HIV-1 SPECIFIC T-CELL RESPONSES IN CHRONIC HIV INFECTED CHILDREN DURING CONTINUOUS TREATMENT AND STRUCTURED TREATMENT INTERRUPTIONS (STI)

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SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS IN MEDICAL SCIENCE IN THE DEPARTMENT OF PAEDIATRICS, UNIVERSITY OF KWAZULU-NATAL

AUTHOR'S DECLARATION

This study represents original work by the author. It has not been submitted previously to this or any other University. Where use was made of the work of others, it has been duly acknowledged in the text.

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PRESENTATIONS

1. **The 2nd South African AIDS Conference**

   Durban, South Africa.

   Title: HIV-1 Specific T-Cell Responses in Chronic HIV-1 Paediatric Subjects Undergoing Continuous HAART and Structured Treatment Interruptions.

   June 2005 – Poster Presentation

2. **The 3rd IAS Conference on HIV Pathogenesis and Treatment**

   Rio de Janeiro, Brasil

   Title: HIV-1 Specific T-cell Responses in Chronic HIV-1 Paediatric Subjects Undergoing Continuous HAART and Structured Treatment Interruptions.

   July 2005 - Poster Presentation

3. **Research Faculty Day, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal**

   Durban, South Africa.

   Title: HIV-1 Specific T-cell Responses in Chronic HIV-1 Paediatric Subjects Undergoing Continuous HAART and Structured Treatment Interruptions.

   September 2005 - Poster Presentation

4. **The 3rd South African AIDS Conference**

   Durban, South Africa.

   Title: The Association of HIV-1 Specific T-Cell Responses and Drug-Resistance in Chronic Paediatric Infection Before and During Antiretroviral Therapy (Continuous and Structured Treatment Interruptions).

   June 2007 - Poster Presentation

5. **The 4th IAS Conference on HIV Pathogenesis and Treatment**
   Sydney, Australia
   Title: The Association of HIV-1 Specific T-Cell Responses and Drug-Resistance in Chronic Paediatric Infection Before and During Antiretroviral Therapy (Continuous and Structured Treatment Interruptions).
   July 2007 - Poster Presentation

6. **Research Faculty Day, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal**
   Durban, South Africa.
   Title: The Association of HIV-1 Specific T-Cell Responses and Drug-Resistance in Chronic Paediatric Infection Before and During Antiretroviral Therapy (Continuous and Structured Treatment Interruptions).
   September 2007 - Poster Presentation

ACKNOWLEGDEMENTS

I wish to thank the following people who have helped me make this achievement of obtaining this degree possible:

Dr Photini Kiepiela (for supervising the laboratory work and the first draft of this thesis);

Professor Thumbi Ndung'u (for reviewing and supervision of this thesis);

Professor Raziya Bobat (Department of Paediatrics – UKZN);

Dr Gupreet Kindra (Clinician for the study);

Zetu Gwamanda (Study counselor);

The HPP team;

Professor Bruce Walker (USA) and Professor Philip Goulder (UK) for their motivation and guidance during my studies;

The children involved in this study and to their supportive mothers and guardians, a very big thank you;

This study was funded by BMS–Secure the Future;

A special thank you to my very supportive family who encouraged me during the time of my studies.

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LIST OF ABBREVIATIONS

3TC	:	2',3'-dideoxy-3'-thiacytidine
ABC	:	Abacavir
ACTG	:	AIDS Clinical Trials Group
AIDS	:	Acquired Immune Deficiency Syndrome
APC	:	Allophycocyanin
ARV	:	Antiretroviral
AZT	:	Azidothymidine
BCIP	:	Bromo-Chloro-Indoylphosphate
BSC	:	Biosafety Cabinet
CAT	:	Continuous Treatment Arm
CD	:	Cluster of Differentiation
CDC	:	Centre for Disease Control
CTL	:	Cytotoxic T-Lymphocytes
DHHS	:	Department of Health and Human Sciences
DMSO	:	Dimethylsulfoxide
DNA	:	Deoxyribonucleic Acid
DSMB	:	Data Safety and Monitoring Board
EDTA	:	Ethylenediaminetetraacetic acid
ELISPOT	:	Ezyme-linked ImmunoSPOT
FBC	:	Full Blood Count
FCS	:	Fetal Calf Serum
FITC	:	Fluorescein Isothiocyanate
HAART	:	Highly Active Antiretroviral Therapy
HIV	:	Human Immunodeficiency Virus
HIV-RNA	:	Human Immunodeficiency Virus – Ribonucleic acid
HLA	:	Human Leukocyte Antigen
ICF	:	Instrument Cleaning Fluid
ICS	:	Intracellular Cytokine Staining

IFN-Y	:	Interferon gamma
MGH	:	Massachusetts General Hospital
MMX	:	Master Mix
Mn2+	:	Manganese ion
NBT	:	Nitroblue Tetrozolium Chloride
NHP	:	Normal Human Plasma
NNRTI	:	Non-nucleoside Reverse Transcriptase Inhibitors
NRTI	:	Nucleoside Reverse Transcriptase Inhibitors
NVP	:	Nevirapine
OLP	:	Overlapping Peptide
PARV	:	Paediatric Antiretroviral
PBMC	:	Peripheral Blood Mononuclear Cells
PBS	:	Phosphate Buffered Saline
PCR	:	Polymerase Chain Reaction
PE	:	Phycoerythrin
PerCP	:	Peridinin Chlorophyll Protein
PHA	:	Phytohaemagglutinin
PMT	:	Photomultiplier Tube
PMTCT	:	Prevention of Mother to Child Transmission
QS	:	Quality Standard
RBC	:	Red Blood Cell
SFCs	:	Spot Forming Cells
SMART	:	Strategies for the Management of Antiretroviral Therapy
STI	:	Structured Treatment Interruption
TAT	:	Treatment Interruption Arm
TI	:	Treatment Interruption
TRIS	:	Tris(hydroxymethyl)aminomethane

ABSTRACT

BACKGROUND

Sub-Saharan Africa has the highest number of HIV-infected individuals and limited treatment programs. The use of Highly Active Antiretroviral Therapy (HAART) has resulted in a considerable decrease in morbidity and mortality among HIV-infected individuals. Long-term use of HAART has several limitations relating to cost, drug toxicity and adherence. Structured Treatment Interruption (STI) has been proposed as a therapeutic approach which limits the exposure to continuous HAART, but retains the benefits thereof. The role of HIV-specific T-cell responses in the control of viraemia has not been well studied in children and it is not clear when these responses become detectable or whether they are associated with improved viral control. Furthermore, antiretroviral drug resistance is well documented in adults infected with HIV-1 clade B virus but comparable information is lacking for chronic paediatric clade C virus infection. This pilot study focused on a chronic HIV-infected paediatric cohort from Durban, South Africa, to assess the immunologic and virologic responses in perinatal HIV-infected children undergoing STI.

METHODS

Thirty chronic HIV-infected treatment naïve children were enrolled and randomised into either the treatment interruption or continuous treatment group. Longitudinal measurements of viral loads and CD4 percentages were done at scheduled intervals. Peripheral blood mononuclear cells (PBMCs) were screened for cytotoxic T-lymphocyte (CTL) gamma interferon (IFN-γ) enzyme-linked immunospot (ELISpot) assay responses using 410 peptides which spanned the entire HIV-1 clade C proteome. Intracellular cytokine staining (ICS) was done to distinguish between IFN-γ Gag-specific T-helper and cytotoxic T cell responses. Pre-HAART drug resistance mutations testing and HLA typing were done for all children.

RESULTS

There was a significant increase in the median CD4 percentage after HAART was introduced. Six children randomized to the STI arm did not undergo treatment interruption because their viral loads remained detectable at the time of scheduled interruption. Most HIV proteins were

targeted in this paediatric cohort. Gag was the most frequently targeted HIV-1 protein (93.1%). In both treatment groups, there were broadening of T-cell responses, however, the magnitude of T-cell responses decreased over time on HAART. Drug-resistant mutations were detectable in 4/29 children before initiation of HAART.

CONCLUSION

In this pilot study, the HIV-1-specific $CD8^+$ and $CD4^+$ T-cell responses were detected before and during HAART. Although the treatment interruption period was short, there were no adverse outcomes in either the continuous or treatment interruption groups in terms of death or other clinical outcomes. This study suggests that it is important to continue to explore alternative treatment strategies in order to reduce cost and toxicity as well as to enhance adherence.

CHAPTER ONE

1. INTRODUCTION

"*In all human affairs . . . there is a single dominant factor – 'TIME'. To make sense of the present state of science, we need to know how it got like that: we cannot avoid an historical account . . . . To extrapolate into the future we must look backwards a little into the past*" -- JM. Ziman

The human immunodeficiency virus (HIV) has generated two and a half decades of research in trying to understand how this pathogen interacts with its human host, leading to infection and disease outcomes. Although important concepts such as the complex interaction between human leukocyte antigen (HLA) types and the virus to modulate immune responses have emerged; as well as major technological advances that have allowed the study of T-cell dynamics, there still remain many unanswered questions (Rowland-Jones *et al*, 2003). Despite many accompanying conflicting debates and conflicts, more than two decades of HIV research has helped us better understand this infection and how to prevent and treat it. However, no vaccine has yet been shown to either prevent infection or control viral replication i.e. preventative or prophylactic vaccine.

1.1 EPIDEMIOLOGY OF HIV GLOBALLY, REGIONALLY AND SOUTH AFRICA

More than twenty five years have passed since HIV was discovered and shown to be the cause of AIDS. However, the disease burden of the HIV/AIDS pandemic and the consequent human suffering continue to grow steadily (Garber *et al*, 2004).

HIV is the leading cause of death worldwide especially among those in the 15-59 year age group (UNAIDS/WHO Worldwide HIV and AIDS Summary, 2007). It is considered a threat to the economic well-being, social and political stability of many nations (UN Population Division, 2005 and UNAIDS, 2006). Globally, the number of people living with HIV rose

from around 8 million in 1990 to almost 33 million in 2007. Statistical reports have indicated a decrease in the estimated number of people living with HIV from 39 million in 2005 to 33 million in 2007. It was estimated that 2.1 million HIV-infected persons died in 2007 of which 1.7 million were adults and 0.33 million were child deaths worldwide (Kaiser Family Foundation: HIV/AIDS Policy Fact Sheet, 2007).

Regional statistics at the end of 2007 indicated that sub-Saharan Africa has 22.5 million adults and children living with HIV/AIDS. New HIV infections in adults and children were 1.7 million and 1.6 million deaths were reported in adults and children. More than 11 million children in Africa have been orphaned by AIDS. The estimated number of people living with HIV in sub-Saharan Africa was 69% in 2007 (Kaiser Family Foundation: HIV/AIDS Policy Fact Sheet, 2007).

These alarming figures have highlighted sub-Saharan Africa as the hardest hit region in the world with the highest number of individuals infected with HIV-1. At least 22.5 million people (adults and children) in sub-Saharan Africa are infected with HIV with an adult prevalence of 5% thus making HIV/AIDS the leading cause of death in sub-Saharan Africa.

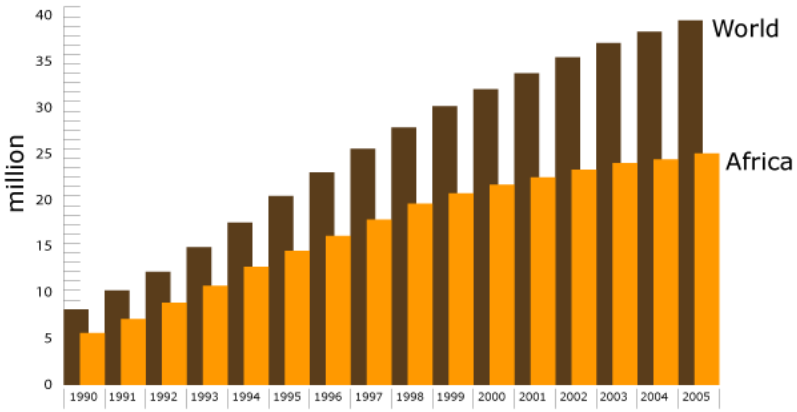


Figure 1. The number of people living with HIV in the world (shown in brown bars) in contrast to the number of people living with HIV in Sub-Saharan Africa (shown in orange bars).

Adapted from: Worldwide AIDS and HIV Statistics including Deaths, 2007 [www.advert.org/worldstats.htm (Accessed 12 June 2007)]

South Africa is experiencing one of the most severe AIDS epidemics in the world. By the end of 2005, it was estimated that almost 1000 deaths occurred every day. AIDS deaths have become alarmingly common in South Africa and almost 71% of all deaths are among those aged 15-49 years (UNAIDS, 2007). The South African Department of Health Study estimated that 29.1% of pregnant women were living with HIV in 2006. KwaZulu Natal recorded the highest rates (39.1% prevalence in 2006) followed by Mpumalanga and Free State (32.1% and 31.1% respectively). The most rapid increase in South Africa's HIV prevalence occurred from 1993-2000, during which time the country was distracted by major political changes. There has been much progress, with the introduction of South African National HIV Treatment Programme and antiretroviral treatment started in 2004, although the distribution of drugs has been relatively slow.

It is unfortunate that this region had to bear the brunt of more than five hundred thousand infants who became HIV infected prior to the introduction of the mother-to-child prevention programs. In addition, due to civil conflicts and wars, large numbers of refugees are produced and they are at greater risk of contracting the disease (Girard *et al*, 2006). The estimated population for South Africa was 47.4 million at mid-year 2006, with 15.3 million children between the ages of 0-14 years. KwaZulu-Natal (KZN) has the largest share of the South African population, with just over 20% of the total population living in the province (SA News, 2006).

The estimated HIV prevalence among antenatal clinic attendees for KZN was 33.5% in 2001, which increased to 39.1% in 2006 with the highest estimation of 40.7% in 2004. This increase occurred despite the South African High Court who ordered the Government to make the drug nevirapine available to pregnant women to help prevent the transmission of HIV to their unborn babies (HIV and AIDS Stats, 2005).

In 2005 it was estimated that at least 2 million children in Sub-Saharan Africa were living with HIV. They represented more than 85% of all children living with HIV worldwide (UNAIDS, 2006). The majority of these children would have become infected with HIV during pregnancy or through breastfeeding from their mothers who are HIV infected. In many

developed countries, steps were taken to virtually eliminate Mother To Child Transmission (MTCT). Sub-Saharan Africa continues to be severely affected by this problem and this is due to the lack of drugs, services, information and shortage of testing facilities.

HIV infections are alarmingly common among children in South Africa especially because women who are HIV positive are still not receiving antiretroviral treatment that could prevent HIV transmission to their unborn babies. Government antenatal surveys indicated that there were 260 000 children below the age of fifteen years, living with HIV in 2006 (Department of Health, 2006). Children living with HIV are highly vulnerable to illness and death unless they are provided with paediatric antiretroviral treatment. Unfortunately there is a great shortage of antiretroviral treatment in South Africa. The AIDS Law Project in Gauteng, estimated that 50 000 children in South Africa were in need of antiretroviral treatment at the beginning of 2006 but only 10 000 were receiving them (Mail and Guardian Online, 2006). These statistics underline the urgency for the development of an HIV vaccine, in one of the populations most severely affected.

Despite the introduction of the PMTCT programs and the start of the South African Government's treatment programs, the high levels of new infections occurring in South Africa reflects the difficulties that are being faced by the AIDS education and prevention campaigns. In addition, the high number of deaths in the country reflects the continuing lack of antiretroviral treatment availability. South Africa has had a turbulent past and there are many possible reasons why South Africa has been so badly affected by AIDS, these include poverty, social instability, lack of government action, stigma and discrimination, prohibitive cost of antiretroviral drugs, delivery delays, insufficient counseling of HIV infected individuals and lack of health-care professionals.

1.2 PATHOGENEISIS

1.2.1 HIV structure

Like all viruses, HIV cannot grow or reproduce on its own. It must infect the cells of a living organism in order to make new copies of itself. Outside the human cell, it exists roughly as spherical particles (called virions) and these particles are too small to be seen through an ordinary microscope but they can be seen clearly with an electron microscope.

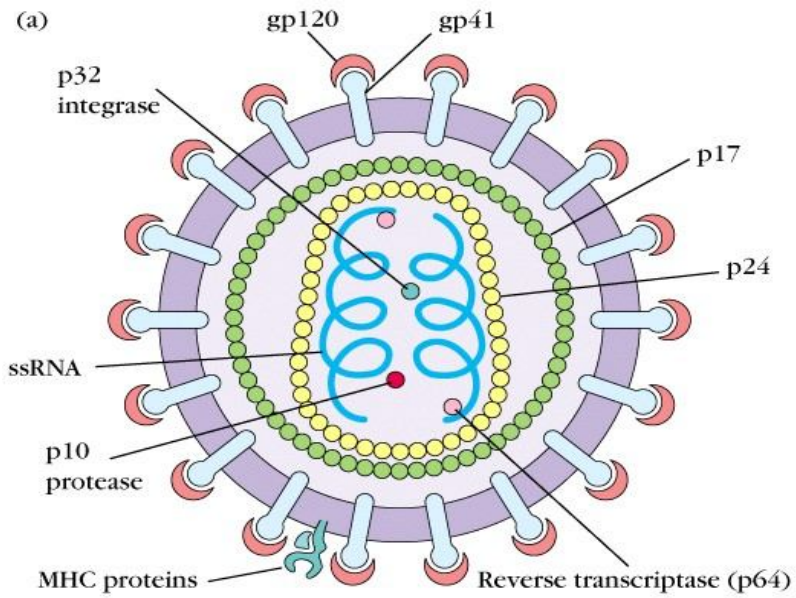


Figure 2. Cross-sectional schematic diagram of HIV virion

Adapted from: http://tutor.lscf.ucsb.edu/instdev/sears/immunology/chapter19/figure19-08a (Accessed 18 April 2007)

The virion surrounds itself with a coat of fatty material known as the viral envelope. From this membrane, there are 72 glycoprotein (gp) projections composed of the proteins gp41 and gp120. Gp41 is a transmembrane molecule that crosses the lipid bi-layer of the viral envelope. Gp120 is non-covalently associated with gp41 and it serves as the viral receptor for the CD4

host cells. The matrix is the region found just below the viral envelope and this is composed of the protein p17. The conical shaped viral core (capsid) comprises of the p24 protein and is located below the p17 region. There are three enzymes found inside the viral core, which are required for HIV replication called reverse transcriptase (p64), integrase (p32) and protease (p10).

1.2.2 HIV genome

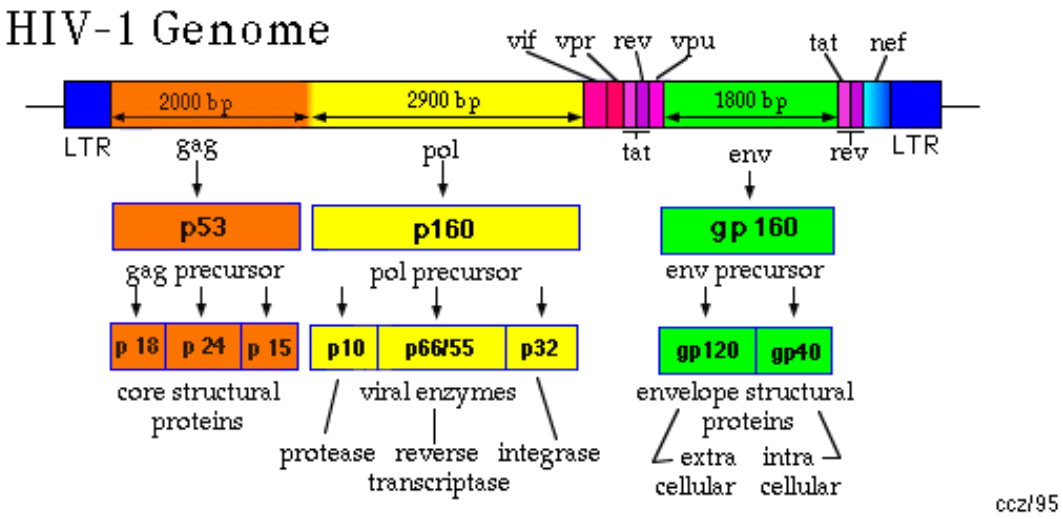


Figure 3. The HIV genome with proteins specific to HIV
Adapted from: www.yale.edu/bio243/HIV/newhiv.html
(Accessed 17 April 2007)

HIV's genetic material consists of two identical strands of RNA which are found within the core of the virion. HIV has nine genes, three of which contain the information needed to make structural proteins for new virus particles. These are the *gag* (group specific antigen gene), *pol* (polymerase gene) and the *env* (envelope gene). The other six genes code for the proteins that control the ability of HIV to infect a cell, produce new copies of the virus or to cause disease. These genes are known as *tat* (encodes transactivator protein), *rev* (encodes a regulator of

expression of viral protein), *vif* (associated with viral infectivity), *vpu* (viral protein U), *vpr* (viral protein R) and *nef* (encodes negative regulator protein).

1.2.3 The origins and diversity of HIV

Although the origins of HIV are unclear, Robert Gallo and Luc Montagnier discovered the HIV-1 virus around 1983-1984 and believed that it was the viral cause of a new disease (Cichocki, 2007). HIV-1 is predominant worldwide and mutates very easily. There are different strains of HIV-1 and they are categorized in groups and subtypes called clades.

The origins of HIV and AIDS have captured the attention of many scientists ever since the disease first came to light in the early 1980s. The first recognizable cases of AIDS occurred in the USA (New York and San Francisco) where a number of homosexual men suddenly developed rare opportunistic infections (Gottlieb *et al*, 1981). However, these infections were resistant to the normal treatment. It then became obvious that these men were suffering from a common syndrome, which occurred in a setting of markedly reduced circulating $CD4^+$ T-cells. The suspicion that AIDS has an infectious cause was confirmed in 1983-1984, when the isolation of a retrovirus from the blood of patients with AIDS was reported (Barre-Sinnoussi *et al*, 1983). Barre-Sinnoussi and Montagnier had obtained several isolates of the human retrovirus and it was their findings that led to the award of The Nobel Prize in Physiology or Medicine in 2008 for the discovery of the "Human Immnodeficiency Virus" (The Nobel Prize in Physiology or Medicine, 2008). These findings allowed for the development of a reliable test for HIV infection: looking for the presence of circulating immunoglobulin-g antibodies specific for HIV which provided the basis for screening of donated blood and for large-scale epidemiological studies (Levy *et al*, 1984).

HIV is a lentivirus. Like all viruses of this type, it attacks the immune system. Lentiviruses are part of a larger group of viruses known as retroviruses. The name "lentivirus" means "slow virus" because these viruses take a long time to produce any noticeable adverse effects in the body. They have been found in a number of different animals, which includes cats, sheep, horses and cattle. It is generally believed that HIV-1 is a descendant of the simian immunodeficiency virus (SIV), which is a lentivirus that affects monkeys. One of the earliest

documented cases of HIV-1 infection was identified in a stored plasma sample collected from an adult male in 1959 living in what is now the Democratic Republic of Congo (Barre-Sinoussi *et al*, 1983). Analysis of this sample in 1998 suggested that HIV-1 was introduced into humans around 1931 (Korber *et al*, 2000).

1.2.4 The natural course of HIV-1 infection

The progression of the HIV infection may be measured through various outcome measures such as the rate of decline in the $CD4^+$ T-cell count, increase in plasma viral load and the HIV associated opportunistic infections (Paranjape, 2005).

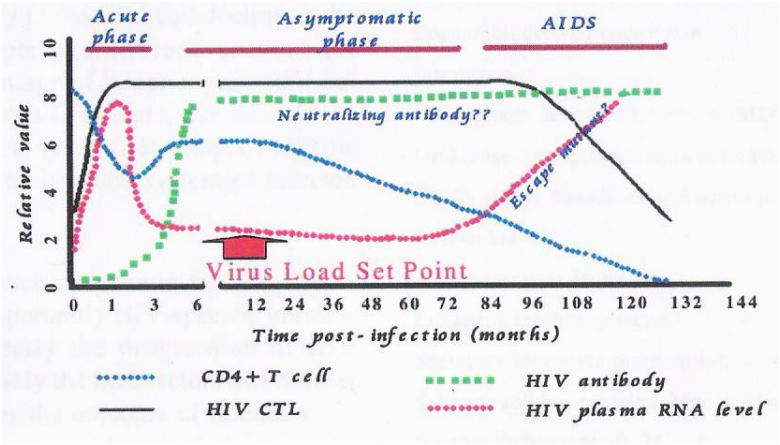


Figure 4. The natural course of HIV-infection.

The course of infection can be categorized into three phases namely: the acute phase, the asymptomatic phase and the AIDS phase

Adapted from: www.journaids.org/hivaidsoverview (accessed 12 June 2007)

1.2.4.1 The acute phase

Early events during acute HIV-1 infection are critical in determining the course of disease progression. Since there are no HIV-1 specific antibodies detectable during this early phase, diagnosis of acute HIV-1 infection is often missed in the majority of cases as other viral

infections are assumed to be the cause of symptoms (Altfeld and Walker, 2001). Antibodies to HIV p24 antigen begin to appear within several weeks of acute infection. The p24 antigen levels once present persist and then increase during active viral replication (Quin, 1994). The principle impact of HIV infection on the immune system is the destruction of the $CD4^+$ T-lymphocytes. Patients with an initial diagnosis of HIV-infection should have $CD4^+$ T cell measurements performed approximately every six months to see if a declining trend is noted (Gupta *et al*, 2004).

During HIV-1 infection, anti-viral CTL responses develop and persist. Unfortunately, CTL escape mutations are selected, thus helping the virus evade immune surveillance (Allen *et al*, 2000 and Goulder *et al*, 2001). The virus replicates extensively during the acute phase of infection with viral titers reaching extraordinary levels (Little *et al*, 1999). However, these high levels are short-lived thus indicating that the immune system can generate immune responses that could control the replication of the virus. Viremia declines before reaching a set-point (Altfeld and Walker, 2001).

1.2.4.2 Asymptomatic phase

The asymptomatic phase of HIV infection (chronic HIV infection) is where the viral load reaches a viral set point and the HIV antibody level tends to plateau at high levels, however the CD4 cell count is the hallmark of HIV infection and the rate of loss in each person is unique (Gupta *et al*, 2004).

1.2.4.3 AIDS phase

The AIDS defining stage of HIV-1 infection has a rapid decline in the CD4 cell count with an increase in viral load if an escape mutant is present. This occurs in conjunction with other opportunistic infections as the immune system has weakened tremendously. Early processes that try to contain the pathogen during the interim period when adaptive immune responses are not yet ready are known as innate immune responses. The innate immune responses may play a major role in initial containment of infection and hence may be crucial in the acute HIV

infection phase. Almost all HIV infected individuals mount HIV-specific immune responses. However, only few can control the viremia successfully for prolonged periods (Paranjape, 2005).

The immune system is a complex network of cells and chemicals and its mission is to protect us against foreign organisms and substances. During HIV-1 infection, anti-viral CTL (Cytotoxic T lymphocyte) responses develop and persist. CTL escape mutants are selected and this helps the virus to evade immune surveillance (Mahnke *et al*, 2006).

1.2.5 Clinical Staging of HIV/AIDS

HIV disease staging and classification systems are critical tools for tracking and monitoring the HIV epidemic which provides important information to clinicians for clinical management. The CDC and WHO are the two major classification systems that is currently in use. The clinical staging and case definition of HIV for resource settings were developed by the WHO in 1990 and was revised in 2007 (World Health Organization, 2007). The immunologic criteria for diagnosing advanced HIV in adults and children five years or older with confirmed HIV infection is having a "CD4 count less than 350 per mm^3 of blood in an HIV infected adult or child" and for children younger than five years of age is:

a) CD4 percent less than 30 among those younger than 12 months
b) CD4 percent less than 25 among those aged 12- 35 months and
c) CD4 percent less than 20 among those aged 36-59 months

1.2.6 Natural immune control

Individuals who have been infected with HIV for more than 20 years and maintain viral loads below the detection levels or at low levels, without the need of antiretroviral drug therapy, clearly suggest that immune control of HIV is indeed possible. Viral and genetic factors

together with the host immune responses contribute significantly to the delay in HIV-1 disease progression in HIV-1 infected long-term nonprogressors (LTNPs) (Baoz *et al*, 2002).

Studies of many chronic viral infections in which containment rather that eradication is the outcome suggests that the quality of the immune system plays a central role in viral control. Some LTNPs appear to have strong cytotoxic T-lymphocyte (CTL) responses as well as T-helper responses directed against HIV (Altfeld *et al*, 2001).

It is evident that human leukocyte antigen (HLA) class I genes are associated with the ability to mediate strong immune pressure against HIV-1 (Elrefaei *et al*, 2004; Garber *et al*, 2004 and Altfeld *et al*, 2001). Specific HLA alleles have been associated with slow (eg. HLA-B*57 and B*27) or rapid (HLA-B*35) disease progression (Garber *et al*, 2004 and Altfeld *et al*, 2001). It is presumed that the diversity and specificity of HIV-1 epitopes presented by protective HLA alleles result in enhanced recognition and viral control by CTLs.

1.2.7 Natural killer cell response to HIV

In most viral infections, cell-mediated immune responses play a pivotal role in arresting and/or eliminating the infecting agent (Miller, 2002) with the outcome of many of these viral infections being containment rather that eradication (Altfeld and Rosenberg, 2000). This includes cytotoxic responses of Natural Killer (NK) cells as well as $CD4^+$ and $CD8^+$ T-cells.

Natural Killer cells are bone marrow derived and do not require thymic processing. They kill other cells within approximately four hours of exposure. Cytotoxic T-cells differ from NK cells as they require exposure to antigens. In HIV-1 infection, NK cells are able to recognize virus infected cells and mount an antibody-dependent cell-mediated cytotoxity (ADCC) response against the human cells that express the viral envelope epitopes (Miller, 2002).

1.2.8 Immune Pathology

1.2.8.1 $CD4^+$ T-cell response

The negative correlation of strong HIV-1 specific $CD4^+$ T helper cell responses and viral load indicates that $CD4^+$ T helper cells may also play an important role in the immune response to HIV-1 (Rosenberg *et al*, 1997).

Virus-specific $CD4^+$ helper-cells (Th) play an important role in the induction and maintenance of effective $CD8^+$ T-cell function in adults (Altfeld *et al*, 2001; Wilson 2006 and Kuhn *et al*, 2001). $CD4^+$ helper-cells also play an important role in early immune response to HIV, but HIV infection leads to the progressive loss of $CD4^+$ T-cells and their function, more specifically $CD4^+$ T-cells have been shown to be preferentially depleted in the gut (Lim *et a*l, 1993) early after infection (Lacabaratz-Porret *et a*l, 2004; Altfeld *et al*, 2001). Studies in adults have shown that HIV-specific $CD4^+$ T–cell responses may be detected during early infection (Lacabaratz-Porret *et al*, 2004), but they are lacking during chronic infection, except in cases of long-term non-progressors (Lacabaratz-Porret *et al*, 2004 and Jansen *et al,* 2006).

A study performed on acutely HIV-1 infected adults (Altfeld *et al*, 2001) has shown that the CTL responses are weak and these findings were consistent with the cross-sectional data from another study (Dalod *et al*, 1999) where the breadth of responses were less extensively characterized. Individuals who are treated in early HIV-1 infection may benefit from strategies which are aimed at enhancing virus-specific CTL responses, whereas individuals who are treated later in HIV-1 infection may benefit from the strategies aimed at enhancing HIV-1 specific T-helper cell functions so as to broaden the CTL responses which will help combat the viral diversity (Altfeld *et al*, 2001).

Effective immune control in animal models of chronic viral infections is associated with an adaptive immune response that includes $CD4^+$ and $CD8^+$ T-cell responses (Ramduth *et al*, 2005). Antiretroviral therapy in chronic HIV-1 infection has resulted in increased $CD4^+$ cell counts but it has not resulted in the restoration of strong or persistent virus-specific $CD4^+$ T-cell proliferative responses (Rosenberg *et al*, 1997).

1.2.8.2 $CD8^+$ T-cell response

In studies like the murine lymphocytic choriomeningitis virus model, there is indication that maintenance of effective CTLs does require the presence of virus-specific T-helper cells (Matloubian *et al*, 1994). The inverse association between virus-specific $CD4^+$ cell responses and viral load and the association between strong HIV-1 specific CTL and T-helper cell responses clearly suggest that $CD4^+$ T-cells are required for the maintenance of effective CTL response in HIV-1 infected individuals (Rosenberg *et al*, 1997; Kalams *et al*, 1999).

$CD8^+$ T-cells contribute to the partial immune control of HIV-1 in acute, chronic and the long-term non-progressing infection (Borrow *et al*, 1994 and Koup *et al*, 1994). The anti-viral effect of these $CD8^+$ T-cells is mediated by effector mechanisms, which include the release of lytic granules and the release of cytokines and chemokines (Harty *et al*, 2000). The HIV-specific $CD8^+$ T-cells play a major role by suppressing HIV replication. The diversity and specificity of these HIV-specific $CD8^+$ T-cells are important for the efficiency of the immune response as well as control of the virus that bears immune escape mutations (Buseyne *et al*, 2006).

Data from animal models have shown that the depletion of $CD8^+$ T-cells is associated with an increase in viral load (Matloubian *et al*, 1994) and the inverse correlation between the HIV-specific CTLs and viral loads was seen in untreated HIV-1 chronic infection (Ogg *et al*, 1998). Most HIV-1 infected individuals who have poor control of viremia, progress to AIDS without antiretroviral therapy despite the antiviral activity of the CTLs in control of viremia (Altfeld *et al*, 2001).

Studies show that there are differences in the $CD8^+$ T-cell repertoire of responses to the whole HIV-1 genome from adult patients during the acute and chronic phase of HIV-1 infection (Goulder *et al*, 2001 and Alter *et al*, 2007).

Immunohistochemical studies on mucosal immune cells in the gut of adults have shown that the $CD4^+$ lymphocyte subset is depleted and the $CD8^+$ lymphocyte subset is expanded in

AIDS subjects (Rodgers *et al*, 1986; Budhraja *et al*, 1987; Jarry *et al*, 1990). The increase in mucosal $CD8^+$ lymphocytes appears to parallel the expanded blood $CD8^+$ populations as a result of HIV infection. The importance of $CD8^+$ T-cells in host protection has been increasingly recognized and the loss of $CD4^+$ T-cells in HIV implies an increased role for $CD8^+$ T-cells in host defense (Scott *et al*, 1991).

The initial HIV-specific $CD8^+$ T-cell response in acute adult HIV-1 infection is of low magnitude and is narrowly directed against a limited number or epitopes. However, these responses broaden during the prolonged antigen stimulation in the chronic phase of the HIV-1 infection (Altfeld *et al*, 2001; Dalod *et al*, 1999; Oxenius *et al*, 2000). Viral replication tends to wane during the later stages of HIV-1 infection, although the HIV-specific $CD8^+$ T-cell responses are generally broader and there is a vigorous immune response in the chronic phase of infection. These findings have suggested that the quality and specificity of the initial $CD8^+$ T-cell responses may be associated with initial control of the viral replication as apposed to the magnitude of the responses (Pantaleo *et al*, 2004).

Some studies have shown that in chronic HIV-1 infection, the $CD8^+$ T-cell responses are directed against HIV-1 Gag in subjects with low viral loads and slow disease progression (Addo *et al*, 2003; Gray *et al*, 1999; Kiepiela *et al*, 2007). $CD8^+$ T-cells cannot adequately suppress HIV-1 infection by themselves which leads to subsequent impairment of the $CD8^+$ T-cells (Migueles *et al*, 2002; Lichterfeld *et al*, 2004). Evidence in studies with animal models and HIV-1 clade B infection in humans indicate that long-term maintenance of effective cytotoxic T lymphocyte activity is dependent on the functionality of the $CD4^+$ T-cells (Kalams *et al*, 1999; Cao *et al*, 2003). The preferential targeting of HIV-1 Gag responses indicates the importance of immune containment (Ramduth *et al*, 2005).

1.3 Pediatric HIV infection and treatment

Acquired immunodeficiency syndrome is now believed to be the leading cause of death in sub-Saharan Africa and the fourth biggest killer in the world (UNAIDS/WHO Worldwide HIV and AIDS Summary, 2007). Most HIV-1 infected subjects live in Africa, and women of child-bearing age are disproportionately affected.

The HIV epidemic has grave implications for children, particularly in Africa where HIV prevalence is high (Chakraborty *et al,* 2005). In adults, the clinical manifestations of HIV-1 infection and the course of immunologic and virologic markers differ from that of children. Most children acquire HIV-1 infection perinatally and their immune systems are immature at the time of infection and are exposed to high-levels of viral replication.

In developed countries, studies have shown that children infected with HIV-1 perinatally, progress rapidly to AIDS (within a year), before the introduction of HAART. The median time to reach the AIDS stage is ~7 years and a minority of children remain clinically asymptomatic beyond their childhood and some even into adolescence (Goulder *et al*, 2001).

Earlier studies on HIV-1 specific CTL responses among perinatally infected children indicated that these CTL responses emerged later in the course of HIV-1 infection and were much weaker than the responses that are seen in adults (Feeney *et al*, 2003b; Buseyne *et al*, 1993; Buseyne *et al*, 1998a and Luzuriaga *et al*, 1995). However, these studies relied on techniques that were less sensitive than some techniques used currently. Recent studies have shown that CTL responses can be detected very early in infants (Lohman *et al*, 2005 and Thobakgale *et al*, 2007). However, these responses are not clearly associated with clinical outcomes and the finding that the older infants had stronger CTL responses which may be due to age-related maturation (Lohman *et al*, 2005).

Little is known about the magnitude and breadth of paediatric HIV-1 specific CTL responses beyond infancy and early childhood. In the USA, a large proportion of HIV-1 infected children are now entering the second or third decade of life. This is mainly due to the

changing demographics of paediatric HIV-1 in developed countries (Feeney *et al*, 2003a). Antiretroviral interventions to reduce Mother-To-Child-Transmission (MTCT) and HAART therapy have played a major role in reducing the HIV-1 transmission rates in developed countries.

Although substantial improvement had been made with adults being treated with ARVs in sub-Saharan Africa, progress for children has lagged behind (Sutcliff *et al*, 2008). The South African National HAART programs which began in 2004 have proven to be effective but delays in HAART initiation are experienced. New guidelines for the PMTCT programs were launched in South Africa in February 2008 (Alcorn, 2008). However, in many of the provincial hospitals this implementation was expected to begin in June 2008. It is delays like these that increase the number of new infections and the number of subjects waiting to be treated with HAART.

Only a small proportion of HIV-1 infected children are now entering or have already entered the second decade of their lives. Most of these children have already started antiretroviral therapy. Data regarding the HIV-1 specific immune responses in these perinatally infected children whose immune systems have matured in the presence of HIV-1 is limited. The relationships between the frequency of HIV-specific T-cells, treatment strategies and viral replication have been well established in adult HIV-1 infection but are less well established in older chronic HIV-1 infected children (Buseyne *et al*, 2005).

1.3.1 HAART in children

Highly Active Anti-retroviral Therapy (HAART) has significantly improved HIV-associated morbidity and mortality (Boschi *et al*, 2004). Although HAART does not eradicate HIV from the infected individual, there are various strategies, which have been proposed to facilitate the management of individuals on therapy. HAART has however revolutionized the treatment of HIV, but has its drawbacks in the manifestation of side effects and becomes ineffective when the virus develops resistance.

The global impact of the HIV epidemic on children worldwide has been devastating. Statistics have shown that South Africa is one of nine countries where the mortality of children less than 5 years of age is increasing at an estimated annual rate of 1,6% (UNAIDS, 2007) and in the last decade has orphaned far too many children. South Africa has one of the highest antenatal HIV prevalence rates in the world where most of the infections in children result from perinatal transmission. PMTCT, one of the largest antiretroviral treatment programmes has mitigated this problem. This intervention programme is currently an effective strategy for reducing the size of the paediatric HIV epidemic (Coetzee *et al*, 2005; Rollins *et al*, 2007).

The use of HAART has resulted in a considerable decrease in morbidity and mortality among HIV-1 infected adults and children worldwide. To replicate these successes in less developed countries where HIV-1 is endemic has certainly proved to be a challenge not only for governments but nongovernmental organizations and agencies (Chakraborty *et al*, 2003). The World Health Organisation (WHO) estimated that only 3% to 4% of HIV- infected children in Africa who require immediate treatment are actually receiving it (UNAIDS, 2007).

HAART by itself cannot eradicate HIV from the infected infant or child, but it can certainly improve the quality and prolong lives. HIV infected children on HAART reconstitute their immune system similar to adults. However, children have a more rapid increase in naïve $CD4^+$ T-cells and normalize their $CD8^+$ T-cell repertoire better than adults and this is due to the increased function of their thymus (Hazra *et al*, 2005)). Without antiretroviral treatment in Africa, the mortality rate of HIV-infected children 2 years of age was estimated to be 60% and 75% in children before the age of 5 (Nicoll *et al*, 1994 and Spira *et al*, 1999).

Without HAART the development of AIDS in HIV-infected infants and children occurs more rapidly and this is due to their immature immune system, which poorly suppresses HIV. In contrast to adults, AIDS typically averages 10-12 years from the time of infection to the development of AIDS (Reute, 2006). The initiation of antiretroviral therapy is preceded by the clinical staging of the HIV-infected individual, however immunological staging for children is important. CD4 testing is important but not essential by itself for the initiation of HAART in children and therefore should be used in conjunction with their clinical staging. The

measurement of CD4 percentage is recommended in infants and young children because the CD4 absolute count is associated with specific levels of immuno-suppression and this tends to change with age.

A step-by step guide which is dependent on age has been established for the management of HIV-infected children on antiretroviral therapy. All children <1 year with clinic WHO staging 2, 3 and 4 or who have a CD4% of ≤ 35 or an absolute CD4 cell count <1500 cells/ mm^3 should start antiretroviral therapy. Children between the ages of 1-5 years with clinic WHO stage 3 and 4 or who have a CD4 count ≤ 20% as well as children older than 5 years with a clinical WHO stage 3 and 4 or a CD4 count of <15% or who have an absolute CD4 count of <200 cells/ mm^3 should be treated with antiretroviral therapy. Co-trimoxazole prophylaxis is given to HIV-infected children if they have a CD4 count of < 25% – 35% depending on their age and in HIV exposed children < 12 months until the HIV is excluded.

In the absence of antiretroviral therapy, HIV-infected children die before their fifth birthday (Newell *et al*, 2004) and vertically HIV-infected infants progress to AIDS or death in the first year of life (Gray *et al*, 2001). Although only 2/20 HIV-1 infected infants had viral loads less than 10, 000 copies/ml and a CD4% of > 30, by the age of 2 years without antiretroviral therapy, 85% of HIV-1 infected infants on the Paediatric Early HAART And Structured-Interruption Study (PEHSS), in KwaZulu-Natal, met with the current WHO criteria to initiate HAART within 12 months of infection (Thobakgale *et al*, 2007).

HAART used alone, despite the benefits, has proved to be insufficient to completely eradicate viral reservoirs. Under current guidelines, HAART has to be administered throughout life. However, drug-induced toxicities and issues relating to treatment adherence as well as the cost of providing continuous HAART to a large number of HIV-1 infected individuals has proven difficult for most developing countries. Some HIV-infected individuals refuse treatment and some spontaneously interrupt the prescribed HAART for short and sometimes long periods of time.

1.3.2 HIV-1 specific $CD4^+$ and $CD8^+$ T-cell responses

A study that focused on acute HIV-1 infected infants, reported that HIV-1 specific $CD4^+$ T-cell responses were detected in a 6 day old untreated infant suggesting that in-utero priming of $CD4^+$ T cells against HIV-1 does occur (Ramduth *et al*, 2008). This study also indicated that there was a greater $CD4^+$ T-cell proliferative response seen in infants who had undergone antiretroviral treatment as apposed to the untreated infants, where the $CD4^+$ T-cell responses were virtually absent.

The longitudinal assessment of HIV-specific T-cell responses in HIV-1 acute paediatric infection is currently being undertaken. Preliminary data indicates that Gag-specific $CD4^+$ T-cell responses in acutely HIV-1 infected infants are weak or undetectable in the first six months of life (Thobakgale *et al*, 2007).

Many studies have examined the $CD4^+$ T-cell responses in both the acute HIV-1 infected (Chakaraborty *et al*, 2005; Luzuriaga *et al*, 2000) and chronic (Feeney *et al*, 2003b; Feeney *et al*, 2004) HIV-1 infected infants and children. However, in chronic HIV-1 paediatric infection, the assessment of CTL responses in children undergoing STIs, is yet to be determined.

Although Buseyne *et al*, (1998b) and Sandberg *et al*, (2003) demonstrated that HIV-specific $CD8^+$ T-cell responses can be detected in children before the age of 6 months, Thobakgale *et al*, (2007) proved that these HIV-specific $CD8^+$ T-cell responses can be detected in infants from the first days of life. However these responses from the paediactric studies indicated that they were weaker than in adults (Buseyne *et al*, 1998b; Sandberg *et al*, 2003).

Studies with paediatric subjects showed that they mount a very weak and narrow $CD8^+$ T-cell response to HIV-1 Nef epitopes as compared with adult subjects (Chandwani *et al*, 2004; Buseyne *et al*, 2006). The frequency of detectable $CD8^+$ T-cell responses in children was low. Using the ELISPOT assay, the magnitude of these responses was documented to be low (Buseyne *et al*, 2006).

Data from a study has shown that HIV-1 specific $CD8^+$ responses in late childhood are similar in both the magnitude and breadth to those in adults (Feeney *et al*, 2003b). In perinatal HIV-1 infection, the immune system of the newborn is immature at the time of primary infection and is therefore exposed to prolonged high-levels of viral replication. There is decreased IFN-γ production by the $CD8^+$ T-cells in young children and this may lead to the impairment of their antigen-presenting cells (Adkins *et al*, 2004; Marshall-Clarke *et al*, 2000; Rouzioux *et al*, 1997). Studies have shown that low HIV-specific IFN-γ producing CD8+ T-cell responses persist in children 6 months of age (Scott *et al*, 2001) to children 4 years of age (Sandberg *et al*, 2003).

The frequency of HIV-specific IFN-γ producing $CD8^+$ T-cells has been shown to be dependent on age and unrelated to plasma viral loads or CD4 and CD8 T-cell levels in children not receiving HAART as compared to children receiving HAART, where the plasma viral load is an important determinant in the frequency of HIV-specific IFN-γ producing $CD8^+$ T-cells (Buseyne *et al*, 2005).

1.4 Review on Structured treatment interruptions

The long-term use of HAART in patients is not only expensive but difficult for patients to follow as the long-term use generates drug toxicity. Therapeutic approaches, which seek to limit the exposure to ARVs, but retain the benefits of continuous therapy, have highlighted the area of investigation of Structured treatment interruptions (STI) (Azzoni *et al*, 2003).

STI has been suggested as a possible alternative to continuous therapy. STI involves the alternating "on-and-off" cycles of HAART in order to enhance the use of HAART (Lisziewicz *et al*, 2002). Figure 5 is an illustration of the immunological rationale for STI in controlling the virus using HAART. It has been hypothesized that a brief and slow increase in HIV viremia, which results from the suspension of HAART, might boost the HIV-specific $CD8^+$ and $CD4^+$ T-cells (Altfeld and Walker, 2001). With subsequent structured treatment (indicated by the shaded regions), in figure 5, the viral levels have decreased to low levels and

the CTL and T-helper responses have increased over time. This is an indication that the CTL and T-helper responses have control over the viral replication.

After the subsequent suppression of the virus with the re-introduction of HAART, the $CD8^+$ T-cells might be able to have better control of the virus. If the use of STIs leads to strengthening of the immune system, this would alleviate ARV therapy hence there would be no side-effects and less chance of the development of resistance.

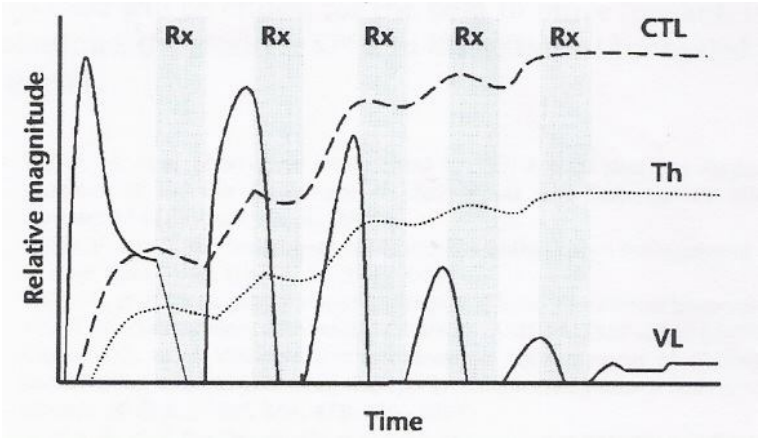


Figure 5. Illustrates the possibility of STIs in HIV-1 acute infection that would lead to immune boosting and subsequent control of viremia without the use of drugs
Adapted from: Marcus Altfeld, 2001

There have been several studies that have assessed the clinical, immunologic and virologic outcomes following STIs in adults treated with HAART during chronic infection (Altfeld *et al*, 2001). Viral levels were shown to have approximated pre-treatment levels (Ruiz *et al*, 2000; Hatano *et al*, 2000) and HIV-1 specific $CD4^+$ and $CD8^+$ T-cells responses were enhanced (Papasavvas *et al*, 2000).

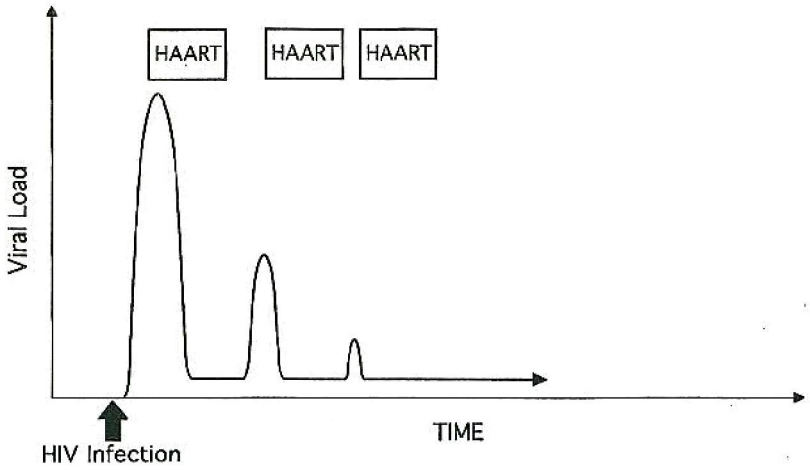
A loss of CD4 T-lymphocytes and a rapid viral rebound is usually experienced in patients who discontinue HAART (Papasavvas *et al*, 2000). Subsequent studies have shown that STIs in

adults acutely infected with HIV induce HIV-specific responses mediated by the $CD4^+$ and $CD8^+$ T-cells, which may enhance control of viremia (McFarland *et al*, 2003). The "Berlin patient" was probably the first recognized example of the STI concept. He received antiretroviral therapy before complete seroconversion, indicated by Western Blot and had stopped ARV therapy two weeks after starting and then resumed therapy a week later after his viral load had rebounded from < 500 copies/ml to 8000 copies/ml. He resumed ARV therapy for a period of three months and then stopped ARV therapy and his viral load remained undetectable for more than two years. The presence of increased levels of HIV-specific $CD4^+$ T-cell responses in the "Berlin patient" led researchers to speculate that the treatment interruption cycles had allowed for the viral antigen to be re-exposed and thus stimulated the HIV-specific cellular immune responses (Ball, 2003).

Studies have shown that a minority of chronic HIV-1 infected individuals are able to suppress the virus after STIs without HAART. However, rapid declines were observed in the $CD4^+$ T-cell counts with an inversely proportional viral load during STIs (Youle *et al*, 2000). An STI regime of HAART appears to be most beneficial during the acute phase of HIV-1 infection because of the development of enhanced HIV-1 specific cellular immune responses, which could alter the viral set point (Oxenius *et al*, 2002). Results have been encouraging thus far for patients on HAART who had structured treatment interruptions during the acute phase but the benefits in chronic HIV-infection are less clear (Rosenberg *et al*, 2000 and Lori *et al*, 2000).

Although the outcome of STIs is qualitatively different in acute and chronic HIV-1 infection, it was originally thought that STIs in HIV-1 infected subjects would be successful. However patients lost control and had to restart therapy (Altfeld & Walker, 2001). Early ARV therapy in acute HIV-1 infection generates strong HIV-specific $CD4^+$ T-cell responses and the viral load decreases with each HAART cycle (Fig 6a) whereas ARV therapy in chronic HIV-1 infection does not lead to restoration of these responses and a detectable viral load is evident (Fig 6b).

(6a)



(6b)

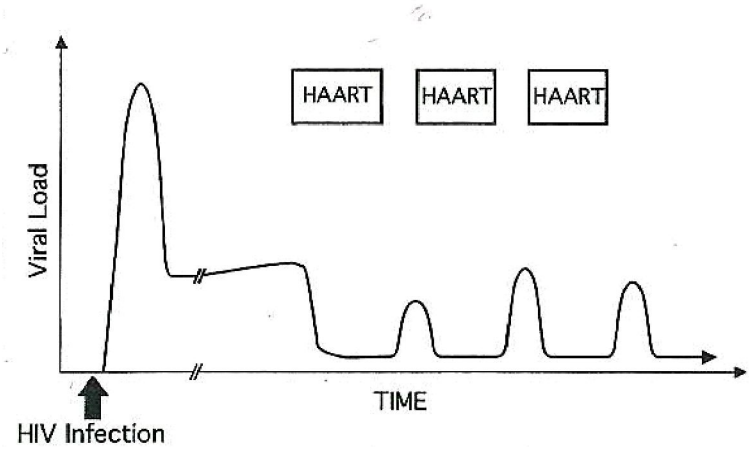


Figure 6: a) STI in acute and early HIV-1 infection. The goal is to achieve long-term immune control of viral replication. b) STI in chronic HIV-1 infection responding to HAART. The goal here is to reduce the toxicity and cost of drugs.

Adapted from: Lisziewicz and Lori, 2002

Pilot studies on small cohorts showed that STI in chronic HIV-1 infection can augment virus specific immune responses in most individuals and these augmented responses were associated with lower levels of plasma virus (Altfeld *et al*, 2001), but these studies were undertaken in adults. In contrast while control was not long lived, STIs in adult chronic infection has shown to be detrimental (The SMART Study Group, 2006), however the use of STIs in children with chronic HIV-1 infection, has not been widely investigated. Although STIs have been used in many adult acute and chronic studies, the use in children has been limited especially when investigating immune responses. There were limited studies performed by Feeney *et al.* (2003a) and Thobakgale *et al*. (2007).

1.5 HIV Vaccines

Although effective medical treatments for HIV exist, it is generally acknowledged that an HIV vaccine is the only alternative that would bring this pandemic to a halt. Currently, no effective vaccine has been developed.

HIV infection results in a progressive decline in $CD4^+$ T-cell number and function, thus resulting in an increased risk of opportunistic infections (Elrefaei *et al*, 2004). The HIV-1 epidemic still remains a global challenge with rapid and continual spread, especially in the developing countries. Despite the existence of HAART, which has helped to curb the rates of disease progression, the mortality rate due to the HIV-1 epidemic, is astoundingly high (Garber *et al*, 2004). The high prevalence amongst the antenatal clinic attendees has brought overwhelming insight into the burden of new HIV infections that occur in developing countries. Therefore the challenge is to design a vaccine that would be safe, effective, affordable and simple to implement in developing countries.

However, despite the enormous amount of research already done with regards to vaccine development, success has proven difficult. This is due to the complex structure of the HIV envelope glycoprotein, which is resistant to antibody neutralization and has the capacity to rapidly evolve to escape from whatever antibody responses are mounted by the infected individuals (Garber *et al*, 2004). The rapid evolution of HIV within infected individuals is

one of the factors that have contributed significantly to the slow progress in the development of an effective HIV vaccine.

In February 2003, the AIDSVAX vaccine that targets the gp120 region of the HIV genome was declared a failure in North America as there was not a statistically significant reduction of HIV infection within the study population (Francis *et al*, 2003). In November 2003, the AIDSVAX vaccine failed in Thailand for the very same reason as in North America (McCarthy *et al*, 2003) The ALVAC vCP1521 canary pox vector/AIDSVAX prime-boost vaccine trial in Thailand provided interesting results in that CTLs from the volunteers were able to kill PBMCs infected with primary isolates of HIV (Kantakamalakul *et al*, 2004). It was also the first vaccine to induce cross-clade functional CTL response.

In December 2004, the V520 vaccine trial was introduced in North and South America, the Caribbean and Australia. This vaccine was intended to prompt the body to produce T-cells that kill HIV-1 infected cells. In fact the V520 vaccine failed to confer protection against HIV-1. It actually increased the odds of becoming infected with HIV-1 (White, 2008).

In February 2008, a major vaccine trial in South Africa, which used the Merck MRKAd5 vaccine, was brought to a complete halt after vaccines in the sister trials in Australia and the USA were declared ineffective as the vaccine which contained three genes in a weakened adenovirus, had made the study participants in the vaccine arm more likely to become infected with HIV compared to placebo recipients in the vaccine trial (Health News, 2008).

It is hypothesized that for a vaccine to be effective, $CD8^+$ T-cell (CTL) based AIDS vaccines will probably need to suppress the extent of HIV replication from the earliest stages of infection, in order to limit the generation of immune escape variants. Furthermore the CTL-based AIDS vaccine will need to elicit responses against multiple HIV epitopes (Altman *et al*, 2004).

1.6 The SMART study

The Strategies for the Management of Antiretroviral Therapy trial was designed to investigate the use of episodic antiretroviral therapy as compared to the continuous antiretroviral therapy. The protocol for the trial involved interrupting therapy when the CD4 count was 350 cells/ml and to resume therapy when the CD4 count fell below 250 cells/ml. The theory behind this trial was that the association between CD4 count and the risk of clinical disease from a large number of studies suggested that people who start antiretroviral therapy early in HIV-1 infection could safely interrupt therapy for prolonged periods of time without obvious harmful consequences. (The SMART Study Group, 2006).

The SMART Trial was the largest treatment strategy trial ever undertaken. It began in 2002 and enrolled 5, 472 participants from 318 clinic sites in 33 countries. In January 2006, the Data and Safety Monitoring Board (DSMB) recommended that the trial stop due to the fact that the risk of disease progression to the participants in the episodic treatment arm of the trial was twice as high as the participants in the continuous therapy arm of the trial. There was also an increase in major complications such as cardiovascular, kidney and liver diseases in the participants on the intermittent therapy arm. The DSMB promptly advised that all participants restart therapy.

1.7 Rationale of the Paediatric Antiretroviral (PARV) study

At the time that this study was initiated, there was little evidence for the non-effectiveness of structured treatment interruptions (STI) in chronic HIV-1 paediatric infection. Most of these HIV-infections in children are acquired through perinatal transmission, (i.e. during the development of their immune system) therefore the clinical manifestations of the infection and the course of immunological and virological markers may differ from those of adults. Clinical efficacy of HAART in children has been well documented in industrialized countries, (Viani *et al*, 2004; Gortmaker *et al*, 2001; Van Rossum *et al*, 2002). Although there had been a few

studies of STI in adult cohorts, studies of STI in children particularly in African countries, had not been well investigated.

Therefore this pilot study focused on a chronic HIV-1 paediatric cohort from Durban (KwaZulu-Natal, in South Africa) to assess the immunological and virological responses in perinatal HIV-infected children undergoing structured treatment interruptions (STIs) and to determine whether treatment interruptions would effectively boost and broaden the CTL responses in chronic HIV-1 paediatric infection.

The aim of this study was to assess the magnitude and breadth of HIV-specific T-cell responses before and during HAART in chronic HIV-1 infection in children and to describe the pattern of these responses in children who underwent Structured Treatment Interruptions. The enrolled children were followed up for a period of 24 months. The objectives of this study were to determine the CD4 percentages and estimation of the viral load for all visits, to characterize HIV-1 specific $CD4^+$ and $CD8^+$ T-cell responses in these chronic HIV-1 infected children as well as to assess viral resistance patterns before they started HAART, during HAART and during the treatment interruptions, and finally to determine the response to antiretroviral treatment and whether HIV-specific T-cell responses were associated with control of viraemia.

1.8 Specific Aims

- To characterize HIV-1 specific $CD4^+$ and $CD8^+$ T-cell responses in this cohort
- To determine changes in the CD4 % and viral load during HAART and STI
- To assess viral resistance patterns before, during HAART and during STIs
- To determine response to HAART and whether HIV-specific T-cell responses were associated with control of viraemia

1.9 Hypotheses and Rationale

Hypothesis: Short-term HAART in chronically infected children boosts HIV-specific T helper responses, and therefore improved immune control of HIV is reached during STI.

Rationale:

1. The course of HIV-1 infection differs significantly between adults and children. Children, in part due to the immaturity of the immune system at the time of infection, typically control viraemia poorly. In part, this is a result of impairment of T-cell function.

2. HAART initiated in chronic paediatric infection, but not in chronic adult infection, has been shown to increase levels of HIV-specific CD4+ T helper responses (Feeney *et al*, 2005). Thus, short-term HAART, followed by STI, might be expected to allow older children to generate more effective HIV-specific T-cell responses than without the period on HAART.

3. Structured Treatment Interruptions provide an attractive alternative to continuous therapy, which has its own particular problems in paediatric infection, in particular compliance/adherence, availability of paediatric formulations of anti-HIV drugs, and the need to frequently alter drug dose to keep track with growth and weight changes in childhood.

4. A major concern of STI is the possibility that the "on/off" cycles of treatment may result in more drug resistant mutations compared to continuous therapy.

1.10 Ethical Approval

Ethical approval for the study was obtained in 2002 from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal. Ethics Ref: E147/02.

CHAPTER TWO

2.1 STUDY DESIGN

Thirty children who satisfied the inclusion criteria were enrolled into the study. Upon enrollment, they were randomized to either the Treatment Interruption Arm (TAT) or the Continuous Treatment Arm (CAT).

Figure 7 is a schematic representation of the study design. All children received the triple antiretroviral therapy combination (AZT/3TC/ABC) for six months. The children randomized to the CAT Arm, continued with the regimen to the end of the study period (two years), whereas those randomized to the TAT Arm, stopped the triple antiretroviral treatment if their viral load was undetectable. Viral loads were monitored weekly.

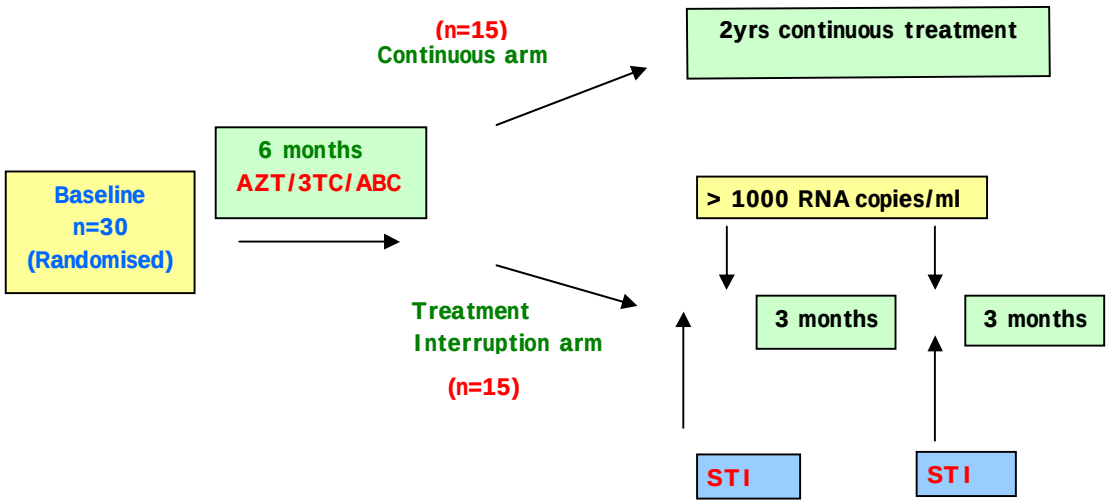


Figure 7. Schematic representation of the design of study.

The course of antiretroviral therapy was resumed for a period of three months if participant viral load increased by one log value. These cycles continued throughout the study period (two years).

After six months of antiretroviral treatment, children in the TAT arm, who had a detectable viral load, continued with antiretroviral treatment for a further six months (received treatment for a period of 48 weeks). After forty-eight weeks of antiretroviral treatment, children who had an undetectable viral load followed the interruption protocol with repeated cycles. Children with detectable viral loads after 48 weeks of antiretroviral treatment were screened for antiretroviral drug-resistance mutations.

The visits for all children were scheduled as follows:

- CAT Arm – baseline (pre-HAART); 12 weeks; 24 weeks; 48 weeks; 72 weeks and the final visit at 96 weeks.
- TAT Arm – baseline (pre-HAART); 12 weeks; 24 weeks; 48 weeks; 72 weeks; weekly during the treatment interruptions and a final visit at 96 weeks.

2.1.1 Study site and inclusion criteria for enrolment

Chronic HIV-1 infected children attending the Family Clinic at King Edward VIII Hospital in Durban were recruited between January 2004 and November 2004, after they met the inclusion criteria for the study.

Inclusion Criteria for Study:

- Chronic HIV-1 infected children with post test counseling and informed consent of a responsible parent or guardian
- Age: 2 – 12 years
- Resides at a fixed address and within an accessible area in the Durban Metropolitan region.

- ART naïve (Infants who had received a single dose nevirapine in the MTCT program were not excluded)
- To have had no other illness at the time of enrolment
- Must be in category B according to the CDC guidelines

2.1.2 Informed Consent

Informed consent was obtained from either the participant's parent or guardian. (Appendix 1)

2.2 SAMPLE COLLECTION AND LABORATORY METHODS

2.2.1.1 Blood sample collection

Approximately 8mls of whole blood sample were collected in a 10ml EDTA anticoagulant tube at the baseline visit for the following tests: a CD4 cell count, full blood count (FBC), isolation of DNA, isolation of plasma for RNA-PCR, isolation of peripheral blood mononuclear cells (PBMCs) for use of ELISpot screening and Intracellular Cytokine Staining (ICS). Where insufficient amounts PBMCs were recovered, the ELISpot and ICS tests were performed at the next visit. Figure 8 is an illustration of the different tests that were performed on the blood sample of each participant.

Blood samples were collected at the following time points: 12 weeks, 24 weeks, 48 weeks, 72 weeks and 96 weeks for the children who were randomized to the continuous treatment arm of the study. The children who were randomized to the structured treatment interruption (STI) arm of the study, had blood samples collected at the 12 week and 24 week time points for immunology studies. Following the STIs, ~5mls of whole blood sample was collected weekly for a CD4 cell count and viral load measurement.

2.2.1.2 Laboratory Testing Criteria

a) CD4 cell counts and viral load measurements were performed for all children at all timepoints. This included the time off treatment period.

b) The ELISPOT test was performed at baseline, 12 weeks, 24 weeks, 48 weeks and 96 weeks for the children in the continuous treatment group. The children in the treatment interruption group had ELISPOT tests done at baseline, 12 weeks, 24 weeks, 48 week, 96 weeks and at all timepoints (where PBMCs were available) during the period off treatment.

c) The ICS test was performed for all children at varying timepoints due to the availability of PBMCs.

d) HIV resistance tests were performed on all samples obtained at baseline, during the treatment interruption periods and where drug resistance was suspected.

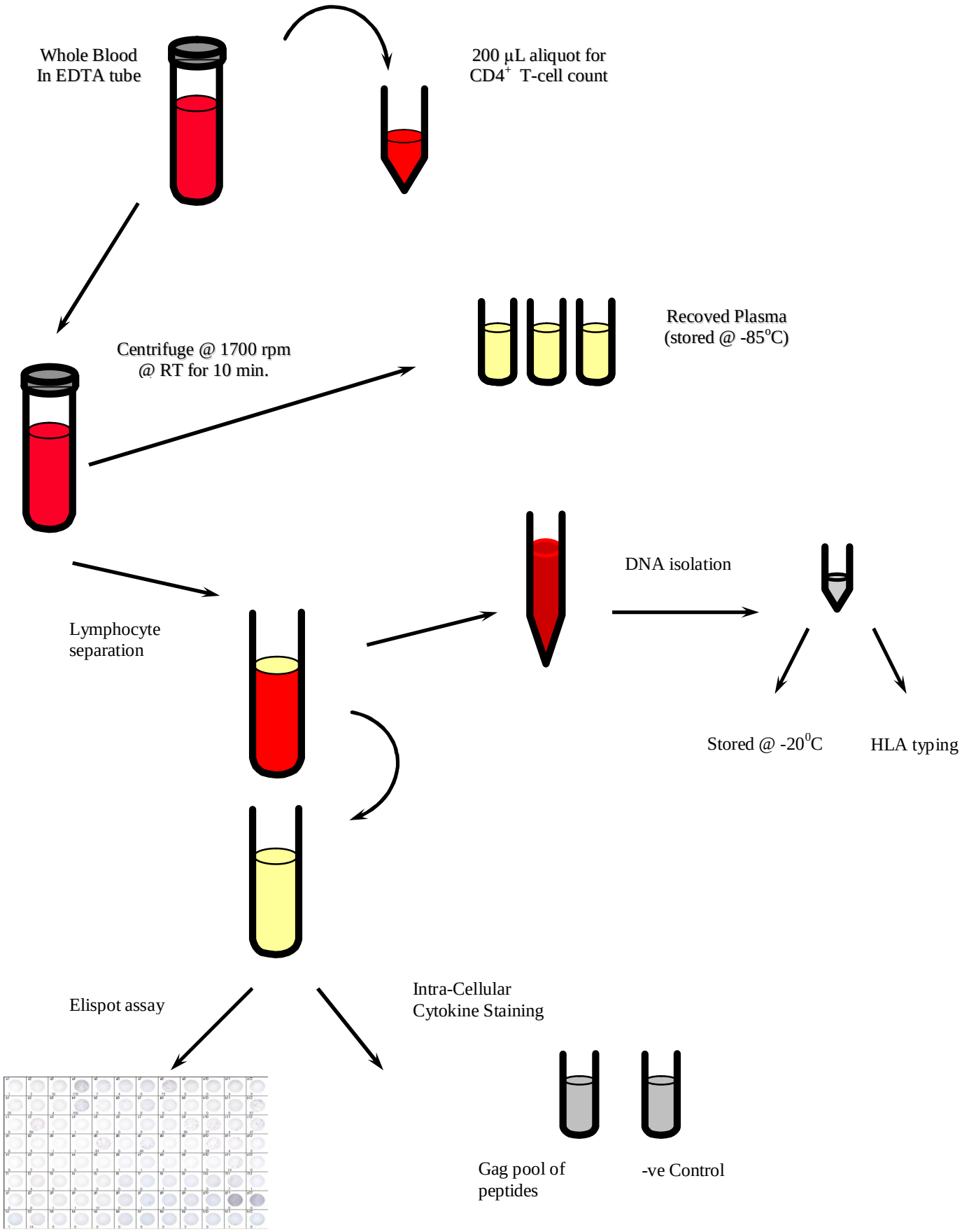


Figure 8. Schematic representation of the tests performed on the participant's blood sample

2.2.2 $CD4^+$ T-cell count

Principle: Immunophenotyping involves the identification and enumeration of targeted cells in whole blood samples using immune cell markers expressed by the cell. Fluorochrome-labeled antibodies bind specifically to the leukocytes of the whole blood. The erythrocytes were lysed prior to flow cytometry analysis on a FACS Calibur flow cytometer (Becton Dickenson Immunocytometry Systems, San Jose, CA).

Safety: All blood specimens, reagents and controls were considered biohazardous and infectious. When working within the biosafety cabinets, a Howie lab coat including double gloves, were worn. When using the FACS calibur a lab coat, gloves and eye goggles were worn when loading samples. The antibody reagents contain sodium azide as a preservative. The Lysing Solution contains diethylene glycol and formaldehyde.

Sample preparation: The BD MultiTEST^{TM} CD3 fluorescein isothiocyanate (FITC)/ CD8 phycoerythrin (PE)/CD45 peridinin chlorophyll protein (PerCP)/CD4 allophycocyanin (APC) is a four-color direct immunofluorescene reagent used with the TruCOUNT Tubes which contains a freeze-dried pellet of fluorescent beads. This kit was stored at 4^{o}C.

- The antibody and the required number of TruCOUNT tubes were removed from the fridge and allowed to stand for a few minutes at room temperature.
- A 1:10 dilution of working FACS Lyse solution was prepared (Appendix 2).
- The TruCOUNT tube was labeled with the sample identification number and the date.
- The antibody was vortexed for ~5 seconds and 20µL was pipetted into each of the TruCOUNT tube. The tube was gently tapped to allow the antibody to mix with the bead pellet.
- The whole blood was then vortexed and 50µL was pipetted into the respectively labelled TruCOUNT tube. The TruCOUNT tubes were capped, vortexed for ~3 seconds. The tubes were then covered with tin foil (to allow for the incubation for 15 min at room temperature in the dark).

- On completion of the incubation, 450µL of the working FACS Lyse Solution was added to each tube. The tubes were capped and vortexed for ~3 seconds. The tubes were then covered with tin foil for 15 min at room temperature (to allow for the Red blood cells to lyse).
- The remaining blood was kept in the BSC until the CD4 assay had been completed. On completion of the analysis, the samples were discarded appropriately.

Internal quality control: The IMMUNO-TROL™ cells from Beckman Coulter are an assayed, lysable whole blood quality control sample that was processed with every batch of test samples. The IMMUNO-TROL™ cells provided optimization of the instrument settings and performance.

Calibration of Equipment: The CaliBRITE™ Beads from Becton Dickenson was used to calibrate the FACS Calibur flow cytometer. Calibration allowed the photomultiplier tube (PMT) voltages and the fluorescence compensation to be set and also checked the sensitivity of the instrument.

Analysis of samples: The samples were analyzed on the FACS Calibur flow cytometer with the MultiTEST SOFTWARE (version1. 1.2). Manual gating of each sample was performed if necessary. A printout out for each of the test sample and control sample was generated.

Cleaning and shutdown of the FACS Calibur Flow Cytometer: Once all the samples had been analysed, the flow cytometer automatically goes into a cleaning program. On completion of the cleaning cycle, the flow cytometer is depressurized and a worklist summary report was printed and filed. The analyzed samples were removed from the flow cytometer, capped and discarded into the biohazardous waste disposal buckets. The tube with distilled water was replaced so that the electrode was always kept wet.

2.2.3 Separation and storage of plasma

The whole blood samples were centrifuged at 1700 rpm for 10 minutes at room temperature. The plasma was recovered with a sterile Pasteur pipette under sterile conditions and aliquoted into sterile internal thread cryovials and stored at -85^0C until required.

2.2.4 HIV-1 RNA-PCR Quantification (viral load)

The viral loads for this pilot study were initially performed at the Africa Centre Laboratory. Due to the changes within the Africa Centre Department, the viral loads were no longer performed at the Africa Centre Laboratory. Continuation of the viral load testing was performed at the HPP Laboratory from November 2004 until the end of the study.

2.2.4.1 NucliSens®: Lysis buffer, magnetic extraction reagents and NucliSens EASYQ HIV-1 version -1.2 (Biomerieux)

Principle: The Nucleic acid extraction method is based on magnetic silica particles. Nucleic acids bind to the silica particles under high salt concentrations. The silica acts as a solid phase and non-nucleic acid components are removed by several wash steps using the miniMAG. The nucleic acids are eluted from the solid phase. NucliSens EasyQ HIV-1 v1.2 reagents are used for the nucleic acid amplification and detection assay for the quantitative determination of HIV-1 RNA in human plasma. The HIV-1 viral loads that can be measured in this assay range from 51 – 5,390,000 RNA copies/ml.

EASYQ HIV-1 NucliSens Magnetic Extraction:

200µl of plasma sample was added to a 2ml aliquot of NucliSens Lysis Buffer in a 15ml Falcon tube. This was vortexed and left to incubate for 10mins at room temperature. 220µl of elution buffer was then added to the calibrator (to reconstitute the lyophilized pellet) and this was vortexed thoroughly. 20µl of the reconstituted calibrator was added to the test sample and

vortexed followed by the addition of 50µl of vortexed silica. The tube was then vortexed thoroughly and incubated for 10mins at room temperature, thereafter centrifuged for 2mins at 1500g. The supernatant was then removed with a pastette and 400µl of wash buffer 1 was pipetted into the sample tube. The wash buffer was mixed with the pellet and this was then transferred to a sterile 1.5ml starsted tube using a sterile pastette. After 30 rotations on the miniMag, the supernatant was removed and the wash step was repeated. After 30 rotations, the supernatant was removed and two wash steps were done with 500µl wash buffer 2. After the supernatant was removed, the final wash was done with 500µl wash buffer 3. The supernatant was removed after 15 rotations on the miniMag. 25µl of the elution buffer was added to the tube and incubated at 60^0C for 5mins on a shaking incubator. This was followed by the removal of the silica using the miniMag. The extracted RNA was then transferred to a clean 1.5ml starsted tube.

Preparation of mastermix:

ENZYME: 45µl of Enzyme Diluent was added to the lyophilized pellet. After a gentle mix, the tube was left at room temperature for 15mins.

PRIMERS: 90µl of Primer Diluent was added to the lyophilized pellet and vortexed immediately.

Amplification and Detection:

5µl of the extracted RNA was aliquoted into the correctly labeled 8-tube strip. This was followed by the addition of 10µl of the primer. The strip was placed in the NucliSens EasyQ Incubator and the programmed for 1min at 65^0C, then 2mins at 41^0C. Thereafter 5µl of the enzyme solution aliquoted into the each of the caps of the strip, the strip was then capped and centrifuged (pulse spin) in the Mini-Strip Centrifuge. The strip was then transferred to the NucliSens EasyQ Analyser for amplification and detection. The results of the test samples were printed in a numerical format.

Internal quality control: The EASYQ HIV-1 NucliSens Magnetic Extraction is designed such that each run comprises of 8 tests. There were no individual control samples; however, the calibrator was incorporated with the test sample. If the calibrator failed then that particular test sample was repeated.

2.2.4.2 Cobas amplification HIV-1 Monitor test, version -1.5 (Roche Diagnostics)

Principle: The COBAS AMPLIFICATION HIV-1 MONITOR TEST, version-1.5 is an in vitro nucleic acid amplification test for the quantification of HIV-1 RNA in human plasma on the COBAS AMPLICOR™ ANALYZER. The Test can quantitate HIV-1 RNA over the range of 50-750 000 copies/mL by using a combination of two specimen preparation procedures namely the Standard and the UltraSensitive procedures (Roche Diagnostics, Germany).

The Cobas amplification HIV-1 monitor test is based on five major processes:

- Specimen preparation
- Reverse Transcription (the target RNA is transcribed into cDNA)
- PCR Amplification (target cDNA used HIV-1 specific complimentary primers)
- Hybridization (of the amplified products to the oligonucleotide probes specific to the target)
- Detection (of the probe-bound amplified products by colorimetric determination)

Standard procedure (HIM test)

Preparation of mastermix (in pre-amplification room)

100µL of the HIV-1 Mn^{2+} solution was added to one vial of the HIV-1 MMX solution and mixed well by inverting the tube (working HIV-1 MMX). 50µL of the working HIV-1 MMX was added to each of the 12 A-tubes of the A-ring. The A-ring was stored in the fridge (up to 4 hours) in a reseal-able bag, until use.

HIV-1 RNA Extraction from the controls and test samples (Performed in the HPP Virology Laboratory in a Biological Safety Cabinet)

A 2ml starsted tube was used for each of the three controls (Negative, Low Positive and High Positive Controls) and test samples processed. The precipitate that formed in HIV-1 LYSIS Buffer upon storage at 2-8^{0}C, was dissolved (by warming it up to room temperature). The working Lysis Buffer was prepared by adding 100µl of HIV-1 MONITOR Quantitation Standard (HIV-1 QS) to the vial of HIV-1 Monitor Lysis Reagent. The working Lysis Buffer was mixed well and 600µl was aliquoted into each tube. 200µL of Normal Human Plasma (NHP) was added to each of the control tubes and 200µL of patient's sample to the respective tubes. 50µL of the controls were added to the respective control tubes.

All tubes were vortexed and incubated for 10 minutes. 800ul of 100% iso-propanol was added to each tube which was then vortexed and centrifuged for 20 minutes. The supernatant was discarded and the pellet was washed with 1ml of fresh 70% ethanol (Appendix 3). The tubes were vortexed and centrifuged for 5 minutes. The supernatant was discarded and the pellet was clearly visible. Thereafter 400µL of HIV-1 Monitor Specimen Diluent was added to each tube. 50µL of each processed controls and specimens were added to the appropriately labeled A- tube in the A-ring (which contained the working MMX solution).

Detection and Quantitation of HIV-1 RNA: The A-ring was transferred to the Roche Cobas Amplicor Machine where the process of reverse transcription, amplification, hybridization and detection was performed. The COBAS AMPLICOR HIV-1 MONITOR TEST KIT quantitates the HIV-1 viral RNA and utilizes the HIV-1 Quantitation Standard which was added to the test specimen of a known concentration. The results of the controls and test samples were printed in an exponential format.

Internal quality control: The COBAS AMPLICOR HIV-1 MONITOR TEST KIT is designed such that each run (12 tests) would include three controls (Negative, Low positive and High positive control) and nine test samples. All controls must be within the range stipulated by the COBAS AMPLICOR HIV-1 MONITOR TEST KIT. If any of the controls

failed to meet the stipulated criteria of the TEST KIT, the entire assay would have to be repeated.

2.2.5 DNA isolation

DNA was isolated from the sample with the PUREGENE™ DNA ISOLATION Kit (Gentra Systems, Minneaplois, USA). The Kit consisted of five processes namely;

Red Blood Cell Lysis: 9ml of RBC Lysis Solution (Appendix 4) was added to ~3ml of the blood sample (after the PBMCs were removed). The tubes were inverted and left to incubate for 10 minutes at room temperature. After centrifugation at 2000 rpm for 10 minutes, the supernatant was poured out. (This step was performed twice, to ensure the recovery of the white blood cells only).

Cell Lysis: The excess supernatant was removed with a fine tip Pasteur pipette. After which the pellet was disrupted by vigorous motion of the tube along the surface of a tube rack. 3mls of Cell Lysis Solution was added to re-suspend the cells. The sample was left to incubate at room temperature for at least 24 hours.

Protein Precipitation: The proteins were precipitated with the addition of 1mL Protein Precipitation Solution to the cell lysate. This was vortexed for ±20 seconds and centrifuged at 2000 rpm for 10 minutes. The precipitated proteins formed a tight, dark brown to reddish pellet.

DNA Precipitation: The supernatant which contained the DNA was removed with a Pasteur pipette, and put into a clean tube (labeled with the sample identification no. and date), which contained 3mls of 100% iso-propanol (the precipitated protein pellet was left behind). The tubes were inverted ± 30 times, until white threads of DNA formed a visible clump. If no strand of DNA was seen, it was noted on the side of the tube. The DNA formed a small visible white pellet at the bottom of the tube after centrifugation at 2000 rpm for 3 minutes. The supernatant was poured off and the pellet was then washed with a 3ml 70% ethanol solution (Appendix 3). The supernatant was poured off again and the excess drained on clean

absorbent paper after it was centrifuged at 2000 rpm for 1 minute. The DNA pellet was left to air dry for ± 15 minutes.

DNA Hydration: Approximately 50 µl of DNA hydration solution was added to each tube. (This was based on the actual size of the DNA pellet). Samples were left at 4^0C for at least 24 hours before it was pulse centrifuged and aliquoted into pre-labeled (with the specific sample identification no, date of receipt and the specific time point) 1.5 ml eppendorf tubes and stored in at -20^0C until required.

DNA Measurement: Prior to the use of the required DNA samples, the samples were removed from the -20^0C freezer and thawed at room temperature for a few minutes and then gently vortexed at a low speed. A sample volume of 1 ul of DNA was measured at 260/280nm using a Nanodrop (ND-1000 Spectrophotometer - Inqababiotec™, Pretoria, South Africa). A ratio of 1.8 was regarded as pure DNA.

2.2.6 Peripheral blood mononuclear cells (PBMCs)

2.2.6.1 Separation of PBMCs

Principle: The HISTOPAQUE-1077 reagent was used for the isolation of mononuclear cells by density-gradient centrifugation. The blood sample was layered onto the HISTOPAQUE-1077. During centrifugation, the erythrocytes and granulocytes were aggregated by the polysucrose and rapidly sediment; whereas the lymphocytes and other mononuclear cells remained at the plasma- HISTOPAQUE-1077 interface. The extraneous platelets were removed by the low speed centrifugation during the wash steps (Sigma-Aldrich, INC. St. Louis, USA).

Safety: HISTOPAQUE-1077 solutions are harmful. It causes sensitization by inhalation and skin contact. A Howie coat and double gloves were worn when working with these solutions and effort was made not to breathe the vapours.

Reagent preparation: The Histopaque-1077; PBS (Appendix 5) with antibiotics (Appendix 6) and R10 (Appendix 7) media were removed from the fridge and left at room temperature to warm. The plasma from the whole blood samples were removed and stored prior to the separation of the PBMCs. The separation was based on a ratio of 1:1:1 of the HISTOPAQUE-1077 : PBS+ANTIBIOTICS : BLOOD SAMPLE.

Sample preparation: An equal volume of Histopaque-1077 was added to a sterile 15ml Falcon tube. The blood sample (buffy coat + red blood cells) was diluted with an equal volume of PBS+antibiotics (Appendix 6). This was mixed well and carefully layered onto the Histopaque-1077. The tubes were centrifuged at room temperature for 30 minutes at 1500 rpm with slow start and the brakes off.

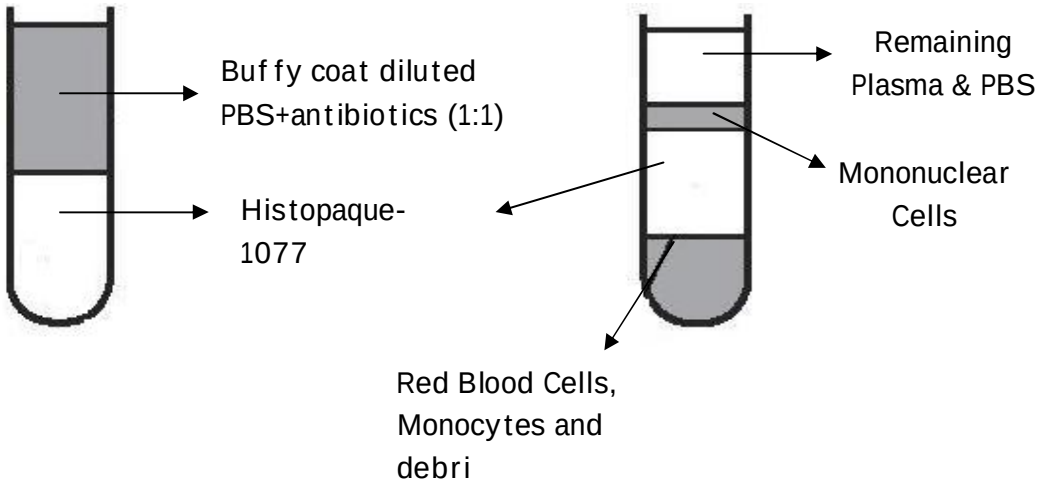


Figure 9. Illustration of the blood sample layered on the Histopaque® - 1077

The mononuclear cell layer (opaque interface) was transferred into a sterile 15mL Falcon tube. PBS with antibiotics was added up to a volume of 13mL. The tubes were gently inverted and then centrifuged at 1500 rpm for 10 minutes at room temperature (first wash of the PBMCs). When decanting the supernatant it was ensured that the cell pellet was not disrupted. If red blood cells were present after the first wash step, a few drops of sterile distilled water was added to lyse the red blood cells followed by the immediate addition of PBS with

antibiotics for the second wash of the PBMCs. The supernatant was decanted and the tubes were tapped gently to break up the cell pellet and then resuspended with 10 mL of R10 medium (Appendix 7). The PBMCs was enumerated prior to the performance of tests performed.

2.2.6.2 Enumeration of PBMCs

PBMCs were initially counted with the Haemocytometer. This method proved to be time consuming and there was variation with the cell counts of samples. With the increase in the number of samples, the Guava Cell Counter (Guava Technologies INC, Hayward, CA 94545-2991, USA) was purchased. The Guava Cell Counter proved to be useful for cell viability, counts over a wide range of cell concentrations and consistency.

2.2.6.2.1 Haemocytometer – Cell counting

Principle: It is important to establish the concentration of the cells that you are working with. There are many applications that require specific cell counts eg. Intracellular Cytokine Staining, ELISPOT assays, freezing cells etc.

Procedure: The isolated PBMCs were resuspended in 10 ml of R10 medium. The cells were thoroughly mixed with a pipette aid and a 10 ml pipette. The tube was then vortexed and 50µl of the cell suspension was aliquoted into a sterile 1.5 ml eppendorf. 50 µl of Trypan Blue was then added to the eppendorf and was then vortexed. 10µl of the diluted cell suspension was transferred into the well of the haemocytometer and was then placed under the microscope and the cells were counted.

The cells were counted in two of the nine squares as shown in Figure 10. Cells were not counted if they touched the line that bordered the square or if they were between the two little squares as shown in Figure 10.

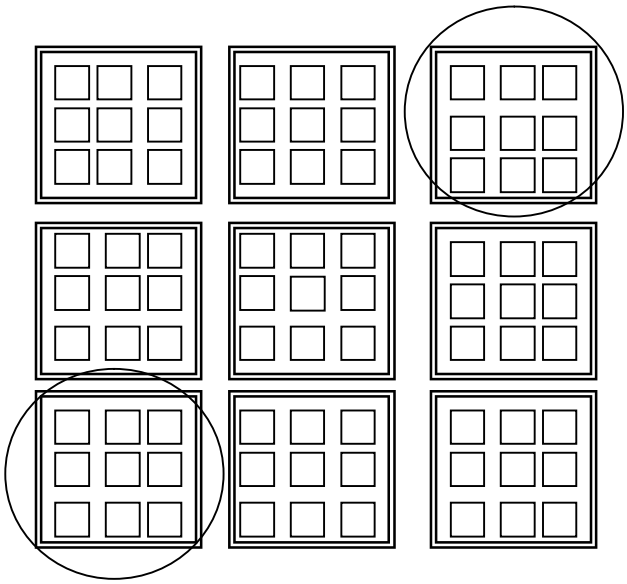


Figure 10. Schematic representation of the haemocytometre used for the counting of PBMCs.

Enumeration of the cells:

Cells per ml = the average count per square x dilution in trypan blue x volume of stock suspension x 10^4

Example: First square = 55 cells

Second square = 49 cells

Average of both squares = 52 cells

x 2 for the dilution in trypan blue = (52 x 2) = 104 cells

x 10ml (in which the cells were reconstituted)

x 10^4 (for the volume of the haemocytomer chamber)

= 52 x 2 x 10 x 10^4 cells/ml

= 104 x 10^5 cells/ml

= 10.4 x 10^6 cells/ml

2.2.6.2.2 Guava ViaCount – Cell counting

Maintenance: The machine was turned on and left for at least 15 minutes to warm up. The waste bottle was emptied and »8ml of 2% Virkon (Appendix 8) was added.

Guava Check: A 1:20 dilution of the Guava Check Beads (25 ml Beads to 475 ml Diluent) was freshly prepared and run three times on the Guava Cell Counter. The Guava Check Program was selected. Each reading of the beads must flag in green. If this was not the case, the clean cycle was selected and Guava Check Beads were rerun. If the Guava Check failed, the Guava Beads were re-prepared and read three times.

Preparation of the samples: A 1:10 dilution of the PBMCs was prepared (20 ml of PBMCs to 180ml of Counting Solution). The diluted sample was vortexed thoroughly and incubated for 8 minutes at room temperature.

Acquisition: The GuavaVia Count program was selected. The instrument was cleaned with Instrument Cleaning Fluid (ICF) on the clean cycle. The prepared samples were loaded individually on the instrument, the required patient data was entered and the cells were then acquired. The instrument was cleaned twice with ICF and once with sterile distilled water before it was switched off.

2.2.6.3 Freezing of PBMCs

Purpose: Freezing of PBMCs was to allow for assays to be performed at a later date.

Safety: A Howie coat and gloves were worn as well as cryogenic gloves and a face shield or goggles when working with the frozen cells and when using the liquid nitrogen freezer.

Procedure: 20ml of PBMCs was added to 180ml of Guava ViaCount Reagent. The PBMCs count was obtained using the Guava Cell Counter. The 15ml falcon tube that contained the cells was centrifuged in a chilled (4^0C) centrifuge for 5mins at 1700 rpm. The supernatant was removed with a volume of 200ml left behind. The pellet of cells was then resuspended in this volume. Based on the cell count, the cells were frozen at a concentration of 10 million cells per vial in a 1ml volume using 10% DMSO added to 100% Fetal Calf Serum. To reduce shock to the cells, half the quantity of freezing solution was added as FCS on its own and the cells were thoroughly suspended in this formula. Thereafter the FCS with DMSO was added in a drop-wise manner whilst gently shaking the tube. The cryovials were immediately transferred to the Mr. Frosty stratacooler box (which contained 100% isopropanol) and this was placed in the –85% freezer overnight. The following day the cryovials were transferred to the liquid nitrogen freezer.

2.2.7 Intracellular Cytokine Staining (ICS)

DAY 1

The isolated PBMCs were counted and resuspended to a final concentration of 1 X 10^6 cells/ml. A positive control sample (where 10 µl of PHA was added) and a negative control sample (where 10 µl of R10 media was added) were prepared with each test sample.

A mastermix solution was prepared (per tube) with the following:

- 1µl of anti-CD28 antibody (Becton Dickenson Immunocytometry Systems, San Jose, CA)
- 1µl of anti-CD49d antibody (Becton Dickenson Immunocytometry Systems, San Jose, CA)
- 500µl of R10 (Appendice 7)

The mastermix was vortexed and 500µl was aliquoted into each tube. 10µl of the Gag-pool of peptides was added to the test sample and 10µl of PHA to the positive control tube. 500µl of the PBMCs suspended in R10 media at a concentration of 1 X 10^6 cells/ml was added to each

tube. The tubes were incubated for 60 minutes at 37 ^{0}C in a 5% CO_2 incubator. (The lids of the FACS tubes were loosened to allow the cells to breathe). Following the 60 minute incubation, 10µl of 1mg/ml Brefeldin A (Sigma Chemical Co., St Louis, USA) was added to each tube. (Brefeldin A prevented the secretion of cytokine IFN-γ during the cell activation to be transported outside of the cell). The tubes were incubated for 4.5 hours at 37 ^{0}C in a 5% CO_2 incubator (a total of 6 hours from the start). The tubes were then refrigerated at 4^{0}C overnight and processed the following day.

DAY 2

2 mls of sterile PBS was added to each tube to resuspend the cells. The tubes were centrifuged at 2000 rpm for 8 minutes.

Wash Step:

The tubes were removed as soon as the spin was complete (cells would die if left longer that the specified time) and the PBS was removed by the inversion of the rack over the waste container (making sure not to disrupt the pellet).

The tube with pellet was vortexed gently. The wash step with PBS was repeated twice.
After the second wash, a mastermix solution of the following fluorescent cell markers was prepared:

- 2µl anti-CD8-APC antibody (Becton Dickenson Immunocytometry Systems, San Jose, CA)
- 1µl anti-CD4-PE antibody (Becton Dickenson Immunocytometry Systems, San Jose, CA)
- 47µl chilled PBS

50µl of antibody mastermix was aliquoted into each tube. The tubes were gently vortexed and incubated at 4^{0}C in the dark for 15 minutes. Followed by the wash step.

2mls of PBS was added to each tube and centrifuged at 2000 rpm for 8 minutes. The tubes were removed as soon as the spin was completed and the PBS was removed by the inversion

of the rack over the waste container (be sure not to disrupt the pellet). 100µl of Fix/Perm Medium A (Caltag Laboratories, Burlingame, CA) was aliquoted into each tube and vortexed gently. The tubes were incubated for 15 min at room temperature in the dark (covered with tin foil), followed by the wash step.

2mls of PBS was added to each tube and centrifuged at 2000 rpm for 8 minutes. The tubes were removed as soon as the spin was completed and the PBS was removed by the inversion of the rack over the waste container (be sure not to disrupt the pellet). 100µl of Cell Permeabilization Medium B (Caltag Laboratories, Burlingame, CA) and 6µl of anti-IFN-γ (Becton Dickenson, USA) was aliquoted into each tube. The tubes were vortexed gently and incubated for 20 mins at 4 ^{0}C in the dark, followed by the wash step.

2mls of PBS was added to each tube and centrifuged at 2000 rpm for 8 minutes. The tubes were removed as soon as the spin was completed and the PBS was removed by the inversion of the rack over the waste container (be sure not to disrupt the pellet). The tubes were gently vortexed and then resuspended in 200µl of PBS. The samples were acquired and analysed on the FACS Calibur Flow Cytometer.

2.2.8 ELISPOT Assay

Freshly isolated PBMCs were incubated overnight in a 96-well polyvinylidene difluoride-backed plates (MAIP S45, Millipore) with 410 overlapping peptides (OLP) that spanned the entire HIV-1 genome (Gag, Pol, Vif, Vpr, Vpu, Rev, Tat, Env and Nef). The PBMCs were screened in an Enzyme-Linked Immunospot (ELISPOT) assay, which was specific for IFN-g production. The amount of IFN-g secreted, was measured using an ELISA-based method. The ELISPOT plates were read using an automated reader (Autoimmune Diagnostics – Germany).

Overlapping peptides: The panel of 410 overlapping peptides (OLPs) (15-18 amino acids in length and overlapped by 10 amino acids) were synthesized at the MGH Peptide Core Facility on an automated peptide synthesizer (MBS 396; Advanced Chemtech, Louisville, Ky.). The OLPs were arranged to form a matrix screen, where a positive response in two individual wells correlated to an individual peptide sequence.

The Peptide Matrix: The Gag, Pol, Env and Nef regions were represented as individual peptide matrices while the Rev, Tat, Vif, Vpr and Vpu regions were pooled into a combined accessory and regulatory protein peptide matrix as described previously for B Clade by Addo et al. (2003). Figure 11 is a representation of an ELISPOT plate showing the pooled peptides as well as some selected optimal optimals and the controls.

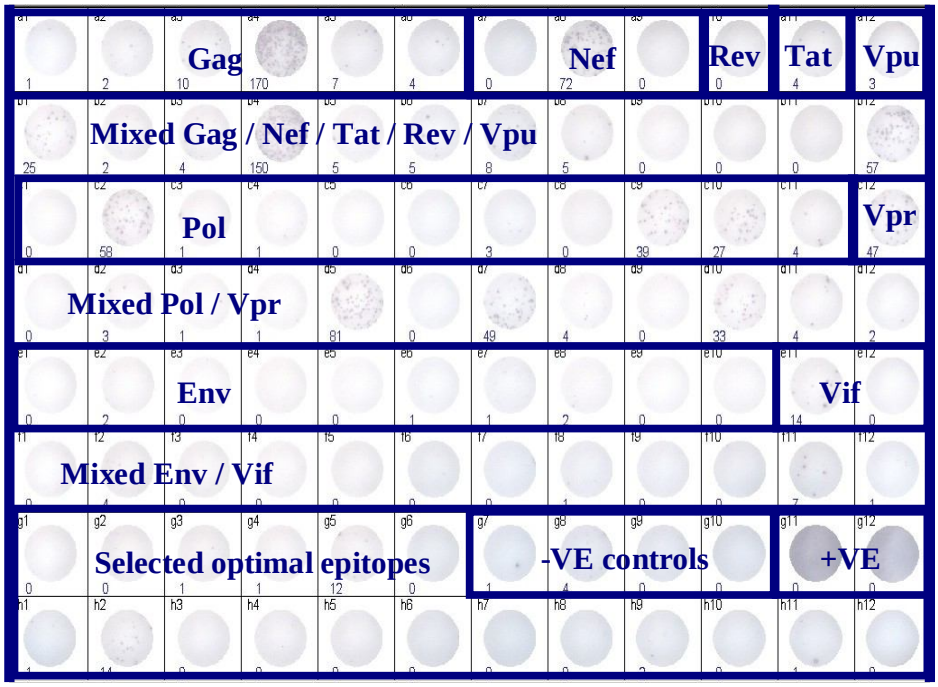


Figure 11. Pooled peptides in a matrix format on an ELISPOT plate that spanned the HIV genome.

Peptide pools: The number of pools per protein matrix and the number of peptides per pool depended on the total number of peptides spanning each protein.

Table 1. The no. of peptides in each protein within the HIV-1 genome

HIV-1 Proteins	No. of Peptides	HIV-1 Proteins	No. of Peptides
Gag	66	**Pol**	133
Neg	27	**Vpr**	11
Rev	13	**Env**	113
Tat	12	**Vif**	24
Vpu	9		

The concentration of each peptide within a pool was 200μg/ml. Wells containing PBMC's and R10 medium were used as negative controls and they were run in quadruplicate. Phytohaemaglutinin A (PHA) was added at a final concentration of 1.8μg/ml and run in duplicate to serve as a positive control.

Positive Responses on ELISPOT Assay: Within the matrix, each peptide is contained in two different wells such that a positive response in two corresponding wells is linked to a single peptide (refer to figure 11). A positive response to a peptide was defined as response of ≥ 100 spot forming cells (SFC's) / million PBMC's after subtraction of the background (mean SFC's within the 4 negative wells to which PBMC's were added and no peptide). Responses were excluded in the analysis if background exceeded 100 SFC's / million PBMC's or if the level of the peptide-specific response was less than the mean of the control wells plus three times the standard deviation of the IFN-γ production (SFC's) within the control wells.

Confirmation of Responses: Recognition of a pool of peptides was then reconfirmed using individual peptides in an IFN-γ ELISPOT assay.

PROCEDURE

DAY 1

5μl of anti-human IFN-γ (Mabtech) was diluted with 10ml of Phosphate Saline Buffer (Appendix 5). The solution was vortexed thoroughly and 100μl was coated into the wells of the sterile 96-well polyvinylidene diflouride plate (Multiscreen, Millipore, Bedford, USA) with a multichannel pipettor. The plates were incubated overnight at 4^0C.

DAY 2

The plates were removed from the fridge and washed six times with blocking buffer (Appendix 9). ~100µl of blocking buffer per well of each plate removed the unbound antibody. After the last wash step, 50µl of R10 medium was added to each well of the ELISPOT plate. For every patient's cells tested, two wells contained 10µl of Phytohaemaglutinin A (PHA) at a concentration of (33.3µg/ml) as positive controls and four wells without any peptide as negative controls. 10µl of each peptide (33.3µg/ml) giving a final concentration of 2µg/ml was added to each well. 100µl of freshly isolated PBMCs suspended in R10 medium at a final concentration of 0.5 X 10^6 cells/ml was added to each well. The plate was incubated overnight at 37^0C in 5% CO_2 incubator.

DAY 3

The cells were discarded into a disinfectant container which contained 2% Virkon (Appendix 8). The plate was washed six times with PBS in the ELISPOT washer. 100µl of diluted Biotinylated anti-human IFN-γ (5µl of antibody to 10ml of PBS) was added to each well. The plate was incubated in the dark (covered with tin foil) at room temperature for 90 minutes. The plate was washed six times with PBS in the ELISPOT washer. 100µl of diluted Streptavidin-alkaline phosphatase conjugate (5µl of antibody to 10ml of PBS) was added to each well. The plate was incubated in the dark (covered with tin foil) at room temperature for 45 minutes. The plate was again washed six times with PBS in the ELISPOT washer.

Colour Development Reagents: 100µl of Bromo-Chloro-Indoylphosphate (BCIP) (Appendix 10) and 100µl Nitroblue Tetrozolium Chloride (NBT) (Appendix 11) was diluted in 10 ml of 1M TRIS Buffer (pH = 0.3) (Appendix 12).

100µl of the colour development reagent was added to each well of the plate which was incubated at room temperature until blue spots were seen (± 15 minutes). The development process was stopped by washing the plate at least six times under running water. The plate was left to air-dry on absorbent tissue paper. The ELISPOT plates were read using an automated reader (Autoimmune Diagnostics – Germany).

Table 2. The instrument settings on the ELISPOT reader

Parameter	Instument settings
Intensity	10 (min selected)
Size	108
Gradient	1 (min selected)
Emphasis	Tiny
Max growth	30
Max shrinking	10
Max position change	50
Correction method	Find any ring shaped structure

2.2.9 Human Leukocyte Antigen (HLA) typing

Principle : The Dynal RELI™ SSO HLA Test (Dynal Biotech, Bromborough, UK) is a DNA based typing kit which provides a low to medium resolution HLA typing. Refining the genotype to the allele level was performed using the Dynal Biotech sequence-specific priming kits for the HLA-A, HLA-B and HLA-C loci. The test is based on four major processes i.e: (1) PCR target amplification; followed by (2) hybridization of the amplified products to immobilized sequence-specific oligonucleotide probes; (3) the detection of the probe-bound to the amplified product on nylon membrane strips by color formation; and (4) results interpretation. The HLA typing was performed according to the manufacture's instructions and all the DNA samples were sent to the South African National Blood Transfusion Service where the assays were undertaken by personnel in the Transplant Laboratory.

PCR Amplification: 15µl of DNA (13-15 ng/µl ~ approximate 200ng/reaction) was added to the cocktail of 15µl of $MgCl_2$ solution and 30µl of the HLA master mix, for each allele (HLA-A, HLA-B and HLA-C), in sterile PCR tubes. A positive control (15µl of positive DNA control) and negative control (15µl of de-ionized water) was prepared, with each amplification assay set up. The tubes were capped and placed in the thermocycler and amplified on a selected program. 60µl of denaturation solution was pipetted into each PCR tube and incubated for 10 minutes at room temperature.

Hybridization and Detection of the Strips: The labeled strips were placed in the typing tray with the probe lines facing upwards. 5ml of the pre-warmed working hybridization buffer (Appendix 13) was added to each strip followed by the 70µl of the correctly labeled PCR product. The strips were incubated for 30 minutes at 50^0C on a shaking platform.

Two wash steps followed after the solution in each well was aspirated from each well. The first wash step was with 5ml of ambient wash buffer (Appendix 14) for 5 minutes at room temperature and second wash step with 5ml of pre-warmed stringent wash buffer (Appendix 14) for 15 minutes at 50^0C.

5ml of Conjugate Solution (Appendix 15) was added and the strips were incubated for 15 minutes after the stringent wash buffer was aspirated. Two ambient wash steps were performed each with 5ml of ambient wash buffer and an incubation of 5 minutes after the conjugate solution was aspirated. 5ml of working citrate buffer (Appendix 16) was added after the wash buffer was aspirated and this was incubated for 5 minutes.

After the citrate buffer was aspirated, 5ml of working substrate buffer (Appendix 17) was added to each well and incubated for 8 minutes. This was followed by two wash steps, each with 5ml of de-ionized water and incubated for 5 minutes. 5ml of Citrate buffer was added to each well after the de-ionized water was aspirated.

Results Analysis: The strips were kept moist for the result analysis and then stored at 8^0C. The nylon membrane strips were analyzed using the RELI-Scan™ and Dynal Pattern Matching Program (PMP).

2.2.10 HIV drug resistance and resistant mutations

Resistance testing was performed on stored frozen plasma samples from thirty children enrolled to this study. The samples were sent to the National Institute for Communicable Diseases in Gauteng, South Africa where the assays were undertaken by personnel in the laboratory of Professor Lynn Morris.

Genotyping: Sequencing of the HIV-1 *pol* gene was conducted using an in-house assay. Viral RNA was isolated from the stored plasma samples using the MagNa Pure automated system (Roche Diagnostics, Indianapolis, IN). A 1.7Kb fragment spanning the *pol* gene was amplified by nested PCR using the Thermoscript™ RT-PCR System.

PCR Primers:

First round primers -

G25REV (5'GCAAGAGTTTTGGCTGAAGCAATGAG3')

IN3 (5'TCTATVCCATCTAAAAATAGTACTTTCCTGATTCC3')

Second round primers –

AV150 (5'GTGGGAAAGGAAGGACACCAAATGAAAG3')

PolM4 (5'CTATTAGCTGCCCCATCTACATA3')

Amplification conditions:

Both the first and second PCR amplification conditions were identical.

1 cycle	: 94^0C	: 2 minutes
10cycles	: 94^0C	: 10 seconds
	: 50^0C	: 30 seconds
	: 68^0C	: 2 minutes
25 cycles	: 94^0C	: 15 seconds
	: 50^0C	: 30 seconds
	: 68^0C	: 2 minutes
		: 20 seconds for elongation on every cycle
Final step	: 68^0C	: 7 minutes

Sequencing: The PCR products were sequenced (codons 1-99 of protease and codons 1-350 of reverse transcriptase) by using the BigDyeTerminators and an ABI 310 DNA Sequencer (Applied Biosystems, Foster City, CA).

Analysis: Consensus sequences from all genotyped subjects were aligned and manually edited using the Sequencher version 4.5 program (GeneCodes, Ann Arbor, MI). Multiple alignments were performed using Clustal X. Phylogenetic analysis of nucleic acid sequences was performed with Mega version 3.1. Reference sequences were downloaded from Los Alamos (www.hiv.lanl.gov). Genotypic resistance was defined as the presence of mutations associated with impaired drug susceptibility or virologic response as specified by the WHO surveillance list of mutations (Shafer *et al*, 2006)

CHAPTER THREE

3.1 RESULTS

3.1.1 Demographics and characteristics of participants

Thirty chronically HIV-1 infected children were enrolled in the pilot study. Immunological tests were performed on all children. However, these tests were not performed at all timepoints due to either insufficient peripheral blood mononuclear cells (PBMCs), disenrolment of children due to treatment non-adherence or sensitivity to the antiretroviral treatment.

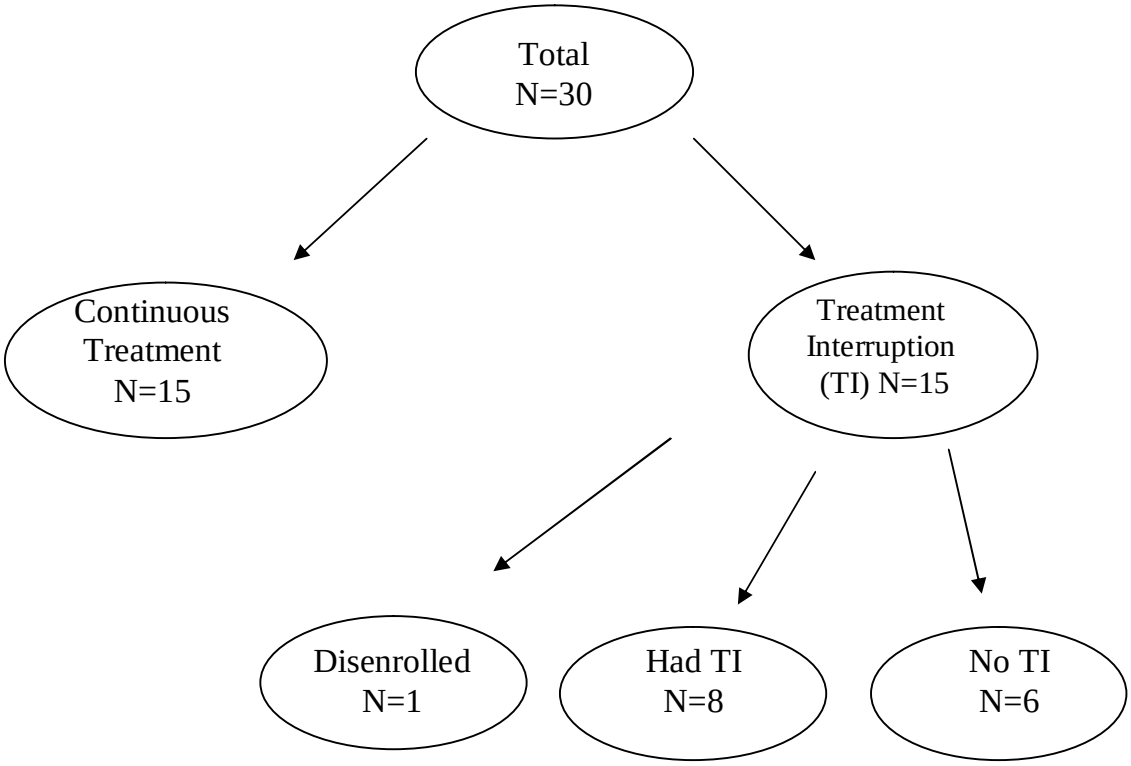


Figure 12. Illustrates the number of children enrolled and their subsequent treatment modalities.

Thirty children were enrolled in the study. Fifteen children were randomized to the continuous treatment group and fifteen to the treatment interruption (TI) group. One

(6.7%) child from the Treatment Interruption group of the study was disenrolled after two weeks due to sensitivity to the antiretroviral agent Abacavir (ABC). Eight (53.3%) children had treatment interruptions whilst the other six (40%) children did not undergo any treatment interruptions due to their detectable viral loads i.e. never reaching undetectable viral loads.

The gender and age as well as the pre-treatment immunologic and virologic characteristics of the enrolled HIV-1 chronically infected children in the two arms of the study are depicted in Table 3. Statistical analysis of significance (*P values*) was calculated using the Mann Whitney test on GraphPad Prism. The *P values* indicated that there was no statistical significant difference with regard to gender, age, %CD4 T-cells, %CD8 T-cells and HIV-RNA log viral load, between the continuous therapy and treatment interruption arms of the study.

Table 3. Demographic and clinical variables of the study participants in the continuous treatment group and the treatment interruption group at the pre-HAART timepoint

Characteristics	Total	Continuous Treatment Group	Treatment Interruption Group	*p value*
No. of Subjects	30	15	15	-
Males	15	8	7	-
Females	15	7	8	-
Age in months: median (range)	60 (24-144)	72 (24-132)	58 (24-144)	0.2354
$CD4^+$ T-cell % : median (range)	18 (7-29)	18 (7-26)	17 (8-29)	0.9337
$CD4^+$ T-cell count: median (range)	567 (218-1842)	567 (218-913)	533 (245-1842)	0.8682
$CD8^+$ T-cell %: median (range)	47 (19-73)	46 (35-66)	47 (19-73)	0.7394
$CD8^+$ T-cell count: median (range)	1569 (559-8133)	1816 (559-2871)	1451 (808-8133)	0.6186
HIV-RNA log10: median (range)	4.73 (3.60-6.00)	4.69 (3.62-6.00)	4.85 (3.60-5.83)	0.6934

3.1.2 $CD4^+$ T-cell percentage and viral load estimation

Immunological and virological parameters have been proposed as potential surrogate markers for viral replication (Ondoa *et al*, 2005). Immunological monitoring is an essential component in HIV clinical management. Monitoring tests assist in not only defining the stage of the infection but the need for antiretroviral therapy and monitoring the treatment thereof. HIV-specific $CD4^+$ T-cells are likely to be the first to be activated in response to the virus however they become infected by the HIV and are destroyed. The $CD4^+$ T-cell count and HIV-RNA (viral load) are considered to be the end-point parameters to monitor the efficacy of antiretroviral treatment (Mellors *et al*, 1997).

3.1.2.1 $CD4^+$ T-cell percentage and viral load estimation before starting and during early treatment with HAART

Immune restoration Disease (IRD) may occur when the CD4 cell count rises rapidly during the first few months of antiretroviral therapy. Figures 13a and 13b demonstrate the %CD4 T-cells and the log viral load respectively for all patients at the pretreatment phase and during the initial treatment phase at 12 and 24 weeks.

(13a) **(13b)**

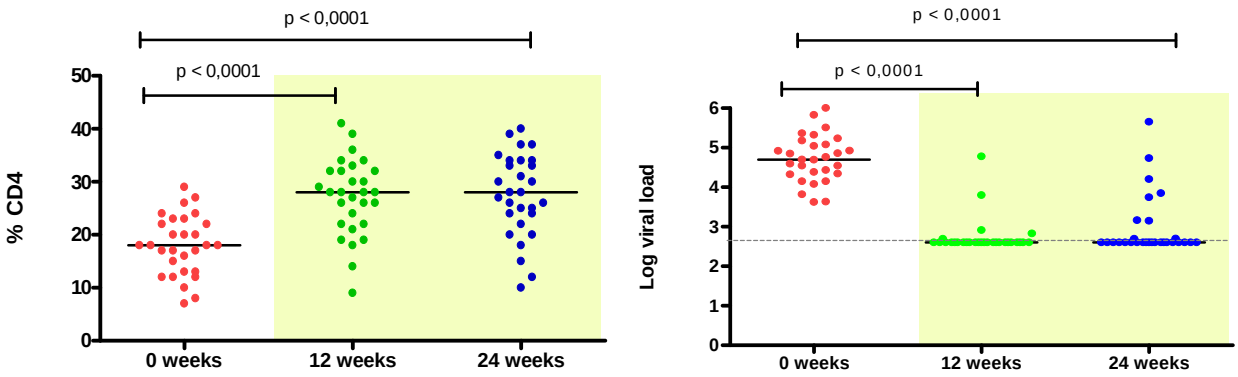


Figure 13. Comparison of the %CD4 (a) and Log viral load (b) of the pretreatment phase (0 weeks) to the initial treatment phase (12 and 24 weeks). The yellow background indicates the treatment phase.

The median %CD4$^+$ T-cells increased from 18% before ARV treatment to 27% at 12 weeks after ARV treatment (*p value* < 0.0001). %CD4$^+$ T-cell recovery was maintained at 24 weeks (*p value* < 0.001). The median viral load decreased from log 4.70 to levels below detection limits by 12 weeks (*p value* < 0.0001) and was maintained at 24 weeks (*p value* < 0.0001). As depicted in figure 13b, 5/29 children had detectable viral loads at 12 weeks (>log 2.60) and 9/29 at 24 weeks (>log 2.60). Hence not all children reached undetectable viral load levels by 24 weeks of ARV treatment. A possible reason why there was detectable virus at 24 weeks of ARV treatment could be that the initial viral load may have been extremely high (eg. in millions) so that it would have taken much longer than 24 weeks to reach undetectable levels, however this was the case for only one child as the viral loads for the other children were less than log 5. Three other possibilities include a) that the ARV treatment would have killed most of the susceptible virus, hence giving resistant virus a greater chance to replicate b) that the children may not have been adherent to the antiretroviral treatment and c) is that the HAART regime of 3 NRTIs was not the ideal one.

3.1.2.2 $CD4^+$ T-cell percentage and viral load estimation in the continuous treatment group

Figures 14a and 14b demonstrate the effect of continuous ARV therapy on $\%CD4^+$ T-cells and viral load over a two-year period in HIV-1 chronically infected children (n=21).

(14a)

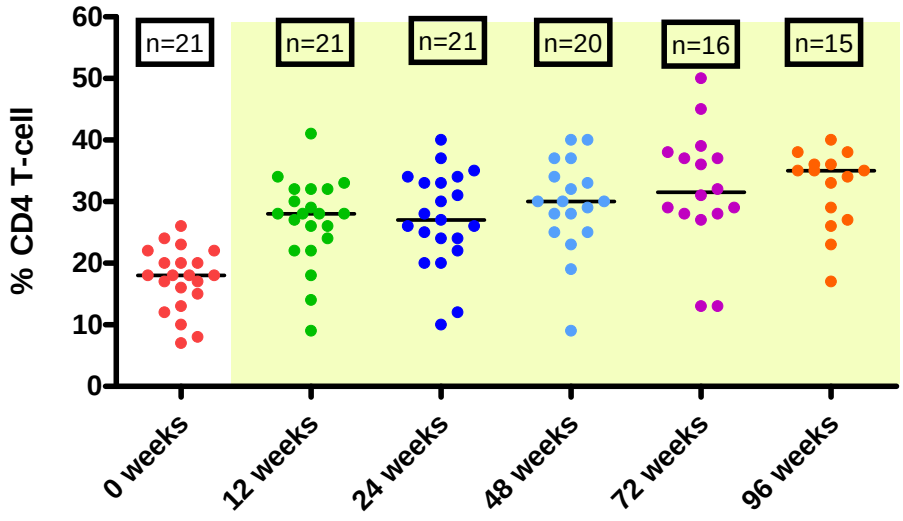


Figure 14(a). Demonstrates the pretreatment $\%CD4^+$ T-cell in relation to the $\%CD4^+$ T-cell over the two-year study period for the twenty-one children who received continuous ARV therapy (this includes the six children who did not have treatment interruptions but were randomized to the treatment interruption arm). The yellow background indicates the treatment phase. The change in the *n* values at 48, 72 and 96 weeks, indicates the children who were disenrolled due to treatment non-adherence and development of drug resistance.

(14b)

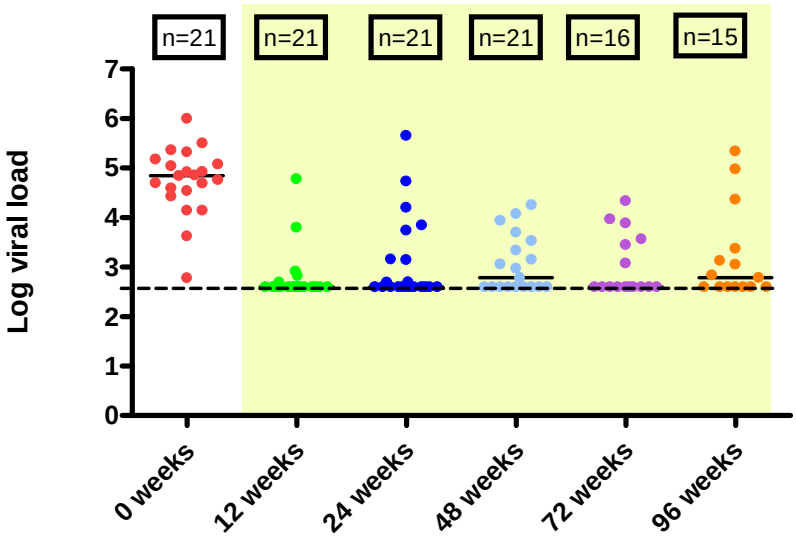


Figure 14(b). The pre-treatment log viral load is shown in relation to the log viral loads over the two year study period for the twenty-one children who received continuous ARV therapy (this includes the six children who did not have treatment interruptions but were randomized to the treatment interruption arm). The yellow background indicates the treatment phase. The change in the *n* values at 48, 72 and 96 weeks, indicates the children who were disenrolled due to treatment non-adherence and development of drug resistance.

The median %$CD4^+$ T-cell increased from 18% before antiretroviral treatment to 35% at 96 weeks (fig 14a). Six children (PARV 04, PARV 06, PARV 15, PARV 17, PARV 21 and PARV 30) although randomised to the treatment interruption group, did not have any treatment interruptions due to their viral loads being detectable at 24 weeks. They therefore received continuous treatment for the study period. Two of the six children were monitored until 96 weeks and together with the other four children they were referred and subsequently monitored on the National Roll-Out Program.

PARV 04, a five year old female child who had a 24% pre-HAART CD4% which increased to ≥30% up until 48 weeks on treatment. The pre-HAART viral load was log

4.76 which decreased to undetectable levels (log 2.09) at 12 weeks but was detectable (log 2.38) at 24 weeks therefore this child had to continue with the ARV treatment for another six months but the viral load was still detectable (log 2.97) at 48 weeks. Resistance testing was performed at this timepoint. The M184V mutation to 3TC had developed. This child also had a pre-HAART drug mutation G190EG to NNRTI.

PARV 06, a four year old male child, had a low CD4 count (8%) and a high viral load (log 5.5) before starting antiretroviral treatment. Despite this severe deterioration of the immune system, with the antiretroviral therapy, a good increase of CD4 count (18%) was noted at 12 weeks after starting antiretroviral treatment. Although the CD4% maintained ~20%, the viral load decreased to undetectable levels (log 2.09) at 12 weeks but was detectable (log_{10} = 4.20) and (log_{10}= 4.08) at 24 and 48 weeks respectively. It was discovered that this child had contracted another viral infection about the 18^{th} week on ARV treatment, and with a detectable viral load, scheduled treatment interruptions were not implemented. Resistance testing was performed and five new mutations (T215Y, M184V, M41L, D67N and K70R) had developed, because PARV 06 had no drug mutations prior to starting HAART.

PARV 15, an eight year old female child, had a CD4 count of 16% prior to starting HAART which increased to ≥26% during the 96 weeks of follow-up. The pre-HAART viral load was log 4.92 but reached undetectable levels at 12 weeks (log 2.09). At 24 weeks the viral load increased to log 3.15 and continued to increase at 26 weeks (log 3.26), 48 weeks (log 3.53) until 50 weeks (log 4.59). Resistance testing was performed and M184V mutation had developed, since PARV 15 had no drug mutations before starting HAART. However, at 72 weeks, the viral load reached undetectable levels (log 2.09) but was detectable again (log 4.98) at 96 weeks.

PARV 17, a two year old male child, had a CD4 count of 17% and a viral load of log 4.85 before starting HAART. His CD4 count increased to 26% at 12 weeks on ARV treatment but his viral load did not reach undetectable levels (log 3.80). At 24 weeks his CD4 count was 24% and his viral load increased to log 5.65. A confirmation test was

performed at 28 weeks and his viral load was log 5.77. Resistance testing indicated that there were 4 mutations (M184V, K219N, D67N and K70R) that developed, as PARV 17 did not have any drug mutations before starting HAART.

PARV 21, a three year old female child, had a CD4 count of 12% and a viral load of log 5.17 before starting HAART. Although her CD4 counts increased significantly until the end of the study period 96 weeks (≥24%), her viral load was undetectable at 12 weeks on treatment but increased thereafter throughout the rest of the study period. There were no drug mutations before starting HAART but three mutations (M184V, D67N and K70R) had developed after initiation of HAART.

PARV 30 had a CD4 count of 18% before starting HAART. Although the CD4 count fluctuated a 10% increase was noted after treatment commenced. The pre-HAART viral load was log 5.04; however the viral load did not reach undetectable levels after treatment had commenced. Resistance testing indicated that M184V and Y181C drug mutations were present after the treatment had started as there were no drug mutations present before starting HAART.

For most children (in this cohort) receiving continuous ARV therapy, % $CD4^+$ T-cells increased steadily over the study period from a median of 18% to 35% in 13/15 children (Figure 14a). Two subjects had < 25% $CD4^+$ T-cells even at 96 weeks of ARV therapy.
One of the children had a very low CD4% before starting HAART and the other was treatment non-adherent. ARV therapy suppressed viraemia to undetectable levels as early as 12 weeks post therapy and remained for the duration of the two-year study period in some children, whilst in some children undetectable levels were never obtained even after two years, due to drug resistant mutations arising. In other cases, children had suppressed viraemia, but there was a viral load rebound detected as a result of non-adherence resulting in drug resistant mutations

3.1.2.3 $CD4^+$ T-cell percentage and viral load estimation in the treatment interruption group

Figures 15a and 15b demonstrate the effect of structured treatment interruptions on $\%CD4^+$ T-cells and viral load over a two-year period in HIV-1 chronically infected children (n=8).

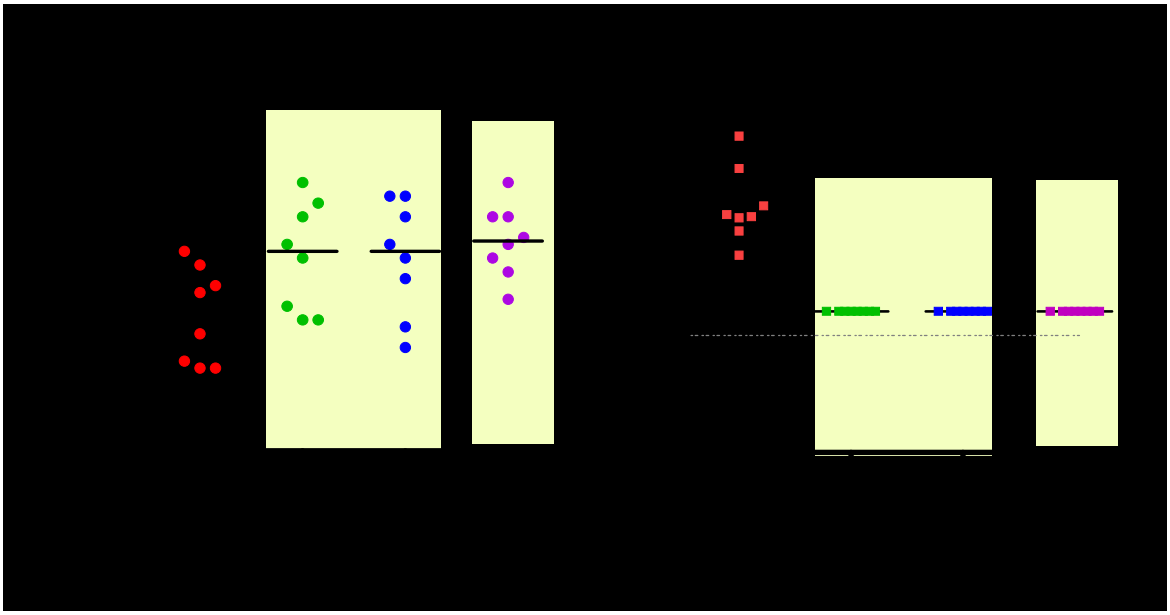


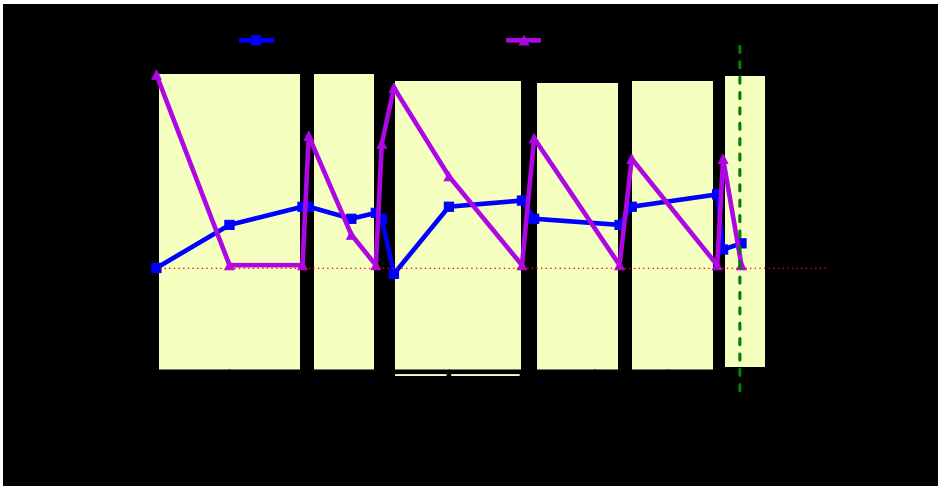
Figure 15. Comparison of the pre-treatment %CD4 (a) T-cells and log viral load (b) in relation to the %CD4 and log viral load over the two-year study period for the children randomized to the treatment interruption arm.

Although there were viral rebounds and small changes in the CD4 counts, overall at the end of the 2 year follow-up period, the CD4 counts and viral loads were consistent with good outcome. The CD4 count in the continuous treatment group was 18% at baseline and 35% at the end of the follow-up period and in the treatment interruption group it was 20% at baseline and 30% at the end of the follow-up period. The log viral load decreased from 4.7 in the continuous group and 4.3 in the treatment interruption group to undetectable levels at the end of the 2 year follow-up period.

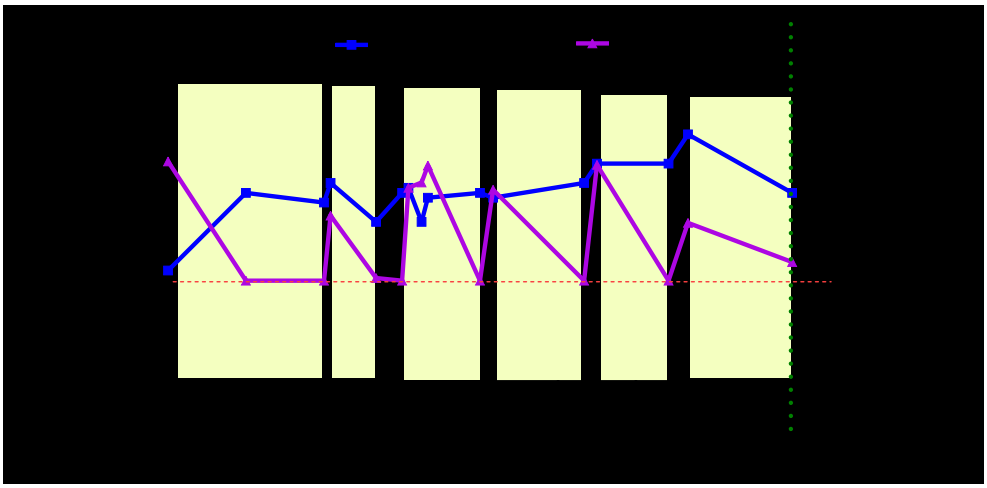
3.1.2.4 $CD4^+$ T-cell percentage and viral load estimation in the eight children who had treatment interruptions

Eight of the fifteen children who were randomized to the treatment interruption group had successful treatment interruptions. The interruptions were of a short duration (~ a week) for 5 children and varied (2-4 weeks) for the other 3 children. Figure 16 shows the longitudinal data for the eight children who had treatment interruptions.

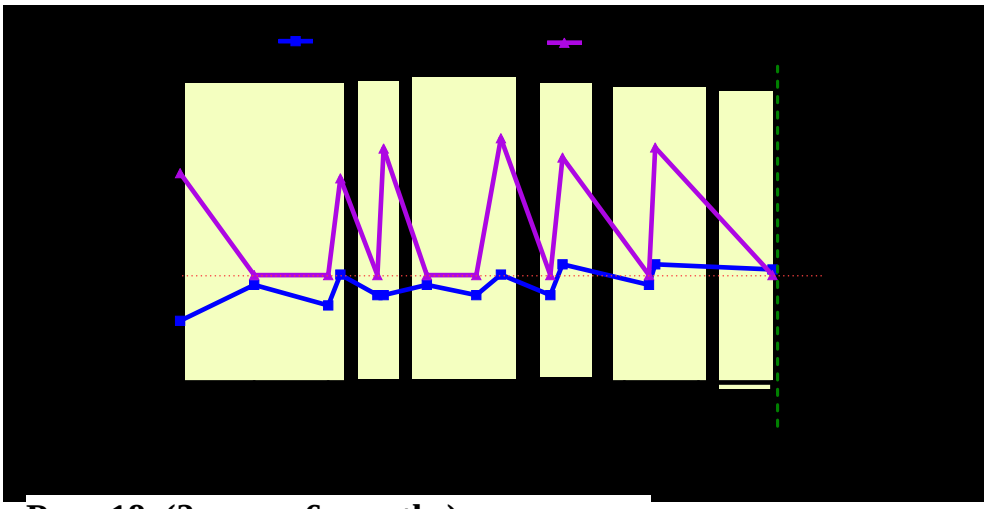
(16a) **Parv 03 (2 years, 7 months)**



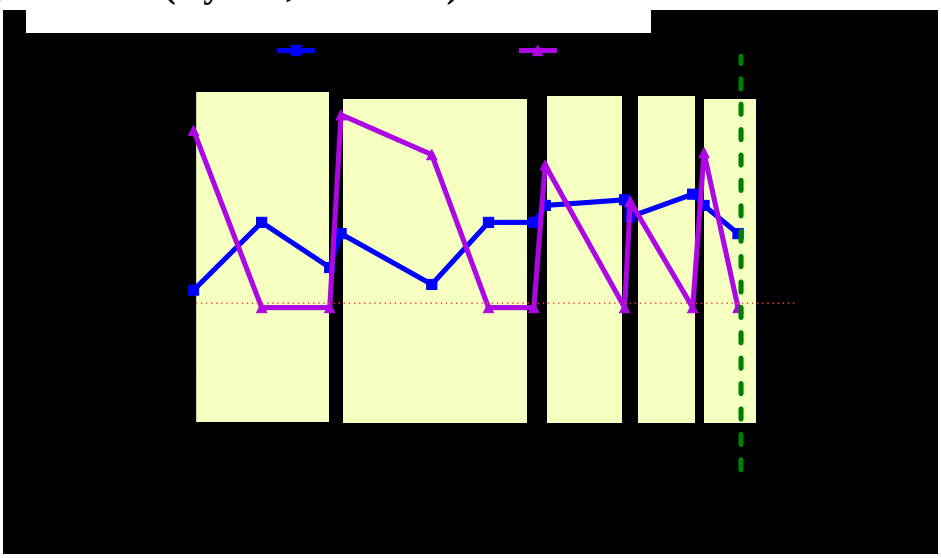
(16b) **Parv 05 (2 years)**



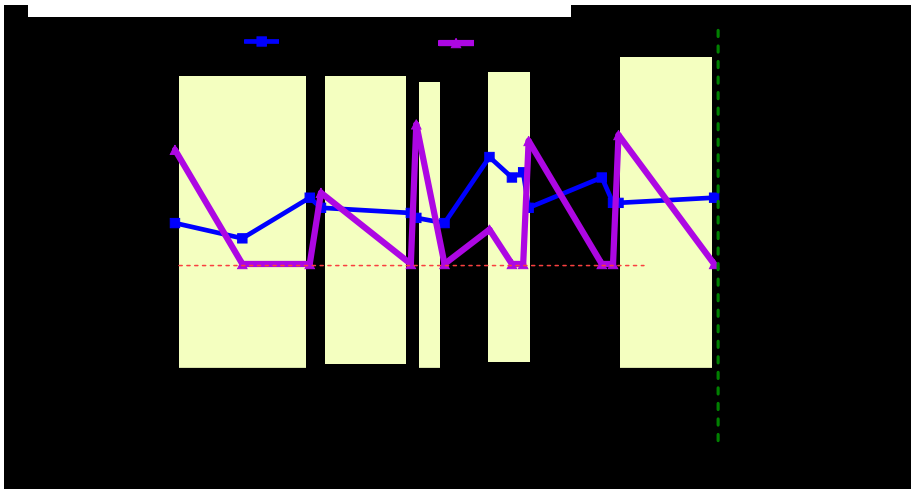
(16c) **Parv 14 (6 years)**



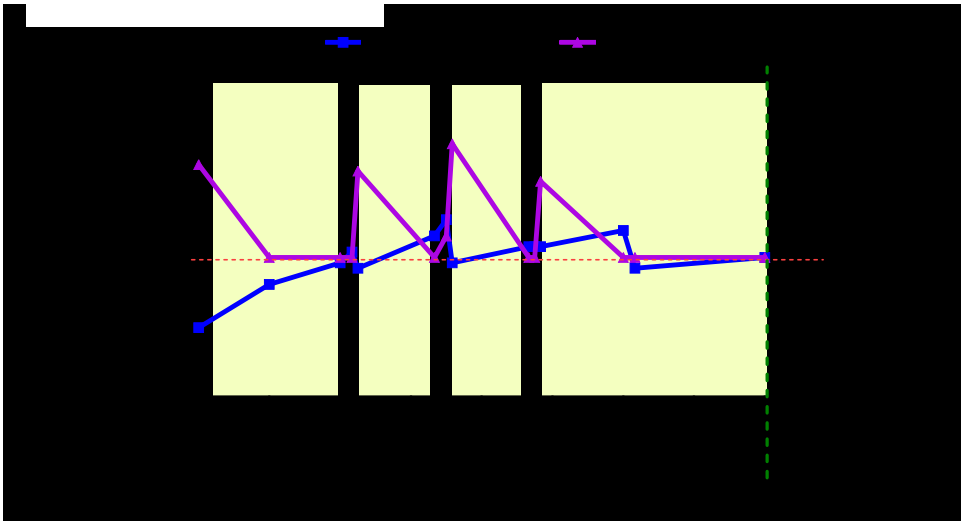
(16d) **Parv 18 (2 years, 6 months)**



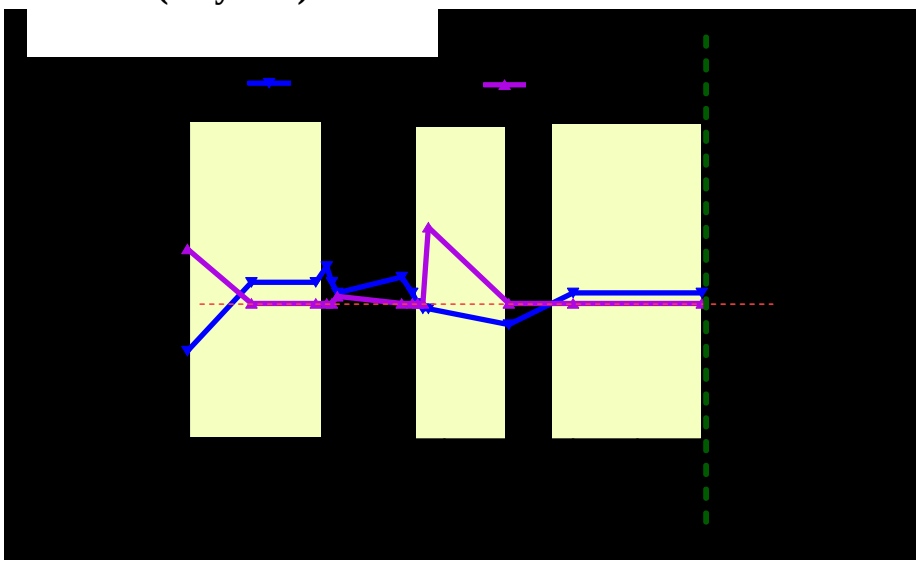
(16e) **Parv 19 (4 years)**



(16f) **Parv 26 (5 years)**



(16g) **Parv 28 (12 years)**



(16h) **Parv 29 (8 years)**

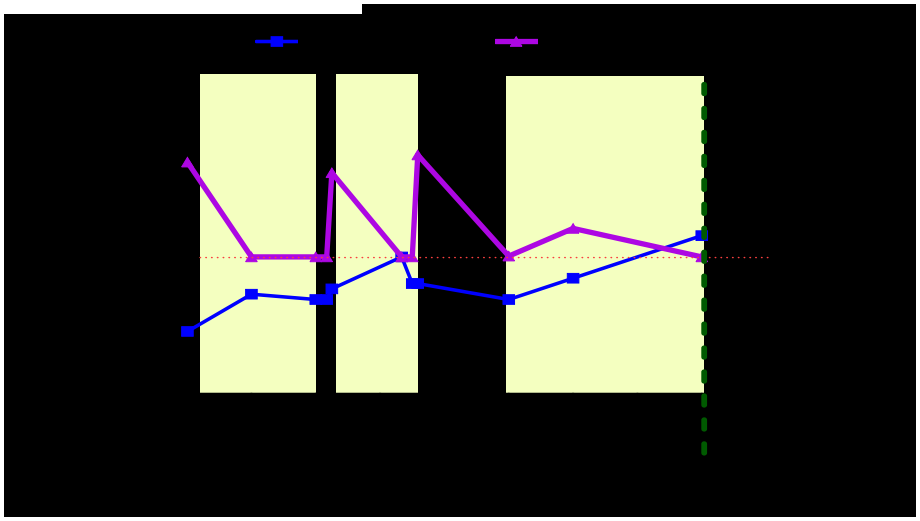


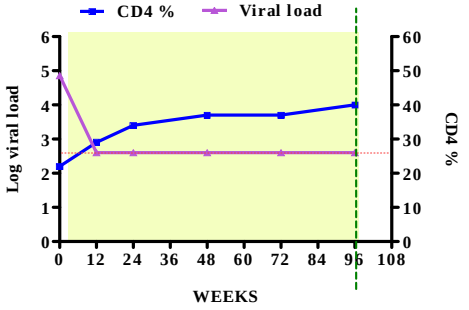
Figure 16. Longitudinal CD4% and log viral load of the eight children who had treatment interruptions. (a) PARV 03 (b) PARV 05 (c) PARV 14 (d) PARV 18 (e) PARV 19 (f) PARV 26 (g) PARV 28 and (h) PARV 29. The yellow background indicates the treatment phase. The age of the child represented, is the age when enrolled to the study at 0 weeks.

PARV 03, PARV 05 and PARV 14 had 5 treatment interruptions. PARV 18 and PARV 19 had 4 treatment interruptions. PARV 26 had 3 and PARV 28 and PARV 29 had 2 treatment interruptions. A steady increase was seen in the %CD4 T-cells for all eight children however, the viral rebounds were almost to baseline levels during the treatment interruptions.

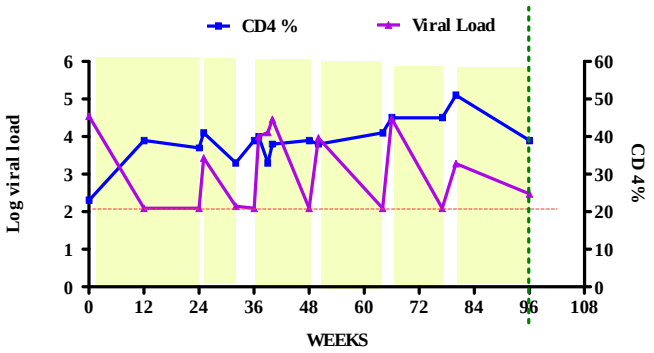
3.1.2.5 Different CD4$^+$ T-cell percentage and viral load estimation in the study cohort

Figures 17a-d show examples of individual patients, from the continuous and structured treatment interruption arms, and the effects on the %CD4 as well as the log viral load, over the two study period.

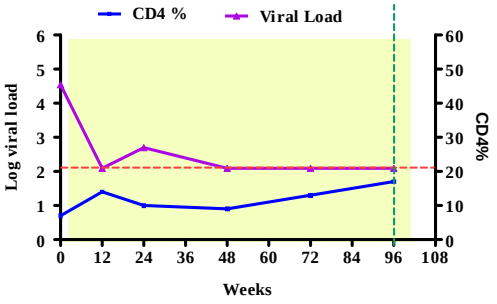
(17a)



(17b)



(17c)



(17d)

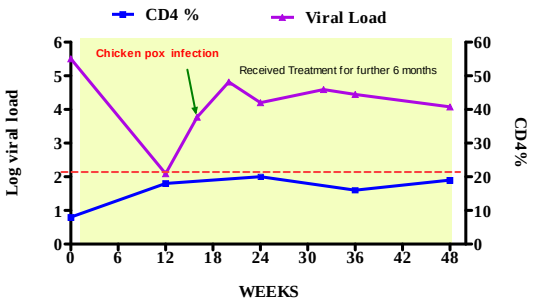


Figure 17a. The CD4% and Log Viral load of PARV 27 (11 years), who was randomized to the continuous treatment group. The CD4% increased once treatment commenced (from 0 weeks) and steadily continued to rise throughout the study. The log viral load decreased from log 4.8 to an undetectable level and maintained undetectable levels throughout the study.

Figure 17b. In comparison to PARV 27, PARV 05 (2 years, 1 month) was randomized to the treatment interruption group and had five successful treatment interruptions. The CD4% increased once treatment commenced and despite the treatment interruptions, the CD4% did not reach pre-treatment levels. A steady increase throughout the study was noted. The log viral load decreased to an undetectable level by 12 weeks and was maintained at 24 weeks. The log viral load rebounded to detectable levels during the interruption periods hence treatment recommenced within a week of the interruptions for three months. The yellow indicates treatment phase.

Figure 17c. **The CD4% and Log Viral load of PARV 08 (4 years, 2 months)**. Although the Log viral load decreased and maintained undetectable levels by twelve weeks on antiretroviral therapy, there was a viral blip at 24 weeks. This was due to a Lower Respiratory Tract Infection (LRTI). This child had a low CD4 count (7%) at the pre-HAART visit. Although the CD4% increased very slowly to 17% at 96 weeks, this child maintained undetectable levels of viremia with antiretroviral therapy.

Figure 17d. **The CD4% and Log Viral load of PARV 06 (4 years, 2 months).** This child was randomized to the Treatment Interruption group but did not have treatment interruptions. Although the viral load was undetectable at 12 weeks, when tested at 16, 20 and 24 weeks, the viral load was detectable. During this time it was established that this child had a chicken pox infection. Subsequently the viral load was still detectable at 48 weeks and resistance testing was carried out and the results indicated that resistance had developed. Therefore it was decided by the study clinician and the Principal Investigators that it would in the best interest of the child to dis-enroll the child from the study and as the National Roll-Out Programme had just started it would be good to start the child with the regime that included a Protease Inhibitor.

In this study there were no differences between the continuous and treatment interrupted groups with regards to the CD4 counts and viral load estimations at the end of the 2 year follow-up period. In addition there were no adverse outcomes in either of the groups in terms of death or other clinical outcomes.

3.2 ELISPOT T-CELL IMMUNE RESPONSES

3.2.1 T-cell immune responses before HAART

The enzyme-linked immunospot (ELISPOT) is a relatively simple assay for the assessment of pathogen-specific T-cell immune responses. The ELISPOT assay can detect virus-specific T-cells secreting cytokines at a single cell level (Czerkinsky *et al*, 1998 and Lalvani *et al*, 1997). Interferon-gamma (IFN-g) is the cytokine most frequently used to detect the antigen-specificity of specific $CD8^+$ T-cells. Inter-leukin-2, another cytokine which is also secreted by CTL T-lymphocytes, is secreted at a much lower frequency compared to the secretion of IFN-g (Letsch and Scheibenbogen, 2003).

Comprehensive screening ELISPOT assays detecting IFN-g secretion have been used to evaluate the magnitude, breadth and specificity of HIV-specific responses in several cohorts of HIV-infected individuals (Currier *et al*, 2002; Addo *et al*, 2003; Feeney *et al*, 2003a; Kiepiela *et al*, 2004 and Novitsky *et al*, 2002).

Comprehensive screening using pooled peptides (arranged in a matrix format) which spanned the entire HIV-1 proteome was followed by confirmation of individual overlapping peptides (OLPs), if a positive pool was detected. The optimal peptides are shorter in length (shorter in amino acid) as compared to the OLP length and found within the sequence of the OLP. The optimal peptides were tested based on the individual's HLA type (Appendix 18).

Figure 18 shows the percentage frequencies of the HIV proteins targeted by twenty two of the chronically HIV-1 infected children in this study who were screened prior to taking antiretroviral therapy.

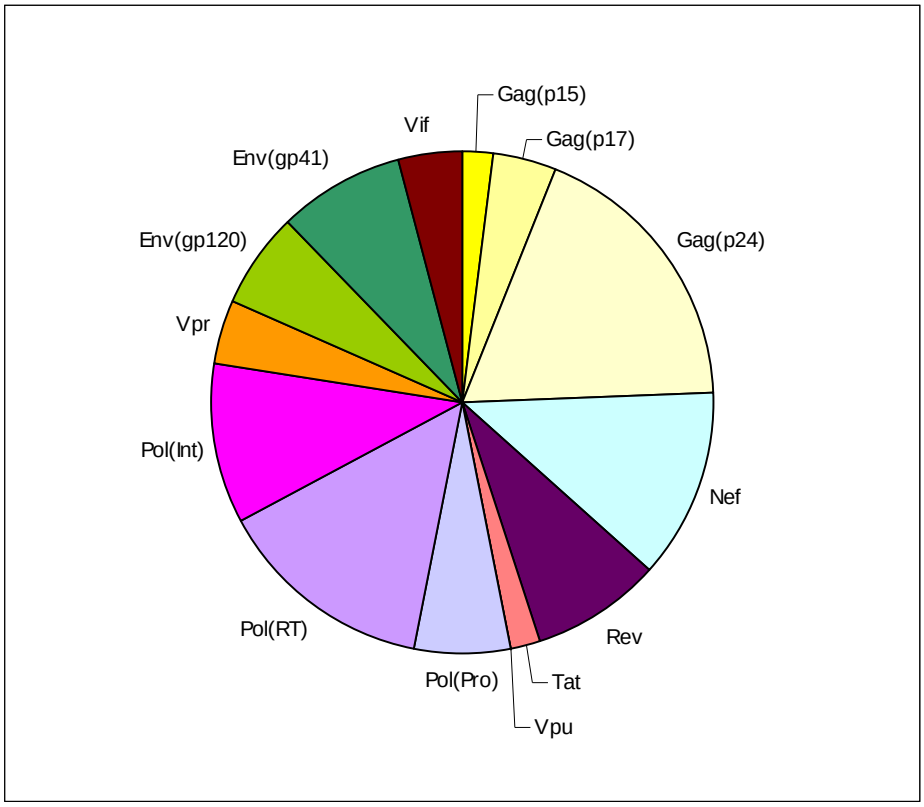


Figure 18. The percentage frequencies of the individual proteins targeted within the HIV-1 proteome prior to starting treatment in 22 chronically HIV-1 infected children.

In this cohort, the Pol HIV-1 protein was most frequently targeted (30%) followed by the total Gag HIV-1 protein (24%), Env HIV-1 protein (14%), Nef HIV-1 protein (12%), Rev HIV-1 protein (8%), Vif and Vpr HIV-1 proteins (4%) each and the least or not targeted HIV-1 proteins were from Tat and Vpu with 2% and 0% respectively.

The Pol and Gag HIV-1 proteins were most frequently targeted in this chronic HIV-infected paediatric cohort. Hence the frequencies of the individual subunits indicated that the p24 region was the most dominant subunit of the Gag HIV-1 protein (18%) as compared to the p15 and p17 regions which were less frequently targeted (2%) and (4%) respectively. The Pol (RT) region had a frequency of 14% followed by Pol (Int) and Pol (Pro) with (10%) and (6%) respectively.

Fifteen of twenty two (68.2%) children screened at baseline (prior to antiretroviral therapy), responded to at least one peptide in the p24 Gag region. The Gag p24 was the most frequently recognized subunit in the protein. Three children (13.6%) responded to the Gag (p17) subunit and only one (0.5%) child responded to the Gag (p15) subunit.

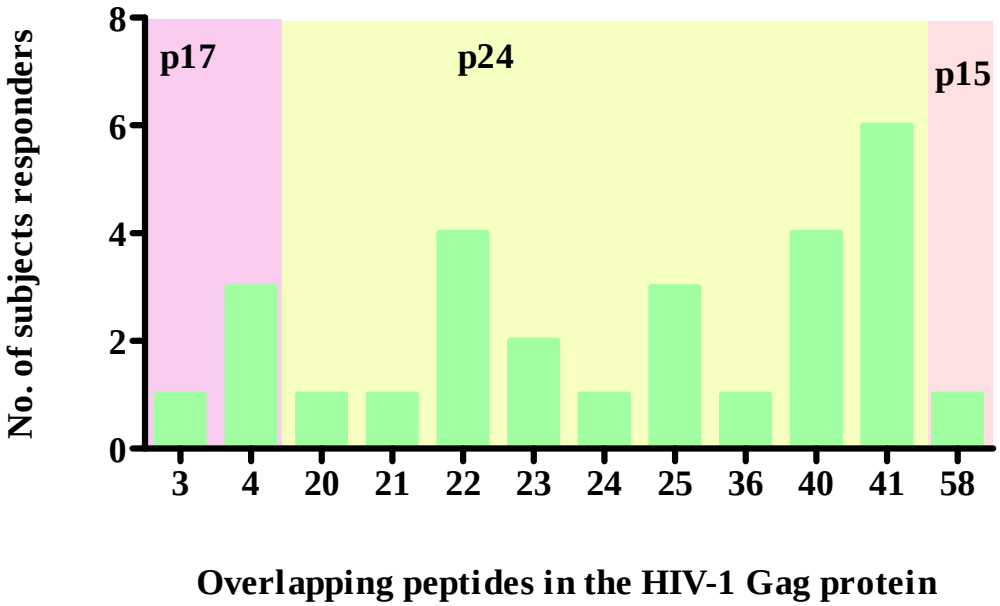
Figure 19a highlights the number of children who responded to the overlapping peptides (OLPs) in the Gag (p15), Gag (p17) and Gag (p24) subunits. OLP #41 (YVDRFFKTLRAEQATQDV) dominated the detectable responses by six children. OLP #41 has been associated with T-helper responses (Ramduth *et al*, 2009). These 6 children also had a positive $CD4^+$ T-cell response when tested with the pool of Gag peptides by the Intracellular Cytokine Staining (ICS) assay. Four children responded to OLP #22 (WVKVIEEKAFSPEVIPMF) and #40 (GPKEPFRDYVDRFFKTLR).

Figure 19b shows that only 6 peptides were targeted within the HIV-1 Env protein. OLPs #293 (VMGNLWVTVYYGVPVWK) and #325 (IRSENLTNNAKTIIVHL) are from the Env gp120 subunit. OLP #362 (IVQQQSNLLRAIEAQQHM), #394 (QRGWEALKYLGSLVQYWGL), #395 (LGSLVQYGWLELKKSAVSLL) and #396 (ELKKSAVSLLDTIAIAVA) are from the gp41 subunit of the Env protein.

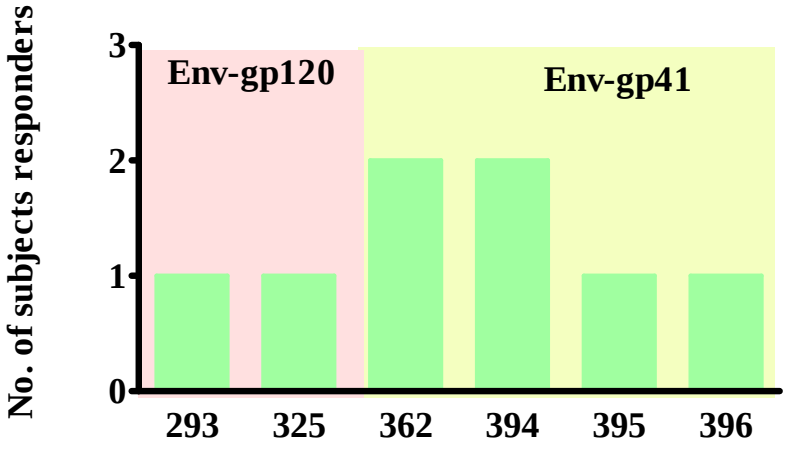
In figure 19c, OLP #281 (ELKQEAVRHPFRPWLHGL) and #283 (GLGQYIYETYGDTWTGV) were targeted from the Vpr protein and OLP #411 (LQTGERDWHLGHGVSIEW) and #412 (HLGHGVSIEWRLRRY) were targeted from the Vif protein. These accessory proteins were less frequently targeted in this HIV-1 chronically infected cohort. Overall 15 peptides were targeted within the HIV-1 Pol protein as shown in figure 19d.

Eleven OLP were targeted within the regulatory HIV-1 proteins. Six children targeted OLP# 84 (NYTPGPGVRYPLTFGWCF) from the Nef protein as seen in figure 19e. The Rev and Tat peptides were less frequently targeted.

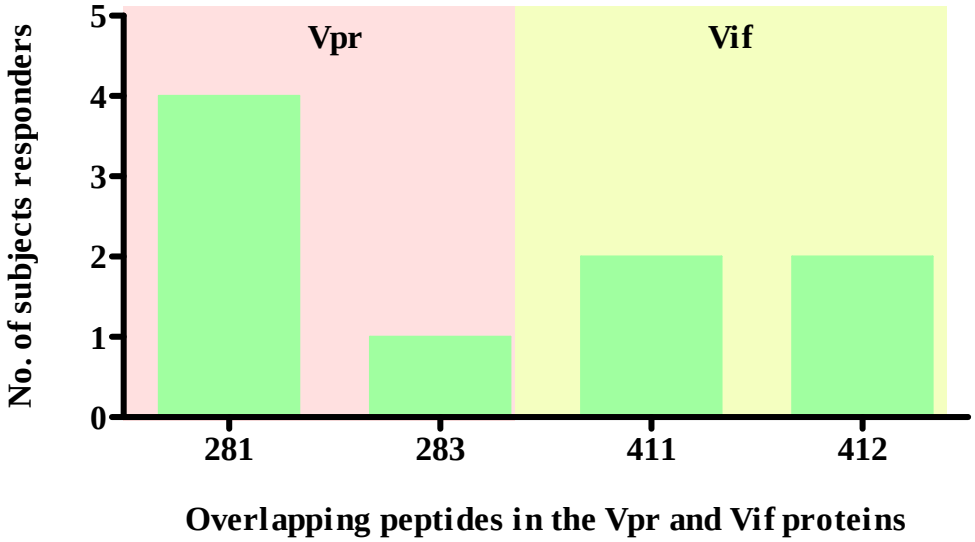
(19a)


No. of subjects responders
8
6
4
2
0
p17
p24
p15
3
4
20
21
22
23
24
25
36
40
41
58
Overlapping peptides in the HIV-1 Gag protein

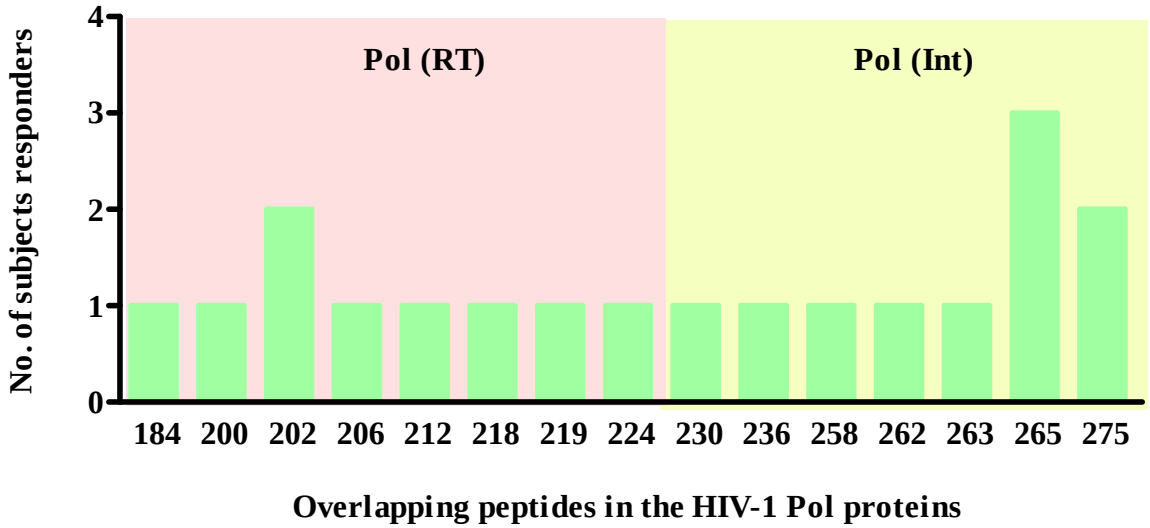
(19b)


No. of subjects responders
3
2
1
0
Env-gp120
Env-gp41
293
325
362
394
395
396
Overlapping peptides in the HIV-1 Env protein

(19c)


Vpr
Vif
No. of subjects responders
0
1
2
3
4
5
281
283
411
412
Overlapping peptides in the Vpr and Vif proteins

(19d)


Pol (RT)
Pol (Int)
No. of subjects responders
0
1
2
3
4
184
200
202
206
212
218
219
224
230
236
258
262
263
265
275
Overlapping peptides in the HIV-1 Pol proteins

(19e)

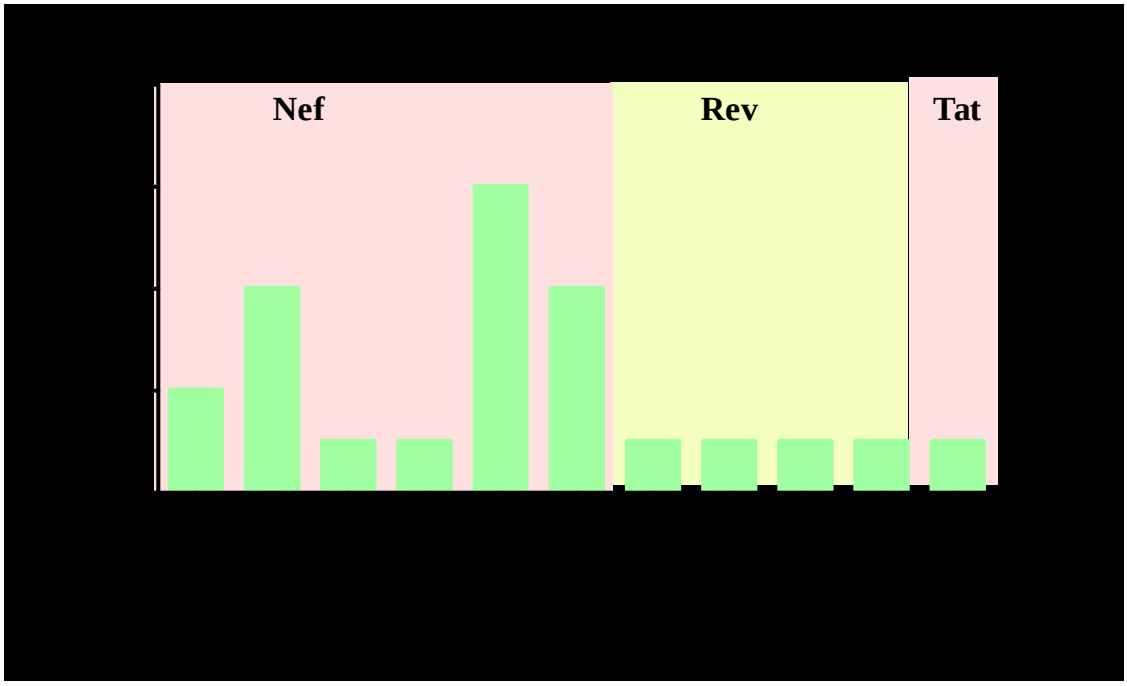


Figure 19. The number of children who responded to overlapping peptides in the HIV-1 genome: a) Gag (p15), Gag (p17) and Gag (p24) protein. b) Env (gp120) and Env (gp41) protein. c) Vpr and Vif protein. d) Pol (RT) and Pol (Int) protein. e) Nef, Rev and Tat protein.

HIV-specific $CD4^+$ and $CD8^+$ T-cells play an important role in the control of HIV-1 infection. Studies have failed to show a correlation between the magnitude and breadth of HIV-specific IFN-γ CTL responses with viral load (Addo *et al*, 2003, Buseyne *et al*, 2005; Ogg *et al*, 1998 and Lacabratz-Porret *et al*, 2003). Figures 20a & 20b depict the 22 children that were screened for CTL responses and HIV-1 RNA plasma viral load before starting antiretroviral treatment. Figure 20a shows that there was no significant correlation between the magnitude of CTL immune response and the HIV-1 RNA plasma viral load. The Pearson's correlation test calculated the $r^2 = 0.02805$ and $p = 0.4563$. Figure 20b shows that there was no significant correlation between the number of OLPs targeted from all the HIV-1 proteins and the HIV-1 RNA plasma viral load. The Pearson's correlation test calculated the $r^2 = 0.02395$ and $p = 0.4916$.

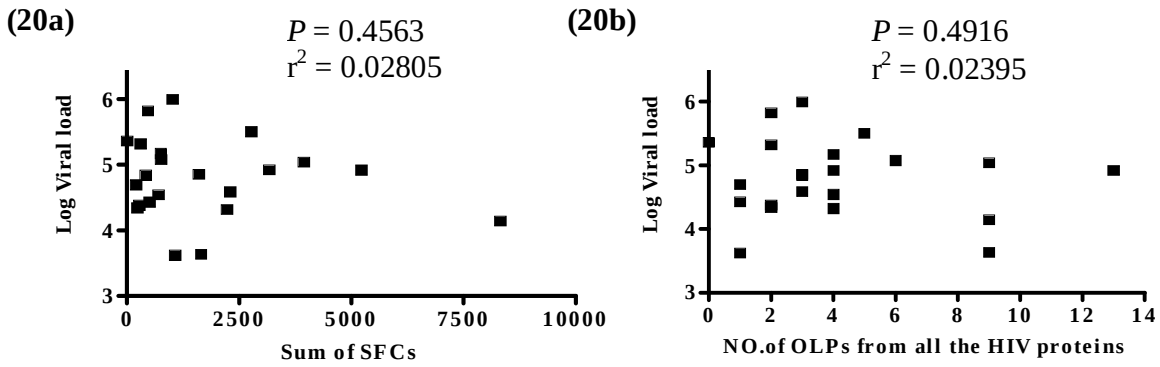


Figure 20: a) Shows the magnitude and b) the breadth of the CTL Immune responses for the twenty two chronically HIV-1 infected children screened before starting antiretroviral therapy. These results are consistent with those from other studies (Lacabaratz-Porret *et al,* 2003 and Plana *et al,* 2004) which also found no significant correlation between viral load and the total magnitude of HIV-1 specific responses before starting HAART.

3.2.2 T-cell immune responses in the continuous treatment group

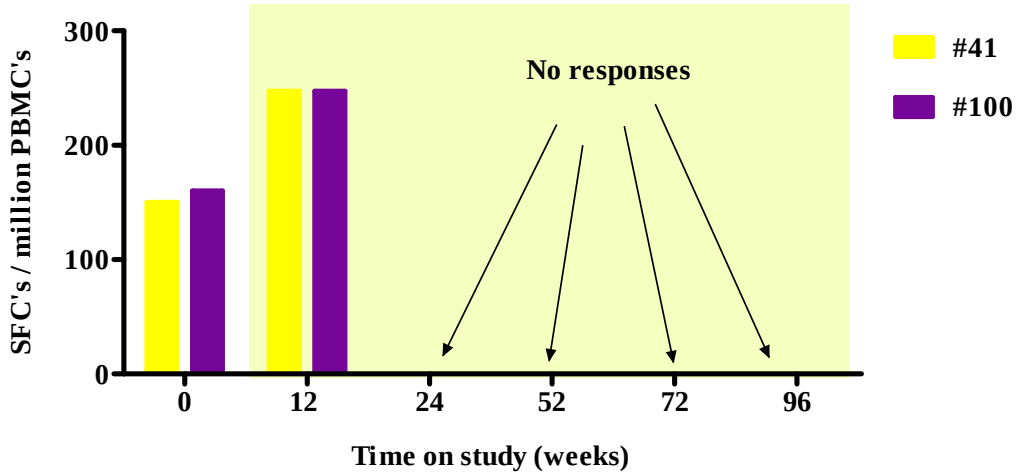
Fifteen children were randomized to the continuous treatment arm of the study. The CTL HIV-specific immune responses waned and disappeared in only one child (6.7%) (PARV 09) whose viral load level reached undetectable limits during HAART. The optimal immune response was detected at 24 weeks on HAART and disappeared thereafter. The HLA type for PARV 09 was A4301/6802 B15/15 Cw03/1801. Although the B-alleles were typed and reported as two digits alleles, the responses suggest that B1503 and B1510 were the optimal B-alleles.

Figures 21a and 21b indicate the overlapping peptides and optimal peptides respectively to which PARV 09 responded before starting HAART (0 weeks) and when treatment commenced. The yellow block indicates commencement and duration of treatment. A

response to the sequence of OLP #41 Gag (p24) and the sequence of OLP #100 (Rev) was made before starting HAART as well as at 12 weeks while on treatment. There were no detectable responses at the subsequent visits.

The optimal epitopes B1510-IL9 in Rev, Cw03-YL9 in Gag (p24) and Cw18-ER15 in Gag (p24) were detected at 12 and 24 weeks on HAART and then disappeared. Optimal epitopes B1503-FY10 in Tat, B1503-VI10 in Pol (Int) and B1510-IL9 also in Pol (Int) were also detected at 24 weeks on HAART.

(21a)



(21b)

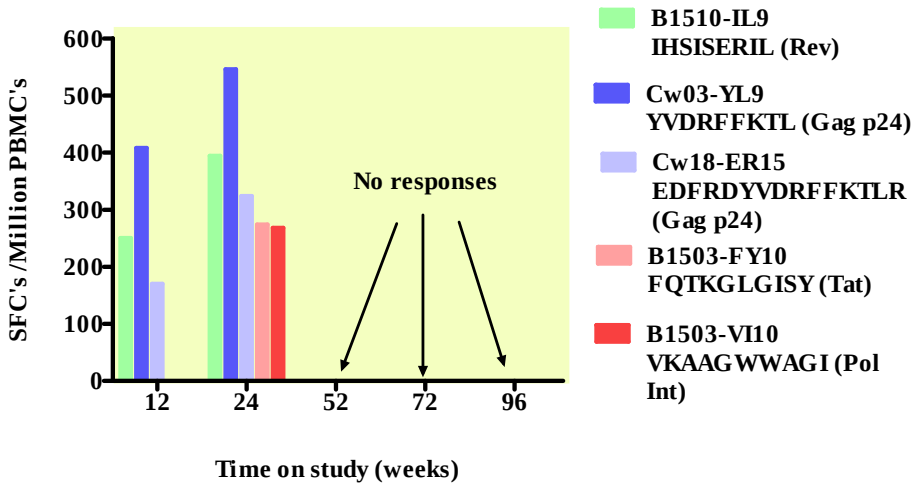


Figure 21. (a) The OLP response increased marginally but disappeared over time on HAART. (b) The Optimal peptide response increased as well as new epitopes targeted but disappeared over time on HAART. The background indicates the treatment phase.

PARV 13 had CTL responses and detectable viral loads from baseline until the 48 week follow-up visit. Due to treatment non-adherence, treatment in this child was stopped. Although this child was disenrolled after the 48 week follow-up visit, this child was clinically managed by the study clinician.

PARV 16 had no CTL HIV-specific immune responses before and during HAART, except for optimal immune responses Cw02-AY15-ULAACWWAGIQQEFGIPY (Pol-Int) and Cw18-ER15-EDFRDYVDRFFKTLR (Gag p24) at the 12th week follow-up visit.

Twelve of 15 children (80%) in the continuous treatment group of the study had CTL HIV-specific immune responses before and during HAART. Seven of these 12 children (58.3%) had undetectable viral loads while the remaining 5 children (46.7%) had varying detectable viral load levels. Overall all 12 children in the continuous treatment group targeted almost all of the HIV proteins (Gag, Pol, Nef, Rev, Tat, Vif, Env and Vpr) during HAART with the exception of the Vpu HIV-1 protein. Table 4 depicts the 12 children who had varying CTL immune responses during HAART. Due to the insufficient number of PBMC's, the ELISPOT assay was not performed at baseline (0 weeks) for PARV 02, PARB 08, PARV 24 and PARV 25. However, the first available immune responses were shown. The CTL immune responses are shown before and/or during HAART. Overall broadening of the CTL immune responses were seen in a majority of the children although they were on HAART.

Table 4: CTL immune responses to HIV-1 proteins before and/or during HAART

		Gag	Pol	Nef	Env	Rev	Tat	Vif	Vpr	Vpu
PARV 02	8 weeks	+	+	+	+	+				
	96 weeks	+	+	+	+	+	+		+	
PARV 07	0 weeks	+		+					+	
	96 weeks		+	+					+	
PARV 08	2 weeks			+						
	96 weeks	+	+	+						
PARV 10	0 weeks	+	+	+		+		+		
	96 weeks	+	+	+	+	+		+		
PARV 11	0 weeks		+							
	96 weeks		+				+	+		
PARV 12	0 weeks		+	+					+	
	96 weeks		+	+	+				+	
PARV 20	0 weeks	+				+		+		
	72 weeks	+	+				+	+		
PARV 22	0 weeks		+							
	96 weeks		+							
PARV 23	0 weeks	+								
	96 weeks	+						+		
PARV 24	24 weeks		+	+						
	96 weeks									
PARV 25	12 weeks		+							
	96 weeks	+	+				+			
PARV 27	0 weeks		+	+						
	96 weeks		+	+	+		+			

3.2.3 T-cell immune responses in the treatment interruption group

The rationale for structured treatment interruptions (STIs) is based on immune stimulation in the form of auto-vaccination as a result of re-exposure to HIV during the viral rebound. The hypothesis forming the basis of STI in patients with primary infection is that the preserved immunity could control the viral replication once the treatment had stopped. For patients with chronic infection, the treatment interruptions may allow the stimulation of T-helper and cytotoxic T-helper (CTL) response (Bongiovanni *et al*, 2006). The HIV-1 $CD8^+$ T-cell responses can be detected in children, however they tend to be much weaker than that of adults (Chandwani *et al*, 2004).

The CTL responses of the eight children who had treatment interruptions are demonstrated in Figure 22. Figures 22(a-d) show the responses of the children who had five treatment interruption cycles, figure 22(e & g) and figure 22(f & h) had two and four treatment interruption cycles respectively. As can be seen from figures 22(a-d, f) at certain STI cycles the ELISPOT could not be performed due to insufficiency of PBMC availability. The difference in the number of STI cycles was due to the criteria of undertaking an STI cycle with respect to undetectability of viral load for 3 months (12 weeks) in individual patients before commencement of an STI.

Seven of these eight children (Figure 22 a-c & e-h) responded to the Gag protein as well as to Nef, Pol, Vpr and Env proteins within the HIV-1 proteome. Three of these seven children, (Figure 22a-c), on availability of PBMC's responded only to the Gag protein. PARV 05 (Figure 22a), the Gag response was detectable before starting HAART and although the assay was not performed for the first three treatment interruptions due to insufficient cells to perform the assay, the response was still clearly seen at the STI 4 (~3000 SFCs) and a weaker response at STI 5. PARV 18 (Figure 22c) reflected similar results. In this patient the Gag response was seen from the second STI as the assay was not performed prior to this timepoint due to insufficient cells to perform the assay. PARV 14 (Figure 22b) had no response before starting HAART and was not tested during the first and second treatment interruptions due to

insufficient cells however a response (~280 SFCs) was made at the STI 4. There was no response made during STI 5.

PARV 03 (Figure 22d) was the only child who was randomized to the treatment interruption arm who did not make a response to the Gag protein. However a Nef and a very weak Pol response were made prior to starting HAART. Due to insufficient cells at STI 1 and STI 2, the assay was not performed. A weak Pol response was still evident at STI 3 with no responses at STI 4. At the STI 5, a weak Nef and a strong Pol (~ 3000 SFCs) were made.

In three patients ELISPOTs were able to be set up at baseline and all STI time points eg. PARV 29 (Figure 22e), PARV 28 (figure 22g) and PARV 26 (figure 22h). A weak immune response targeting Gag and Pol proteins was detected prior to starting antiretroviral therapy and in addition to the subsequent STIs, a Nef response was made in PARV 09 (figure 22e). Stimulation of CTL response was clearly depicted at the last treatment interruption.

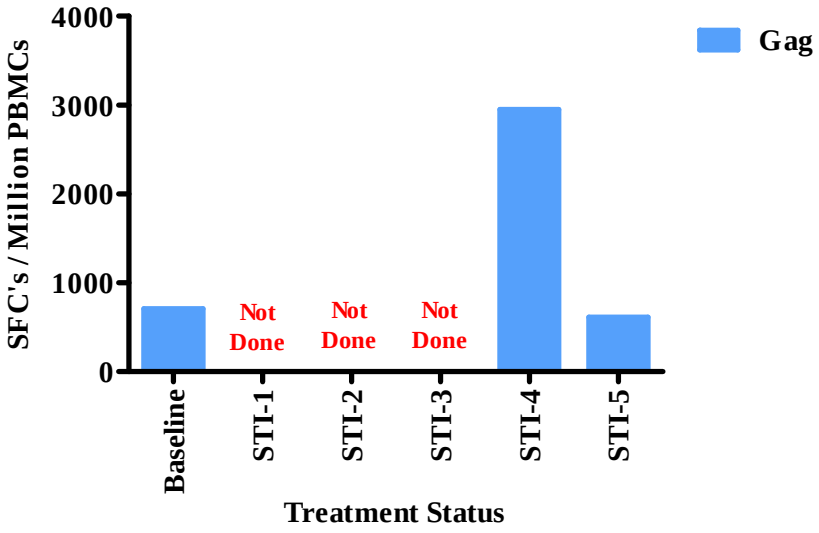
PARV 19 (Figure 22f) had CTL responses to Gag, Pol and Nef at baseline, lost the Gag and Nef responses at STI 2, had no CTL responses at STI 3 while CTL responses to Gag, Nef and Pol re-appeared at STI 4 at a higher magnitude compared to the baseline CTL responses to the same three HIV proteins.

PARV 28 (figure 22g) had three main responses i.e. Gag, Pol and Vpr at all time points with Gag being the highest magnitude. The Env response is lost at the STI 1 and Nef at the STI 2.

The most consistent CTL responses in PARV 26 at all time points were Gag and Vpr with Nef and Env CTL responses being detected only at baseline.

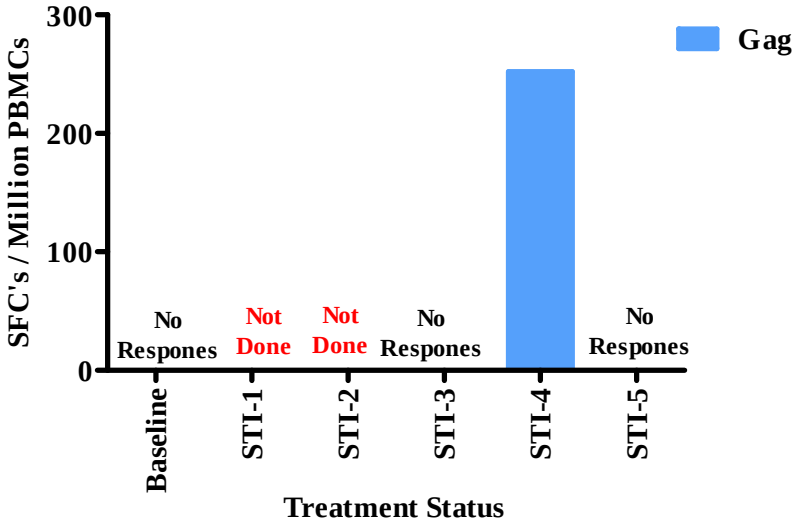
PARV 05 (2 years 1month)

(22a)



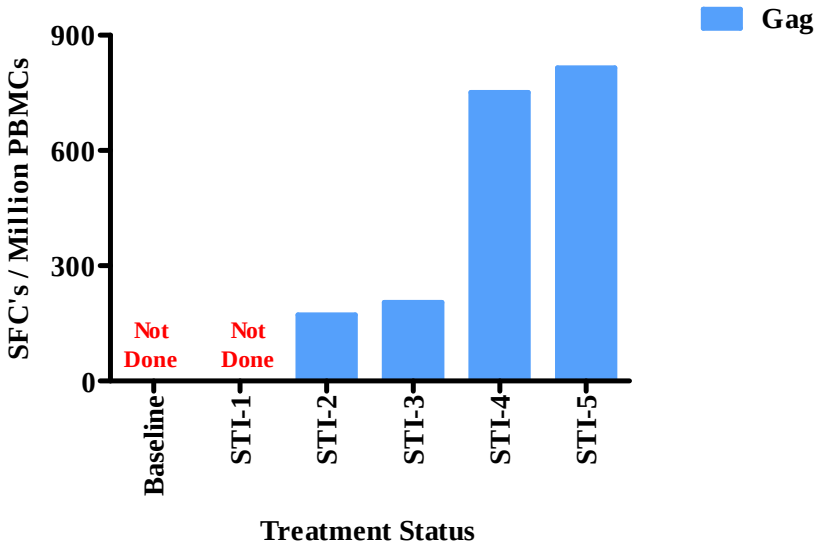
PARV 14 (6 years)

(22b)



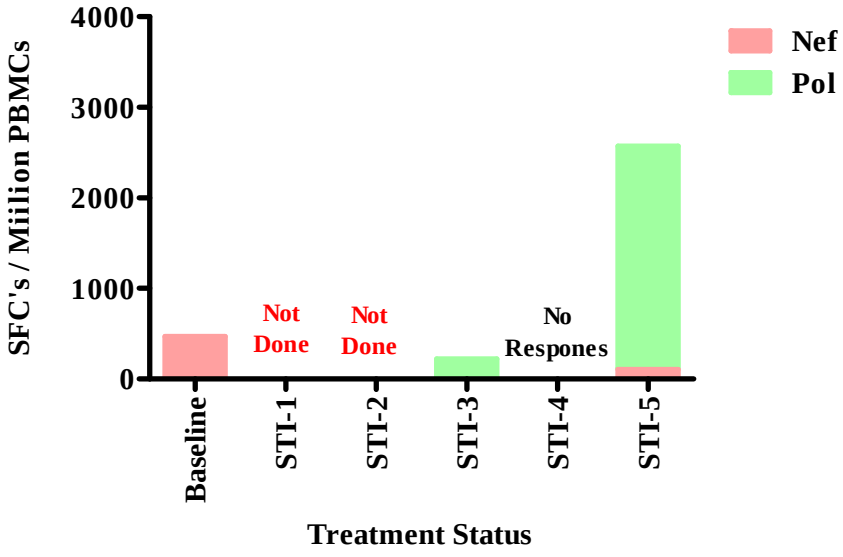
PARV 18 (2 years 6 months)

(22c)



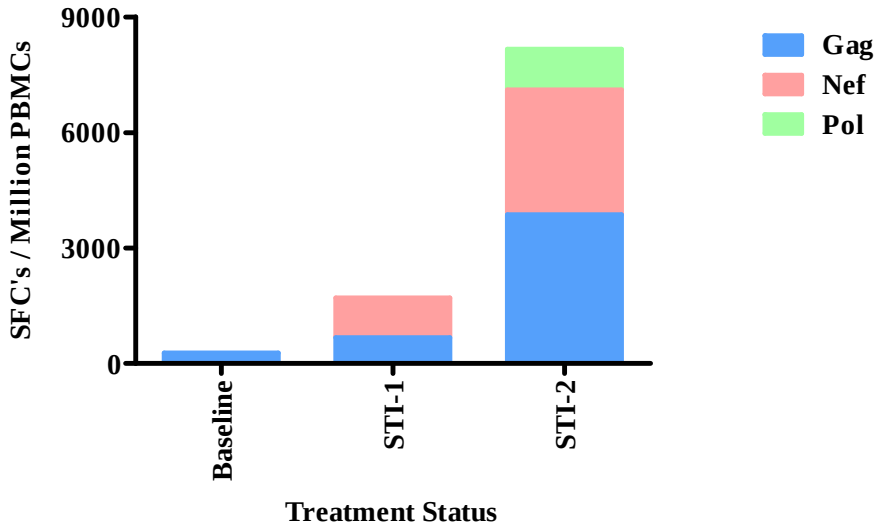
PARV 03 (2 years 7 months)

(22d)



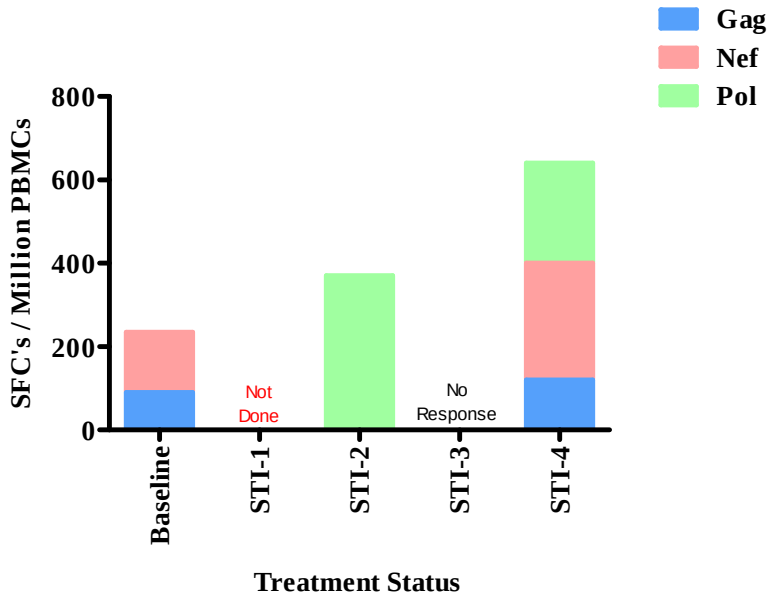
PARV 29 (8years)

(22e)



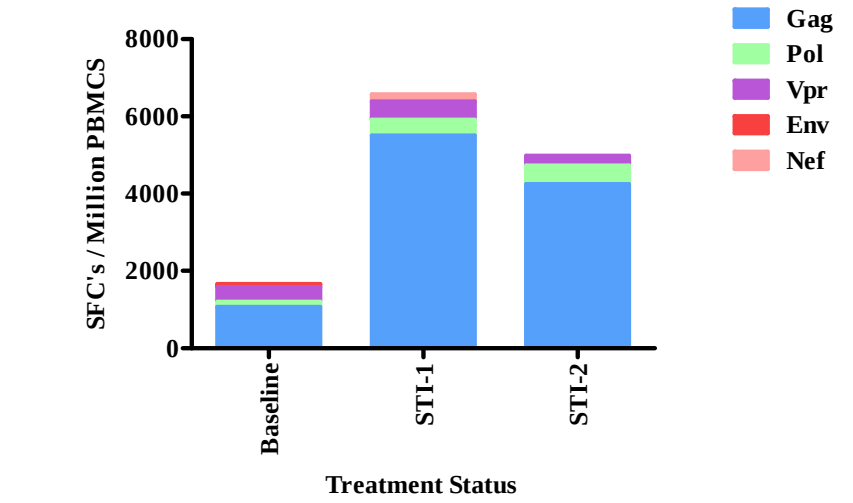
PARV 19 (4 years)

(22f)



PARV28 (12 years)

(22g)



PARV 26 (5 years)

(22h)

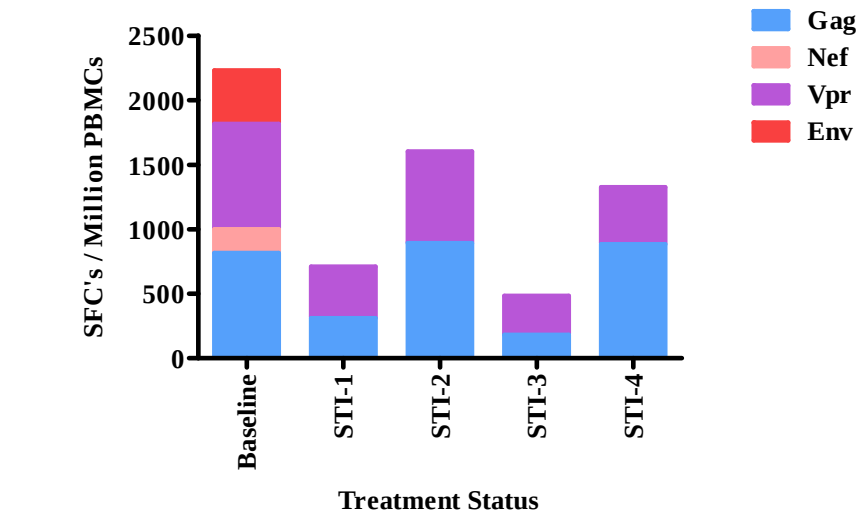


Figure 22. Demonstrates the protein responses in the eight children who had treatment interruptions. Fig. 22(a-d) shows the subjects who had five treatment interruption cycles, Fig. 22(e & g) and Fig. 22(f & h) had two and four treatment interruption cycles respectively.

3.3 INTRACELLULAR CYTOKINE STAINING (ICS)

The virus-specific $CD8^+$ T-cell response in acute HIV-1 infection is low in magnitude and is directed against a limited number of epitopes (Dalod *et al,* 1999). However during chronic HIV-1 infection, a vigorous immune response, which is due to the broadening of antigen stimulation, occurs (Streeck *et al,* 2007).

The quality and specificity of the initial $CD8^+$ T-cell responses may be associated with control of the initial viral replication (Pantaleo *et al,* 2004). Several studies have shown that $CD8^+$ T-cell responses mainly targeted against HIV-1 Gag are associated with low viral loads (Streeck *et al,* 2007; Gray *et al,* 1999; Kiepiela *et al,* 2007). In this study, virus-specific immune responses were tested in twenty-two of twenty-nine chronically infected children using intracellular cytokine staining (ICS). Responses were tested against the HIV-1 Gag protein since Gag is the most conserved region within the HIV-1 genome and as it has previously been shown that immune responses against Gag are important in the control of viremia.

3.3.1 IFN-γ ICS in children before starting HAART

Although all 29 children were tested during the study period, only 7 children (24%) were tested prior to starting HAART (figure 23). This was due to the insufficient amount of PBMC's obtained for the test as other tests were also undertaken in order of priority. Table 5 shows the seven children in their respective randomized treatment group. Comparison of Gag specific T-cell responses using ELISPOT and the ICS assays revealed that in six of the seven children (85.7%) there was at least one response to an OLP in the Gag region using the ELISPOT assay and this corresponded to a positive $CD8^+$ and $CD4^+$ T-cell response by the ICS assay. Only one child did not make a response to a HIV-1 Gag OLP in the ELISPOT assay and this was in keeping with the negative response to the $CD8^+$ and $CD4^+$ T-cell on the ICS assay. Hence there was a correlation between the two assays as far as Gag specific $CD8^+$ T-cell responses were concerned. This is depicted in Table 5.

Table 5. Comparison of ELISPOT and ICS T-cell responses in the seven children that had sufficient PBMC's for ICS testing before starting HAART. The ELISPOT Gag overlapping peptides and the ICS Gag pool of peptides are highlighted.

	Randomization Group	**Gag #OLP Response: ELISPOT**	**Gag pool $CD8^+$ Response: ICS**	**Gag pool $CD4^+$ Response: ICS**
PARV 09	Continuous Treatment	YVDRFFKTLRAEQATQDV	+	+
PARV 19	Treatment Interruption	GKVSQNYPIVQNLQGOMV	+	+
PARV 20	Continuous Treatment	WVKVIEEKAFSPEVIPMF GPKEPFRDYVDRFFKTLR AFSPEVIPMFTALSEGA	+	+
PARV 21	Treatment Interruption	YVDRFFKTLRAEQATQDV GPKEPFRDYVDRFFKTLR	+	+
PARV 23	Continuous Treatment	WVKVIEEKAFSPEVIPMF	+	+
PARV 24	Continuous Treatment	None	-	-
PARV 30	Treatment Interruption	PVGDIYKRWIILGLNKIV	+	+

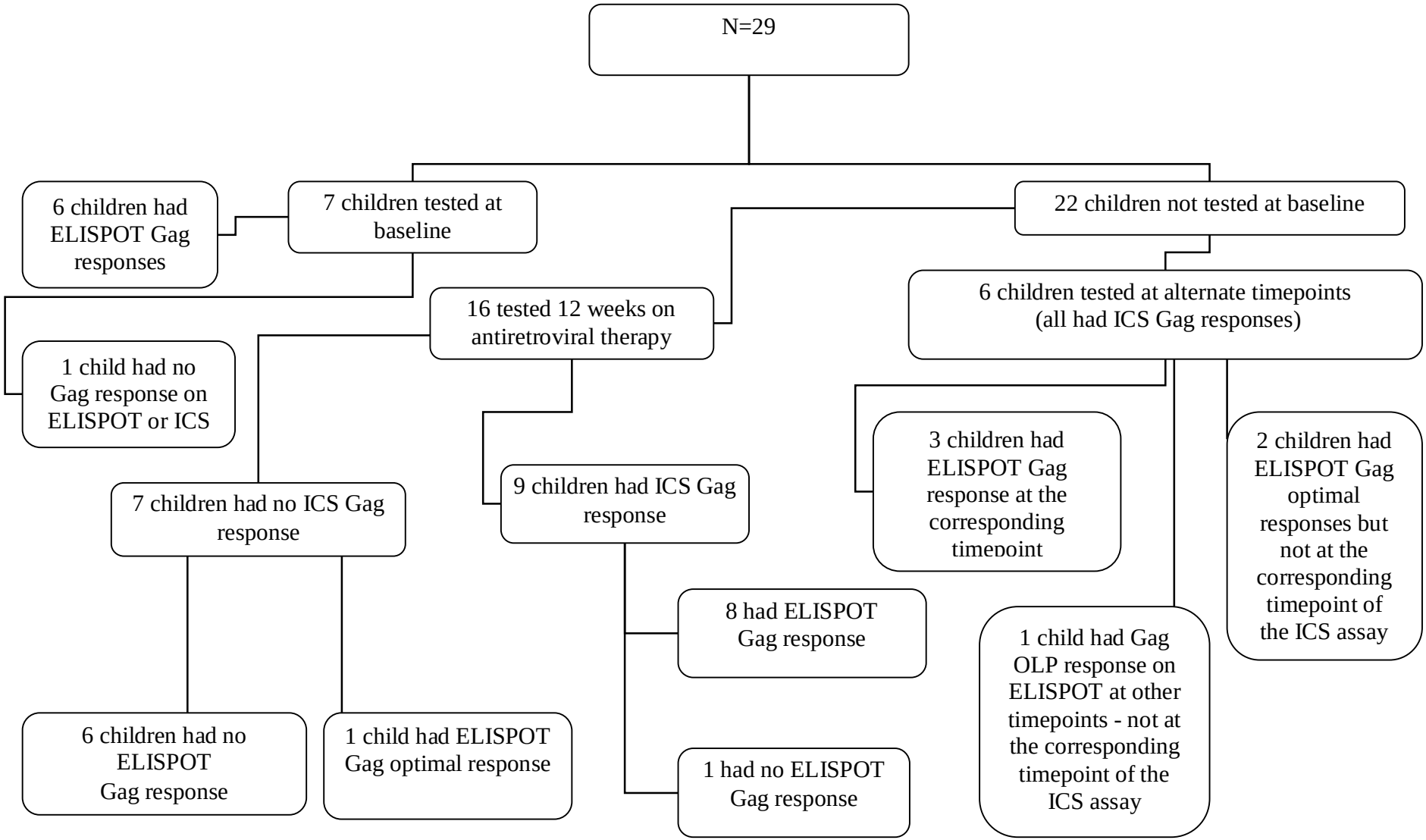


Figure 23. A schematic representation of the Intracellular Cytokine Staining and comparison to the ELISPOT tests in twenty-nine children.

3.3.2 $CD8^+$ IFN-γ ELISPOT and ICS

PBMCs from twenty-two children were tested by intracellular cytokine staining (ICS) during the study (figure 23). Sixteen of 22 (72.7%) children were tested at the 12 weeks timepoint (on treatment) whilst the remaining six children were tested at random timepoints whenever PBMC's were available.

Nine of sixteen children (56.2%) tested at 12 weeks, had a positive $CD8^+$ and $CD4^+$ T-cell response for the ICS assay, while 8/9 children had a gag response on the ELISPOT assay. A possible reason for the one child not having a gag response on the ELISPOT assay could be due to the low magnitude of the ELISPOT response (below the cut off point of detection as the limit of the assay). We have previously found that ELISPOT responses > 500 SFC's will be detectable by ICS (Ramduth *et al*, 2005).

In the rest of the seven of 16 children at the 12 weeks timepoint there was a correlation between the two assays whereby no Gag specific responses were detected in 6/7 children while in the remaining one child (PARV 06) there was a Gag specific response to optimal peptides A02-SL9-SLYNTVATL and B44-SL9-SEGATPQDL with a negative ICS response.

All six children tested at alternate timepoints during the study had a positive Gag response for the ICS assay, while only three of the children had Gag specific ELISPOT response at the corresponding timepoint. The other three children did not have both assays done simultaneously due to insufficient PBMC's despite making Gag specific responses by one of the assays. Hence the responses obtained cannot be compared.

A comparison of the ICS (Gag pool of peptides) between the pre-HAART and the 48 weeks on antiretroviral therapy samples was undertaken (Figure 24). As can been seen in Figure 24, there was no difference in Gag-specific $CD8^+$ T-cell response before or 48 weeks after antiretroviral treatment was started. Likewise, no difference in $CD4^+$ T-cell Gag-specific responses was seen before or 48 weeks after antiretroviral treatment was started.

3.3.3 Comparison of $CD8^+$ IFN-γ ICS before starting HAART to 48 weeks on HAART

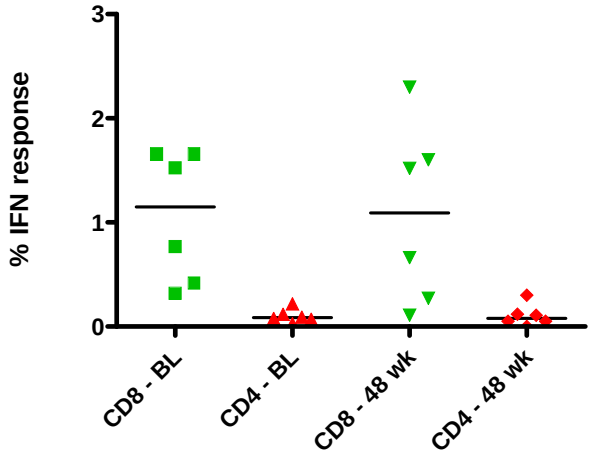


Figure 24. $CD8^+$ and $CD4^+$ T-cell responses in seven children who were tested at baseline (before starting HAART) and at 48 weeks on HAART.

The median %IFN-g response at baseline was (1.15%) and (0.09) for the $CD8^+$ and $CD4^+$ T-cell respectively. The median % IFN-g response at 48 weeks was (1.09%) and (0.08) for the $CD8^+$ and $CD4^+$ T-cell respectively. The benefits of HAART have been clearly documented in several clinical studies. Despite the control of viral replication and increases in the $CD4^+$ T-cells, HAART does not eradicate the virus and it does not reconstitute the HIV-specific immune responses in the infected individual (Finzi *et al*, 1999 and Pitcher *et al*, 1999).

Intracellular cytokine staining (ICS) of PBMCs was undertaken at different timepoints, on one of the children (PARV 09) who was randomized to the continuous treatment group of the study. IFN-g production by Gag specific $CD8^+$ T-cells, at the pre-HAART timepoint was 0.23% (figure 25c); 0.075% at 52 weeks (Figure 26c) and 0.13% at 72 weeks of antiretroviral treatment (Figure 27d). IFN-g production by Gag specific $CD4^+$ T-cells, at the pre-HAART timepoint was 0.085% (figure 25f); 0.06% at 52 weeks (figure 26f) and 0.068% at 72 weeks of antiretroviral treatment (figure 27j).

The decrease in the production levels of IFN-g Gag-specific $CD8^+$ T-cells was evident after the initiation of HAART in this child, as the viral load decreased to levels below the detection limits. However, there was little difference in Gag-specific $CD4^+$ T-cell responses before or during antiretroviral treatment

HIV-specific $CD4^+$ T-cell response has shown to be absent or severely impaired in the majority of HIV-1 chronic infections (Shedlock *et al*, 2003). This generally leads to the waning of $CD8^+$T-cell responses that require help from the $CD4^+$ T-cells.

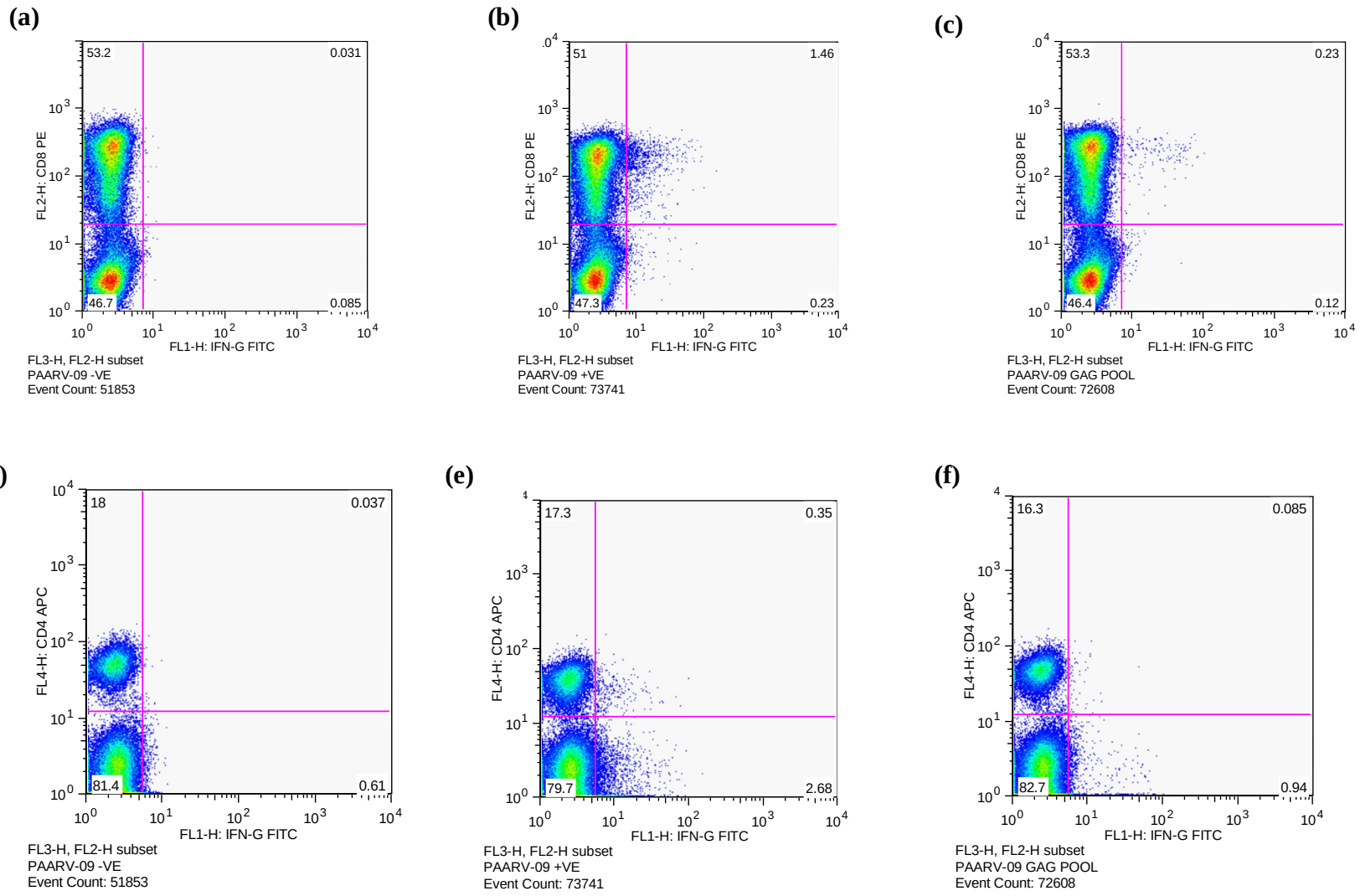


Figure 25. ICS for Parv 09 at baseline (pre-HAART)

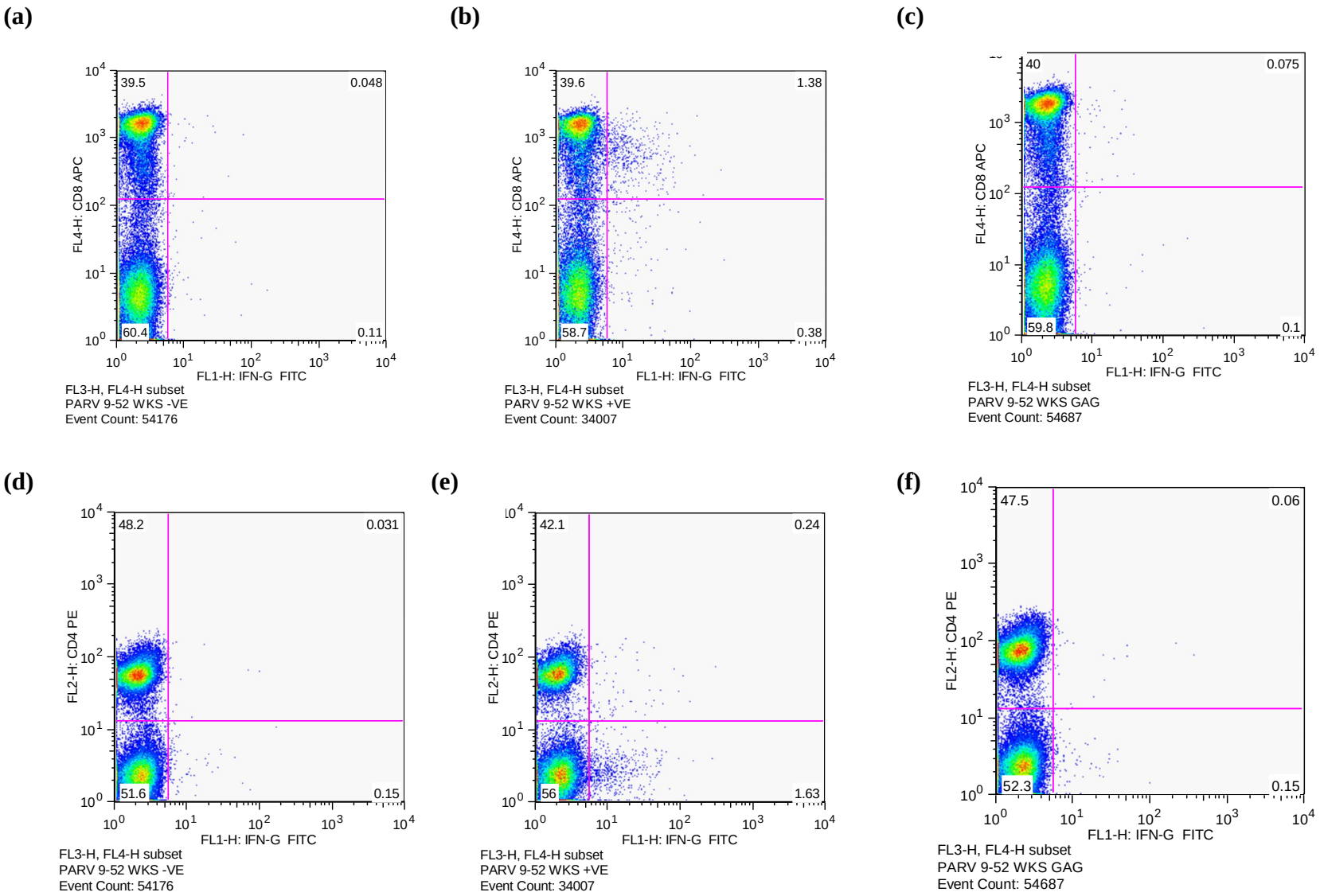


Figure 26. ICS for Parv 09 at 52 weeks on treatment

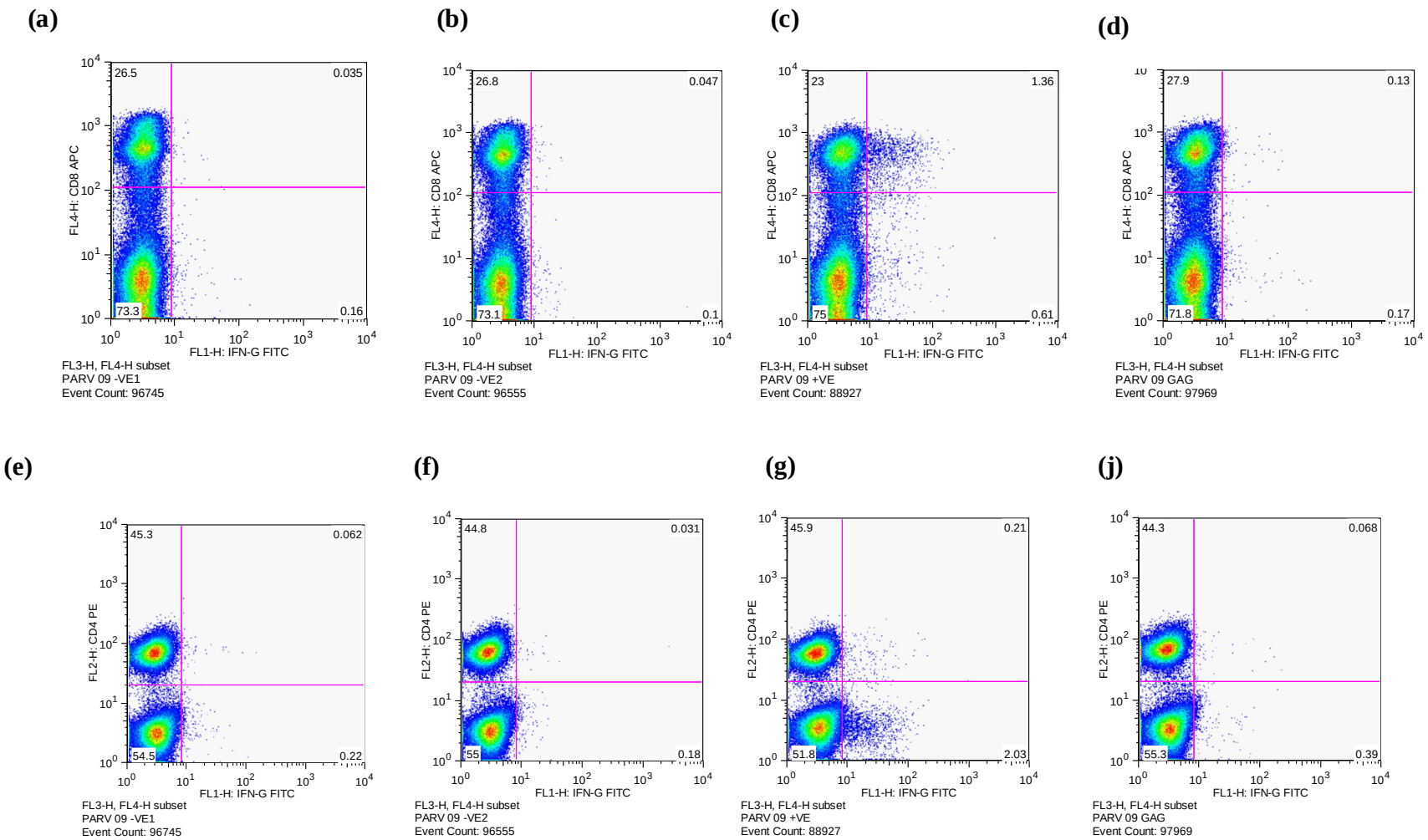


Figure 27. ICS for Parv 09 at 72 weeks of treatment

3.4 HIV-1 DRUG MUTATIONS AND RESISTANCE

Cytotoxic T-lymphocyte cell (CTL) responses develop and persist during HIV-1 infection in therapy naïve subjects. The virus however evades immune surveillance and this is due to CTL escape mutations (Allen *et al,* 2000; Goulder *et al,* 2001). Resistance to antiretroviral drugs in previously untreated HIV-infected people is a growing problem with public health implications (Oette *et al,* 2004). Transmitted or acquired resistance prior to a combination of HAART may compromise the effectiveness of nevirapine (NVP) or other NNRTI based HAART regimes in particular when NVP is provided for the prevention of mother-to-child transmission (MTCT). HIV-1 drug resistant mutations detected during structured treatment interruptions have generally been selected by agents with relatively prolonged intracellular half-lives and for which a single-base pair change in HIV-1 confers high-level drug resistance (Oette *et al,* 2004).

3.4.1 Drug Resistant Mutations before starting HAART

Thirty children (fig 7) were treated with a combination of three nucleoside reverse transcriptase inhibitors (NRTI). These were AZT, ABC and 3TC. Resistance testing was performed on frozen plasma samples for all children prior to starting antiretroviral treatment. Drug mutations to NRTIs and NNRTIs were tested. Three children (PARV 03, PARV 05 and PARV 18) were reported to have had single-dose Nevirapine (NVP) at birth. Twenty-six of thirty children had no drug resistance mutations before starting HAART. Table 6 indicates the four of thirty children who had at least one resistant mutation to NRTIs and/or NNRTIs prior to starting HAART. There was no significant difference with regards to viral loads with the four children who had pre-HAART drug mutations as compared to the other 26 children who did not have a pre-HAART drug resistant mutation. PARV 02 and PARV 18 had one NRTI resistant mutation each and PARV 04, PARV 14 and PARV 18 had one NNRTI mutation each. It is possible that these children were infected with this resistant strain of HIV-1. PARV 18 received a single dose NVP at birth and it is likely that this children developed resistance to the NVP as the mutation was detected at all time points tested and not after initiating HAART.

Table 6. Drug resistance mutations in four children prior to starting HAART

		DRUG MUTATIONS	
No.	**Sample ID**	**NRTI**	**NNRTI**
1	PARV 02 [a]	M41L	None
2	PARV 04 [b]	None	G190EG
3	PARV 14 [b]	None	V179D
4	PARV 18 [b]	T69N	Y181C

[a] randomized to the continuous treatment group
[b] randomized to the treatment interruption group

Only one child (PARV 02) had a baseline drug mutation (M41L) to the NRTI regime. Once antiretroviral treatment commenced, the viral load was undetectable up until the last visit at 96 weeks. It was reported that this child had defaulted with the treatment program hence allowed for a viral rebound to occur. A confirmation test was done a month later after the child had restarted antiretroviral treatment. The viral load decreased significantly (data is not shown).

Four of fifteen children (PARV 11, PARV 12, PARV 16 and PARV 20), who were randomized to the continuous treatment group, developed drug mutations after starting the triple NRTI combination. Interestingly these 4 children were not the same that had the pre-HAART drug resistant mutations. Although the viral loads decreased for each child, it did not reach undetectable levels. The M184V drug mutation (to the 3TC ARV drug) was the only mutation that was evident in all of these four children.

Three of the fifteen children randomized to the treatment interruption group, had a drug mutation prior to starting HAART (table 6). The viral load for PARV 04 had reached undetectable levels at 12 weeks (on treatment) but thereafter continued to increase despite the ARV treatment. Due to detectable viral load levels at 24 weeks, this child did not have a treatment interruption, hence continued with the treatment program. Although PARV 14 and

PARV 18 had drug mutations prior to starting HAART, the viral loads were undetectable at 24 weeks, hence they had five and four treatment interruptions respectively. PARV 14 developed resistance to 3TC and the drug mutation M184V was detected after the first treatment interruption. Although the viral loads were undetectable, the M184V and V179D mutations were evident at successive timepoints (26, 33, 52, 62 and 77 weeks). PARV 18 had two drug mutations prior to starting HAART. However, this did not affect the four treatment interruptions this child had due to the undetectable viral loads. The mutations were present at all timepoints (26, 42, 62, 77 and 90 weeks) tested.

3.4.2 Drug Resistant Mutations after initiating HAART

PARV 13 was forced to stop the treatment program. Due to social-economic problems, this child was non-adherent to the ARV treatment. This however would have resulted in serious implications of developing drug resistance. PARV 13 was monitored by the clinician and social-welfare.

Fourteen of the twenty nine children (48.3%) who were monitored longitudinally, developed drug resistant mutations. Interestingly as table 7 indicates, 4/14 children (28.6%) developed drug resistant mutations whilst their viral loads were undetectable and they also underwent treatment interruptions.

Three of 4 children (75%) developed only the M184V drug resistant mutation. Treatment interruption did not have an affect on the acquisition of this mutation as PARV 12 and PARV 15 had continuous treatment and still acquired the M184V mutation. The D67N, K70R as well as the M184V drug resistant mutations were common in seven children (PARV 06, PARV 11, PARV 16, PARV 17, PARV 20, PARV 21 AND PARV 30)

Table 7. Children who developed drug resistant mutations during the study

No.	PATIENT ID	NRTI MUTATIONS	NNRTI MUTATIONS
1	PARV 03[C]	M184V	
2	PARV 04	M184V	G190EG
3	PARV 05[C]	M184V, K65R	
4	PARV 06	M184V, M41L, D67N, K70R, T215Y, K219EKQ	
5	PARV 11	M184V, D67N, K70R	
6	PARV 12	M184V	
7	PARV 14[C]	M184V	V179D
8	PARV 15	M184V	
9	PARV 16	M184V, D67N, K70R, Y115FY, T215IT, K219EQ, G333E	
10	PARV 17	M184V, D67N, K70R, K219N	
11	PARV 20	M184V, D67N, K70R, T215IV, K219Q	
12	PARV 21	M184V, D67N, K70R	
13	PARV 29[C]	M184V	
14	PARV 30	M184V, D67N, K70R	

[C] Had treatment interruptions

3.4.3 3TC Drug Resistance Mutation

The M184V mutation confers high-level resistance to 3TC and this was the most dominant mutation in this cohort. Although this mutation is associated with a significant reduction in the HIV's ability to replicate, it has been hypothesized that continuation of 3TC despite documented resistance, could provide an overall benefit (Castagna *et al*, 2006). Resistance to 3TC is associated with the substitution of isoleucine for methionine at position 184 of HIV-1 RT region, which results in a single base pair change (ATG–ATA). The M184I variant is rapidly replaced by the variant M184V during 3TC therapy (Sarafiano *et al*, 1999).

Fourteen of the thirty children (46.7%) enrolled in this study developed the M184V mutation (10 children received continuous treatment while 4 children had treatment interruptions). There was no difference in the response to the treatment in the 14 children who developed the M184V mutation during their treatment as compared to those children who did not develop the M184V mutation. This mutation was not present in any of the thirty children before starting HAART as resistance testing was performed on pre-HAART samples (table 7).

CHAPTER FOUR

DISCUSSION

The Human Immunodeficiency Virus (HIV) has long been demonstrated to be the causative agent of AIDS. Despite the tremendous advances in the research field such as the development of life-extending antiretroviral drugs and understanding the cause and course of the disease, HIV continues to wreak havoc on a global scale especially in resource-poor countries where devastating consequences are seen. Without an effective prophylactic and therapeutic HIV-1 vaccine, which is cost-effective, HIV-1 will remain a global public health catastrophe.

The long-term use of HAART is expensive and difficult for many patients (especially infants and children) to follow and manage. Drug toxicity is generated as a result of constant drug intake (Lisziewicz *et al*, 2002). Therefore the implementation of Structured Treatment Interruptions (STI) was initially thought to be a promising intervention for the treatment of HIV-1 infection, which offered a cheap and less toxic alternative to continuous antiretroviral therapy. Previous studies that involved STIs in chronic HIV-1 infection focused mainly on adult subjects. Results obtained in January 2006, evaluated by the Data Safety and Monitoring Board (DSMB) from one of the largest STI studies in the world, The Strategies for the Management of Antiretroviral Therapy (SMART Trial), suggested that there was an increase in major complications such as cardiovascular diseases, kidney and liver diseases as well as reported cases of deaths as a result of structured treatment interruptions. The DSMB thereby recommended that the SMART Trial be stopped and that all participants resume continuous therapy. Based on these findings, this pilot study stopped all further treatment interruptions

The mean age of the participants in the SMART Trial was 46 years. In contrast the average age of the children in the current study was 5 years old. Studies of this nature have been limited in children. Therefore this randomized, controlled pilot study was performed to assess the use of STIs in chronically HIV-1 infected children in KwaZulu Natal, South Africa. This pilot study did not complete the full assessment of the use of STIs in this cohort and this was due to the outcome of the findings in the SMART study as mentioned above. All the children who were randomized to the STI arm of the study and had treatment interruptions, resumed

continuous therapy and the children were then followed-up on their routine scheduled visits until the end of the study period.

Although investigators indicated that the clinical outcomes in the drug conservation arm (structured interruption arm) of the SMART study were inferior, the factors that contributed to such adverse findings in this major trial is still being investigated. However, a major difference between the SMART Trial and this pilot study is the fact that there were no deaths reported in this cohort and the quality of life in these children has improved significantly. However, it should be noted that clinical and laboratory monitoring in the SMART trial was much more stringent and this could account for the differences between the two studies.

The results in this study showed good clinical response to HAART in the majority of the children. An exception to this was the side effect of one of the antiretroviral regimes, Abacavir which causes "hypersensitivity" and has been reported to cause severe illness and/or death. Previous studies have demonstrated that the HLA-B*5701 allele, which is most common in Caucasian populations, has a high predictive value for this reaction (Mallal *et al*, 2002).

PARV 01, a five-year old male child, was enrolled to this pilot study, experienced this "hypersensitivity" to Abacavir within the first week of starting the triple nucleoside combination. HAART was immediately suspended in this child and although the CD4 percentage of this child remained above the National Roll-Out cut-off value, he was still managed clinically by the study clinician but was not part of the pilot study analysis. Interestingly, this child did not possess the HLA-B*5701 allele.

There is now significant research evidence to indicate which antiretroviral drug combinations are best in terms of reducing ill health and extending survival. The anti-drug classes include: nucleoside analogues, non-nucleoside reverse transcriptase inhibitors (NNRTI) and protease inhibitors (PI) (Appendix 15).

In South Africa the first and second line antiretroviral-drug regime currently includes two nucleoside analogues and one NNRTI. This study however, used a recommended alternative

regime that consisted of three nucleoside analogues (i.e. AZT, 3TC and Abacavir). This was because the study design had been decided upon at least 3-4 years before the South African Government Roll-Out ARV Programme. At that time this was the best possible regime for children. However, due to lack of funding this pilot study was only commenced in January 2004.

A triple nucleoside combination is usually considered to be administered to HIV-infected individuals if they have a low viral load or if there are concerns regarding treatment adherence. However the major advantage of a triple nucleoside regime is that they are simple, more tolerable, involves fewer pills and causes fewer problems with drug interactions and side effects.

Patients on antiretroviral therapy demonstrate a decrease in viral load levels often to undetectable levels while improving their immune system with a $CD4^+$ T-cell count rise. It is not uncommon for patients to have increasing viral loads while on treatment with a steady increase in $CD4^+$ cell count. The discordance between T-cell counts and viral load is often referred to as the Disconnect Syndrome (Berger, 2002). This is an observation of the difference between the viral and immunological laboratory measures which includes the viral load and the CD4 cell counts.

Disappointing news in 2003 regarding this triple nucleoside regime only got worse in 2004 by which time this pilot study had already began. This disappointment began with preliminary data with the triple nucleoside regime in the AIDS Clinical Trials Group (ACTG) 5095 Study, where a high rate of early virologic failure and rapid development of resistance was evident (Gulick *et al*, 2004). As a result of this ACTG 5095 Study, the Department of Health and Human Services (DHHS) guidelines did not recommend the triple nucleoside reverse-transcriptase combination of AZT/3TC/ABC, unless no other treatment options are available.

Overall this study showed that the CD4 percentages increased markedly during the first 12 weeks (3 months) of starting antiretroviral therapy. These results are in keeping with the data obtained from the retrospective studies with HIV-infected children in Mombasa, Kenya (Song

et al, 2007) and in Thailand (Puthanakit *et al,* 2005). A report from West Kenya that followed up on children from 9 different clinics, indicated that there was a significant increase in the CD4 percentages after treatment was initiated with the greatest increase noted during the first 30 weeks.

The current study of STIs in chronic HIV-1 infected children showed that in the 8 children who had treatment interruptions, 50% (4/8) of the children had viral rebounds to their pre-HAART baseline levels during the treatment interruptions and small changes (decrease in number) were noted in the CD4 percentages during the treatment interruptions. As shown in figure 15 (a & b), the overall CD4 counts and viral load measurements at the end of the 2 year follow-up period were consistent with good outcomes. There were no differences with regards to the outcomes of the CD4 counts and viral load measurements in the continuous treatment group and treatment interruption group. These results are consistent with previous studies (Ruiz *et al,* 2000; Papasavvas *et al,* 2000; Hatano *et al,* 2000 and Ortiz *et al,* 2001).

Safety was a primary concern when treatment interruptions were performed in this study. The study protocol mandated the first treatment interruption after 24 weeks (6 months) of the triple antiretroviral regime, only if the viral load was undetectable (i.e. <400 HIV-RNA copies/ml). Treatment resumed if there the viral load increased to ≥1000 HIV-RNA copies/ml.

In almost all of the treatment interruption studies performed, treatment interruption cycles were standardized for the participants within the cohort. The Swiss-Spanish study used 4 HAART cycles of 2 weeks "off" HAART and 8 weeks "on" HAART. Other studies used 7 days "on" HAART followed by 7 days "off" HAART (Dybul *et al,* 2001) while another study used 1-month "off" HAART followed by 1-month "on" HAART (Ortiz *et al,* 2001). This study focused on the individual child and therefore the length of the treatment interruption was variable. The study protocol to resume therapy was guided by the viral load criteria of ≥1000 HIV-RNA copies/ml and not the $CD4^+$ T-cell percentage as described in other studies (Thobakgale *et al,* 2007; Chakraborty *et al,* 2003). Some studies used the viral load as the treatment resumption criteria. In these studies, treatment resumed if the viral load was >100, 000 copies/ml (Ortiz *et al,* 2001 and Molto *et al,* 2004). It was not established whether the

outcome of this study would be any different, if the criteria for resuming HAART after a treatment interruption was changed, firstly to increase the viral load cut-off to 100, 000 HIV-RNA copies/ml (log 5.00) or secondly to include the CD4 percentages, like in the above mentioned studies.

In this study no death or major opportunistic infections were observed during the course of treatment. STIs may be able to reconstitute the immune system in some chronic infected individuals, but this outcome is both variable and difficult to predict. The quality of the immune reconstitution during chronic infection appears to be different from acute infection. The immune control of HIV replication during treatment interruption in acute HIV-1 infection is better achieved than in chronic HIV-1 infection, where the HIV rebounds are usually higher (Altfeld *et al*, 2001).

The screening of the overlapping HIV-1 peptides in an IFN-gamma ELISPOT assay at baseline revealed that the majority of these perinatally chronic HIV-1 infected children mounted HIV-1 specific CD8+ T-cell responses that were weak and narrowly directed to a limited number of epitopes. These findings are consistent with prior studies which included perinatally HIV-1 infected children (Luzuriaga *et al*, 1995; Luzuriaga *et al*, 2000; Scott *et al*, 2001; Spiegel *et al*, 1999 and Spiegel *et al*, 2000). However, these findings, contrast with the data obtained in a study (Feeney *et al*, 2003a) where young perinatally HIV-1 infected children mounted broad and vigorous CTL responses which targeted multiple epitopes within the HIV proteome. There was a significant difference with regards to the age of the children in the above mentioned study as compared to the PARV study. The average age of the children in above mentioned study (Feeney *et al*, 2003a) was 10 years with a range of 6-17 years. In the PARV study the average of the children was 5 years with a range of 2-12 years. With regards to HAART, 16 children were on HAART and only 2 children were treatment naïve (Feeney *et al*, 2003a). The possible reasons for the differences of the $CD8^+$ T-cell HIV-1 responses in the two cohorts could be due to the age of the children as well as the treatment status.

In this study, all the children had detectable $CD8^+$ T-cell responses directed towards the Gag and/or to at least one other protein of the virus before starting antiretroviral therapy. The Gag (p24) protein was the most frequently recognized subunit protein in this chronic HIV-1 infected cohort (Figure 16). Preferential targeting of Gag by the $CD8^+$ T-cells as compared to targeting other proteins within the viral genome is the fact that Gag may tolerate less sequence diversity because it is the most highly conserved protein in the HIV proteome (Ramduth *et al,* 2005; Feeney *et al*, 2003a; Kaushik *et al*, 2005 and Streeck *et al,* 2007).

Although the Gag p24 subunit of the protein was most frequently targeted in this study, the overall highest total contribution in the HIV-1 proteome was the Pol protein followed by Gag, Nef and Env proteins. Feeney *et al*, (2003b), showed the order of magnitude of the most frequently targeted proteins were Gag, Nef and Pol in perinatally HIV-1 infected children. Differences were observed in the relative contribution of each protein to the anti-HIV-specific $CD8^+$ T-cell responses between acute and chronic therapy naïve children. The order of magnitude in a paediatric acute study was Nef protein followed by Gag and then Env proteins (Thobakgale *et al,* 2007). In contrast Fu *et al,* (2007), showed that Gag, Pol and the Nef were the most frequent targets in chronic adult HIV-1 infection.

With the exception to the Vpu protein, which was not targeted by any of the children, the regulatory and accessory proteins were infrequently targeted. In particular, the early expressed proteins Tat and Rev were recognized by minority of the children in the cohort. These findings are consistent with data from other studies (Feeney *et al*, 2003b and Ching *et al,* 2007).

There was no correlation between the total sum of $CD8^+$ T-cell responses and plasma viral load before starting antiretroviral therapy (Figure 18A and 18B). The IFN-γ ELISPOT assay does not permit the differentiation between $CD8^+$ and $CD4^+$ T-cell responses. Therefore the IFN-γ ICS assay which allows for the simultaneous quantification of both $CD4^+$ and $CD8^+$ T-cell responses was performed.

Results from our laboratory indicate that the majority of the chronically HIV-1 clade C infected children, maintained HIV-specific $CD8^+$ and $CD4^+$ T-cells that produced IFN-gamma. Preferential targeting of Gag by the $CD8^+$ and $CD4^+$ T-cells were associated with control of viremia in these chronic HIV-infected children (Ramduth *et al,* 2005).

Virus-specific $CD4^+$ T-cell responses are important in maintaining effective immunity to a number of viral infections (Rosenberg *et al,* 1997) but are usually undetectable during the chronic phase of HIV-1 infection. The reason is probably because these $CD4^+$ T-cells are preferentially targeted by HIV-1 (Douek *et al,* 2000). In this study, majority of the children (21/29) had a HIV-1 specific $CD4^+$ T-cell response to the Gag pool of peptides in the intracellular cytokine staining assay while in twenty of these children there was also a Gag response on the ELISPOT assay. Although baseline (pre-HAART) ICS testing was performed in seven children, testing of the remaining twenty two children, at varying timepoints, indicated that IFN-γ $CD4^+$ and $CD8^+$ HIV-1 Gag-specific T-cell responses were still detectable despite being on HAART. Repopulating the T-helper lymphocytes after HAART involves naïve $CD4^+$ T-cells in children (de la Rosa and Leal, 2003) with a small increase in memory $CD4^+$ T-cells which reflects on the thymic capacity (Gibb *et al,* 2000).

The effects of HAART on the virus specific $CD4^+$ and $CD8^+$ T-cell responses showed that the $CD4^+$ and $CD8^+$ T-cell responses waned over time on HAART, however in 3 children, the $CD4^+$ and $CD8^+$ T-cell responses disappeared whilst on HAART.

Although the IFN-γ ICS data was limited to a small sample size and infrequent timepoints, a positive association between the HIV-1 specific $CD4^+$ and $CD8^+$ T-cell responses was seen. The magnitude of the $CD8^+$ T-cell responses was higher than that of the $CD4^+$ T-cell responses and this was also seen in other studies (Ramduth *et al,* 2005 and Lacabaratz *et al,* 2003). The magnitude of these responses increased during the treatment interruptions and decreased during the treatment phase.

The M184V mutation (associated with 3TC resistance) was a frequently (36%) selected mutation both in the continuous treatment group as well as in those children who had

treatment interruptions. 3TC is known to have a longer half-life in the cell and not necessarily in the blood. Although the M184V mutation was a frequently selected mutation, it was found only after repeated treatment interruptions in the treatment interruption arm of the study. Similar findings were obtained in the Swiss cohort (Yerly *et al*, 2003) with the median time on HAART being 27 months (range 9-45 months) as compared to this study which was a 24 month study period.

The V179D mutation associated with NVP resistance was identified in one child (PARV 14) prior to starting HAART and this mutation was maintained through the study period. The Y181C mutation associated with NVP resistance was evident in PARV 18 who was reported to have received a single-dose of NVP at birth. Drug resistant mutations are common especially in South Africa as single-dose NVP provides prophylaxis for the prevention-of-mother-to-child-transmission (PMTCT) of HIV infection (Kurb *et al*, 2007).

A study which looked at STIs in adult patients with multi-drug resistance HIV found that the STI was associated with greater progression of the disease and did not confer immunologic or virologic benefits or improve the overall quality of life (Pai *et al*, 2009). In comparison, all of the HIV-1 chronically infected children in this pilot study were reported to have improved quality of life. There were no reports of deaths. There was an increase in their CD4 percentages and decrease in the number of HIV-RNA copies/ml. There were two children who had baseline drug resistant mutations who had treatment interruptions. Other studies have indicated that resistance in HIV-1 infection in infants is not only due to transmission of drug resistant virus but continued therapy is also associated with drug resistance (Noe *et al*, 2005).

Although HAART significantly delays disease progression and prolongs life expectancy in children, viral suppression is often incomplete or transient in treated individuals and this was due to various factors such as host immune response impairment and viral resistance (Feeney et al, 2005). In this study there were 6 children who maintained a detectable viral load throughout the study period of 24 months. A further 7 children who reached an undetectable viral load during the study period (most of them by twelve weeks on HAART) had detectable viral loads thereafter.

The mortality rates of untreated HIV-infected children are extremely high, especially within the first 5 years of life (Nicoll *et al*, 1994, Spira *et al*, 1999, Taha *et al*, 2000). The majority of the children on this study survived past that age without HAART and this suggest that these children were paediatric slow progressors.

This pilot study that focused on HIV-1 specific T-cell responses in chronic HIV-1 infected children during continuous treatment and treatment interruption periods had several limitations. Firstly as this was one of the first studies which focused on chronic HIV-1 infection in children, the protocol used for the STI was not ideally suited. The STIs probably started too early after the initial treatment phase of the study before the children had a chance to stabilize and this was evident by the fact that 6/14 children in the treatment interruption group did not reach undetectable viral load levels by 24 weeks of treatment. Secondly this study used viral load monitoring to dictate treatment interruptions and the treatment interruption periods were very short for at least 5/8 children. This does indicate that this is not a true "structured treatment interruption." Thirdly there was a variation in the age of children (2-12 years). Due to the small sample size of the study, it was difficult to distinguish age groups as older children may have stronger immune responses as compared to the younger children. Fourthly it was difficult to obtain adequate blood sample from all the children, and this made it impossible to do all assays at all time points. Lastly as I had no access to the WHO staging and other clinical data to categorically state that there were no differences according to the study arm even though immunologic and virologic measurements suggested so.

CONCLUSIONS

The course of HIV-1 infection in infants and children is different to that in adults, and it is therefore important that monitoring, care and treatment of these HIV-1 infected infants and children be tailored and optimized accordingly. Children are not all the same, and there are obviously age-related differences which affect adherence. For children of school going age, adherence to regimes that require them to take doses at school can be difficult as it could lead to their HIV status being disclosed. Childhood infections such as measles and chickenpox can have very severe health implications for HIV-positive children and immediate medical advice should be sought if they have been exposed to or infected with these diseases.

There were no statistically significant differences with regards to age, gender, pre-HAART CD4 percentages and pre-HAART log viral loads, between the structured treatment interruption group and the continuous treatment group in this pilot study. Fourteen children (48.28% of the children) in this study had a ≥10% increase in their CD4 percentage by twelve weeks on HAART.

Most HIV proteins were targeted by the CTLs in this chronic paediatric HIV-1 infected cohort. The Gag (p24) was the most dominant protein which was targeted by a majority of the children screened at baseline (pre-HAART). The Pol and Nef proteins were also frequently targeted. Both Gag-specific $CD4^+$ and $CD8^+$ T-cell responses were evident in the cohort. Gag specific $CD8^+$ T-cell responses were of greater magnitude as compared to the Gag-specific $CD4^+$ T-cell responses. The presence of the HIV-1 specific $CD4^+$ and $CD8^+$ T-cell responses were evident with and without HAART. Although the pre-HAART HIV-specific T-cell responses were narrow and weak, broadening of T-cell responses was evident in both treatment groups. The data in this pilot study indicates that structured treatment interruptions in this cohort were of a short duration due to the fast rebound of viral load upon interruption of HAART.

The M184V resistant mutation (3TC) was the most frequently detected mutation in this cohort. This M184V mutation was detected in children during treatment interruptions as well as in children who were randomized to the continuous treatment arm of the study. Despite having the M184V resistant mutation, viraemia was controlled and the $CD4^+$ T-cell percentages were stable.

Antiretroviral therapy has been shown to be effective in older infants and children, many of whom are living longer and healthier lives. Although HAART has reduced HIV mortality significantly, prolonged treatment that suppresses the plasma viremia does not eradicate HIV and therefore, therapy must be continued throughout life in HIV-infected individuals. Overall there were no deaths in this cohort during the study period and all the children were reported to be clinically well at the end of the study.

In this pilot study we used viral load monitoring to dictate treatment interruptions and found that the time off treatment was very short. The final outcome with regards to the CD4 and viral load, at the end of the 2 year follow up period, indicated that there was no difference between the continuous and treatment interruption groups. It is not possible to make any definitive statements on the use of treatment interruptions due to the small sample size, however, this study suggests that it is important to continue to explore alternative treatment strategies to reduce cost and toxicity and to enhance adherence.

CHAPTER FIVE

APPENDICES

1. Informed Consent Form

INFORMATION GIVEN TO SUBJECTS

Title of study: Assessment of Intermittent and Continuous Anti-retroviral Therapy regimens used for HIV-infected infants and children in Durban, South Africa

You have been informed that your child is HIV positive and the doctor and nurse/ counsellor would have explained to you the importance of the test result and about HIV infection. We request your permission for your child to take part in a study. This study will look at giving children medicines to treat the HIV infection. The doctor will also explain the HIV infection and how the medicines may help your child. The medicines we plan to use have already been tested and shown to be effective in adults and children in other places in the world. We will give your child three types of medicines for 6 months. The doctor will explain the details of each of these medicines and how they should be taken. You will also be informed about the number of times your child has to come to the clinic and what blood tests need to be done.

At the end of 6 months, we will do blood tests to see whether the medicines are helping your child. If they are, your child will be entered into the second part of the study. If the medicines are not helping, we will stop them, but continue to look after your child in our clinic.

In the second part of the study, your child will take part in either one of two arms, which will occur by chance. In one part of the study, all the children will continue to take the medicines in the same way as the first 6 months. In the other part of the study, we will stop your child's medicines when blood results show us that he/she is doing very well. We then give the body a chance to "rest" from the medicines. During this time, we will do more blood tests (to check on the virus and the body's response to the infection), so you will need to attend the clinic regularly. If at any stage, we are concerned about your child's blood results, we will re-start the medicines. Both systems of treatment will continue for 2 years.

Any questions you have will be answered before you agree to allow your child to take part in the study. The study will last about 2 years and your child will also be offered ongoing free treatment with the same medicines, if the child is doing well on the medicines and you have been attending the clinic regularly, as requested by the doctors.
Your child will visit the clinic about 12 times during the study. We will pay for any additional travelling expenses. We cannot guarantee that the treatment will work. You

will have to sign to indicate your agreement to take part in the study. You have the right to withdraw from the study at any time and this will not affect the treatment your child is entitled to. We will continue seeing your child at the clinic.
There are several things in the study that may cause inconvenience or discomfort. Firstly blood will be taken from your child regularly to check the state of the HIV tests and to look for side-effects of the medicines. There may be pain and bruising at the site of the injection. The study medicines may cause some side-effects such as a skin rash, fever and swelling of the glands. Your child will be seen by the doctor and carefully examined at each clinic visit. You may also bring the child to the clinic any time you notice any problem.

G.6 INFORMED CONSENT FOR INCLUSION IN A STUDY (NOT TO BE USED FOR PHARMACEUTICAL STUDIES) A TRANSLATION INTO THE HOME LANGUAGE OF THE SUBJECT MUST BE PROVIDED.

Title of study: Assessment of Intermittent and Continuous Anti-retroviral Therapy regimens used for HIV-infected infants and children in Durban, South Africa

G.6.1 I,(Name)

hereby consent to the following Procedure and/or Treatment being conducted on myself or the person indicated in G.6.4 below:

G.6.2 I acknowledge that I have been informed by:

(Name) Prof R Bobat

concerning the possible advantages and possible adverse effects which may result from the abovementioned procedure and/or treatment and of the ways in which it is different from the conventional procedure and/or treatment.

I,(Name)

hereby acknowledge that I understand and accept that this study involves research and the "Information to Patients" leaflet has been handed to me in connection with this study.

G.6.3 I agree that the above procedure and/or treatment will be carried out and/or supervised by

(Name) Prof R Bobat
G.6.4 I acknowledge that I understand the contents of this form, including the information provided in the "Information to Patients" leaflet and as the

PARENT GUARDIAN OTHER (specify)

freely consent to the above procedure and/or treatment being conducted on:

(Name)

G.6.5 I am aware that I may withdraw my consent at any time without prejudice to further care.

Signed: Date:

Subject/Parent/Guardian

Signed: Date:

Witness

Signed: Date:

Informant

Signed: Date:

Researcher

For illiterate subjects:

Date:

Mark with a 'X'

Independent witness:

Date:

Details of independent witness:

1. Title plus name:

Telephone number:

For additional information with respect to ethical aspects of this study, contact the Postgraduate Administration office on (031) 260 4416 (Fax/Tel). E-mail: postgrad-med.nu.ac.za

2. Working FACS Lysing Solution (1:10 Dilution)

50µl 10X FACS Lysing Solution (BD Biosciences, San Jose, USA)

450µl Sterile distilled water

Method: 50µl of the 10X FACS Lysing Solution was diluted in 450µl of sterile distilled water. 450µl of the working FACS Lysing Solution was used for each test.

3. 70% Ethanol

(a) **70% Ethanol (Viral load Assay – for 12 tests)**

4.5ml Sterile distilled water

10.5ml 99.7% Ethanol (Merck, Gauteng, South Africa)

Method: 4.5ml of Sterile distilled water was added to 10.5ml of 99.7% Ethanol. 1ml of the 70% was used for each test.

(b) **70% Ethanol (DNA Isolation)**

3ml Sterile distilled water

7ml 99.7% Ethanol (Merck, Gauteng, South Africa)

Method: 4.5ml of Sterile distilled water was added to 10.5ml of 99.7% Ethanol. 1ml of the 70% was used for each test.

4. Red Blood Cell Lysis Buffer

7.7g NH_4Cl (Sigma-Aldrich Inc, St. Louis, USA)

0.84g $NaHCO_3$ (Sigma-Aldrich Inc, St. Louis, USA)

1 L Sterile Distilled water

Method: 7.7g NH_4Cl and 0.84g NaHO3 was dissolved in sterile distilled water. The buffer was mixed well and stored at room temperature.

5. Phosphate Buffer Saline (PBS)

1 Phosphate Buffered Saline (Dulbecco A) Tablet (Oxoid Limited, Hamshiper,England)
100 ml Sterile distilled water

Method: One Phosphate Buffered Saline (Dulbecco A) Tablet was dissolved in 100 ml sterile distilled water. The pH of the PBS solution was adjusted to approximately 7.2. The solution was autoclaved for 10 minutes at 115^0C and stored at room temperature.

6. Phosphate Buffer Saline (PBS) + Antibiotics

1L Phosphate Buffer Saline
1% of 100X Penstrep/Fungizone (P/S/F) (Biowhittaker, Cambrex Bio Science, Walkersville, USA)

Method: 10 ml of the already prepared PBS was removed and discarded. 10 ml of a 1% Penstrep/Fungizone (P/S/F) solution was added to the remaining PBS. The solution was mixed well and stored at 4^0C.

7. R10 Media

500 ml RPMI (Biowhittaker, Cambrex Bio Science, Walkersville, USA)
50 ml Fetal Calf Serum (FCS) (Delta Bioproducts, Johannesburg, South Africa)
5 ml of 200mM L-glutamine (Biowhittaker, Cambrex Bio Science, Walkersville, USA)
5 ml of 100X Penstrep/Fungizone (P/S/F) (Biowhittaker, Cambrex Bio Science, Walkersville, USA)
5 ml of 1M Hepes Buffer (Biowhittaker, Cambrex Bio Science, Walkersville, USA)

Method: 65 ml of RPMI was removed from the original 500 ml quantity. 50 ml of heat inactivated and filtered FCS, 5ml of 200mM L-glutamine, 5ml of 100X

Penstrep/Fungizone (P/S/F) and 5ml of 1M Hepes Buffer was added. The media was mixed well and stored at 4^0C.

8. 2% Virkon Solution

200g of Virkon Powder (Antec International, Sudbury, Suffolk)

10 L Water

Method: 200g of Virkon Powder was dissolved in 10 L of water.

9. Blocking Buffer

1L Phosphate Buffer Saline

10 ml Fetal Calf Serum (FCS) (Delta Bioproducts, Johannesburg, South Africa)

Method: 10ml of PBS from the already prepared 1L bottle was removed and discarded. 10ml of heat inactivated, filtered FCS was added to the remaining PBS. The 1% solution was mixed well and stored at 4^0C.

10. BCIP (5-bromo-4-chloro-3-indoly/phosphate)

0.015g BCIP (BioRad Laboratories, Hercules, CA)

1 ml DMF (Merck, Wadeville, Gauteng, South Africa)

Method: 0.015g of BCIP powder was dissolved in 1ml of DMF. The final concentration was 15mg/ml. The solution was mixed well and stored at 4^0C.

11. NBT (Nitroblue Tetrazolium Chloride)

0.03g NBT (BioRad Laboratories, Hercules, CA)

700 µl DMF (Merck, Wadeville, Gauteng, South Africa)

300 µl Sterile distilled water

Method: 0.03g of NBT powder was dissolved in 700 µl DMF and 300 µl of sterile distilled water was added. The solution was mixed well and stored at 4^0C.

12. 1M TRIS Buffer

12.1g TRIS (BioRad Laboratories, Hercules, CA)

1L Sterile distilled water

Method: 12.1g of TRIS powder was dissolved in 1L of sterile distilled water with the use of a magnetic stirring bar. The pH was adjusted to 9.3 with concentrated Hydrogen Chloride (HCl). The solution was autoclaved for 10 minutes at 115^0C and stored at room temperature.

13. Working Hybridization Buffer

213ml Di-ionised Water

55ml SSPE Concentrate (Dynal Biotech, Bromborough, UK)

7ml SDS Concentrate (Dynal Biotech, Bromborough, UK)

Method: 7ml of the SDS Concentrate (was heated to 50^0C prior to use) and 55ml of the SSPE Concentrate (was heated to 50^0C prior to use) was added to 213ml of de-ionised water. The buffer was mixed well and heated to 50^0C to ensure that all the components were in solution. The buffer was stored at room temperature for 3 months.

14. Ambient Wash Buffer and Stringent Wash Buffer

1228.5ml Di-ionised Water

65ml SSPE Concentrate (Dynal Biotech, Bromborough, UK)

6.5ml SDS Concentrate (Dynal Biotech, Bromborough, UK)

Method: 6.5ml of the SDS Concentrate and 65ml of the SSPE Concentrate was added to 1228.5ml of de-ionised water. The buffer was mixed well and then divided into two separate containers. 275ml was used for the Stringent wash buffer and 1025ml was used for the Ambient wash buffer. Both buffers were stored at room temperature for 3 months.

15. Working Conjugate Solution

5.3ml Ambient Wash Buff

16µl Streptavidin-Horseradish Peroxidase Conjugate (Dynal Biotech, Bromborough, UK)

Method: A cocktail of 5.3ml ambient wash buffer and 16µl of Streptavidin-Horseradish Peroxidase Conjugate was prepared for each strip. This was prepared just before use.

16. Working Citrate Buffer

570ml De-ionised Water

30ml Citrate Concentrate (Dynal Biotech, Bromborough, UK)

Method: 30ml of Citrate Concentrate was added to 570ml of de-ionised water. The working citrate buffer was mixed well and stored at room temperature for 3 months.

17. Working Substrate

4.4ml Substrate A (Dynal Biotech, Bromborough, UK)

1.1ml Substrate B (Dynal Biotech, Bromborough, UK)

Method: A cocktail of 4.4ml of Substrate A and 1.1ml of Substrate B was prepared for each strip just before use.

18. Human Leukocyte Antigen Types

ID	A1	A2	B1	B2	C1	C2
PARV 001	02	74	1510	44	0202	03
PARV 002	29	74	15	35	04	04
PARV 003	0301	2301	1503	5802	0202	0602
PARV 004	24	68	0702	1510	0304	08
PARV 005	23	29	08	44	03	1403
PARV 006	02	29	44	44	07	07
PARV 007	23	29	14	4201	0802	1701
PARV 008	02	30	45	58	06	16
PARV 009	4301	6802	15	15	03	1801
PARV 010	29	3001	4202	57/570301	07	1701
PARV 011	0301	3001	1503	5802	0202	0602
PARV 012	3001	6601	4202	5802	0602	1701
PARV 013	02	29	1302	1401	0602	08
PARV 014	03	03	4501	5802	0602	06
PARV 015	2301	4301	1503	5802	0202	0602
PARV 016	01	3402	1503	8101	0202	1801
PARV 017	23	3203	1503	1503	0202	0202
PARV 018	02	2301	1510	5801	07	1601
PARV 019	23	66	1510	5802	0602	1601
PARV 021	2301	6802	1510	44	3	3
PARV 020	0202	4301	1503	5703	07	18
PARV 021	2301	6802	1510	44	3	3
PARV 022	6802	7408	14	41	0802	1701
PARV 023	3001	3201	5703	5802	1801	0602
PARV 024	02	2301	0801	1503	02	04
PARV 025	3402	6802	1503	44	4	4
PARV 026	2	33	1516	4201	1601	1701
PARV 027	2301/24	3201	1503	1503	0202	03
PARV 028	23	6802	0801	4201	03	1701
PARV 029	23	23	0801	1503	0202	03
PARV 030	03	23	0801	41	07	1701

19. Anti-HIV Drugs Names

Anti-HIV drug class	Common names	Brand names
Nucleoside analogues	AZT, ziduvodine	Retrovir™
	DdI, didanosine	Videx™
	3TC, lamivudine	Epivir™
	D4T, stavudine	Zerit™
	Abacavir	Ziagen™
NNRTI (Non nucleoside reverse transcriptase inhibitors)	Efavirenz	Sustiva™
	Nevirapine	Viramune™
PI (Protease inhibitors)	Lopinavir/ritonavir	Kaletra™
	Indinavir	Crixivan™
	Nelfinavir	Viracept™
	Saquinavir (hard gel capsule)	Invirase™
	Saquinavir (soft gel capsule)	Fortovase™
	ritonavir	Norvir™

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