IMMUNE ACTIVATION AND CIGARETTE SMOKE EXPOSURE AS POTENTIAL DETERMINANTS OF FAILURE OF HAART IN THE SETTING OF MOTHER-TO-CHILD TRANSMISSION.

by

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I DEDICATE THIS DISSERTATION TO MY PARENTS, RICHARD AND ANNAH, AND TO ALL MY FAMILY.

SUMMARY

Persistent immune activation, even in the setting of virologically-suppressive HAART, is a hallmark of chronic immunodeficiency virus type (HIV-1) infection and a major force driving HIV-1 replication and progression to AIDS. Little is known about immune activation profiles and the effect of therapy in children infected with HIV-1 subtype C.

The objectives of this study were to i) investigate and compare levels of circulating biomarkers of immune activation in a cohort of mothers (n=46) infected with HIV-1 subtype C relative to those of 20 healthy controls; ii) compare the biomarkers of immune activation between mothers and their HIV-infected children (n=46); iii) monitor the effects of virologically suppressive and non-suppressive HAART immune activation profiles in a subgroup of children (n=28) and iv) determine the effects of active smoking as well as maternal smoking on the biomarkers of immune activation in the mothers and their children, respectively.

Multiplex bead array, ELISA and immunonephelometric procedures were used to measure plasma levels of the following biomarkers of immune activation: soluble CD14 (sCD14), beta 2 microglobulin (β2M), C-reactive protein (CRP), interferon gamma (IFNγ), monokine induced IFNγ, IFNγ-inducible protein 10 (IP10), tumour necrosis factor α (TNFα), macrophage inflammatory protein α and β (MIP-1α and β), transforming growth factor β (TGFβ), interleukin-1 receptor antagonist (IL-2 Ra), granulocyte- and granulocyte macrophage colony stimulating factors (G-CSF and GM-CSF), and several interleukins including IL-2, IL-4, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, and IL-17.

Relative to the healthy control subjects, almost all of the circulating biomarkers of immune activation, including sCD14, β2M, CRP, MIG, IP10, IFNγ, TNFα,TGFβ and several of the interleukins and colony stimulating factors, were elevated in the HIV-infected mothers (P=0.0346-P<0.0001). The biomarker levels of the children were generally lower than those of the mothers with the exception of β2M which was significantly higher (P=0.001). Virologically suppressive HAART caused decreases in sCD14, β2M and MIG in a subgroup of the children (P=0.0286-P<0.0001) while sCD14 and MIG were increased in the treatment failure groups relative to the

suppressed groups (P=0.0407-0.0002). An unexpectedly high number of mothers were smokers (21.7%); however no significant differences were observed between the non-smokers and smokers. In children exposed to maternal smoking, TGFβ levels were higher (P=0.0288).

In conclusion, although somewhat lower than those of their mothers, HIV-infected children were found to have high levels of a range of circulating biomarkers of immune activation in the setting of higher viral loads. Successful HAART with virological suppression and increased levels of CD4+ T lymphocytes, was associated with significant decreases in some of the biomarkers, notably sCD14, beta 2 microglobulin and MIG, in children. Maternal smoking was associated with a significant increase in the concentration of immunosuppressive TGFβ in children, however further studies in larger groups are necessary.

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ABBREVIATIONS

AIDS - Acquired immune deficiency syndrome

AZT - Azidothymidine

CCR5 - C-C chemokine receptor type 5

cART - Combination antiretroviral therapy

CD - Cluster of differentiation

CMV - Cytomegalovirus

CRF - Circulating recombinant form

CRIs - Co-receptors inhibitors

CTLs - Cytotoxic T lymphocyte

CXCR4 - C-X-C chemokine receptor type 4

DNA - Deoxyribonucleic acid

FIs - Fusion inhibitors

HAART - Highly active antiretroviral therapy

HCV - Hepatitis C virus

HIV - Human immunodeficiency virus

HLA-DR - Human leukocyte antigen D-related

HSRC - Human science research council

IFN - Interferon

IL - Interleukin

INIs - Integrase inhibitors

LPS - Lipopolysaccharides

LTNPs - Long-term nonprogressor

MDM - Monocyte-derived macrophage

MHC - Major histocompatibility complex

MIP - Macrophage inflammatory protein

NNRTIs - Non-nucleoside reverse transcriptase inhibitors

NK cells - Natural killer cells

NKT cells - Natural killer T cells

NRTIs - Nucleoside reverse transcriptase inhibitors

pDS - Plasmacytoid dendritic cells

PD-1 - Programmed cell death-1

PIC - Preintergration complex

PIs - Protease inhibitors

RANTES - Regulated on activation, normal T cell expressed and secreted

RNA - Ribonucleic acid

RT - Reverse transcriptase

SIV - Simian immunodeficiency viruses

TGF - Transforming growth factors

TNF - Tumor necrosis factors

TLR - Toll-like receptors

UNAIDS - United Nations Programme on acquired immune deficiency syndrome

UNICEF - United Nations International children's emergency fund

WHO - World Health Organization

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CHAPTER 1

LITERATURE REVIEW

1.1. Literature review

1.1.1. HIV/AIDS Statistics.

Human Immunodeficiency virus (HIV) infection continues to be a major global public health issue with an estimated 35.3 million people worldwide living with the virus in 2012.[1] An estimated 2.3 million people were newly infected with HIV, representing a 33% decline in the number of new infections worldwide, while the numbers of AIDS deaths also declined from 2001 to 2012 with 1.6 (1.4-1.9) million reported in 2012; down from 2.3 (2.1-2.6) million in 2005.[2] These trends are depicted in Figure 1.

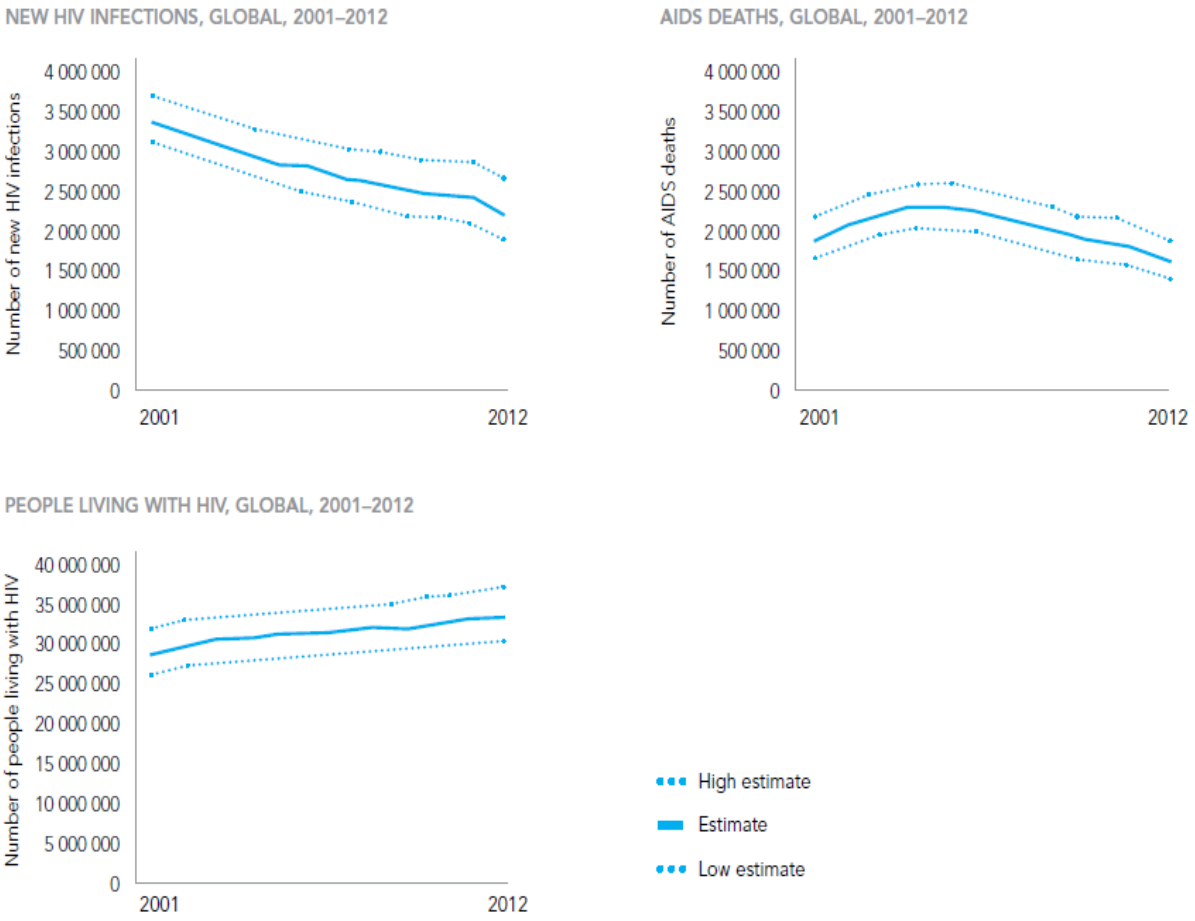

Fig 1. Numbers living with HIV, newly HIV infected, and AIDS death, 2001-2012, globally. Permission for use of figures was provided by UNAIDS (UNAIDS Report, 2013). [2]

Sub-Saharan Africa is the most affected region with nearly 1 in every 20 adults living with HIV. Sixty nine percent of all people living with HIV, live in this region.[1] More women than men are living with HIV in this area, and young women aged 15-24 years are as much as eight times more likely than their male counterparts to be HIV-

positive.[3] In addition, each year around 1.5 million women living with HIV become pregnant [4] and without antiretroviral drugs, there is a 15 to 45% chance that their child will also become HIV-infected.[5] Mother-to-child transmission (MTCT) is when a HIV-positive mother passes the virus to her child during pregnancy, delivery or breastfeeding.[5] In 2011, approximately 330,000 children under the age of 15 became infected with HIV and an estimated 230,000 died from HIV/AIDS.[6] Almost all of these infections were as a result of mother-to-child transmission of children living in Sub-Saharan Africa.[6]

The total number of people living with HIV in South Africa increased from an estimated 4 million in 2006 to 6.4 million by 2013. In a survey on HIV done in South Africa in 2012, HIV-prevalence was highest in Kwazulu-Natal, followed by Mpumalanga, Free State and then North West Province; while Western Cape, Northern Cape and Limpopo have the lowest HIV-prevalence.[7] The overall prevalence (%) by province is shown in Fig 2.

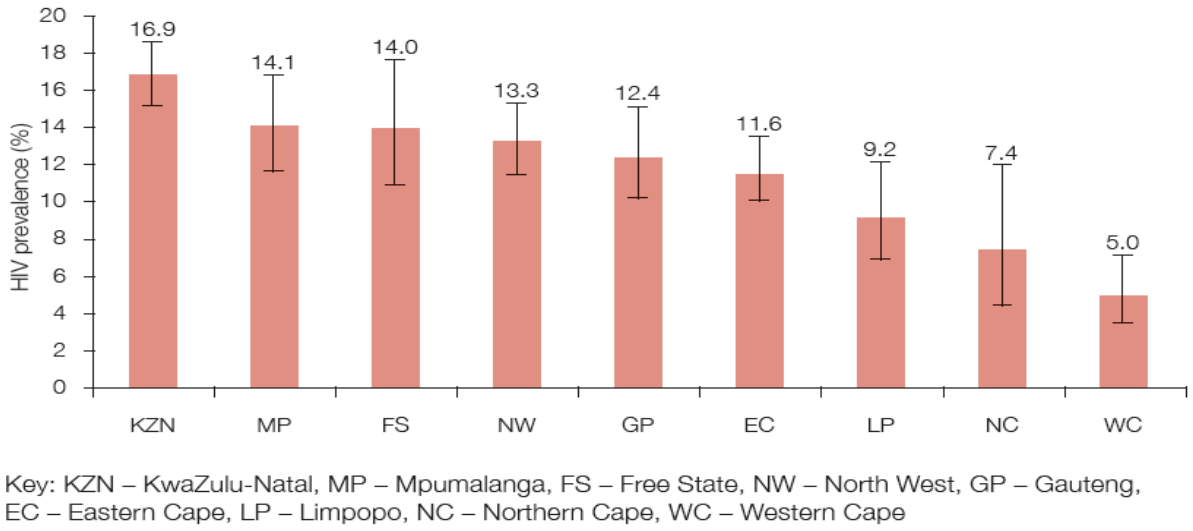

Fig 2. Overall HIV prevalence by province, South Africa, 2012. Permission to use the figure was provided by HSRC.[7]

These statistics demonstrate that the burden of HIV/AIDS is primarily in sub-Saharan Africa and even though worldwide trends of the disease seem to be decreasing, South Africa still shows increasing numbers. Although quite alarming, increased prevalence may just be an indicator that fewer people are dying from HIV-infection because of effective treatment strategies. However, the high incidence reported in the recent national HIV HSRC (Human Science Research Council) survey, may

indicate that South Africans are complacent about HIV. Worryingly, this latest survey showed plummeting condom use, increases in multiple concurrent partnerships and more boys having sex at a younger age.[7]

1.1.2. Origin of HIV & AIDS.

HIV is a lentivirus, which mainly attacks certain cells of the immune system. Lentiviruses are in turn part of a larger group of viruses known as retroviruses. They possess a ribonucleic acid (RNA) core, encoded in the viral envelope.[8] The first recognised cases of AIDS were officially reportedly in the 1980s. It is now generally accepted that HIV is a descendant of a simian immunodeficiency virus (SIV) because certain strains of SIV bear a very close resemblance to HIV-1 and HIV-2, the two types of HIV.[9]

HIV-1 and HIV-2 infections cause immune suppression and progression to acquired immunodeficiency syndrome (AIDS), a condition that is accompanied by a profound decrease in the number of CD4 + T cells and characterised by the susceptibility to infection with opportunistic pathogen.[10,11] HIV-2 infection is generally characterised by a longer asymptomatic stage, lower plasma HIV-2 viral loads, and a lower mortality rate compared with HIV-1.[12] HIV-2 infection is endemic in West Africa and although HIV-2 has only limited spread outside this area, some sporadic cases have been reported elsewhere in Africa. The HIV-2 appears to be significantly less virulent than HIV-1.[13]

1.1.3. Subtypes of HIV-1.

HIV-1 is characterised by extensive and dynamic genetic diversity, wherein distinct subtypes are expanding in different geographical regions.[14] There are four major phylogenetic groups of HIV-1 variants: group M (Main), group O (Outlier), group N (non-M/non-O), and group P.[15,16] The main group of HIV-1 variants, group M, accounts for the majority of infections in the worldwide HIV-1 epidemic and can be further subdivided into phylogenetic subtypes or clades (A to K) and circulating recombinant forms (CRFs). CRFs emerge from two different viral strains that fuse

together to form a new genetic variant or viral strain in the same individual.[17] The average intersubtype genetic variability of the HIV-1 variant group M is 15% for the *gag* gene and 25% for the *env* gene.[18]

The most prevalent HIV-1 genetic forms are subtypes A, B and C, where subtype C accounts for almost >80% of all HIV-1 infections worldwide.[14] Subtype A recombinant variants are predominant in areas of central and eastern Africa (Kenya, Tanzania, Uganda and Rwanda) and in eastern European countries.[13] Subtype B is the main genetic form in western and central Europe and North America. However, with increasing immigration and globalisation, >40% of new infections in Europe are presently non-B Africa and Asian variants.[14] Subtype C viruses are predominant, accounting for >80% of all global HIV-1 infections. Indeed, clade C has become the epicentre of the HIV pandemic through its uncontrolled spread throughout Botswana, Zimbabwe, Malawi, Zambia, Namibia, Lesotho, South Africa, India, Nepal and China.[19] Although the reason for the high prevalence of HIV subtype C is not known, Gordon *et al*, speculated that it may be related to host, viral, or socioeconomic factors. At the viral level it has been suggested that C viruses may be more stable, while their protease gene may have increased catalytic activity relative to the other subtypes.[20]

1.1.4. Structure and molecular features of HIV.

HIV virions contain a virus capsid, which is composed of the following layers; *a)* the major capsid protein p24, *b)* the nucleocapsid protein p7/p9, *c)* diploid single-stranded RNA genome, *d)* the three viral enzymes, protease, reverse transcriptase (RT) and integrase. The matrix protein p17, which is located underneath the virion envelope, is the one surrounding the viral capsid.[21]

The major viral structural proteins and enzymes are encoded by 3 different major HIV genes arranged in the orders 5'-*gag-pol-env*-3', and a series of genes, *vif, vpr, vpu, tat, rev* and *nef* encode for accessory and regulatory proteins. The first open reading frame of the *gag* gene encodes the matrix protein, p17, the major capsid, p24, and the nucleocapsid protein, p7/p9. The second open reading frame *pol* gene encodes the viral enzymes protease, reverse transcriptase and integrase.[22,23] A

diagram of the HIV-1 genome organisation and reverse transcriptase (RT) structure is shown in Fig 3.

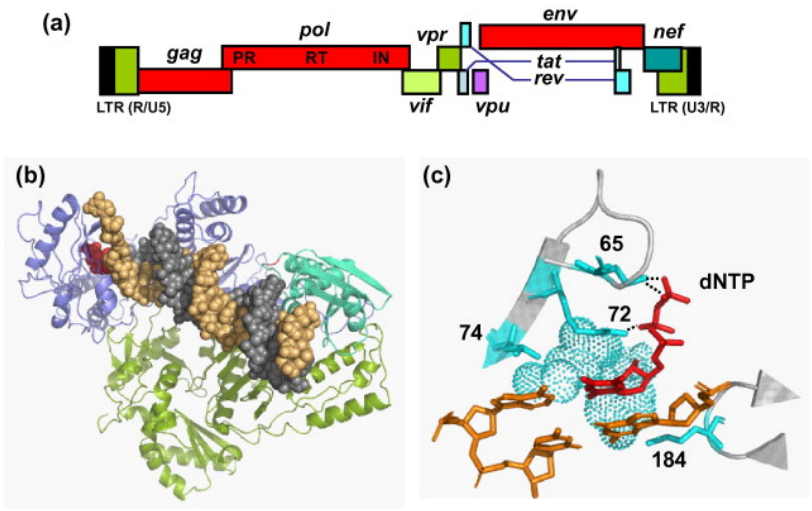

Fig.3 HIV-1 genome organisation and RT structure. (a) Major genes (*gag,pol,env*) and regulatory genes (*vif,vpr,vpu,tat,rev,nef*) in the HIV-1 genome. In the *pol* gene, antiretroviral drug targets such as protease (PR), reverse transcriptase (RT) and intergrase (IN) are encoded. (b) Crystal structure of the ternary complex of HIV-1 RT, double-stranded DNA and incoming nucleotide. (c) The nucleotide binding site with the side chain of Lys^{65} and Arg^{72} form a hydrogen bond with the phosphate group of the incoming nucleotide. Stick representation used to show the location of Lys^{65}, Arg^{72}, Leu ^{74} and Met^{184} (blue) and primer nucleotides (orange). Permission to use the figures was provided by Hang et al., 1998.[24]

1.1.5. HIV entry and replication in the host cell.

The first stage of HIV-1 infection is viral attachment. On the surface of the virion, viral glycoprotein (Env) gp120 binds to the primary receptor, CD4 and co-receptor, typically either the CCR5 or CXCR4 chemokine receptors, which are present on susceptible cells such as T lymphocytes and macrophages. Subsequently, the binding of gp120 to the CD4 and co-receptor cause env gp41 to undergo a conformational change that induces fusion of the viral membrane with the target cell membrane.[25,26]

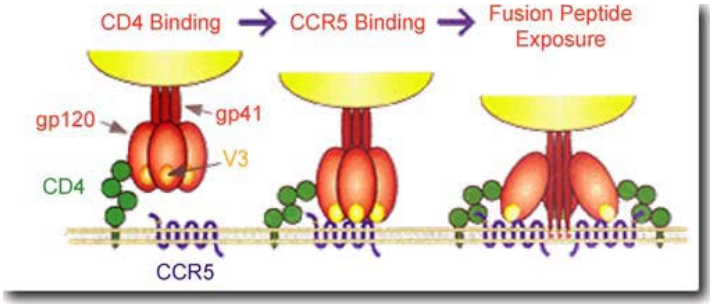

Fig 4. A representation of HIV-1 entry into target cells. Viral gp 120 binds to primary receptor CD4 and with the co-receptors CCR5 or CXCR4 in the target cell. The signal induced by the complex promotes fusion of the viral membrane to fuse with the target cell membrane. Permission to use the figure was provided by Chan *et al*., 1998.[25]

After attachment and fusion of the viral envelope with the target cell membrane, the viral capsid enters the cytoplasm, where it uncoats, releasing the viral genome and accessory proteins.[27] HIV-1 envelope proteins and regulatory/accessory proteins are encoded by single and multiple spliced mRNAs.[27] The viral enzyme reverse transcriptase transcribes the single-stranded, positive sense RNA genome of HIV-1 into a proviral DNA precursor consisting of both viral and host cellular proteins, that results in the formation of the preintergration complex (PIC). The preintergration complex (PIC) then traffics to the nucleus where the HIV-1 integrase promotes insertion of viral cDNA into the host cell genome.[28]

In the late stage of viral life cycle, Gag and Gag-pol polyprotein precursors are synthesised and transported by an unknown mechanism to the plasma membrane. The gag protein of the retroviruses directs the assembly of the virion particles that are subsequently released from the infected cells.[29] The assembled Gag protein complex induces membrane curvature, leading to direct formation and budding of virus-like particles.[30] Immediately after budding, the viral protease cleaves the Gag and Gag-pol polyprotein precursors to its mature Gag and Pol proteins, resulting in the generation of mature, infectious virions which are now capable of initiating a new round of infection.[31]

1.1.6. HIV transmission and the risk factors.

The primary method of spread of HIV infection worldwide is through sexual exposure. Globally, in the areas of highest HIV prevalence, approximately 70% of the overall sexual transmission occurs through heterosexual intercourse as the primary mode of transmission.[32] Anal intercourse when compared with vaginal intercourse and oral intercourse, carries the highest risk of HIV transmission for both receptive and insertive partners; this may be due to the high density of lymphoid follicle target cells for HIV in rectal mucosa and it is more susceptible to abrasions than the vaginal mucosa.[33] In addition, the risk factor is estimated to be between 5 and 18 times higher than the risk factor from receptive vaginal intercourse.[36,37] Intravenous drug users have high rates of infection with HIV and other blood-borne viruses, such as hepatitis C virus.[36] Risk factors of transmission include unsafe injecting practices, such as injection with a contaminated needle and sharing needles and syringes.[37-39]

More than 3.2 million of HIV-infected positive children worldwide are infected via mother-to-child transmission, which can occur during pregnancy, delivery or breastfeeding.[40] It has been estimated that the probability of vertical transmission during gestation and delivery in the absence of any preventive intervention ranges from 30%-45%.[41] In South Africa, the implementation of an effective national prevention of mother-to-child transmission (PMTCT) programe has, however, decreased the transmission rates from 9.6% in 2008 to 2.8% in 2011.[42]

A number of co-factors affect the risk of vertical transmission through breastfeeding, including duration, and pattern of breastfeeding, maternal breast health, and high HIV viral load. According to the results of two meta-analyses and a randomised control trial, the probability of mother-to-child transmission of HIV through breastfeeding ranges between 9% to 14%.[43] This may be explained by regional differences in breastfeeding practices. Coovadia et al (2007) found that in Kwazulu Natal, South Africa 14.1% of exclusively breastfed infants were infected with HIV-1 by age 6 weeks and 19.1% by 6 months; the risk was significantly associated with maternal CD4 cell counts below 200 cells/µl and birth weight less than 2500g.[44]

1.1.7. Pathogenesis of HIV-1 infection.

Once HIV infection is established, the virus replicates rapidly until viremia – the amount of circulating virus in the host's blood – reaches a peak. This acute phase of HIV-infection persists for several weeks and is characterised by a dramatic change in peripheral CD4 counts and HIV viral load. This is followed by a stage of relative equilibrium between viral replication and the host immune response.[45] At this stage, the immune response against HIV-1 is evident and viremia stabilizes to its set-point.[45] This is followed by a chronic asymptomatic phase that can last for an average of a decade or more, even in the absence of treatment. CD4 cell turnover and viral replication remain active despite the relative clinical latency of HIV-infection.[46] Unless treatment is initiated, most of the HIV-infected individuals will have progressive loss of CD4 lymphocytes and perturbation of immune functions [Figure 4]. At a level below 350 CD4+ T cells/µl of blood, HIV-1 infected patients can experience opportunistic infections.[47,48] During HIV-infection, the rate of CD4 decline correlates with the level of HIV RNA in plasma, genetic and immune functions, co-infections as well as viral genetic variability, while a high viral load in plasma causes the high rapid progression to AIDS.[49]

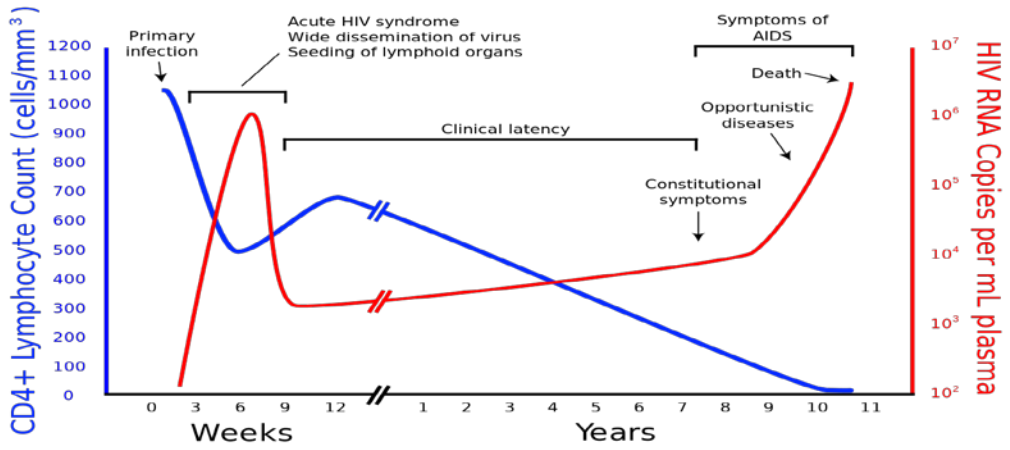

Fig 5: Typical course of HIV infection. During the early period after primary infection there is widespread dissemination of virus and a sharp decrease in the number of CD4 T cells in peripheral blood. An immune response to HIV ensues, with a decrease in viremia followed by a prolonged period of clinical latency. The CD4 T cell count continues to decrease during the following years, until it reaches a critical level below which there is a substantial risk of opportunistic infections and malignancy. Permission to use the figure was provided by Murillo, 2012.[50]

1.1.8. Mechanism of T-cell depletion.

As compared to T-cells from HIV-uninfected individuals, several studies showed that T-cells from HIV-1 infected patients are more prone to cell death.[51] The mechanisms leading to CD4+ T cell depletion are various and involve direct cytopathic effects of HIV-1 replication, killing by cytotoxic T lymphocytes (CTLs), effects of viral proteins and bystander apoptosis [52,54] which will all be discussed in turn.

1.1.8.1 Direct and indirect effect of HIV-1.

Cell death is caused by the disruption of the cell membrane through the process of viral budding and/or by the cellular toxicity induced by the accumulation of RNA/DNA and protein from virus after direct infection of CD4+ T cells by HIV-1.[55] Syncytia are giant multinucleated cells. They rapidly undergo apoptosis through the mitochondrial pathway and are hardly detectable in HIV-1 infected patients. Syncytia are formed by expression of env protein on the surface of infected cells allowing binding to cells expressing CD4 molecules though a virological synapse that leads to cell-to-cell fusion.[56,57] The env protein can induce cell death via different mechanisms which activate the mitochondrial pathway.[58] The Fas/Fas ligand (FasL) system is a key cellular apoptotic pathway that plays a major role in HIV-mediated cell death,[59] and is important in regulation of lymphocyte survival and antigen-induced cell death.[60] Infected cells, through the effect of nef, also up-regulate the expression of FasL, whereas the sensitivity of the cell to Fas-mediated apoptosis is mediated by vpu molecules. The expression of Bcl-2 in primary monocyte-derived macrophage is clearly upregulated by Tat protein. Consequently, an anti-apoptotic response by Bcl-2 may play a significant role in allowing HIV to establish a reservoir of actively replicating virus in the cells of the monocyte/macrophage lineage.[57]

1.1.8.2. Killing by cytotoxic T lymphocytes.

Cytotoxic T lymphocyte (CTLs)-mediated killing of infected cells provide potent defences against virus infection and intracellular pathogens. However, CTLs have a

dark side — their lytic machinery can be directed against self tissues in autoimmune disorders, transplanted cells during graft rejection and host tissues to cause graft-versus-host disease, which is one of the most serious diseases related to CTL function.[61-63] Studies have shown that as soon as 2 to 6 hours after infection, HIV-infected cells become targets for CTLs.[64,65] The transition rate at which HIV-infected cells become recognised as target cells for CTLs is very fast and much higher than the typical decline rate of viral load after drug treatment (HAART).[66] Interestingly, down-regulation of MHC-1 molecules on HIV-infected cells is one of the mechanisms initiated by HIV to partially evade killing by CTLs.[6768] The nef protein mediates the down-regulation of MHC-1 molecule which starts as early as 12 hours post-infection.[64]

1.1.8.3. Bystander apoptosis.

Apoptosis of uninfected bystander cells is a key element of HIV pathogenesis and believed to be the driving force behind the selective depletion of CD4+ T cells leading to immunodeficiency.[69,70] It is evident from studies over the years that direct infection is not sufficient to account for the entire loss of CD4 cells in HIV infections.[71] Some of the key factors that regulate indirect mechanisms of bystander T cell death include the following: syncytium formation between infected and uninfected cells, triggering of apoptotic pathways in uninfected cells by soluble HIV-1 gene products or by infected macrophages expressing Fas-ligand, and cytokine dysregulation, such as overproduction of TNF-α, leading to T cell death.[72,73]

1.1.9. Treatment of HIV/AIDS.

At this time, there is no cure for HIV/AIDS, but medications are effective in suppressing HIV replication and preventing the complications caused by immunodeficiency.[74] In 1986, Yarchoan and his colleagues, discovered the first anti-HIV drug zidovudine (AZT), a nucleoside inhibitor of the reverse transcriptase enzyme. This drug was evaluated in a placebo-controlled clinical trial in patients with late-stage disease [75] in 1987 and revealed 1 death in the AZT treatment group compared to 19 deaths in the placebo group.[76] This trial led to the implementation of

HIV treatment with a single drug, but it soon became evident that the treatment response was short-lived.

The discovery of several classes of antiretroviral drugs resulted in the successful development of combination antiretroviral therapy (cART), also known as highly active antiretroviral therapy (HAART). This therapy has resulted in a dramatic increase in the survival of HIV-infected patients, reductions in opportunistic infections, and morbidity and mortality associated with HIV infection.[77] HAART involves the combination of three or more drugs, most commonly reverse transcriptase inhibitors with or without additional agents. The treatment is highly effective, decreasing viral replication and halting the progression of infection to AIDS and is also associated with partial restoration of the immune system.[78]

There are currently several groups of antiretroviral drugs, targeting viral proliferation and multiplication; these are nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs/NtRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors (FIs), co-receptors inhibitors (CRIs) and integrase inhibitors (INIs). Although these antiretroviral drugs effectively reduce HIV replication, they do not completely cure the disease.[79-81]

The objective of developing drugs that will completely eliminate HIV has faded with the recognition of latently infected T lymphocytes with an extremely long half-life.[82] The existence of these cellular reservoirs prevents the eradication of HIV with currently available antiretroviral therapy. As a result, HAART must be continued indefinitely to maintain virological suppression in HIV-1 infected patients.[83]

1.1.10. HIV treatment failure.

Treatment failure occurs when HAART cannot control the HIV-infection, occurring due to poor adherence to HAART, drug resistance, poor absorption of drugs, inadequate dosing, and drug-drug interactions.[84] It can be categorized as virological failure, immunologic failure, or clinical failure, the latter due to the occurrence of new opportunistic infections.[84] Virologic failure is defined by persistently detectable viral loads exceeding 1000 copies/ml plasma after at least 6 months of using HAART while immunological failure occurs where there is a persistent CD4 level below 100

cell/mm^3 in adults and 200 cell/mm^3 or <10% in children.[85] Limited data exist to confirm the specific threshold to define virological treatment success, (i.e., <50 copies/ml) but the benefits of viremic control are clear.[86]

1.1.11. Drug Resistance.

Unfortunately, the effectiveness of ART can be markedly reduced by the emergence of drug resistance. Drug resistance refers to the ability of HIV to adapt (by mutation) so that the virus can survive and multiply in the presence of drugs that would normally be therapeutically effective.[87] HIV-1 drug resistance is a major factor in the failure of ART.[88] There are two main forms of HIV resistance. Primary or transmitted resistance occurs when an individual is infected with a strain of HIV-1 already resistant to one or more antiretroviral drugs by either a partner or a baby by its mother.[89] Secondary or acquired resistance occurs when resistance develops while on antiretroviral therapy.[90]

Some of the factors that play a role in the rapid and widespread emergence of resistance that is seen during HIV infection are related to the life-cycle and replication of the virus.[87] The HIV reverse transcriptase (RT) enzyme is prone to errors when copying viral RNA into DNA. According to some estimates, HIV RT results in one error in each HIV genome per round of replication.[87,91] translating into roughly 1 mutation for every 2000 nucleosides.[87]

HIV also has an exceptionally high rate of replication. This high rate of replication coupled with the high rate of errors for RT means that numerous HIV “variants” are rapidly formed and propagated.[87] Other factors that may contribute to the development of HIV drug resistance in patients taking antiretroviral treatment include lack of adherence to HIV treatment by patients and insufficient drug levels due to inferior potency, wrong dose, poor absorption, rapid clearance and/or drug interactions.[92]

1.1.12. Immune activation.

In HIV infection the immune response is correlated with the presence of circulating chemokines and anti-inflammatory and pro-inflammatory cytokines, as well as other biochemical markers of immune activation, which differ quantitatively and qualitatively with disease progression.[93-95] Persistent immune activation is hallmarked by activation of various cell types of the innate and adaptive immune system. This occurs, during persistent infection, when the immune system continues to respond to the virus by producing antibodies and activating killer T-cells.[96,97] Persistent immune activation and inflammation, despite sustained HAART-mediated viral suppression, have emerged as a major challenge of the modern HIV treatment era.[98] While immune activation, inflammatory, and coagulation markers typically decline during suppressive HAART, they remain abnormally elevated in many HIV-infected individuals and predict subsequent mortality and non-AIDS morbidities, such as cardiovascular disease.[98]

This persistent activation is likely to be initiated predominantly at the level of innate immunity, particularly involving plasmacytoid dendritic cells (pDC) through Toll-like receptor (TLR) stimulation, involved in the activation of adaptive HIV-specific immune responses (humoral and cellular).[99] Plasmacytoid DC act as effector cells and secrete high levels of mediators of immune activation, enhancing activation of peripheral T cells, B cells, NK cells, NKT cells and myeloid cells, as well as increasing the levels of the proinflammatory cytokines TNF, IL-6, IL-1β and the chemokines MIP-1α, MIP-1β and RANTES, as well as immunosuppressive indoleamine-2,3-dioxygenase and transforming growth factor beta-1 (TGF-β1).[100,101]

These cytokines regulate the function of T cells by directing T cell polarization and up-regulating of expression of MHC class II and other T cell-stimulating molecules. The resultant production of pro-inflammatory cytokines, especially TNF, drives T-cell activation and activation-induced cell death.[102-104] Replication and progression of HIV-1 to AIDS is therefore driven by excessive immune activation.[105]

HIV-associated immune activation was first described in HIV-infected individuals with lymphadenopathy in whom there were increases in the levels of expression of markers of immune activation (HLA-DR, CD38) and proliferation (Ki-67) in both $CD4^+$ and $CD8^+$ T cells. A decline in circulating $CD4^+$ T cell counts and development of

AIDS were associated with the increases in the numbers of HLA-DR$^+$ and CD38$^+$ CD8$^+$ T lymphocytes.[106]

Chronic immune activation with high production of proinflammatory cytokines characterizes the chronic phase of HIV infection,[107,109] and is also responsible for clonal deletion [109] and the loss of peripheral CD4 + T cells over time.[110,111]

Several studies investigated biomarkers of immune activation in HIV infected patients. Malherbe *et al* (2014) who investigated these markers in patients with HIV-1 subtype C, found that there was increased levels of soluble CD14 (sCD14), beta-2 microglobulin (β2M), transforming growth factor beta (TGFβ), CXCL9 and CXCL10 in the HAART naïve study cohort relative to HIV uninfected controls. Soluble CD14 and β2M are markers of cellular activation, while TGFβ is an immunosuppressive cytokine.[112] These findings were consistent with those of others who also found that β2M was positively correlated with viral loads and inversely with CD4%.[10,113] These biomarkers as well as others were also investigated in the study that formed part of this dissertation.

1.1.13. Possible causes of immune activation.

Microbial translocation during HIV infection was first described in 2006, when it was demonstrated that bioactive microbial products were significantly elevated in plasma from HIV-infected individuals.[114] Microbial lipopolysaccharides (LPS) and nucleic acid directly stimulate the activation of both the innate and adaptive immune systems.[114] During the acute phase of HIV-infection, the dysfunction of the intestinal barrier with resultant leakage of microbial products contributes to systemic immune activation[115] and persistently high levels of plasma LPS and bacterial DNA throughout the course of the infection.[116]

As mentioned above, HIV-1 infection induces a barrier defect of the intestinal mucosa, which is closely linked to immune activation and CD4+ T cell depletion.[117-119] Epithelial damage may result from decreased expression of the epithelial repair tight junction gene, thereby increasing epithelial permeability.[115] The gut mucosa is a protective barrier to harmful luminal pathogens, and also plays a major role in the absorption of nutrients. Disruption of this important barrier will therefore lead to

malabsorption and enteropathy[120] The loss of Th17 CD4+ T cells that are normally present in the gut appears to be particularly relevant to the pathogenesis of HIV infection. Microbial translocation and impairment of Th17 function are likely to contribute to chronic immune activation.[121]

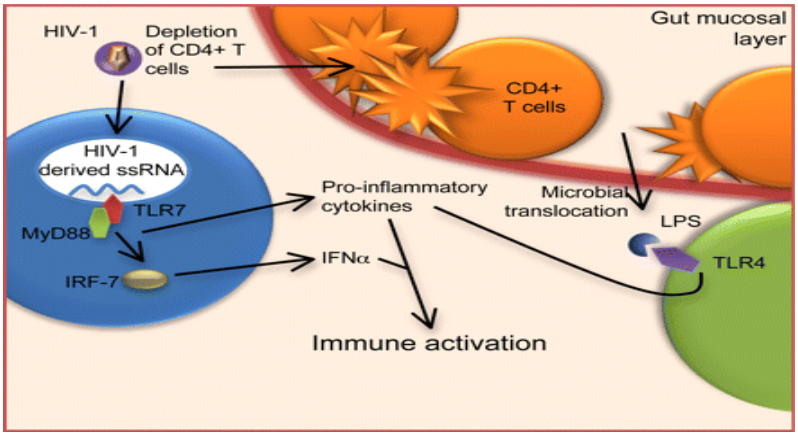

Fig 6. A representation of microbial products (LPS) leaking out of the gut due to a breach in the mucosal barrier that occurs as a result of depletion of CD4+ cells during HIV infection. Permission to use the figure was provided by Chang et al., 2008.[122]

During HIV disease, the development of opportunistic infections may also have an impact on the disease progression to AIDS.[123] Co-infection such as CMV (cytomegalovirus) and HCV (hepatitis C virus) is common in HIV-infected individuals. CMV-specific T cell responses are 3-5 fold higher in HIV-infected individuals than in uninfected individuals.[124-126] Immune activation in HIV-infected patients can be reduced by using valganciclovir treatment, an antiviral medication with action against CMV and other herpes viruses.[127]

A study recently reported by Shaun and colleagues demonstrated that even in virally suppressed individuals, HIV-specific cytotoxic T lymphocytes (CTLs) are inefficient at eliminating the infected CD4+ T cells in which HIV replication occurs.[128] Increases in the production of proinflammatory cytokines (e.g. TNF-α, IL-1, and others) caused by non-antigen specific bystander activation of B- and T-lymphocytes, render them prone to activation-induced cell death by up-regulation of apoptosis related molecules (CD95, TRAIL, DR4/5) on the surface of T-cells.[129,130] Dysfunctionality of the T regulatory cell subset, in comparison with other T-cell subsets, may also favour activation of Th 17 cells.[131,133]

Apart from loss of CD4+ T cells, immune exhaustion also results in impairment of function of effector T cells, which become progressively less responsive, losing both their effector functions and proliferative capability due to the persistence of antigenic stimulation and inflammatory status,[133] resulting in a progressive decrease in several T cell functions such as cytokine secretion, proliferation, and cytotoxic potential, loss of control over viral replication e.g. CMV and EBV (Epstein–Barr virus).[134] The expression of PD-1 (Programmed cell death 1), a marker of immune exhaustion in both CD4+ T and CD8+ T cells, is associated with HIV viral load and loss of CD4+ T cells.[135] A study conducted by Zhang *et al*. [136] has reported that in long-term nonprogressors (LTNPs), who maintain CD4+ T cell counts despite active viral replication, the expression of PD-1 is lower on HIV-specific CD8+ T cells, compared to individuals with progressive HIV-infection.

1.1.14. Consequences of cytokines on HIV-infection.

As mentioned earlier, cytokines are a family of proteins that play a role in inflammatoion and immune activation and in viral infection.[137] In HIV pathogenesis, cytokines play a major role by regulating viral replication as well as orchestrating innate and adaptive immune responses.[137] *In vitro* studies, as well as *in vivo* observations, have identified cytokines as important factors regulating the immunological and virological mechanisms involved in HIV persistence of subjects receiving suppressive HAART, resulting in a failure to completely eradicate HIV.[138] There are two well-described barriers to HIV-eradication: (i) viral replication may occur in an anatomical reservoir inaccessible to antiretroviral drugs such as the gastrointestinal tract, lymph nodes and central nervous system; and (ii) HIV can also persist as proviral DNA in long-lived cellular reservoirs integrated into the host genome.[139,140]

During the course of HIV-1 infection secretion of T-helper type 1 (Th1) cytokines, such as interleukin-2 (IL-2) and antiviral interferon (IFN)-gamma, is generally decreased,[141] whereas production of T helper type 2 (Th2) cytokines, IL-4, IL-10, proinflammatory cytokines (IL-1, IL-6, IL-8) and TNF-α, is increased.[142] Such abnormal cytokine production contributes to the pathogenesis of the disease by impairing cell-mediated immunity. A number of cytokines have been shown to

modulate HIV-1 replication in both CD4 T lymphocytes and cells of macrophage lineage *in vitro*.[143,144] HIV-inducted cytokines include: TNF-alpha, TNF-beta, IL-1 and IL-6, which stimulate HIV-1 replication in T cells and monocyte-derived macrophages (MDM); IL-2, IL-7 and IL-15, which upregulate HIV-1 in T cells; and macrophage-colony stimulating factor, which stimulates HIV-1 replication in MDM. HIV-suppressive cytokines include: IFN-alpha, IFN-beta and IL-16, which inhibit HIV-1 replication in T cells and MDM, and IL-10 and IL-13, which inhibit HIV-1 in MDM.[145,146] Bifunctional cytokines such as IFN-γ, IL-4 and granulocyte-macrophage colony-stimulating factor have been shown to have both inhibitory and stimulatory effects on HIV-1 replication. The beta-chemokines, macrophage-inflammatory protein (MIP)-1α, MIP-1β and RANTES are important inhibitors of macrophage-tropic strains of HIV-1,[147,148] whereas the alpha-chemokine, stromal-derived factor-1, suppresses infection of T-tropic strains of HIV-1.[149]

Immunosuppressive cytokines such as IL-10 and transforming growth factor-beta (TGF-β) secreted by regulatory T cells, NKT cells and myeloid cells, might also contribute to the establishment of viral latency by dampening T cell activation and HIV production, thereby creating the necessary immune-virological conditions for the establishment of latent infection.[150,151]

High levels of the cytokine IL-7, a cytokine that plays a role in homeostasis and survival of CD4+ T cells, may also promote the survival of these cells during HAART.[152] Conversely, proinflammatory cytokines IL-2, TNF-alpha and IL-6 may favour HIV persistence by exacerbating low levels of ongoing viral replication in lymphoid tissues even after prolonged therapy.[153]

1.1.15. Immune activation in HIV-infected children

Both pro-inflammatory and regulatory cytokines and other circulating biomarkers are produced during persistent immune activation in chronic HIV infection. Some of these have been investigated in children, but the data are sparse. Persaud *et al* demonstrated that plasma concentrations of tumour necrosis factor, granulocyte-macrophage colony-stimulating factor, interleukin 1β, IL-2, and IL-8 were substantially higher in perinatally infected children despite being on HAART.

Concentrations of sCD14, a predictor of HIV disease progression and mortality in adults, were also higher in the HIV infected group relative to the perinatally exposed, but uninfected control group.[154] It has also been reported that IL-2 is associated with recovery of CD4 + T cells while HIV viral loads correlated with levels of IFNγ, IL-4 and IL-10 in perinatally infected Asian children receiving HAART.[155] Little is known, however, about the immune activation profiles of children infected with HIV-1 subtype C. Children with this type of HIV-infection were therefore incorporated in the study cohort that was investigated in the current study.

1.1.16. Clinical consequences of immune activation

Effective antiretroviral regimens for the treatment of HIV infection have increased life expectancy, and prolonged survival is accompanied by an increased frequency of non-AIDS related co-morbidities in these individuals.[156] For children that have been perinatally infected, exposure to the virus starts in the uterus and continues through growth, puberty and development.[156] Atherosclerotic cardiovascular disease (CVD) is a leading comorbidity and cause of mortality among HIV-infected adults (2).[156] CVD risk factors such as dyslipidaemia, insulin resistance and central fat redistribution are also observed in HIV-infected children.[156-159] The aetiology of the increased risk for CVD is probably multifactorial, however, there is mounting evidence to support that inflammation and immune activation are likely to be major drivers of atherosclerosis in HIV-infected patients.[160] Chronic immune activation may also play a role in HIV-related complications such as malignancies and neurological diseases.[161-163]

1.1.17. Effect of cigarette smoking on HIV-infection.

Cigarette smoke has been reported to contain >7300 different chemicals including stable and unstable reactive oxygen and reactive nitrogen species, mutagenic hydrocarbons such as benzo[a]pyrene, and other toxicants, including heavy metals.[163] Chronic inhalation of cigarette smoke and consequent unrelenting exposure to these various toxicants result in suppression of critical innate and adaptive host defences in the upper and lower airways due not only to direct

cytotoxicity, but also to oxidative inactivation of intracellular signalling mechanisms. This, in turn, results in increased susceptibility to various respiratory pathogens, especially *Streptococcus pneumoniae* (the pneumococcus) and *Mycobacterium tuberculosis*.[163] Pulmonary host defences which are compromised by exposure to cigarette smoke include: i) the highly orchestrated ciliary beating of ciliated respiratory epithelium; ii) the phagocytic activity of alveolar macrophages; iii) the antigen-presenting functions and anti-viral activities of myeloid and plasmacytoid dendritic cells respectively; iv) the anti-viral and anti-tumour activities of NK and iNKT cells; and v) the protective activities of T and B lymphocytes.[163]

Somewhat paradoxically, however, both HIV infection and cigarette smoking cause systemic activation of circulatory neutrophils, which, in both cases, exacerbates immunosuppression via the increased generation of anti-proliferative reactive oxygen species and release of the granule enzyme, arginase, as well as via upregulation of expression of the programmed cell-death ligand 1 (PD-L1) which induces apoptosis of T cells following interaction with its receptor, PD-1.[163-165] To date it appears that the issue of possible interactive, suppressive effects of smoking and HIV infection on pulmonary host defences have not been addressed.[166] Nonetheless, even in the absence of supporting evidence, it seems reasonable to assume that the combination of HIV and smoking is likely to be particularly ominous with respect to interactive suppression of pulmonary host defences.

With respect to antiretroviral chemotherapy, it is conceivable, though not proven, that smoke-derived mutagens may cause genetic modifications in pro-viral HIV-1 DNA integrated into the genome of the infected host, favouring the emergence of drug-resistant mutants. In the case of antimicrobial chemotherapy, exposure of *Pseudomonas aeruginosa* to cigarette smoke and other environmental mutagens has been reported to promote antibiotic resistance [63,64], while smoking has been reported to promote treatment failure and recurrence of disease in patients with tuberculosis.[167,168] Chronic systemic activation of neutrophils associated with both HIV infection and smoking may also predispose to antiretroviral drug/antibiotic resistance via exaggerated generation of mutagenic reactive oxygen species by neutrophils.[163]

Worldwide, it is well recognised that HIV-infected individuals have higher rates of cigarette smoking than their non-infected counterparts.[163,166] However, in South Africa, the overall prevalence of current smoking in HIV-infected patients has been reported to be 15%, which is similar to the frequency of smoking in the general population in this country.[169] In addition to predisposing to respiratory infections, HIV-infected individuals who smoke are at particularly high risk for the development of non-AIDS defining illnesses, including cardiovascular and pulmonary disorders such as chronic obstructive pulmonary disease, as well as various non-AIDS-related malignancies such as lung cancer.[166] The associated increased morbidity and mortality, even in the face of successful HAART, underscores the magnitude of the threat posed by smoking in the setting of HIV infection. Although the mechanisms which underpin the increased susceptibility of HIV-infected individuals who smoke for development of degenerative diseases and cancer remain uncertain, sustained inflammatory/oxidative stress due to chronic systemic activation of neutrophils may be implicated.

1.2. PURPOSE OF THE STUDY

1.2.1. Objectives

The primary objectives of this study were to investigate and compare:

i. the levels of circulating biomarkers of immune activation in a cohort of mothers (n=46) infected with HIV-1 subtype C relative to those of 20 healthy controls.
ii. the biomarkers between mothers and their HIV-infected children (n=46).
iii. the effects of virologically suppressive and non-suppressive HAART on immune activation profiles in a subgroup of children (n=28).
iv. the effects of active smoking and passive maternal smoking on the biomarkers of immune activation in the mothers and their children, respectively.

CHAPTER 2

MATERIALS AND METHODS

2.1. METHODOLOGY.

2.1.1. Study design.

It was a retrospective study because the immune activation and cigarette smoke determinants were measured on samples that had already been collected.

2.1.2. Study population.

HIV-infected mothers and their infants presenting for care at the Paediatric Immunology Clinic at Kalafong Academic Hospital in Pretoria, South Africa, after failed prevention of mother-to-child transmission (PMTCT) were included in this study. Ethics approval was granted by the Research Ethics Committee of the Faculty of Health Sciences at the University of Pretoria (Ethics Committee Approval No. 159/2009 and 175/2013). All mothers gave informed consent on behalf of themselves and their infants.

Eighty-six mother-infant pairs were recruited at the time infants presented to the clinic for initiation of highly-active antiretroviral treatment (HAART) and followed prospectively for up to 24 months as part of a HIV drug resistance study. Of these, 46 mother-infant pairs had adequate pre-initiation plasma samples for inclusion in this study (named the mother-infant group). Twenty-eight infants had adequate follow-up samples at month 6 (± 2 months) and at month 12 (± 2 months) post-initiation (named the longitudinal group). Children were evaluated at 6 and 12 months for the presence of virological treatment failure, defined as the presence of HIV viral load >1000 copies/ml at two separate occasions at least 4 weeks apart despite adherence counselling. Twenty healthy, HIV-uninfected, age-matched, females were included to serve as controls for the mothers. Due to the difficulty in obtaining blood samples from healthy, HIV-uninfected, age-matched infants, each participant infant served as his or her own control.

The median age of the mothers was 27.3 years (IQR 24.8 – 30.3) and of the controls 26 years (IQR 23.7-32.5). A median of 14 months (IQR 5 – 21) had elapsed between the birth of their infants and enrolment in the study. Thirty-eight mothers had received single-dose nevirapine and 11 had also received AZT (for between 7 and 192 days) as part of PMTCT. Only one mother had been started on HAART

(tenofovir, lamivudine, efavirenz) at the time of study entry. None of the mothers had any signs of active opportunistic infections at the time of study enrolment.

The median age of the infants in the mother-infant group was 12.7 months (IQR 6.8 – 20.7). The male to female ratio was 1:1,47. Thirty-four (74%) infants had received nevirapine after birth (for between 7 and 270 days) and only seven infants had been on formula milk. The infants in the longitudinal group were slightly younger with a median age of 9.8 months (IQR 4.9 – 20.5) and a male to female ratio of 1:1. Twenty-six (93%) infants had received nevirapine after delivery and only 1 had been on formula milk. All infants in the longitudinal group were started on HAART in the form of abacavir, lamivudine and either ritonavir-boosted lopinavir (n=25) or efavirenz (n=3). Fifteen infants started HAART at the time of the first study visit, 12 started with one month of the first study visit, and one only started HAART after 3.8 months. One infant defaulted treatment in the first month and was re-initiated a month later. All infants were on co-trimoxazole prophylaxis and four had a diagnosis of tuberculosis around the time of HAART initiation (between 14 days before and 40 days after HAART).

Whole blood samples were collected in EDTA vacutainers, processed within 24 hours to separate the plasma component by centrifugation, and stored at -70°C for up to 24 months. CD4+ T-lymphocyte counts (CD4+) (Beckman Coulter SA (Pty) Ltd) and HIV-1 RNA (VL) (Nuclisens HIV-1 Viral Load Assay v1.2 or v2.0) were measured by standard flow cytometric and PCR-based procedures respectively, according to manufacturer's instructions. These assays were performed by Ms Gisela van Dyk, Dept Immunology, University of Pretoria.

2.2. METHODS.

2.2.1. Preparation of plasma samples.

Whole blood samples were collected in EDTA vacutainers, processed within 24 hours to separate the plasma component by centrifugation, and stored at -70°C for up to 24 months.

2.2.2. Measurement of biomarkers of inflammation (cytokines/chemokines) in the plasma.

The following cytokines (IL-1β, IL-1 receptor antagonist, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-17, eotaxin, G-CSF, GM-CSF, IFNγ, IP10, MCP-1, MIP-1α, MIP-1β, TNF, MIG and TGF-β) were tested in the plasma of patients and controls using the multiplex bead suspension array assays (Bio-RAD Laboratories Inc, Hercules, California). The Bio-Plex suspension array system employs multiplex technology that used up to 100 colour-coded bead sets, each of which was conjugated with a specific reactant. Each reactant was specific for a different target molecule.

Bio-Plex cytokine assays were designed in a capture sandwich immuno-assay format. Antibody specifically directed against the cytokine of interest was covalently coupled to colour-coded 5.6 µm polystyrene beads. The antibody-coupled beads were allowed to react with a sample containing an unknown amount of cytokine, or with a standard solution containing a known amount of cytokine. After performing a series of washes to remove unbound protein, a biotinylated detection antibody specific for a different epitope on the cytokine was added to the beads. The reaction mixture was detected by the addition of streptavidin-phycoerythrin (streptavidin-PE) (50µl), which binds to the biotinylated detection antibodies. The contents of each well were drawn up into the flow-based Bio-Plex suspension array system, which identifies and quantitates each specific reaction based on bead colour and fluorescence. The magnitude of the reaction was measured using fluorescently labeled reporter molecules associated with each target protein. Unknown cytokine concentrations were automatically calculated by Bio-Plex Manager™ software using a standard curve derived from a recombinant cytokine standard.

The sample for TGF-β measurement was prepared according to manufacture specifications. Briefly, 10µl of 1N hydrochloric acid (HCl) was added to an aliquot of plasma followed by neutralization with 1.2N sodium hydroxide (NaOH)/0.5 HEPES. Seventy five µl of sample diluent was added to make a dilution of 1/16 of the sample.

2.2.3. Measurement of soluble CD14 in plasma.

Human sCD14 was measured utilizing the Abcam ELISA kits (Abcam Cambridge, MA, USA), following the manufacturer's specifications. The sandwich ELISAs employ specific capture antibodies coated on a 96-well plate. Standards and samples were pipetted into the wells; the target protein in the standards and samples binds to the immobilized antibody. The wells were washed and the biotin-labeled detection antibody was then added. After washing away the unbound biotinylated antibodies, HRP-conjugated streptavidin was pipetted into the wells, followed by a colourimetric substrate solution. The intensity of colour development in the wells was proportional to the amount of target protein bound. The concentration of the analytes was calculated using a standard curve that was generated via serial dilutions of the standard (220-6.8ng/mL) concentrations.

2.2.4. Measurement of β2-microglobulin and CRP in plasma.

β2-microglobulin and CRP were analysed using a Dade Behring BNII nephelometer (Siemens Healthcare Diagnostics). Polystyrene particles coated with monoclonocal antibodies to CRP or β2-microglobulin, were agglutinated when mixed with sample containing the analytes. The amount of light scatter detected by the nephelometer was proportional to the concentration of β2-microglobulin or CRP. The concentration of the analytes was calculated using a standard curve that was generated via the serial dilutions of the standard (1.09-2.53 mg/L) concentrations.

2.2.5. Measurement of cotinine in plasma of HIV-infected mothers.

Cotinine levels in the stored plasma samples were measured by using a Calbiotech ELISA (Spring Valley, California). This kit was a solid phase competitive ELISA. The samples and cotinine enzyme conjugate were added to the wells coated with anti-cotinine antibody. Cotinine in the samples competes with a cotinine enzyme (HRP) conjugate for binding sites. Upon addition of the substrate, the intensity of colour was inversely proportional to the concentration of cotinine in the samples. The

concentration of cotinine was calculated using a standard curve that was generated via the serial dilutions of standard (0-100ng/mL) concentrations.

2.3. DATA ANALYSIS AND STATISTICS.

All data followed a non-normal distribution and are presented as the median, minimum, and maximum concentrations (range) for each of the measured cytokines/chemokines, as well as for cotinine. Median concentrations of each parameter were compared between cohorts using the Mann-Whitney test for independent groups. Statistical significance was set at $P \leq 0.05$.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 RESULTS

3.1.1 Demographic data of paired mothers and children at baseline

These data are shown in Table 1 (page 37) for the entire cohort (n=46), as well as for the subgroup (n=33) for which flow cytometric analysis of $CD4^+$ and $CD8^+$ T cells was available. The children had significantly higher $CD4^+$- and $CD8^+$ T cell counts than the mothers, as well as significantly higher HIV viral loads. The CD4% was, however, not significantly different between the two groups.

3.1.2 Comparison of immune activation profiles of mothers and adult controls

These results are shown in Table 2 (page 38). Relative to the control group, the mothers showed significant increases (P=0.0233-P<0.0001) in the concentrations of almost all of the tested circulating biomarkers of immune activation with the exception of MIP-1α. Other cytokines that were also found to be increased in mothers relative to the controls are shown in Table 3 (page 39). These are G-CSF, GM-CSF, IL-2, IL-4, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-17 and eotaxin.

3.1.3 Comparison of immune activation profiles of mothers and their children

These are shown in Table 4 (page 40). Beta 2 microglobulin was found to be significantly increased, while the other markers (IFNγ, TNFα, TGFβ, ILRa) were found to be significantly decreased in the children relative to the mothers. Other cytokines that were also found to be decreased in children relative to the mothers are shown in Table 5 (page 41). These are GM-CSF, IL-2, IL-4, IL-6, IL-9, and IL-17.

Four children with tuberculosis (TB) were identified in the group of children. When their values were omitted from the data analysis, no significant changes were observed.

3.1.4 Longitudinal follow-up of a subgroup of the children.

Twenty eight children were followed longitudinally from baseline to 12 months and stratified thereafter according to treatment response with viral loads below and above 1000 copies/ml, representing virological suppression and non-suppression, respectively. These results are shown in Table 6 (page 42).

Relative to the baseline group, sCD14, beta 2 microglobulin and MIG were decreased in the children on virologically-suppressive antiretroviral therapy at 6 months and 12 months. Soluble CD14 and MIG were significantly increased in the treatment failure group relative to the virologically suppressed group at 6 months and 12 months. Beta 2 microglobulin was significantly decreased in the treatment failure group at 12 months, yet it still reached higher levels than the suppressed group. IP10 was unexpectedly low in the treatment failure group at 6 months. The data for the remaining biomarkers are shown in Table 7 (page 43). No significant differences were observed in these.

Significant increases in the circulating CD4 counts and significant decreases in the HIV viral load were observed in the suppressed groups at 6 and 12 months (Table 6). Omitting the values of the children with TB from the data analysis had no significant effects on the data showed.

3.1.5 The effects of active and maternal smoking on the immune activation profiles of the mothers and their children.

Of the 46 mothers of the study cohort, 10 were smokers (21.7% of study population). The effects of active smoking in this study group are shown in Table 8 (page 44). No significant differences were observed between smokers and non-smokers, except for the CD8 count which was increased in the group of smokers. The effects of maternal smoking on the immune activation profiles of children are shown in Table 10 (page 46). TGFβ was significantly increased in the smoke-exposed children while increased trends, albeit not attaining statistical significance, were observed for sCD14, CRP, MIG, HIV viral loads and the CD8 T cell count. The data for the remaining cytokines are shown in Tables 9 and 11 (page 45 and 47). No significant differences were observed in these.

3.2. Discussion

According to the data, the mothers had relatively low $CD4^+$ T cell counts (median, 330 cells/µl) and a high HIV viral load (median, 61000 copies/ml). With the exception of one, these individuals were not on HAART. The children at baseline had higher $CD4^+$ T cell counts than the mothers (median, 774 cells/µl) - it is however difficult to attribute any significance to these differences as lymphocyte counts in infants are higher than those of adults.[170] The CD4% was, however, not significantly different

between the two groups. The HIV viral load in the children was markedly increased, a finding which is in agreement with previous reports which found that HAART naïve children tend to have higher viral loads relative to those of adults.[171,172] The reason for this may be that their immune system is still relatively immature and less effective in suppressing HIV replication.

Relative to the healthy controls, the HIV-infected, adult women (mothers of the children) showed increases in almost all of the circulating biomarkers of immune activation. The biomarkers that were raised included inflammatory cytokines (IFNγ, TNFα) chemokines (MIG, IP10, MIP-1β), plasma markers of cellular activation (sCD14 and β2-microglobulin), CRP, the anti-inflammatory cytokine, TGFβ, and ILRa. As indicated, several interleukins and colony-stimulating factors were also found to be increased in this cohort. These findings are consistent with those of others, who also found increases in soluble IL2R, soluble TNFR1, neopterin and soluble UPAR (soluble urokinase type plasminogen activator receptor) in HAART naïve, HIV-infected subjects.[10,112,113]

These trends of immune activation were also observed in HIV-infected children. Persaud et al (2014) reported that perinatally HIV-infected children, showed increases in circulating TNF, GM-CSF, IL1-β, IL-2 and IL-8, despite being on HAART. They further stated that sCD14, a predictor of HIV disease progression and mortality in adults, was also increased in this cohort relative to exposed, but HIV-uninfected controls.[154] Another study on children indicated that IL-2 was associated with recovery of CD4 T cells, while HIV viral load correlated with IFNγ, IL-4 and IL-1.[155]

Relative to their mothers, the children at baseline showed significantly higher levels of beta 2 microglobulin. This observation is consistent with a report that this marker is higher in neonates and infants up to one year than in adults.[173] Levels of IFNγ, TNFα, TGFβ, ILRa and several other interleukins were however lower in the children than those of their mothers, which again point to the relative immaturity of the children's immune system. These findings are consistent with those of Lilic et al (1997) who found that in healthy children, cytokine production is decreased or altered when compared to those of adults, and that this may result in suboptimal immune responses and an increased susceptibility to infection.[174]

CD4 counts were significantly increased and viral loads markedly decreased in children who received virologically-suppressive HAART (suppressed groups) at 6 months and 12 months. Children in these groups also showed significantly lower levels of sCD14, beta 2 microglobulin and MIG following HAART. This is consistent with the study of Malherbe et al (2014) who showed that successful HAART was associated with significant decreases in the concentrations of several markers of immune activation including beta2 microglobulin, MIG and IP10.[112] Soluble CD14 and MIG were significantly higher in the treatment failure groups compared to the suppressed groups at the 6 and 12 month time points. Unexpectedly IP10 was very low in the children with treatment failure at 6 months which may be explained by variability in our relatively small study group. According to Malherbe et al (2014) persistently elevated levels of the biomarkers of immune activation in patients failing therapy in the setting of a marked reduction in these markers in patients on successful HAART may not only be useful to monitor immune activation during HAART, but may also distinguish between good and poor responders.[112]

A high percentage of mothers were active smokers (21.7%). This was unexpected as it is well known that Africans, particularly females, are less likely to smoke than their American and European counterparts. A recent South African based-study of Waweru et al, for example, observed that only 7.5% of the African, HIV-infected women were current smokers.[175]

With the exception of an increase in the circulating CD8+ T cell count in smokers as shown by others,[176] no significant differences were observed between the immune activation profiles of active smokers and non-smokers. Associations may have been obscured by the effects of high HIV viral load or by low levels of tobacco exposure. However further studies in a larger patient cohort are necessary. Children exposed to maternal smoking, however, showed significantly increased levels of TGFβ as well as trends, albeit not reaching statistical significance, for increased levels of sCD14, CRP and MIG, as well as higher CD8+ T cell counts and HIV viral loads. TGFβ is an anti-inflammatory cytokine with protective functions; however overexpression of it, may lead to pathogenic manifestations.[181] TGFβ, through its profound and broad inhibitory effects on antiviral defense mechanisms, including a key role in progressive lymphoid tissue fibrosis during HIV-infection, may facilitate more rapid

progression of virus infection resulting in increased in susceptibility to opportunistic infections and malignancies.[177,178]

3.3. Conclusions

Although somewhat lower than those of their mothers, HIV-infected children were found to have high levels of a range of circulating biomarkers of immune activation in the setting of higher viral loads. The concentrations of these biomarkers were differentially affected following administration of HAART to the children, decreasing in some cases, specifically sCD14 and MIG, and associated with viral suppression. With the exception of $CD8^+$ T cell counts, cigarette smoking did not significantly alter the circulating biomarker profiles of the HIV-infected mothers. Maternal smoking was, however, associated with a significant increase in the concentration of immunosuppressive TGF-β1 in the children, while a trend towards increases in the concentrations of sCD14, CRP, MIG, as well as the HIV viral load and circulating $CD8^+$ T cell count, albeit statistically insignificant in all cases, was evident. While seemingly consistent with an adverse effect of maternal smoking on immune activation, interpretation is tempered by the small number of smoke-exposed infants in the study.

Table 1. Demographic data of paired mothers and their children.

	Mothers [Median (Range)] n=46	Children [Median (Range)] n=46	P-value
Age	27.3 years (24.8-30.3)	12.7 months (6.8-20.7)	**<0.0001**
CD4 count (Cells/µl)	330[+] (115-744)	774[++] (66-2,856)	**<0.0001**
CD4 T cells (%)	22[+] (7-37)	21[++] (7-45)	0.8852
CD8 count (Cells/ µl)	740[+] (265-2,942)	1,675[++] (181-6,301)	**<0.0001**
CD4/CD8 ratio	0.4[+] (0.1-1)	0.5[++] (0.1-2)	0.2879
HIV-Viral Load (Copies/ml)	70,000[+] (240-3,400,000) 61,000[⊕] (240-3,400,000)	1180,000[++] (500-26,000,000) 980,000[⊕] (440-50,000,000)	**<0.0034** **<0.0001**

[+]Available data of 33 patients

[++]Available data of 32 patients

[⊕] Data for the whole group

Table 2. Comparison of the circulating biomarkers of immune activation of mothers at baseline and adult controls.

	Mothers Baseline n=46 [Median(Range)]	Adult Controls n=20 [Median(Range)]	P-value
sCD14 (ng/ml)	8,068 (0.00-19,431)	5,971 (853-8,220)	**<0.0011**
β2 M (µg/ml)	2.84 (0.847-6.86)	1.360 (1.02-3.77)	**<0.0001**
CRP (µg/ml)	1.8 (0.173-10.3)	0.8 (0.173-7.5)	**0.0242**
MIG (pg/ml)	2,143 (595-19,734)	190.4 (84.9-2,932)	**<0.0001**
IP10 (pg/ml)	6,866 (2267-64,710)	1,075 (690-12,005)	**<0.0001**
IFNγ (pg/ml)	1924 (510-5,109)	854 (310-2,010)	**<0.0001**
TNFα (pg/ml)	654 (168-1,412)	373 (212-756)	**<0.0001**
MIP-1α (pg/ml)	38 (14-352)	34.96 (26-382)	0.5802
MIP-1β (pg/ml)	166 (75-325)	91.5 (70.23-185)	**<0.0001**
TGF-β (pg/ml)	22,480 (52.8-33,685)	10,960.7 (3,157.6-31,806)	**0.0093**
IL-1Ra (pg/ml)	1,495 (3.6-3,437)	628 (263-1,526)	**<0.0001**

Abbreviations: sCD14 (soluble CD14), β2M (β2-microglobulin), IFNγ (interferon gamma), MIG (monokine induced IFNγ), IP10 (IFN-γ-inducible protein 10), TNFα (tumour necrosis factor α), MIP-1 α and β (macrophage inflammatory protein α and β), TGF-β (transforming growth factor β), IL-1 Ra (IL-1 receptor antagonist).

Table 3. Comparison of the circulating biomarkers of immune activation of mothers at baseline and adult controls.

	Mothers Baseline n=46 [Median(Range)]	Adult Controls n=20 [Median(Range)]	P-value
G-CSF (pg/ml)	765 (375-1,392)	586 (480-1,054)	**<0.0001**
GM-CSF (pg/ml))	148.2 (4.94-397)	8.615 (3.4-168)	**<0.0001**
IL-1β (pg/ml	26.2 (2-8,291)	20.2 (13-8,291)	0.1404
IL-2 (pg/ml)	59 (13.65-135)	41 (2.45-2,342)	**<0.0001**
IL-4 (pg/ml)	27 (9-68)	16.7 (9.5-32.6)	**<0.0001**
IL-6 (pg/ml)	85 (19-1,044)	47 (23-88)	**0.0346**
IL-7 (pg/ml)	82.19 (17-180)	73.77 (47-112)	**0.0051**
IL-8 (pg/ml)	95.23 (27-1,056)	55.31 (38-85)	**<0.0290**
IL-9 (pg/ml)	82 (26-252)	38.3 (18-87)	**<0.0001**
IL-10 (pg/ml)	44.68 (2-155)	28.3 (11.4-56)	**<0.0001**
IL-12 (pg/ml)	132 (4-427)	93.66 (20-161)	**0.0005**
IL-13 (pg/ml)	44.23 (24-200)	28 (19-49)	**0.0022**
IL-17 (pg/ml)	332 (88-576)	222 (62-420)	**<0.0001**
Eotaxin (pg/ml)	595 (393-1,116)	371 (139-790)	**<0.0001**

Abbreviations: G-CSF (granulocyte colony stimulating factor); GM-CSF (granulocyte macrophage colony stimulating factor).

Table 4. Comparison of the circulating biomarkers of immune activation of mothers and children at baseline.

	Mothers Baseline n=46 [Median(Range)]	Children Baseline n=46 [Median(Range)]	P-value
sCD14 (ng/ml)	8,128 (0-19,431)	9,741 (481-20,448)	0.0820
β2 M (µg/ml)	2.84 (0.847--6.86	4 (1.8-7.4)	**<0.001**
CRP (µg/ml)	2 (0.2-79)	5 (0.2-144)	0.0950
MIG (pg/ml)	2,491 (595-19,734)	3,808 (1,058-13,818)	0.7830
IP10 (pg/ml)	6,867 (2,267-64,710)	7,171 (1,054-636,325)	0.8607
IFNγ (pg/ml)	1,924 (510-5,109)	1,207 (211-3,770)	**<0.0001**
TNFα (pg/ml)	654 (168-1,412)	421 (93-1,224)	**<0.0001**
MIP-1α (pg/ml)	38 (14-352)	46 (16-569)	**0.0132**
MIP-1β (pg/ml)	166 (75-325)	163 (46-872)	0.8667
TGF-β (pg/ml)	22,480 (52.8-33,685)	12,627 (67-30,336)	**<0.0001**
IL-1Ra (pg/ml)	1,604 (606-86,729)	904 (201-2,913)	**<0.0001**

Table 5. Comparison of the circulating biomarkers of immune activation of mothers and children at baseline.

	Mothers Baseline n=46 [Median(Range)]	Children Baseline n=46 [Median(Range)]	P-value
G-CSF (pg/ml)	766 (375-1,392)	682 (236-1,751)	0.0873
GM-CSF (pg/ml)	172 (35—24,876)	85 (7-24,236)	**0.0124**
IL-1β (pg/ml)	24 (4-55)	24 (6-89)	0.1780
IL-2 (pg/ml)	61 (14-135)	39 (5-116)	**<0.0001**
IL-4 (pg/ml)	27 (9-68)	22 (5-43)	**<0.0001**
IL-5 (pg/ml)	36 (6-80)	37 (11-83)	0.3027
IL-6 (pg/ml)	84 (19-259)	64 (28-1,012)	**0.0202**
IL-7 (pg/ml)	85 (17-180)	86 (27-240)	0.3045
IL-8 (pg/ml)	95 (27-246)	101 (38-1,231)	0.5348
IL-9 (pg/ml)	82 (25-197)	61 (20-200)	**0.0007**
IL-10 (pg/ml)	48 (7-4,750)	51 (10-148)	0.5713
IL-12 (pg/ml)	139 (8-895)	136 (23-367)	0.6200
IL-13 (pg/ml)	45 (24-200)	47 (20-196)	0.7756
IL-17 (pg/ml)	334 (88-576)	209 (17-462)	**<0.0001**
Eotaxin (pg/ml)	597 (392-1,116)	528 (120-1,011)	0.0796

Table 6. A subgroup of the children at baseline (ART naïve) and children at second visit (6 months) and third visit (12 months) (both on ART) stratified according to treatment failure.

	Baseline children (n=28) [Median (Range)]	Children at 6 months: Virologically suppressive ART (Suppressed) n=20 [Median (Range)]	Children at 6 months: Virologically non-suppressive ART (Failing) n=8 [Median (Range)]	Children at 12 months: Virologically suppressive ART (Suppressed) n=22 [Median (Range)]	Children at 12 months: Virologically non-suppressive ART (Failing) n=6 [Median (Range)]
sCD14 (ng/ml)	9,838 (481-18,591)	7,270 (889-15,466)	11,114 ** 6,062-18,591)	6,669 * (734-13,241)	5,887 (817-12,511)
β2 M (µg/ml)	4 (1.8-8.8)	2 * (1.7-5)	3 (1.9-5)	2.5 * (1.3-4.5)	2.9 * (1.9-4)
CRP (µg/ml)	4 (0.2-144)	1.3 (0.2-42)	1.7 (0.6-49)	1.6 (0.2-43)	9 (0.2-27)
MIG (pg/ml)	4,665 (847-14,037)	1,441 * (611-11,355)	3,289 (269-12,258)	813 * (385-6,081)	2,142 ** (384-11,926)
IP10 (pg/ml)	9,014 (1,054-64,710)	6,023 (1,335-18,749)	2,531 * ** (1,446-7,282)	4,614 (997-16,679)	4,789 (1,567-8,942)
IFNγ (pg/ml)	1,671 (148-3,770)	1,822 * (811-5,108)	1,738 (558-3,668)	1,415 (764-2,491)	1,506 (965-2,336)
TNFα (pg/ml)	527 (81-1,224)	636 (337-1,421)	634 (2,367-1,167)	547 (285-894)	798 (381-1,167)
MIP1α (pg/ml)	39 (9-569)	41 (22-676)	52 (36-256)	40 (22-352)	35 (24-75)
MIP1β (pg/ml)	176 (47-872)	164 (66-872)	173 (100-300)	165 (55-300)	138 (81-226)
TGFβ (pg/ml)	12,489 (67-30,336)	13,419 (39-23,940)	9,198 (149-22,691)	14,117 (1,021-18,364)	14,017 (134-19,422)
IL1 Ra (pg/ml)	1,132 (201-2,913)	1,345 (555-3,375)	1,306 (370-2,404)	1,162 (548-1,874)	1,121 (720-1,888)
CD4 count (cells/µl)	778 (66-2,856)	1,724* (31-6,401)	1,576 (343-2,860)	1,731* (635-3,828)	1,329 (662-3,753)
CD8 count (cells/µl)	1,649 (181-9,764)	1,819 (25-5,984)	2,709 (1,278-6,191)	1,785 (44-2,792)	2,216 (808-6,787)
Viral Load (copies/ml	2600,000 (500-220,000,000)	25* (25-200)	4,100* ** (1,620-3,000,000)	25* (25-320)	195,700 (3,200-8,000,000)

Abbreviations: ART: antiretroviral treatment; Failing: treatment failure

* For comparison with baseline values; p<0.05

** For comparison with the same time-point, virologically suppressed group; p<0.05

Table 7. A subgroup of the children at baseline (ART naïve) and children at second visit (6 months) and third visit (12 months) (both on ART) stratified according to treatment failure.

	Baseline children (n=28) [Median (Range)]	Children at 6 months: Virologically suppressive ART (Suppressed) n=20 [Median (Range)]	Children at 6 months: Virologically non-suppressive ART (Failing) n=8 [Median (Range)]	Children at 12 months: Virologically suppressive ART (Suppressed) n=22 [Median (Range)]	Children at 12 months: Virologically non-suppressive ART (Failing) n=6 [Median (Range)]
G-CSF (pg/ml)	607 (212-1,751)	659 (268-1,268)	765 (428-1,140)	567 (59-773)	681 (333-1,233)
IL-1β (pg/ml)	23 (4-89)	26 (13-793)	28 (13-36)	23 (9-35)	21 (17-41)
IL-2 (pg/ml)	53 (15-9,935)	58 (16-132)	59 (14-108)	45 (16-90)	51 (39-96)
IL-4 (pg/ml)	23 (5-42)	27 (13-65)	30 (12-42)	21 (11-40)	27 (15-37)
IL-5 (pg/ml)	34 (6-80)	38 (11-80)	48 (22-78)	34 (11-58)	36 (18-88)
IL-6 (pg/ml)	62 (18-1,012)	62 (21-1,044)	63 (37-139)	60 (27-96)	64 (28-143)
IL-7 (pg/ml)	73 (17-218)	87 (42-254)	119 (43-204)	74 (30-143)	89 (40-221)
IL-8 (pg/ml)	107 (34-1,231)	129 (35-1,000)	122 (48-216)	97 (41-197)	115 (63-193)
IL-9 (pg/ml)	75 (18-200)	97 (21-304)	105 (39-153)	73 (28-132)	81 (36-135)
IL-10 (pg/ml)	31 (5-138)	36 (15-134)	50 (19-147)	35 (13-100)	28 (16-148)
IL-12 (pg/ml)	79 (3-467)	74 (41-256)	128 (35-285)	93 (26-174)	106 (42-252)
IL-13 (pg/ml)	39 (21-200)	40 (23-78)	59 (22-156)	39 (22-97)	45 (23-84)
IL-17 (pg/ml)	240 (48-30,153)	297 (136-554)	305 (85-470)	237 (93-406)	266 (87-377)
Eotaxin (pg/ml)	570 (224-1,116)	521 (318-890)	581 (373-833)	519 (341-930)	539 (418-740)

Table 8. Effects of active smoking on immune activation profiles of mothers.

	Mothers		P-value
	Non-smokers n=36 [Median (Range)]	Smokers n=10 [Median (Range)]	
sCD14 (ng/ml)	7,893 (1233-19,431)	8,554 (5265-19,431)	0.9575
β2 M (µg/ml)	3 (0.9-7)	3 (0.9-5)	0.5491
CRP (µg/ml)	2 (0.2-10)	3 (0.2-9)	0.6329
MIG (pg/ml)	2,143 (595-19,734)	1,943 (465-5,603)	0.8417
IP10 (pg/ml)	6,342 (2,776-64,710)	7,439 (1,824-24,421)	0.8003
IFNγ (pg/ml)	2,013 (509-9,992)	1,779 (992-2,491)	0.3053
TNFα (pg/ml)	658 (169-1,412)	556 (368-888)	0.1043
MIP1α (pg/ml)	40 (14-199)	32 (25-352)	0.1395
MIP1β (pg/ml)	168 (75-325)	137 (98-235)	0.1096
TGFβ (pg/ml)	20,995 (52.8-33,686)	23,485 (13,153-27,170)	0.2961
IL1 Ra (pg/ml)	1646 (739-3,437)	1,287 (606-1,962)	0.1767
CD4 count (cells/µl)	323 (115-744)[+]	349 (225-573)[++]	0.2523
CD8 count (cells/µl)	696 (267-2,600)[+]	1,383 (316-2,942)[++]	**0.0386**
Viral load (copies/ml)	51,000 (820-3,400,000)	(116,000 (240-520,000)	0.8836

[+]Available data of 26 patients

[++]Available data of 7 patients

Table 9. Effects of active smoking on immune activation profiles of mothers.

	Mothers		P-value
	Non-smokers n=36 [Median (Range)]	Smokers n=10 [Median (Range)]	
G-CSF (pg/ml)	796 (375-1,392)	715 (595-1,387)	0.9999
GM-CSF (pg/ml)	190 (35-24,876)	110 (58-24,876)	0.1101
IL-1β (pg/ml)	27 (4-54)	23 (15-35)	0.2534
IL-2 (pg/ml)	60 (14-134)	57 (39-92)	0.3582
IL-4 (pg/ml)	30 (9-68)	25 (18-41)	0.2929
IL-5 (pg/ml)	36 (6-81)	31 (19-48)	0.3795
IL-6 (pg/ml)	85 (19-1,043)	78 (50-108)	0.3053
IL-7 (pg/ml)	85 (17-180)	67 (45-142)	0.5315
IL-8 (pg/ml)	93 (27-247)	95 (52-149)	0.7900
IL-9 (pg/ml)	85 (26-198)	69 (41-132)	0.2206
IL-10 (pg/ml)	48 (7-155)	33 (20-63)	0.2972
IL-12 (pg/ml)	133 (8-8,954)	139 (70-191)	0.8734
IL-13 (pg/ml)	49 (25-116)	38 (24-200)	0.2749
IL-17 (pg/ml)	333 (87-576)	317 (215-447)	0.7192
Eotaxin (pg/ml)	581 (393-932)	671 (454-1,116)	0.3445

Table 10. Effects of maternal smoking on immune activation profiles of children.

	Children of non-smoking and smoking mothers		P-value
	Non-smokers n=36 [Median (Range)]	Smokers n=10 [Median (Range)]	
sCD14 (ng/ml)	9,615 (481-14,620)	11,924 (5427-20,448)	0.1172
β2 M (µg/ml)	4 (1.8-7.3)	4 (2.5-7)	0.4477
CRP (µg/ml)	4 (0.173-144)	9 (1.9-37)	0.1232
MIG (pg/ml)	3,836 (1,057-13,818)	5,182 (1,639-8,768)	0.4716
IP10 (pg/ml)	7,014 (2,092-636,325)	8,448 (1,054-10,918)	0.8899
TNFα (pg/ml)	400 (92-1,224)	514 (207-762)	0.3465
MIP1α (pg/ml)	46 (16-569)	46 (28-77)	0.8681
MIP1β (pg/ml)	155 (46-872)	183 (155-616)	0.8463
TGFβ (pg/ml)	11,916 (67-22,879)	17,972 (4,932-30,336)	**0.0288**
IL1 Ra (pg/ml)	853 (201-2,913)	963 (501-1,579)	0.4802
CD4 count (cells/µl)	645 (66-2,856)	1,181 (233-2,600)	0.5879
CD8 count (cells/µl)	1,559 (297-5,731)	2,968 (181-6,301)	0.0695
Viral load (copies/ml)	780,000 (440-26,000,000)	1,960,000 (5,200-50,000,000)	0.1580

Table 11. Effects of maternal smoking on immune activation profiles of children.

	Children of non-smoking and smoking mothers		P-value
	Non-smokers n=36 [Median (Range)]	Smokers n=10 [Median (Range)]	
G-CSF (pg/ml)	699 (236-1,751)	665 (382-1,189)	0.3395
GM-CSF (pg/ml)	86 (7-24,876)	84 (23-14,036)	0.9448
IL-1β (pg/ml)	25 (6-89)	20 (17-38)	0.9999
IL-2 (pg/ml)	36 (5-116)	51 (14-60)	0.1178
IL-4 (pg/ml)	22 (5-43)	25 (12-32)	0.4464
IL-5 (pg/ml)	37 (12-83)	36 (19-72)	0.9338
IL-6 (pg/ml)	65 (28-164)	60 (29-1,012)	0.6182
IL-7 (pg/ml)	87 (27-240)	82 (52-221)	0.9558
IL-8 (pg/ml)	100 (37-1,231)	134 (51-261)	0.3756
IL-9 (pg/ml)	59 (20-200)	77 (43-87)	0.2991
IL-10 (pg/ml)	57 (10-147)	43 (19-133)	0.9890
IL-12 (pg/ml)	137 (23-3,670)	128 (49-315)	0.8463
IL-13 (pg/ml)	51 (20-196)	45 (24-130)	0.6378
IL-17 (pg/ml)	207 (17-462)	214 (108-303)	0.5242
Eotaxin (pg/ml)	524 (120-1,011)	545 (418-1,006)	0.5795

LIMITATION AND STRENGTHS

OF THE STUDY

Limitations and strengths of the study

Limitations of the study

Relatively small numbers of HIV-infected mothers who smoke, as well as smoke-exposed infants;

The relative short duration of administration of HAART to the study infants.

Insurmountable difficulty in recruiting a control group of healthy age-matched infants;

In keeping with the previous limitation, an inability to establish reference ranges for the various test biomarkers for infants.

Strengths of study

Given the success of the South African prevention of mother to child transmission program (PMTCT) with MTCT rates now very low, the prevalence of perinatally infected children is very low. In this context this study has relatively large numbers.

Children are difficult to keep in care. Seen in this context, relatively large numbers (n=28) returned for the longitudinal study.

Confirmation of the apparent utility of several biomarkers in monitoring the response to HAART in infants;

The apparent adverse effects of maternal smoking on biomarkers of immune activation in smoke-exposed HIV-infected infants. Although these must be considered as preliminary findings, the current study should serve as a template for future, more definitive studies.

CHAPTER 4

REFERENCES

4.1. REFERENCES.

1. WHO Fact Sheet on HIV/AIDS (updated October 2013). Available at http://wwww.who.int/mediacentre/factsheets/fs360/en/. Accessed 3 April 2014.
2. UNAIDS report on the global AIDS epidemic 2013 (updated October 2013). Available at file:///C:/Users/User/Downloads/AIDS%2013_FactSheet_Global_en.pdf Accessed 6 May 2014.
3. UNAIDS report on the global AIDS epidemic (2010). Available at http://www.unaids.org/globalreport/global_report.htm. Accessed 3 April 2014.
4. WHO/UNAIDS/UNICEF (2011) “Global HIV/AIDS Response: Epidemic update and health sector progress towards Universal Access 2011”. Available at: http://www.avert.org/preventing-mother-child-transmission-hiv.htm#athash.W3bNx55C.dpuf. Accessed 16 April 2014.
5. WHO (2010) "Antiretroviral drugs for treating pregnant women and preventing HIV-infection in infants’ recommendation for a public health approach 2010 version". Available at http://whqlibdoc.who.int/publications/2010/9789241599818_eng.pdf?ua=1. Accessed 22 May 2014.
6. UNAIDS (2012) Global report: "UNAIDS Report on the Global AIDS Epidemic". Available at http://www.unaids.org/en/media/unaids/contentassets/documents/epidemiology/2012/gr2012/20121120_UNAIDS_Global_Report_2012_with_annexes_en.pdf. Accessed 3 April 2014.
7. South Africa National HIV Prevalence, Incident and Behaviour Survey, 2012. Available at http://www.hsrc.ac.za/uploads/pageContent/4565/SABSSM%20IV%20LEO%final.pdf. Accessed 23 April 2014.
8. Worobey M, Telfer P, Souquière S, Hunter M, Coleman CA, Metzger MJ, Reed P, Makuwa M, Hearn G, Honarvar S, Roques P, Apetrei C, Kazanji M, Marx PA (2010). "Island Biogeography Reveals the Deep History of SIV." Science New York **329**: 5998.

9. Hayami M, Ido E, Miurant T (1994). "Survey of simian immunodeficiency virus among non human primate population." Curr Top Microbiol Immunol **188**: 1-20.
10. Nyamwenya S, Towned J, Zaman A, Steele SJ, Jeffries D, Rowland-Jones S, Whittle H, Flanagan KL, Jaye A (2012). "Are plasma biomarkers of immune activation predictive of HIV progression: a longitudinal comparison and analyses in HIV-1 and HIV-2 infections?" PLoS One **7**: e44411.
11. Drylewicz J, Matheron S, Lazaro E, Damond F, Bonnet F, Siomn F, Dabis F, Brun-Vezinet F, Chene G, Thiebaut R (2008). "Comparison of viro-immunological marker changes between HIV-1 and HIV-2- infected patients in France." AIDS **22**: 457-468.
12. Marlink R, Kanki P, Thior I, Travers K, Eisen G, Siby T, Traore I, Hsieh CC, Dia MC, Gueye EH, Hellinger J, Gueye-NDiaye A, Sankale JL, N'Doye I, MBoup S, Essex M (1994). "Reduced rate of disease development after HIV-2 infection as compared to HIV-1." Science New York **265**: 1587-1590.
13. Piot P, Bartos M, (2002). The epidemiology of HIV and AIDS. In: AIDS in Africa, 2nd edn (Essex M, MBoup S, Kanki PJ, Marlink PJ, Tlou SD, Eds). Kluwer Academic/ Plenum Publishers, New York, USA. 200-17
14. Hemelaar JE, Gouws PD, Osmanov S (2006). Global and regional distribution of HIV-1 genetic subtype and recombinants in 2004. AIDS **20**:13-23.
15. Ayouba AS, Souquieres B, Njinku PM, Martin MC, Muller-Trutwin P, Roques F, Barre-Sinoussi P. Mauclere F, Simon, Nerrienet E (2000). HIV-1 group Namong HIV-1-seropositive individuals in Cameroon. AIDS **14**: 2623-2625.
16. Gurtler LJ, Eberle A, von Brunn S, Knapp HP, Hauser L, Zekeng JM, Tsague E, Selegny, Kaptue (1994). A new subtype of human immunodeficiency virus type 1 (MVP-5180) from Cameroon. J. Virol. **68**: 1581-1585.
17. McCutchan FE (2000). "Understanding the genetic diversity of HIV-1." AIDS **14**: S31-44.
18. Janssens WL, Heyndrickx K, Fransen J, Motte M, Peeters JN, Nkengasong PM, Ndumbe E, Delaporte JL, Perret C, Atende P, Van der Groen G (1994). "Genetic and phylognetic analysis of env subtype G and H in Central Africa." AIDS Res. Hum Retrovir **10**: 877-879.

19. Simon F , Mauclere P, Roques P, Loussert-Ajaka I, Muller-Trutwin MC, Saragosti S, Georges-Courbot MC, Barré-Sinoussi B, Brun-Vézinet F (1998). "Identification of a new human immunodeficiency virus type 1 distinct from group M group O." Nature Medicine **4**: 1032-1037.
20. Gordon M, Den OT, Bishop K, Coovadia HM, Madurai L, Engelbrecht S, Janse van Rensburg E, Mosam A, Smith A, Cassol S (2003). "Molecular characteristics of human immunodeficiency virus type-1 subtype C viruses from Kwazulu-Natal, South Africa: Implications for vaccine and antiretroviral control strategies." J Virol **4**: 2587-2599.
21. Bukrinsky MI, Haggerty S, Dempsey MP, Sharova N, Adzhubel A, Spitz L, Lewis P, Goldfarb D, Emerman M, Stevenson M. (1993). "A nuclear localization signal within HIV-1 matrix protein that govern infection of non-diving cells." Nature Medicine **365**: 666-668.
22. Coffin JM (1992). "Structure and classification of retroviral. In: The retroviridae Levy JA, ed." Plenum press New York **1**: 19-50.
23. Levy JA (1993). "Pathogenesis of human immunodeficiency virus infection" Micrbiol Rev **57**: 183-289.
24. Huang H, Chopra R, Verdine GL, Harrison SC (1998). "Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance" Science **282**: 1669–1675.
25. Chan DC, Fass D, Berger JM, Kim PS (1997). "Core structure of gp41 from the HIV envelope glycoprotein." Cell **89**: 263-273.
26. Chan DC, Kim PS. (1998). "HIV entry and its inhibition." Cell press **89**: 681-684.
27. Collins DR, Collins K. (2014). "HIV-1 accessory proteins adapt cellular adaptors to facilitate immune evasion." PLoS Pathogens **10**: e1003851.
28. Telesnitsky A, Goff S. (1997). "Reverse transcriptase and the generation of retroviral DNA. In: Reverse transcription and evolution in: Reverse transcriptase. Coffin J.M., Hughes SH. and Varmus HE. (ed)." Cold Spring Harbor Laboratory Press, New York.
29. Granowitz C and Goff SP **(**1994). "Substitution mutations affecting a small region of the Moloney murine leukemia virus MA gag protein block assembly and release of virion particles". Virology **205:** 336-344.

30. Braaten D, Aberham C, Franke EK, Yin L, Phares W, Luban J (1996). "Cyclosporine A-resistant human immunodeficiency virus type 1 mutants demonstrate that Gag encodes the functional target of cyclophilin A" J Virol **70**: 5170–5176.
31. Bukrinskaya AG, Ghorpade A, Heinzinger NK, Smithgall TE, Lewis RE, Stevenson M (1996). "Phosphorylation-dependent human immunodeficiency virus type 1 infection and nuclear targeting of viral DNA" Proc Natl Acad Sci USA **93**: 367–371.
32. Gayle H (2000). "An overview of the global HIV/AIDS epidemic, with a focus on the United States" Aids **2**: S8-17.
33. Levy JA (1993). "The transmission of HIV and factors influencing progression to AIDS". Am J Med **95**: 86-100.
34. Varghese B, Maher JE, Peterman TA, Branson BM, Steketee RW (2002). "Reducing the risk of sexual HIV transmission: Quantifying the per-act risk for HIV on the basis of choice of partner, sex act, and condom use". Sex Transm Dis **29**: 38-43.
35. Grulich AE, Zablotska I (2010). "Commentary: Probability of HIV transmission through anal intercourse". Int J Epidemiol **39**: 1064-5.
36. World Health Organization, United Nations Office on Drugs and Crimes, Joint United Nations Programme on HIV/AIDS (2008). "Policy guidelines for collaborative TB and HIV services for injecting and other drug users: An integrated approach". Geneva, Switzerland: Available at http://whqlibdoc.who.int/publications/2008/9789241596930_eng.pdf. Accessed 11 August 2014.
37. Baggaley RF, Boily MC, White RG, Alary M (2006). "Risk of HIV-1 transmission for parenteral exposure and blood transfusion: A systematic review and meta-analysis". AIDS **20**: 805-12.
38. Bruneau J, Daniel M, Abrahamowicz M, Zang G, Lamothe F, Vincelette J (2011). "Trends in human immunodeficiency virus incidence and risk behavior among injection drug users in Montreal, Canada: A 16-year longitudinal study". Am J Epidemiol **173**: 1049-58.
39. Santibanez SS, Garfein RS, Swartzendruber A, Purcell DW, Paxton LA, Greenberg AE (2006). "Update and overview of practical epidemiologic

aspects of HIV/AIDS among injection drug users in the United States". J Urban Health **83**: 86-100.

40. UNAIDS report on the global AIDS epidemic (2013). Available at http://www.who.int/hiv/data/epi_core_dec2014.png?ua=1. Accessed 21 July 2014.
41. Lehman DA and Farquhar C (2007). "Biological mechanisms of vertical human immunodeficiency virus (HIV-1) transmission". Rev Med Virol **17**: 381-403.
42. Barron P, Pillay Y, Doherty T, Sherman G, Jackson D, Bhardwaj S, Robinson P, Goga A (2012). "Eliminating mother-to-child HIV transmission in South Africa." Bull World Health Organ **91**: 70-74.
43. Nduati R, John G, Mbori-Ngacha D, Richardson B, Overbaugh J, Mwatha A, Ndinya-Achola J, Bwayo J, Onyango FE, Hughes J, Kreiss J (2000). "Effect of breastfeeding and formula feeding on transmission of HIV-1: a randomized clinical trial". JAMA **283**: 1167-74.
44. Coovadia HM, Rollins NC, Bland RM, Little K, Coutsoudins A, Bennish ML, Newell ML (2007). "Mother-to-child transmission of HIV-1 infection during exclusive breastfeeding in the first 6 months of life: an intervention cohort study". Lancet **369**: 1107-16.
45. Castro KG, Ward JW, Slutsker L, Buehler JW, Jaffe HW, Berkelman RL (1993). "Revised classification-system for HIV-Infection and expanded surveillance case-definition for AIDS among adolescents and adults." Clinical Infect Dis **17**: 802-810.
46. Neumann AU, Ho DD, Perelson AS, Chen W, Leornard JM, Markowitz M (1995). "Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection." Nature Medicine **373**: 123-126.
47. Lefrene JJ, Morand-Joubert L, Mariotti M, Bludau H, Burghoffer B, Petit JC, Roudot-Thoraval F (1997). "Even individuals considered as long-term nonprogressors show biological signs of progression after 10 years of human immunodeficiency virus infection." Blood **90**: 1133-1140.
48. Balotta C, Bagnarelli P, Riva C, Valenza A, Antinori S, Colombo MC, Sampaolesi R, Violin M, de Pasquale MP, Moroni M, Clementi M, Galli M (1997). "Comparable biological and molecular determinants in HIV type 1-

infected long-term nonprogressors and recently infected individuals." AIDS Res. Hum Retrovir **13**: 337-341.

49. O'Brien TR, Blattner W, Waters D, Eyster E, Hilgartner MW, Cohen AR, Luban N, Hatzakis A, Aledort LM, Rosenberg PS, Miley WJ, Kroner BL, Goedert JJ (1996). "Serum HIV-1 RNA levels and time to development of AIDS in the Multicenter Hemophilia Cohort Study." JAMA **276**:105-10.
50. Murillo W (2012). "Drug resistance and molecular epidemiology" Karolinska Institutet **9**: 1-39.
51. Gougeon ML, Lecoeur H, Dulioust A, Enouf MG, Crouvoiser M, Goujard C, Debord T, Montagnier L. (1996). "Programmed cell death in peripheral lymphocytes from HIV-infected persons: increased susceptibility to apoptosis of CD4 and CD8 T cells correlates with lymphocyte activation and with disease progression." J Immunol **156**: 3509-3520.
52. Meyaard L, Otto S, Keet IP, Roos MT, Miedema F (1992). "Programmed death of T cells in HIV-1 infection." Science New York **257**: 217-219.
53. Groux H, Torpier G, Monté D, Mouton Y, Capron A, Ameisen JC (1992). "Activation-induced death by apoptosis in CD4+ T cells from human immunodeficiency virus-infected asymptomatic individuals." J Exp Med **175**: 331-340.
54. Badley AD, Pilon A, Landay A, Lynch DH. (2000). "Mechanisms of HIV-associated lymphocyte apoptosis." Blood **96**: 2951-2964.
55. Varbanov M, Espert L, Biard-Piechaczyk M (2006). "Mechanisms of CD4 T-cell depletion triggered by HIV-1 viral proteins." AIDS Rev **8**: 221-236.
56. Siegel RM (2006). "Caspases at the crossroads of immune-cell life and death." Nat Rev Immunol **6**: 308-317.
57. Alimonti JB, Ball T, Fowke KR (2003). "Mechanisms of CD4+ T lymphocyte cell death in human immunodeficiency virus infection and AIDS." J Gen Virol **84**: 1649-1661.
58. Perfettini JL, Castedo M, Roumier T, Andreau K, Nardacci R, Piacentini M, Kroemer G. (2005). "Mechanisms of apoptosis induction by the HIV-1 envelope." Cell Death Differ **12**: 916-923.
59. Katsikis PD, Wunderlich ES, Smith CA, Herzenberg LA, Herzenberg LA (1995). “Fas antigen stimulation induces marked apoptosis of T lymphocytes

in human immunodeficiency virus–infected individuals." J Exp Med **181**: 2029–2036.

60. Nagata S (1997). "Apoptosis by death factor." Cell **88**: 355–365.
61. Kaslow RA, Carrington M, Apple R, Park L, Muñoz A, Saah AJ, Goedert JJ, Winkler C, O'Brien SJ, Rinaldo C, Detels R, Blattner W, Phair J, Erlich H, Mann DL (1996). "Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection." Nature Medicine **2**: 405–411.
62. Kim EY, Veazey R, Zahn R, McEvers KJ, Baumeister SH, Foster GJ, Rett MD, Newberg MH, Kuroda MJ, Rieber EP, Piatak M Jr, Lifson JD, Letvin NL, Wolinsky SM, Schmitz JE. (2008). "Contribution of CD8+ T cells to containment of viral replication and emergence of mutations in Mamu-A*01-restricted epitopes in Simian immunodeficiency virus-infected rhesus monkeys." J Virol **82**: 5631–5635.
63. Leslie A, Mattews P, Listgarten J, Carlson JM, Kadie C, Ndung'u T, Brander C, Coovadia H, Walker BD, Heckerman D, Goulder PJR (2010). "Additive contribution of HLA class I alleles in the immune control of HIV-1 infection." J Virol **84**: 9879–9888.
64. Sacha JB, Chung C, Rakasz EG, Spencer SP, Jonas AK, Bean AT, Lee W, Burwitz BJ, Stephany JJ, Loffredo JT, Allison DB, Adnan S, Hoji A, Wilson NA, Friedrich TC, Lifson JD, Yang OO, Watkins DI. (2007). "Gag-specific CD8+ T lymphocytes recognize infected cells before AIDS-virus integration and viral protein expression." J Immunol **178**: 2746–2754.
65. Sacha JB, Buechler M, Newman LP, Reed J, Wallace LT, Loffredo JT, Wilson NA, Watkins DI (2010). "Simian immunodeficiency virus-specific CD8+ T cells recognize Vpr- and Rev-derived epitopes early after infection." Proc Natl Acad Sci U SA **106**: 9791–9796.
66. Sacha JB, Giraldo-Vela. JP., Buechler MB, Martins MA, Maness NJ, Chung C, Wallace LT, León EJ, Friedrich TC, Wilson NA, Hiraoka A, Watkins DI. (2009). "Gag- and Nef-specific CD4+ T cells recognize and inhibit SIV replication in infected macrophages early after infection." Proc Natl Acad Sci USA **106**: 9791–9796.

67. Collins K, Chen B, Kalams S, Walker B, Baltimore D (1998). "HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes." Nature Medicine **391**: 397–401.
68. Swigut T, Alexander L, Morgan J, Lifson J, Mansfield KG, Lang S, Johnson RP, Skowronski J, Desrosiers R. (2004). "Impact of Nef-mediated downregulation of major histocompatibility complex class I on immune response to simian immunodeficiency virus." J Virol **78**: 13335–13344.
69. Hurtrel B, Petit F, Arnoult D, Muller-Trutwin M, Silvestri G, Estaquier J (2005). "Apoptosis in SIV infection." Cell Death Differ **12**: 979–990.
70. WHO (2013). "Global update on HIV treatment 2013: results, impact and opportunities". Available at http://apps.who.int/iris/bitstream/10665/85326/1/9789241505734_eng.pdf. Accessed 17 June 2013.
71. Yarchoan R, Klecker R, Weinhold KJ, Markham PD, Lyerly HK, Durack DT, Gelmann E, Lehrman SN, Blum RM, Barry DW (1986). "Administration of 3´-azido-3´-deoxythymidine, an inhibitor of HTLV-III/LAV replication, to patients with AIDS or AIDS-related complex." Lancet **1:** 575-580.
72. Fischl MA, Richman D, Grieco MH, Gottlieb MS, Volberding PA, Laskin OL, Leedom JM, Groopman JE, Mildvan D, Schooley RT (1987). "The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. A double-blind, placebo-controlled trial." N Engl J Med **317**: 185-191.
73. Gulick MD, Roy M, John W MPH, Mellors MD, Diane Havlir MD, Joseph J, Eron MD, Charles Gonzalez MD, Deborah Mcmahon, MD, Douglas D, Richman MD, Fred T. Valentine MD, Leslie Jonas BS, Anne Meibohm PHD, Emilio A, Emini PHD, Jeffrey A, Chodakewitz MD (1997). "Zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy." N Engl J Med **11**: 734-739.
74. Walensky RP, Paltiel A, Losina E, Mercincavage LM, Schackman BR, Sax PE, Weinstein MC, Freedberg KA. (2006). "The survival benefits of AIDS treatment in the United States." J Infect Dis **192**: 11-19.
75. King Jr JT, Justice A, Roberts MS, Chang CC, Fusco JS (2003). "Collaboration in HIV outcomes research – U.S. Program Team. Long-term

HIV-AIDS survival estimation in the highly active antiretroviral therapy era " Med. Decis. Making **23**: 9–20.

76. Kitchen CM, Kitchen S, Dubin JA, Gottieb MS. (2001). "Initial virological and immunologic response to highly active antiretroviral therapy predicts long-term clinical outcome" Clin. Infect. Dis **33**: 466–472.
77. Valenti WM (2001). "HAART is a cost-effective and improves outcomes" AIDS Reader **11**: 260–262.
78. Chun TW, S. L., Mizell SB, Ehler LA, Mican JA, Baseler M, Lloyd AL, Nowak MA, Fauci AS. (1997). "Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy." Proc Natl Acad Sci USA **94**: 13193-13197.
79. Siliciano JD , D Finzi J. K., Quinn TC, Chadwick K, Margolick JB, Kovacs C, Gange SJ, Siliciano RF (2003). "Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells." Nat Med **9**: 727-728.
80. Aldous JL, Haubrich R (2009). "Defining treatment failure in resource-rich setting." Curr Opin HIV AIDS **4**: 459-466.
81. WHO (2013). "Definitions of clinical, immunological and virological failure for the decision to switch ART regimens". Available at http://www.who.int/hiv/pub/guidelines/arv2013/art/WHO_CG_table_7.15.pdf. Accessed 17 June 2014.
82. Hatano H, Hunt P, Weidler J, Coakley E, Hoh R, Liegler T, Martin JN, Deeks SG. (2006). "Rate of viral evolution and risk of losing future drug options in heavily pre-treated, HIV-infected patients who continue to receive a stable, partially suppressive treatment region." Clin Infect Dis **43**: 1329-1336.
83. Zdanowicz MM (2006). "The Pharmacology of HIV Drug Resistance." Am J Pharm Educ. **70**: 100.
84. Tobin N, Frenkel L (2002). "Human immunodeficiency virus drug susceptibility and resistance testing." Pediatr Infect Dis J **21**: 668–683.
85. Goh WC, Markee J, Akridge RE, Meldorf M, Musey L, Karchmer T, Krone M, Collier A, Corey L, Emerman M, McElrath MJ. (1999). "Protection against human immunodeficiency virus type 1 infection in persons with repeated exposure: evidence for T cell immunity in the absence of inherited CCR5 coreceptor defects." J Infect Dis **179**: 548–557.

86. Easterbrook PJ (1999). "Long-term non-progression in HIV infection: definitions and epidemiological issues." J Infect Dis **38**: 71–73.
87. Preston BD, Poiesz B, Loeb LA (1988). "Fidelity of HIV-1 reverse transcriptase." Science New York **242**: 1168-1171.
88. Martinez-Picado J, DePasquale MP, Kartsonis N, Hanna GJ, Wong J, Finzi D, Rosenberg E, Gunthard HF, Sutton L, Savara A, Petropoulosi CJ, Hellmanni N, Walker BD, Richman DD, Siliciano R, D'Aquila RT (2000). "Antiretroviral resistance during successful therapy of HIV type I infection." Proc Natl Acad Sci USA **97**: 10948–10953.
89. Barqasho B1, Nowak P, Tjernlund A, Kinloch S, Goh LE, Lampe F, Fisher M, Andersson J, Sönnerborg A (2009). "Kinetics of plasma cytokines and chemokines during primary HIV-1 infection and after analytical treatment interruption." HIV Medicine **10**: 94–102.
90. Roberts L, Passmore J, Williamson C, Little F, Bebell LM, Mlisana K, Burgers WA, van Loggerenberg F, Walzl G, Djoba Siawaya JF, Karim QA, Karim SS. (2010). "Plasma cytokine levels during acute HIV-1 infection predict HIV disease progression." AIDS **24**: 819–831.
91. Shebl FM, Yu K, Landgren OO, Goedert JJ, Rabkin CS (2012). "Increased levels of circulating cytokines with HIV-related immunosuppression." AIDS Res. Hum Retrovir **28**: 809–815.
92. Meier A, Alter G, Frahm N, Sidhu H, Li B, Bagchi A, Teigen N, Streeck H, Stellbrink HJ, Hellman J, Lunzen J, Altfeld M (2007). "MyD88-dependent immune activation mediated by human immunodeficiency virus type 1-encoded Toll-like receptor ligands." J Virol **81**: 8180-8191.
93. Vabret N, Bailly-Bechet M, Najburg V, Muller-Trutwin M, Verrier B, Tangy F (2012). "The biased nucleotide composition of HIV-1 triggers type 1 interferon response and correlates with subtype D increased pathogenicity." PLoS One **7**: e33502.
94. Hunt PW (2012). "HIV and Inflammation: Mechanisms and Consequences." Curr HIV/AIDS Rep. **9**: 139-147.
95. Megjugorac NJ, Young H, Amrute SB, Olshslsky SL, Fitzgerald-Bocarsly P (2004). "Virally stimulated plasmocytoid dendritic cells produce chemokines and induce migration of T and NK cells." J Leukoc Biol **75**: 504-514.

96. French MA, King MS, Tschampa JM, da Silva BA, Landay AL (2009). "Serum immune activation markers are persistently increased in patients with HIV infection after 6 years of antiretroviral therapy despite suppression of viral replication and reconstitution of CD4+ T cells." J Infect Dis **200**: 1212–1215.

97. Appay V, Sauce D (2008). "Immune activation and inflammation in HIV-1 infection: causes and consequences." J Pathol **214**: 231–241.

98. Herbeuval JP, Grivel JC, Boasso A, Hardy AW, Chougnet C, Dolan MJ, Yagita H, Lifson JD, Shearer GM (2005). "CD4+ T-cell death induced by infectious and non-infectious HIV-1: role of type 1 interferon-dependent, TRAIL/DR5-mediated apoptosis." Blood **106**: 3524-3531.

99. Cassol E, Malfeld S, Mahasha P, van der Merwe S, Cassol S, Seebregts C, Alfano M, Poli G, Rossouw T (2010). "Persistent microbial translocation and immune activation in HIV-1 infected South Africans receiving combination antiretroviral therapy." J Infect Dis **202**: 723-733.

100. Collini P, Noursadeghi M, Sabroe I, Miller RF, Dockrell DH (2010). "Monocyte and macrophage dysfunction as a cause of HIV-1 induced dysfunction of innate immunity." Curr Mol Med **10**: 727-740.

101. Hazenberg MD, Otto S, van Benthem BH, Roos MT, Coutinho RA, Lange JM, Hamann D, Prins M, Miedema F. (2003). "Persistent immune activation in HIV-1 infection is associated with progression to AIDS." AIDS **17**: 1881-1888.

102. Giorgi JV, Detels R (1989). "T-cell subset alteration in HIV-infected homosexual men: AIDS Multicenter AIDS cohort study." Clin Immunol Immunopathol **52**: 10-18.

103. Cossarizza A, Ortolani C, Mussini C, Borghi V, Guaraldi G, Mongiardo N, Bellesia E, Franceschini MG, De Rienzo B, Franceschi C. (1995). "Massive activation of immune cells with an intact T cell repertoire in acute human immunodeficiency virus syndrome." J Infect Dis **172**: 105–112.

104. Norris PJ, Pappalardo BL, Custer B, Spotts G, Hecht FM, Busch MP (2006). "Elevations in IL-10, TNF-alpha, and IFN-gamma from the earliest point of HIV Type 1 infection." AIDS Res Hum Retrovir **22**: 757–762.

105. Picker LJ (2006). "Immunopathogenesis of acute AIDS virus infection." Curr Opin Immunol **18**: 399–405.

106. Cossarizza A, Mussini C, Mongiardo N, Borghi V, Sabbatini A, De Rienzo B, Franceschi C. (1997). "Mitochondria alterations and dramatic tendency to

undergo apoptosis in peripheral blood lymphocytes during acute HIV syndrome" AIDS **11**: 19–26.

107. Franceschi C, Franceschini MG, Boschini A, Trenti T, Nuzzo C, Castellani G, Smacchia C, De Rienzo B, Roncaglia R, Portolani M, Pietrosemoli P, Meacci M, Pecorari M, Sabbatini A, Malorni W, Cossarizza A. (1997). "Phenotypic characteristics and tendency to apoptosis of peripheral blood mononuclear cells from HIV+ long term non progressors." Cell Death Differ **4**: 815–823.
108. Malherbe G, Steel HC, Cassol S, De Oliveira T, Seebregts CJ, Anderson R, Cassol E, Rossouw TM (2014). "Circulating biomarkers of immune activation distinguish viral suppression from nonsuppression in HAART-treated patients with advanced HIV-1 subtype C infection". Mediators Inflamm 10:198413.
109. Kamat A, Misra V, Cassol E, Ancuta P, Yan Z, Li C, Morgello S (2012). "A Plasma Biomarker Signature of Immune Activation in HIV Patients on Antiretroviral Therapy". PLoS ONE **7**: e30881
110. Brenchley JM, Prince DA, Schacker TW, Asher TE, Silvestri G, Rao S, Kazzaz Z, Bornstein E, Lambotte O, Altmann D, Blazar BR, Rodriguez B, Teixeira-Johnson L, Landay A, Martin JN, Hecht FM, Picker LJ, Lederman MM, Deeks SG, Douek DC (2006). "Microbial translocation is a cause of systemic immune activation in chronic HIV infection." Nature Medicine **12** 1365–1371.
111. Estes JD, Harris L, Klatt NR, Tabb B, Pittaluga S, Paiardini M, Barclay GR, Smedley J, Pung R, Oliveira KM, Hirsch VM, Silvestri G, Douek DC, Miller CJ, Haase AT, Lifson J, Brenchley J.M (2010). "Damaged intestinal epithelial integrity linked to microbial translocation in pathogenic simian immunodeficiency virus infections." PLoS Pathogens **6** e1001052.
112. Wallet MA, Rodriguez C., Yin L, Saporta S, Chinratanapisit S, Hou W, Sleasman JW, Goodenow MM. (2010). "Microbial translocation induces persistent macrophage activation unrelated to HIV-1 levels or T-cell activation following therapy." AIDS **24**: 1281–1290.
113. Guadalupe M, Reay E., Sankaran S, Prindiville T, Flamm J, McNeil A, Dandekar S. (2003). "Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial

delay in restoration following highly active antiretroviral therapy." J Virol **77**: 11708-11717.

114. Li Q, Dual L, Estes JD, Ma ZM, Rourke T, Wang Y, Reilly C, Carlis J, Miller CJ, Haase AT. (2005). "Peak SIV replication in resting memory CD4+T cells depletes gut lamina propria CD4+T cells." Nature **434**: 1148-1152.
115. Mattapallil JJ, Douek D., Hill B, Nishimura Y, Martin M, Roederer M. (2005). "Massive infection and loss of memory CD4+T cells in multiple tissues during acute SIV infection." Nature **434** 1093-1097.
116. Mhiri C, Belec L., Di Costanzo B, Georges A, Gherardi R. (1992). "The slim disease in African patients with AIDS." Trans R Soc Trop Med Hyg **86**: 303-306.
117. Paiardini M, Frank I., Pandrea I, Apetrei C, Silvestri G. (2008). "Mucosal immune dysfunction in AIDS pathogenesis." AIDS Rev **10**: 36-46.
118. Chang J, Altfeld, M (2008). "TLR-mediated immune activation in HIV" Blood **113** 269-270.
119. Saravolatz L, Neaton J, Sacks L, Deyton L, Rhame F, Sherer R (1996). "CD4+ T lymphocyte counts and patterns of mortality among patients infected with human immunodeficiency virus that were enrolled in community programs for clinical research on AIDS." Clin Infect Dis **22**: 513-520.
120. Deeks, S.G (2011). “The pathogenesis of persistent HIV-associated inflammation during long-term antiretroviral therapy”, 6th IAS Conference on HIV Pathogenesis, Treatment, and Prevention, Rome, Italy. Available at http://pag.ias2011.org/session.aspx?s=91. Accessed 17-20 July 2011.
121. Buzón J, Massanella M, Llibre M, Esteve J, Dahl A, Puertas V, Gatell M, Domingo J, Paredes P, Sharkey R, Palmer M, Stevenson S, Clotet M, Blanco B, Martinez-Picado J (2010). "HIV-1 replication and immune dynamics are affected by raltegravir intensification of HAART-suppressed subjects" Nature Medicine **16**: 460-465.
122. Sylwester, A. (2005). "Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects " J Exp Med **202**: 673-685.
123. Schacker, T (2011). "Chronic Inflammation in HIV Disease". CROI, Boston, Massachusetts, Hynes Convention Centre. Available at

http://retroconference.org/2011/data/files/webcast_2011.htm. Accessed 1 May 2014.

124. Shan L, Deng K, Shroff NS, Durand CM, Rabi SA, Yang HC, Zhang H, Margolick JB, Blankson JN, Siliciano RF. (2012). "Stimulation of HIV-1-specific cytolytic T lymphocytes facilitates elimination of latent viral reservoir after virus reactivation." Immunity **36**: 491–501.

125. Estaquier J, Idziorek T, De Bels F, Barré-Sinoussi F, Hurtrel B, Aubertin AM, Venet A, Mehtali M, Muchmore E, Michel P (1994). "Programmed cell death and AIDS: Significance of T-cell apoptosis in pathogenic and nonpathogenic primate lentiviral infections." Proc. Natl. Acad. Sci. USA. **91**: 9431–9435.

126. Finkel T, Tudor-Williams. G, Banda N, Cotton M, Curiel T, Monks C. Baba T, Ruprecht R, Kupfer A (1995). "Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes." Nat. Medicine **1**: 129–134.

127. Favre D, Mold J, Hunt PW, Kanwar B, Loke P, Seu L, Barbour JD, Lowe MM, Jayawardene A, Aweeka F, Huang Y, Douek DC, Brenchley JM, Martin JN, Hecht FM, Deeks SG, McCune JM. (2010). "Tryptophan catabolism by indoleamine 2,3-dioxygenase 1 alters the balance of TH17 to regulatory T cells in HIV disease." Sci Transl Med **2**: 32-36.

128. Favre D, Lederer S, Kanwar B, Ma ZM, Proll S, Kasakow Z, Mold J, Swainson L, Barbour JD, Baskin CR, Palermo R, Pandrea I, Miller CJ, Katze MG, McCune JM. (2009). "Critical loss of the balance between Th17 and T regulatory cell populations in pathogenic SIV infection." PLoS Pathogens **5**: e1000295.

129. Khaitan A, Unutmaz D. (2011). "Revisiting immune exhaustion during HIV infection." Curr HIV/AIDS Rep **8**: 4–11.

130. Wherry EJ, Blattman J., Murali-Krishna K, van der Most R, Ahmed R. (2003). "Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment." J Virol **77**: 4911–4927.

131. Day CL, Kaufman DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, Mackey EW, Miller JD, Leslie AJ, DePierres C, Mncube Z, Duraiswamy J, Zhu B, Eichbaum Q, Altfeld M, Wherry EJ, Coovadia HM, Goulder PJR, Klenerman P, Ahmed R, Freeman GJ, Walker BD (2006). "PD-1 expression

on HIV-specific T cells is associated with T-cell exhaustion and disease progression." Nature **443**: 350–354.

132. Zhang JY, Zang Z, Wang X, Fu JL, Yao J, Jiao Y, Chen L, Zhang H, Wei J, Jin L, Shi M, Gao GF, Wu H, Wang FS. (2007). "PD-1 up-regulation is correlated with HIV-specific memory CD8+ T-cell exhaustion in typical progressors but not in long-term nonprogressors." Blood. **109**: 4671–4678.
133. Clerici M (2010). "Beyond IL-17: new cytokines in the pathogenesis of HIV infection." Current Opinion in HIV and AIDS. **5**: 184–188.
134. Katsikis PD, Mueller YM, Villinger F (2011). "The cytokine network of acute HIV infection: a promising target for vaccines and therapy to reduce viral setpoint?" PLoS Pathogens. **7**: e1002055.
135. Richman DD, Margolis D., Delaney M, Greene WC, Hazuda D, Pomerantz RJ (2009). "The challenge of finding a cure for HIV infection." Science New York **323**: 1304–1307.
136. Trono D, Van Lint C, Rouzioux C, Verdin E, Barré-Sinoussi F, Chun TW, Chomont N. (2010). "HIV persistence and the prospect of long-term drug-free remissions for HIV-infected individuals." Science. **329**: 174–180.
137. Clerici M, Shearer G. (1993). "A TH1—>TH2 switch is a critical step in the etiology of HIV infection." Immunol Today **14**: 107–111.
138. Esser R, Von Breiesen H, Brugger M, Ceska M, Glienke W, Muller S, Rehm A, Rubsamen-Waigmann H, Andreesen R (1991). "Secretory repertoire of HIV-infected human monocytes/macrophages." Pathobiology **59**: 219–222.
139. Zack J, Arrigo S, Weitsman S, Go A, Haislip A, Chen I (1990). "HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure." Cell **61**: 213–222.
140. Sonza S, Maerz A., Deacon N, Meanger J, Mills J, Crowe S (1996). "Human immunodeficiency virus type 1 replication is blocked prior to reverse transcription and integration in freshly isolated peripheral blood monocytes." J Virol **70**: 3863–3869.
141. Esser R, Glienke W, Andreesen R, Unger R, Kreutz M, Rubsamen-Waigmann H, von Briesen H (1998). "Individual cell analysis of the cytokine repertoire in human immunodeficiency virus-1-infected monocytes/macrophages by a combination of immunocytochemistry and in situ hybridization." Blood **91** 4752–4760.

142. Foli A, Saville M, May L, Webb D, Yarchoan R (1997). "Effects of human immunodeficiency virus and colony-stimulating factors on the production of interleukin 6 and tumor necrosis factor alpha by monocyte/macrophages." AIDS Res. Hum Retrovir **13**: 829–839.

143. Kornbluth R, Keek K, Richman D (1998). "CD40 ligand (CD154) stimulation of macrophages to produce HIV-1-suppressive beta-chemokines." Proc Nat Acad Science, USA **95**: 5205–5210.

144. Fantuzzi L, Canini I, Belardelli F, Gessani S (2001). "HIV-1 gp120 stimulates the production of beta-chemokines in human peripheral blood monocytes through a CD4-independent mechanism." J Immunol **166**: 5381–5387.

145. Greco G, Fujimura S, Mourich D, Levy J (1999). "Differential effects of human immunodeficiency virus isolates on beta- chemokine and gamma interferon production and on cell proliferation." J Virol **73**: 1528–1534.

146. Brooks DG, Trifilo MJ, Edelmann KH, Teyton L, McGavern DB, Oldstone MB (2006). "Interleukin-10 determines viral clearance or persistence in vivo." Nature Medicine. **12**: 1301–1309.

147. Ejrnaes M, Filippi C, Martinic MM, Ling EM, Togher LM, Crotty S, von Herrath MG. (2006). "Resolution of a chronic viral infection after interleukin-10 receptor blockade." J Exp Medi **203**: 2461–2472.

148. Surh CD, Sprent J. (2008). "Homeostasis of naive and memory T cells." Immunity. **29**: 848–862.

149. Oguariri RM, Brann T, Imamichi T (2007). "Hydroxyurea and interleukin-6 synergistically reactivate HIV-1 replication in a latently infected promonocytic cell line via SP1/SP3 transcription factors." J Biol Chemist **282**: 3594–3604.

150. Persaud, D, Patel K, Karalius B, Rainwater-Lovett K, Ziemniak C, Ellis A, Chen YH, Richaman D, Siberry GK, Van Dyke RB, Burchett S, Seage GR, Luzuriaga K (2014) "Influence of age at virologic control on peripheral blood human immunodeficiency virus reservoir size and serostatus in perinatally infected adolescents". JAMA Pediatr **10**: 1560.

151. Jones BM, Chiu SSS, Wong WHs, Lim WWL, Lay Y (2005) "Cytokine profiles in human immunodeficiency virus-infected children treated with highly active antiretroviral therapy". Med Gen Med **7**: 71.

152. Miller TL, Borkowsky W, DiMeglio LA, Dooley L, Geffner ME, Hazra R, McFarland EJ, Mendez AJ, Patel K, Siberry GK, Van Dy**ke** RB, Worrell CJ,

Jacobson DL **(**2012**)** "Metabolic abnormalities and viral replication are associated with biomarkers of vascular dysfunction in HIV-infected children**"**. HIV Med **13**: 264-275.

153. Sánchez Torres AM, Munoz Muniz R, Madero R, Borque C, García-Miguel MJ, De José Gómez MI (2005) "Prevalence of fat redistribution and metabolic disorders in human immunodeficiency virus-infected children". Eur J Pediatr **164**: 271-276.
154. Miller TL, Orav EJ, Lipshultz SE, Arheart KL, Duggan C, Weinberg GA, Bechard L, furuta L, Nicchitta J, Gorbach SL, Shevitz A **(**2008**)** "Risk factors for cardiovascular disease in children infected with human immunodeficiency virus-1". J Pediatr **153**: 491-497.
155. Miller TL, Grant YT, Almeida DN, Sharma T, Lipshultz SE **(**2008**)** "Cardiometabolic disease in human immunodeficiency virus-infected children". J Cardiometab Syndr **3**: 98-105.
156. Sainz T, Álvarez-Fuente M, Navarro ML, Diaz L, Rojo P, Bláues D, de José MI, Ramos JT, Serrano-Vilaar S, Martinez J, Medrano C, Muñoz-Fernández MA, Mellado MJ **(**2014**)** "Structural changes of the arterial wall appear in first decades of life during HIV-infection". Acqui Immune Defic Syndr **65**: 42-49.
157. Yoshioka M, Bradley WG, Shapshak P, Nagano I, stewart RV, Xin KQ, Srivastava AK, Nakamura S (1995) "Role of immune activation and cytokine expression in HIV-1 associated neurologic diseases". Adv Neuroimmunol **5**: 335-58
158. Chiappini E, Berti E, Gianesin K, Petrara MR, Galli L, Giaquinto C, de Martino M, De Rossi A (2014) "Pediatric human immunodeficiency virus infection and cancer in the highly active antiretroviral treatment (HAART) era". Cancer Lett **347**: 38-48.
159. Feldman C, Anderson R. (2013). "Cigarette smoking and mechanisms of susceptibility to infectious of the respiratory tract and other organ systems." J infect **67**: 169-184.
160. Cloke T, Munder M, Bergin P, Herath S, Modolell M, Taylor G, Müller I, Kropf P (2013). "Phenotypic Alteration of Neutrophils in the Blood of HIV Seropositive Patients." PLoS One **8**: e72034.

161. Bowers NL, Helton E, Huijbregts RPH, Goepfert PA, Heath SL, Hel Z (2014). "Immune Suppression by Neutrophils in HIV-1 Infection: Role of PD-L1/PD-1 Pathway." PLoS Pathogens **10**: 1371.

162. Pacek LR, Crum R (2014). "A review of the literature concerning HIV and cigarette smoking: Morbidity and mortality, associations with individual- and social-level characteristics, and smoking cessation efforts." Addict Res and Theo **10**: 1–14.

163. Tachfouti N, Nejjari C, Benjelloun MC, Berraho M, Elfakir S, El Rhazi K, Slama K (2011). "Association between smoking status, other factors and tuberculosis treatment failure in Morocco." J Tuber and Lung Dis **15**: 838-843.

164. Yen YF, Yen MY, Lin YS, Lin YP, Shih HC, Li LH, Chou P, Deng CY (2014). "Smoking increases risk of recurrence after successful anti-tuberculosis treatment: a population-based study." J Tuber and Lung Dis **18**: 492-498.

165. Peer N, Bradshaw D, Laubscher R, Steyn K (2009). "Trends in adult tobacco use from two South African demographic and health surveys conducted in 1998 and 2003". S Afr Med J **99**: 744–9.

166. Paiardini M, Müller-Trutwin M (2013) "HIV-associated chronic immune activation". Immunol Rev **254**: 78-101.

167. d'Ettorre G, Paiardini M, Ceccarelli G, Silvestri G, Vullo (2011) "HIV-Associated Immune Activation: From Bench to Bedside. AIDS Res Hum Retroviruses". AIDS Res Hum Retroviruses **27**: 355-64.

168. Van Eeden SF, Hogg JC (2000) "The response of human bone marrow to cigarette smoking". Eur Respir J 15: 915-921.

169. Reynolds NR (2009) "Cigarette smoking and HIV: more evidence for action". AIDS Edic Prev **21**: 106-121.

170. Shearer WT, Rosenblatt HM, Gelman RS, Oyomopito MS, Plaeger S, stiehm ER, Wara DW, Douglas SD, Luzuriaga K, McFarland EJ, Yogeu R, Rathore MH, Levy W, Graham BL, Spector SA (2003) "Lymphocyte subsets in healthy children from birth through 18 years of age: The pediatric AIDS clinical trials group P1009 study". J Allergy Clin Immun **112**: 973-980.

171. Abrams EJ, Weedon J, Steketee RW, Lambert G, Bamji M, Vrown T, Kalish ML, Schoenbaum EE, Thomas PA, Thea DM (1998) "Association of Human Immunodeficiency Virus (HIV) Load Early in Life with Disease Progression among HIV-Infected Infants". J Infect Dis **178**:1 01-108.

172. Muechhoff M, Prendergast AJ, Goulder PJR (2014) "Immunity to HIV in early life". Front Immunol **5**: 391.

173. Liappis N (1988) "Referenzwerte der β2-mikroglobulin-konzentration im serum von kindern". Klin Pädiatr **200**: 67-69.

174. Lilic D, Cant AJ, Abinun M, Calvert JE, Spickett G (1997) "Cytokine production differs in children and adults". Ped Res **42**: 237-240.

175. Waweru P, Anderson R, Steel H, Venter WDF, Murdoch D, Feldman C (2013) "The prevalence of smoking and the knowledge of smoking hazards and smoking cessation strategies among HIV positive patients in Johannesburg, South Africa". S Afr Med J **103**: 858-860.

176. Valiathan R, Miguez MJ, Patel B, Arheart KL, Asthana D (2014) "Tobacco smoking increases immune activation and impairs T-cell function in HIV infected patients on antiretrovirals: A cross-sectional pilot study". PLoS ONE **9**: e97698.

177. Lotz M, Seth P (1993) "TGF beta in HIV infection". Ann NY Acad Sci **685**: 501-511.

178. Zeng M, Smith AJ, Wietgrefe SW, Southern PJ, Schacker TW, Reilly CS, Estes JD, Burton GF, Silvestri G, Lifson JD, Carlis JV, Haase AT (2011) "Cumulative mechanisms of lymphoid tissue fibrosis and T cell depletion in HIV-1 and SIV infections". J Clin Invest **121**: 998-1008.