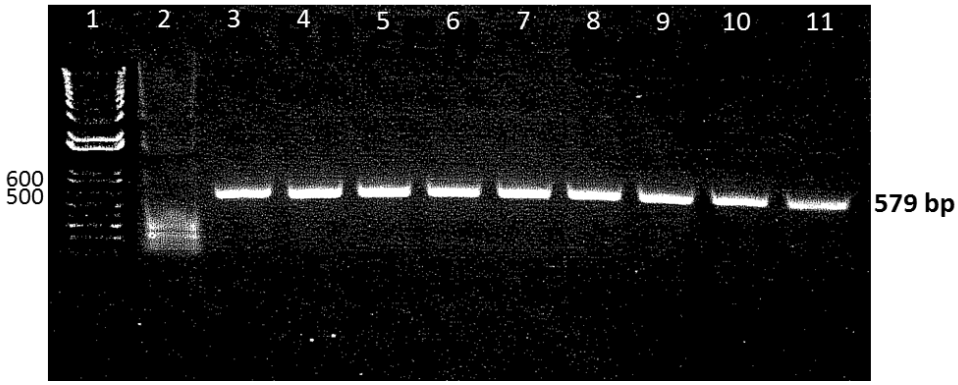


Figure 5.10. Agarose gel depicting successful amplification of HIV-1 *vif* variants contained within pCR2.1 vector, which were also digested with EcoRI and NotI enzymes for subsequent cloning into the pCRV1 vector. Lanes 1-2 depict a 100 base pair DNA ladder while lanes 3-11 depicts the 579 base pair HIV-1 *vif* amplicons.

5.8 Site Directed Mutagenesis of Vif Position 36

Site-directed mutagenesis of each of four samples was achieved by primer extension which involved incorporating mutagenic primers in two independent, nested PCRs per sample as depicted on the 2% agarose gel in Figure 5.11.

The two separate fragments that were created, were successfully combined in a 3rd PCR using outer primers to create the final product which contained the mutated internal sequence specifically serine mutated to lysine or lysine mutated to serine at position 36 (Figure 5.12A)

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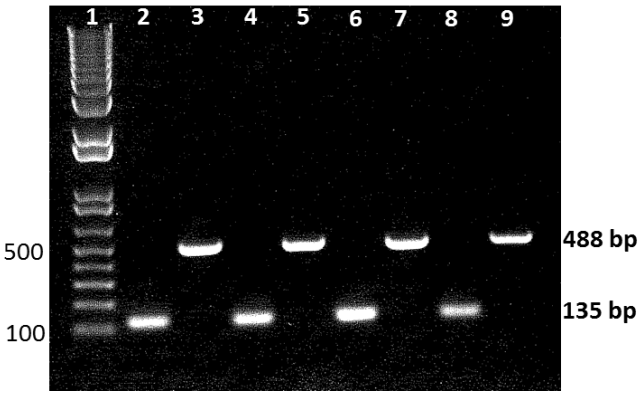


Figure 5.11 Agarose gel depicting successful amplification of four HIV-1 *vif* variants, each amplified as two separate fragments by specific mutagenic primers. Lane 1 is a 100 base pair DNA ladder. Lanes 2 and 3 represent one HIV-1 *vif* variant amplified as two separate fragments and so on.

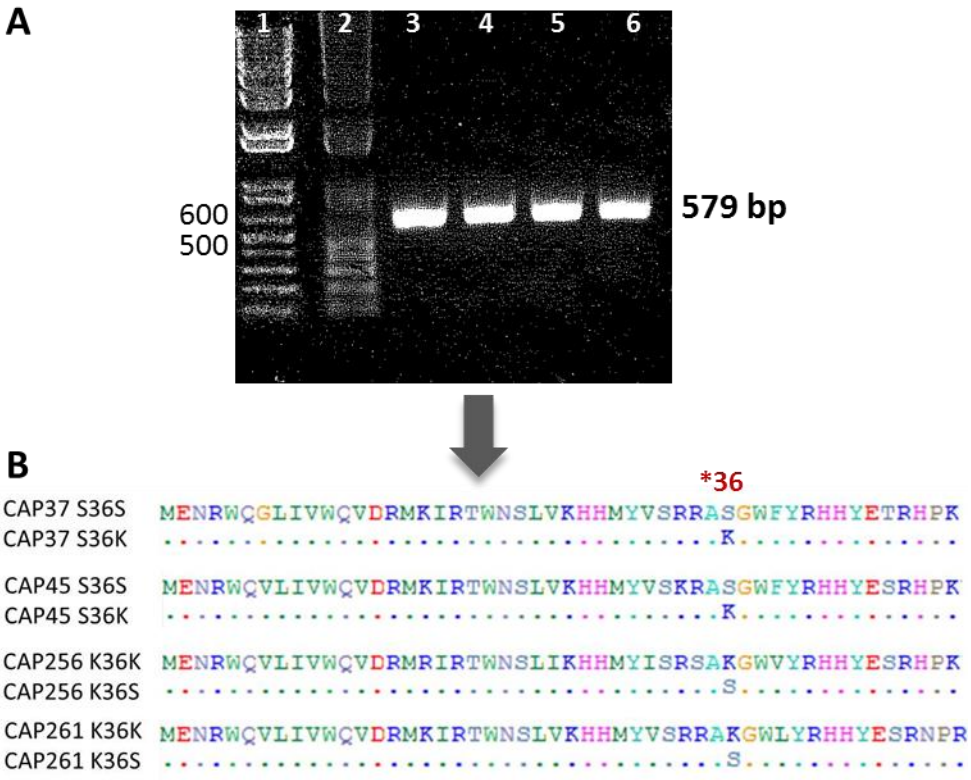


Figure 5.12 [A] Successful recombination of PCR fragments (depicted in Figure 5.11) to create the final products (lanes 3-6) which now contained the mutation at position 36, [B] specifically serine mutated to lysine or lysine mutated to serine as confirmed by a sequence alignment. Lane 1 of the agarose gel depicts a 100 base pair DNA ladder.

5.9 Functional activity of Vif alleles against APOBEC3G *H186R* Genotypes

HIV-1 Vif counteracts APOBEC3G by inducing proteasome mediated degradation of the host protein. Although HIV-1 Vif has been genotypically characterised, knowledge on the phenotypic function remains limited, particularly in the HIV-1 subtype C setting. Additionally, the influence of APOBEC3G genetic variants on the function of HIV-1 Vif remains to be elucidated.

For this reason we cloned a subset of the subtype C vif alleles from our study cohort into the pCRV1 expression vector to test their function *in vitro*. From the pool of 392 *vif* sequences, 16 unique *vif* plasmids were selected for functional testing as described in Materials and Methods section 2.7.6. An additional 4 *vif* sequences were selected for site directed mutagenesis at position 36 following our Vif sequence analysis also described in section 2.7.6. CAP37 and CAP45 were selected as they expressed a Serine at position 36 while CAP256 and CAP261 were selected for expressing a Lysine at postion 36. Serines were then mutated to a Lysines while Lysines were mutated to Serines (Figure 5.12). We focused on amino acid 36 as it is located in the N-terminal region of Vif which has been shown to interact with APOBEC3G.

5.9.1 Vif Expression

The 16 pCRV1 *vif* expression plasmids showed considerable variation in their levels of expression when transfected into 293T cells. To confirm that the variation observed was not a result of diminished antibody recognition by the rabbit polyclonal anti-Vif serum, each of the Vif variants were FLAG tagged and Vif expression detected once more by rabbit monoclonal anti-FLAG serum. Results confirmed that there was indeed substantial variation in expression levels of the 16 Vif variants (Figure 5.13).

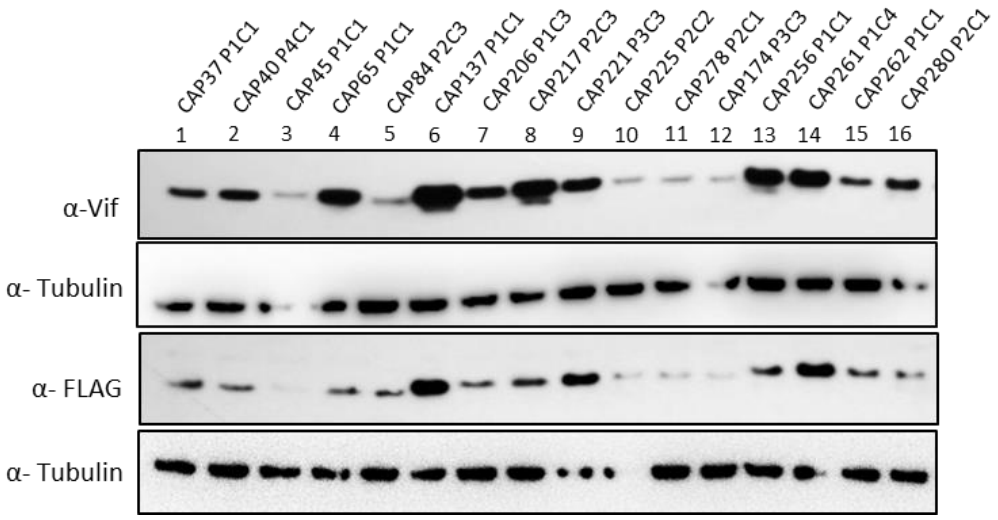


Figure 5.13 Both tagged and untagged pCRV1 *vif* expression plasmids display varying levels of expression upon transfection in 293T cells.

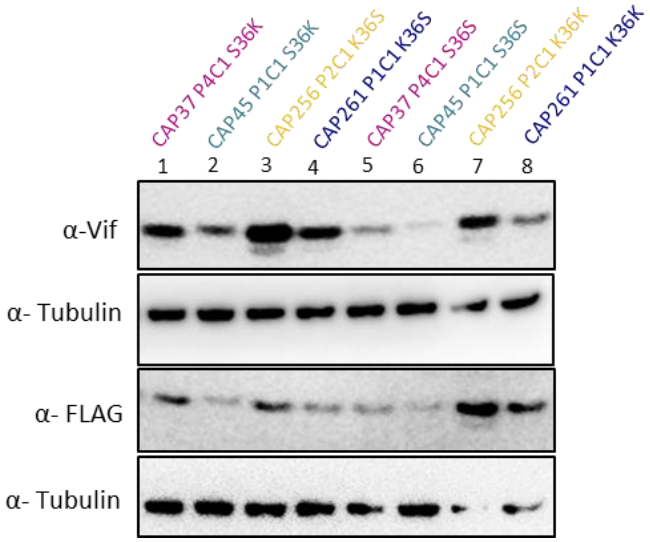


Figure 5.14 Expression profile of both tagged and untagged *vif* plasmids that were subjected to site directed mutagenesis at position 36. Serine was mutated to a Lysine in 2 samples (lanes 1 and 2) while Lysine was mutated to Serine in an additional 2 samples (lanes 3 and 4). Lanes 4 – 5 contain the corresponding wild type *vif* plasmids. Varying levels of expression can be observed upon transfection in 293T cells.

Varied expression profiles were also examined for *vif* plasmids that were subjected to mutagenesis at position 36 (Figure 5.14). Interestingly, mutating a Serine to a Lysine (S36K) (lanes 1 and 2) resulted in an increase in expression as compared to the corresponding wild type *vif* plasmids (lane 5 and 6). There was no observable difference in expression when Lysine was replaced with a Serine (K36S) (lanes 3 and 4) compared to the corresponding wild type plasmids (lanes 7 and 8). All Vif expression assays were repeated and a consistent trend was observed.

5.9.2 APOBEC3G Degradation

Vif variants were then tested for their ability to degrade APOBEC3G *186H* and *186R* variants by co-tranfection in 293T cells. Analysis by Western blotting showed that the subtype C Vif variants preferentially targeted the wild type APOBEC3G *186H* allele rather than the *186R* variant (Figure 5.15). Vif variants mutated at position 36 were also tested for the activity against APOBEC3G alleles. Although Vif expression plasmids again seemed to favourably degrade the wild type (WT) APOBEC3G *186H* allele, the level of degradation did not seem to be influenced by the amino acid (Serine or Lysine) at position 36. (Figure 5.16).

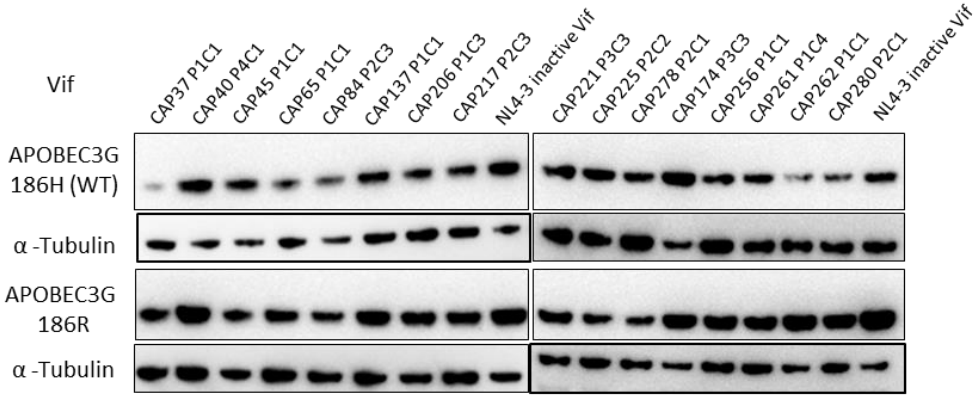


Figure 5.15 Degradation of APOBEC3G *186H* (WT) and *186R* in the presence of Vif expression plasmids when transfected in 293T cells. Subtype C Vifs seem to favourably degrade the wild type (WT) APOBEC3G 186 H allele.

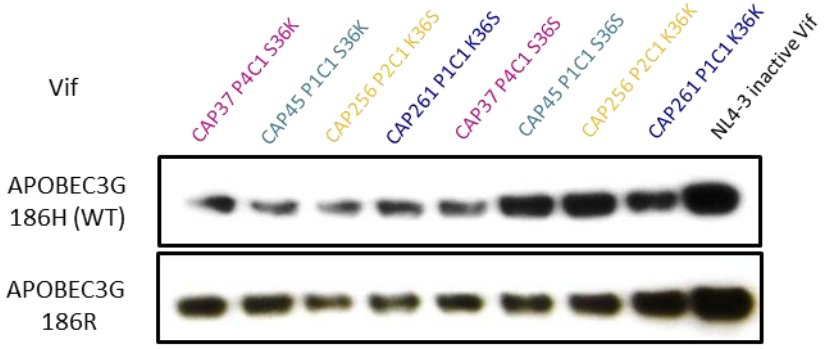


Figure 5.16 Degradation of APOBEC3G *186H* wild type (WT) and *186R* in the presence of mutant Vif expression plasmids when transfected in 293T cells. Vif expression plasmids seem to favourably degrade the WT APOBEC3G 186 H allele. The level of degradation does not seem to be influenced by the amino acid at position 36. Vif WT and corresponding site directed mutant are depicted in the same colour.

5.10 Impact of Subtype C Vifs on Infectivity in the Presence of APOBEC3G *186H* and *186R*

The anti-APOBEC3G phenotype of Vif was tested by measuring rescue of infectivity by HIV-1 (WT) (NL4-3), HIV-1ΔVif (NL4-3 with a deletion of Vif - NL4-3ΔVif) and HIV-1 ΔVif complemented with a subtype C Vif (NL4-3ΔVif + Vif_C) in the absence or presence of APOBEC3G (Figure 5.18). In the absence of APOBEC3G, HIV-1 WT, HIV-1ΔVif and HIV-1ΔVif + Vif_C established 100% infectivity (relative to HIV-1 WT). However, upon introduction of APOBEC3G WT, infectivity of HIV-1 WT decreased by 50% while the infectivity of HIV-1ΔVif was completely abolished, but infectivity of HIV-1ΔVif was restored to 50% upon supplementation with Vif_C. The same pattern of infectivity and rescue was observed in the presence of APOBEC3G *186R*.

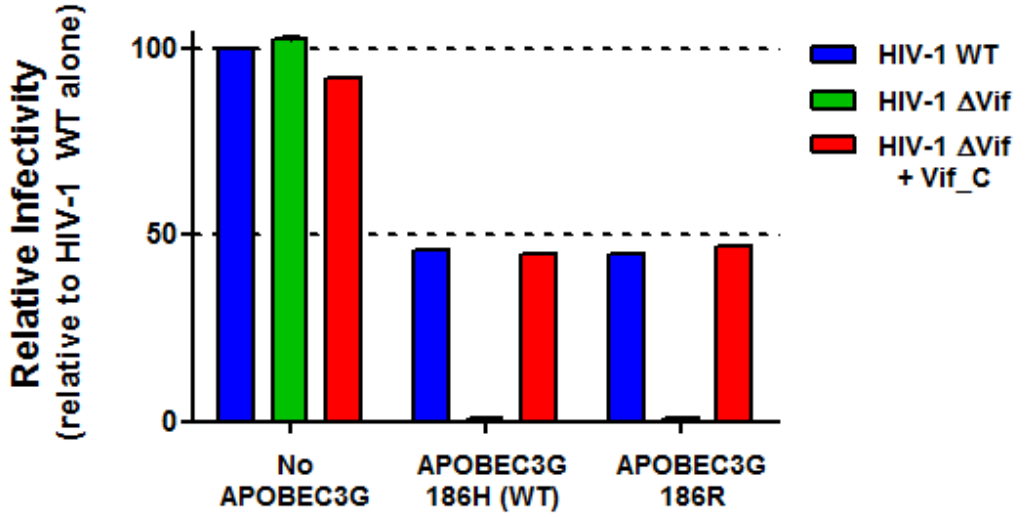


Figure 5.17 Anti-APOBEC3G phenotype of Vif. HIV-1 WT, HIV-1ΔVif, HIV-1ΔVif + Vif_C displayed 100% infectivity when APOBEC3G was lacking. Addition of either APOBEC3G WT or *186R* resulted in a 50% decrease in infectivity of HIV-1 WT and eradication of HIV-1ΔVif infectivity which was rescued to 50% by supplementation with Vif_C. Infectivity was assessed at 48 hours post-transfection on TZM-bl reporter cells. Infectivity is expressed relative to the infectivity of HIV-1 WT alone.

We also tested the infectivity of HIV-1ΔVif (NL4-3ΔVif) supplemented with all selected 16 subtype C Vifs in the presence of APOBEC3G *186H* and *186R* (Figure 5.19). As described previously, the infectivity of viral stocks was quantified 48 hours post-transfection by use of TZM-bl reporter cells. The NL4-3 WT, NL4-3ΔVif, NL4-3 WT without APOBEC3G were used as assay controls.

Results showed that 14 out of 16 subtype C Vifs had greater activity against APOBEC3G 186WT compared to APOBEC3G *186R* allele. There were 7 Vifs that displayed a >10% difference in activity between APOBEC3G 186WT and *186R* variants. Additionally the tested subtype C Vifs appear to have greater ability to degrade APOBEC3G 186WT particularly if the Vif was derived from a patient who carried the *186H/H* (WT) genotype. However, if Vif was derived from a patient who carried the *186R/R* genotype, its ability to counteract both the *186H* or *186R* alleles was similar. Interestingly, these subtype C Vifs exhibited contradictory activity to that of NL4-3 Vif which had greater activity against the APOBEC3G *186R* variant. In the absence of Vif, the infectivity of NL4-3ΔVif was abolished.

In addition, we tested the infectivity of HIV-1ΔVif (NL4-3ΔVif) supplemented with Vifs that were subjected to site directed mutagenesis at residue 36, in the presence of APOBEC3G *186H* and *186R* (Figure 5.20). As above, the infectivity of viral stocks was quantified 48 hours post-transfection by use of TZM-bl reporter cells and the NL4-3 WT, NL4-3ΔVif, NL4-3 WT without APOBEC3G were used as assay controls. Vifs expressing a serine at position 36 appeared to have a greater tendency to degrade the APOBEC3G *186H* (WT) allele while a Lysine at this position displayed a greater or equal ability to degrade the APOBEC3G *186R* variant.

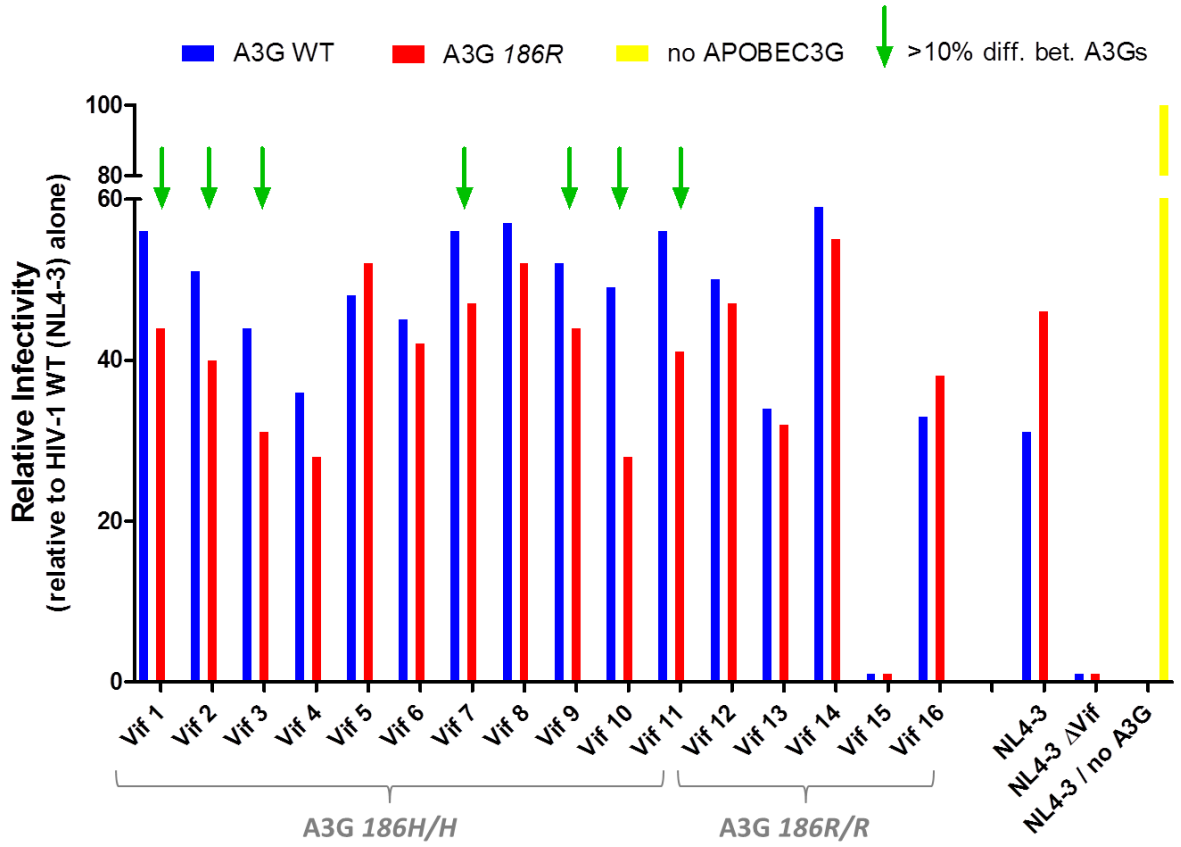


Figure 5.18 Activity spectrum of subtype C Vifs against the APOBEC3G *186H* (WT) and *186R* alleles. Infectivity of HIV-1 ΔVif (NL4-3ΔVif) supplemented with 16 subtype C Vifs was measured 48 hours post-transfection using TZM-bl reporter cells. Vifs 1 – 11 were derived from patients carrying the *186H/H* genotype while Vifs 12 – 16 were derived from those carrying the *186R/R* genotype. NL4-3 WT, NL4-3ΔVif, NL4-3 WT without APOBEC3G were controls.

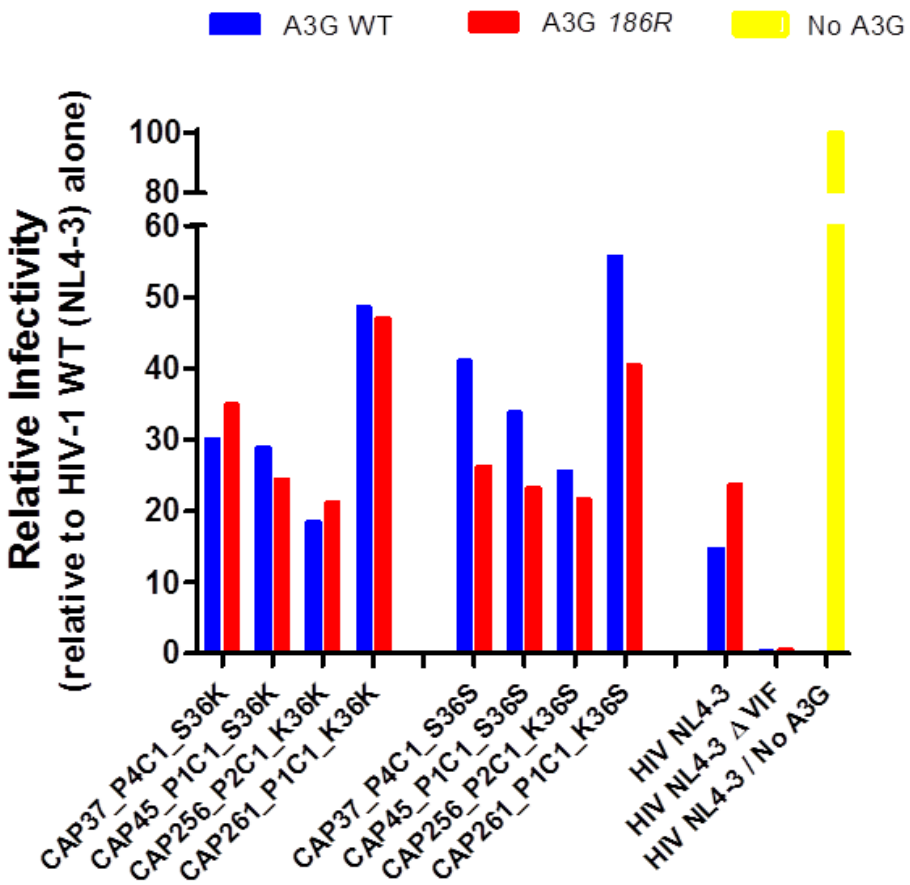


Figure 5.19 Activity spectrum of subtype C Vifs mutated at position 36 against the APOBEC3G *186H* (WT) and *186R* alleles. Infectivity of HIV-1ΔVif (NL4-3ΔVif) complemented with subtype C Vifs expressing either lysine or serine at position 36 was measured 48 hours post-transfection using TZM-bl reporter cells. NL4-3 WT, NL4-3 ΔVif, NL4-3 WT without APOBEC3G were used as controls.

CHAPTER SIX

Discussion and Conclusions

6.1 *APOBEC3G* is Dysregulated in Primary HIV-1 Infection

Cytidine deaminases represent a recently discovered class of novel intrinsic factors that are involved in cellular defense against viruses. Most studies that have characterized these proteins have employed *in vitro* experimental systems that have led to insight into how these proteins work, raising the hope that this knowledge could lead to new therapeutic strategies against HIV-1. Relatively few studies have investigated the contribution of cytidine deaminases to viral control *in vivo*. In this study, we focused on the characterization of *APOBEC3G*; the best studied of the cytidine deaminases. In particular, we were interested in describing the contribution of *APOBEC3G* to viral control during the critical primary infection phase, as well as understanding the kinetics of *APOBEC3G* expression pre- and post-infection. Primary HIV-1 infection is characterized by rapid viral replication during the acute phase, which is followed by resolution of peak viremia to a hypothesized viral set point. Previously, it has been shown that an arm of innate immunity, namely NK cells, dominates early in HIV-1 infection and then declines as virus specific T cells and antibody responses become established (Alter et al. 2007). We thus wondered whether we might see a similar mobilization of intrinsic antiviral host factors such as *APOBEC3G* characterized by its increased expression. Further, we hypothesized that at this critical phase of infection, *APOBEC3G* expression levels might negatively correlate with viremia and positively with CD4+ T cell counts. However, HIV-1 infection is associated with profound immune dysregulation (Munier and Kelleher 2007) and it is unclear if this dysregulation extends to intrinsic antiviral host factors such as APOBEC3G. Therefore, in this study we investigated how primary HIV infection may affect *APOBEC3G* expression.

Our data show that *APOBEC3G* mRNA levels are lower in HIV+ve primary infection PBMCs compared to HIV–ve PBMCs. Previously, a number of studies have also investigated mRNA levels of *APOBEC3G* in PBMCs of HIV-1-uninfected individuals versus during chronic HIV-1 infection. In agreement with our findings, the earlier studies showed that *APOBEC3G* expression levels were higher in HIV-uninfected individuals when compared to HIV infected individuals (Jin et al. 2005; Cho et al. 2006; Gandhi et al. 2008). Our study comparing *APOBEC3G* mRNA in HIV-1–ve and HIV-1 primary infection samples suggests that *APOBEC3G* expression is rapidly downregulated upon HIV-1 infection. To test the possibility that individuals with lower expression of *APOBEC3G* are more susceptible to HIV-1 infection, we quantified *APOBEC3G* mRNA levels in pre-infection samples of 13 seroconverters and compared these levels to baseline *APOBEC3G* mRNA levels of persistently seronegative participants. We found no significant differences between these two groups, suggesting that *APOBEC3G* mRNA levels per se are not associated with protection against HIV-1 infection.

We then addressed the question of whether *APOBEC3G* expression is downregulated upon HIV-1 infection, by comparing matched pre- and post-seroconversion samples from the 13 seroconverters. Our data show that *APOBEC3G* expression is actively downregulated upon HIV-1 infection as the primary infection samples had lower *APOBEC3G* levels than the pre-infection samples. Our results therefore lend further support to previous observations that HIV-1 has specific mechanisms for counteracting APOBEC3G, as a possible immune evasion strategy. We cannot also rule out the possibility that HIV-1 infection is associated with redistribution away from peripheral blood of cellular components that are enriched for *APOBEC3G* or that HIV-1 specifically targets such cells. Previous studies show that immune evasion against APOBEC3G is mediated by HIV-1 Vif, and that it is two-fold, involving

translational and posttranslational inhibitory effects on *APOBEC3G* (Stopak et al. 2003). There have been no previous reports showing that HIV-1 inhibits *APOBEC3G* expression at the transcriptional level. Whether the *APOBEC3G* mRNA reduction seen in HIV+ve samples in this study is tied to or independent of the Vif ubiquitin proteasome degradation mediated via HIV-1 Vif may require further investigation but our results suggest that a third mode of HIV-1 APOBEC3G inhibition may involve down-regulation of mRNA expression. This observation is in contrast to *in vitro* studies where HIV-1 infection does not appear to affect *APOBEC3G* mRNA levels (Sheehy et al. 2002; Kao et al. 2003; Stopak et al. 2003).

We did not find a correlation between *APOBEC3G* mRNA levels and viral load or CD4 counts in this primary infection cohort. This finding is in agreement with at least two other studies, albeit performed in chronic infection settings (Cho et al. 2006; Gandhi et al. 2008). One study found a statistically significant inverse correlation between *APOBEC3G* mRNA levels and viral load and a positive correlation with CD4+ T cell count in chronic HIV-1 infection but it is worth noting that in that study the investigators stimulated PBMCs with antibodies before RNA isolation. This difference in processing and handling of samples may thus explain the unique findings of that study. Given the significant downregulation of *APOBEC3G* mRNA seen in HIV-1 infected PBMCs compared to HIV uninfected ones, it is perhaps not surprising that we did not find a correlation between *APOBEC3G* mRNA levels and viral load or CD4+ T cell counts. HIV appears to have evolved efficient mechanisms for counteracting APOBEC3G even at the transcriptional level thus inhibiting its contribution to antiviral control in most instances. Further studies may be required to assess whether stabilization of *APOBEC3G* mRNA in HIV-1 infection could result in improved correlation with markers of disease progression such as viral load and CD4 counts. Further studies may

also investigate *APOBEC3G* expression in specific subsets of cells within PBMCs that are specifically targeted by HIV-1.

6.2 *APOBEC3G* Genetic Variants Influence CD4+ T cell Counts and Plasma Viral Load

In this study, we also investigated the extent of genetic variation within the *APOBEC3G* gene in a South African study cohort. The Southern African sub-region continues to be the epicentre of the HIV epidemic. Although the number of new infections has decreased in this region by more than 25% in recent years, analyses estimates that approximately 5.6 million people remain HIV-infected in South Africa and the incidence remains unacceptably high (UNAIDS 2012; UNAIDS 2012). Despite these statistics, relatively few studies in southern Africa have attempted to define AIDS restriction genes, defined as genes with polymorphic variants that influence outcome of exposure to HIV-1 or disease progression. AIDS restriction genes and their frequencies may vary according to ethnic background (An et al. 2004; O'Brien and Nelson 2004; Donfack et al. 2006). Besides, there is evidence that AIDS restriction genes that affect the rate of disease progression may have differential effects at distinct phases of infection (Gao et al. 2005). We therefore took the opportunity within a well characterized HIV–ve cohort at high risk for HIV infection to investigate the contribution of *APOBEC3G* gene variants to susceptibility and viral control in an area where HIV-1 subtype C dominates. We described here the frequencies of several SNPs in this cohort and identified several novel SNPs that have not been described before.

It was earlier reported that a genetic variant of *APOBEC3G*, the *H186R* mutation, influenced HIV-1 pathogenesis and accelerated the rate of progression to AIDS in African Americans infected with HIV-1 subtype B (An et al. 2004). This mutation was identified in our cohort,

with a frequency of approximately 30%, and our data shows that this association extends to southern Africans infected with HIV-1C, where the mutation was associated with significantly increased viral loads and decreased CD4+ T cell counts. These associations are detectable during primary infection and become more prominent with progression to early chronic infection. The presence of this polymorphism within *APOBEC3G* may affect its activity or levels of expression by altering its interaction with other proteins, or modifying its editing functions, thus influencing HIV-1 replication (MacGinnitie et al. 1995; Jarmuz et al. 2002; Mariani et al. 2003; An et al. 2004). However, in this study, we were unable to link this polymorphism with expression levels (p=0.695), possibly as a result of the sample number being too small. However, given the lack of correlation between *APOBEC3G* mRNA levels and viral load or mRNA levels, it is doubtful that this polymorphism acts by merely affecting *APOBEC3G* expression.

Our data however, indicates that this SNP is out of Hardy Weinberg Equilibrium (HWE) (p=0.04) in our study population. We were able to eliminate genotyping error as a reason for this, as we have both re-sequencing and TaqMan genotyping confirmation. Further, genotypes that were obtained were consistent between duplicates and free of contamination as the negative controls did not amplify. When HWE was calculated separately for HIV+ve and HIV-ve individuals we found that the HIV+ve group conformed to HWE (p>0.05) while the HIV-ve group were out of HWE (p=0.03). Therefore, the distortion seems to be due to the excess of the *GG* genotype in the negative group (26/31). While not significant, this may suggest that the minor allele is protective against infection in homozygotes (OR=0.54; p=0.23) but not heterozygotes (OR=0.9, p=0.75) using the *AA* as the reference group. This may be the reason for the distortion in HWE in the negative group where we see an excess of *GG* genotypes. This suggests that this mutation may reduce susceptibility to HIV infection,

but upon infection becomes detrimental and accelerates disease progression. A larger sample size will be required to resolve this issue.

APOBEC3G expression may vary within cell types and it is important to consider and define the inter-individual distribution of *APOBEC3G* expression within different PBMC subpopulations and to determine the changes within the same subpopulations during infection. We therefore compared the distribution of *APOBEC3G* in PBMCs and CD4 cells from healthy controls and in chronically HIV-1 infected subjects. Our results showed that *APOBEC3G* mRNA expression was augmented in the CD4+ T cell subpopulation. *APOBEC3G* mRNA levels tended to be higher in CD4+ T cells as compared to levels in PBMCs in the healthy donors (p=0.08) as well as in chronically infected subjects (p=0.02) Although sample numbers were too few, the data suggests that APOBEC3G levels are augmented in cells primarily targeted by HIV-1 as an essential factor in the anti-viral immune response. A study by Koning *et. al*. (2009) showed that *APOBEC3G* expression was higher in B cells and T cells as compared to monocytes (Koning et al. 2009). However, further studies are necessary to investigate *APOBEC3G* expression in a larger sample cohort and in other cell subsets.

6.3 The APOBEC3G *H186R* Polymorphism Does Not Influence Hypermutation

An abundance of APOBEC3G induced hypermutation in HIV-1 infected individuals has been associated with decreased viral loads and increased CD4+ T cell counts (Pace et al. 2006; Land et al. 2008; Vazquez-Perez et al. 2009; Kourteva et al. 2012), however, other studies report contradictory findings (Gandhi et al. 2008; Ulenga et al. 2008). Additionally, APOBEC3G footprints have been identified in treated and untreated patients at different clinical stages of infection (Rose and Korber 2000; Janini et al. 2001; Kieffer et al. 2005; Pace et al. 2006; Keele et al. 2008; Kijak et al. 2008), in infected infants (Koulinska et al. 2003; Amoedo et al. 2011; De Maio et al. 2012), as well as in long term non-progressors and elite suppressors (Huang et al. 1998; Alexander et al. 2000; Wei et al. 2004; Bailey et al. 2006). On the other hand, sub-lethal levels of APOBEC3G-mediated DNA editing may also promote HIV-1 evolution by contributing to its diversification thus resulting in the emergence of drug resistance and immune escape (Mulder et al. 2008; Wood et al. 2009; Kim et al. 2010).

HIV-1 subtype C derived Vif proteins have been shown to have the highest neutralising activity against APOBEC3G (Iwabu et al. 2010) substantiating an earlier finding that the extent of G to A hypermutation was less frequent in viral genomes of subtype C origin as compared to other subtypes (Janini et al. 2001). APOBEC3G mediated DNA editing *in vivo* may be influenced by the presence of APOBEC3G polymorphisms as well as by variation within Vif. Studies thus far have reported no difference in levels of hypermutation for APOBEC3G genetic variants that were studied (Pace et al. 2006; De Maio et al. 2012), however hypermutated proviruses, attributable to both APOBEC3G and -3F, have been identified in individuals with complete or partially defective Vif variants (Simon et al. 2005).

Given the high frequency of the *H186R* polymorphism and its association with disease progression in our study cohort, we were curious to determine the effects of this APOBEC3G variant on hypermutation. We focused on the HIV-1 *env* gene as it has been documented to have high rate of hypermutation (Yu et al. 2004; Suspene et al. 2006).

Phylogenetic analysis of proviral HIV-1 Env sequences revealed that all sequences were subtype C derived with a calculated mean interpatient diversity of 15.6% at the amino acid level. The highest diversity within HIV-1 Env was observed in patient sample CAP84 which diverged by 14.3% from a consensus subtype C reference sequence. Interestingly, further analyses of sequences for investigation of APOBEC hypermutation revealed that CAP84 was significantly hypermutated ($p<0.0001$). Closer inspection of CAP84 revealed that the sequence contained numerous stop codons, a known characteristic consequence of APOBEC3G hypermutation. Further, this patient carried the APOBEC3G *186H/H* (wild type) genotype and had a viral load set point of 8485 copies/ml and experienced episodes of viral control with RNA copies below 2,000 copies/ml during follow-up, including at the time of evaluation (1,710 copies/ml) suggesting a degree of viral inhibition and control of viral replication.

Additionally, we stratified the sample set according to *H186R* genotypes to determine if there was a difference in the level of hypermutation for the APOBEC3G variants. However, our data showed that there was no association between *H186R* genotype and the number of hypermutations present as reported previously (Pace et al. 2006; De Maio et al. 2012).

6.4 APOBEC3F-related Hypermutation Occurs More Frequently than APOBEC3G changes and Associates Inversely with HIV-1 Viremia

The Hypermut 2.0 tool that was used to assess APOBEC3G hypermutation also provides data on APOBEC3F-induced hypermutation. As this data was already available, out of interest we analysed and compared differences between the levels of APOBEC3G and -3F hypermutations for the HIV-1 *env* sequences. Surprisingly, we found that overall that was a greater number of APOBEC3F hypermutations as compared to those induced by APOBEC3G but there was also a significant correlation between the number of APOBEC3G and -3F-induced mutations that were present. Most studies have shown that APOBEC3G is a significantly more powerful inhibitor of HIV-1 replication and causes a greater frequency of G-to-A mutations than APOBEC3F ((Bishop et al. 2004; Wiegand et al. 2004; Zennou and Bieniasz 2006)). However, in keeping with our findings, one study showed that APOBEC3F may function as the major contributor to HIV-1 hypermutation in vivo ((Liddament et al. 2004).

Other studies have also shown that APOBEC3F-related hypermutation can occur more frequently than APOBEC3G changes at specific regions in the HIV-1 genome, the largest being the *pol* PR region (Janini et al. 2001; Armitage et al. 2008; Amoedo et al. 2011). Interestingly, it has also been demonstrated that APOBEC3G and APOBEC3F seem to be co-ordinately expressed and co-regulated in a range of human tissues (Bishop et al. 2004; Wiegand et al. 2004; Kourteva et al. 2012) which may substantiate the correlation between the number of APOBEC3G and -3F induced hypermutations that we observed. However, while APOBEC3G expression has previously been shown to correlate with the amount of G to A mutations (Ulenga et al. 2008), to date there is no evidence that APOBEC3F expression influences the extent of hypermutation.

We next tested for an association between hypermutation and markers of disease progression. Our data showed that there was a significant negative correlation between the levels of APOBEC3F induced hypermutation and viral load. APOBEC3F hypermutation also showed a significant positive correlation with CD4+ T cell count. However, no such significant correlation was noted for APOBEC3G. Contradictory to our findings, most previous studies have reported that APOBEC3F is actually the less potent inhibitor of HIV-1 (Liddament et al. 2004; Miyagi et al. 2007; Mulder et al. 2008; Chaipan et al. 2013). Interestingly, it has been shown that Vif can only partially restore the infectivity of viruses produced in the presence of APOBEC3F, suggesting that APOBEC3F is less susceptible to Vif than APOBEC3G ((Liddament et al. 2004). However, contradictory findings suggest that APOBEC3F is fully susceptible to inhibition by Vif ((Bishop et al. 2004)). Additionally, Liddament et. al further showed that decreases in HIV infectivity attributable to APOBEC3F and APOBEC3G were additive, suggesting that these proteins can function independently.

The interesting findings in our study cohort may be also explained in part by the high frequency of the APOBEC3G *H186R* polymorphism that exists exclusively within African populations, and has been demonstrated by us and others (An et al. 2004) to accelerate disease pathogenesis, thereby inferring loss of APOBEC3G editing function. This may therefore prompt a somewhat larger contribution of APOBEC3F to cytidine deaminase editing and hypermutation in our South African study cohort. APOBEC3F has been understudied in the HIV-1 subtype C setting particularly within African populations and certainly requires further investigation.

6.5 HIV-1 Subtype C Vif Displays Substantial Diversity But Also Contains Several Highly Conserved Motifs

HIV-1 Vif plays a crucial role in establishing and maintaining infection *in vivo* and functions primarily to suppress the APOBEC3 anti-viral function. Even though HIV-1 subtype C is the most prevalent clade globally particularly in regions that bear the world's greatest HIV burden (Buonaguro et al. 2007), this strain of the virus is still relatively understudied. Studies of genetic diversity and characteristics of South African HIV-1 subtype C viruses can provide vital information that can inform vaccine or novel immunotherapeutic designs, particularly at sites involved in host interaction. Previous studies that have characterized subtype C HIV-1 Vif have described only a limited number of sequences (Scriba et al. 2001; Bell et al. 2007; Jacobs et al. 2008).

In this study we cloned and sequenced HIV-1 *vif* from 26 study participants and generated a total of 392 HIV-1 *vif* clonal sequences for analysis. HIV-1 Vif sequences displayed notable diversity with intra-individual sequence differences ranging between 0.1 - 4.9% at the amino acid (aa) level and 0.1 - 2.8% at the DNA level and inter-individual diversity ranging from 6.2 - 19.2% (aa) and 4.7 - 11.4% (DNA). A previous study on a Ugandan cohort of subtypes A, D and an A/D mosaic, reported intra-individual differences of ≤ 6% (aa) or ≤ 4% (DNA) and inter-individual variability of ≤ 28% (aa) or ≤ 16% (DNA) (Wieland et al. 1997). These levels of diversity were comparable to but slightly higher than that observed in our subtype C cohort.

Additionally, consensus *vif* sequences from each individual diverged by 1.8 - 7% (DNA) and 3.8 - 13.8% (aa) from a consensus subtype C reference sequence. A study on subtype B HIV-1 *vif* showed a divergence of 3 - 9% from the prototype HIV-1 NL4-3 *vif* allele ((Simon et al.

2005). These data confirm that *vif* genes have relatively high *in vivo* genetic variability. HIV-1 Vif divergence perhaps occurs as an adaptive response to the selection pressure exerted by APOBEC3G. It is also probable that the sequence diversity displayed by HIV-1 Vif may result in differential activity against APOBEC3G which has also been tested in the current study.

Despite the substantial degree of diversity that was observed, several highly conserved motifs were present within the HIV-1 subtype C Vif proteins analysed in study and were in agreement with previous studies (Scriba et al. 2001; Bell et al. 2007; Jacobs et al. 2008). Interestingly, all of the conserved motifs are key sites involved in the Vifs interaction with APOBEC3G or cullin-5 and the E3 ubiquitin ligase complex which ultimately result in the degradation of the host restriction factor. Conservation of these functional motifs suggests that HIV-1 Vif strives to maintain its primary activity to counteract the obstructive anti-viral effect of APOBEC3G. Additionally, these stretches of conserved residues represent attractive drug target sites.

6.6 APOBEC3G *H186R* Genotypes do not Influence *in Vivo* HIV-1 Vif Sequence Diversity but Demonstrate an *in Vivo* and *in Vitro* Association with Vif Residues 36 and 96

Data from the present study as well as a previous study (An et al. 2004) showed an association between the APOBEC3G *186R/R* polymorphism and accelerated progression to AIDS, however the mechanism remains unknown. Studies also indicate that natural sequence variation at a number of pivotal residues can influence the Vif-APOBEC interaction (Santa-Marta et al. 2005; Simon et al. 2005). We were therefore interested in determining whether the *186R/R* polymorphism influences the diversity of HIV-1 Vif and/or the evolution of

specific Vif variants which may consequently alter the interaction between Vif and APOBEC3G.

To determine whether APOBEC3G variation influences the overall diversity of HIV-1 Vif we compared intra-patient diversities among the APOBEC3G *H186R* genotype groups. However, our data shows that the *H186R* genotype does not have an impact on overall HIV-1 Vif diversity per se.

We also assessed the frequency of amino acid residues at each position in our HIV-1 Vif sequence alignment and concentrated on sequences that belonged to the *186H/H* (wild type) and the *186R/R* (mutant) groups. There was a striking difference in the amino acid profile at residues 36 and 93 for each of the genotype groups. The most common amino acid at these two positions differed approximately five fold between the *186R/R* group and the *186H/H* group. At position 36, there was a strong preference for Lysine (K) in APOBEC3G *186R/R* individuals and for Serine (S) in *186H/H* individuals, while at position 93 there was a strong preference for an Arginine (R) or a Serine (S) for APOBEC3G *186H/H* and *186R/R* individuals respectively. Comparison of Vif positions 36 and 93 between other HIV-1 subtypes indicated that the amino acid frequency and distribution at these loci in the studied Vif sequences is similar to other subtype C Vif sequences. Notably, at position 36, the predominant residue for subtype C is a Serine (S), while all other subtypes typically express a Lysine (K) at this site. At position 93, Arginine (R) is the predominant amino acid expressed in all subtypes.

Although, there was no association between the APOBEC3G *H186R* genotype and overall Vif diversity, the latter data suggests that the APOBEC3G polymorphism may have an

impact on the evolution of individual Vif residues; however it is difficult to speculate about how this might influence viral pathogenesis.

We therefore proceeded to investigate the phenotypic effect of altering Vif residue 36, by site directed mutagenesis, on degradation of APOBEC3G variants as well as its impact on infectivity in the presence of the APOBEC3G 186WT (H) and *186R* alleles. We chose to focus on residue 36 as it is located in the amino-terminal domain of Vif which has been shown to play a critical role in binding of APOBEC3G (Simon et al. 2005; Schrofelbauer et al. 2006; Mehle et al. 2007; Russell and Pathak 2007; Yamashita et al. 2008). While no specific pattern of degradation of APOBEC3G was observed with the presence of either a Serine or Lysine at Vif position 36 by Western blot, HIV-1 infectivity assays showed that Vifs expressing a Serine at position 36 had a stronger propensity to degrade the APOBEC3G *186H* (WT) allele whereas those expressing a Lysine at this position had a greater or equal ability to degrade the APOBEC3G *186R* variant. These findings further suggest that the evolution of individual Vif residues is shaped by polymorphisms within APOBEC3G, in this case *H186R*. Thus, it seems likely that HIV-1 Vif adapts to ensure optimal degradation of the APOBEC3G variant that it encounters. It may also be possible that residue 36 acts in synergy with residue 93 in controlling Vifs antagonism of APOBEC3G; however this remains to be investigated.

Interestingly, a recent study (De Maio et al. 2011) observed a correlation between the *APOBEC3G* polymorphism C40693T, which has been associated with an increased risk of infection in a cohort of Caucasian adults (Valcke et al. 2006), and a Vif variant E45D which lies in a region involved in the Vif-APOBEC3G interaction. The residue at position 45 is significant to Vif interaction with the APOBEC3 proteins, as it has been shown that the

naturally occurring *E45G* variant is able to counteract APOBEC3F but not APOBEC3G (Simon et al. 2005; Mulder et al. 2008). This concordance further highlights that Vif substitutions are may be selectively influenced by host APOBEC3G genetic variants.

6.7 HIV-1 Vif Adapts to Preferentially Counteract the APOBEC3G Wild Type *186H* Allele

Investigations on primate models of SIV infection demonstrate that adaptation of lentiviruses to their host is influenced by cellular restriction factors that create an obstruction to viral replication. Specifically, it has been reported that in reacting to changes and polymorphisms within APOBEC3G, SIV adapts through evolution of its Vif protein which is required to re-target the restriction factor for destruction (Malim and Emerman 2008; Compton et al. 2012). Further, Vif degrades APOBEC3G in a species specific manner. The specificity of Vif is indicative of its amazing adaptability to varying environments (Mariani et al. 2003; Bogerd et al. 2004; Xu et al. 2004).

Moreover, HIV-1 is known to adapt to host immune pressure as demonstrated by studies that show the presence of positively selected amino acid changes in various viral proteins (Liang et al. 2007; Liang et al. 2008; Peters et al. 2008). Additionally, there is subtype variation in the frequency of positive selection sites in Envelope and Pol sequences from various geographical locations, suggesting that HIV-1 subtypes in different parts of the world evolve in response to specific immunogenetic profiles of the host population (Liang et al. 2007; Avila-Rios et al. 2009). The most well documented example of HIV-1 adaptation to host immune pressure is that of specific and predictable viral adaptation to HLA class I mediated CTL pressure, which results in selection of immune escape mutations (Phillips et al. 1991; Goulder et al. 2001; Draenert et al. 2004), that hinder viral peptide binding to HLA molecules

and ultimately restrict the processes of antigen presentation to CTLs. Further, depending on their costs to viral fitness, some CTL escape mutations upon transmission can revert to wild type in the absence of the selecting HLA allele or may also select for compensatory mutations that restore viral fitness (Friedrich et al. 2004; Leslie et al. 2004; Liu et al. 2007).

In this study we tested patient derived subtype C HIV-1 *vif* alleles for their ability to counteract the action of APOBEC3G *H186R* variants from a South African cohort. Additionally, infectivity of HIV-1ΔVif (NL4-3ΔVif) supplemented with 16 patient derived subtype C Vifs was measured in the presence of either APOBEC3G *186H* or *186R* expression plasmids.

Our findings show that subtype C Vifs appear to have greater ability to degrade the wild type APOBEC3G 186 H allele particularly if the Vif was derived from a patient who carried the *186H/H* genotype. However, if Vif was derived from a patient who had the*186R/R* genotype, its ability to counteract both the *186H* or *186R* alleles was similar. Our preceding association of the *186R/R* polymorphism with accelerated disease pathogenesis implies loss of anti-viral function of this variant. Thus, preferential degradation of the wild type APOBEC3G *186H* allele, corroborates adaptation of Vif to target the more active and hostile APOBEC3G *186H* allele.

Our study demonstrates that just as HIV-1 has the remarkable capability to adapt across species as well as the ability to adapt to immune pressure exerted by HLA alleles, so too exists the possibility that the virus also adapts to intrinsic immune pressure exerted by host restriction factors, presumably in a predictable and specific way.

6.8 Study Limitations

Although the research project has achieved its aims, there were several unavoidable limitations. Firstly, the sample size of the study population was relatively small and a larger cohort may have strengthened the findings of the study.

Secondly, *APOBEC3G* mRNA levels were measured in PBMCs as opposed to specific HIV target cells such as T cells, DCs and macrophages. We are aware that *APOBEC3G* expression may vary within cell types and that it is important to define these cell subsets in which *APOBEC3G* expression is enriched or naturally expressed. As mentioned we were unable to perform extensive experiments to assess *APOBEC3G* expression in the different cell types due to the scarcity of samples as well as low cell counts, of approximately 5 million cells of the samples which were available. However, two recent studies performed a detailed examination of *APOBEC3* expression in haemopoetic cell subsets, in a range of human tissue and in lymphocyte subsets (Koning et al. 2009; Refsland et al. 2010).

Additionally, *APOBEC* hypermutation was not assessed against the patient's own founder virus but against a consensus subtype C reference sequence. Although it is acceptable to use an appropriate subtype consensus reference sequence when assessing hypermutation in a set of unrelated sequences, the dataset would have been more informative if longitudinal samples in an intrapatient set was analysed with the reference representing the most common form in the first sampled time point. Although, there is evidence that hypermutated proviral HIV-1 DNA sequences can be demonstrated by bulk PCR methods (Pace et al. 2006), some may argue that the study of hypermutation by bulk sequencing is not ideal and truly informative, and that hypermutation is best studied in individual clonal sequences. It may therefore be

necessary to repeat the hypermutation experiments by cloning or 3D PCR (Suspene et al. 2005) to confirm our findings.

We were also unable to carry forward all study samples in which APOBEC3G expression was measured, for assessment of *APOBEC3G* hypermutation. We were therefore unable to perform a complete analysis of the relationship between the degree of hypermutation and the levels of *APOBEC3G* mRNA expression. However, it has been reported before that *APOBEC3G* expression correlates with the extent of G to A mutations (Ulenga et al. 2008).

HIV-1 Vif sequences were only characterised at a single time point at 36 months post infection. A longitudinal analysis of Vif sequences from each patient would have provided further insight into the evolution and adaptation of Vif from acquisition to chronic infection. Additionally, even though there was considerable variation in Vif expression levels this was not standardised before investigating their effects on APOBEC3G degradation. This is a limitation of our study and needs to re-assessed in the future using Vifs that are similar or matched for their levels of expression.

6.9 Conclusions

In conclusion, we have shown that HIV-1 infection is associated with rapid downregulation of *APOBEC3G* expression at the transcriptional level. Studies to decipher the mechanisms involved and to possibly develop means for counteracting this are needed. During primary infection, *APOBEC3G* expression levels in PBMCs do not correlate with viral loads or CD4+ T cell counts. Importantly, this is the first study to describe genetic polymorphisms with *APOBEC3G* in an African setting, where HIV-1C prevalence and incidence rates are extremely high. Genetic variants of *APOBEC3G* were shown to significantly affect early and late HIV-1 pathogenesis. This is the first study that indicates that *APOBEC3G* may be an important AIDS restriction gene during the primary phase of HIV.

We also found that *APOBEC3G* genetic variation did not contribute to the differential development of hypermutation. Interestingly, APOBEC3F induced hypermutation was more frequent than that of APOBEC3G and associated inversely with HIV-1 viraemia, suggesting that APOBEC3F may in fact play a greater role in HIV-1 subtype C pathogenesis particularly in African populations.

In an effort to understand the mechanisms by which *APOBEC3G* genetic variants affect HIV-1 pathogenesis our investigation of HIV-1 Vif revealed that although this gene displays considerably diversity it also maintains several conserved regions essential for its interaction with and antagonism of APOBEC3G. There was no association between *H186R* genotype and HIV-1 Vif diversity; however, this *APOBEC3G* variant did associate with changes at specific Vif residues, 36 and 93. HIV-1 subtype C Vifs also demonstrated adaptation to preferentially target and counteract the functionally intact wild type *186H* allele, indicating that Vif characteristics are certainly shaped by the host genotype. Taken together these data

exemplify the existence of the co-evolutionary arms race that exists between HIV-1 Vif and the host protein APOBEC3G.

This is the first extensive study on the role of APOBEC3G in HIV-1 subtype C infection within the South African setting. The study emphasises that the dynamic of the APOBEC3G-Vif interplay may vary within different populations, based on genetic variants that exist within the population. In terms of clinical relevance, the APOBE3G-Vif interaction exposes a lucrative target for the design of drug therapy, however there remains controversy about which counterpart should be targeted to achieve optimal antiviral effects. Inhibition of Vif would seem to be the obvious and best strategy as this would allow to APOBEC3 proteins to freely inhibit and destroy the virus. However it is interesting, that some scientists have also proposed that inhibiting APOBEC proteins and reducing the rate of APOBEC induced mutations, would reduce sublethal editing and thus evolution of HIV-1, thereby increasing vulnerability of the virus to adaptive immune response as well as decreasing the emergence of drug resistant strains. Nevertheless, our study has raised the possibility of population specific differences that influences this virus-host interaction, which may need to be considered in drug therapy design, but further studies are needed to devise the best strategy.

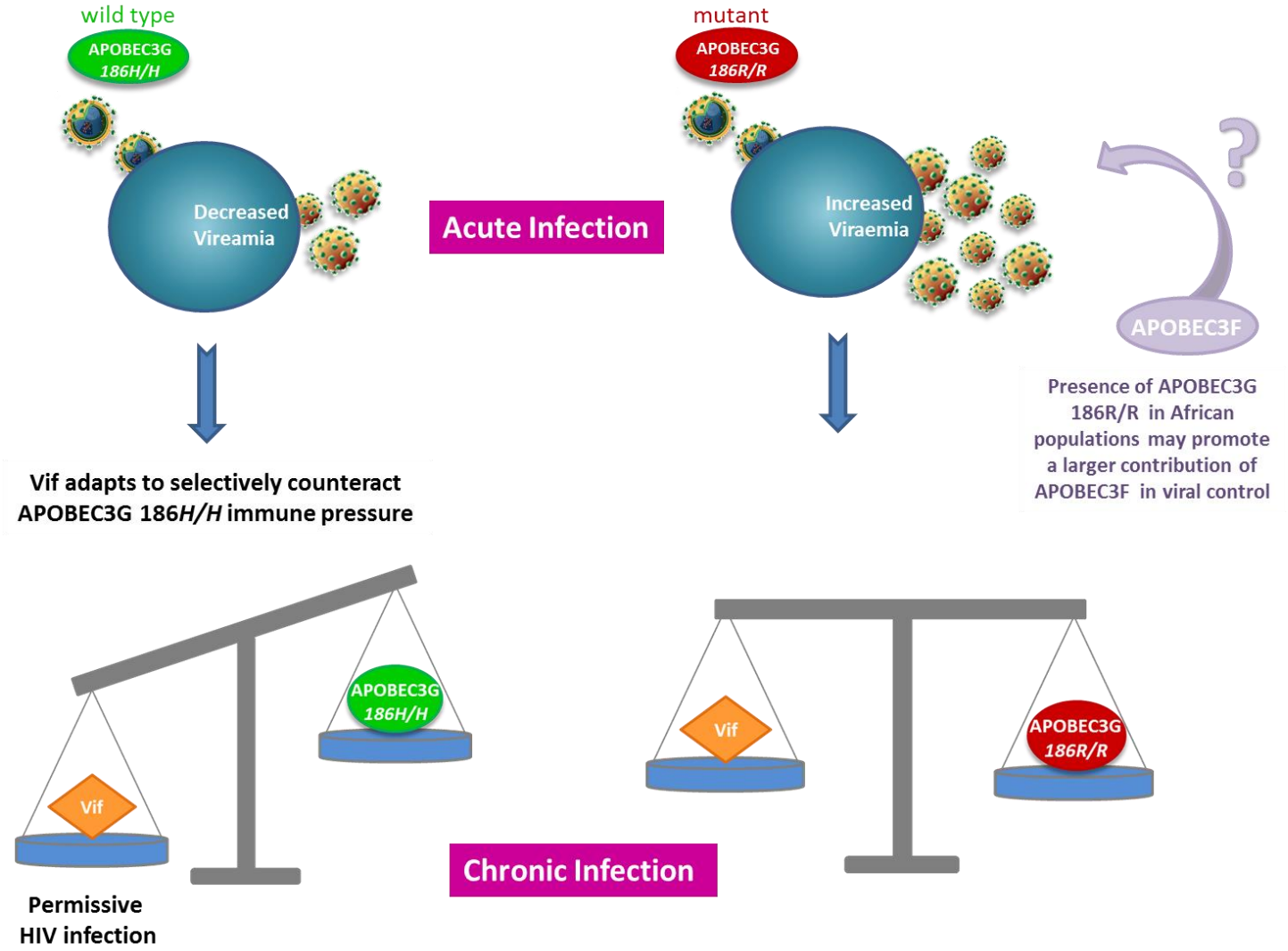
6.10 Future Directions

This study has raised many significant leads that need to be explored in future studies. Firstly, a study to assess APOBEC3 hypermutations at a clonal level in a longitudinal sample set would provide further evidence about the roles of APOBEC3G and -3F in HIV-1 subtype C infection. Further a more comprehensive investigation on the influence of APOBEC3F on HIV-1 subtype C infection in a cohort of African ethnicity would be necessary to determine if

this host restriction factor may be playing a more crucial role in restricting HIV-1 infection in this population.

Additionally, an interesting study would be to characterise Vif sequences longitudinally to determine the degree and effects of selective pressure being exerted by APOBEC3G on the viral protein over time from initial infection. Functional studies of Vif adaptation to APOBEC3G variants would need to be extended to a larger sample set. It would also be interesting to extend the functional studies to assess Vif adaptation to APOBEC3G variants in a longitudinal manner.

Additionally, the effects of amino acids at positions 36 and 93 on Vif function also need to be investigated further in a larger group of samples. The mutations at these positions will also have to be introduced and tested simultaneously to determine whether they act in a synergistic manner to influence Vif function.



An overall model summarising the effects of APOBEC3G variants on HIV-1 viraemia and their interaction with HIV-1 Vif and the possible role of APOBEC3F in HIV-1 subtype C infection as suggested by the findings of this study.

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APPENDIX