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E-Selectin, and markers of HIV disease severity, inflammation and coagulation in treatment- naïve individuals living with HIV

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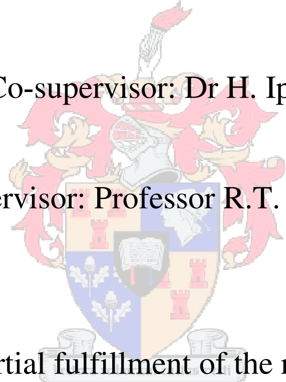

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DECLARATION

I, the undersigned, hereby declare that the work contained in this assignment in my original work and that I have not previously submitted it, in its entirety or in part, at any university for a degree.

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ABSTRACT

Background: E-selectin is an adhesion molecule that is expressed on the surface of activated endothelial cells. During inflammation the endothelial cells are activated, trafficking cells of the immune system through the endothelial wall to the point of inflammation. Human Immunodeficiency Virus (HIV) infection causes continuous and long term activation of the immune system and has an increased incidence of cardiovascular disease. Selectins play an important role in atherosclerotic plaque formation as continuous activation leads to plaque formation and eventual plaque rupture with subsequent thrombosis and the initiation of a cardiac event. The aim of this study was to determine the levels of E-selectin in an HIV infected and control population and to correlate these levels with markers of HIV disease severity, inflammation and coagulation in anti-retroviral treatment (ART)-naïve HIV infected individuals.

Methods: E-selectin levels were determined using ELISA in 180 participants from an HIV prevention and testing clinic in Crossroads, Cape Town. There were 114 HIV infected cases and 66 HIV negative controls. These levels were compared with each other and correlated to various other markers associated with HIV disease severity (viral load and CD4+count), inflammation (white cell count (WCC), high sensitivity C-reactive protein (hsCRP), %CD38/8, albumin and IgG) and coagulation (fibrinogen and D-dimer).

Results: A total of 75% of the females tested positive for HIV compared to 37% of the males. Statistics comparing HIV status with WCC, CD4+count, %CD38/8, albumin, IgG, hsCRP and D-dimer found significant differences ($p<0.01$) between the two groups. No differences in E-selectin ($p=0.84$) and fibrinogen ($p=0.65$) levels were found between the cases and the controls. When E-selectin was compared with all the analytes tested, significant correlations were found with age ($p=0.02$) and gender ($p=0.01$). Albumin ($p=0.05$) showed a significant correlation with E-selectin in the control group. The correlation with the WCC ($p=0.07$) in the HIV infected group neared significance.

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Conclusion: No significant difference in E-selectin levels was found between the HIV positive and negative control group and no correlations were found with E-selectin and the markers of disease severity, inflammation and coagulation. Thus we found E-selectin to be a poor marker of inflammation in this setting. As age and gender are established markers of CVD and males have higher E-selectin levels than females, the lack of significance may be due to our sample population's young age (mean 31 years) or the fact that 70% of the cohort was female. Thus significant endothelial damage may not yet have taken place to increase E-selectin levels. In addition, this HIV group was predominantly in the chronic stage of infection, therefore the increase in E-selectin levels may have occurred earlier during the acute infection.

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ABSTRAK

Agtergrond: E-selektien is ‘n adhesie molekule wat teenwoordig is op die sel oppervlakte van geaktiveerde endoteelselle. Gedurende inflammasie word die endoteelselle geaktiveer en selle van die immuunstelsel beweeg deur die endoteellaag na die area van inflammasie. Menslike-immuungebreksvirus (MIV) veroorsaak aanhoudende en langdurige aktiveering van die immuunstelsel. Selektiene speel ‘n baie belangrike rol in arterosklerotiese plaak vorming, aangesien aanhoudende immuun aktivering bydra tot plaak vormasie. Uiteindelik raak die plaak onstabiel en breek oop met gevolglike bloedklont vorming en die aanvang van ‘n kardiale gebeurtenis. Die doel van die studie was om die vlak van E-selektien vas te stel in MIV infeksie en negatiewe kontroles en dit te korreleer met merkers van die graad van MIV infeksie, inflammasie en bloedstolling in anti-retrovirale terapie (ART) naïef MIV geïnfekteerde individue.

Metodes: E-selektien vlakke is bepaal met behulp van ensiem gekoppelde immunosorbent toets (EGIST) in 180 deelnemers wat ‘n MIV voorkomings en toetsings kliniek in Crossroads, Kaapstad bygewoon het. Daar was 114 MIV positiewe gevalle en 66 MIV negatiewe kontroles. Die gevalle en kontroles is gekorreleer met mekaar en verskeie ander merkers wat geassosieer word met die graad van MIV infeksie (virale lading en CD4+telling), inflammasie (witsel telling (WST), hoë-sensitiewe C-reaktiewe proteïen (hsCRP); %CD38/8, albumien en IgG) asook bloedstolling (fibrinogeen en D-diemer).

Resultate: ‘n Totaal van 75% vroulike deelnemers in vergelyking met 37% manlike deelnemers het positief getoets vir MIV. Statistiese korrelasies wat MIV status vergelyk het met die WST, CD4+telling, %CD38/8, albumien, IgG, hsCRP en D-diemer vlakke het noemenswaardige verskille ($p<0.01$) getoon tussen die twee groepe. Geen verskille in E-selektien ($p=0.84$) en fibrinogeen ($p=0.65$) vlakke is gevind tussen the gevalle en kontroles nie. Toe E-selektien vergelyk is met al die analiete, is betekenisvolle korrelasies gevind met ouderdom ($p=0.02$) en geslag ($p=0.01$). Albumien ($p=0.05$) het betekenisvolle korrelasie getoon met E-selektien in

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die kontrole groep. En die WST (p=0.07) het 'n tendens van korrelasie met E-selektien getoon in die MIV geïnfekteerde group.

Gevolgtrekking: Ons het geen betekenisvolle verskille in die E-selektien vlakke gevind tussen die twee groepe, of toe dit vergelyk is met die merkers van die graad van MIV infeksie, inflammasie en bloedstolling nie. Dus het ons vasgestel dat E-selektien 'n swak merker van inflammasie in hierdie omstandighede is. Aangesien ouderdom en geslag vasgestelde merkers van kardiovaskulêre siekte is en mans hoër E-selektien vlakke het teenoor vrouens, mag die gebrek aan betekenisvolle verskille as gevolg van die toets populasie se jong ouderdom (gemiddeld 31 jaar) wees, of die feit dat 70% van die deelnemers vroulik was. Ons vermoed dat endoteel skade nog nie ernstig genoeg was om verhoogte vlakke van E-selektien te weerspïeel nie. Daarbenewens, was hierdie MIV groep oorheersend al in die chroniese fase van infeksie, dus kon verhoogte vlakke van E-selektien dalk in die vroëer stadium van akute infeksie plaasgevind het.

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TABLE OF CONTENTS

List of Abbreviations **I-IV**

List of Figures **V-VI**

List of Tables **VII**

Acknowledgements **VIII**

Introduction **IX**

Section I: Literature Review **1-40**

1.1 Human Immunodeficiency Virus (HIV) Pathogenesis 2-15
1.1.1 History of HIV infection 2-4
1.1.2 HIV structure 5
1.1.3 Immune Response 6-11
1.1.3.1. Innate immune response (0-12 hours after exposure to pathogens) 6- 7
1.1.3.2 Adaptive Immune response (1-5 days post exposure to pathogens) 8-11
1.1.3.2.1 Humoral immune response 9-10
1.1.3.2.2 Cell-mediated immune response 10-11
1.1.4 HIV infection 11-16
1.1.4.1 Acute phase 12-13
1.1.4.2 Chronic Infection 14-15
1.1.4.3 Leaky Gut 15-16
1.1.5 HIV and anti-retroviral treatment 17-18

Stellenbosch University http://scholar.sun.ac.za

1.2. HIV Disease Severity 18-19

1.2.1 CD4 18-19

1.2.2 Viral Load 19

1.3 Inflammation 20-32

1.3.1 HIV, inflammation and aging 20-21

1.3.2 Inflammation, atherosclerosis and cardiovascular Disease 21-22

1.3.3 CD38 expression on CD8+ T lymphocytes 23-24

1.3.4 Immunoglobulin G (IgG) 24-25

1.3.5 Albumin 26-27

1.3.6 High Sensitivity C-reactive protein (hsCRP) 27

1.3.7 Thrombosis 28-32

1.3.7.1 Fibrinogen 30

1.3.7.2 D-dimer 31-32

1.4 Selectins 33-37

1.4.1 Selectins- Introduction 33

1.4.2 Structure 34

1.4.3 Function 35 -36

1.4.4 Soluble E-selectin 36

1.4.5 Endothelial Dysfunction 36-37

1.4.6 Cellular adhesion molecules and cancer 37

1.4.7 Opportunistic infections 37

1.5 Studies of E-selectin in HIV 38-40

1.6 Other studies performed on the present study cohort 40-45

1.7 Hypothesis 46

1.8 Aims of study 46

1.9 Objectives 46

Stellenbosch University http://scholar.sun.ac.za

Section II: Materials and Methods **47-44**

2.1 Sample stability 48
2.2 Sample size and ethical considerations 48-50
2.2.1 Inclusion Criteria 49
2.2.2 Exclusion Criteria 50

2.3 Assay Methods 50-55
2.3.1 HIV status determination: screening and confirmation 50-51
2.3.2 E-selectin 51-52
2.3.3 White cell count 52
2.3.4 CD4+count 53
2.3.5 CD8 and CD38/8 53-54
2.3.6 HIV viral load 54
2.3.7 Fibrinogen 54
2.3.8 D-dimer 55
2.3.9 High-sensitivity C-reactive protein (hsCRP) 55
2.3.10 Immunoglobulin G (IgG) 55-56
2.3.11 Albumin 56

2.4 Statistical Analysis 56

Section III: Results **57-77**

3.1 Characteristics of the study population 58-59
3.2 Analytes tested: HIV positive compared to HIV negative subjects 60-70
3.2.1 White cell count (WCC) 60
3.2.2 CD4+count 61
3.2.3 %CD38/8 62
3.2.4 Albumin 63
3.2.5 IgG 64

Stellenbosch University http://scholar.sun.ac.za

3.2.6 Fibrinogen 65

3.2.7 D-dimer 66

3.2.8 Log_{10}(hsCRP) 67

3.2.9 Log_{10}(E-selectin) 68-70

3.2.9.1 Log_{10}(E-selectin) compared to gender 69

3.2.9.2 E-selectin corrected for gender 70

3.3 E-selectin correlations with all the parameters tested in the cohort as a whole and separately as HIV positive and HIV negative populations 72-73

3.4 E-selectin compared to the CD4+count at different Levels 74-75

3.5 Biplot 76-77

Section IV: Discussion **79-87**

Strengths and limitations 87

Section V: Conclusion **88-89**

Section VI: Bibliography **90-103**

Appendix I: Ethics for this study

Appendix II: Consent forms

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LIST OF ABBREVIATIONS AND SYMBOLS

Ab	Antibody
ADA	Adenosine deaminase
ADMA	Asymmetric dimethylarginine
Ag	Antigen
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of variance
APC	Allophycocyanin
APCs	Antigen presenting cells
ART	Antiretroviral Treatment/Therapy
B-cell	Bone-marrow cell
BCG	Bromocresol-green
BD	Becton Dickinson
CA	California
CCR5	Co-chemokine receptor 5
CD	Cluster of differentiation
CDC	Centre for Disease Control
cDNA	copy Deoxyribonucleic acid
CMV	Cytomegalovirus
CRP	C-reactive protein
CVD	Cardiovascular disease
CXCR4	Co-expression chemokine receptor 4
DDAH	Dimethylarginine dimethylaminohydrolase
DVT	Deep vein thrombosis
E, P, L-selectin	Endothelial, Platelet, Leukocyte selectin
EGF	Epithelial growth factor
EGIST	ensiem gekoppelde immunosorbent toets
ELISA	Enzyme linked immunosorbent assay
F(ab')2	Fragment of a monoclonal antibody
FACS	Fluorescence Activated Cell Sorter
FBC	Full blood count

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FCS	Fetal Calf Serum
FDPs	Fibrin degradation products
FITC	Fluorescein isothiocyanate
FLCs	Free light chains
F-value	Degree of freedom value
GCP	Good clinical practice
GIT	Gastro intestinal tract
GP120	Glycoprotein 120
GP41	Glycoprotein 41
HAIG	HIV Activation and Inflammation Group
Hb	Haemoglobin
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
HPT	HIV prevention and testing
HREC	Human Research Ethics Committee
HRP	Horseradish peroxidase
hsCRP	High sensitivity C-reactive protein
ICAM-1	Intercellular adhesion molecule -1
ICH	International Conference on Harmonization
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
JUPITER	Justification for the use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin
K_2-EDTA	Di-potassium Ethylene diaminetetraacetic acid
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
LUCs	Large unstained cells
MHC	Major Histocompatibility
MIV	Menslike-immuungebreksvirus
NF-κB	Nuclear factor kappa-light chain enhancer of activated B-cells
NOS	Nitric oxide synthase

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OD	Optical Density
oxLDL	Oxidized low density lipoprotein
p24	Protein 24
PC	Principle component
PCA	Principle component analysis
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEG	Polyethylene glycol
PerCP	Peridinin chlorophyll protein
p-value	Probability value
RNA	Ribonucleic acid
rpm	Revolutions per minute
r-value	Correlation coefficient value
SA	South Africa
SD	Standard deviation
SELE-gene	Selectin E- gene
sE-selectin	Soluble endothelial selectin
SIV	Simian Immunodeficiency Virus
SIVcpz	SIV chimpanzee
SIVmac	SIV macaques
SMART	Strategies for Management of Antiretroviral Therapy
SPE	Serum protein electrophoresis/electrophoretic
TB	Tuberculosis
T-cell	Thymus cell
Th1, Th2	T1, T2, Helper cells
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumour necrosis factor
UCT	University of Cape Town
UK	United Kingdom
VCAM-1	Vascular cell adhesion molecule -1
vWF	von Willebrands factor
WCC	White cell count

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WST	Witsel telling
WHO	World Health Organization
α	Alpha
β	Beta
κ	Kappa
λ	Lambda
γ	Gamma
®	Registered Trademark
™	Trademark
Inc	Incorporated
°C	Degree Celsius
µL	microliter
g/L	grams per Liter
L	Liter
mg/L	milligrams per Liter
mL	Milliliter
mm	Millimeter
mm^3	Cubic millimeter
ng/mL	nanograms per milliliter
nm	nanometer

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LIST OF FIGURES

Section I: Literature Review

Figure 1.1: The spread of Simian Immunodeficiency Virus to humans 4

Figure 1.2: HIV viral structure 5

Figure 1.3: Cells that form part of the innate immune response 7

Figure 1.4: Cells that play a role in the adaptive immune response 9

Figure 1.5: CD4+ and CD8+ T cell binding to their corresponding MHC class molecule on the antigen presenting cell 10

Figure 1.6: HIV infection, replication and release of virions into host extracellular fluids 12

Figure 1.7: Co-receptors needed for HIV attachment and fusion into corresponding leukocytes 14

Figure 1.8: Consequences of HIV induced immune activation 15

Figure 1.9: The 3 stages of HIV disease progression 16

Figure 1.10: Formation and rupture of an atherosclerotic plaque 23

Figure 1.11: Diagrammatic representation of an antibody and its light (κ and λ) and heavy chains 25

Figure 1.12: The function of fibrinogen in the formation of atherosclerotic plaques 31

Figure 1.13: The formation of D-dimer molecules through the process of fibrinolysis 32

Figure 1.14: Depicts the three different selectins 33

Figure 1.15: Rolling and slowing of leukocytes, entering the endothelium and moving to the point of inflammation 35

Section II: Materials and Methods

Figure 2.1: E-selectin ELISA showing the colour change after the addition of the stop reagent 52

Stellenbosch University http://scholar.sun.ac.za

Section III: Results

Figure 3.1 HIV negative compared to HIV positive participants 58

Figure 3.2 HIV status according to gender 59

Figure 3.3 WCC in the HIV positive population compared to HIV negative population 60

Figure 3.4 CD4+count in the HIV negative population compared to HIV positive population 61

Figure 3.5 %CD38 on CD8 expression in the HIV negative compared to HIV positive population 62

Figure 3.6 Albumin levels in the HIV positive population compared to HIV negative population 63

Figure 3.7 IgG levels in the HIV positive population compared to HIV negative population 64

Figure 3.8 Fibrinogen levels in the HIV positive population compared to HIV negative population 65

Figure 3.9 D-dimer levels in the HIV positive population compared to HIV negative population 66

Figure 3.10 Log_{10}(hsCRP) level in the HIV positive population compared to HIV negative population 67

Figure 3.11 Log_{10} (E-selectin) levels in HIV positive population compared to HIV negative population 68

Figure 3.12 E-selectin compared to gender 69

Figure 3.13 E-selectin corrected for gender 70

Figure 3.14 E-selectin levels at the WHO cut-off point (500 cells/mm^3) for ART initiation 74

Figure 3.15 E-selectin levels at the South African Department of Health cut-off point (350 cells/mm^3) for ART initiation 75

Figure 3.16 Biplot Summary of the comparison of all the analytes tested in HIV positive and HIV negative populations 78

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LIST OF TABLES

Section III: Results

Table 3.1: The comparison of the means of all the analytes determined in the HIV positive and negative populations 71

Table 3.2: E-selectin correlations with other parameters tested; like hypothesis (P<0.05 statistically significant) 72

Table 3.3: Comparing the WCC to the differential count 73

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INTRODUCTION

The World Health Organization (WHO) recognized the Human Immunodeficiency Virus (HIV) epidemic for the first time in 1983, and since then a global estimate of approximately 78 million people has been infected with the virus and approximately 39 million people have died from Acquired Immunodeficiency Syndrome (AIDS). At the end of 2013, it was estimated that 35 million people were living with HIV globally. However, this figure varies greatly between countries and regions, with Sub-Saharan Africa being affected the most, with nearly 1 in every 20 adults living with HIV, accounting for 71% of the world's HIV infected individuals.[1]

By December 2012, an estimated 25 million people in sub-Saharan Africa were living with HIV of which 6.1 million people (17.9%) were living in South Africa. However, this prevalence varies between provinces, with Kwazulu–Natal having the highest prevalence of approximately 40%. Provinces with the lowest prevalence of HIV infected individuals are the Western Cape (18.2%) and Northern Cape (17.0%). In the same year, an estimated 1.6 million new infections and 1.2 million AIDS-related deaths were documented.[2,3]Thus it is evident that this disease places a huge burden on social and economic resources with South Africa spending over R11 billion annually on its HIV and AIDS programs[4]. However, since the rollout of anti-retroviral treatment (ART), morbidity has dramatically reduced, with HIV now being a manageable disease allowing individuals to have a longer lifespan.[5] Recently, unexpected inflammatory conditions such as cardiovascular disease (CVD) and thrombosis have made an appearance in HIV-infected individuals. This is thought to be due to ongoing inflammation in HIV that cannot be resolved by ART.[5]

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SECTION I: LITERATURE REVIEW

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1.1 HUMAN IMMUNODEFICIENCY VIRUS (HIV) PATHOGENESIS:

1.1.1 History of HIV infection

Acquired Immune Deficiency Syndrome (AIDS) was first recognized as a new disease in 1981 when 5 young, previously healthy homosexual men in Los Angeles presented with unusual opportunistic infections and rare, aggressive cancers such as Karposi Sarcoma. By the end of that year, 270 cases of severe immunodeficiency were reported in homosexual men, and 121 of these had died.[6] In January 1982 the first AIDS clinic was established in San Francisco and on September 24 of that year, the term AIDS was used for the first time. The first definition of AIDS was at that time described as “a disease at least moderately predictive of a defect in cell-mediated immunity, occurring in a person with no known case for diminished resistance to that disease”. A retrovirus now known has Human Immunodeficiency Virus type 1 (HIV-1) was identified as the causative agent. In 1983 the Centre for Disease Control (CDC) reported that the most cases on AIDS were among homosexual men with multiple sexual partners, intravenous drug users, Haitians and haemophiliacs.[6] It was suggested that AIDS may be caused by an infectious agent (HIV-1) that spreads by means of sexual, percutaneous, perinatal routes and/or through exposure to blood or blood products.[6,7]

In 1986 a morphologically similar, but antigenically distinct virus was found to cause AIDS in subjects from Western Africa.[8] This new virus was called Human Immunodeficiency Virus type-2 (HIV-2), and was found to be distantly related to HIV-1, but more close related to a simian virus that caused immunodeficiency in captive macaque monkeys.[9] Following this, additional viruses collectively termed Simian Immunodeficiency Viruses (SIVs) were found in various primates from sub-Saharan Africa, including African green monkeys, sooty mangabeys, mandrills and chimpanzees. However these viruses appeared to be nonpathogenic in their natural hosts. Later, close simian relatives of HIV-1 and HIV-2 were found in chimpanzees and sooty mangabeys.[10,11] This provided evidence that AIDS had emerged in both humans and macaques monkeys as a result of cross-species infections.

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Subsequent studies confirmed that SIVmac was not a natural pathogen in macaques, but had been generated accidentally in the United States primate centers by inoculating various species of macaques with blood and/or tissue from naturally infected sooty mangabeys monkeys.[12,13] It became clear the HIV-1 and HIV-2 were a result of zoonotic transfers of viruses infecting primates in Africa.[7]

Many lentiviruses have been identified, but the chimpanzee strain of SIV (SIVcpz) has been most studied for its close genetic relationship to HIV-1. Chimpanzees are known to hunt and kill other mammals, including monkeys, and this is most likely how they became infected.[7] SIVcpz was initially thought to be harmless in their natural host as none of the captive chimpanzees who where naturally infected with SIVcpz suffered from immunodeficiency.[14] In addition SIV infected sooty mangabeys and African green monkeys showed no sign of disease despite the presence of a high viral load in blood and lymphatic tissue. It was then surmised that all naturally occurring SIV infections were nonpathogenic.[15]

In 2009, Keele et al found that after correcting for age, SIVcpz infected chimpanzees had a 10 to 16-fold increased risk of death as compared to uninfected chimpanzees. SIVcpz infected females were less likely to give birth and had a much higher infant mortality rate. Post-mortem analysis revealed significant Cluster of differentiation (CD)4+T-cell depletion and histological findings consisted with end-stage AIDS. These findings indicated that SIVcpz was pathogenic to its natural host and that the virus has a substantial negative effect on the health, reproduction and lifespan of all infected chimpanzees.[16]

It has long been suspected that HIV-1 originates from chimpanzees. HIV-1 is not just one virus, but consists of four distinct lineages, termed group M, N, O and P. Group M (Main) is the first discovered HIV-1 form and the cause of the current HIV pandemic. Forthcoming evidence produced found that both HIV-1 and SIVcpz originated from chimpanzee, thus identifying chimpanzee as the original reservoir for human HIV-1 infections.[7] Figure 1.1 depicts the spread of SIV to humans.

It remains unclear exactly how humans acquired HIV-1, but it is thought that transmission occurred through cutaneous or mucous membrane exposure after bushmeat hunting.[17]

HIV-2 infection has remained isolated in Western Africa and prevalence rates are declining.[18] Infection with HIV-2 is associated with lower viral loads, lower transmission and almost no mother-to-infant transmission.[19] This infection seldom progresses to AIDS and the origin of HIV-2 infection is thought to be from the sooty mangabey.[10]

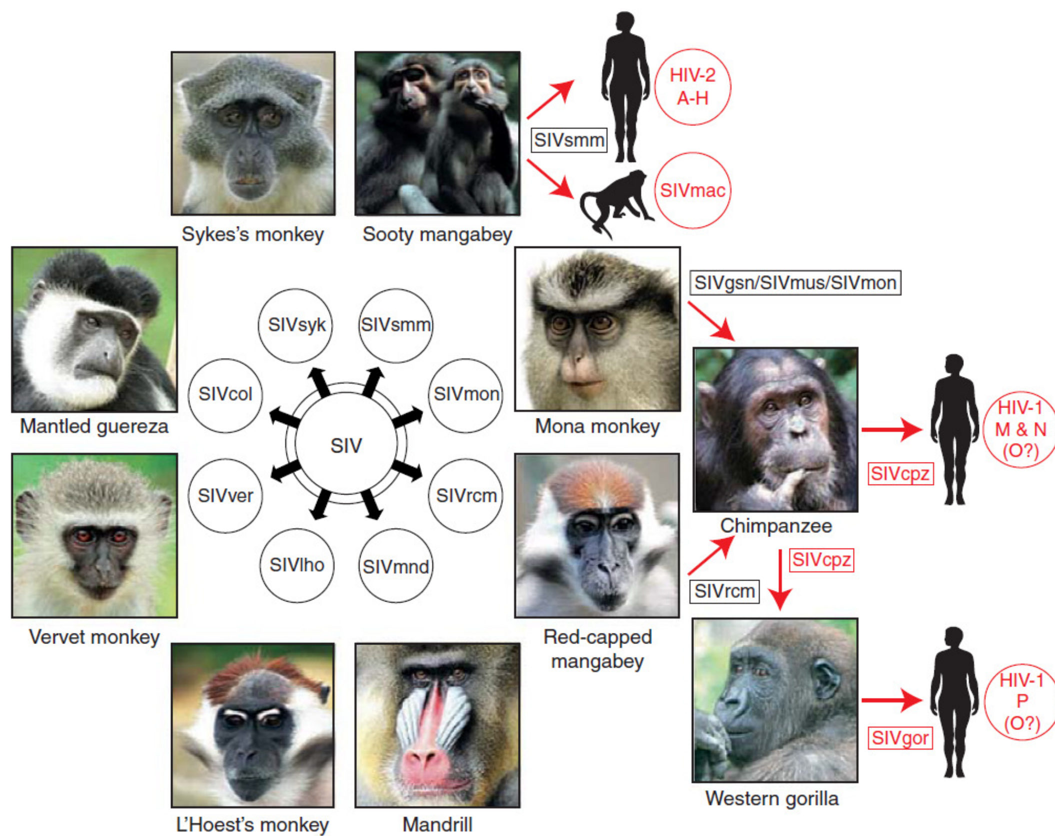

Figure 1.1: The spread of Simian Immunodeficiency Virus to humans[7]

1.1.2 HIV structure

HIV is a retrovirus belonging to the family of lentiviruses. It contains two single strands of ribonucleic acid (RNA) in its core on which nine viral genes are found which code for 15 individual proteins such as for viral structure, enzymes, regulatory and accessory proteins.[20,31]

HIV is a spherical particle with a diameter of 1/10 000 millimeter (mm). Unlike other viruses, it does not contain a cell wall or nucleus in which its genome is kept, but is surrounded by a viral core that contains the capsule protein p24.[20]

The outer coat of the virus is called the viral envelope and is made up of 2 lipid layers and some proteins forming "spikes" on the outer layer. Among these proteins are glycoprotein 120 (gp120) and the transmembrane glycoprotein gp41. Gp120 is used during viral/host cell attachment and gp41 is used for fusing the viral particle with the host cell.[20]

HIV also contains matrix proteins e.g. gp17 which is situated between the envelope and the core. The protein 24 (p24) capsule contains the viral RNA and enzymes needed for replication (Figure 1.2).[20]

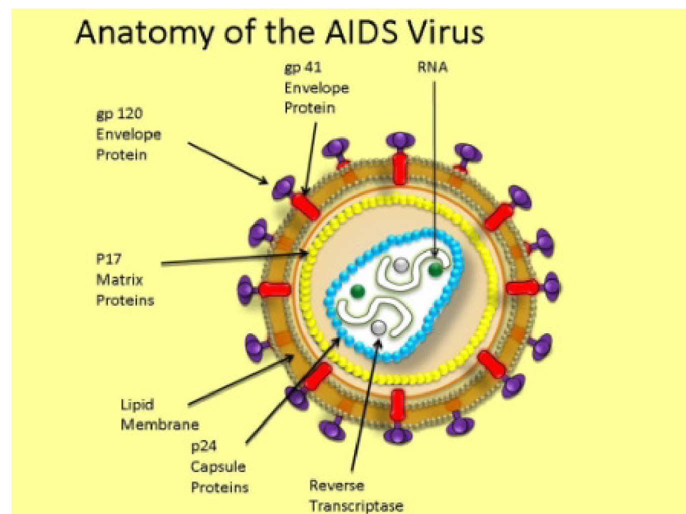

Figure 1.2: HIV viral structure[20]

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1.1.3 Immune Response

The human body has two lines of defense namely the innate and the adaptive immune responses.

1.1.3.1 Innate immune response (0-12 hours after exposure to pathogens) (Figure 1.3)

The innate immune system consists of cells and proteins that are always present and ready to mobilize and fight microbes locally at the site of infection. The cells of the innate immune system recognize foreign pathogens in a generic way which allows them to provide immediate protection and does not confer long-lasting immunity as afforded by the adaptive immune system. The major functions of the innate immune system include:

1. Recruiting immune cells to the sites of infection via the production of cytokines.
2. Activating the complement cascade to opsonize pathogens, activating other immune cells and promoting the clearance of dead cells or antibody complexes.
3. Activating the adaptive immune system by means of antigen presentation.
4. Acting as a physical and chemical barrier to invading pathogens.[23]

The first line of defense is usually the epithelial surface that is impermeable to most pathogens, its lack of blood vessels and sebaceous glands creates an unfavorable environment for survival and shedding of the skin helps to remove microbes that have become attached to it. In the gastrointestinal and respiratory tract, peristaltic movements, cilia and mucous trap and remove infectious agents. In the gut, normal flora can restrict the growth of infectious agents by competing for nutrients, secreting toxic substances or adhering to the epithelial walls preventing pathogens from colonization.[21] Therefore maintenance of an intact epithelial barrier is critical as a first-line defense against pathogens.

Cells of the innate immune system can capture and destroy cellular debris, foreign particles or microbes. These cells include: dendritic cells, neutrophils, macrophages, eosinophils, basophils, mast cells and natural killer cells.[25]

The initial immune response is responsible for acute inflammation and is caused by the release of cytokines from nearby cells such as dendritic cells, macrophages, histiocytes, Kupffer cells and mastocytes. The cytokines released during inflammation attract phagocytes mainly neutrophils which release further cytokines that attract lymphocytes.[22]

The complement system is another part of the immune system that consists of plasma proteins, synthesized by the liver. This system recruits inflammatory cells and enhances the ability of antibodies to clear the pathogen or marks it by coating it allowing for opsonization or destruction by leucocytes.[22]

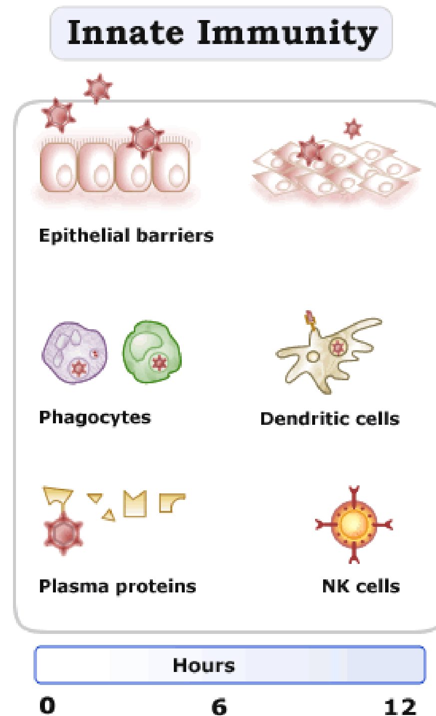

Figure 1.3: Cells that form part of the innate immune response[23]

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1.1.3.2 Adaptive Immune response (1-5 days post exposure to pathogens) (Figure 1.4)

The adaptive immune response is the second of two immune strategies in the prevention and elimination of foreign pathogens. Unlike the innate immune system, the adaptive immune system is highly specific towards pathogens and involves immunological memory after the first pathogen encounter. The immunological memory allows for a quicker response on the second encounter. Acquired or adaptive immunity is so called because it "acquires" pathogen specific receptors from each encounter during the organism's life and adapts to prepare the organism for future encounters. The adaptive immune response consists of humoral and cell-mediated immunity.[23]

Antigens are any substance that provokes an adaptive immune response. The cells responsible for the adaptive immune response are the lymphocytes which can be divided into two classes: the humoral (antibody producing Bone-marrow (B)-cells) and cell-mediated (Thymus (T)-cell) immune response. The adaptive immune system is activated when the invading pathogen produces a threshold of antigen that activates the innate system's dendritic cells to produce and present antigen to naïve B- and T- cells.[23]

The function of the adaptive immune response is:

1. To recognize pathogenic antigens,
2. To generate a response that will quickly and effectively eliminate the invading pathogen or infected cells,
3. To develop memory B- and T-cells against the pathogen to prevent against future invasions.[23]

Lymphocytes comprise 20-40% of the body's leucocytes of which approximately 2% are circulating in the peripheral blood. The remainder is found in tissues of the lymphatic system such as the spleen and lymph nodes. B- and T-cells are morphologically indistinguishable until after activation.[23]

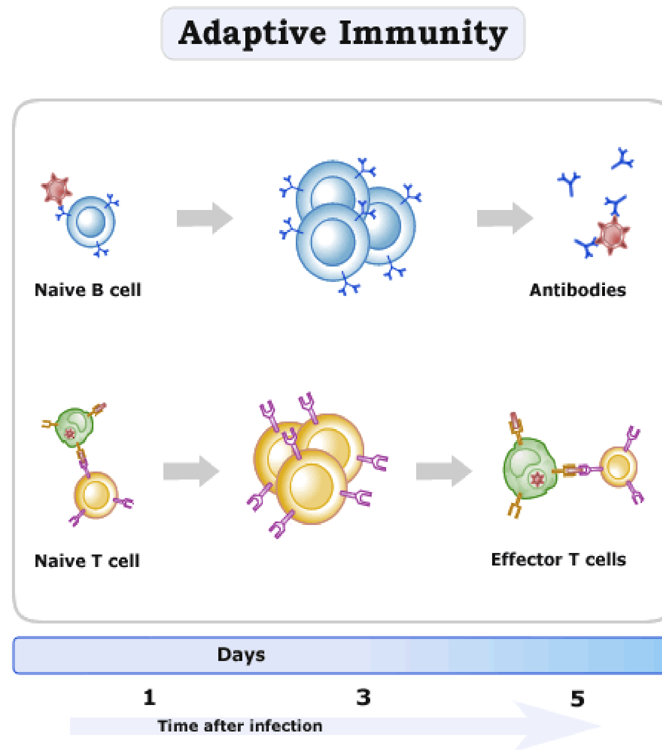

Figure 1.4: Cells that play a role in the adaptive immune response[23]

1.1.3.2.1 Humoral immune response

The bone marrow produces immature B-cells that migrate to the lymph nodes and spleen where they encounter foreign particles (antigens) that are presented by the dendritic cells. There the cells respond by becoming effector cells (plasma cells) producing antibody, or long lived memory cells. Activated B-cells secrete antibodies which are also called immunoglobulins. There are five types of immunoglobulins: IgA, IgM, IgG, IgD and IgE. Each is primed to neutralize a different type of antigen or pathogen either by coating the pathogen with antibody allowing it to be engulfed by macrophages, or by coating it allowing for opsonization. Antibodies are secreted by the plasma cells which die after 2-3 days with some becoming long-lived antigen specific memory B-cells.[22]Apart from the function mentioned above, B-cells are required to initiate T-cell immune response. This occurs via B- and T-cell antigen interactions. The B-cell first engulfs the foreign particle and then presents it to the T-

cell via the B-cell receptor (which is a type of immunoglobulin) and causes it to differentiate into effector cells. Thus the B-cells are essential for optimal T-cell activation.[24] Furthermore, studies have shown that a deficiency in B-cells leads to a dramatic decrease in thymocyte number and diversity, significant defects within the spleen dendritic cell and T-cell compartments, Peyer patches are absent, follicular dendritic cell networks are in disarray, the marginal zone and macrophages are depleted and show decreased cytokine expression. Thus the B-cells are important for immune system development and maintenance.[24]

1.1.3.2.2 Cell-mediated immune response (Figure 1.5)

As part of the innate immune response, pathogens are engulfed by dendritic cells, which then migrate to the T-cell rich lymph nodes. During migration the dendritic cells mature and lose their engulfing ability. They present the engulfed pathogen as small pieces (antigens) on its cell membrane to naïve T-cells which are then primed and activated to become either CD8+ cytotoxic T-cells or CD4+T-helper cells depending on the major histocompatiblity (MHC) class (either I or II respectively) that is presented along with the antigen.[22]

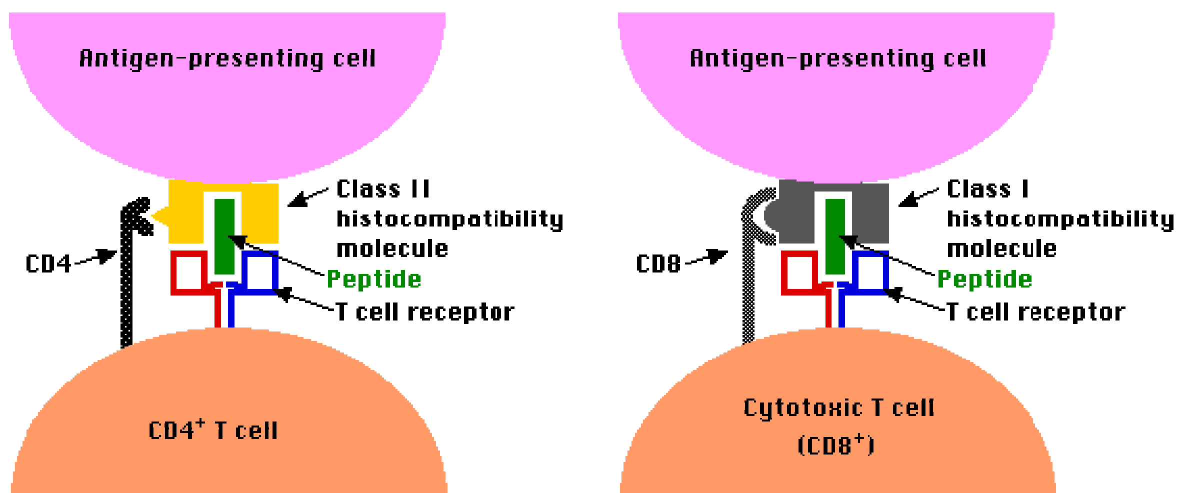

Figure 1.5: CD4+ and CD8+T cell binding to their corresponding MHC class molecule on the antigen presenting cell.[25]

CD8+ cytotoxic T-cells cause the death of virally infected, damaged or dysfunctional cells. They are activated when their T- cell receptor binds strongly to the antigen presented on the MHC class I of the infected cell. CD8 is predominantly found on cytotoxic T-cells but is also found on natural killer cells and dendritic cells.[26] They are able to kill infected and dysfunctional cells and once the problem is solved, most die or become memory cells.

CD4+T-helper cells have no cytotoxic or phagocytic abilities; therefore they cannot kill infected cells or clear dead cells away. They "manage" the immune response by directing other cells to perform their tasks. T helper cells recognize antigen bound to MHC class II, causing the cell to produce cytokines that influence the activity of many other cells such as antigen presenting dendritic cells and cytotoxic T-cells.[22] CD4+T-helper cells can be further divided into two classes namely T1 helper cells and T2 helper cells each eliminating different pathogens.

(a) Th1 produces interferon-gamma (INF-γ) activating the bactericidal properties of macrophages and activate B-cells to produce antibody needed for opsonization.
(b) Th2 produces Interleukin 5 (IL-5), priming eosinophils to clear parasites and IL-4 to prime B-cells to perform immunoglobulin class switching.

Most of the T helper cells will die leaving a few to become memory cells.[22]

CD4 is a glycoprotein found on the surface of T-helper cells, monocytes and dendritic cells and assists the cell in binding more tightly to its T-cell receptor when "communicating with its antigen" presenting cell.[29]

1.1.4 HIV infection

The initial infection with HIV may or may not be associated with a flu-like illness. However it is associated with changes in the immune system such as elevated makers of immune activation and apoptosis of CD8+ and CD4+T-cells, B-cells, natural killer

cells and monocytes followed by a gradual depletion of CD4+T-lymphocytes, leading to immunosuppression and opportunistic infection.[27,33,5]

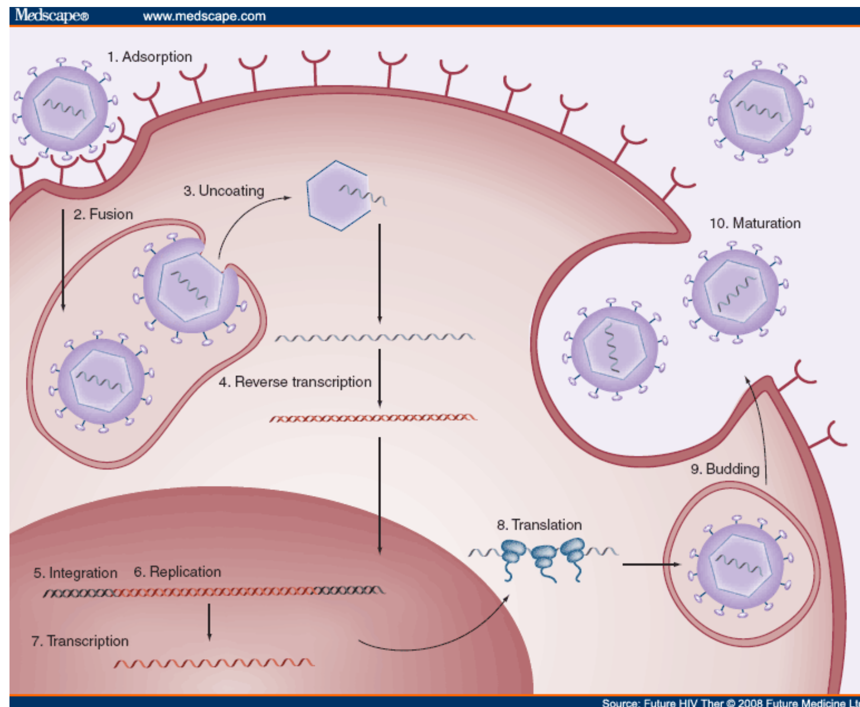

Figure 1.6: Diagrammatic representation of HIV infecting a CD4+T-cell, taking over its mechanisms to reproduce its own viral particles and releasing them into the extracellular fluids of the host.[28]

1.1.4.1 Acute phase (Figure 1.6)

Acute infection occurs over a period of 6 to 12 weeks during which seroconversion takes place and HIV antibodies are detected in the peripheral blood. HIV infects mainly the CD4+T-lymphocytes and to a lesser extent monocytes, macrophages and dendritic cells.[20,33,29]

Antigen presenting cells (APCs) such as dendritic cells line mucosal sites, trap and engulf the virus.[30,31,5] The dendritic cells then present the viral antigen to naïve T-cells which proliferate and present antigen to the B-cells which are primed against the viral antigen.

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Pro-inflammatory cytokines and chemokines released by dendritic cells and other immune cells in the infected area facilitate the recruitment of inflammatory cells to the site of infection.[30] Phagocytes (neutrophils, monocytes/macrophages) and other innate role players (such as natural killer cells) are initially recruited in an attempt to eliminate and prevent further infection of the invading virus.[31] This leads to the production of a broad range of pro-inflammatory cytokines known as the cytokine storm. Despite the activity of numerous cells and mediators, the innate immune system is unable to bring the infection under control.[30]

The virus spreads rapidly to the lymph nodes, where a massive expansion of infectious viral particles occurs, associated with a dramatic increase in peripheral blood virus. During the acute phase of the infection, activation of the immune system is crucial for the initial containment of the virus. Cytotoxic T-lymphocytes are formed that actively kill infected cells and suppress viral replication leading to the establishment of a “viral set-point”.[30]

HIV infection may occur either by the transmission of cell-free virions or by means of cell-to–cell transmission. Cell-to-cell transmission has been shown to be the fastest and most effective means of infection.[32] HIV infection starts when the virus gains entry into the CD4+T-cells by attaching itself to the surface of these cells. However, it has been found that the presence of the CD4+ molecule alone is insufficient to ensure HIV’s fusion with other immune cells i.e. dendritic cells and monocytes, which need the presence of co-receptors. There are two chemokine co-receptors that have been identified i.e. co-chemokine receptor 5 (CCR5) and co-expression chemokine receptor 4 (CXCR4) to play an important role if HIV/cell fusion (Figure 1.7). CCR5 is present on a broad range of immune cells including monocytes, macrophages and T- lymphocytes, but not T-cell lineages, as these express predominantly CXCR4.[33,5]

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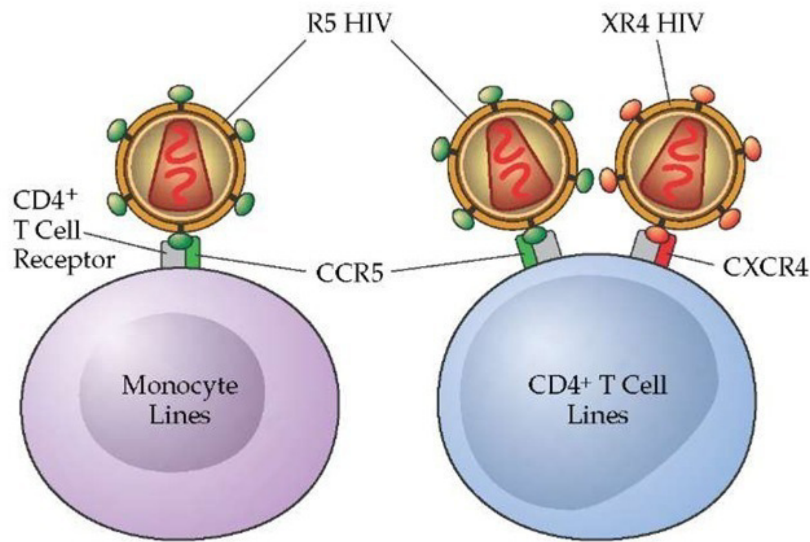

Figure 1.7: Co-receptors needed for HIV attachment and fusion with corresponding leukocytes.[34]

1.1.4.2 Chronic Infection

Chronic infection is the period from 3 months post infection until the establishment of AIDS when a high plasma viral load and a CD4+T-lymphocyte count less than 350 cells/mm^3 are present.[33]

Proliferation of B- and T-cells leads to the production of effector cells and antibodies, which in turn contribute to the partial control of the infection.[30] However, continuous stimulation of the lymph nodes to produce B- and T-cells leads to hyperplasia and fibrosis of the lymph nodes, impairing their function and contributing to the gradual decline in T- and B-cell proliferation and activation. During chronic HIV infection, the ongoing exposure and presentation of HIV proteins and other pathogens in a pro-inflammatory environment leads to continuous activation of CD4+T-cells resulting in the T-cells being "primed" for apoptotic death. This leads to a gradual depletion of CD4+T-cells and is correlated with disease progression.[30,5] Figure 1.8 shows the generalized immune activation induced by HIV.[35] The presence of certain chemokines and cytokines has been associated

with disease progression and HIV pathogenesis e.g. cytokines such as tumour necrosis factor (TNF) -alpha (α) and –beta (β) which have been shown to enhance HIV replication, whereas others, such as IFN-α and-β have been shown to have an inhibitory effect on HIV replication.[33,31]

The lack of clearance of viral particles together with the burden of other foreign pathogens results in the ongoing activation of both T- and B-cells and ultimately the exhaustion of the immune system.[30]

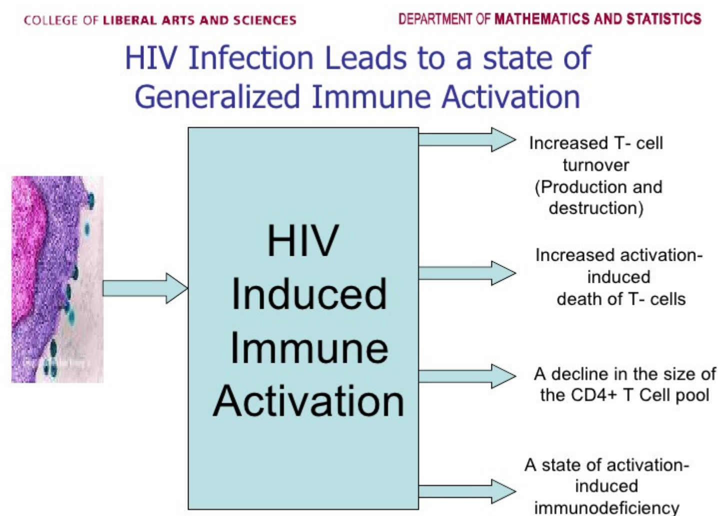

Figure 1.8: Consequences of HIV induced immune activation.[35]

1.1.4.3 Leaky Gut

During the chronic state of infection, the frequency of peripheral circulating HIV infected T-cells is low (0.01%-1.0%) and insufficient to account for the gradual decline in CD4+T-cells. Thus it has been proposed that one of the main causes for the CD4+T-cell decline is infection at the mucosal sites where activation and apoptosis are due to constant microbial infection from a “leaky gut”.[5]

The main cells needed for establishing HIV infection are the CCR5+ CD4+ activated T-cells, which are found in lymphoid tissues and mucosal lymphoid tissues such as

the gastrointestinal tract (GIT). In the GIT, activated memory CD4+T-lymphocytes are primed with the c. o-receptor CCR5, making them the ideal target for HIV infection[5] This massive CD4+T-cell depletion in the mucosal tissues results in the disruption of the gut mucosal barrier, leading to loss of barrier integrity and translocation of normal intestinal flora to the lamina propia and nearby lymph nodes, eliciting a secondary immune response.[5,47]

Additionally, the loss of memory CD4+T-cells lining the GIT mucosa is not reversed by ART, so the "leaky gut" remains a site for ongoing inflammation due to microbial infection.[47] HIV infection is associated with elevated levels of lipopolysaccharide (LPS) which is an indicator of microbial translocation and directly correlates with immune activation. The presence of LPS leads to the production of pro-inflammatory cytokines with dendritic cells, monocytes and lymphocytes being primed and activated establishing a pro-inflammatory environment.[5]

Figure 1.9 shows the stages of HIV infection with changes in levels of CD4+ cells and HIV viral load.

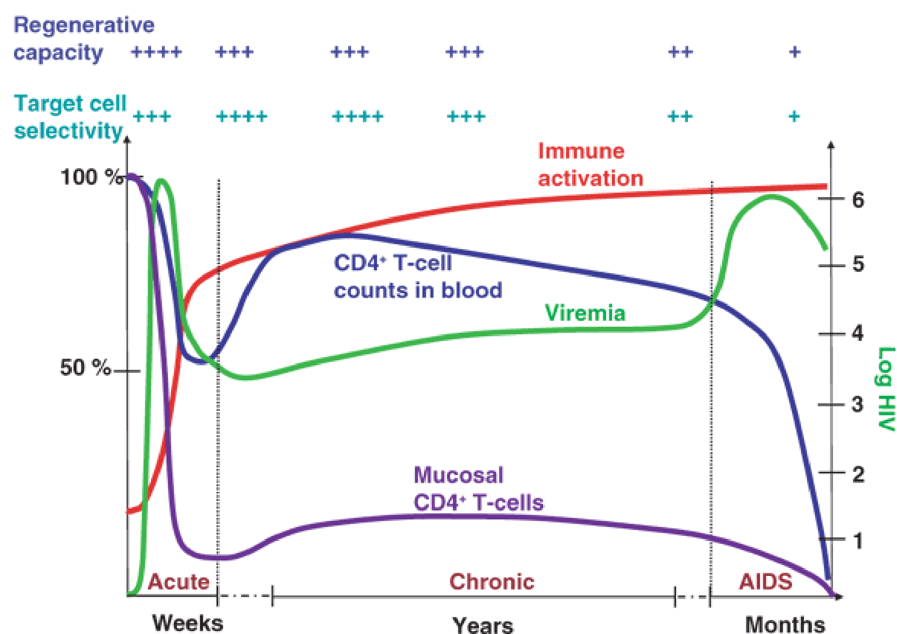

Figure 1.9: The 3 stages of HIV disease progression.[33] Of note is the ongoing immune activation even in the chronic stage.

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1.1.5 HIV and anti-retroviral treatment (ART)

ART is currently unable to cure HIV infection; this is partly due to the virus being able to create viral reservoirs which are insensitive to ART. These reservoirs may consist of long-lived infected cells, cells containing latent virus or ongoing cycles of low level replication or a combination of the above. In a study performed by Sigal et al it was found that when single viral infection occurs in the presence of an ART tenofovir (nucleotide reverse transcriptase inhibitor), the anti-retroviral drug is able to kill the virus. However, when multiple viral infections per cell occur, the drug is overwhelmed and unable to kill all the newly formed viral particles, causing some to escape and become insensitive to the ART.[36]

The CD4+count is an important marker of HIV disease progression and has been the main means of determining ART initiation and monitoring treatment response. CD4+count is predictive of several diseases associated with increased mortality such as cryptococcal meningitis and disseminated cytomegalovirus (CMV) disease.[36] In 2013, the WHO consolidated guidelines recommended that ART should be initiated for all patients with a CD4+count of ≤500 cells per cubic millimeter (mm^3), and that ART be immediately initiated in children up to 5 years regardless of the CD4+count.[38]

HIV viral load predicts HIV related mortality and accurately detects virological failures before immunological or clinical deterioration is evident.[37] Therefore, in 2013, the WHO recommended the measuring of the HIV viral load as the preferred approach to treatment monitoring.[38] Several studies have suggested that the value of CD4+count for routine monitoring is questionable during a state of viral suppression when viral load results are available.[37] Malawi and South Africa rely on HIV viral load for long-term monitoring. In South Africa it has been recommended that the CD4+count measurement be stopped once a patient has been stable on ART for at least 1 year.[39]

A study from South Africa following HIV infected individuals on ART for 10 years showed that most patients who were virologically suppressed maintained CD4+counts of > 200 cells/mm^3.[37]

Guidelines recommended by the South African HIV Clinicians Society states that patients being monitored with viral loads, once the CD4+count is > 200 cells/mm^3 and the viral load is suppressed, need not continue with CD4+count testing, although CD4+count testing is recommended should virological or clinical failure occur.[39]

A study performed by Govender et al found that there was a large portion (34% of individuals tested) of HIV infected individuals with very high viral loads (> 10 000 copies per milliliter (mL)) that did not qualify for ART since their CD4+counts were > 350 cells/mm^3. This suggested that CD4+count at the time of HIV diagnosis is a poor predictor for HIV transmission risk. Therefore it has been recommended that the CD4+count threshold for ART initiation should be replaced by the viral load threshold. Their results confirmed that a large portion (± 50%) of asymptomatic individuals had low CD4+counts and high viral loads qualifying immediately for ART.[40]

Previous 2010 South-African guidelines recommended that ART be initiated at CD4+count of ≤ 350 cells/mm^3 in non-pregnant individuals, adolescents and adults.[39,41] In January 2015 the South African department of Health adopted the 2013 WHO recommendation to initiate ART when the CD4+count reaches ≤ 500 cells/mm^3; however the use of viral load as opposed to CD4+count has not yet been considered.[42]

1.2 HIV Disease Severity

1.2.1 CD4+count

HIV infects the CD4+T-lymphocytes, takes control of its mechanisms in order to produce viral particles, leaving it unable to perform its function resulting in

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progressive depletion of T-cells expressing CD4.[43] The CD4+count is still the most widely used indicator of disease progression to AIDS. CD4+count does not give an idea of the amount of viral particles in the blood, but it does give an idea of the strength of the immune system and its ability to fight disease. The severity of immune depletion is reflected by three measures:

(1) The absolute CD4 cells/mm^3 of blood,

(2) The %CD4 expressing cells amongst the lymphocytes and

(3) The CD4/CD8 ratio.[43]

The absolute CD4+count is favoured above the other two. Normal range for CD4+count is 500-1200 cells/mm^3.[43]

1.2.2 Viral Load

Viral load is a numerical value that quantifies the amount of viral RNA particles per milliliter of blood. A higher viral load correlates with a more severe disease progression. Viral loads are used to monitor the effectiveness of ART in immunocompromised individuals. A viral load of >100 000 copies/mL within 6 months after infection increases the likelihood of developing AIDS within 5 years. In contrast, a viral load of <10 000 copies/mL during the early stages of infection decreases the risk of developing AIDS.[44] A viral load is considered to be undetectable if it measures below 50 copies per/mL.[44]

Individuals are most contagious in the early stages of infection, when the virus replicates uncontrollably while the immune system has not yet responded appropriately to the invading virus. There is some controversy as to whether or not an individual is contagious when the viral load is undetectable. The viral load is only measured in blood, and not in other body fluids such as semen or vaginal secretions, while the blood viral load is undetectable; the same cannot be said for other body fluids that may serve as viral reservoirs.[45]

1.3 Inflammation

A direct consequence of T-cell activation is the increase of intracellular nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-*k*B), which enhances the transcription of integrated virus producing new virions which can then be integrated in other targets, further promoting immune activation and HIV replication.[5] Activation of T-cells also affects their turnover such as differentiation from naïve to effector cells and apoptosis. While a large number of T-cells end up dying upon activation, the dynamics of activation, expansion and apoptosis are different for CD8+T-cells which are able to undergo extensive expansion upon activation, establishing a stable pool of resting memory cells, whereas CD4+T-cells die almost immediately after being activated.[5]

1.3.1 HIV, inflammation and aging

Several immunological factors characterized by HIV infection also coincide with features observed in non-HIV infected elderly individuals. During aging a reduction in T-cell renewal along with the shortening of telomeric endpoints are seen. This is thought to be a consequence of aging over a lifetime which translates into a general decline of the immune system and gradual immunosenescence.[5] This may be partially responsible for the increased incidence of infectious diseases such as influenza, pneumonia, meningitis and sepsis in the elderly, in addition to an increased incidence of non-infectious diseases such as cancers, CVD, liver and renal disorders.[5,33] Studies have shown that HIV-infected individuals present with a reduced bone mineral content and bone formation rate along with an early onset of osteoporosis and increased areas of atherosclerosis when compared to the general non-HIV infected population[33]. In addition, HIV-infected individuals present with a variety of symptoms associated with cognitive deterioration such as memory loss and dementia usually associated with old age.[5] A central theme underlying all these conditions is chronic immune activation and inflammation which has been shown to be associated with this systemic ageing of physiological functions. IL-6 has been directly associated with the development of age-related disorders such as osteoporosis,

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cognitive decline and frailty; and increased levels of TNF-α and IL-1β have been associated with atherosclerosis in the elderly.[33]

Many of these conditions, usually associated with aging, have been described as occurring earlier in younger individuals with HIV infection. They are associated with higher levels of inflammatory markers such as IL-6, C-reactive protein (CRP) and markers of coagulation such as D-dimer, that lead to increased mortality in a young population.[33] The Strategies for Management of Antiretroviral Therapy (SMART) study group showed that markers of inflammation such as IL-6 and D-dimer were independent predictors of all-cause mortality including CVD in patients who were clinically asymptomatic with good viral load levels on ART.[46,47]

Thus, an accelerated process of immunosenescence, "inflamm-aging" takes place during HIV infection which ultimately leads to an earlier onset of immunodeficiency and "old-age" related disorders.[5]

1.3.2 Inflammation, atherosclerosis and cardiovascular disease (Figure 1.10)

According to Saidi et al CVD is the most prevalent and major cause of death worldwide and places a huge burden of cost on the health care system.[54]

The pathogenesis of atherosclerosis is a chronic and complex process involving the participation of various cells, growth factors, cytokines and vasoregulatory molecules and can remain asymptomatic for many years.[48,49]An atherosclerotic plaque consists of the accumulation of intracellular and extracellular oxidized low density lipoprotein (oxLDL), macrophages, smooth muscle cells, T-cells, proteoglycan, collagen, calcium and necrotic debris. The earliest histopathological sign of atherosclerosis is the intimal accumulation of fatty streaks due to oxLDL cholesterol.[48] Figure 1.10 shows the gradual increase of an atherosclerotic plaque over time until it ruptures.

According to a review by Mu et al, it appears that HIV causes endothelial damage and increases atherosclerotic plaque formation through its increasing effect on the triglyceride level as HIV causes a hypertriglyceridaemia.[50,51] HIV progression has been shown to positively correlate with elevated levels of IFN-γ which has been associated with hypertriglyceridaemia.[51] Furthermore, the introduction of protease inhibitors in ART has also been shown to up-regulate CD36, which is a scavenger receptor that mediates the uptake of cholesterol into macrophages turning them into foam cells that contribute to the atherosclerotic plaque formation.[51] Bobryshev showed that HIV directly contributes to inflammation and plaque formation as immunohistochemical staining revealed significantly more HIV infected dendritic cells are found within an atherosclerotic plaque as compared to the amount of dendritic cells in the atherosclerotic plaques of non-HIV infected individuals.[52] The envelope protein gp120 expressed by HIV, has also been found to be an important mitogen during endothelial smooth muscle cell proliferation serving as a causative agent in vascular lesion formation and the start of an atherosclerotic plaque.[53]

Rupture of the atherosclerotic plaque is due to the destabilization of the outer fibrous cap. Activated macrophages residing within the plaque secrete pro-inflammatory cytokines such as IFN-γ and matrix degrading products which over time accumulate and when the fibrous cap's threshold is reached, the plaque destabilizes and ruptures, exposing blood flow to its lipid core which is highly thrombogenic. The thrombogeneity of the lipid core contributes to the high levels of functionally active tissue factor which directly stimulates the coagulation pathway and platelet aggregation. As a result a fibrin-platelet clot or thrombus is formed within the artery causing an ischaemic area posterior to the clot, leading to acute coronary syndrome.[53] Mortality due to ischaemic heart disease decreases with early treatment and increases patients long term survival significantly.[54]

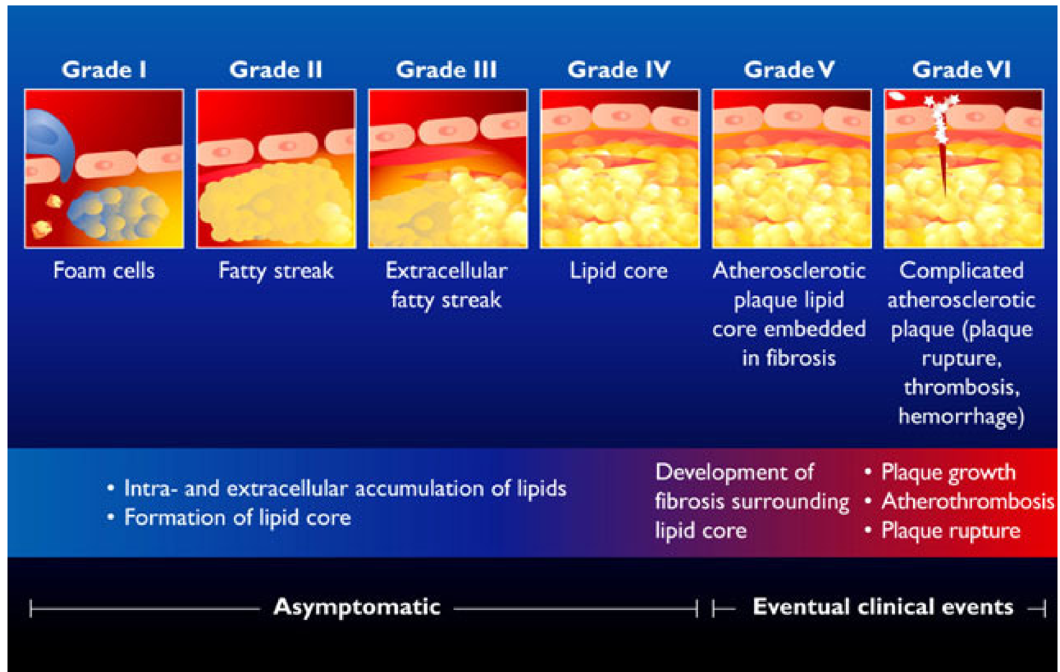

Figure 1.10: Formation and rupture of an atherosclerotic plaque.[55]

1.3.3 CD38 expression on CD8+ T lymphocytes

CD38 is a glycoprotein found on the surface of immune cells such as CD4+, CD8+ and B-lymphocytes.[56] The loss of CD38 function is associated with impaired immune response. CD38 is a marker of cell activation and when expressed on CD8+T-cells it represents a primed activated cytotoxic CD8+T-cell.[57] In the 1990's Giorgi et al observed that higher levels of activated T-cells together with the levels of expressed CD38 marker on CD8+T-cells it coincided with an adverse prognosis for infected individuals.[58]

A study by Benito et al on HIV-infected ART-naïve individuals and ART treated HIV-infected individuals found that the expression of CD38 of CD8+T-lymphocytes correlated with disease progression, independently of the CD4+count. ART treatment reduced the CD38 expression on the CD8+T-lymphocytes in parallel to the viral load, thus CD38 correlated strongly with plasma viral load. However, the CD38 did not normalize completely, indicating that a residual HIV replication still exists. The study also found that CD8+T-cells were higher in the treatment-naïve HIV

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individuals as compared with those on ART. Over time the gradual decline of CD38+ expression on CD8+T-lymphocytes declined with the viral load in HIV infected individuals on ART suggests that CD38/8 cells can be used as a marker of viral replication.[59]

1.3.4 Immunoglobulin G (IgG) (Figure 1.11)

HIV infection elicits an antibody response with multiple isotypes against the proteins encoded by the HIV genes. These antibodies can be unswitched antibody i.e. IgM, and class switched antibody isotypes i.e. IgG, IgA and IgE. In humans IgG has four isotypes: IgG1, IgG2, IgG3 and IgG4. Different HIV-specific IgG antibodies appear approximately 3-4 weeks after infection. Anti-HIV IgG concentrations increase soon after their detection of approx 3-4 weeks and decrease in concentration at approximately 10-12 weeks after infection. The decrease in IgG is due to a decrease in IgG3, which is the first isotype to appear against the viral envelope p24.[60,61] It has been suggested that the early immunoglobulins, IgM and IgG responses against HIV infection does not control the virus replication and therefore is not responsible for the initial decline in the plasma viral load.[60]

In the initial stages of HIV infection, the virus is only confronted by components of the innate immune system, such as the complement system. Several weeks after the infection, the adaptive immunity is fully activated which is reflected by the presence of anti-HIV antibodies and activated T-cells.[62] The most common route for an antigen to stimulate B-cells to undergo class switching is in the lymph nodes. Inflammatory signals from infected mucosal tissue causes the production of B-cell activating factor and IL-10 which can also activate naïve B-cells to undergo class switch recombination.[60]

Tomaras et al found that that HIV transmission severely affected the overall B-cell response, which resulted in an ineffective antibody response. They also found that although protective antibody isotypes may be produced at a later stage of infection, they are ineffective in controlling the infection at the time that they are produced.[60]

Yilmaz et al found that a higher prevalence of atherosclerosis is seen in HIV infected individuals as compared to age matched HIV negative individuals. This risk seemed to be independent of the usual risk factors indicated for CVD.[66] Reports by da Cunha[63] and Glass [64] indicated that HIV-infected individuals had higher circulating levels of IgG against oxLDL. They investigated whether or not HIV viral levels correlated with IgG concentrations. Their results indicated that chronic HIV infection (viral load) correlated strongly with an increased total IgG concentration which indicated disease progression,[66,65] but weakly with markers of inflammation such as hsCRP. The major increase in IgG was due to IgG directed against oxLDL. They also found that a higher IgG was associated with aggravated atherosclerosis indicating its harmful effects. Additionally they described that the HIV cases had higher hsCRP levels than the controls. An alternative reason such as gut bacterial translocation was also excluded as causes for increased IgG levels. [66]

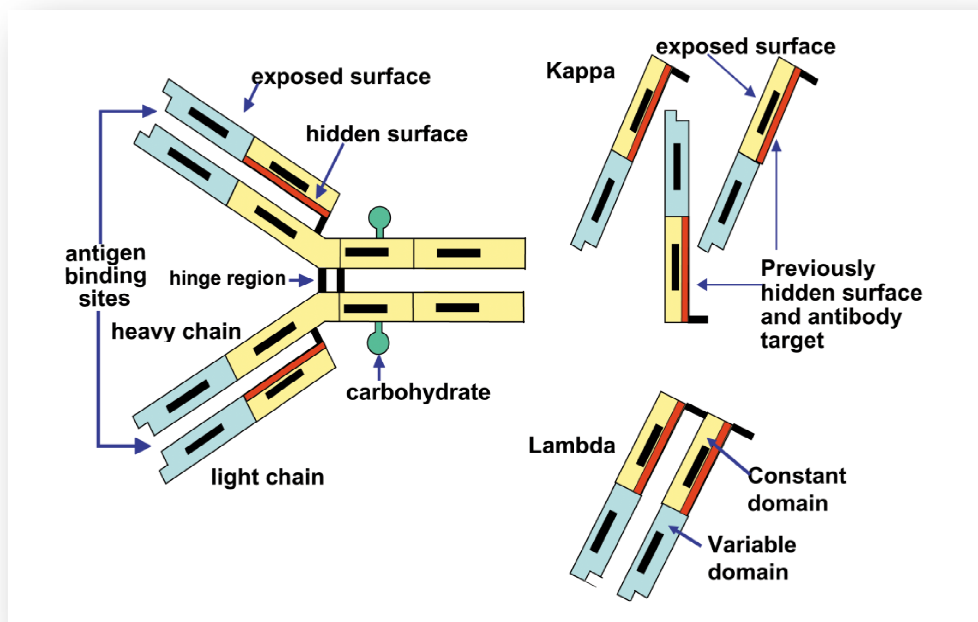

Figure 1.11: Diagrammatic representation of an antibody and its light and heavy (κ and λ) chains.[67]

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1.3.5 Albumin

Albumin is a plasma protein that is exclusively synthesized by the liver and has a half life of 15-20 days. Approximately 38-45% of albumin is intravascular. Elimination of albumin occurs in the muscle, skin, kidney, liver and intestinal tract.[68] Reduced albumin concentrations have been reported in states of chronic infection such as HIV. [68,69] Although serum albumin is not a specific marker for HIV infection it has been found to be the strongest predictor of mortality.[70] Increased catabolism of albumin due to inflammation, worsening of nutritional status and degradation on the liver due to chronic infection has been reported as causes for a reduction in the plasma albumin.[68,69,70] Several studies have indicated that a low albumin concentration is a strong predictor of mortality in various acute and chronic illnesses and that in HIV infected individuals with hypoalbuminaemia (albumin < 35 grams per liter (g/L)) is associated with a more rapid progression to AIDS.[69,71] Bisaso et al reported that serum albumin concentration is a function of the rate of its synthesis, distribution and degradation, due to its long half life a steady decline in albumin suggests a clinically significant deterioration in the liver and thus albumin has prognostic value in determining disease progression independently of the traditional HIV disease severity markers; CD4+counts and viral loads.[68,70]

It has been reported that albumin levels are good predicators of survival in patients with CD4+counts of < 200cells/mm^3 with the risk of death increasing 8-fold with levels of albumin < 35 g/L compared to individuals with albumin levels of > 45 g/L.[70]

A study by Oluwumi and Olatunji, found that serum albumin can be used to monitor response to treatment, as post- treatment albumin levels correlated positively with post treatment weight and CD4+counts up to 700 cells/mm^3. Increased albumin levels also correlated positively with the duration of treatment.[70]

Metha et al performed a study in which it was found that individuals appeared to have a lower albumin concentration after HIV seroconversion as compared to before

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HIV seroconversion and that an albumin concentration of <35 g/L was associated with a faster HIV disease progression. This suggested that the low albumin levels are a consequence of the HIV infections rather than reflecting a mere inherently low albumin of the individuals.[71]

1.3.6 High Sensitivity C-reactive protein (hsCRP)

hsCRP is an acute phase reactant protein produced by the liver in response to IL-6. In inflammation, bacterial infection or trauma, CRP levels can increase dramatically up to a 100-fold.CRP is used to assess the activity of an inflammatory situation. hsCRP can be used to clinically assess chronic low grade inflammation that may be useful in cardiovascular risk assessment as the test is able to measure across the assessment range of 0.2-10 milligrams per Liter (mg/L).[72] A hsCRP level of <2.0 mg/L has a reduced risk of cardiovascular episode.[72] Studies have shown an association between CRP, atherosclerosis and acute CVD and found that hsCRP may be a better predictor of cardiovascular events than some other inflammatory biomarkers. The difference between CRP and hsCRP is in the detectable limit of the protein. CRP assays have a lower detectable limit of 3-5 mg/L, whereas hsCRP assays are able to measure CRP as low as 0.2 mg/L.[73]

A study called the Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) trial used 17800 participants middle aged to elderly with low to intermediate risk of cardiovascular disease and treated them with Rosuvastatin (cholesterol lowering drug) or placebo. They found that Rosuvastatin significantly reduced the hsCRP and LDL-cholesterol and thereby reducing cardiovascular events by 44% in the cases compared the controls. Thus the study suggested that hsCRP levels could be used to assess individuals that may benefit from using statin therapy.[73,74]

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1.3.7 Thrombosis

Many thromboembolic complications have been associated with HIV infection such as deep vein thrombosis (DVT), thrombotic microangiopathy and retinal venous thrombosis. Numerous studies have shown that venous thromboembolic events are two -to ten-fold higher in HIV patients than in healthy controls of comparable age.[51] Furthermore it has been found that the incidence of venous thrombosis is associated with HIV severity with significantly more thrombotic events in individuals with a CD4+count < 200 cells/mm^3. Thromboembolic events have also been associated with certain ART regimes, which have shown to increase thrombotic events from 0.19% before the introduction of protease inhibitors to 1.07% after the use of protease inhibitors.[51]

A hypercoagulation state also seems to exist in HIV infection. Fibrinogen is a well described as an acute phase reactant and platelets interact with fibrinogen resulting in platelet activation and the formation of fibrin. The degradation of fibrin results in the generation of soluble fibrin fragments such as D-dimer. Elevated D-dimer has shown to be a strong predictor of mortality and cardiovascular risk in HIV infection.[47] Studies have also shown that many other coagulation factors and markers of endothelial cell wall damage such as lupus anticoagulant, anticardiolipin antibodies, von Willebrands factor (vWF), soluble thrombomodulin, E-selectin, tissue-type plasminogen activator, angiotensin- converting enzyme and endothelin are also increased over the course of HIV infection.[47,51] Furthermore HIV gp120 induces tissue factor expression in the vascular smooth muscle cells which further affects the arterial wall thrombogeneity.[51]

Studies such as those performed by Jeremiah et al have shown that D-dimer strongly correlates with CD4+counts in HIV infected individuals both on and off ART.[75] Additionally, recent studies have shown that elevated D-dimer levels are strongly associated with mortality after ART initiation and that HIV infected individuals with increased levels of D-dimer should be aggressively monitored after ART initiation.[47]

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The SMART study Group initiated a trial from 2002 – 2006 which compared different strategies for ART and the adverse effects that these strategies may present. Two strategies were evaluated: the current continuous viral suppression strategy and a drug conservation strategy, in which ART would be administered in an interrupted manner depending on the CD4+cell count of ≤ 250 cells/mm^3. Since then, several studies have been performed on the samples that were collected from the 5472 participants. One study of note investigated the all-cause mortality associated with biomarkers such as D-dimer.[76]

The SMART study found that levels of biomarkers such as hsCRP and D-dimer were highly significant (probability value (p) < 0.0001 and $p = 0.005$) respectively when deaths were compared with the controls. The strongest risk gradient with mortality was evident for D-dimer. The D-dimer unadjusted odds ratios for the highest versus the lowest quartile was 6.1 (95% confidence interval, 2.0-18.6; $p = 0.001$) which significantly associated D-dimer with all-cause mortality. The risk of death was significantly increased (24%) for D-dimer when the drug conservation group was compared to the viral suppression group. D-dimer levels were significantly higher in participants not on ART compared to those on ART with a viral load of 400 copies/mL or lower. Other markers such as hsCRP did not vary significantly according to use of ART and viral load at study entry. D-dimer levels were lower for men and for participants with higher CD4+counts.[76]

The SMART study's results indicated that D-dimer was strongly associated with all-cause mortality in all the different scenarios mentioned above. D-dimer increases were also related to increased viral loads one month after study entry. Findings suggest that HIV-induced activation of inflammatory and coagulation pathways has an adverse effect on all-cause mortality among patients with relatively preserved CD4+counts (≥ 400 cells/mm^3) and that interruption of ART may further increase this risk by raising D-dimer levels.[76]

Since the SMART study various articles has been published testing different scenarios of HIV infection and ART for D-dimer risk in all-cause mortality.[77,78,79]

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A study based on the SMART study performed by Boulware et al recruited 1397 participants who were ART–naïve. Their results on some biomarkers tested indicated adverse outcomes with higher levels of D-dimer, CRP and IL-6 prior to ART initiation and 1 month after enrollment with immediate ART initiation. This was in keeping with the SMART study group's results. Their results suggested that D-dimer, CRP and IL-6 could be useful in identifying those ART-naïve patients at higher risk of AIDS or death after ART initiation.[77]

1.3.7.1 Fibrinogen (Figure 1.12)

Fibrinogen, also known as factor I, is a soluble plasma protein synthesized by the liver which circulates in the blood as a disulfide-bonded dimer of 3 subunit chains. It is also one of 13 coagulation factors needed for normal clotting of blood.[80] Circulating fibrinogen has a half-life of 3-5 days and is transformed by thrombin into a fibrin clot in the event of tissue damage.[81,82] Figure 1.12 shows the activation of the clotting cascade after the rupture of an atherosclerotic plaque.

Fibrinogen is involved blood clotting and the management of inflammation. At normal levels, fibrinogen is beneficial to the body; however at chronically increased levels fibrinogen attacks the endothelial wall and causes inflammation, thus promoting the development of atherosclerosis by clot formation. Excess fibrinogen is also able to obstruct blood vessels which may form clots that can become thrombi.[83] Inflammation anywhere in the body triggers the production of fibrinogen which then travels to where inflammation is present and produces cytokines that recruit other immune mediators to deal with the problem. However increased fibrinogen causes inflammation.[83] Many studies have shown that fibrinogen is elevated in diseases such as acute and chronic inflammatory diseases, nephritic syndromes and liver diseases and that elevated fibrinogen levels are associated with atherosclerosis and CVD (fibrinogen > 3.43 g/L). Studies have also shown that an increased fibrinogen is associated with a 7-fold increase in all-cause mortality.[83]

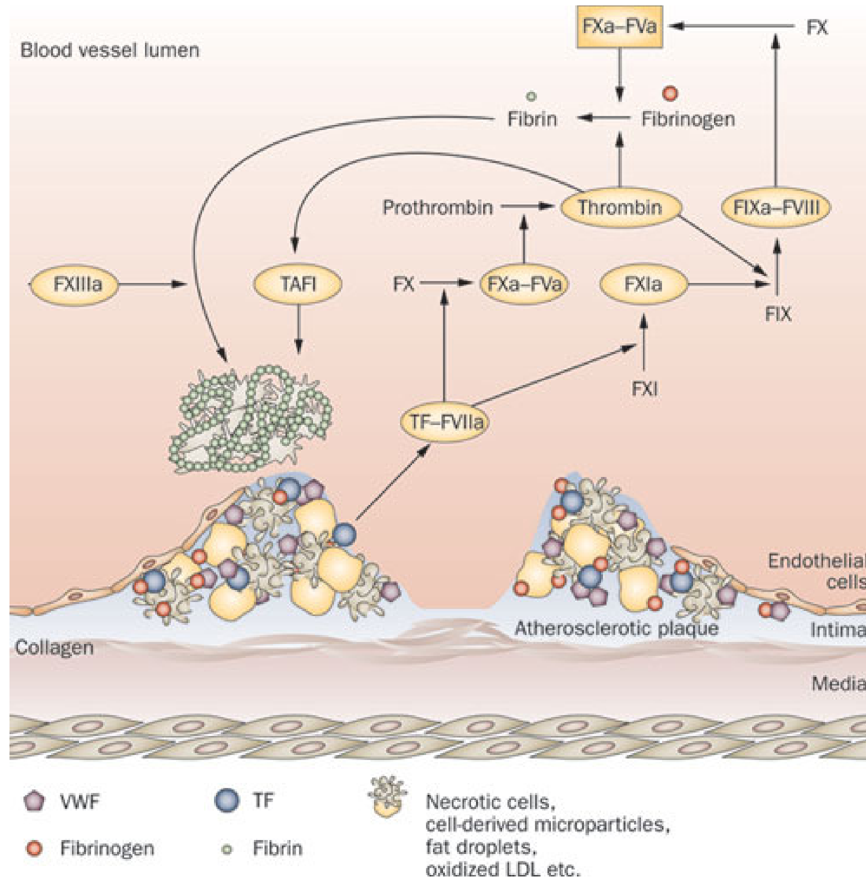

Figure 1.12: The function of fibrinogen in the formation of atherosclerotic plaques.[84]

1.3.7.2 D-dimer (Figure 1.13)

D-dimer formation is the last step in the coagulation cascade after thrombin has cleaved soluble fibrinogen to become a cross-linked gel-like fibrin clot. D-dimer is formed when the protein plasmin cleaves the soluble fibrin clot (fibrinolysis) into various small polymers i.e. fibrin degradation products (FDPs). D-dimer is so called, because after plasmin has cleaved the fibrin clot it leaves two D-domains and one E-domain of the original fibrinogen molecule intact.[85,86] Figure 1.13 shows the formation of D-dimer from a fibrin molecule. D-dimer is therefore a product of normal fibrin degradation, and in healthy individuals it is usually present in a negligible amounts of 0.1-0.2 mg/L. The absence of D-dimer therefore excludes intravascular clot formation and increased levels of D-dimer suggest ongoing thrombotic/fibrinolytic processes.[87] The advantages of measuring D-dimer over

thrombin, is that it is resistant to ex vivo activation, is relatively stable and has a long half-life of 4-6 hours[88] in the circulation of healthy individuals[89]. Elevated levels of D-dimer are seen in all haematological disorders that cause coagulation activation such as acute venous thromboembolism, ischaemic CVD and cancer. Studies have shown that moderately increased D-dimer levels reflect minor increases in blood coagulation and that chronically increased D-dimer levels are associated with coronary heart disease.[89] Furthermore, studies have shown that CVD risk and prognosis is associated with increased levels of D-dimer subtypes.[89] Other studies have shown that an increase in D-dimer is associated with a 1.7-fold increased risk of CVD and that elevated D-dimer is associated with all-cause mortality in apparently healthy individuals.[86]

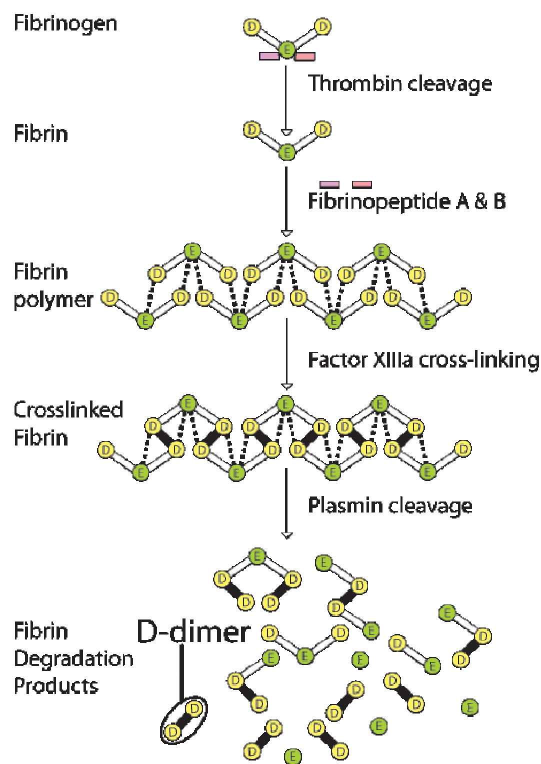

Figure 1.13: The formation of D-dimer molecules through the process of fibrinolysis from a cross-linked fibrin thrombus once the coagulation cascade has completed.[90]

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1.4 Selectins

1.4.1 Selectins- Introduction

Selectins are a group of cell adhesion molecules that are expressed on the surface of leukocytes and activated endothelial cells during periods of inflammation or stress. E-Selectin is encoded by the Selectin E (*SELE)*-gene found on chromosome 1 which synthesises E-selectin from de novo proteins, appearing 3-5 hours after the stimulus and peaking up to 12-hours after exposure to the stimulis.[93] According to Ley and Telen, these molecules bind to glycoprotein ligands that are found on endothelial cells, platelets and leukocytes.[91,93] E-Selectin is also expressed on the skin and bonemarrow.[93]

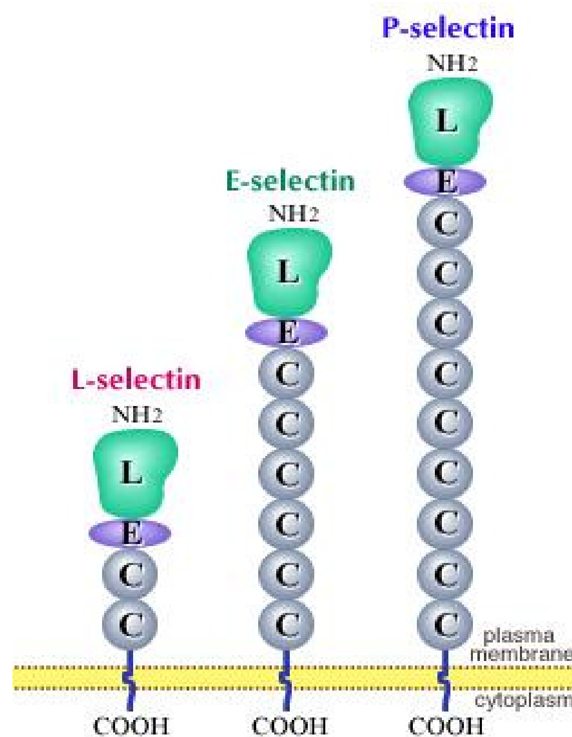

Figure 1.14: Depicts the three different selectins indicating from the top their N-terminus (NH_2), calcium dependent lectin domain (L), epidermal growth factor –like domain (E) the 2, 6 and 9 consensus repeat units (C) and the transmembrane domain.[92]

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1.4.2 Structure

As shown in figure 1.14, all the members of the selectin family share a similar structure namely: an N-terminal, calcium dependent lectin domain, an epidermal growth factor (EGF)-like domain a variable number of consensus repeat units (2, 6 and 9 for L-, E- and P-selectin respectively), a transmembrane domain and an intracellular cytoplasmic tail. The transmembrane and cytoplasmic parts are not conserved across the selectins and allows for targeting different compartments.[93,97] Although they share common elements, their tissue distribution and binding kinetics are different, reflecting their different roles in various pathophysiological processes.[91,97]

L (Leukocyte) selectin is the smallest of the selectins and found on all granulocytes, monocytes and mainly leukocytes. P (Platelet) selectin is the largest and is stored in the alpha granules and Weibel-Palade bodies of endothelial cells and expressed on activated platelets and endothelial cells. E (endothelial) selectin is only expressed on the endothelium during periods of inflammation.[91]

Selectins show significant homology among themselves, except for the transmembrane and cytoplasmic domains, and between species. The lectin domain which binds to sugars is the most conserved, which suggests that the three selectins bind in a similar fashion to sugar molecules. The transmembrane and cytoplasmic domains are highly conserved between the species but not across the selectins. These parts of the molecules are responsible for targeting different compartments such as P-selectin to the secretory granules, E-selectin to the plasma membrane and L-selectin to the tips of the microfolds on leukocytes.[91] During inflammation these selectins traffic the cells of the adaptive immune system, T- and B-lymphocytes and platelets from the peripheral blood towards the point of inflammation. This slow downstream movement of leucocytes along the endothelium is known as leukocyte rolling (Figure 1.15). Each of the 3 selectins can mediate leukocyte rolling under the appropriate conditions.[91,97]

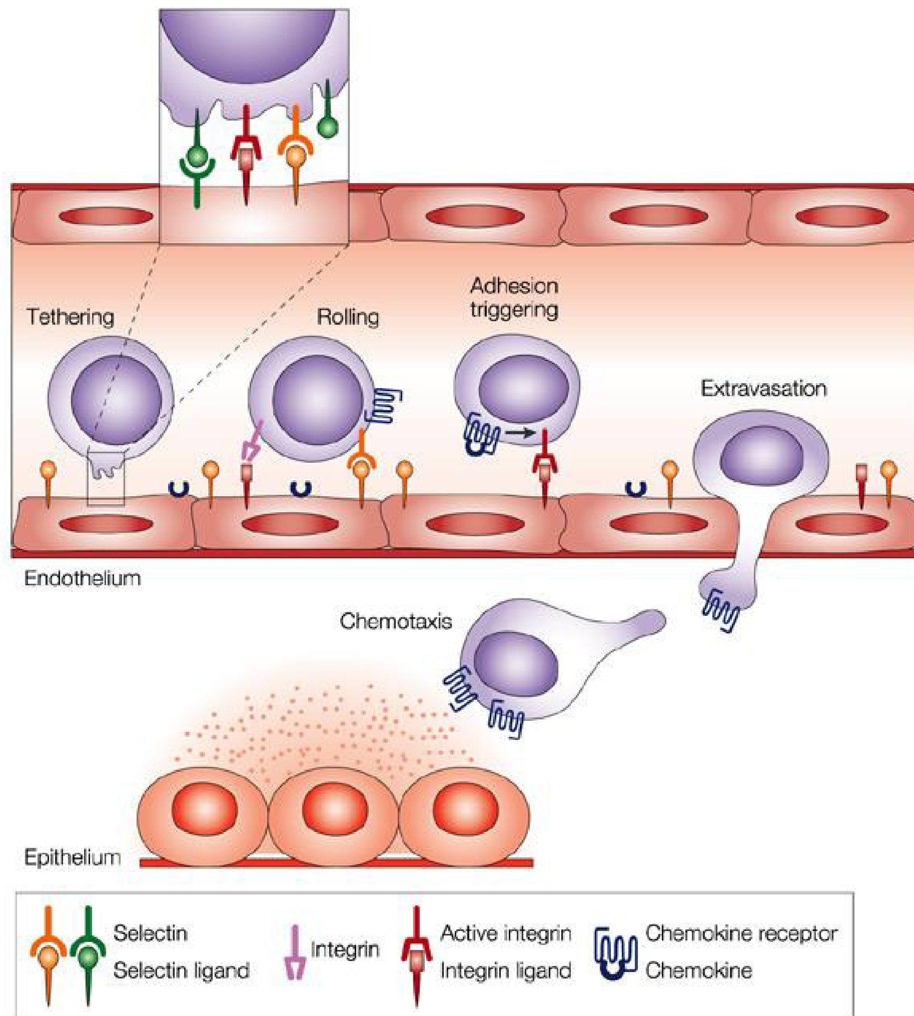

Figure 1.15: Showing the rolling and slowing of the leukocytes, entering the endothelium moving to the point of inflammation[94]

1.4.3 Function

Selectins are involved in lymphocyte homing during acute and chronic inflammation, including post-ischaemic inflammation in muscle, kidney, heart, skin inflammation, atherosclerosis, glomerulonephritis and erythematosus.[91]

During inflammation the local release of cytokines IL-1 and TNF-α by damaged cells induces the over-expression of E-selectin on endothelial cells of nearby blood vessels. Leukocytes in the blood expressing the correct ligand will bind to the E-

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selectin's lectin-like domain with low affinity. As the inflammatory response continues, chemokines released by the injured tissue enter the blood vessels and activate the rolling leukocytes, which are now able to tightly bind to the endothelial surface and begin making their way into the tissue. P-selectin has a similar function, but is expressed on the endothelial cell surface within minutes as it is stored within the cell rather than produced on demand.[91]

Monocytes infiltrate beneath the endothelium where they undergo differentiation to macrophages which phagocytose the LDL-cholesterol and transform to foam cells. These foam cells then produce cytokines, growth factors, reactive oxygen species and matrix degradating enzymes which sustain the atherosclerotic process. The intensity of oxLDL- cholesterol is a major stimulus for ongoing inflammatory processes. The accumulation of foam cells contribute to intimal thickening and lesion formation which in turn contribute to the enhancement of the ongoing inflammatory process.[48,95]

1.4.4 Soluble E-Selectin

E-selectin is bound to the endothelial cells and is expressed during a period of inflammation. However the process of inflammation releases cytokines such as TNF-α that promotes endothelial cell apoptosis, after which they release their ligands and receptors such as E- and P-selectin, vascular cell adhesion molecule -1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) into the blood circulation. Free floating E-selectin is then known as soluble E-selectin (sE-selectin) which can be measured by means of enzyme-linked immunosorbent assay (ELISA).[96]

1.4.5 Endothelial Dysfunction

Inflammation and endothelial dysfunction play an important role in the pathogenesis of atherosclerosis. Adhesion molecules enable leukocytes to adhere to the endothelial cell wall which is essential for an inflammatory response. HIV is associated with the inflammatory activation of the vascular wall as HIV infected macrophages secrete

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large amounts of TNF-α which activates the endothelial cells leading to leukocyte adhesion.[95]

1.4.6 Cellular adhesion molecules and cancer

During the process of metastasis, tumour cells invade the surrounding tissues to reach and penetrate the vascular endothelium. Once the tumour cells enter the circulatory system the "rogue" cancer cells are subjected to shear forces and the immune response which affect their ability to metastasize.[97] Only the tumour cells that are able to overcome the impact of the immune response will adhere by means of selectins to the vascular endothelium of distant organs, migrate and successfully colonize these sites.[97] It is believed that platelets form adhesive clusters with the circulating tumour cells and mask them from the immune response. The platelet-tumour cell cluster is then able to adhere to the vascular wall by means of selectins and adhesion molecules. Activated platelets are able to secrete vascular endothelial growth factor which promotes vascular hyperpermeability allowing tumour cells to more readily migrate to surrounding tissues.[97] Selectins mediate the initial tethering and rolling events during leukocyte accumulation to sites of inflammation, similarly selectins facilitate cancer metastasis.[97]

1.4.7 Opportunistic infections

Cellular adhesion molecules are found circulating in all healthy individuals; however several studies have reported increased selectin levels during various diseases such as infections, autoimmune diseases and various malignancies.[99] HIV-infection causes numerous immune system abnormalities such as leukocyte dysfunction, elevated circulating adhesion molecules i.e. soluble ICAM-1, soluble L- and E-selectin which may lead to altered cell-to-cell interactions.

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1.5 Studies of E-selectin in HIV

Previous studies have shown that E-selectin levels increase in HIV-infection; however E-selectin expression is tightly regulated NF-κB. NF-κB is transcriptionally silent until it comes in contact with inflammatory cytokines or reactive oxygen species.[98] As the expression of E-selectin requires an intact active κB site, Cota-Gomez et al investigated whether Tat-mediated E-selectin up-regulation is able to activate NF-κB independently of inflammatory cytokines.[98]A cell culture study found that HIV Tat protein increased E-selectin expression by up to 87%. This up-regulation was similar to TNF up-regulation of E-selectin via the NF-kB pathway.[98]

In a study by Nordoy et al it was determined that HIV-infected subjects with symptomatic CMV infection had significantly lower E-selectin and ICAM-1 levels than HIV-infected subjects with no CMV infection and the HIV-negative control group.CMV is known to infect endothelial cells and it has been determined that CMV infected endothelial cells show a lack of up-regulation and expression of adhesion molecules. They also found that in HIV infection only serum VCAM-1 levels were raised but not ICAM-1 or E-selectin levels. The elevated VCAM-1 levels may be explained by the presence of VCAM-1 on activated endothelium, dendritic cells, macrophages and renal proximal tubili cells, all of which are essential for HIV infection. TNF-α has been found to induce sE-selectin VCAM-1 and ICAM-1 in endothelial cells[99]

Kristoffersen et al examined the levels of circulating markers of endothelial dysfunction in HIV-infected subjects before and after the initiation of ART and found no difference in E-selectin and sVCAM-1 levels between the case and control group (HIV-negative) before ART initiation. However 2 to 14 months after ART initiation, E-selectin, sVCAM-1 sICAM-1 and hsCRP levels were significantly decreased. They also found that ART significantly increased the levels of total cholesterol, triglycerides and HDL-cholesterol which are major contributors to the formation of atherosclerotic plaques.[100]

A further study by Rönsholt et al measured endothelial markers over a long term (12 years) period in treated HIV-infected individuals found that HIV-infected subjects still had signs of vascular inflammation compared to the healthy controls. They found that IgA levels were lower than the controls; however there was no difference in IgM and IgG levels. None of the parameters tested, namely β-2 microglobulin, IL-8, TNF-α, sICAM-1, sVCAM-1, sE-selectin and sP-selectin correlated with the CD4+count or the viral load. However, the study was performed on 70 HIV-positive participants most of whom were male (68/70) and 16 (14/16 were male) age and gender matched HIV-negative controls. They proposed that their results were due to the case and control numbers being too small to yield any significant results.[101]

Graham et al measured levels of endothelial biomarkers in HIV-infected treatment-naïve Kenyan women who had advanced HIV infection. Testing commenced 6 and 12 months after these women initiated ART and they found that E-selectin levels were significantly reduced (13.7 ± 6.3 nanograms per millilitre (ng/mL)) ($p < 0.001$) 6 months after initiation of ART. However at 12 months, there was no significant ($p = 0.29$) reduction in E-selectin levels (25.2 ± 5.2 ng/mL) as compared to the baseline results (24.2 ± 8.1 ng/mL).[102]

Another important study by Graham et al examined levels of endothelial biomarkers in HIV treatment-naïve women, from before seroconversion to the acute infection (0-119 days after estimated date of infection) and chronic infection (120 days and more after estimated date of infection). They observed that HIV infection does lead to endothelial activation with plasma levels of cellular adhesion biomarkers such as soluble ICAM-1, VCAM-1 and E-selectin being significantly increased from the acute infection phase through the chronic infection phase. However, E-selectin was the only parameter whose levels were significantly increased only during the acute infection stage and then steadily reduced to baseline levels as the chronic infection period continued, whereas the other markers continued to increase. They also found that E-selectin levels do not correlate with the viral set point during the first chronic infection period and that there was no difference in E-selectin levels in women who

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died or had disease progression versus infected women who had no disease progression.[103]

Calza et al examined the levels of various endothelial biomarkers in HIV-negative (controls) and HIV-positive (cases) subjects who were either ART-naïve or on ART. They found that E-selectin levels were significantly higher in group A (HIV-positive ART-naïve subjects) and B (HIV-positive subjects on continued ART) compared to group C (negative controls) and that these levels were associated with an elevated viral load and lower CD4+count. E-selectin levels were also higher in the HIV treatment-naïve group compared to the ART group. They also found that higher levels of endothelial markers were associated with increased levels of triglycerides, LDL-cholesterol and a more advanced age and that ART may reduce the levels of some adhesion molecules by limiting viral replication. [104]

1.6 Other studies performed on the present study cohort

The samples collected for our study were from ART-naïve individuals attending an HIV prevention and testing (HPT) clinic in Crossroads, Cape Town, South Africa. In depth detail will be provided on the sample collection in the Methods section. We will briefly describe some of the other studies performed on this cohort.

As previously mentioned, HIV infection causes chronic inflammation and immune activation, which is associated with B-cell dysfunction. Previous studies have described an increased incidence of abnormal serum protein electrophoresis patterns such as monoclonal bands in HIV.[107] One of the characteristics of HIV infection is the presence of polyclonal hypergammaglobulinaemia and increased IgG levels.[109] There has been some controversial evidence whether or not a higher or lower prevalence of monoclonal, oligoclonal and polyclonal gammopathies is found in HIV.[107]

Activated T-cells interact with B-cells to induce heavy chain immunoglobulin class switching from IgM to IgG. Thus HIV-infection is also characterized by polyclonal

hypergammaglobulinaemia. A study by van Vuuren et al at our institution in 2010 studied the serum protein electrophoretic (SPE) patterns of 368 HIV-infected subjects *on* ART (not our cohort). They found that 3.2% had monoclonal bands and 3.8% had oligoclonal banding. This population was unique in that they were younger than the cohorts used in previous studies. They found that these abnormal protein electrophoretic patterns were associated with shorter duration of ART, increased total protein and gamma fraction levels, but not with CD4+ or viral load counts.[105]

Zemlin et al then studied the (SPE) patterns on our treatment-naïve HIV infected cohort to determine if there was a correlation with markers of disease severity and the value of polyclonal hypergammaglobulinaemia as a marker of generalized B-cell stimulation.[106] Their results were correlated with the markers of disease severity namely CD4+count, viral load, IgG and albumin. Significant differences were found for IgG, the gamma fraction in SPE and CD4+counts in the HIV-positive cases as compared the HIV-negative controls. Controls were found to have a 90% normal SPE pattern. Amongst the cases, 44% presented with polyclonal hypergammaglobulinaemia, 27% with an abnormal gamma-globulin region requiring immunofixation. The immunofixation revealed that 12.5/27% had oligoclonal bands on a polyclonal background and 10.3/27% had polyclonal hypergammaglobulinaemia and 4/27% had small monoclonal bands of IgG kappa (κ) type. CD4+counts and albumin were lower in the polyclonal and abnormal electrophoresis patterns compared to the normal SPE patterns. IgG levels were higher in the polyclonal and abnormal SPE patterns compared to the normal SPE patterns. Their results indicated remarkably high incidence of abnormal SPE patterns in the cases compared to the controls. Only 4% of the cases were found to have monoclonal bands where as the other cases had either oligoclonal bands or polyclonal hypergammaglobulinaemia. Polyclonal hypergammaglobulinaemia correlated significantly with a lower CD4+count. No association was found between the viral load and SPE groups, suggesting that viral load is not the underlying cause for B-cell stimulation in this HIV-infected group, and further supporting the concept of generalized immune activation as an important driving force in disease progression. Thus this study highlighted the occurrence of generalized B-cell

stimulation, and the inverse correlation that exists between polyclonal patterns and the CD4+count in untreated HIV infection.[106]

Serum free light chains (FLCs) are part of the immunoglobulins secreted by B-cells and are either, κ or lambda (λ) type. Increased levels are as a result of imbalanced light or heavy chain immunoglobulin production. Imbalanced immunoglobulin production is associated with various disorders, benign and malignant such as multiple myeloma and abnormal ratios of κ to λ have been associated with risk of disease progression. Abnormal κ and λ ratios are also found in other B-cell malignancies such as chronic lymphocytic leukaemia and non-Hodgkins syndromes.[107]

Further research on this cohort showed significantly increased protein levels in the HIV positive group due to an increased gamma fraction and albumin was significantly decreased. Significant negative correlations were found between albumin and κ and λ FLCs, which suggested that with increased disease severity there were low albumin levels, and B-cell dysfunction due to an increased presence of FLCs. No significance was found between albumin and FLC ratio, suggesting that the hypergammaglobulinaemia was polyclonal in nature. Strong positive correlations were found between the gamma fraction and κ and λ FLC values, and κ/λ FLC ratio, most likely reflecting the increased globulins, supporting the concept of B-cell hyperactivity. Significant difference was found in the CD4+counts for the HIV positive group and both κ and λ chains showed significant inverse correlation with the CD4+count, with the lowest CD4+counts having the highest FLC levels. Positive correlations were also noted between the κ and λ FLC and the viral load. However, the FLC ratio was not influenced by the viral load, which supports the finding of polyclonal B-cell activation in HIV. Creatinine levels for all the subjects were also tested to exclude renal impairment as a cause for raised FLC. All the subjects presented with normal renal function. (Unpublished data)

Asymmetric dimethylarginine (ADMA) is an established marker of endothelial dysfunction, cardiovascular and renal disease. It functions as an endogenous inhibitor

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of nitric oxide synthase (NOS). Nitric oxide is an anti-atherogenic molecule that functions as an endothelium-derived relaxing factor, and prevents platelet adherence and aggregation, suppresses smooth muscle cell proliferation and inhibits leukocyte adhesion. It has been proposed that elevated ADMA occurs as a result of impaired dimethylarginine dimethylaminohydrolase (DDAH) activity which normally mediates the degradation of ADMA. Impaired DDAH activity may result from various stress-inducing factors such as oxLDL and pro-inflammatory cytokines. The decrease in DDAH activity causes ADMA to accumulate resulting in a decreased NOS activity and a loss in vaso-protective properties. Hudson et al performed a study on this cohort to determine if ADMA levels would be increased and whether these would correlate with markers of disease progression and immune activation.[108] They determined in HIV infection, ADMA levels were significantly higher than in controls. ADMA levels correlated negatively with CD4+counts but not with the viral loads and significant correlations were found between ADMA and markers of cellular immune activation namely %CD38/8, IgG and adenosine deaminase (ADA). No correlation was found between ADMA and hsCRP and IL-6.[108] They concluded that ADMA is elevated early in the course of HIV infection due to the young (32 years) age of the study group that had well preserved CD4+counts and were treatment-naïve. ADMA concentrations correlated inversely with the CD4+count, but not with the viral load, suggesting that other factors besides viral infection is contributing to the ongoing inflammation.[108]

The enzyme ADA catalyses the breakdown of adenosine to inosine and is up-regulated during chronic inflammatory conditions. Previous work has suggested that an increased ADA level may be used as a diagnostic marker for AIDS and subsequent studies have shown that ADA levels were increased in the serum of HIV-positive intravenous drug users and this correlated with stage of disease.[109] Increased ADA (marker of cellular immunity) activity correlates with disease progression and IgG concentrations which is a useful indicator of the humoral immune response since B-lymphocyte activation is a characteristic if HIV infection. B-cell activation is well described in the context of HIV. A study was performed by Ipp et al on our cohort in which markers from the adaptive immune response comprising of both the cellular

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arm (depicted by ADA levels) and the humoral arm (depicted by IgG levels) were investigated to assess ongoing stimulation of the immune system in this clinically healthy, asymptomatic ART-naïve HIV-infected population with well preserved CD4+counts. They found that ADA, IgG, lipopolysaccharide binding protein (LBP) and soluble CD14 were significantly increased. Serum ADA and total IgG correlated significantly with the %CD38/8 and ADA correlated with both %CD38/8 and the viral load. ADA and IgG both correlated inversely to the CD4+count. Significant increases in IgG levels were seen between the groups with CD4+counts of 350-500 and >500 cells/mm^3. The %CD38/8 was significantly increased in the HIV-infected group as compared to the controls and this correlated inversely with the CD4+count and directly with the levels of ADA, IgG and LBP. Importantly even subjects with CD4+counts of >350 cells/mm^3 had statistically higher levels of ADA, IgG, %CD38/8, LBP and sCD14.Thus this study showed that by testing markers of inflammation, individuals with well preserved CD4+counts can benefit from earlier ART intervention, rather than waiting for the CD4+count to decrease to less than 350 cells/mm^3.[109]

Immune activation is an important prognostic factor in HIV infection and peripheral blood cytopaenia is a complication of immune activation in HIV. Cytopaenias occur due to pro-inflammatory cytokines, HIV-infected T-cells and malignant bone marrow infiltration that suppress haemopoiesis. Currently testing for immune activation is not a routine practice for monitoring HIV disease progression. Full blood counts (FBC) are regularly requested prior or directly after ART initiation due to the prevalence of cytopaenias, opportunistic infections and adverse drug effects. Cytopaenias include anaemia, leukopaenia, neutropaenia, lymphopaenia and thrombocytopaenia. Leukopaenia in HIV is thought to be due to decreased lymphocyte and neutrophils counts and studies have shown that these correlated with markers of immune activation in HIV. Thus the lower the white cell count (WCC), the higher the immune activation (%CD38/8).[110] A FBC consists of a WCC, haemoglobin (Hb), platelet and white cell differential count. The white cell differential count consists of a neutrophil, lymphocyte, monocyte, eosinophil and basophil count. Large unstained cells (LUCs) do not generally form part of the WCC,

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but is available on request. LUCs are made up of paediatric lymphocytes, peroxidise-negative blasts, hairy cells, plasma cells and virally activated lymphocytes.[110,111]

A study performed by Vanker et al found significant differences between %LUCs, %CD38/8, CD4+counts and LBP in the HIV infected group compared to the uninfected group. They proposed that increased levels of %LUCs indicate increased immune activation and correlated with markers of disease progression due to decreasing CD4+count. An inverse correlation was found between %LUCs and CD4+count. No correlations were found between %LUCs or %CD38/8 as compared to the viral load, suggesting that the viral load is not the main contributor to the sustained CD8+T-lymphocyte activation. Furthermore, %LUCs and %CD38/8 correlated positively with LBP supporting the "leaky gut" concept as the cause for ongoing immune activation. They proposed that %LUCs may be a valuable inexpensive marker to test innate and adaptive immune stimulation in clinically asymptomatic, HIV treatment-naïve subjects.[111]

A subsequent study performed by Vanker et al examined the significance of FBC parameters in HIV-infected individuals compared to uninfected controls and whether these markers correlated with markers of disease progression and immune activation.[110] They found significant differences for %CD38/8, CD4+counts, WCC counts, Hb concentrations, absolute neutrophils counts, absolute lymphocytes and LUC in the HIV-infected group compared to the uninfected group. No significance was found between the platelet counts of the HIV-infected group compared to the uninfected group. A significant correlation was found between %CD38/8 compared to LUC and WCC. %CD38/8 and Hb presented with a statistically significant inverse correlation along with CD4+count and LUCs. Significant correlations were also found between CD4+count and absolute lymphocyte and neutrophil count and the WCC. The WCC was significantly lower and the LUCs was significantly higher in the HIV-infected group compared to the controls, furthermore both parameters correlated with the CD4+count and %CD38/8. They proposed that FBC may be a cost effective method to determine immune activation in our setting.[110]

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1.7 Hypothesis

As the incidence of cardiovascular disease is increased in HIV infection, we hypothesised that E-selectin levels will be increased in ART- naïve HIV infected individuals due to a state of chronic inflammation and endothelial cell activation. We further hypothesised that the levels of E-selectin would be directly proportional to markers of disease severity, inflammation and coagulation.

1.8 Aim of study

The aim of this study was to determine the levels of E-selectin in an ART-naïve HIV-infected population compared to uninfected controls and to correlate these levels with markers of disease severity, inflammation and coagulation.

1.9 Objectives

1. Measure levels of E-selectin in ART-naïve HIV infected individuals and healthy controls.
2. Correlate levels of E-selectin with markers of HIV disease severity (CD4+count and viral load).
3. Correlate levels of E-selectin with markers of inflammation (WCC, %CD38/8, hsCRP, albumin and IgG) and coagulation (D-dimer and fibrinogen).

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SECTION II: MATERIALS AND METHODS

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2.1 Sample stability

Tests performed on di-potassium Ethylenediaminetetraacetic acid (K_2-EDTA) lavender top tubes such as FBC, viral load, CD4, CD8 and %CD38/8 were analysed as soon as possible after collection, as their sample stabilities are as follows:

White cell count: <24 hours at room temperature[112]

Absolute CD4+count: ≤ 4 days at room temperature ±21ºC[113]

Absolute CD8+count: ≤ 2 days (48 hours) at room temperature ±21ºC[114]

Viral Load: ≤ 30 hours at room temperature ±21ºC[115]

Test that were performed on tri-sodium citrate blue top tubes were fibrinogen and D-dimer. These were analysed as soon as possible as their sample stabilities are as follows:

Fibrinogen: ≤ 24 hours at room temperature ±25ºC[116]

D-dimer: ≤ 4 hours at room temperature ±25ºC[88]

Tests that were performed on serum samples stored at -80ºC have of the following stabilities:

hsCRP: stable for at least 10 years at -80ºC[117]

IgG: stable for up to 25 years at -25ºC and at -80ºC for an unknown period of time exceeding 25 years[118]

Albumin: stable for up to 25 years at -25ºC and at -80ºC for an unknown period of time exceeding 25 years[118]

E-selectin: stable for an unknown period of time and up to 5 freeze thaw cycles at -80°C.[119]

2.2 Sample size and ethical considerations

HIV- infected individuals not on ART were recruited from an HIV prevention and testing (HPT) clinic, Emavundleni in Crossroads, Cape Town. The clinic is attached to the Institute for Infectious Disease, Desmond Tutu HIV centre, University of Cape Town (UCT) and employs the national algorithm for accredited rapid tests. The

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uninfected controls were also from the same clinic and had similar social demographics. All the participants were Black and of African descent.

This study involved 180 participants, consisting of 114 HIV positive treatment-naive (case) samples and 66 (HIV negative) control samples. This cohort is part of the existing HAIG study (HREC # N07/09/197). Written consent from participating individuals was obtained and ethics clearance for the HAIG study had been granted. This study obtained its own ethical clearance from the University of Stellenbosch (HREC #S14/08/175) and was carried out in accordance with the Declaration of Helsinki and ICH GCP guidelines.

Sample size was calculated using the following formula:[120]

Formula: $n = Z^2 Pq / d^2$:

Where: n = sample size;

$Z = 1.96$ (confidence interval= 0.95%) which is a constant

P = expected prevalence (we used 18.2%= 0.182)[2]

$q = 1\text{-}P$ = complimentary probability= (1 - 0.182 = 0.818)

d = precision (we used 5% = 0.05);

$n = 1.96^2 \times 0.182 \times 0.818 / 0.05^2 = 228.7$ participants. Therefore 229 individuals should ideally participate in this study.

However the final number of participants is 180 due to lack of sufficient information on the other participants.

2.2.1 Inclusion Criteria

1. HIV infection not on ART
2. Clinically healthy/ asymptomatic individuals
3. All subjects were older than 21 years

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2.2.2 Exclusion Criteria

1. Any concurrent infections
2. Tuberculosis (TB) or anti-TB therapy
3. Pregnant individuals

2.3 Assay Methods

2.3.1 HIV status determination: screening and confirmation

The initial screening test used to determine the participants' HIV status was the HIV rapid strip test method (HIV-1/2 Antigen(Ag)/Antibody(Ab) Combo) by Alere$_{TM.}$

The HIV-1/2 Ag/Ab combo is an immunochromotographic test that detects the presence of HIV-1 and HIV-2 antigen p24 and antibody. Specimen is added to the sample pad and mixes with the biotinylated anti- p24 antibody, selenium colloid – antigen conjugate and selenium colloid- anti p24 antibody. Migration of this mixture continues through the solid phase to the avidin, recombinant antigens and synthetic peptides at the patient window sites. If antibody to HIV-1 and/or HIV-2 is present, one red bar will form at the patient HIV antibody window site. If antibody to the HIV-1 and/ or HIV-2 is absent no red bar is formed at the patient HIV antibody window site.

If free non immunocomplexed HIV-1 p24 antigen is present, a red bar at the patient HIV antigen window is formed and no red bar is formed at the patient HIV antigen window site.

To ensure assay validity a control bar is incorporated into the assay device.

The test has a sensitivity of 100% and specificity of 99.49% for the detection of HIV-1 and HIV-2 antibody and antigen when using whole blood in the African population.[121]

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The confirmation test used for participant HIV status determination is the Uni-Gold™ Recombigen®HIV by Trinity Biotech.

Uni-Gold™Recombigen®HIV is an immunochromatographic sandwich immunoassay strip test that employs genetically engineered recombinant proteins that represent the immunodominant regions of the envelope proteins of HIV-1. These proteins are colloid gold linked and are immobilised in the test region of the nitrocellulose strip.
If antibodies to the HIV-1 are present, they will bind with the HIV-1 antigen/colloidal gold reagent and to the antigens immobilised in the test region. A positive result is visible as a pink/red band in the test region. A build-in control will always appear as a pink band in the control region of the strip test. If the test functions correctly, a positive result will be seen as two pink bands, one in the test region and the other in the control region. A negative result is seen when only one line appears in the control region. The test has a sensitivity of 100% and a sensitivity of 99.0% when using whole blood.[122]

2.3.2 E-selectin

Serum samples frozen at -80ºC were thawed and 1:5 dilutions prepared from each sample using 1X standard diluents buffer. E-selectin (CD62E) human (ELISA) kit from **abcam®** (Cambridge, United Kingdom (UK)) was used to determine the E-selectin levels for each sample, standard and control. Testing was performed in duplicate.

A monoclonal human antibody specific for CD62E is coated onto the wells of the microtiter plates. 100 microliter (µL) of Controls, standards and diluted unknown samples were pipetted into these wells in duplicate. Standards, controls and samples were incubated with 50µL biotinylated monoclonal antibody specific for CD62E. After washing, 100µL of the enzyme, Streptavidin horseradish peroxidase (HRP) was added. This binds to the biotinylated antibody bound to the samples, control and standards during incubation. After incubation the wells underwent a washing cycle.

Lastly 100µL 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was added which binds to the Streptavidin HRP enzyme producing a blue colour reaction. After a 20 minute incubation period in the dark. 100µL of stop reagent was added turning the product a yellow colour (Figure 2.1). The intensity of the coloured product is directly proportional to the concentration of E-selectin in the samples. [123] Thereafter the 96 well plate was placed into a spectrophotometer to obtain the optical density (OD) readings for each well. The readings were inserted into an Excel sheet and the concentration for each sample was calculated from a standard curve.[123]The normal range for E-selectin according to the package insert is: 11.78-160.72 ng/mL[123]

Figure 2.1: Showing the colour change from blue to yellow after the addition of the stop reagent.

Other Biochemical parameters that were used to assess thrombosis and HIV disease severity:

2.3.3 White cell count (WCC)

The WCC was analysed using EDTA whole blood on the Siemens ADVIA 2120 (Berlin and Munich, Germany) haematology analyser. The instrument makes use of cytochemistry and flowcytometry techniques and uses a combination of cell size, myeloperoxidase staining and nuclear lobulation to determine the total leukocyte and 6-part differential count. Samples were processed on the same day that they were collected.[124]The normal range for WCC in our laboratory is 4.0-10.0 x10^9 cells/L.

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2.3.4 CD4+count

The CD4+count was determined my means of flow cytometry using EDTA whole blood. The test performed was the Becton Dickinson (BD) MultiTest CD3-fluorescein isothiocyanate (FITC)/CD8 phycoerythrin (PE)/CD45 peridinin chlorophyll protein (PerCP)/CD4 allophycocyanin (APC) reagent together with TruCOUNT tubes (BD Biosciences, San Jose, California (CA)) and analysed by BD Fluorescence Activated Cell Sorter (FACS) Calibur (BD Biosciences, San Jose, CA). Fluororchrome-labelled CD4 antibodies bind specifically to the leukocyte surface antigens. These cells then pass through a laser beam and scatter the laser light. The stained cells fluoresce. The scatter and fluorescence is detected by the instrument that provides information about the cell's size, complexity and relative fluorescence intensity. The fluorescence is gated and the lymphocyte population determined.[125] Sample were processed on the same they that they were collected. The normal range for CD4+count in our laboratory is 500-1200 cells/mm^3.

2.3.5 CD8 and %CD38/8

CD38/8 was determined by means of flow cytometry using EDTA whole blood. 50 µL of blood was incubated with the following monoclonal antibodies: CD8-PerCP, CD38-APC, CD3-FITC (BD Biosciences, San Jose, CA) for 20 minutes at room temperature. The red blood cells were then lysed in BD FACS Lyse and the cells were then washed with staining buffer (2% Fetal Calf Serum (FCS) in Phosphate Buffered Saline) prior to analysis on the BD FACSCalibur (BD Biosciences, San Jose, CA) instrument. CD8 and CD38 antibodies are labelled with different coloured fluorochromes which bind specifically to their corresponding antigens on the lymphocytes. These cells then pass through a laser beam and scatter the laser light. The stained cells fluoresce. The scatter and fluorescence is detected by the instrument that provides information about the cell's size, complexity and relative fluorescence intensity. The fluorescence is then gated and the lymphocyte population displaying both CD8 and CD38 fluorescence on the same lymphocyte is determined. This principle is based on the same principle as CD4+ determination.[125] This is not

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measured routinely in the laboratory and the normal range for %CD38/8 determined by literature is 0.93-7.03% [126]

2.3.6 HIV viral Load

HIV-1 RNA viral load quantifications were performed using 1.0 mL EDTA plasma. The test used was the Nuclisens Easy Q HIV-1 v.1.2 (BioMerieux Incorporated (Inc)., Boxtel, Netherlands) kit which is a nucleic acid amplification test for the quantification of Human Immunodeficiency Virus Type 1 (HIV-1) RNA in human plasma. It is a 3 step process:

(1) Isolate HIV-1 RNA,

(2) Reverse transcript the RNA to copy deoxyribonucleic acid (cDNA),

(3) Simultaneous polymerase chain reaction (PCR) amplification of the target cDNA and the detection of the cleaved dual-labelled oligonucleotide detection probe specific to the target.

HIV-1 RNA concentration is calculated using the COBAS®TaqMan® (Roche Molecular Systems Inc., Basel, Switzerland) analyser which compares the patient HIV-1 RNA concentration to the Quality Control samples containing HIV-1 RNA. The test has a lower detection limit of 40-50 viral copies per milliliter.[127] Samples were processed on the same day as collection.

2.3.7 Fibrinogen

Freshly drawn tri-sodium citrate samples were used for fibrinogen determination. The samples were centrifuged at 4000 revolutions per minute (rpm) for 15 minutes to obtain platelet poor plasma. The ACL TOP (Beckman Coulter, Inc., Fullerton, CA) instrument uses the Clauss method for Fibrinogen determination which is: in the presence of excess thrombin, fibrinogen is transformed into fibrin. And the time it takes for clot formation is inversely proportional to the concentration of fibrinogen in the sample. The reagent used is the fibrinogen-C kit by HemosIL® (Bombay, United States).[128] The normal range for fibrinogen in our laboratory is 2-4 g/L.

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2.3.8 D-dimer

Freshly drawn tri-sodium citrated plasma was used for D-dimer determination. The instrument used for D-dimer determination is the ACL TOP (Beckman Coulter, Inc., Fullerton, CA) The HemosIL D-Dimer HS 500 (Bombay, United states) is an automated latex enhanced turbidometric immunoassay. The Reagent is a suspension of polystyrene latex particles of uniform size coated with the F(ab')2 fragment of a monoclonal antibody highly specific for the D-Dimer domain included in fibrin soluble derivatives. The use of the F(ab')2 fragment allows a more specific D-Dimer detection avoiding the interference of some endogenous factors like Rheumatoid Factor. The coated latex particles agglutinate. The degree of agglutination is directly proportional to the concentration of D-dimer in the sample and is determined by measuring the decrease of transmitted light caused by the aggregates.[129] The normal range for D-dimer in our laboratory is 0.0-0.25 mg/L.

2.3.9 High-sensitivity CRP

The IMMAGE hs-CRP (Beckman Coulter Inc., Fullerton, CA) is a latex enhanced immunoturbidimetric assay based on the peak rate principle. When an antigen-antibody reaction occurs between CRP in a sample and anti-CRP antibody, which has been sensitized to latex particles, agglutination occurs. This agglutination is detected as an absorbance change, with the change being proportional to the quantity of CRP in the sample. The actual concentration is then determined by interpolation from a calibration curve prepared from calibrators of known concentration.[130,131] Samples were frozen at -80ºC until analysis. The normal range for hsCRP in our laboratory is 0.0-7.5 mg/L.

2.3.10 Immunoglobulin G (IgG)

Serum IgG levels were determined using the Siemens ADVIA® 1800 (Berlin and Munich, Germany) chemistry analyser. Serum IgG was determined using an automated polyethylene glycol (PEG) – enhanced immunoturbidimetric method.

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Suitably diluted samples were allowed to react with antiserum in the reaction cup, this caused a precipitate to form which was measured turbidimetrically at 340/694 nanometer (nm). The absorbance measurement was compared to the standard curve obtained from the standard calibration curve, and the IgG concentration was calculated.[132] Samples were frozen at -80ºC until analysis.The normal range for IgG in our laboratory is 7-16 g/L.

2.3.11 Albumin

Serum albumin levels were determined using Siemens ADVIA® 1800 (Berlin and Munich, Germany) chemistry analyser. Serum albumin binds quantitatively to Bromocresol-green (BCG) dye to form an intense green albumin-BCG complex that is measured by endpoint reaction at 596/694nm. The intensity of the green complex is directly proportional to the concentration of albumin in the serum sample.[133,134] Samples were frozen at -80ºC until analysis. The normal range for albumin in our laboratory is 35-52 g/L.

2.4 Statistical Analysis

Data was analysed with the help of a statistician using STATISTICA version10 (Statsoft Inc.) and Microsoft® Excel®.

The results of the analysed data were displayed graphically by STATISTICA software. Descriptive statistics were used to analyse each parameter in terms of distribution: minimum, maximum, median, mean, standard deviation (SD) and statistical significance. Pearson correlation coefficient was performed for normally distributed data. The Spearman Rank correlation was performed for non-parametric data. Analysis of variance (ANOVA) was used to compare two or more groups of data that was normally distributed. A biplot was use to summarize the correlation of all the analytes tested within the two populations.

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SECTION III: RESULTS

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3.1 Characteristics of the study population

This cohort consisted of 180 participants with a mean age of 31 years. All were of Black ethnicity. There were 66 HIV negative participants and 114 were HIV positive. All HIV positive participants were newly diagnosed and ART-naïve. Figure 3.1 shows breakdown of the cohort according to gender.

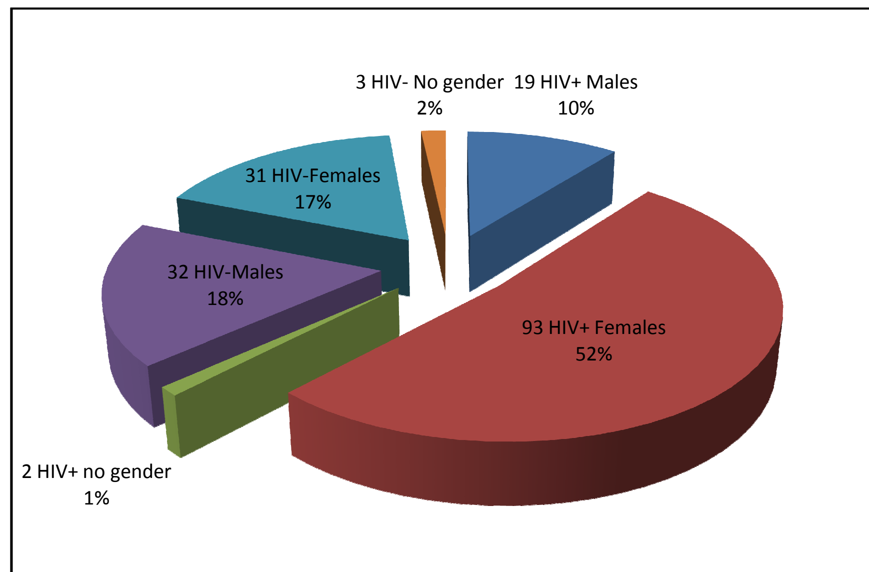

Figure 3.1: HIV negative compared to HIV positive participants

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Our cohort consisted of 124 females compared to 51 males. The skewed gender distribution may be due to the fact that females are more prone to take part in studies. The fact that more females who presented for testing were actually positive may suggest that females were more concerned about their HIV status. A total of 75% of this cohort's females tested HIV positive compared to only 37% of the males. (Figure 3.2)

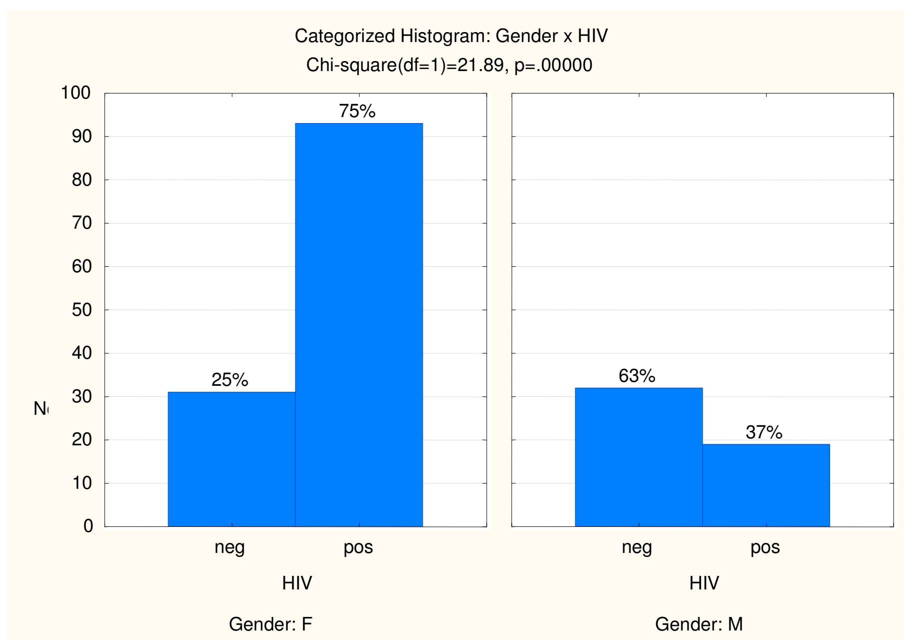

Figure 3.2: HIV status according to gender

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3.2 Analytes tested: HIV positive versus HIV negative subjects

3.2.1 White cell count (WCC)

The normal range for WCC in our laboratory is 4.0-10.0 x10^9 cells per liter(L).

The HIV positive population's WCC ranged from 1.81-13.17 x10^9 cells/L and the HIV negative population ranged from 2.11-10.94 x10^9 cells/L. When the mean (± SD) WCC of the HIV negative population (6.07 ± 1.96 x10^9 cells/L) was compared to the mean (± SD) of the HIV positive population (4.92 ± 1.68 x10^9 cells/L) there was a significantly lower WCC in the HIV positive population ($p < 0.01$).

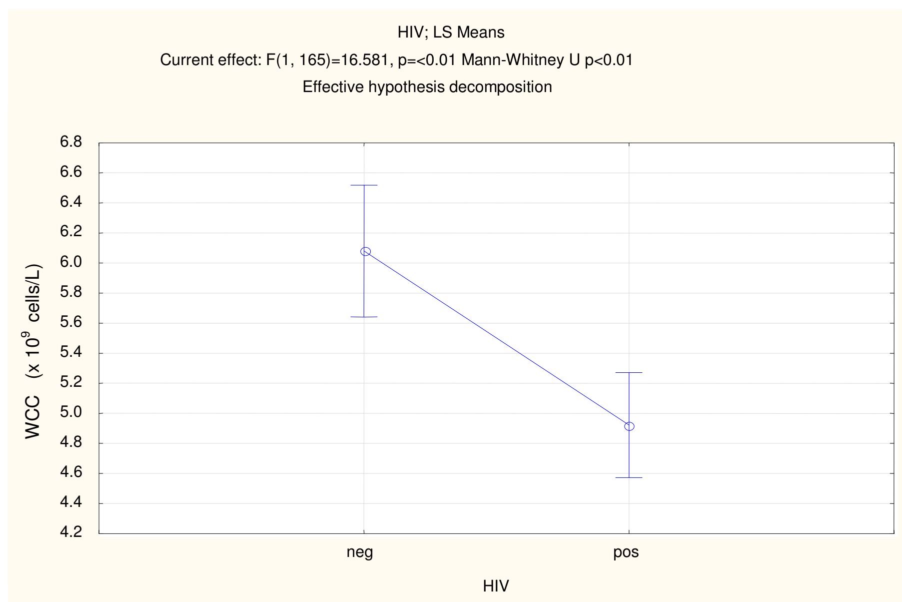

Figure 3.3: WCC in the HIV positive population compared to HIV negative population.

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3.2.2 CD4+ count

The normal range for CD4+count in our laboratory is 500-1200 cells/mm^3.

The HIV positive population's CD4+count ranged from 14-980 cells/mm^3 and the HIV negative population ranged from 272-1691 cells/mm^3. When the mean (± SD) of the HIV negative population (823.9 ± 255.84 cells/mm^3) was compared to the mean (± SD) of the HIV positive population(394.8 ± 216.38 cells/mm^3) there was a significantly lower CD4+count in the HIV positive population ($p < 0.01$).

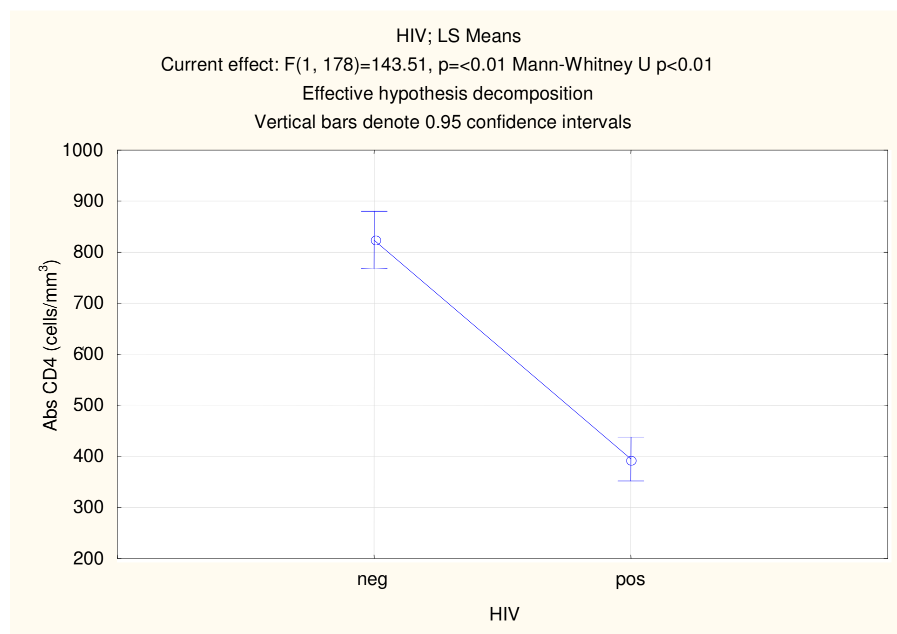

Figure 3.4: CD4+count in the HIV negative population compared to HIV positive population.

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3.2.3 %CD38/8

The normal range for %CD38/8 according to literature is 0.93-7.03% [126]

The HIV positive population's %CD38/8 ranged from 11.29-50.47% and the HIV negative population ranged from 1.48-25.06%. The mean (± SD) %CD38/8 of the HIV positive population (30.88 ± 19.59%) was significantly higher than the mean (± SD) of the HIV negative population (13.27 ± 11.79%) ($p < 0.01$).

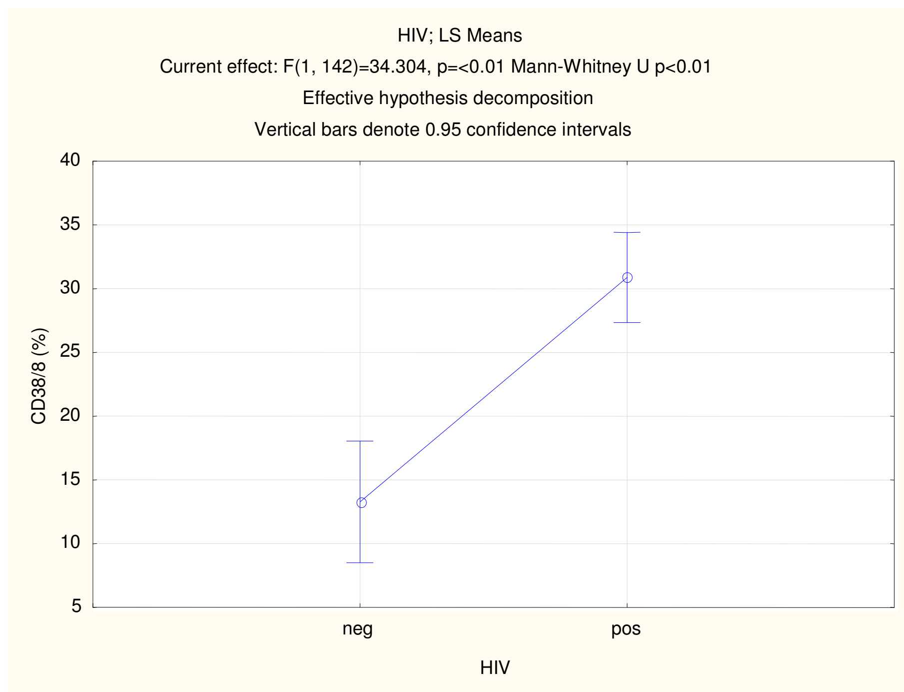

Figure 3.5: %CD38 on CD8 expression in the HIV negative compared to HIV positive population.

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3.2.4 Albumin

The normal range for albumin in our laboratory is 35-52 g/L.

The albumin in the HIV negative population ranged from 38-51 g/L and in the HIV positive population it ranged from 31-48 g/L. The mean (± SD) in the HIV positive group (40 ± 8 g/L) was significantly lower than the mean (± SD) in the HIV negative group (44 ± 7g/L) ($p < 0.01$).

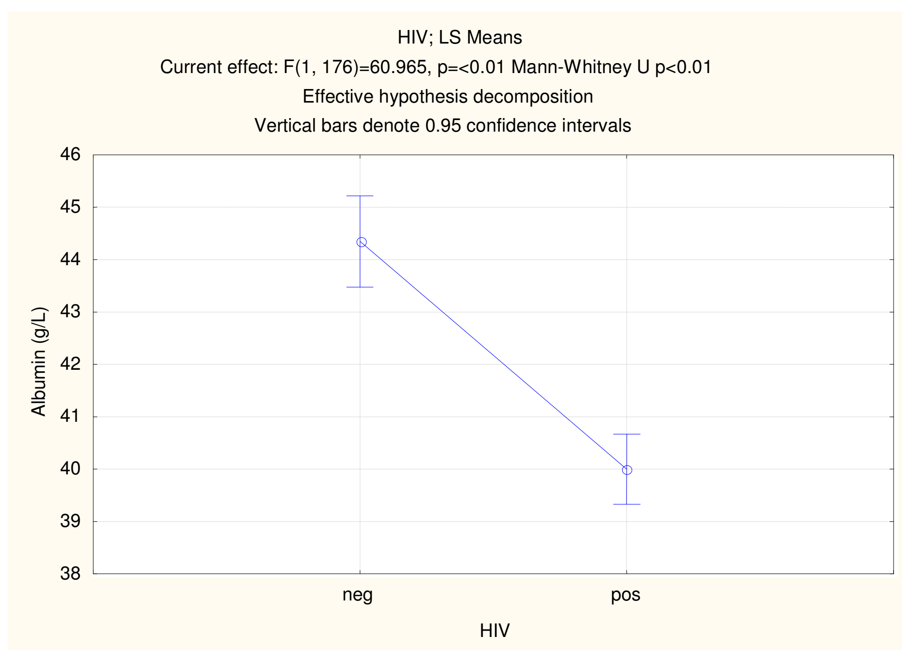

Figure 3.6: Albumin levels in the HIV positive population compared to HIV negative population

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3.2.5 IgG

The normal range for IgG in our laboratory is 7-16 g/L.

The range of IgG in the HIV positive and HIV negative populations were 11.6-63.0 g/L and 10.3-30.0 g/L respectively. The mean (± SD) in the HIV positive group (28.4 ± 11.1 g/L) was significantly higher than the mean (± SD) in the HIV negative group (16.9 ± 4.3 g/L) ($p < 0.01$).

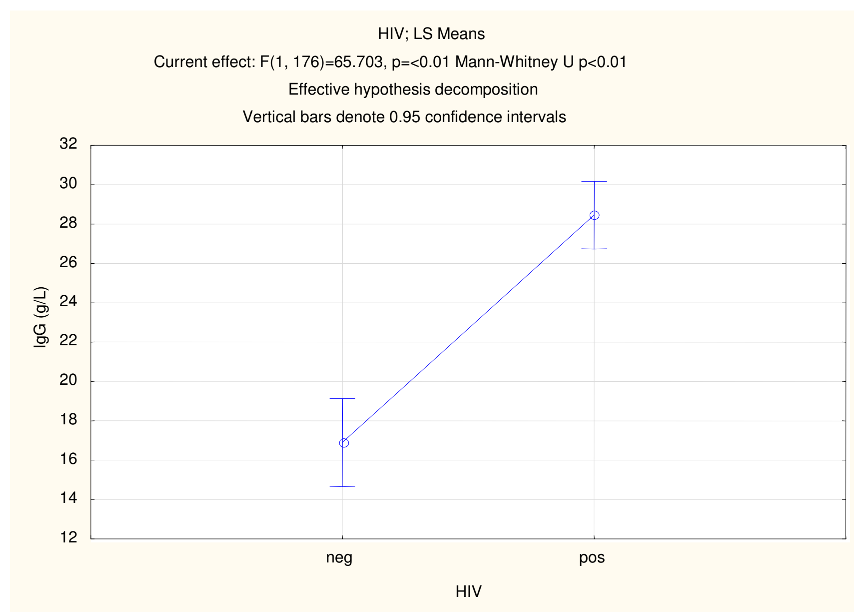

Figure 3.7: IgG levels in the HIV positive population compared to HIV negative population.

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3.2.6 Fibrinogen

The normal range for fibrinogen in our laboratory is 2-4 g/L.

The fibrinogen levels in the HIV positive population ranged from 1.8-4.4 g/L and in the HIV negative population it ranged from 0.8-4.2 g/L. When the mean (± SD) fibrinogen from the HIV negative population (2.85 ± 0.66 g/L) was compared to the mean (± SD) of the HIV positive population (2.81 ± 0.61 g/L) no significant difference was found (p = 0.65).

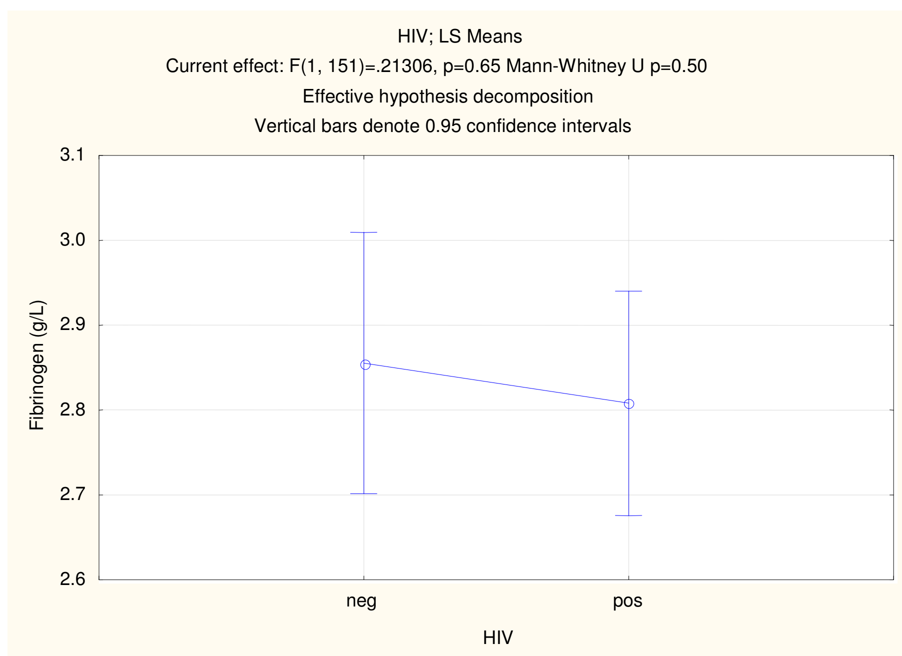

Figure 3.8: Fibrinogen levels in the HIV positive population compared to HIV negative population.

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3.2.7 D-dimer

The normal range for D-dimer in our laboratory is 0.0-0.25 mg/L.

D-dimer levels in the HIV positive population ranged from 0.20-4.07 mg/L and in the HIV negative population they ranged from 0.2-6.94 mg/L. When the mean (± SD) D-dimer level of the HIV negative population (0.22 ± 0.1 mg/L) was compared to the mean (± SD) of the HIV positive population (0.32 ± 0.22 mg/L) a significant increase was found in the HIV positive group ($p<0.01$)

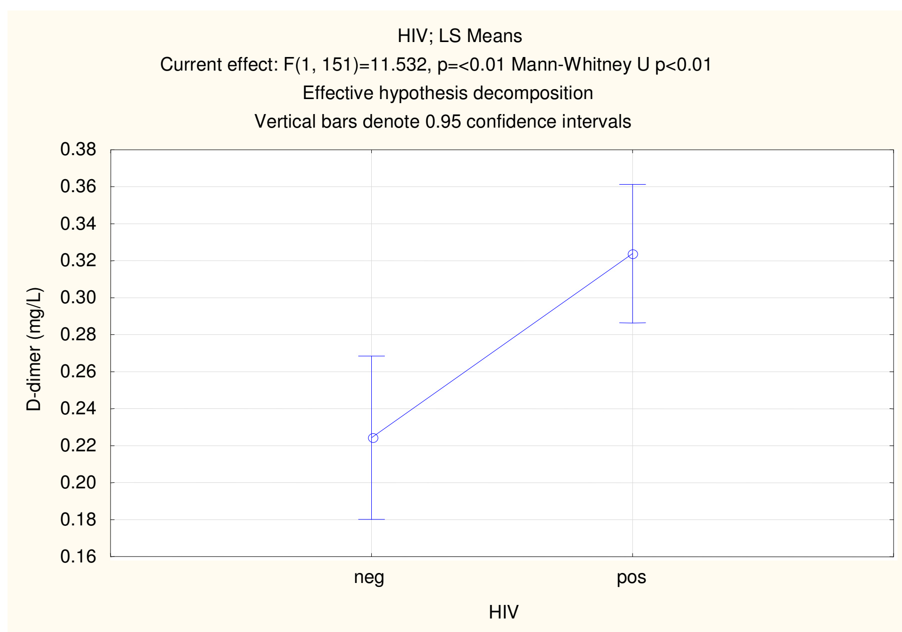

Figure 3.9: D-dimer levels in the HIV positive population compared to HIV negative population.

3.2.8 Log $_{10}$ (hsCRP)

The normal range for hsCRP in our laboratory is 0.0-7.5 mg/L.

Levels of hsCRP in the HIV positive population ranged from 0.2-70.7 mg/L and in the HIV negative population they ranged from 0.2-25.39 mg/L. Log transformation was used as the data was highly skewed data. The data obtained for hsCRP had a very wide range and was mostly skewed towards the lower limit of normal for both populations, by performing log transformation on the data the range for hsCRP was narrowed considerably thereby making it easier to interpret a significance pattern. Comparing the log transformed mean (± SD) for hsCRP level of the HIV negative group (0.29 ± 0.56 mg/L) to the HIV positive group (0.56 ± 0.6 mg/L) we found that there was a significant increase $p < 0.01$ in the HIV positive group.

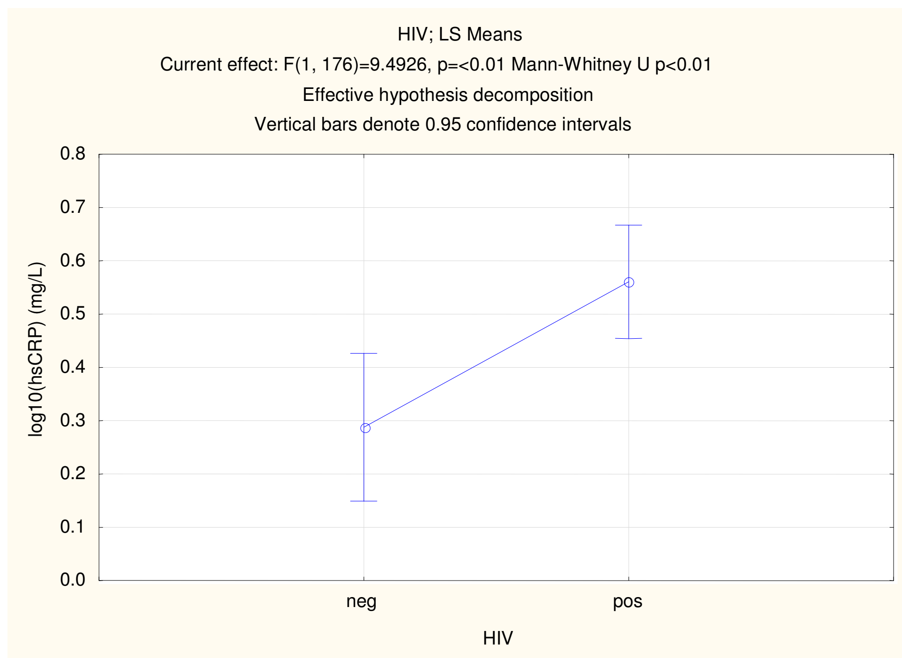

Figure 3.10: Log_{10}(hsCRP) level in the HIV positive population compared to HIV negative population.

3.2.9 Log_{10}(E-selectin)

The normal range for E-selectin according to the manufacturer's package insert is 11.78-160.72 ng/mL.[123]

E-selectin levels in the HIV positive population ranged from 25.25-483.0 ng/ml with a median of 129.9 ng/mL and in the HIV negative group they ranged from 48.63-391.5 ng/mL with a median of 125.7 ng/mL. The data was log transformed as the results varied over a wide range and were skewed towards the upper limit of the normal range. By log transforming the data the range was narrowed and the data made less skew so as to interpret it more easily. Comparing the mean (± SD) log_{10}E-selectin level of the HIV negative group (2.1 ± 0.18 ng/mL) to the HIV positive group (2.09 ± 0.2 ng/mL) we found that there was no significance (p = 0.84) in the HIV positive group compared to the negative group.

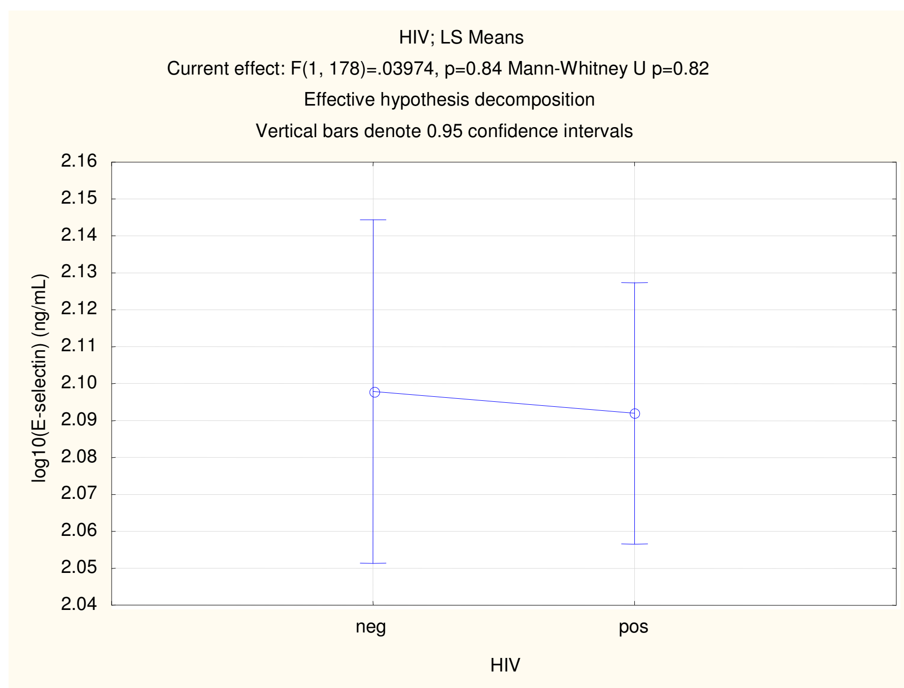

Figure 3.11: Log_{10}(E-selectin) levels in HIV positive population compared to HIV negative population.

3.2.9.1 Log_{10}(E-selectin) compared to gender

When comparing Log_{10}E-selectin to gender it was found that males had a significantly higher level of E-selectin than females (p = 0.01).

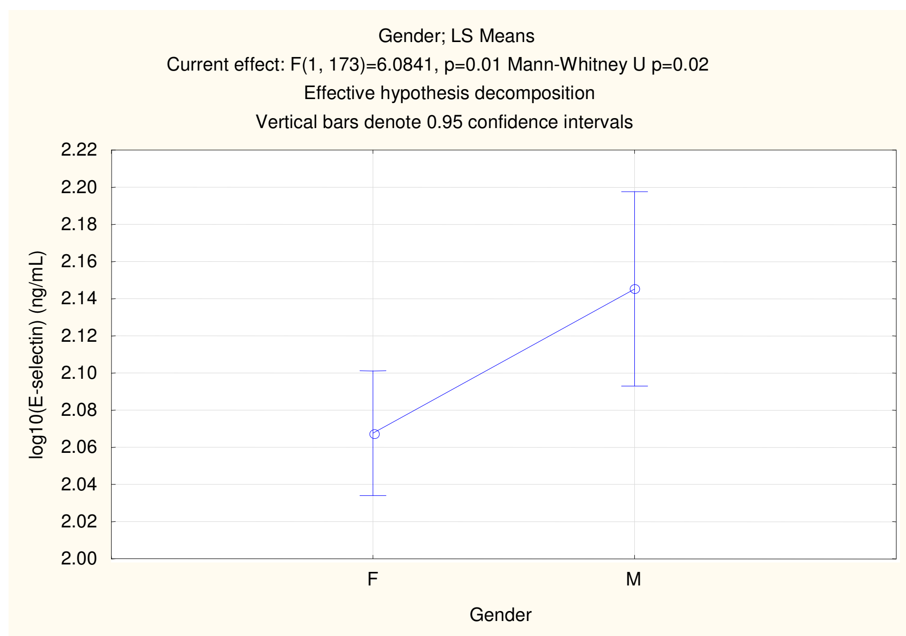

Figure 3.12: Log_{10}(E-selectin) compared to gender

3.2.9.2 E-selectin corrected for gender

Even after correcting E-selectin for gender, no significance was found between the HIV positive and the HIV negative population. However males still had a higher level of E-selectin regardless of their HIV status.

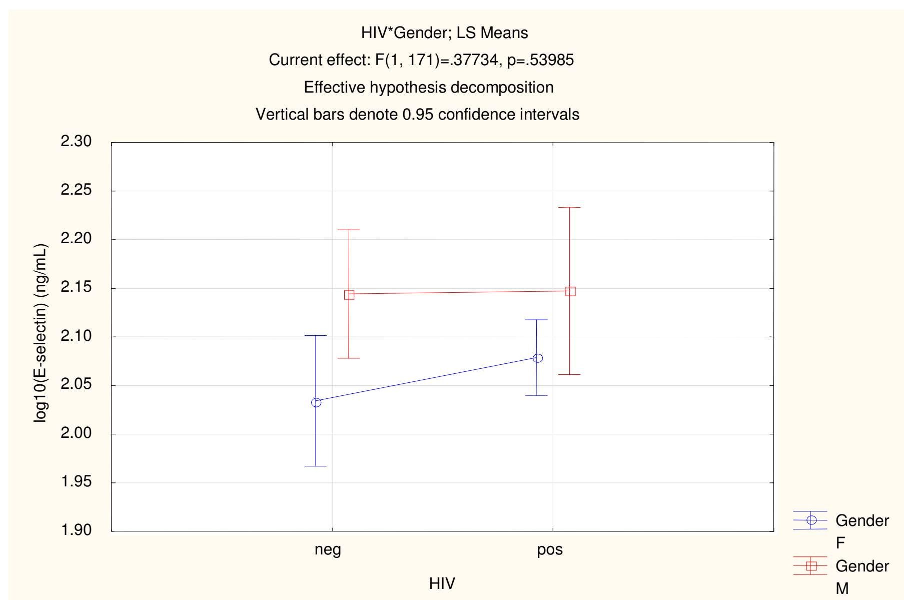

Figure 3.13: Log_{10}(E-selectin) in HIV positive compared to HIV negative populations corrected for gender

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Table 3.1: Shows the comparison of the means of all the analytes determined in the HIV negative and positive populations.

Analytes	**Normal range**	**HIV+ mean and SD**	**HIV- mean and SD**	**F- value**	**P- value**
WCC ($x10^9$cells/L)	4.0-10.0	4.92±1.67	6.08±1.96	F(1,165)=6.581	<0.01*
CD4+count (cells/mm^3)	500-1200	394.8 ±216.4	823.9±255.8	F(1,178)=143.51	<0.01*
%CD38/8	0.93-7.03	30.9±19.6	13.2±11.8	F(1,142)=34.304	<0.01*
Albumin (g/L)	35-52	40±3.7	44±3.4	F(1,176)=60.965	<0.01*
IgG (g/L)	7-16	28.4±11.1	16.9±4.3	F(1,176)=65.703	<0.01*
Fibrinogen (g/L)	2-4	2.81±0.61	2.85±0.66	F(1,151)=0.21306	0.65
D-dimer (mg/L)	0.0-0.25	0.32±0.21	0.22±0.09	F(1,151)=11.532	<0.01*
Log_{10} hsCRP (mg/L)	-	0.56±0.58	0.29±0.56	F(1,176)=9.4926	<0.01*
hsCRP (mg/L)	0.0-7.5	8.15±11.32	4.08±5.33	-	<0.01*
Log_{10} E-selectin (ng/mL)	-	2.09±0.2	2.1±0.18	F(1,178)=0.03974	0.84
E-selectin (ng/mL)	11.78-160.72	135.9±60.7	137.0±63	-	0.84

3.3 E-selectin correlations with all the parameters tested in the cohort as a whole and separately as HIV positive and HIV negative populations (Table 3.2)

In the entire cohort, E-selectin did not correlate significantly with any markers of HIV disease severity (CD4+count and viral load), inflammation (%CD38/8, hsCRP and IgG) or coagulation (fibrinogen and d-dimer)

In the HIV positive population, E-selectin leaned towards significance with WCC (p = 0.07, correlation coefficient value (r) = 0.18).

In the negative population, E-selectin only correlated significantly with albumin (p = 0.05, r = 0.25).

Table 3.2: E-selectin correlations with other parameters tested; like hypothesis ($p<0.05$ statistically significant)

Analytes compared to E-selectin	Spearman r-value for *both* groups	Spearman p-value for *both* groups	Spearman r-value for HIV positive	Spearman p-value for HIV positive	Spearman r-value for HIV negative	Spearman p-value for HIV negative
CD4+count	-0.06	0.39	-0.14	0.13	0.03	0.83
Log viral Load	-0.03	0.80	-0.03	0.80	-	-
%CD38/8	-0.07	0.41	-0.02	0.88	-0.04	0.81
Albumin	0.09	0.21	0.04	0.66	0.25	0.05*
IgG	-0.06	0.42	-0.04	0.67	-0.15	0.22
hsCRP	0.08	0.31	0.07	0.44	0.07	0.60
D-dimer	0.03	0.73	-0.00	0.99	0.06	0.62
Fibrinogen	0.11	0.19	0.15	0.16	0.03	0.79
WCC	0.10	0.21	0.18	0.07	-0.05	0.69

As E-selectin leaned towards significance with WCC in the HIV positive population (p = 0.07), we further correlated E-selectin with the differential white blood cells to determine if this correlation was due to neutrophils or lymphocytes. The correlation was statistically significant for both groups, but as the Spearman r-value was higher (closest to 1.0) for the neutrophils, suggesting that these cells contributed the greatest to the WCC correlation (Table 3.3).

Table 3.3: Comparing the WCC to the differential count

WCC compared to differential count.	Spearman values for both groups		Spearman values for HIV positive group		Spearman values for HIV negative group	
	r-value	p-value	r-value	p-value	r-value	p-value
Neutrophils	0.87*	<0.01	0.83*	<0.01	0.90*	<0.01
Lymphocytes	0.51	<0.01	0.44	<0.01	0.51	<0.01
Monocytes	0.64	<0.01	0.59	<0.01	0.67	<0.01
Eosinophils	0.18	0.02	0.29	<0.01	0.09	0.47
Basophils	0.47	<0.01	0.48	<0.01	0.37	<0.01

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3.4 E-selectin compared to the CD4+count at different levels (Figures 3.14 and 3.15)

As CD4+count of 350 cells/mm^3 was used until recently as the South African cut-off point for ART initiation and a CD4+count of 500 cells/mm^3 was recommended in 2013 by the WHO; we investigated E-selectin levels at both these cut-off points to determine whether a correlation existed at these CD4+count levels.

At both CD4+counts ≤ 500 cells/mm^3 and >500 cells/mm^3 and ≤ 350 cells/mm^3 and > 350 cells/mm^3, E-selectin showed no significance ($p = 0.45$ and $p = 0.63$ respectively)

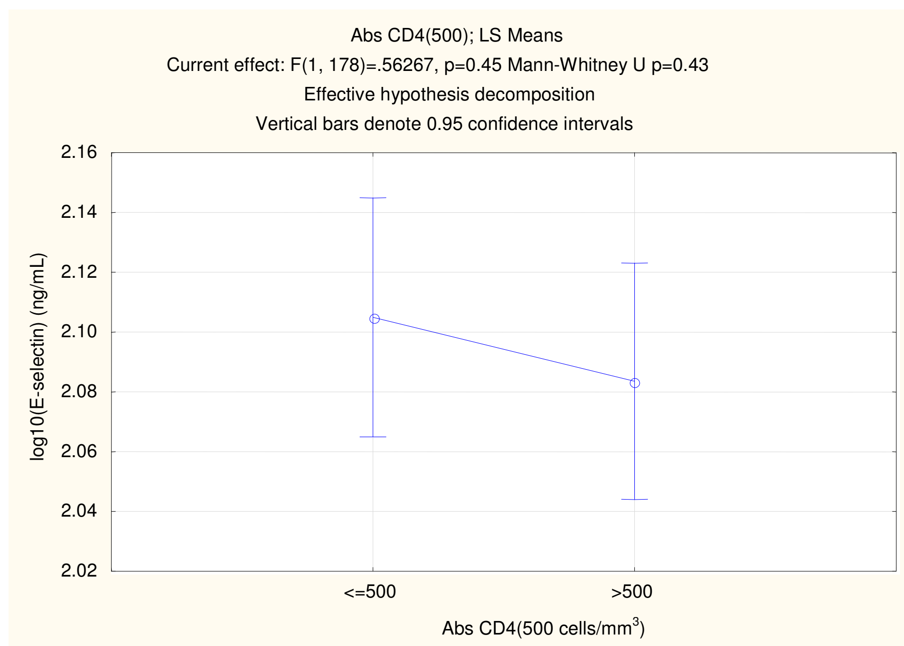

Figure 3.14: E-selectin levels at the WHO cut-off point (500 cells/mm^3) for ART initiation.

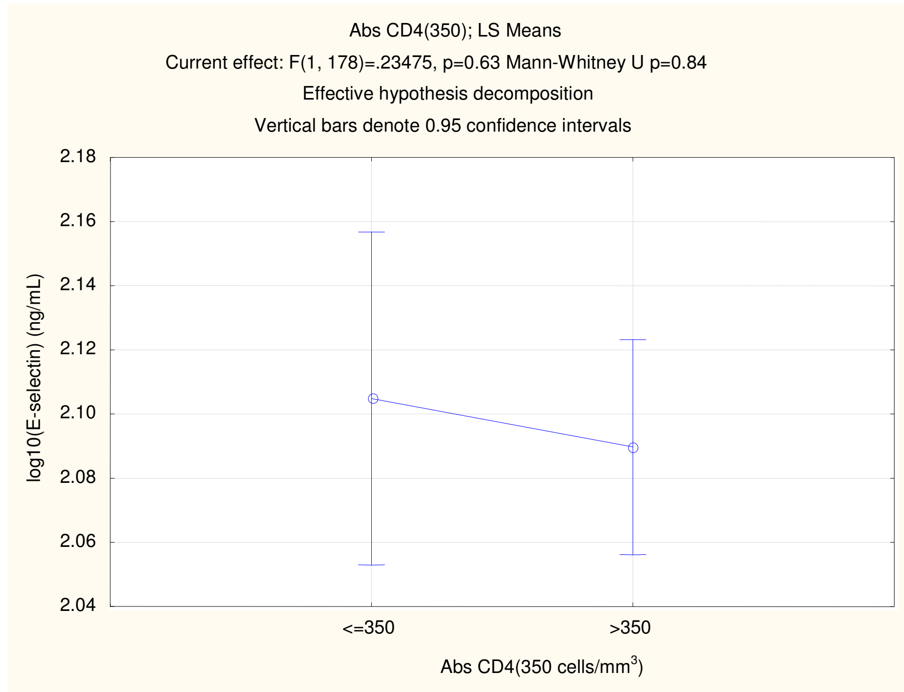

Figure 3.15: E-selectin levels at the previous (2010-2014) South African department of Health CD4 cut-off point (350 cells/mm^3) for ART initiation

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3.5 Biplot (Figure 3.16)

A Biplot is a graphical representation based on principal component analysis (PCA) that depict the relationships between analytes as well as the separation of HIV positive and HIV negative relationship to the analytes.

The x-axis denotes the first principle component (PC) and the y-axis the second principle component of the PCA. The x-axis is denoted by PC1 and the y-axis by PC2.

The red diamonds indicate all the HIV negative cases and the red circle indicates where most of HIV negative cases are on the biplot. The blue dots indicate the HIV positive cases and the circle indicate where most of the HIV positive cases are on the biplot with respect to the HIV negative cases (red circle).The positions of the blue and red circles relative to the directions of the vectors indicate which analyte separates the HIV group.

The vectors (→) indicate the direction of increasing/decreasing concentration for each analyte. Vectors in the same direction indicate positive correlations with each other, while vectors in the opposite directions indicate negative or inverse correlations and vectors that are perpendicular (90° or 270°) to the HIV population indicate no correlation with the HIV group.

The vectors that are at angles 0° and 180° have the greatest correlation of 1 or -1 thus the Spearman (r) is either in direct (1) or inverse (-1) correlation. The closer the angle of a vector (→) is to 90° or 270° the smaller the correlation an analyte has with what it is being compared to.[135]

(a) Albumin has a negative correlation suggesting that in HIV infection albumin levels decrease when compared to the controls.

(b) CD4+count has a negative correlation suggesting that in HIV infection the CD4+count decreases when compared to the controls.

(c) According to this plot, fibrinogen and hsCRP have no correlation with HIV infection. Although the ANOVA analysis for hsCRP did indicate a positive degree of significance ($p < 0.01$) with the HIV positive group, the degree of variance/difference between the two groups (48%) (degree of variance >48% is considered significant) was low between the HIV positive and HIV

negative group that the biplot regards hsCRP as having no correlation with HIV infection. However since their vectors are pointing in the same direction and so close to each other, the biplot indicates a significant positive correlation ($r = 0.49$ and $p < 0.01$) between fibrinogen and hsCRP regardless of HIV infection.

(d) %CD38/8 shows a strong positive correlation suggesting that as HIV infection progress the %CD38/8 expression will increase, but to a lesser extent than IgG.

(e) IgG shows a very strong positive correlation (almost 0 degrees) indicating that as HIV infection progress the IgG levels will directly increase.

(f) Also as both IgG and %CD38/8 vectors are pointing in the same direction and so close to each other, a positive correlation exists between the two. Thus as %CD38/8 increases so the IgG levels will increase in similar proportions in HIV disease progression.

(g) Albumin and %CD38/8 show inverse correlation as their vectors are pointing in opposite directions on the same plain. This suggests that as albumin decrease so the %CD38/8 will increase in similar proportions within the HIV infected group.

(h) Albumin is also showing and inverse correlation with IgG but to a lesser extent than %CD38/8.

(i) CD4+count and albumin also indicate slight positive correlations with each other, suggesting that as the CD4+count decreases so will the albumin levels.

(j) CD4+count also show an inverse correlation towards IgG and %CD38/8. Indicating that as CD4+count decreases the %CD38/8 and IgG levels will increase.

The analytes not shown (E-selectin, D-dimer, WCC and viral load) on the biplot were not well represented by the two principle components (controls and cases) No correlations were performed with viral load, as viral load was only tested in the HIV positive group and thus could not be compared with the HIV negative group.

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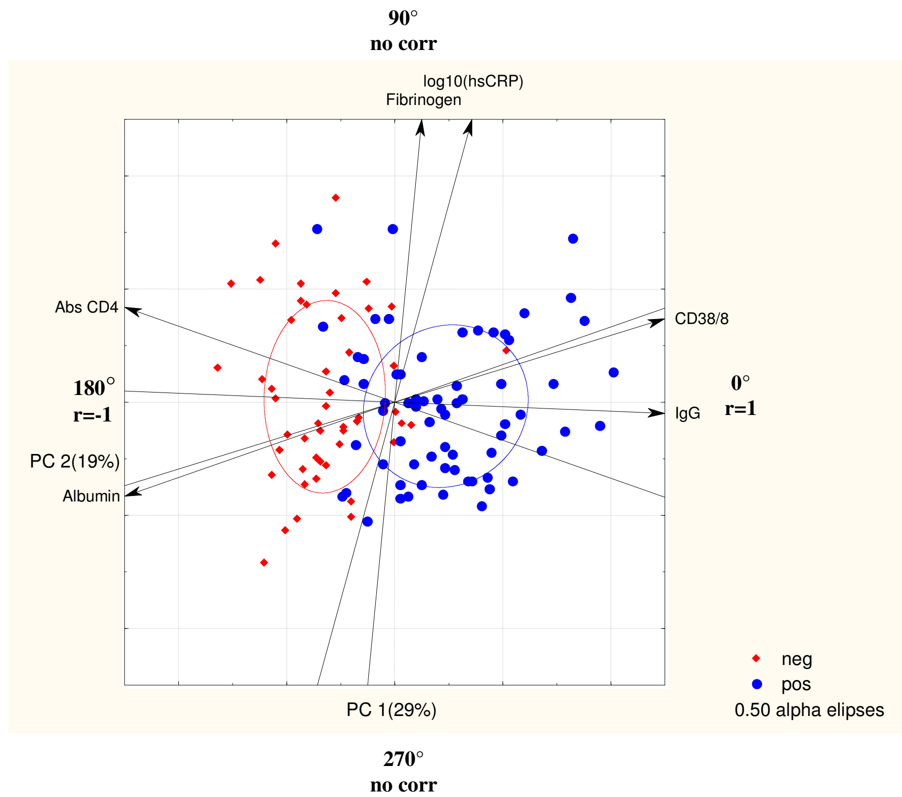

Figure 3.16: Summary of the comparison of all the analytes tested in HIV positive and HIV negative populations.

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SECTION IV: DISCUSSION

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The aim of this study was to determine E-selectin levels in HIV infected individuals, compare them to levels in controls and correlate with markers of HIV disease severity, inflammation and coagulation.

Ongoing inflammation in HIV infection is an important cause of disease progression and is associated with an increased risk of inflammation-associated conditions such CVD and cancer. Various parameters of inflammation and immune activation, such as the D-dimer and hsCRP have been studied previously and shown to be independent risk factors for the development of heart disease [46]. The JUPITER study determined that by reducing CRP the risk of CVD could be dramatically (44%) reduced.[74]

Importantly it was shown that ART does not repair all the immune damage that has already occurred and recommendations for the earlier initiation of ART have resulted.[36] In resource- limited settings the roll-out of ART remains a challenge and therefore,[143] it becomes important to identify patients who may be at increased risk of these conditions.

A previous study performed by our study group on the same untreated HIV infected and uninfected cohort, found significantly increased levels of ADMA in the HIV group, a known CVD risk marker.[108] It was hypothesized that this young, (mean age 31 years) cohort might have other markers of CVD risk as a result of heightened levels of inflammation. The underlying pathophysiology of CVD is atherosclerosis with oxLDL being engulfed by macrophages to form foam cells and being deposited in the subendothelial space.[48] This leads to endothelial dysfunction. E-selectin is an adhesion molecules preferentially found on endothelial cells and a marker of endothelial dysfunction.[95] Furthermore, cytokines released during the inflammatory process activate endothelial cells, resulting in the up-regulated expression of E-selectin.[93] We hypothesized therefore that E-selectin would be increased in our untreated HIV infected group.

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Previous studies have found that E-selectin expression is affected by HIV status. A cell culture study performed by Cota-Gomez et al demonstrated that the HIV Tat protein is able to directly up-regulate E-selectin expression through binding with NF-κB.[98] However, this was a tissue culture-based study which could detect increased levels of expression at a cellular level, whereas our study measured serum levels from individuals, reflecting systemic peripheral blood levels.

Another study found that there was no difference in the E-selectin levels of HIV-infected individuals compared to HIV negative controls. However after ART initiation, E-selectin levels decreased in the infected group.[100] A study by Graham et al found that E-selectin levels were significantly reduced 6 months after ART initiation, however after 12 months of treatment there were no differences in the E-selectin levels compared to baseline levels[102], suggesting that E-selectin is a positive acute phase reactant normalizing with a decrease in viral load and inflammation due the ART.

Another study determined that E-selectin levels were increased in the acute phase of HIV infection, but not in the chronic phase when HIV treatment-naïve women were followed up from seroconversion through to chronic infection.[103] This study is an important finding and could explain the lack of increased E-selectin in our study group. Our HIV infected group although clinically well, may have presented in the chronic stage of their infection, i.e. were not close to the time of seroconversion. Calza et al determined that E-selectin levels were increased in both an ART-naïve group and a group on ART compared to HIV negative controls and that the E-selectin levels correlated with elevated viral loads and decreased CD4+count.[104] However, the HIV positive treatment naïve group were much older (median age 40 years) and contained 5/50 females compared to the HIV negative control group with median age of 32 years and 13/51 females.[104] And since gender (males have naturally higher E-selectin than females) and increasing age has been shown to correlate with increased levels of E-selectin and this study did not correct for gender or age, it is possible that the correlation found was due to age and gender.

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Another study by Nordoy et al found that E-selectin levels were decreased in HIV infection with symptomatic CMV infection compared to HIV infection with no CMV and HIV negative controls.[99] A long term study by Rönsholt et al found that HIV–infected individuals on ART still had increased signs of vascular inflammation and that markers such as E-selectin did not correlate with the CD4+count or the viral load[101] suggesting that E-selectin is a marker of ongoing vascular inflammation.

The value of our study is that we recruited clinically healthy, asymptomatic HIV-infected individuals with relatively well preserved CD4+counts who had not yet been exposed to ART. In addition, this is the first study to our knowledge that correlated E-selectin with markers of immune activation or inflammation and coagulation. All the participants (cases and controls) were recruited from the same HPT clinic in Crossroads, Cape Town and were all of the same ethnicity and similar socio-economic background. The participants were not taking any medication such as anti-tuberculosis treatment, and to our knowledge had no concurrent infections.

Descriptive statistics showed that our cohort consisted mainly of females (123/180) of which the cases had (93/114) females. We hypothesized that this discrepancy in gender may be due to the fact that females are more willing to participate in trials. The fact that the percentage was so much higher in the infected group may be due to the fact that many of these women were concerned that they may be infected and thus presented for testing. A total of 75% of the females tested positive compared to 37% males. The mean age of the total cohort was 31 ±8 years, with the mean age for the HIV positive group being slightly higher (32 ± 7.7 years) than in the HIV negative group (29.4 ±8.3 years).

As expected, analysis of markers tested of disease severity found that the CD4+count was significantly lower in the HIV-infected group ($p < 0.01$) compared to the control group. This supports the concept that our HIV-infected group was already in the chronic phase of infection with significant depletion of CD4+T-cells, most likely due to ongoing activation and destruction of immune cells.

Analysis of the markers tested for inflammation found that hsCRP was significantly higher ($p = 0.01$) in the HIV-infected group compared to the controls. This suggests that increased inflammation was present in our HIV-infected group. This may also be seen as a marker of increased cardiac risk in this population.[74] Albumin was significantly lower ($p < 0.01$) in the HIV-infected group compared to the controls. As albumin is a negative acute phase reactant which is decreased in inflammation, this further supported the presence of ongoing inflammation. In addition, hsCRP showed a significant inverse correlation with albumin ($r = -0.32$ and $p < 0.01$) supporting the value of these markers in this setting. IgG was found to be significantly increased ($p < 0.01$) in the HIV-infected group compared to the controls, suggesting the presence of marked B-cell activation and possibly indicating a response to ongoing bacterial translocation due to the “leaky gut”.[47] Previous studies on our cohort have found that there is indeed a heightened humoral immune response with increased polyclonal patterns on SPE[106] and increased levels of FLCs.[107] The %CD38 expressed on CD8+T-cells was significantly higher ($p < 0.01$) in the HIV-infected group compared to the controls suggesting that significant cellular activation is taking place due to the viral load and ongoing immune stimulation.

Analysis of the markers tested for coagulation indicated that there was no difference ($p = 0.65$) in the fibrinogen levels of the HIV-infected group compared to the controls. Both groups had fibrinogen levels that were within the normal laboratory ranges. Fibrinogen is an acute phase reactant and therefore may not be raised at this more chronic stage of the infection. Importantly, the D-dimer was significantly higher ($p < 0.01$) in the HIV-infected group compared to the controls, which suggests that increased fibrinolysis and thrombosis was taking place in the HIV-infected group. The SMART study found that D-dimer was an independent risk factor for all-cause mortality,[46] suggesting that our study group may be at increased risk. In addition, D-dimer levels in our study showed inverse relations with the CD4+count and albumin ($r = -0.35$ and $r = -0.41$ respectively, both $p < 0.01$) and positive correlations with the viral load, hsCRP and IgG ($r = 0.34$, 0.27 and 0.41 respectively, all $p < 0.01$). Thus the D-dimer would be a valuable marker of immune activation and possible risk for thrombosis in this setting. It is recommended that the D-dimer

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be developed as a point-of-care test for the identification of individuals who may need earlier treatment interventions.

E-selectin did show a significant positive correlation ($r = 0.18$, $p = 0.02$) with age, which supports the age-related increase in E-selectin levels. This can possibly be explained by the oxidative stress theory of age; that older individuals experience more oxidative stress on tissues such as endothelial cells causing the up-regulation of adhesion molecules such as E-selectin.[136] The increased presence of soluble E-selectin is thought to be a protective function. By actively shedding adhesion molecules the effect of oxidative stress and tissue damage is limited by preventing leukocytes to unnecessarily adhere to the endothelium and transmigrate causing endothelial damage.[137] E-selectin was also compared to the cohort's gender and it was found that males had significantly higher ($p = 0.01$) levels compared to females. Again, male gender is a known CVD risk factor so this result is not unexpected. However after correcting E-selectin for gender, no difference was found for E-selectin levels in the HIV-infected group compared to the controls and males still had higher E-selectin levels regardless of their HIV status.

Both the significance of age and gender with E-selectin has been demonstrated in various other studies.[138,139,140] After the age of forty years, the risk of CVD is increased up to 49% in males and 32% in females with males being 3.4 times more susceptible due to their naturally higher expression of E-selectin.[138] Atherosclerosis is a chronic inflammatory and immune disease beginning in early childhood with plaque formation continuing throughout life. Previous studies determined that certain E-selectin polymorphisms increase the expression and affinity of E-selectin thereby accelerating plaque formation and progression to CVD.[138,139,140] However none of these studies investigated the expression of E-selectin at a young age as in our cohort or in the setting of HIV infection. When E-selectin was correlated with all the markers of disease severity, inflammation and thrombosis in our entire cohort, we found no correlation with any of the parameters tested. We also correlated E-selectin with all these parameters in the HIV-infected and control group separately and found

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that E-selectin correlated significantly (p = 0.05) with albumin in the control group and neared significance with the WCC (p = 0.07) in the HIV positive group.

The WCC was significantly decreased in the HIV infected group which may reflect both a decreased CD4+count and decreased neutrophil count. We therefore further correlated the WCC with the subsets of the differential count to determine which leukocyte was contributing to this correlation. Interestingly, the WCC correlated most strongly with the neutrophil count. This may reflect the increased trafficking and consumption of neutrophils at sites of infection or inflammation, effectively removing them from the circulation.

The 2013 WHO guidelines recommend a CD4+count cut-off of 500 cells/mm^3 for ART initiation[38] and until recently, South Africa used a cut-off of 350 cells/mm^3.[41] Since January 2015 the South African Department of Health adopted the 2013 WHO recommended guidelines of 500 cells/mm^3 for ART initiation.[42] We studied E-selectin levels at both these cut-offs and found no significant difference in E-selectin levels at either (p = 0.45 and p = 0.65 respectively). This may suggest that systemic levels of E-selectin are tightly controlled and may only be increased at the site of vessel wall damage or inflammation; or as suggested by Graham et al[103]that E-selectin is only raised during the acute infection of HIV and not after seroconversion has taken place.

Furthermore studies have found that CMV is a common co-infection with HIV infection, reaching a prevalence of up to 51% in Africa. CMV viral replication is associated with accelerated HIV disease progression.[142,141] It was also determined that the incidence of CMV transmission is higher in females compared to males.[142]A study by Nordoy et al found that CMV downregulates the expression of E-selectin by rendering the endothelial cell insensitive to inflammatory cytokines.[99] Unfortunately we did not determine CMV status in our study.

Previous studies have found that there is a high level of inflammation in our population, even in our uninfected cases. Zemlin et al found that 10% of the cases

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had polyclonal increases in the gamma region on SPE.[106] Our study population is from Crossroads, Cape Town which is known to have a low socio-economic setting where children may often not receive the adequate nutrition that is needed for sustained growth and strong immunity. A review on the immune system of African children found that malnutrition and micronutrient deficiency from infancy may cause irreversible damage to the immune system allowing for individuals to have an immune compromised system from early childhood. This predisposes individuals to opportunistic infections which lead to altered immunity, early senescence of the immune system and a depleted naïve CD4+T-cell pool.[143]A lack of micronutrients such as Vitamin A and zinc causes immunological defects with decreased dendritic cell function and thymic cell dysfunction. This malnutrition may lead to an underdeveloped intestinal system which is a site for bacterial attachment and infiltration causing an inflammatory environment. This may irreversibly damage the gut mucosal barrier leading to the "leaky gut" theory from infancy. This irreversible damage caused by malnutrition persists even if adequate nutrition is obtained later in life.[143]

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Strengths and Limitations:

This study has several limitations. The samples obtained for this study were frozen at -80ºC. However, according to the literature, the analytes we studied are stable at this temperature provided there are no freeze-thaw cycles. Evidence of CVD / atherosclerosis was not obtained clinically, and therefore, the predictive value of the markers for the development of these conditions would require longitudinal, follow-up studies. There are no clinically relevant cut-off points for endothelial biomarkers and assays used by us and other studies are not standardized. We had incomplete data on potential confounders such as hypertension and diabetes, which may be related to endothelial activation and risk of CVD.

Another limitation to our study is unknown time of participant seroconversion.

Additionally, we did not test the participants for concurrent infections such as CMV but assessed them based on medical history and as being clinically asymptomatic at time of inception. Thus it may be possible that a large number of our HIV infected group may be co-infected with asymptomatic CMV which could explain the lack of E-selectin significance between the two study populations.

We also did not test for TB but only excluded subjects with known TB. We were not able to exclude undiagnosed TB in our cohort.

Another limitation to our study was that even though the size of our study population was large compared to other studies it did not reach the proposed study size to allow for good statistical predictive value.

However, our study had much strength. Our cohort was a relatively homogenous population, all from the same socio-economic and ethnic background. They were all young so the likelihood of other age-related risk factors such as hypertension and diabetes is decreased. All subjects were unaware of their HIV status when they presented at the clinic, and ART-naive, so the immune system had not yet been modulated by ART. Blood was taken immediately, some tests were performed immediately and serum was stored at -80°C with no freeze-thaw cycles.

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SECTION V: CONCLUSION

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This is the first study to our knowledge that correlated levels of E-selectin with markers of HIV disease severity, inflammation and coagulation. We can conclude that E-selectin is not a valuable marker during the chronic stage of the HIV infection. We recommend that tests such as the D-dimer be utilized to identify individuals who may be at increased risk of disease progression or heart disease in HIV infection.

Although HIV-infection leads to increased risk of CVD and we have determined that our cohort indeed has such a risk, we found no significant difference in E-selectin levels between HIV-infected and non-infected individuals. Future studies that take into account the time of seroconversion may find E-selectin to be a better marker of early, acute inflammation and follow-up studies, monitoring the development of CVD or atherosclerosis plaque development in this cohort would be of value.

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APPENDIX I

Approval Notice

Response to Modifications- (New Application)

28-Oct-2014
Hoffman, Madelein M

Ethics Reference #: S14/08/175

Title: **E-Selectin, other cell adhesion molecules and markers of infammation in treatment - naïve individuals living with HIV.**

Dear Ms Madelein Hoffman,

The **Response to Modifications -** ***(New Application)*** received on **20-Oct-2014**, was reviewed by members of **Health Research Ethics Committee 1** via Expedited review procedures on **28-Oct-2014** and was approved.
Please note the following information about your approved research protocol:

Protocol Approval Period: **28-Oct-2014 -28-Oct-2015**

Please remember to use your **protocol number** (**S14/08/175**) on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:
Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.
Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Helene Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.
For standard HREC forms and documents please visit: www.sun.ac.za/rds

If you have any questions or need further assistance, please contact the HREC office at 0219389657.

Included Documents:

HREC New application form
Investigator CV (Erasmus)
Investigator declaration (Hoffman)
MOD_Protocol

Investigator CV (Ipp)
Investigator CV (Zemlin)
Participant information leaflet and consent form
Investigator declaration (Ipp)
MOD_Cover letter response to Modifications
Ethics letter
Protocol Synopsis
HREC general checklist
Investigator declaration (Zemlin)
Protocol
Investigator CV (Hoffman)
Investigator declaration (Erasmus)

Sincerely,

Franklin Weber
HREC Coordinator
Health Research Ethics Committee 1

Investigator Responsibilities

Protection of Human Research Participants

Some of the responsibilities investigators have when conducting research involving human participants are listed below:

1.Conducting the Research. You are responsible for making sure that the research is conducted according to the HREC approved research protocol. You are also responsible for the actions of all your co-investigators and research staff involved with this research.

2.Participant Enrolment. You may not recruit or enrol participants prior to the HREC approval date or after the expiration date of HREC approval. All recruitment materials for any form of media must be approved by the HREC prior to their use. If you need to recruit more participants than was noted in your HREC approval letter, you must submit an amendment requesting an increase in the number of participants.

3.Informed Consent. You are responsible for obtaining and documenting effective informed consent using **only** the HREC-approved consent documents, and for ensuring that no human participants are involved in research prior to obtaining their informed consent. Please give all participants copies of the signed informed consent documents. Keep the originals in your secured research files for at least fifteen (15) years.

4.Continuing Review. The HREC must review and approve all HREC-approved research protocols at intervals appropriate to the degree of risk but not less than once per year. There is **no grace period.** Prior to the date on which the HREC approval of the research expires, **it is your responsibility to submit the continuing review report in a timely fashion to ensure a lapse in HREC approval does not occur.** If HREC approval of your research lapses, you must stop new participant enrolment, and contact the HREC office immediately.

5.Amendments and Changes. If you wish to amend or change any aspect of your research (such as research design, interventions or procedures, number of participants, participant population, informed consent document, instruments, surveys or recruiting material), you must submit the amendment to the HREC for review using the current Amendment Form. You **may not initiate** any amendments or changes to your research without first obtaining written HREC review and approval. The **only exception** is when it is necessary to eliminate apparent immediate hazards to participants and the HREC should be immediately informed of this necessity.

6.Adverse or Unanticipated Events. Any serious adverse events, participant complaints, and all unanticipated problems that involve risks to participants or others, as well as any research-related injuries, occurring at this institution or at other performance sites must be reported to the HREC within **five (5) days** of discovery of the incident. You must also report any instances of serious or continuing problems, or non-compliance with the HRECs requirements for protecting human research participants. The only exception to this policy is that the death of a research participant must be reported in accordance with the Stellenbosch Universtiy Health Research Ethics Committee Standard Operating Procedures www.sun025.sun.ac.za/portal/page/portal/Health_Sciences/English/Centres%20and%20Institutions/Research_Development_Support/Ethics/Application_package All reportable events should be submitted to the HREC using the Serious Adverse Event Report Form.

7.Research Record Keeping. You must keep the following research-related records, at a minimum, in a secure location for a minimum of fifteen years: the HREC approved research protocol and all amendments; all informed consent documents; recruiting materials; continuing review reports; adverse or unanticipated events; and all correspondence from the HREC

8.Reports to the MCC and Sponsor. When you submit the required annual report to the MCC or you submit required reports to your sponsor, you must provide a copy of that report to the HREC. You may submit the report at the time of continuing HREC review.

9.Provision of Emergency Medical Care. When a physician provides emergency medical care to a participant without prior HREC review and approval, to the extent permitted by law, such activities will not be recognised as research nor will the data obtained by any such activities should it be used in support of research.

10.Final reports. When you have completed (no further participant enrolment, interactions, interventions or data analysis) or stopped work on your research, you must submit a Final Report to the HREC.

11.On-Site Evaluations, MCC Inspections, or Audits. If you are notified that your research will be reviewed or audited by the MCC, the sponsor, any other external agency or any internal group, you must inform the HREC immediately of the impending audit/evaluation.

APPENDIX II

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT: Modulation of activation and apoptosis of immune cells from HIV-infected individuals with the neurotransmitter Vasoactive Intestinal Peptide (VIP) and other biomediators

REFERENCE NUMBER: N07/09/197

PRINCIPAL INVESTIGATOR: Dr Hayley Ipp/ Dr Richard Glashoff

ADDRESS: Medical Virology; 8th Floor Clinical Building; Tygerberg Hospital Campus

CONTACT NUMBER: 021 938 9356

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Health Research Ethics Committee (HREC) at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

- **Selection of Study Volunteers** This study will enroll about 200 volunteers who have been attending the clinics attached to the Desmond Tutu HIV Centre, have CD4 counts greater than 200, are 21 years or older, and who have not yet received antiretroviral drugs.
- About 100 volunteers will also be recruited from trials that are being set up for HIV- negative people.
- *The research on the blood will take place at Tygerberg Hospital, Department of Pathology*
- **Reason for the study** The drugs (anti-retrovirals) that are now available for the treatment of HIV have saved many people' s lives and continue to do so on a daily basis. However, we still need to look for different ways to help people who are HIV positive, live for longer, before they need to take the antiretroviral drugs. If a person's CD4+ T cell count is less than 350, it is very important to get the antiretroviral treatment, however, for those people whose count is still greater than 350, we will be looking for new ways in the laboratory to keep the count at this higher level, and will be checking on the function of the thymus which is the factory for the production of these cells.

Study Procedures
If you decide to take part in the study, after you read, discuss and sign or mark this form, the following procedures will take place:

- You will undergo a medical examination and short medical history.
- You will be asked a number of questions about how you may have been exposed to HIV and when you think you may have become HIV-infected. If you have completed this questionnaire on a previous study with the same investigators, you will not need to complete it again.
- You will also be asked questions about any medicines that you are taking.
- If you are a woman, your urine will be tested to see if you are pregnant. If you are or become pregnant, you will be referred to prenatal care and to programs for Prevention of Mother to Child Transmission (PMTCT) where you will be able to get medicines to prevent your baby from getting HIV.

What will happen to your blood? Up to 10 teaspoons of blood i.e. 50ml (one or 5 small tubes) will be drawn from your arm. The blood will then be sent to the laboratory at Tygerberg Hospital where the red blood cells will be separated from the white blood cells. The cells from the immune system will then be tested in the laboratory, by mixing them with different medications to see which medicine is the best at keeping the cells alive.

- The results of these tests are for research purposes only and cannot be made available to you.
- No genetic testing will be performed.

Will you benefit from taking part in this research?

Benefits: You may benefit by taking part in the study from receiving counseling and a medical examination. You or others may benefit in the future from information learned in this study. You may get some personal satisfaction from being part of research on HIV. If you are a woman, and you become pregnant, you will be referred for prenatal care and for PMTCT.

Risks and/or Discomforts: There may be some discomfort involved in drawing blood for the tests which sometimes can cause pain and bruising where the needle goes into your arm. When blood is being drawn, you may feel dizzy or faint, but this is not common.

Confidentiality
Your participation in the study, all information collected about you as well as all results of laboratory tests will be private and not available to others outside of the people listed below. You will have your own special identity number known only to you and the clinic staff. They are required to respect your confidentiality. Your identity will not be disclosed in any publication or presentation of this study. Your blood specimens will only be identified by your study number. Any documents containing your name will be locked away in a secure place.

Will you be paid to take part in this study and are there any costs involved?

No you will not be paid to take part in the study but your transport and meal costs will be covered for each study visit. There will be no costs involved for you, if you do take part.

Is there any thing else that you should know or do?

- You can contact Dr Hayley Ipp…………………………..….. at tel …021 9389356…………………… if you have any further queries or encounter any problems.
- You can contact the **Health Research Ethics Committee** at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I ……………………………….………… agree to take part in a research study entitled *(insert title of study)*.

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....Crossroads...............…........…………….. on (*date*) …………..……….. 2010

....

Signature of participant **Signature of witness**

Declaration by investigator

I *(name)* ……………………………………………..……… declare that:

- I explained the information in this document to …………………………………..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use a interpreter. (*If an interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) ....Cross roads...........…........…………….. on (*date*) …………...……….. 2010.

....

Signature of investigator **Signature of witness**

Declaration by interpreter

I *(name)* ……………………………………………..……… declare that:

- I assisted the investigator (*name*) ………………………………………. to explain the information in this document to (*name of participant*) ……………..…………………………….. using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) ....…........……………. on (*date*) …………..……………….

....

Signature of interpreter **Signature of witness**

INCWADANA YEENKCUKACHA ZOMTHATHI-NXAXHEBA NOXWEBHU LOKUVUMA

ISIHLOKO SEPROWUJEKTI YOPHANDO: Ukulungiswa nokukhuthazwa kodlamko kunye ne-apoptosis yeeseli ezigonyekileyo zabantu abasuleleke yintsholongwane i-HIV-ngodluliselo olukwimithambo-luvo. I-Vasoactive Intestinal Peptide (VIP) nabanye abaxolelanisi / abalamli kwezobomi.

INOMBOLO YESALATHISI: N07/09/197

UMPHANDI OYINTLOKO: nguGqirha Hayley Ipp/ Dr Richard Glashoff

IDILESI: Medical Virology; 8th Floor Clinical Building; Tygerberg Hospital Campus

INOMBOLO YOQHAGAMSHELWANO: 021 938 9356

Uyamenywa ukuba uthathe inxaxheba kwiprowujekti yophando. Nceda uzinike ithuba lokufunda iinkcukacha ezilapha, ziza kukuchazela konke ngale prowujekti. Nceda ubuze kubasebenzi abakolu phando okanye kugqirha nawuphi na umbuzo ngenxalenye yale prowujekti ongayiqondi kakuhle. Kubalulekile ukuba waneliseke ukuba unengqiqo ngeenkcukacha zale prowujekti nangendlela ongabandakanyeka ngayo kuyo. Kwakhona, ukuthatha kwakho inxaxheba ukwenza **ngokuzithandela** yaye ukhululekile ukuba ungavumi ukuthatha inxaxheba. Xa usithi hayi, akusayi kuchaphazeleka kakubi nangoluphi na uhlobo. Kwakhona ukhululekile ukuba ungarhoxa kwesi sifundo nanini na, nokuba ngoku uyavuma ukuthatha inxaxheba.

Esi sifundo samkelwe **yiKomiti ePhandayo ngeziThethe zeMpilo (HREC) kwiDyunivesithi yaseStellenbosch,** siza kuqhutywa ngokwezikhokelo zezithethe nangokwemimiselo yesiBhengezo seHelsinki samazwe ngamazwe, ngokweziKhokelo zoMzantsi-Afrika zomSebenzi omHle weeKlinikhi nangokweziKhokelo zeziThethe zeQumrhu eliPhandayo ngezoNyango (MRC) malunga noPhando.

Lumalunga nantoni olu phando?

- **Ukukhethwa kwamaVolonti esiFundo** Kwesi sifundo kuza kubhaliswa malunga nama-70 amavolonti ebesoloko esiya eziklinikhi ezisebenzisana neZiko lentsholongwane i-HIV eliyiDesmond Tutu, abanee-CD4 counts ezingaphezulu kwama-200, baneminyaka engama-21 okanye badala kunoko, yaye abakazivumani iziyobizi ezizii-antiretrovirals.
- Amalunga nama-40 amavolonti aza kugaywa ethathwa kwingxilongo eyenziwayo yabantu abangenayo intsholongwane i-HIV.
- *Ukuphanda okwenziwa egazini kwakwenzelwa kwisiBhedlele iTygerberg, kwiSebe leNzululwazi ngezifo.*
- **Isizathu solu phando** Iziyobisi (ii-antiretrovirals) ezifumanekayo ngoku kunyango lwe-HIV zisindise ubomi babantu abaninzi yaye zibanceda imihla ngemihla. Nangona kunjalo, kusafuneka sikhangele iindlela ezahlukileyo zokunceda abantu abanentsholongwane i-HIV ukuze baphile ixesha elide phambi kokuba kufuneke basebenzise iziyobisi ii-antiretroviral. Ukuba eyomntu i-CD4+ T cell count ingaphantsi kwama-200, kubalulekile ukuba makalufumane unyango lwe-antiretroviral, noxa kunjalo, kwabo bane-count esengaphezulu kwama-200, sakukhangela iindlela ezintsha elebhu ukuze le count ihlale ikweli

nqanaba liphezulu, yaye siyathemba oku kunganceda ekuthinteleni ukuqalisa kwemeko emandundu / exhomisa amehlo kaGawulayo.

Inkqubo yesifundo

Ukuba unezigqibo zokuthatha inxaxheba kwesi sifundo, emva kokuba ufundile, xoxa ukuze utyikitye okanye ufake uphawu kolu xwebhu, ezi ndlela zilandelayo zakwenzeka:

- Uza kungena kwingxilongo kubekho nembali emfutshane yezonyango.
- Uza kubuzwa iqela lemibuzo malunga nendlela ogaxeleke ngayo kwi-HIV, nokuba wena ucinga ukuba usulelwe nini na yintsholongwane i-HIV. Xa ugqibile ngeli phepha lemibuzo esifundweni esigqithileyo kunjalo usebenza naba baphandi bakhoyo ngoku, akuzikubakho mfuneko yokuphinda ubhale iinkcukacha kwiphepha eli lemibuzo.
- Kwakhona uza kubuzwa imibuzo ngawo nawaphi na amayeza owasebenzisayo.
- Xa ungumntu obhinqileyo umchamo wakho uza kuxilongwa kukhangelwe ukuba akukhulelwanga na. Ukuba sowukhulelwe okanye uqalisa ukukhulelwa, uza kuthunyelwa kwindawo enonophela abangekabeleki nakwiinkqubo zokuThintela uGqithiselo olusuka kuMama luye eluSaneni (PMTCT), kulapho uza kufumana amayeza athintela ukuba usana lwakho malungabinayo i-HIV.

<u>Kuza kwenzeka ntoni egazini lakho?</u> Amatispuni ali-10 egazi, oko kukuthi ama-50 eemilililithere (enye okanye ezi-5 iityhubhu ezininzi) zakutsalwa kwingalo yakho. Emva koko igazi elo lakusiwa kwilebhu yesiBhedlele iTygerberg apho iiseli zegazi ezibomvu ziza kwahlulwa kwezo zimhlophe. Iiseli eziphuma emandleni okukhusela umzimba zakuxilongwa elebhu, zakuxutywa namayeza ahlukileyo ukuze kubonwe ukuba leliphi elona yeza liphucukileyo ekugcineni iiseli zidlamkile.

- Iziphumo zale ngxilongo zenzelwe uphando kuphela, akunakuzinikwa wena.
- Ayikho ingxilongo yemfuzo / yemvelo eza kwenziwa.

Lukhona uncedo oza kuluzuza ngokuthatha inxaxheba kolu phando?

<u>Uncedo</u>: Ungancedakala ngokuthatha inxaxheba kwesi sifundo ngokuzuza iingcebiso, inkxaso nengxilongo kwezonyango. Wena okanye abanye ningancedakala kwixesha elizayo ngenxa yolwazi olunokufumaneka kwesi sifundo. Ungafumana ukwaneliseka kwesiqu sakho ngokuba yinxalenye yolu phando lwe-HIV. Ukuba ungumntu obhinqileyo, ukuze ukhulelwe, uza kugqithiselwa kwindawo enonophela abantu abaza kubeleka ukwenzela i-PMTCT.

<u>Umngcipheko kunye / okanye ukungonwabi:</u> Kungakhona ukungonwabi okuvayo xa kutsalwa igazi eliza kuxilongwa, ngamanye amaxesha kukho iintlungu kubekho nokugrhuzuka apho inaliti ingenayo kwisikhumba sengalo. Xa litsalwayo igazi ungaziva unesiyezi okanye ungafeyinta, kodwa ezo zehlo azixhaphakanga.

Ukugcinwa kweemfihlelo

Ukuthatha kwakho inxaxheba esifundweni, zonke iinkcukacha eziqokelelweyo ezimalunga nawe kunye neziphumo zengxilongo yaselebhu, zakugcinwa ziyimfihlelo yaye azisayi kufunyanwa ngabantu abangengabo aba bakolu luhlu lungezantsi. Uza kunikwa inombolo esisazisi sakho, iza kwaziwa ngabasebenzi abakolu phando kuphela.

Kulindeleke ukuba iimfihlelo zakho bazihloniphe. Igama lakho alisayi kukhankanywa nakoluphi na upapasho okanye nakoluphi na uzathuzo lwesi sifundo. Igazi lakho lakwaziwa kuphela ngenxa yenombolo esetyenziswe kwesi sifundo. Nawaphi na amaxwebhu anegama lakho aza kutshixelwa emkiswe ayokubekwa endaweni ekhuselekileyo.

Uza kuhlawulwa na ngokuthatha inxaxheba kwesi sifundo, zikhona phofu iindleko ekungenwa kuzo?

Hayi, akuzokuhlawulwa ngokuthatha inxaxheba kwesi sifundo, kodwa iindleko zokuhamba kwakho ngezithuthi nezokutya ziza kuhlawulelwa wena qho xa usityelele ngenxa yesi sifundo. Akukho zindleko uza kungena kuzo wena xa uthatha inxaxheba.

Ikhona enye into omele ukuyazi okanye omele ukuyenza?

- Ungaqhagamshelana noGqirha Hayley Ipp…...........................kule nombolo: 021 9389356…xa uneminye imibuzo okanye usezingxakini ezithile.
- Ungaqhagamshelana **neKomiti yeziThethe zoPhando** kule nombolo: 021-938 9207 xa uxhalabile okanye unezikhalazo ezingakhange ziqwalaselwe kakuhle ngugqirha wakho ophethe isifundo.
- Uza kufumana ikopi yezi nkcukacha nenye ikopi yoxwebhu lokuvuma ukuze uzigcinele.

Isibhengezo somthathi-nxaxheba

Ngokutyikitya ngezantsi, Mna …………………………………..…………. ndiyavuma ukuthatha inxaxheba kwisifundo sophando esinesihloko esithi *(bhala isihloko sesifundo)*.

Ndazisa ukuba:

- Ndilufundile okanye ndalufunda olu lwazi kunye nefomu yemvumelwano kwaye ibhalwe ngolwimi endiliciko nendikhululekileyo kulo
- Bendinalo ithuba lokuba ndibuze imibuzo kwaye yonke imibuzo yam iphendulwe ngokwanelisayo.
- Ndiyakuqonda ukuba ukuthatha inxaxheba kolu phando kube **kukuzithandela kwam** kwaye andikhange ndinyanzelwe ukuba ndithathe inxaxheba.
- Ndingakhetha ukusishiya isifundo nanini na kwaye andisayi kohlwaywa ndingasayi kuqala ndigwetywe nangayiphi indlela.
- Ndisenokucelwa ukuba mandisishiye isifundo phambi kokuba siphele, ukuba ugqirha wesifundo okanye umphandi ukubona kufanelekile oko, okanye ukuba andisilandeli isicwangciso sesifundo ekuvunyelenwe ngaso.

Kutyikitywe e-(indawo) ....ngomhla (we— ngenyanga engu---------) ngo-2010..……….. 2005.

....
Ukutyikitya komthathi-nxaxheba **Ukutyikitya kwengqina**

Isibhengezo somphandi

Mna *(igama)* ……………………………………………… ndazisa ngokubha:

- Ndilucacisile ulwazi olukolu xwebhu ku-…………………..……………...
- Ndimkhuthazile ukuba makabuze imibuzo, nam ndithathe ixesha elifanelekileyo lokuyiphendula.
- Ndiyaneliseka kukuba unengqiqo eyaneleyo ngeengongoma zonke zolu phando njengoko kuchaziwe apha ngasentla.
- Ndisebenzise/andisebenzisanga toliki. (*Ukuba itoliki isetyenzisiwe imele ukuba ityikitye kwisaziso esingezantsi.*

Kutyikitywe e-(indawo) .... ngomhla (we--- kwinyanga engu---------) ngo-2010

....
Ukutyikitya komphandi **Ukutyikitya kwengqina**

Isibhengezo setoliki

Mna *(igama)* ……………………………………………… ndazisa ukuba:

- Ndincede umphandi (*igama*) …………………………. ekucaciseni ulwazi

  olukolu xwebhu ku-(*igama lomthathi-nxaxheba*) ……………………………..

  ndisebenzisa ulwimi lwesi-Afrikaans / lwesiXhosa.

- Simkhuthazile ukuba makabuze imibuzo kwaye sithathe ixesha elifanelekileyo xa besimphendula.
- Ndimxelele ngezona ngongoma zichanekileyo zako konke endikuchazelweyo.
- Ndiyaneliseka kukuba umthathi-nxaxheba ukuqonda ngokupheleleyo okuqulathwe lolu xwebhu lwemvumelwano eyazisiweyo kwaye nemibuzo yakhe yonke iphendulwe ngokwanelisayo.

Kutyikitywe e-(*indawo*) .... ngomhla -(we—- *kwinyanga engu*------) ngo-2010

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Ukutyikitya kwetoliki **Ukutyikitya kwengqina**