The impact of sexually transmitted infections and inflammation in the female genital tract and blood on susceptibility to HIV-1 infection and disease progression

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List of abbreviations

ACD	Acetate citrate dextran
AIDS	Acquired Immune Deficiency Syndrome
APOBEC3G	Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G
BV	Bacterial vaginosis
CAPRISA	Centre for the AIDS Program of Research in South Africa
CCL	Chemokine ligand
CCR5	Chemokine receptor 5
CD	Cluster designation
CFA	Confirmatory factor analysis
CI	Confidence interval
CS	Cellulose sulfate
CTL	Cytotoxic T lymphocyte
CVL	Cervicovaginal lavage
CXCL	CXC chemokine ligand
CXCR4	CXC chemokine receptor type 4
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin
EFA	Exploratory factor analysis
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ESN	Highly HIV-1-exposed persistently seronegative women
FDR	False discovery rate
FGF-2	Fibroblast growth factor 2
FLT3L	Fms-like tyrosine kinase-3 Ligand
G-CSF	Granulocyte colony stimulating factor
GI	Gastrointestinal
GM-CSF	Granulocyte macrophage colony stimulating factor
GRO	Growth related oncogene
HAART	Highly active antiretroviral therapy
HSV-2	Herpes simplex virus type 2
IFN	Interferon
IL	Interleukin
IL-1Ra	IL-1 receptor antagonist
IP-10	IFN-γ-induced protein 10
IQR	Interquartile range

LTR	Long terminal repeat
MCP	Monocyte chemotactic protein
MDC	Macrophage-derived chemokine
MIP	Macrophage inflammatory protein
ml	Millilitre
MSM	Men who have sex with men
N-9	Nonoxynol-9
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NMAbs	Neutralizing monoclonal antibodies
NNRTI	Nonnucleoside reverse transcriptase inhibitors
OR	Odds ratio
PBMCs	Peripheral blood mononucleocytes
PCA	Principal component analysis
PDGF	Platelet-derived growth factor
pg	Picogram
pH	Potential of hydrogen
PrEP	Pre-exposure prophylaxis
RANTES	Regulated upon activation normal T cell expressed and secreted
RNA	Ribonucleic acid
sCD40L	Soluble CD40 ligand
SHIV-1	Simian-human immunodeficiency virus
sIL-2Rα	Soluble IL-2 receptor α
SIV	Simian immunodeficiency virus
SLPI	Secretory Leukocyte Protease Inhibitor
SLS	Sodium Laurel Sulfate
STI	Sexually transmitted infection
T_{CM}	Central memory T cells
T_{EM}	Effector memory T cells
TGF-α	Transforming growth factor α
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
µl	Microlitre

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Abstract

Background. In sub-Saharan Africa, which has the highest prevalence of HIV-1 worldwide, most new HIV-1 infections occur by sexual transmission to women. Recent studies in non-human primates have demonstrated that pro-inflammatory cytokine production in the genital tract is necessary for immune cell recruitment and establishment of simian immunodeficiency virus (SIV) infection following vaginal inoculation. The aims of this study were to evaluate the relationships between inflammation in the female genital tract and (i) susceptibility to HIV-1 infection and (ii) subsequent disease progression in women who became infected. Additionally, genital inflammation was investigated as a mechanism for breakthrough HIV-1 infections in women who became infected even though they were using 1% tenofovir (TFV) microbicide. In the systemic compartment, the level of T cell activation and soluble markers of immune activation during HIV-1 infection are associated with disease outcome. Therefore, the relationships between plasma cytokine concentrations during early HIV-1 infection and disease progression were evaluated.

Methods. The participants of this study included 230 HIV-uninfected women from the CAPRISA 002 cohort who were followed longitudinally for HIV-1 infection, 49 women who were enrolled during acute HIV-1 infection and followed until 12 months post-infection and 166 HIV-uninfected women who were enrolled in the CAPRISA 004 1% TFV microbicide trial (62 of whom later became HIV-1-infected). Cytokine concentrations were measured in cervicovaginal lavage (CVL) and plasma samples from these women using Luminex and ELISA.

Results. It was found that elevated cervicovaginal concentrations of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, GM-CSF) and immunoregulatory IL-10 were associated with increased risk of HIV-1 infection, even in women who were using the highly active antiretroviral 1% TFV microbicide. Following HIV-1 infection, genital cytokine concentrations 6 and 17 weeks post-infection were associated with CD4 depletion (GM-CSF) and higher viral load set-points (pro-inflammatory GM-CSF, IL-1β, IL-6, IL-12p70, MIP-1β and RANTES, T cell homeostatic IL-2 and IL-15 and immunoregulatory IL-10). It was further found that inflammatory cytokines pre-infection were correlated with those post-infection and in fact were not significantly elevated shortly following HIV-1 infection (6 weeks post-infection). Additionally, 7 of the 20 cytokines measured were found to correlate between at least 3 of the 5 time-points assessed, suggesting that the relative degree of genital inflammation in individual women remained relatively constant over time during HIV-1 infection. It was found that cytokine concentrations in the female genital tract were associated with sexually transmitted infections, bacterial vaginosis, vaginal discharge, HIV-1 shedding, blood CD4+ T cell counts, combined oral contraceptive use and the age of study participants. Plasma cytokine concentrations during early HIV-1 infection were found to be predictive of HIV-1 disease progression: Two models including five cytokines each were more strongly predictive of viral load set-point and CD4+ T cell loss than either early infection CD4 counts, viral loads or both combined.

Conclusion. Women who have inflammation in their genital tracts are at increased risk of HIV-1 infection and may experience more rapid HIV-1 disease progression should they become infected. Therefore better management of the causes of cervicovaginal inflammation, particularly in high-risk women, may be essential for prevention of HIV-1 infection and may improve disease prognosis. Plasma cytokine concentrations, which are easily measurable, are predictive of subsequent disease progression and may be useful for evaluating the ability of therapeutic HIV-1 vaccines to control HIV-1 infection.

CHAPTER 1

Chapter 1
Literature Review

1.1 Introduction

HIV-1 is most prevalent in sub-Saharan Africa, where 68% of all HIV-1-infected individuals reside and 72% of AIDS-related deaths occur (Figure 1.1; UNAIDS, 2010). In this region, most new HIV-1 infections occur by sexual transmission to women (Figure 1.2; UNAIDS, 2010). The risk of HIV-1 transmission per heterosexual contact is highly heterogenous and ranges between zero and 8.2%, depending on the setting, type of sex and biological factors in both partners (Boily *et al.*, 2009). In low-income countries, the per-act risk of HIV-1 transmission is 3.8 and 9.5 times higher than in high income countries, for male-to-female and female-to-male transmission, respectively (Boily *et al.*, 2009). In sub-Saharan Africa, women account for approximately 60% of HIV-1-infected individuals and among 15 to 24-year olds, women are up to eight times more likely to be HIV-1-infected than men (UNAIDS, 2010).

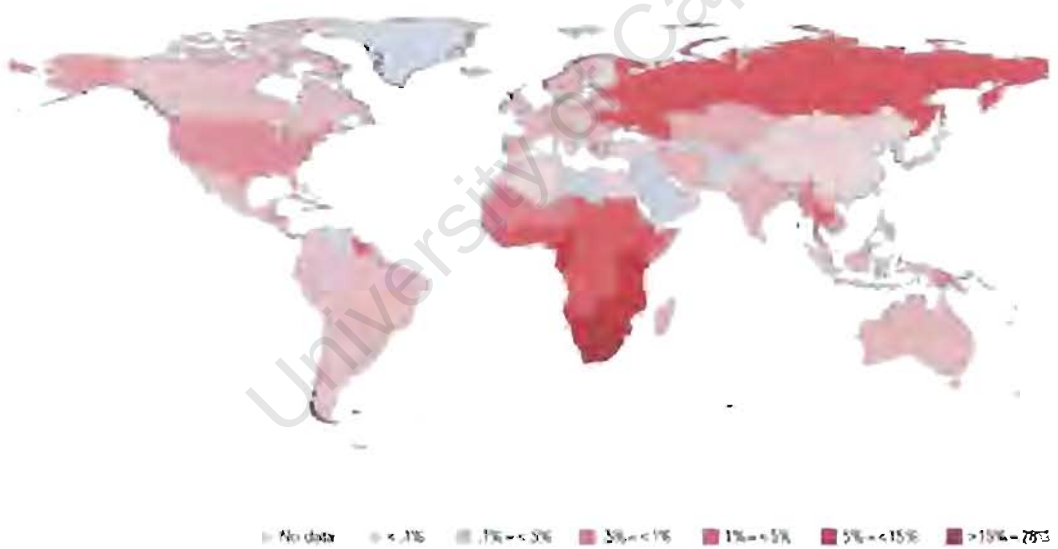


Figure 1.1 Global prevalence of HIV-1 (source: UNAIDS, 2010)

During heterosexual intercourse, the relatively large surface area of the vaginal mucosa is exposed to seminal fluids for long periods, partly explaining why women are more susceptible to infection than men (Hirbod and Broliden, 2007). Additionally, cultural factors may play a significant role in transmission to women in low-income countries, where some women have little control over the conditions of sexual encounters (Heise and Elias, 1995; Hirbod and Broliden, 2007). Thus, although male condom use has

increased worldwide (UNAIDS, 2010), the development of safe and efficacious vaccines and microbicides is a promising intervention strategy for women in particular (Voelker, 2006).

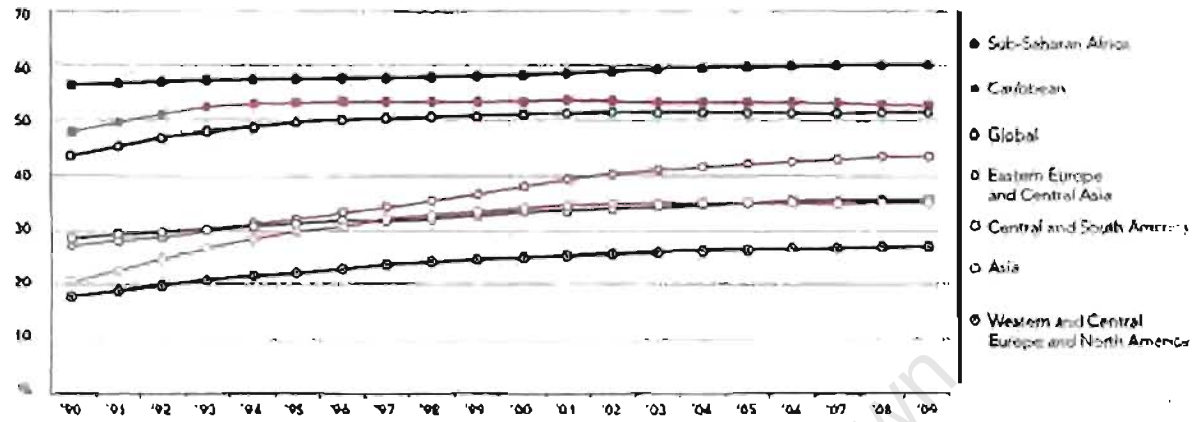


Figure 1.2 Proportion of individuals 15 years and older living with HIV-1 who are women (source: UNAIDS, 2010)

Like HIV-1, other sexually transmitted infections (STIs) are also very prevalent in sub-Saharan Africa (Johnson *et al.*, 2010a) and are associated with increased susceptibility to HIV-1 acquisition and secondary transmission (Hayes *et al.*, 1995; Robinson *et al.*, 1997; Cohen, 2004). Under normal conditions, mucosal innate immunity in the female genital tract provides over 99% protection against HIV-1 infection (Gray *et al.*, 2001). However, the presence of an STI may increase the risk of HIV-1 transmission substantially by disrupting various components of the immune system (Hayes *et al.*, 1995; Robinson *et al.*, 1997; Cohen, 2004). Genital ulceration of the mucosal epithelial barrier is caused by STIs such as syphilis and herpes simplex virus type 2 (HSV-2) and is associated with 5.3 times (95% confidence interval (CI) 1.4-19.6) greater risk of HIV-1 transmission (Boily *et al.*, 2009). STIs also cause inflammation in the genital tract, which may increase the risk of HIV-1 transmission by facilitating HIV-1 replication (Osborne *et al.*, 1989; Swingler *et al.*, 1999; Nkwanyana *et al.*, 2009). Several studies in macaques and humans have further suggested that genital tract inflammation prior to and during the early stages of HIV-1 infection may influence HIV-1 disease progression (Zara *et al.*, 2004; Wang *et al.*, 2005; Bebell *et al.*, 2008; Li *et al.*, 2009; Haase, 2011). In the systemic compartment, the relationship between inflammation and susceptibility to HIV-1 infection has not been investigated, however, chronic inflammation is an important characteristic of HIV-1 infection and is associated with rapid disease progression (Douek *et al.*, 2009).

1.2 The role of cytokines in inflammation

Inflammation is an innate immune process that is essential for microbial control and clearance, however it is also associated with tissue destruction (Svanborg *et al.,* 1999; Rouse *et al.,* 2006), increased susceptibility to infection by HIV-1 and other microorganisms and with progressive HIV-1 disease (Connolly *et al.*, 2005; Rebbapragada and Kaul, 2007). Pro-inflammatory cytokines and chemokines play a central role in initiating and sustaining the inflammatory response by recruiting immune cells from circulation and stimulating their differentiation and activation (Figure 1.3; Charo and Ransohoff, 2006; Dinarello, 2010). The principal effector cells in innate immunity are neutrophils, mononuclear phagocytes and natural killer (NK) cells (Abbas and Lichtman, 2007). These cells, along with epithelial and endothelial cells, produce pro-inflammatory cytokines and chemokines in response to binding of pathogen-associated molecular patterns to pattern recognition receptors, such as toll-like receptors (TLRs), C-type lectins, scavenger receptors and NOD-like receptors (Dinarello *et al.*, 1997; Connolly *et al.*, 2005; Wira *et al.*, 2005; Abbas and Lichtman, 2007; Mirmonsef *et al.*, 2011). Chemokines and pro-inflammatory cytokines then recruit leukocytes by forming chemoattractant gradients and by inducing cellular adhesion molecule expression by vascular endothelial cells. Circulating leukocytes bind to these adhesion molecules, slowly roll along the vascular endothelium, eventually stop and transmigrate through the endothelium and into tissues (Meager, 1999). Inflammation usually involves initial, transient infiltration of neutrophils, followed by longer-persisting monocytes, which differentiate to macrophages as they enter the tissues (Huerre and Gounon, 1996; Abbas and Lichtman, 2007). These phagocytic cells identify, ingest and destroy microbes (Abbas and Lichtman, 2007). Antigen-presenting cells, such as dendritic cells (DCs), convert microbial proteins to peptides (antigen processing) and migrate to the lymph nodes where they present these antigens to T cells to initiate T cell adaptive immune responses (Banchereau and Steinman, 1998). Following antigen-induced activation of naive T cells in the lymph nodes, differentiation into effector T cells occurs. This involves down-regulation of lymph node-homing receptors (CCR7) and up-regulation of adhesion molecules and chemokine receptors. As a result, effector T cells migrate from the lymph nodes, into circulation and into infected/damaged tissues (Weninger *et al.*, 2002).

Some cytokines act as growth factors that stimulate growth and proliferation of immune and non-immune cells (Abbas and Lichtman, 2007). Vascular growth factors, such as vascular endothelial growth factor (VEGF), promote angiogenesis and lymphangiogenesis (blood and lymphatic vessel formation; Tammela *et al.*, 2005). These growth factors have also been found to participate in inflammation. VEGF is induced by pro-inflammatory cytokines, suggesting that VEGF may influence leukocyte trafficking by playing a role in blood and lymphatic vessel function during inflammation (Tammela *et al.*, 2005; Rafii *et al.*, 2003; Cursiefen *et al.*, 2004). In support, VEGF is chemoattractant for monocytes (Ferrara *et al.*, 2003) and overexpression in mouse models leads to abundant angiogenesis and inflammation (Ferrara, 2004). Furthermore, inflammatory reactions in human transplants undergoing rejection are accompanied by

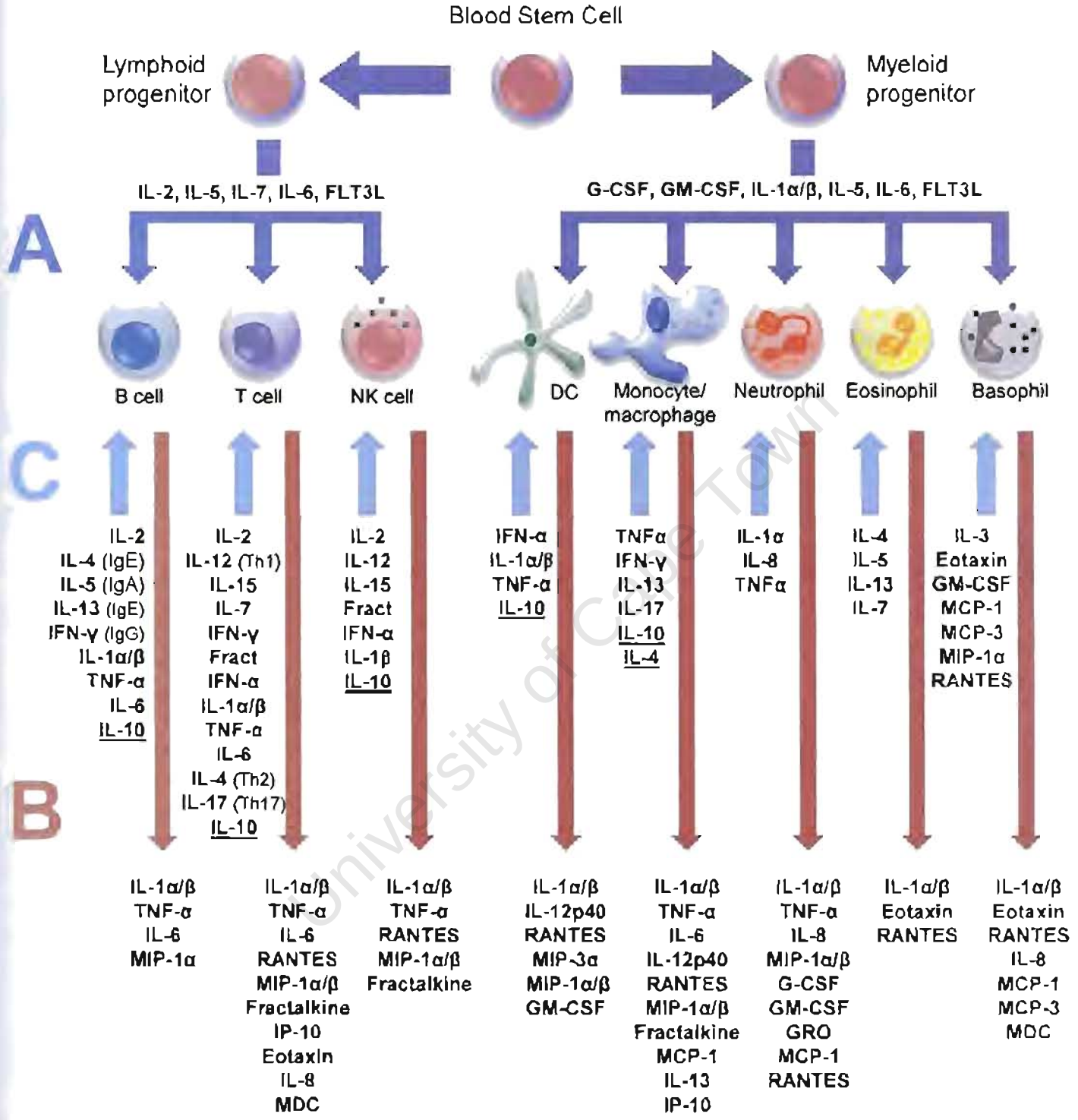


Figure 1.3 Summary of cytokine functions. A) Hematopoietic cytokines stimulate production of immune cells from stem cell progenitors. B) Pro-inflammatory cytokines recruit immune cells to infection sites. C) Adaptive and innate immune mediators stimulate growth, differentiation, activation and survival of immune cells. Underlined cytokines such as IL-4 and IL-10 may also have regulatory functions (Baggiolini *et al.*, 1989; Metcalf , 1986; Sanderson, 1992; Taub *et al.*, 1993a and b; Alam *et al.*, 1994; Harada *et al.*, 1994; Schmidtmayerova *et al.*, 1996; Dinarello *et al.*, 1997; Haas *et al.*, 1999; Baji *et al.*, 2001; Gilliland and Griffin, 2002; Gaffen and Liu, 2004; Sporri and Reis e Sousa, 2005; Wira *et al.*, 2005; Gabay, 2006; Abbas *et al.*, 2007; Couper *et al.*, 2008; Jana and Pahan, 2009; Kelly *et al.*, 2009; Dinarello, 2011).

angiogenesis and VEGF receptor signalling (Kerjaschki *et al.*, 2004). In addition to pro-inflammatory cytokines, VEGF is also induced by other growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (FGF) and transforming growth factors (TGF; Ristimaki *et al.*, 1998; Ferrara, 2004). Anti-inflammatory cytokines, such as IL-10 and IL-1 receptor antagonist (IL-1Ra), regulate inflammatory responses. IL-10 limits production of pro-inflammatory cytokines and chemokines by monocytes and macrophages (Couper *et al.*, 2008). IL-1Ra binds to a receptor for IL-1α and IL-1β and specifically inhibits their pro-inflammatory function (Dinarello, 2009).

Other major classes of cytokines include those with hematopoietic functions and mediators and regulators of adaptive immunity. Hematopoietic cytokines, which are produced by bone marrow stromal cells, leukocytes and other cells, instruct lineage choice of hematopoietic stem cells, promoting their differentiation, proliferation and survival (Abbas and Lichtman, 2007; Rieger *et al.*, 2009; Metcalf, 2008). Hematopoietic cytokines such as granulocyte colony stimulating factor (G-CSF) and granulocyte macrophage (GM)-CSF may also play a role in inflammation by recruiting leukocytes to infection sites (Wira *et al.*, 2005). Adaptive immune cytokines regulate the growth and differentiation of B and T lymphocytes, and recruit, activate and regulate effector cells such as phagocytes and neutrophils during the effector phase of the adaptive immune response. T lymphocytes are the primary producers of adaptive cytokines (Abbas and Lichtman, 2007).

Although cytokines are often classified into one of the five above groups (pro-inflammatory, hematopoietic, growth factors, adaptive immune mediators, regulatory/anti-inflammatory), many have diverse and redundant biological functions and some may function in all of the above arms of the immune response (Connolly *et al.*, 2005).

1.3 The female genital tract

The female genital tract is a unique environment that has the ability to respond rapidly to infections that are generally transmitted in seminal plasma or during sexual contact, whilst at the same time being tolerant to allogenic spermatozoa (Quayle *et al.*, 1989; Kelly *et al.*, 1997; Tremellen *et al.*, 1998). The lower female reproductive tract consists of four regions: (i) the keratinised stratified squamous epithelial introitus (vaginal opening), (ii) the vaginal mucosa, (iii) the external region of the cervix (ectocervix), and (iv) the endocervix (Pudney *et al.*, 2005; Hladik and McElrath, 2008). The vaginal mucosa and the ectocervix, are lined with non-keratinized stratified squamous epithelium (Quayle, 2002; Coombs *et al.*, 2003; Hirbod and Broliden, 2007). The endocervix is lined with a single layer of columnar epithelial cells, which meet the stratified layer of the ectocervix at the squamocolumnar junction (Coombs *et al.*, 2003; Hirbod and Broliden, 2007). This junction may lie on the ectocervix (cervical ectopy) during menarche, in most adolescent women, in parous women and during gestation. However, during reproductive years,

ectropian columnar epithelial cells are replaced by stratified squamous epithelium (metaplasia), forming the epithelial transformation zone. The squamocolumnar junction has usually receded into the endocervix by the onset of menopause (Jacobson *et al.*, 1999; Coombs *et al.*, 2003).

Although the tight junctions between the endocervical columnar epithelial cells provide a mechanical barrier to infection, the multiple layers of squamous cells in the lower reproductive tract and continuous sloughing off of the superficial layer are thought to provide more effective protection (Kaushic *et al.*, 2010). However, the greater surface area of the vaginal mucosa and ectocervix may allow greater access for pathogens (Hladik and McElrath, 2008). Epithelial cells in the genital tract produce a protective moisture-providing, hydrophilic layer of glycoprotein called glycocalyx and a hydrophobic glycoprotein mucus (Lai *et al.*, 2009; Kaushic *et al.*, 2010). Additionally, they release numerous antimicrobial peptides and proteins into the mucosal fluid, express TLRs and secrete inflammatory cytokines in response to antigen binding (Schaefer *et al.*, 2004; Fahey *et al.*, 2005; Hirbod and Broliden, 2007; Kaushic *et al.*, 2010). Leukocytes make up 6-20% of the total cells in the female reproductive tract. T lymphocytes and granulocytes are major components of this population, while B lymphocytes and monocytes are present in small numbers (Givan *et al.*, 1997; Levine *et al.*, 1998). In healthy women, the vaginal mucosa contains relatively few leukocytes: CD8+ T cells are most abundant, while only small numbers of CD4+ T cells, NK cells, macrophages and DCs are present (Pudney *et al.*, 2005). The ectocervical mucosa and transformation zone are potentially more vulnerable to HIV-1 infection as these regions contain larger numbers of CD4+ T cells, macrophages and DCs than the lower genital tract (Levine *et al.*, 1998; Pudney *et al.*, 2005). However, CD8+ T cells and antigen-presenting cells are also abundant in the cervical transformation zone, suggesting the potential for initiation of cellular immunity. The endocervical epithelium contains the lowest numbers of T cells and macrophages, and no DCs (Pudney *et al.*, 2005).

The vagina and ectocervix are non-sterile and contain roughly 10^9 microorganisms/ml (Quayle, 2002; Mirmonsef *et al.*, 2011). *Lactobacillus* species represent the most dominant population of bacterial microorganisms in a healthy genital tract and metabolize glycogen released by vaginal epithelial cells to lactic acid, while some species also produce hydrogen peroxide, a virucidal agent. Lactic acid production creates an acidic environment which is less conducive to colonization by gram negative bacterial pathogens and to HIV-1 infection (Eschenbach *et al.*, 1989).

1.4 HIV-1 transmission across the female genital mucosa

Several mechanisms for HIV-1 transmission across the female genital epithelial barrier have been suggested. The main receptor that is used by HIV-1 for host cell entry is the CD4 molecule (Weiss, 1996). Although genital secretions may contain viruses that use CCR5 as a co-receptor for cell entry (R5-tropic), as well as viruses use CXCR4 for entry (X4-tropic), all studies on sexual transmission have shown that

new infection are established by R5 viruses (Peters *et al.*, 2006; Philpott, 2003). Cell-free HIV-1 may translocate through the vaginal and ectocervical stratified squamous epithelium of the lower genital tract via physical abrasions (Shen *et al.*, 2011). Cell-free virus has also been shown to move between the upper layers of the "leaky" squamous epithelial barrier (Hladik and Hope, 2009). It has been suggested that transcytosis of HIV-1 may occur through polarized columnar epithelial cells and squamous epithelial cells (Bomsel and Alfsen, 2003; Hladik and McElrath, 2008). However, other studies have found that HIV-1 does not penetrate through intact cervical explants (Shattock and Moore, 2003). CD4+ T cells and DCs may also migrate into the vaginal and ectocervical epithelium (Johansson *et al.*, 1999; Pudney *et al.*, 2005; Hladik and Hope, 2009). Resting and activated CD4+ T cells were found to be the predominant targets for simian immunodeficiency virus (SIV) and HIV-1 infection in the genital tract (Zhang *et al.*, 1999). Similarly, the earliest targets of HIV-1 in cervical tissue culture were shown to be memory CD4+ T cells (Gupta *et al.*, 2002). In contrast, macrophages have been identified as the predominant target cells in the vaginal lamina propria (Greenhead *et al.*, 2000). Langerhans cells, which reside in the genital epithelium, express both CD4 and CCR5 and may internalize R5 strains of HIV-1 and transfer the virus to CD4+ T cells (Hladik *et al.*, 2007). However, Langerhans cells have also been shown to bind to HIV-1 via their langerin receptors, which may result in virus endocytosis and degradation (de Witte *et al.*, 2007a). DCs express dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), mannose receptor and heparin sulphate proteoglycan syndecan-3 which may capture HIV-1 (Geijtenbeek *et al.*, 2000; de Witte *et al.*, 2007b). HIV-1 is either endocytosed or transferred to CD4 and CCR5 receptors on DCs, resulting in viral-cell membrane fusion and infection. Endocytosed HIV-1 may be degraded or transferred to CD4+ T cells or macrophages (Geijtenbeek *et al.*, 2000; Turville *et al.*, 2004). As DCs and Langerhans cells mature, CCR5 expression is down-regulated while CXCR4 expression is up-regulated. Therefore, immature DCs and Langerhans cells may play a more important role in R5 HIV-1 transmission (Cunningham *et al.*, 2008).

1.5 Establishment of HIV-1 infection

A productive HIV-1 infection can be divided into three phases: (i) the acute phase, which is characterized by high levels of viral replication and rapid depletion of CD4+ T cells followed by initiation of antiviral immune responses and a decrease in viral load to a set-point level that is maintained for months to years; (ii) the asymptomatic or chronic phase, which is associated with a gradual decrease in viral load and CD4+ T cells; and (iii) the symptomatic phase or AIDS, which is associated with failure of the immune system and disease (Lyles *et al.*, 2000; Fiebig *et al.*, 2003; Brenchley *et al.*, 2004; Mehandru *et al.*, 2004; Centlivre *et al.*, 2007; Stacey *et al.*, 2009).

Several studies have found that, in approximately 75% of HIV-1 sexual transmission events, infection is established by a single transmitted virus (Keele *et al.*, 2008; Salazar-Gonzalez *et al.*, 2008; Abrahams *et*

al., 2009). Factors that have been found to be associated with transmission of more than one founder virus through sexual intercourse include (i) disruption of the mucosal barrier by STIs, (ii) female sex work and (iii) men who have sex with men (MSM; Poss *et al.*, 1995; Long *et al.*, 2000; Haaland *et al.*, 2009; Li *et al.*, 2010). Following sexual transmission of HIV-1, it has been shown that viral RNA is not detectable in blood for approximately 10 days, a period referred to as the eclipse phase (Miller *et al.*, 2005; Haase, 2011). In macaques, it has been shown that SIV expansion occurs in a small population of cells within the cervical/vaginal tissues during this eclipse phase (Li *et al.*, 2009). In support of this, other studies have reported that SIV RNA was detectable in systemic lymphoid tissues 1 day post-infection, but little viral replication was found to occur here until 5-6 days following infection (Abel *et al.*, 2005; Miller *et al.*, 2005). It has been suggested that continued viral expansion in the genital tissues and seeding of the systemic compartment is necessary to achieve a threshold of sustainable infection at distal sites (Miller *et al.*, 2005).

Following the eclipse phase, it has been shown that rapid viral replication occurs and peak viraemia in blood is reached between 21 and 29 days post-infection, at which time HIV-1-infected individuals may present with flu-like symptoms (Haase, 2011). Mucosal tissues, including the gastrointestinal (GI) tract and vagina, have been shown to be important sites of early SIV/HIV-1 replication, with massive depletion of activated CD4+ T cells occurring here during early infection, before significant depletion in blood and lymph nodes (Veazey *et al.*, 2003a; Brenchley *et al.*, 2004; Mehandru *et al.*, 2004). Mucosal tissues are thought to be important sites of continued viral replication and CD4+ T cell loss throughout HIV-1 infection (Veazey *et al.*, 2003a; Picker *et al.*, 2004). By the time peak viraemia has been reached, CD4+ T cell depletion has occurred from the GI tract, a viral reservoir in the lymphatic tissue has been established and activation of the immune system has occurred (Chun *et al.*, 1998; Blankson *et al.*, 2000; Brenchley *et al.*, 2004).

1.6 Inflammation in the female genital tract and susceptibility to HIV-1 infection

There is a large degree of heterogeneity in susceptibility to HIV-1 infection through sexual transmission and some individuals remain uninfected despite high levels of exposure (Kaul *et al.*, 2011). Many studies have investigated biological factors that may influence susceptibility to HIV-1 infection. Inflammation in the female genital tract is one such factor that it thought to increase the risk of sexual HIV-1 transmission (Figure 1.4). Pro-inflammatory cytokines that are involved in immune cell recruitment to the genital tract, activation or differentiation may increase HIV-1 transmission, as HIV-1 replication depends on the presence of immune cell targets, the level of immune cell activation and monocyte differentiation to macrophages or DCs (Zack *et al.*, 1990; Taub *et al.*, 1993a; Taub *et al.*, 1993b; Harada *et al.*, 1994; Dinarello *et al.*, 1997; Swingler *et al.*, 1999; Wira *et al.*, 2005; Gabay *et al.*, 2006; Dinarello, 2009;

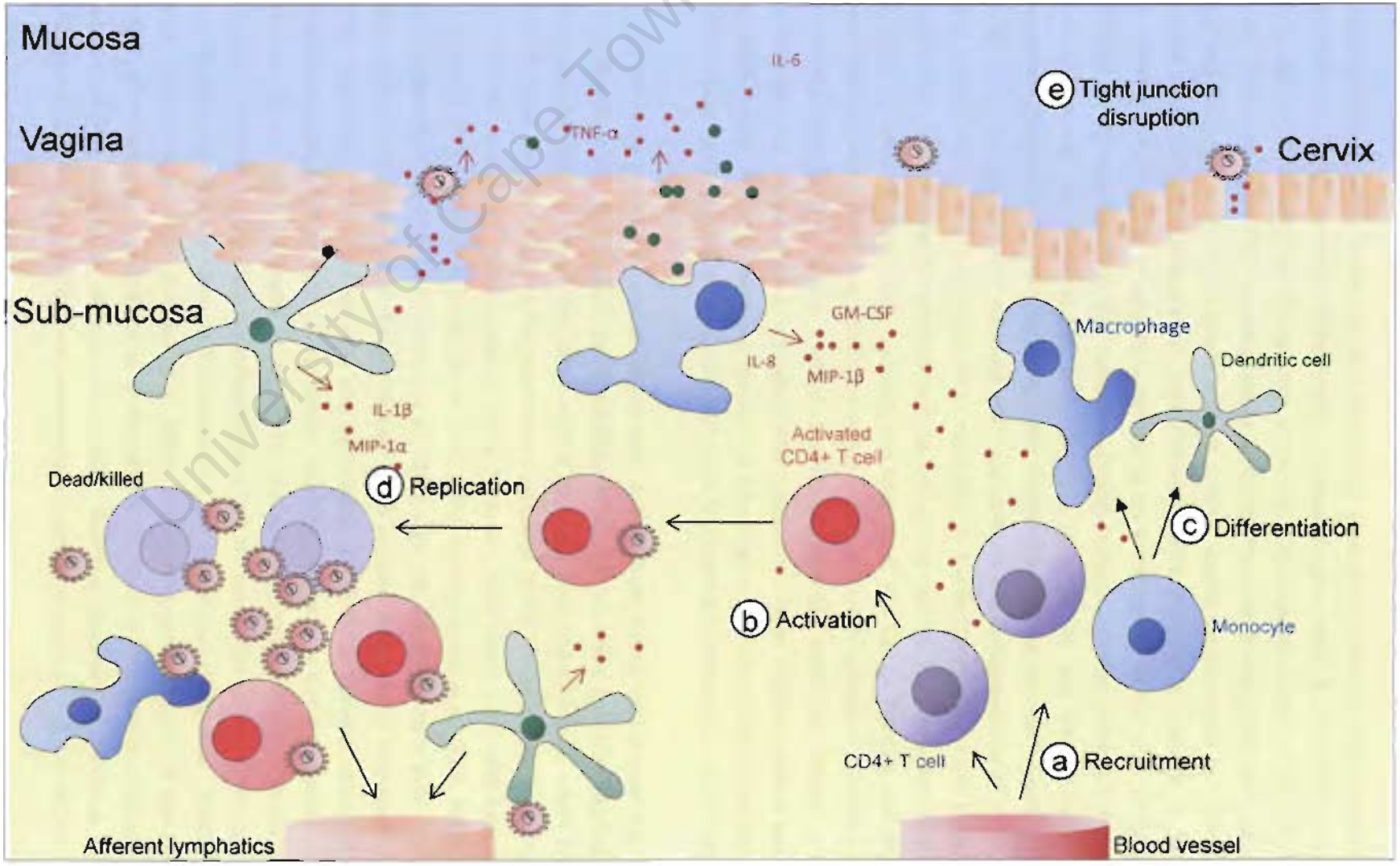


Figure 1.4 Mechanisms by which elevated concentrations of inflammatory cytokines in the female genital tract may increase susceptibility to HIV-1 infection. Inflammatory cytokines and chemokines (a) recruit, (b) activate (c) and stimulate differentiation of immune cells which may act as targets for HIV-1 replication. Certain inflammatory cytokines also direct upregulate HIV-1 replication by activating NK-κB which binds to HIV-1 long terminal repeat (LTR) and induces HIV-1 transcription (d). Inflammatory cytokines may facilitate HIV-1 infection by disrupting tight junctions between endocervical epithelial cells, increasing the permeability of this barrier (e).

Nkwanyana *et al.*, 2009). Unlike DCs and Langerhans cells, whose maturation is accompanied by down-regulation of CCR5, monocyte differentiation to macrophages, which is induced by pro-inflammatory cytokines, is accompanied by up-regulation of the HIV-1 R5 virus co-receptor, CCR5 (Hariharan *et al.*, 1999; Kaufmann *et al.*, 2001; Cunningham *et al.*, 2008). Pro-inflammatory cytokines such as interleukin (IL)-1α/β, IL-6, IL-7 and tumour necrosis factor (TNF)-α induce expression of the transcription factor, nuclear factor (NF)-κB (Osborne *et al.,* 1989; Poli *et al.,* 1990; Blackwell and Christman, 1997; Chene *et al.,* 1999; Niu *et al.*, 2004). In addition to enhancing the expression of various host cell proteins that are involved in inflammation (including cytokines, growth factors, adhesion molecules and acute phase proteins), NF-κB also binds to HIV-1-long terminal repeat (LTR) and directly up-regulates HIV-1 replication (Osborne *et al.,* 1989; Poli *et al.,* 1990; Blackwell and Christman, 1997; Chene *et al.,* 1999; Niu *et al.*, 2004). IL-8 and GM-CSF stimulate HIV-1 replication in macrophages (Lane *et al.,* 2001; Koyanagi *et al.*, 1988) and IL-8 and IL-2 induce HIV-1 replication in T cells (Chun *et al.,* 1998; Lane *et al.,* 2001). Additionally, pro-inflammatory cytokines such as TNF-α may facilitate HIV-1 infection by disrupting tight junctions between epithelial cells, reducing the integrity of this barrier (Madara and Stafford, 1989; Schmitz *et al.*, 1996; Nazli *et al.*, 2010). In rhesus macaques, pro-inflammatory cytokine production and associated recruitment of CD4+ T target cells for SIV infection have been shown to be important prerequisites for establishment of a productive SIV infection in blood following vaginal inoculation (Li *et al.*, 2009; Haase, 2011). Furthermore, inhibition of inflammatory responses using a topically-applied anti-inflammatory agent (glycerol-monolaurate) was found to prevent SIV infection (Li *et al.*, 2009).

There are many potential causes of inflammation in the female genital tract (Figure 1.5). Perhaps the most significant and well studied are pertubations in vaginal microflora [bacterial vaginosis (BV)], and STIs such as syphilis, gonorrhoea, active HSV-2, trichomoniasis and chlamydia, which are associated with elevated concentrations of pro-inflammatory cytokines in the female genital tract (Agrawal *et al.,* 2007; Cohen *et al.,* 1999; Fichorova *et al.,* 2001a; Fitzgerald, 1992; Kiviat *et al.,* 1985; Levine *et al.,* 1998; Yudin *et al.*, 2003; Libby *et al.,* 2008; Novak *et al.,* 2007; Rebbapragada *et al.,* 2007; Reddy *et al.,* 2004; Sturm-Ramirez *et al.,* 2000; van Voorhuis *et al.,* 1996). High-risk sexual activity or even "normal" sex acts may result in microabrasions in the genital tract which increase inflammatory responses (Norvell *et al.*, 1984; Guimaraes *et al.*, 1997; de Jong and Geijtenbeek, 2008). Other potential causes of inflammation include hygiene practices such as antiseptic douching, proteins in seminal plasma and lubricants (Scholes *et al.*, 1993; Fichorova *et al.*, 2001b; Sharkey and Robertson, *2007*; Berlier *et al.*, 2006). Changes in oestrogen and progestin concentrations that are associated with hormone contraceptive use and hormone cycling may influence inflammation in the genital tract (Hunt *et al.,* 1997; Akoum *et al.*, 2000; Prakash *et al.,* 2001; Ghanem *et al.*, 2005). Physiological concentrations of estrogen have been shown to stimulate IL-1β production by endometrial cells, which in turn induces monocyte chemotactic protein (MCP)-1 expression (Akoum *et al.*, 2000). Estrogen treatment of immature DCs increases secretion of IL-6, IL-8 and MCP-1 and progesterone withdrawal results in IL-8 and MCP-1 up-regulation

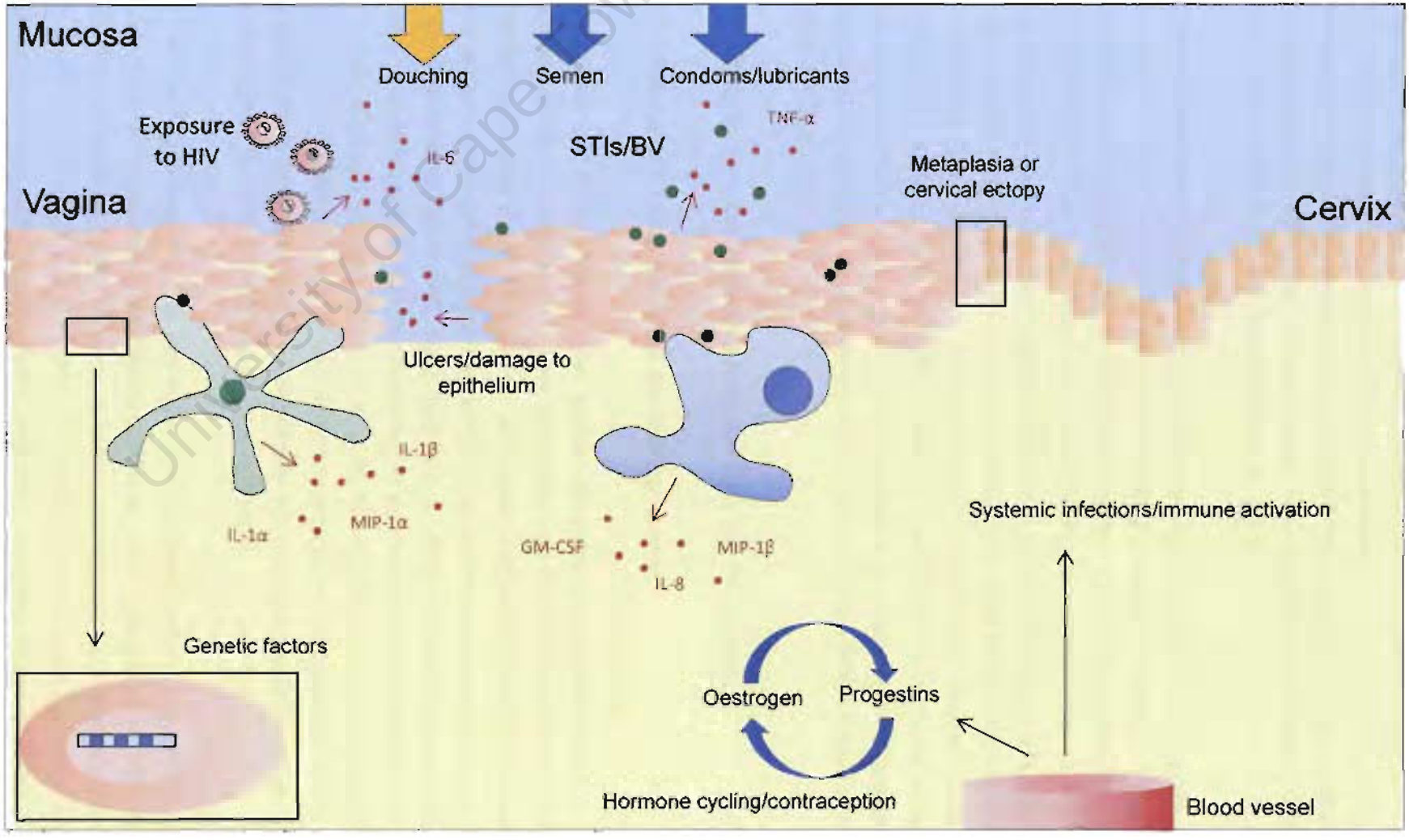


Figure 1.5 Potential causes of inflammation in the female genital tract. Sexually transmitted infections (STIs) and bacterial vaginosis (BV) are associated with increased genital inflammatory cytokine concentrations. Ulcers or microabrasions that are associated with STIs or sexual intercourse may result in inflammatory cytokine induction. Exposure to irritants such as antiseptic douching, seminal plasma proteins, condoms or lubricants have been correlated with increased genital inflammation. Cytological changes at the cervical transformation zone (metaplasia or cervical ectopy), hormone cycling or hormone contraceptive use are associated with changes in local inflammatory cytokine production. Differences in cytokine gene copy number or allelic distribution have been reported within or between populations. Systemic infections or immune activation may influence genital inflammation. Exposure to HIV itself may cause inflammatory responses by binding of HIV-encoded ligands to pattern recognition receptors expressed on immune and non-immune cells in the genital tract.

and recruitment and activation of monocytes and neutrophils (Critchley *et al.*, 2001; Bengtsson *et al.*, 2004). Cytological alterations of the cervix occur as a result of hormone changes and are associated with higher cervicovaginal IL-1β and IL-6 concentrations (Zara *et al.*, 2008). Progesterone-based injectable hormone contraceptive use is associated with increased numbers of inflammatory cells in cervicovaginal fluid, while oral contraceptive use is associated with increased CCR5 expression on CD4+ T cells (Ghanem *et al.*, 2005; Prakash *et al.*, 2001).

Several studies have suggested that demographic or geographic characteristics of women may influence the inflammatory environment in the female genital tract (Gonzalez *et al.*, 2005; Ryckman *et al.*, 2008; Zabaleta *et al.*, 2008). Counter to the increased incidence and prevalence of HIV-1 infection in sub-Saharan Africa, some studies have found that African populations have a higher copy number of CCL3L1, which encodes macrophage inflammatory protein (MIP)-1α and this has been associated with reduced risk of HIV-1 infection (Gonzalez *et al.*, 2005; Field *et al.*, 2009; Liu *et al.*, 2010). White women with normal vaginal flora have been found to have higher cervical concentrations of pro-inflammatory IL-1α and IL-6, anti-inflammatory IL-10 and PDGF-BB compared to black women with normal vaginal flora (Ryckman *et al.*, 2008). Zabaleta *et al.* (2008) similarly reported that the allelic distribution at commonly assayed sites in IL-1β, IL-10 and TNF-α differed significantly between white and black populations.

1.7 Other factors that influence susceptibility to HIV-1 infection

1.7.1 Plasma HIV-1 viral load in the transmitting partner

Blood viral load of the HIV-1-infected partner has consistently been reported to be a significant predictor of HIV-1 transmission. Higher viral loads (>50,000 RNA copies/ml) in the HIV-1-infected partner are associated with transmission, while lower viral loads (<3,500 RNA copies/ml) or effective use of highly active antiretroviral therapy (HAART) by the infected partner are associated with a lower risk of transmission (Quinn *et al.*, 2000; Donnell *et al.*, 2010). Several studies have demonstrated that blood viral loads correlate with HIV-1 shedding in both female genital secretions and semen (Hart *et al.*, 1999; Kovacs *et al.*, 2001; Gumbi *et al.*, 2008; Kalichman *et al.*, 2008), although a few individuals have high viral loads in the genital tract but undetectable blood viral loads (Kovacs *et al.*, 2001). HIV-1 transmission is more likely during acute infection and immunosuppression due to higher viral loads and increased risk of HIV-1 shedding (Ghys *et al.*, 1997).

It has been suggested that STIs may impair immune control of HIV-1, increasing viral load and the associated risk of secondary transmission (Kaul *et al.*, 2002; Schacker *et al.*, 2002; Rebbapragada *et al.*, 2007; Sheth *et al.*, 2008). However, as immunocompromised HIV-1-infected individuals are more susceptible to STIs (Ghys *et al.*, 1997), it is difficult to define cause and effect. Localized cervical gonorrhoea is associated with increased blood HIV-1 viral load, decreased CD4+ T cell counts and increased HIV-1 shedding (Ghys *et al.*, 1997; Mostad *et al.*, 1997; Rotchford *et al.*, 2000; Anzala *et*

al., 2000; McClelland *et al.,* 2001; Nkengasong *et al.,* 2001). CD4+ T cells from individuals who have gonorrhoea have reduced activation and proliferation capabilities compared to CD4+ cells from individuals who do not have gonorrhoea, while gonorrhoea is also associated with reduced CD8+ T cell responses to HIV-1 (Boulton and Gray-Owen, 2002; Kaul *et al.*, 2002). Similarly, HIV-1-infected individuals who have chronic HSV-2 infections have higher blood HIV-1 viral loads, systemic inflammation and reduced HIV-1-specific CD8+ T cell responses (Schacker *et al.,* 2002; Sheth *et al.,* 2008). Furthermore, HSV-2 and HIV-1 shedding are correlated, even in the absence of ulceration (Rebbapragada *et al.,* 2007).

1.7.2 The mucosal epithelial barrier

In the female genital tract, intact epithelia and mucus are protective against HIV-1 infection (Voeller and Anderson, 1992; Coombs *et al.*, 2003). Genital ulcerative diseases such as syphilis and HSV-2 increase susceptibility to HIV-1 infection by providing HIV-1 with access to the submucosa and target cells that are present beneath the epithelial layer and increase the infectiousness of an HIV-1-infected individual by facilitating HIV-1 shedding at the genital mucosa (Plummer and Ndinya-Achola, 1990; Ghys *et al.,* 1997; Gadkari *et al.,* 1998; Kreiss *et al.,* 1989; Celum, 2004; Cohen, 2004; Rebbapragada *et al.,* 2007). It has been suggested that micro-ulceration caused by gonorrhoea or chlamydia infections and punctate mucosal haemorrhages caused by trichomoniasis may also increase HIV-1 transmission (Coombs *et al.,* 2003; Fouts and Kraus, 1980; Rebbapragada and Kaul, 2007; Kilmarx *et al.,* 2001; Rotchford *et al.,* 2000).

Microabrasions in the genital tract may also be caused by sexual activity (Hirbod and Broliden, 2007; Norvell *et al.*, 1984; Guimaraes *et al.*, 1997). Differences in sex hormone levels (Lederman *et al.,* 2006; Marx *et al.,* 1996; Patton *et al.,* 2000), pregnancy, age, or the use of oral contraceptives, intrauterine devices or detergent-based microbicides influence the status of the mucosal epithelia (Hirbod and Broliden, 2007; Iqbal and Kaul, 2008). Oestrogen may reduce the risk of HIV-1 infection by stimulating proliferation and maturation of stratified squamous epithelium in the genital tract and mucin production by endocervical columnar cells (Galand *et al.*, 1971; Haas *et al.*, 1987). Injectable progesterone use, however, is associated with thinned vaginal epithelium and increased susceptibility to SIV infection in primates (Marx *et al.,* 1996). Cervical ectopy, which occurs following menarche, in most adolescents, in parous women and during gestation (Jacobson *et al.*, 1999; Wright and Ferenczy, 2002a), and metaplasia are associated with submucosal inflammation, epithelial erosions and shallow ulcerations (Wright and Ferenczy, 2002b; Coombs *et al.*, 2003). The cervical mucus plug, which functions as a physical barrier to invading pathogens, is absent during ovulation and menstruation (Hirbod and Broliden, 2007).

1.7.3 Changes in vaginal pH

BV occurs when naturally resident vaginal *Lactobacilli* species, particularly those that produce hydrogen peroxide, are replaced by *Gardenella vaginalis*, anaerobic and Gram-negative bacteria and mycoplasmas (Amsel *et al.,* 1983; Eschenbach *et al.,* 1988; Hillier, 1993). The normal acidic pH of the lower female reproductive tract has been found to restrict replication of some strains of HIV-1 (Voeller and Anderson, 1992), however others, particularly non-clade B viruses, have shown enhanced infectivity at low pH (Connor, 2006). Reduced local production of hydrogen peroxide and lactic acid and the subsequent increase in vaginal pH has been suggested to be one of the main reasons for increased HIV-1 transmission in women with BV (Hillier *et al.,* 1998; Klebanoff and Coombs, 1991; Schwebke, 2005). In addition to BV, semen is associated with neutralization of the vaginal pH (Tevi-Benissan *et al.*, 1997).

1.7.4 Antimicrobial peptides

Numerous antimicrobial peptides and proteins are released into the mucosal fluid and have been found to have anti-HIV-1 activity. These include β-chemokines, calprotectin, Secretory Leukocyte Protease Inhibitor (SLPI), trappin-2, lysozome, lactoferrin, defensins, cathelicidin, trappin-2, histones (reviewed by Hirbod and Broliden, 2007), integrins (Davidson and Douglas, 1998) and protegrins (Tam *et al.,* 2000; Kaul *et al.*, 2011). However, many of the studies which identified these factors as potentially protective against HIV-1 infection focused on highly HIV-1-exposed persistently seronegative women (ESN), and possible confounding factors in these studies may not have been considered. Kaul *et al.* (2011) suggested that, because these women often have multiple partners and frequent sexual activity, they are likely to be exposed to semen more often, are more likely to have microabrasions caused by sexual activity and more likely to have STIs, which are associated with changes in the inflammatory environment and/or expression of antimicrobial peptides, compared to HIV-uninfected low-risk women. *In vitro* studies of antimicrobial peptides or cytokine functions are confounded by the fact that many of these molecules have multiple functions in the immune response *in vivo* and their production is regulated by a complex cascade of events. Therefore, each peptide or cytokine may influence HIV-1 replication in more than one way and, although they may inhibit HIV-1 replication *in vitro*, their production may be accompanied by up-regulation of other factors or recruitment of cells that may facilitate HIV-1 replication *in vivo*. For example, the β-chemokines, which are detected at higher concentrations in the genital tracts of ESN women compared to HIV-uninfected low-risk women, bind to CCR5, the co-receptor for R5 HIV-1 isolates, and competitively inhibit HIV-1 binding and infection of a number of cell lines *in vitro* (Cocchi *et al.,* 1995; Gonzalez *et al.*, 2005; Iqbal *et al.*, 2005; Hirbod *et al.*, 2006; Novak *et al.*, 2007). However, these chemokines also recruit target cells for HIV-1 replication *in vivo* (Taub *et al.*, 1993a; Swingler *et al.,* 1999; Wira *et al.*, 2005). ESN women may have higher concentrations of β-chemokines in their genital tracts compared to HIV-uninfected low-risk women because they are more likely to have STIs, which are associated with elevated RANTES concentrations (Rebbapragada *et al.*, 2007; Baltzer, *et al.*, 2009). Macaque studies

have shown that MIP-1α and MIP-1β production in the genital mucosa correlate with influx of DCs and CD4+ T cells that are necessary to establish SIV infection (Li *et al.*, 2009).

Other cytokines that are thought to be associated with protection against HIV-1 infection include the interferons (IFN). Concentrations of IFN-α are elevated in the genital tracts of ESN individuals compared to HIV-uninfected low-risk women (Hirbod *et al.*, 2006). Additionally, IFN-α, IFN-β and IFN-γ induce several proteins that have anti-HIV-1 properties, including apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G), 2',5'-oligoadenylate synthetase, and nitric oxide synthase (Karupiah *et al.*, 1993; Stark *et al.*, 1998; Rebouillat and Hovanessian, 1999; Chen *et al.*, 2006). APOBEC3G is an intracellular host factor that interferes with the early phase of HIV-1 infection (Mangeat *et al.*, 2003). Large amounts of APOBEC3G have been found in cervical biopsies of ESN (Biasin *et al.*, 2007). IFN-α can also stimulate antigen cross presentation between cytotoxic T lymphocytes (CTLs) and DCs (Le Bon *et al.*, 2003). IFN-γ, like IFN-α, is associated with natural resistance to HIV-1 infection (Montoya *et al.*, 2006). Other studies have however shown that IFN-α plays a role in continued CD4+ T cell loss during HIV-1 infection (Herbeuval *et al.*, 2005) and IFN-γ induces HIV-1 replication *in vitro* (Koyanagi *et al.*, 1988; Han *et al.*, 1996).

1.8 Microbicides for preventing HIV-1 transmission to women

Due to biological and cultural factors, women are more vulnerable to HIV-1 infection than men (Heise and Elias, 1995; Hirbod and Broliden, 2007). In sub-Saharan Africa, approximately 60% of HIV-1-infected individuals are women, and among 15 to 24-year olds, women are up to eight times more likely to be HIV-1-infected than men (UNAIDS, 2010). Currently, topical microbicide gels and oral antiretroviral pre-exposure prophylaxis (PrEP) are being explored for prevention of sexual transmission of HIV-1 (Arien *et al.*, 2011). The potential for effective oral PrEP was recently demonstrated by the findings of the phase III Pre-exposure Prophylaxis Initiative (iPrEx) trial that once daily oral Truvada™ (emtricitabine and TFV disoproxil fumerate) reduced HIV-1 incidence among men and transgender women who have sex with men by 44% compared to placebo (Grant *et al.*, 2010). Microbicides are also a promising intervention strategy for women in particular, although development of a safe and efficacious formulation has proved to be challenging (Voelker, 2006).

Earlier candidate microbicides included agents that (i) non-specifically disrupt cellular and microbial membranes (surfactants), (ii) restore the natural acidic protective pH of the vagina (acid buffers) and (iii) interfere with interactions between HIV-1 envelope proteins and cellular receptors (anionic polymers; Arien *et al.*, 2011). However, over the past 20 years, none of these candidate microbicides [Nonoxynol-9 (N-9), C31G/Savvy®, Sodium Laurel Sulfate (SLS), Ushercell/cellulose sulfate (CS), Carraguard/carrageenan and PRO-2000] demonstrated significant protection against HIV-1 in clinical trials (Figure 1.6; Abdool Karim *et al.*, 2010a; Doncel and Clark, 2010; Arien *et al.*, 2011). N-9 and CS were even found to increase the risk of HIV-1 infection by disrupting the vaginal epithelial barrier and by inducing inflammatory cytokine responses (Rustomjee *et al.*, 1999; Fichorova *et al.*, 2001b;

Figure 1.6 Overview of phase II and III microbicide and oral pre-exposure prophylaxis (PrEP) clinical trials (source: Arien *et al.*, 2011)

Mesquita *et al.*, 2009). N-9, a non-ionic surfactant that was commonly included in spermicides and some vaginal lubricants, was shown to disrupt the HIV-1 lipid membrane and inactivate the virus *in vitro* (Klasse *et al.*, 2008). However, multiple applications of N-9 were shown to cause inflammatory cytokine (IL-1α, IL-1β, IL-8 and MIP-1β) up-regulation and NF-κB activation in the genital tract,

resulting in influx of CD68+ macrophages and, as a result, increased HIV-1 replication in infected cells (Fichorova *et al.*, 2001b). N-9 also disrupted the phospholipid membrane of cells and caused non-specific damage to the vaginal epithelium and uterine and cervical tissue (Hillier *et al.,* 2005; Van Damme, 2004; Hoffman *et al.,* 2004). N-9 was further associated with reduced concentrations of SLPI (Fichorova *et al.,* 2001b) and altered vaginal flora (Klebanoff, 1992). CS, a polyanionic microbicide candidate, was found to prevent infection by HIV-1 and a number of other STIs *in vitro* (Christensen *et al.*, 2001; Simoes *et al.*, 2002; Balzarini and van Damme, 2007). However, CS induced NF-κB activity in peripheral blood mononucleocytes (PBMCs), and IL-1α and IL-6 up-regulation in cervical epithelial cell culture supernatants following treatment (Mesquita *et al.*, 2009), although inflammatory cytokine levels were not elevated in cervicovaginal lavage (CVL) from women applying CS (Schwartz *et al.*, 2006). C31G/Savvy®, another surfactant microbicide which showed the ability to block HIV-1 infection in pre-clinical studies, entered phase III clinical trials, but efficacy was not demonstrated because the rate of HIV-1 infection in trial participants was low and the trial was discontinued (Klasse *et al.*, 2008). Analysis of 12 month follow-up data found a trend toward a higher rate of HIV-1 infection in participants using Savvy compared to placebo users, however this was not significant (Feldblum *et al.*, 2008). The surfactant SLS was shown to disrupt enveloped and non-enveloped viruses (Piret *et al.*, 2002). Although SLS was well-tolerated in phase I and II clinical trials, efficacy trials have not been conducted and are not foreseen (Mbopi-Keou *et al.*, 2009; Mbopi-Keou *et al.*, 2010; Arien *et al.*, 2011).

The acidic pH of the lower female genital tract has been found to inhibit HIV-1 replication (Voeller and Anderson, 1992). However, semen and BV can neutralize the pH, increasing susceptibility to HIV-1 transmission (Tevi-Benissan *et al.*, 1997; Hillier *et al.,* 1998; Klebanoff and Coombs, 1991; Schwebke, 2005). Acid buffers, such as BufferGel and Acidform, act as direct acidifying agents. Although BufferGel was shown to have activity against HIV-1, HSV-1 and 2 *in vitro* and *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in mice (Croughan and Behbehani, 1988; Ongradi *et al.*, 1990; Achilles *et al.*, 2002; Spencer *et al.*, 2004), it had no effect on rate of HIV-1 infection in a phase II/IIb clinical trial (Abdool Karim *et al.*, 2011). Acidform is a vaginal lubricant gel with acid buffering properties. Although a phase I clinical trial found no adverse events in women using this gel (Williams *et al.*, 2007), other studies have reported that use of Acidform was associated with mild to moderate vaginal irritation and increased genitourinary symptoms (Amaral *et al.*, 2006; Behets *et al.*, 2008). The use of exogenous lactobacilli is being explored as a method to maintain or restore acidic vaginal pH and prevent BV (Antonio and Hillier, 2003; Senok *et al.*, 2009). Additionally, lactobacilli are being bioengineered to produce antiviral proteins (Chang *et al.*, 2003; Rao *et al.*, 2005; Liu *et al.*, 2007).

PRO-2000 is a polyanionic naphthalene sulphonate polymer that targets the adsorption and fusion process of X4 and R5 HIV-1 isolates (Neurath *et al.*, 2002). PRO-2000 prevented *in vitro* HIV-1 infection of T lymphocytes and macrophages, as well as vaginal infection of macaques by a hybrid simian-human immunodeficiency virus (SHIV-1) (Rusconi *et al.*, 1996; Lewis *et al.*, 2001). PRO-2000 was also shown to be effective at preventing infection by HSV-2 and *N. gonorrhoeae* (Spencer *et al.*,

2004; Keller *et al.*, 2006). Phase I and II safety studies demonstrated that low dose formulations of PRO-2000 (0.5% and 2.0%) were safe, well-tolerated and acceptable (Mayer *et al.*, 2003; Kamali *et al.*, 2010). Although PRO-2000 use was associated with a 30% reduction in the risk of HIV-1 infection in one effectiveness trial, this was not statistically significant (Abdool Karim, 2010b) and findings of a larger study revealed that PRO-2000 had no protective effect (McCormack *et al.*, 2010). Carraguard/carrageenan, which contains sulfated polysaccharides, was shown to inhibit *in vitro* infection of cervical epithelial cells by HIV-1 and trafficking of macrophages from the vagina to the lymph nodes (Pauwels and De Clercq, 1996; Perotti *et al.*, 2003). No adverse events were noted in Phase I and II trials (Elias *et al.,* 1997; Kilmarx *et al.,* 2006; Bollen *et al.*, 2008), but results of the Phase III trial showed no protective effect of Carraguard compared to placebo (Arien *et al.*, 2011).

The focus of microbicide development has now largely shifted from polyanions, surfactants and acid buffers to gels including specific antiretroviral compounds. Among these are entry/fusion inhibitors, reverse transcriptase inhibitors and integrase inhibitors. Many entry/fusion inhibitors, which specifically inhibit HIV-1 entry into target cells, are currently in preclinical development and have been shown to be protective in the SHIV-1-macaque model (Klasse *et al.*, 2008; Arien *et al.*, 2011). These include proteins that inhibit HIV-1 envelope glycoprotein binding to target cell CD4 molecules and co-receptors, CCR5 and CXCR4, and subsequent fusion of viral and target cell membranes (Klasse *et al.*, 2008). Inhibitors can either target host cell receptors or the virus itself. RANTES analogues that aim to block HIV-1 binding to CCR5, while lacking cellular activation and recruitment activity, are under development (Kawamura *et al.*, 2004; Lederman *et al.*, 2004; Cerini *et al.*, 2008; Gaertner *et al.*, 2008; Veazey *et al.*, 2009). Vaginal application of the CCR5 antagonist, Maraviroc, has been shown to protect against local SHIV-1 challenge in macaques (Veazey *et al.*, 2010). Neutralizing monoclonal antibodies (NMAbs) and ligands that bind to CD4 have also been considered as microbicides, although few studies have been conducted (Klasse *et al.*, 2008). NMAbs and small molecule inhibitors that bind to the CD4-binding site on HIV-1 envelope glycoprotein have shown activity against HIV-1 *in vitro* and have been shown to protect macaques against vaginal and rectal SHIV-1 challenge (Veazey *et al.*, 2003b; Tsai *et al.*, 2004; Veazey *et al.*, 2005; Klasse *et al.*, 2008). Integrase inhibitors, which prevent HIV-1 integration, also hold potential as microbicides and have been shown to inhibit HIV-1 infection of DC and CD4+ T cell cultures by cell-free and cell-associated virus (Terrazas-Aranda *et al.*, 2008).

Dianilinopyrimidine derivative TMC-120 (Dapivirine) and UC-781 are nonnucleoside reverse transcriptase inhibitors (NNRTIs) that have been shown to inhibit infection by both cell-free and cell-asssociated HIV-1 (Balzarini *et al.*, 1998; van Herrewege *et al.*, 2004; van Herrewege *et al.*, 2007). Both Dapivirine and UC-781 have anti-HIV-1 activity in DC and CD4+ T cell cultures and Dapivirine blocks HIV-1 infection of sub-epithelial DCs and CD4+ T cells when applied to the apical side in a dual chamber model with a confluent epithelial cell layer (van Herrewege *et al.*, 2004; van Herrewege *et al.*, 2007). A phase I study found that vaginal application of a microbicide containing Dapivirine was safe and well-tolerated (Jespers *et al.*, 2007). Dapivirine loaded into a long-term controlled release

intravaginal ring device is currently being evaluated in a phase II safety trial and the phase III trial is expected to begin this year. A combination gel including MIV-150, an NNRTI, and zinc acetate was shown to be protective against vaginal SHIV-1 infection in macaques (Kenney *et al.*, 2011).

Tenofovir (TFV) disoproxyl fumarate, an adenosine nucleotide analogue that inhibits HIV-1 reverse transcriptase, is widely used in systemic, oral regimens for HIV-1 treatment (Balzarini and Van Damme, 2007). TFV was selected for inclusion in a microbicide gel formulation because not only is this drug efficacious, but it also has a good safety profile, a long half life and HIV-1 does not easily develop mutations against TFV (Barditch-Crovo *et al.*, 2001; Rohan *et al.*, 2010). In contrast to N-9 and CS, treatment of cervical epithelial cell cultures with TFV gel did not result in up-regulation of inflammatory cytokine production and was not associated with NF-κB activation (Mesquita *et al.*, 2009). Furthermore, daily TFV application for 2 weeks was not associated with elevated CVL inflammatory cytokine concentrations (Keller *et al.*, 2010). TFV gel is the first microbicide to confer partial protection against HIV-1 infection. A recent phase IIb clinical trial conducted in KwaZulu-Natal in South Africa demonstrated that a microbicide gel containing 1% TFV prevented 39% of male-to-female sexual transmission of HIV-1 (Abdool Karim *et al.*, 2010a). Currently, the efficacy of daily 1% TFV gel application is being compared to daily oral TFV and daily oral Truvada™ use in the VOICE trial (Minces and McGowan, 2010; Ramjee, 2010). Additionally, delivery of combination anti-retroviral drugs such as TFV and Dapivirine by long-term controlled release intravaginal ring devices is under investigation (Johnson *et al.*, 2010b).

1.9 Management and treatment of STIs as a strategy to reduce rates of HIV-1 infection

Several studies have demonstrated that a number of common STIs are associated with increased susceptibility to HIV-1 infection and increased infectiousness of HIV-1-infected individuals (Stamm *et al.*, 1998; Plummer *et al.*, 1991; Wasserheit *et al.*, 1992; Laga *et al.*, 1993; Cohen, 1998; Cohen *et al.*, 1997; McClelland *et al.*, 2001; Ramjee *et al.*, 2005). Therefore, STIs have likely played a central role in facilitating the spread of HIV-1, and STI management is a key issue for HIV-1 prevention in countries where both HIV-1 and other STIs are prevalent. Currently, STIs are managed syndromically in developing countries including South Africa, on the basis of STI-associated signs and symptoms (World Health Organization, 2003). However, the non-specific nature of the STI syndromes that form the basis for diagnosis and the fact that STIs are commonly asymptomatic, result in over- and under-treatment (Pettifor *et al.*, 2000; Behets *et al.*, 2001; Desai *et al.*, 2003; Pepin *et al.*, 2004; Bogaerts *et al.*, 1999). In South Africa, asymptomatic infections occur in almost 50% of STI-infected women who do not seek healthcare and are therefore untreated (Wilkinson *et al.*, 1999).

Several studies have investigated the effects of different strategies of STI management on reducing the risk of HIV-1 infection. Population-wide treatment of bacterial infections and therapy for HSV-2 were found to be ineffective at reducing HIV-1 infection (Wawer *et al.*, 1999; Kaul *et al.*, 2004; Gray *et al.*, 2001; Celum *et al.*, 2008; Watson-Jones *et al.*, 2008). Although HSV-2 therapy may suppress reactivation, it has been shown that higher numbers of DC-SIGN+ DCs and CCR5+ CD4+ T cells are

observed in the genital tracts of women who have HSV-2, even in the absence of HSV-2 shedding or genital ulceration (Rebbapragada *et al.*, 2007). Therefore, although suppressive therapy may reduce genital ulceration, HSV-2 may induce a persistent state of susceptibility to HIV-1 infection because of the ongoing inflammation it causes. Periodic mass treatment of bacterial STIs in Rakai, Uganda, resulted in a modest decline in the prevalence of some STIs, however all STIs and BV were persistent and the prevalence of symptoms of STIs was similar in both the treatment and control groups (Wawer *et al.*, 1999). The persistence of BV/STIs may have been due to recurrence (Sweet, 1993) or re-infection by partners outside of the intervention. Monthly antibiotic treatment of bacterial STIs in Kenya resulted in a more substantial reduction in the prevalence of STIs, but did not influence HIV-1 acquisition. HIV-1 infection was found to be associated with preceding *N. gonorrhoea* and *C. trachomatis* infections (Kaul *et al.*, 2004). Treatment for HSV-2, which was very prevalent in the participants of these studies, was not included in either the Uganda or the Kenya interventions.

Two of three STI syndromic management interventions, which were implemented in Tanzania, Uganda and Zimbabwe, resulted in no change in HIV-1 acquisition (Grosskurth *et al.*, 1995; Grosskurth *et al.*, 2000; Kamali *et al.*, 2003; Gregson *et al.*, 2007), suggesting that asymptomatic infections may play a role. In addition to causing visible clinical symptoms, such as genital ulceration, which increase susceptibility to HIV-1 infection, certain STIs may be associated with manifestations that may be subclinical, including up-regulated genital tract inflammatory cytokine responses (Bogaerts *et al.*, 1999; Wiesenfeld *et al.*, 2002). In Mwanza, Tanzania, 12 537 individuals from 12 communities were assessed for STIs at baseline and at follow-up 2 years later. Six communities served as the comparison group, while the remaining 6 were included in the intervention group. The intervention included training of existing staff from local healthcare facilities in syndromic diagnosis and treatment of STIs, regular supply of drugs, regular supervisor visits and health education about STIs. At follow-up it was found that the rate of HIV-1 infection was significantly lower in the intervention group, compared to the comparison group (risk ratio: 0.58; Grosskurth *et al.*, 1995). Although the prevalence of newly-diagnosed syphilis and male urethritis were lower in the intervention group in Tanzania, there were no differences in chlamydia and gonorrhoea infection rates among men and STIs among pregnant women were unchanged (Wawer *et al.*, 1999). In Masaka, Uganda, 96 000 individuals were recruited from 18 communities and followed between 1994 and 2000. The intervention in Uganda included training of health care workers in syndromic management of STIs, regular supply of drugs, supervision and health education about STIs. Although syndromic management reduced the incidence of active syphilis and gonorrhoea in study participants, the incidence of chlamydia, genital ulcers, discharge and HIV-1 were unchanged (Kamali *et al.,* 2003). Similarly, in Zimbabwe, implementation of syndromic management of STIs was not associated with reduced incidence of self-reported STI symptoms or HIV-1 incidence (Gregson *et al.*, 2007). This study included 11 980 individuals in 12 communities, with an intervention comprised of health education and strengthened syndromic management services at local health centres.

The conflicting results of these studies may be due to characteristics of the populations in which the studies were carried out. The prevalence of HIV-1 in Tanzania was lower than the prevalence in Uganda, therefore the proportion of new HIV-1 infections attributable to STIs may be lower in a more mature population, such as Uganda. Additionally, the higher prevalence of HSV-2 in Uganda may have reduced the effect of STI treatment since acyclovir was not included in the treatment regimen (Grosskurth *et al.*, 2000; Kamali *et al.*, 2003). More intensive STI interventions are likely to be necessary to achieve a more substantial reduction in STI and HIV-1 infection rates, perhaps integration of both syndromic management strategies and mass treatment targeting both bacterial and viral STIs.

1.10 HIV-1 disease progression

Rates of HIV-1 disease progression vary substantially among HIV-1-infected individuals (Haynes *et al.*, 1996) and progression has typically been classified as rapid, typical or slow (Fauci and Lane, 2005; Pantaleo and Fauci, 1996). Most infected individuals (70-80%) exhibit typical progression and develop AIDS-related illnesses within 6-10 years of infection. Roughly 5% of infected individuals are slow progressors, who are able to naturally suppress viral loads, maintain normal peripheral CD4+ T cell numbers and immune functions, and remain clinically healthy for more than 10 years (Guadalupe *et al.*, 2003; Paroli *et al.*, 2001; Valdez *et al.*, 2002). In contrast, rapid progressors (10-15%) experience fast CD4+ T cell decline and high viral loads and, in the absence of HAART, develop AIDS within a few years of infection (Pantaleo and Fauci, 1996). The US Department of Health and Human Services recommends commencement of HAART treatment based on CD4+ T cell counts (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2011). Although high viral loads and low CD4 counts are associated with rapid progression to AIDS, the factors which influence these changes are poorly understood (Goujard *et al.*, 2006; Mellors *et al.*, 2007; de Wolf *et al.*, 1997; Lepri *et al.*, 1998).

1.10.1 Immune activation and HIV-1 disease progression

Widespread activation of the immune system occurs during HIV-1 infection and is associated with subsequent disease progression (Liu *et al.*, 1997; Giorgi *et al.*, 1999; Geiss *et al.*, 2000; Paroli *et al.*, 2001; Hazenberg *et al.*, 2003; Deeks and Walker, 2004; Deeks *et al.*, 2004; Hunt *et al.*, 2008). During acute HIV-1 infection, peak viraemia is reached and is accompanied by high levels of immune activation and CD4+ T cell depletion, particularly from the GI tract (Guadalupe *et al.*, 2003; Brenchley *et al.*, 2004; Mehandru *et al.*, 2004; Stacey *et al.*, 2009). Although viral load declines following acute HIV-1 infection, immune activation persists, viral replication continues and CD4+ T cells are progressively lost (Hazenberg *et al.*, 2003; Deeks *et al.*, 2004). The acute phase of SIV infection in natural primate hosts (African Green Monkeys and Sooty Mangabeys) is also accompanied by high levels of systemic immune activation, however, unlike HIV-1 and pathogenic SIV infections, this is rapidly attenuated during chronic infection, despite high viral loads (Gordon *et al.*, 2007; Estes *et al.*, 2008).

Increased circulating concentrations of pro-inflammatory cytokines and activation and proliferation of most immune cells in blood, including T cells, B cells, NK cells and macrophages, have been described at various stages of HIV-1 infection (Lane *et al.*, 1983; Alter *et al.*, 2004; Brenchley *et al.*, 2006; Norris *et al.*, 2006; Bebell *et al.*, 2008; Stacey *et al.*, 2009). Immune activation is thought to be the product of inflammatory responses to HIV-1-encoded TLR ligands, the homeostatic response to CD4+ T cell depletion and microbial translocation from the GI tract (Deeks *et al.*, 2004; Brenchley *et al.*, 2006; Meier *et al.*, 2007; Centlivre *et al.*, 2007; Catalfamo *et al.*, 2008). Additionally, reactivation of latent viral infections such as Epstein Bar Virus, Cytomegalovirus and HSV-2 may contribute to immune activation (Doisne *et al.*, 2004). HIV-1 single stranded RNA encodes several ligands which bind to TLR7/8 and activate DCs, monocytes and bystander T cells (Meier *et al.*, 2007). CD4+ T cell depletion induces homeostatic responses in order to replenish this population, however homeostatic cytokines may stimulate inflammatory cytokine production and contribute to immune activation (Catalfamo *et al.*, 2008; Alderson *et al.*, 1991; Damås *et al.*, 2003). During HIV-1 infection, GI tract integrity is reduced by CD4+ T cell depletion in this compartment, preferential Th17 cell loss (which are important for defence against bacteria and fungi at mucosal surfaces), enterocyte apoptosis and structural damage to the epithelial surface during HIV-1 and pathogenic SIV infections (Batman *et al.*, 1989; Sankaran *et al.*, 2005; Brenchley *et al.*, 2008; Cecchinato *et al.*, 2008). Increased GI tract permeability results in movement of microbial products from the lumen into circulation (microbial translocation) in the absence of bacterial, viral or fungal enteropathogens (Batman *et al.*, 1989; Brenchley *et al.*, 2006). CD4+ T cell depletion in the GI tract and microbial translocation also occur during the acute phase of SIV infection in natural hosts, however detection of microbial products in the systemic compartment is transient and controlled during chronic infection (Brenchley *et al.*, 2006; Gordon *et al.*, 2007; Pandrea *et al.*, 2007). Higher concentrations of microbial products in the systemic compartment have been found to be associated with activation of both innate and adaptive immune cells (Brenchley *et al.*, 2006).

1.10.2 T cell depletion

Immune activation is thought to worsen HIV-1 disease progression by providing more activated CD4+ T cell targets for HIV-1 infection, by activation-induced apoptosis of bystander T cells and by excessive proliferation and differentiation of T cells, resulting in clonal exhaustion and drainage of memory T cell pools (Hellerstein and McCune, 1997; Lin *et al.*, 1997; Liu *et al.*, 1997; Brenchley *et al.*, 2003; Hazenberg *et al.*, 2003; Picker *et al.*, 2006; Okoye *et al.*, 2007; Burgers *et al.*, 2009). The contribution of direct infection of CD4+ T cells to depletion of this population is unclear. Although activation of CD4+ T cells provides more targets for HIV-1 infection, it is estimated that HIV-1 infects only a small proportion of these cells (Douek *et al.*, 2009). Activation-induced apoptosis of bystander T cells, a process that regulates immune homeostasis by preventing continued effector functions which may otherwise be harmful, may contribute to CD4+ T cell depletion and worsen disease progression (Lin *et al.*, 1997; Brenchley *et al.*, 2003). Both CD4+ and CD8+ T cell proliferative

capacity and activation states during early and chronic HIV-1 infection are predictive of disease progression (Liu *et al.*, 1997; Giorgi *et al.*, 1999; Hazenberg *et al.*, 2003; Deeks *et al.*, 2004; Burgers *et al.*, 2009; Scriba *et al.,* 2005; Wilson *et al.,* 2004). Immune activation may induce extensive proliferation and differentiation of memory T cells, resulting in premature exhaustion of critical populations (Okoye *et al.*, 2007). It has been suggested that the balance between CD8+ central memory T cells (T_{CM}) and effector memory T cells (T_{EM}) may play an important role in HIV-1 disease progression (Okoye *et al.*, 2007; Burgers *et al.*, 2009; Douek *et al.*, 2009). Higher proportions of CD8+ T_{CM} during early HIV-1 infection are associated with lower viral load set-points, while higher proportions of CD8+ T_{EM} correlate with rapid disease progression (Burgers *et al.*, 2009). T_{CM}, which are a "self-renewing" source for T_{EM}, home to lymph nodes and have comparable cytotoxic abilities to T_{EM} following antigen stimulation (Barber *et al.*, 2003; Sallusto *et al.*, 2004). T_{EM} home to effector sites and serve as the primary targets for HIV-1 infection and destruction (Sallusto *et al.*, 2004). Although these cells have ready effector functions, they are considered terminally differentiated and die following restimulation (Seder and Ahmed, 2003; Riou *et al.*, 2007). It has been suggested that T_{CM} may be more effective at mediating protective immunity because they are long-lived and able to proliferate (Barber *et al.*, 2003; Burgers *et al.*, 2009).

1.10.3 Soluble biomarkers of immune activation

Concentrations of soluble biomarkers of immune activation during chronic HIV-1 infection predict progression to AIDS and/or CD4 decline with a degree of accuracy comparable to that of CD4+ T cell counts and viral load measurements (Mellors *et al.*, 1997; Fahey *et al.*, 1998; Zangerle *et al.*, 1998). Elevated concentrations of neopterin, β2-microglobulin, TNF type II receptor, TNF receptor 75 and soluble IL-2 receptor α-chain (sIL-2Rα) are associated with more rapid disease progression (Fuchs *et al.*, 1988; Hofmann *et al.*, 1989; Honda *et al.*, 1989; Melmed *et al.*, 1989; Fahey *et al.*, 1990; Tsoukas and Bernard, 1994; Stein *et al.,* 1997; Zangerle *et al.,* 1998). Neopterin is a product of macrophages stimulated by IFN-γ (Huber *et al.*, 1984), while β2-microglobulin indicates activation of many immune cells but is not the result of specific cytokine activity (Hofmann *et al.,* 1989; Tsoukas and Bernard, 1994). Soluble TNF receptor concentrations are indicative of HIV-1-specific immune activity, particularly TNF-α (Aukrust *et al.,* 1994; Godfried *et al.,* 1994), whereas sIL-2Rα reflects IL-2 stimulation (Rubin *et al.,* 1985). Although some studies found no correlation between neopterin, β2-microglobulin, or soluble TNF receptors and disease progression (O'Brien *et al.,* 1996; Stein *et al.,* 1997), it was demonstrated that a combination of a single plasma activation marker with CD4+ T cell levels improved the prognostic capability of each. Fahey *et al.* (1998) further found that combination of a plasma activation marker, instead of plasma HIV-1 viral load, with CD4+ T cell count was a more accurate predictor of disease progression.

1.10.4 Circulating cytokines during HIV-1 infection

Several cross-sectional studies have investigated plasma and serum cytokine concentrations during acute HIV-1 infection. It has been shown that the concentrations of IL-2 (Sinicco *et al.*, 1993) and IL-12 (Norris *et al.*, 2006) in plasma or serum are reduced during acute HIV-1 infection, while IFN-α, IFN-γ, IL-1β, TNF-α, IFN-γ-induced protein (IP-10), RANTES, MIP-1α, MIP-1β and IL-10 are elevated relative to HIV-uninfected individuals (Biglino *et al.*, 1996; Norris *et al.*, 2006; Sinicco *et al.*, 1993; von Sydow *et al.*, 1991; Rizzardi *et al.*, 1996; Roe *et al.*, 2007; Ullum *et al.*, 1998). No changes in IL-4, IL-6 or IL-13 concentrations have been observed during acute infection (Biglino *et al.*, 1996; Sinicco *et al.*, 1993; Norris *et al.*, 2006). In contrast, Biglino *et al.* (1996) found slightly reduced TNF-α concentrations and no changes in IL-1β and IL-2 during acute HIV-1 infection. Barcellini *et al.* (1996) found no differences in acute HIV-1 infection IL-10 concentrations and Sinicco *et al.* (1993) observed no changes in TNF-α relative to HIV-uninfected controls. These differences most likely reflect the dynamic nature of cytokine production during the early stages of HIV-1 infection and the timing of sample collection in each study. Stacey *et al.* (2009) recently conducted a comprehensive analysis of the evolution of plasma cytokine concentrations during acute HIV-1 infection, and reported that a pro-inflammatory cytokine storm accompanied peak HIV-1 viraemia and was only later followed by immunoregulatory cytokine production. They reported that IL-15 and IFN-α concentrations increased rapidly, peaking at 6 and 7 days post-infection, respectively, but were only transiently elevated. A large increase in IP-10 was also observed, with this cytokine peaking at 9 days post-infection. TNF-α and MCP-1 concentrations were also rapidly elevated, but high concentrations were sustained. IL-6, IL-8, IL-18 and IFN-γ levels increased more slowly and IL-10 concentrations peaked only after HIV-1 viraemia.

Similar to acute HIV-1 infection, various studies have found that concentrations of IL-2, IL-12 and IFN-γ in plasma were reduced during chronic HIV-1 infection compared to uninfected individuals (Clerici *et al.*, 1993; Maggi *et al.*, 1994; Chehimi *et al.*, 1994; Kedzierska and Crowe, 2001), while plasma concentrations of IL-1β, IL-4, IL-6, IL-8, IL-10 and TNF-α were elevated (Reddy and Grieco, 1989; Clerici *et al.*, 1993; Kobayashi *et al.*, 1990; Lahdevirta *et al.*, 1988; Marshall *et al.*, 1999; Matsumoto *et al.*, 1993; von Sydow *et al.*, 1991).

1.10.5 Systemic cytokines and HIV-1 disease progression

Cytokines play an important role in HIV-1 disease. During HIV-1 infection, down-regulation of circulating cytokines such as IL-2, IL-12 and IFN-γ (which generally elicit strong cellular responses and control HIV-1 replication) and up-regulation of IL-4 and IL-5 and inflammatory cytokines (which reduce cellular responses and promote viral replication) are thought to be associated with HIV-1 disease progression (Clerici and Shearer, 1993; Clerici and Shearer, 1994; Connolly *et al.*, 2005; Macias *et al.*, 2001; Okamoto *et al.*, 1989; Poli *et al.*, 1994). IFN-γ production during HIV-1 infection has been identified as a correlate of better disease prognosis, and was positively associated with

CD8+ T cell and activated NK cell counts (Ullum *et al.*, 1997; Bailer *et al.*, 1999). In SIV-infected macaques, IL-12p70 treatment during acute infection was associated with decreased viral loads, increased IFN-γ production, increased CD8+ NK and T cells, reduced naïve CD4+ T cells expressing homing markers, retention of HIV-1-specific CTL and prolonged survival (Ansari *et al.*, 2002). Similarly, treatment with IL-2 was associated with reduced apoptosis of T cells in HIV-1-infected individuals and improved CD4+ T cells counts (Adachi *et al.*, 1996; Sereti *et al.*, 2002; Natarajan *et al.*, 2002; Kovacs *et al.*, 1995). Deficiencies in IL-2 during HIV-1 infection have been associated with an inability of the immune system to activate antigen-specific CD8+ CTL (Clerici *et al.*, 1993).

During HIV-1-infection, inflammatory cytokines are produced in response to binding of either HIV-1-encoded ligands or microbial products to TLRs (Dinarello *et al.*, 1997; Connolly *et al.*, 2005; Wira *et al.*, 2005; Abbas and Lichtman, 2007; Mirmonsef *et al.*, 2011). Additionally, inflammatory cytokine production is induced by T cell homeostatic cytokines (Catalfamo *et al.*, 2008; Alderson *et al.*, 1991; Damås *et al.*, 2003). Therefore, individuals who have high plasma viral loads, increased GI tract permeability and CD4 depletion are likely to have high concentrations of circulating inflammatory cytokines. Although it is thought that mucosal tissues are the major sites for HIV-1 replication (Veazey *et al.*, 2003a; Picker *et al.*, 2004), elevated concentrations of inflammatory cytokines in the systemic compartment may promote HIV-1 replication by activating immune cells and inducing HIV-1 replication via NF-κB activation (Chene *et al.*, 1999; Osborne *et al.*, 1989; Poli *et al.*, 1990; Niu *et al.*, 2004). Other nuclear factors, such as CCAAT enhancer binding protein and mitogen-activated protein (MAP) kinase, which are responsive to IL-6, may increase HIV-1 replication when activated (Yang *et al.*, 1999; Lee *et al.*, 2002; Henderson and Calame, 1992; Natsuka *et al.*, 1992; Henderson *et al.*, 1996; Lee *et al.*, 2001). Certain inflammatory cytokines, such as TNF-α and IL-7, have also been shown to promote activation-induced CD4+ and CD8+ T cell apoptosis (Badley *et al.*, 1997; Lelievre *et al.*, 2005; Lin *et al.*, 1997; Zheng *et al.*, 1995).

Although some studies have reported that IL-2, IL-12 and IFN-γ reduce HIV-1 replication and slow disease progression, others have shown that these cytokines may rather increase viral replication and promote rapid disease progression. For example, even though IFN-γ is associated with strong cellular responses, this cytokine has been found to synergize with TNF-α *in vitro* in its ability to increase HIV-1 transcription (Han *et al.*, 1996). IL-2 treatment was shown to be associated with increased plasma HIV-1 viral loads (Kovacs *et al.*, 1995). Cytokines such as TNF-α and IL-1β, which are pro-inflammatory, have also been associated with CD4 and/or CCR5 down-regulation on macrophages (Bailer *et al.*, 2000; Herbein *et al.*, 1995). Although β-chemokines, RANTES, MIP-1α and MIP-1β, are pro-inflammatory and recruit target cells for HIV-1 replication (Swingler *et al.*, 1999; Lane *et al.*, 2001; Dufour *et al.*, 2002; Wira *et al.*, 2005), they also competitively inhibit HIV-1 binding to CCR5 (Cocchi *et al.*, 1995; Blanpain *et al.*, 1999). MIP-1β and RANTES concentrations measured in HIV-1-infected individuals were found to correspond to levels that inhibit HIV-1 replication *in vitro* (Cocchi *et al.*, 1995). However, only MIP-1β, and to a lesser extent MIP-1α, but not RANTES were associated with

decreased risk of disease progression (McKenzie *et al.,* 1996; Ullum *et al.,* 1998; Zanussi *et al.,* 1996).

The role of IL-10 in HIV-1 disease progression is also controversial. Although IL-10 is a regulatory cytokine that reduces HIV-1 replication in macrophages (Akridge *et al.*, 1994) and secretion of inflammatory cytokines, IL-1, IL-6, IL-8, IL-12 and TNF-α (reviewed by Moore *et al.,* 2001), this cytokine may contribute to HIV-1 persistence by suppressing effector T cell responses (D'Andrea *et al.*, 1993; Brooks *et al.*, 2006; Ejrnaes *et al.*, 2006). In support of this, it has been demonstrated that serum IL-10 levels increase with disease progression in HIV-1-infected individuals (Styliano *et al.*, 1999). Additionally, frequencies of regulatory T cells in blood, an important source of IL-10 (Couper *et al.*, 2008), were shown to correlate inversely with the magnitude of SIV-specific CTL responses during acute SIV infection, and may contribute to viral persistence (Estes *et al.*, 2006).

T cell homeostatic cytokines, IL-7 and IL-15, may also play a role in HIV-1 disease progression (Fry and Mackall, 2005; Picker *et al.*, 2006). IL-7 selectively induces proliferation of naïve T cells and T_{CM} cells and it has been proposed that high concentrations of IL-7 may disrupt the normal naïve/memory differentiation pathway by inducing memory-like characteristics on naïve cells. Exhaustion or excessive differentiation of memory T cells could reduce the longevity of this population, in addition to its ability to self-renew, expand and differentiate upon antigen-stimulation (Picker *et al.*, 2006). IL-15 induces antigen-independent proliferation and differentiation of T_{EM} from T_{CM} (Geginat *et al.*, 2001; Picker *et al.*, 2006). Thus, elevated IL-15 concentrations during early infection may accelerate the loss of T_{EM}, thereby depleting T_{CM} more rapidly. Additionally, it was demostrated that increased IL-15 concentrations during acute SIV infection led to an up-regulation of CD4 expression on memory CD4+ T cells which increased in their susceptibility to SIV infection (Eberly *et al.*, 2009).

1.10.6 Cytokines in the female genital tract during HIV-1 infection

Although the mechanisms involved in regulation of local immune responses to HIV-1 in the female genital tract are not well understood (Zara *et al.,* 2004), a number of studies have shown that inflammatory cytokines are up-regulated in women with early or chronic HIV-1 infection relative to uninfected women (Belec *et al.*, 1995; Crowley-Nowick *et al.*, 2000; Zara *et al.*, 2004; Bebell *et al.*, 2008; Guha and Chatterjee, 2009; Nkwanyana *et al.*, 2009). Bebell *et al.* (2008) found significantly higher concentrations of IL-6, IL-10 and IL-12 in cervicovaginal fluid from women with acute HIV-1 infection relative to uninfected women. Similarly, Crowley-Nowick *et al.* (2000) showed that IL-10 and IL-12 levels were elevated in genital tract secretions from women with relatively recent infection compared to uninfected women. During chronic HIV-1 infection, genital tract concentrations of IL-1β, IL-6, IL-10 and TNF-α, along with the proportion of inflammatory cells, were found to be elevated relative to uninfected women (Belec *et al.,* 1995; Guha and Chatterjee, 2009). Nkwanyana *et al.* (2009) similarly demonstrated that IL-1β, IL-6 and IL-8 are elevated during chronic infection compared to uninfected women and that elevated inflammatory cytokine concentrations in genital secretions

correlated with higher immune cell counts in cervical cytobrush samples. IL-4, IL-5 and IL-10 expression in the genital mucosa were found to be elevated in women with chronic HIV-1-infection relative to uninfected women, while IL-2 was reduced (Olaitan *et al.,* 1998). Contrary to these findings, Sha *et al.* (1997) reported that IL-2, IL-10, IL-6, TNF-α, IL-1β and IFN-γ levels did not differ significantly in cervicovaginal fluid from women who had chronic HIV-1-infection relative to uninfected women, although HIV-1-infected women with vaginal infections had higher IFN-γ levels. Similarly, Mitchell *et al.* (2008) found no changes in IL-6, IL-8 and IL-1β in HIV-1-infected women after adjusting for concurrent BV, but demonstrated that BV instead was associated with higher IL-1β concentrations in HIV-1-infected women. These findings indicate that changes in cytokine concentrations in women who are HIV-1-infected are likely to be influenced by the presence of sexually transmitted co-infections, which are prevalent in these women (Fennema *et al.*, 1995).

A better understanding of changes in cytokine production in the female genital tract during the earliest phase of HIV-1 infection has been achieved using primate models (Abel *et al.*, 2005; Miller *et al.*, 2005; Li *et al.*, 2009; Haase, 2011). Vaginal inoculation with SIV was shortly followed by increased expression of MIP-3α in the endocervical epithelium and accumulation of DCs (within 1 day; Li *et al.*, 2009). In turn, DCs produced MIP-1α and MIP-1β, which may be responsible for the influx of CD4+ T cells that was observed. In the transformation zone and vaginal mucosa, SIV-infected cells were found to be concentrated in areas containing IL-8-producing cells (Li *et al.*, 2009). Abel *et al.* (2005) demonstrated that robust pro-inflammatory cytokine production (TNF-α, IL-6, IL-8, MIP-1α and IFN-γ) occurred in the vaginal mucosa and cervix within 0-5 days of vaginal inoculation with SIV. However, anti-viral cytokines (IFN-α, IFN-β) were only up-regulated in the vaginal mucosa following SIV dissemination to the lymph nodes and GI tract (6-10 days post-inoculation). Peak production of pro-inflammatory cytokines in the vaginal mucosa and cervix was generally achieved by 6-10 days post-infection, at approximately the same time as peak SIV viraemia in the genital tract, lymph nodes and GI tract. Pro-inflammatory cytokine concentrations were then found to decline by 28 days post-inoculation. Peak production of anti-viral cytokines was only achieved by 14 days post-infection, but similarly declined by 28 days (Abel *et al.*, 2005).

1.10.7 Female genital tract cytokines and HIV-1 disease progression

Previous studies have suggested that pro-inflammatory cytokines in the genital mucosa and submucosa at the time of HIV-1 transmission may play an important role in establishment of HIV-1 infection and dissemination into the systemic compartment (Wang *et al.*, 2005; Bebell *et al.*, 2008; Li *et al.*, 2009). Inflammatory cytokines may directly up-regulate HIV-1 replication in this compartment and recruit and activate target cells for HIV-1 infection (Osborne *et al.*, 1989; Swingler *et al.*, 1999; Nkwanyana *et al.*, 2009). Pro-inflammatory cytokines and chemokines such as IL-1α/β, TNF-α, IL-6, IL-8, IP-10, RANTES and MIP-1α/β are each involved in recruitment of T cells, DCs and/or macrophages to infection sites (Taub *et al.*, 1993a; Taub *et al.*, 1993b; Taub *et al.*, 1993c; Harada *et al.,* 1994; Dinarello *et al.*, 1997; Swingler *et al.,* 1999; Wira *et al.*, 2005; Gabay *et al.*, 2006; Abbas and

Lichtman, 2007; Dinarello, 2009). In rhesus macaque models, it has been shown that pro-inflammatory cytokine production and recruitment of CD4+ T cell targets following vaginal inoculation with SIV was necessary for local viral expansion and subsequent seeding of the systemic compartment (Li *et al.*, 2009; Haase, 2011). Even following intravenous inoculation of SIV, the macaque vaginal mucosa represents an important site of early viral replication and rapid depletion of CD4+ T cells occurs here before significant depletion in the lymph nodes and blood (Veazey *et al.*, 2003a). Furthermore, Wang *et al.* (2005) demonstrated that pre-existing genital inflammation was associated with higher viral load set-point following vaginal inoculation with SIV. In humans, higher concentrations of inflammatory cytokines IL-1β, IL-6 and IL-8 in genital secretions during acute HIV-1 infection were found to correlate with lower blood CD4+ T cell counts at the same time-point (Bebell *et al.*, 2008), while IL-1β levels during chronic HIV-1 infection were associated with higher plasma and cervical HIV-1 viral loads (Zara *et al.*, 2004).

1.11 Study aims and objectives

The overall aim of this study was to investigate the relationships between STIs and inflammation in the female genital tract and blood and (i) susceptibility to HIV-1 infection and (ii) HIV-1 disease progression. In this dissertation, inflammatory cytokine concentrations were measured as markers of inflammation in both compartments.

Rationale

In sub-Saharan Africa, women account for approximately 60% of HIV-1-infected individuals and among 15 to 24-year olds, women are up to eight times more likely to be HIV-1-infected than men (UNAIDS, 2010). Previous studies in macaque models and humans have suggested that inflammation in the female genital tract at the time of SIV or HIV-1 transmission plays an essential role in establishment of infection and possibly disease progression (Zara *et al.*, 2004; Wang *et al.*, 2005; Bebell *et al.*, 2008; Li *et al.*, 2009; Haase, 2011). This thesis focuses on improving our understanding of immune responses in the female genital tract that are associated with susceptibility to HIV-1 infection and disease progression. The development of safe and efficacious microbicides and vaccines for prevention of male-to-female sexual HIV-1 transmission is dependent on achieving a better understanding of immune events at the site where infection is initially established and propagated.

Specific Objective 1

To investigate the effects of common STIs and BV on cytokine responses in cervicovaginal fluid and plasma from HIV-uninfected women. *Hypothesis:* STIs and BV are associated with localized inflammatory cytokine responses in the genital tract and increased susceptibility to HIV infection.

Specific Objective 2

To investigate the relationships between genital inflammatory cytokine concentrations during early HIV-1 infection and markers of HIV-1 disease progression, (i) viral load set-point and (ii) CD4+ T cell depletion. *Hypothesis:* Genital inflammation at the time of HIV-1 transmission is associated with more rapid HIV-1 disease progression by early seeding of the systemic compartment with a higher viral burden.

Specific Objective 3

To investigate the association between plasma cytokine concentrations during early HIV-1 infection and HIV-1 disease progression and to develop plasma cytokine models that are predictive of disease progression. *Hypothesis:* Plasma cytokine concentrations during early HIV-1 infection are predictive of HIV-1 disease progression.

Specific Objective 4

To evaluate the influence of longitudinal TFV microbicide use on inflammatory cytokine concentrations in the female genital tract and to investigate inflammation as a possible correlate of susceptibility to HIV-1 infection in women who became infected despite using TFV. *Hypothesis:* TFV gel use is not associated with elevated inflammatory cytokine concentrations in the genital tract, however inflammation in this compartment is associated with increased susceptibility to HIV-1 infection.

CHAPTER 2

Chapter 2

Impact of sexually transmitted infections and genital tract inflammation on risk of HIV-1 acquisition in high risk HIV-uninfected women in South Africa

2.1 Summary

Sexually transmitted infections (STIs) and bacterial vaginosis (BV), which are prevalent in South African populations, are associated with increased genital inflammatory cytokine responses. Inflammatory cytokines promote HIV-1 replication, recruit and activate immune cell targets for HIV-1 infection, and it has been suggested that genital inflammation may increase susceptibility to sexual HIV-1 transmission. The aim of this study was to evaluate inflammatory cytokine signatures in the female genital tract that are associated with STIs in high-risk HIV-uninfected women and their role in HIV-1 acquisition. Two-hundred and thirty HIV-uninfected women were enrolled and followed monthly for HIV-1 infection. Women were also tested for BV, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, herpes simplex virus type 2 (HSV-2), *Mycoplasma genitalium* and *Treponema pallidum* at enrolment and 6-monthly thereafter. Cervicovaginal lavage (CVL) samples were obtained from each woman at enrolment into the study and the concentrations of 42 cytokines were measured in these samples using Luminex Multiplex Flow Cytometric assays. The relationships genital inflammation and time to HIV-1 infection was evaluated by Cox survival analysis. It was found that women who had *C. trachomatis* infections had significantly elevated concentrations of IL-1α, IL-1β, IL-6, TNF-α, IL-8, MIP-1β, RANTES, G-CSF, FLT3L, TGF-α, IL-2, IL-13 and IL-17 in CVL while women who had gonorrhoea had significantly higher genital concentrations of IL-1α, IL-1β, IL-12p70, TNF-α, eotaxin, MIP-1α, RANTES, G-CSF, FLT3L, VEGF, IL-2, IL-5, IL-15 and IL-17 compared to women who had no STIs or BV. In contrast, women who had BV had a mixed inflammatory profile with significantly elevated CVL concentrations of IL-1β, IL-1α and TNF-β, but also significantly lower concentrations of GRO, IP-10, MDC, MIP-1α, GM-CSF, IL-7 and IFN-γ compared to women who did not have an STI or BV. It was found that women who had asymptomatic STIs had genital inflammatory cytokine concentrations that were comparable to women with symptomatic STIs, which were significantly elevated compared to women who did not have an STI or BV. Women who had an STI or BV did not have elevated plasma inflammatory cytokine concentrations. Genital IL-1β, IL-6, IL-8 and sCD40L concentrations were associated with increased risk of HIV-1 infection, however this was not significant after adjusting for multiple comparisons. These findings suggest that elevated pro-inflammatory cytokine concentrations in the female genital tract, which are associated with STIs and BV, may increase susceptibility to HIV-1 infection.

2.2 Introduction

STIs and BV are very prevalent in populations that are at high risk of HIV-1 infection (UNAIDS, 2010; Johnson *et al.*, 2010a). STIs and altered vaginal flora cause a number of clinical and subclinical manifestations that are associated with increased susceptibility to HIV-1 infection (Cohen *et al.*, 1999; Novak *et al.*, 2007; Rebbapragada *et al.*, 2007; Reddy *et al.*, 2004). Genital ulceration, which is caused by *T. pallidum* and HSV-2 infections, provides HIV-1 with access to the submucosa and HIV-1 target cells that are present there (Plummer and Ndinya-Achola, 1990; Celum, 2004; Cohen, 2004). Increased vaginal pH as a result of perturbations in microflora (BV) may facilitate HIV-1 replication that is usually restricted in a more acidic environment that is associated with healthy lactobacilli-dominated vaginal flora (Voeller and Anderson, 1992; Hillier *et al.*, 1998; Klebanoff and Coombs, 1991; Schwebke, 2005). Additionally, women who have STIs have increased concentrations of inflammatory cytokines in their genital tracts (Fichorova *et al.*, 2001a; Yudin *et al.*, 2003; Novak *et al.*, 2007; Rebbapragada *et al.*, 2007; Reddy *et al.*, 2004; Levine *et al.*, 1998). Although a direct association between cervicovaginal inflammatory cytokine concentrations and susceptibility to HIV-1 infection has not yet been demonstrated, previous studies have suggested that inflammatory cytokines may up-regulate HIV-1 replication by activating NF-κB and by recruiting and activating immune cell targets for HIV-1 infection (Osborne *et al.*, 1989; Swingler *et al.*, 1999; Nkwanyana *et al.*, 2009; Li *et al.*, 2009). Inflammatory cytokines have also been associated with tight junction disruption between epithelial cells, increasing the permeability of the epithelial barrier in the genital tract (Madara and Stafford, 1989; Schmitz *et al.*, 1996; Nazli *et al.*, 2010).

STIs, such as gonorrhoea and chronic HSV-2, are associated with impaired HIV-1-specific immune responses in HIV-1-infected individuals, higher blood HIV-1 viral loads and increased shedding of HIV-1 (Ghys *et al.,* 1997; Mostad *et al.,* 1997; Rotchford *et al.,* 2000; Anzala *et al.,* 2000; McClelland *et al.,* 2001; Nkengasong *et al.,* 2001; Boulton and Gray-Owen, 2002; Kaul *et al.*, 2002; Schacker *et al.,* 2002; Rebbapragada *et al.,* 2007; Sheth *et al.,* 2008). Certain STIs may thus influence HIV-1 disease progression and secondary transmission to HIV-uninfected partners. However, as immunocompromised HIV-1-infected individuals are more susceptible to STIs (Ghys *et al.*, 1997), it is difficult to define the cause and effect relationship.

STI management is thus an important public health priority in countries such as South Africa, where the prevalence of both STIs and HIV-1 are high (UNAIDS, 2010; Johnson *et al.*, 2010a). Currently, most STIs are managed syndromically on the basis of STI-associated signs and symptoms (World Health Organization, 2003). However, two of three of the population-wide syndromic management interventions conducted in Africa found no change in rates of HIV-1 acquisition (Grosskurth *et al.*, 1995; Kamali *et al.*, 2003; Gregson *et al.*, 2007), suggesting that asymptomatic infections may play an important role in susceptibility to HIV-1 infection. Women who have subclinical STIs may have up-regulated inflammatory cytokine responses in their genital tracts which may increase their risk of HIV-1 infection (Bogaerts *et al.*, 1999; Wiesenfeld *et al.*, 2002). Therefore, in order to further our

understanding of genital tract cytokine responses in women who have symptomatic or asymptomatic STIs and to examine the role of inflammation in susceptibility to HIV-1 infection, the concentrations of key pro-inflammatory cytokines, chemokines, hematopoietic cytokines, growth factors, adaptive immune mediators and anti-inflammatory cytokines were measured in cohort of high-risk HIV-uninfected women who were followed longitudinally for HIV-1 infection.

2.3 Materials and methods

2.3.1 Description of study participants

A cohort of 245 high-risk HIV-uninfected women was established in Durban, South Africa, for the longitudinal CAPRISA 002 Acute Infection Study (van Loggerenberg *et al.*, 2008). Of these women, CVLs were available from 230 women at enrolment into the study. Each woman attended monthly evaluations of HIV-1 status for a maximum of 24 months. Time of infection was defined as the mid-point between the last HIV-1 antibody negative test and the first HIV-1 antibody positive test, or as 14 days prior to a positive RNA polymerase chain reaction (PCR) assay on the same date as a negative HIV-1 Enzyme Immunoassay (EIA). This study was approved by the University of KwaZulu-Natal and University of Cape Town Ethics Committees, and all participants provided informed consent.

2.3.2 Screening for sexually transmitted infections

Each woman was screened for STIs and BV at enrolment into the study and 6-monthly thereafter. Following a gynaecological examination, two vulvovaginal swabs were collected from the posterior fornices and lateral vaginal walls of each woman. The presence of abnormal vaginal discharge, ulceration or visible cervical/vaginal inflammation (swelling, redness) was recorded. All specimens were transported to the diagnostic laboratory (Medical Microbiology Laboratory, University of KwaZulu-Natal) within one hour of collection for same day processing. *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium* and HSV-2 were assessed by PCR, *T. vaginalis* by Diamond's culture and PCR. *N. gonorrhoea* and *C. trachomatis* were isolated using the BDProbe Tec^{TM} ET assay (Becton Dickinson Microbiology systems, U.S.A.). One swab was rolled onto a glass slide for Gram staining for diagnosis of BV using Nugents criteria. Although the Nugent score is subjective and requires an experienced slide reader, this remains the gold standard for diagnosis of BV (Sha et al., 2005). Blood specimens were collected for syphilis diagnosis and HSV-2 serology. Serological tests for HSV-2 were directed against antibodies to HSV glycoproteins G-1 and G-2. Syphilis screening was done using the Becton Dickinson Macro-Vue Rapid Plasma Reagin (RPR) card test followed by the *T. pallidum* Haemagglutination test (Omega ImmuTrep TPHA test) to identify antibodies against syphilis.

2.3.3 Cytokine Measurements

CVL samples for cytokine measurements were collected as follows: Sterile normal saline (10ml) was used to repeatedly bathe the cervix and allowed to pool in the posterior fornix, where it was then aspirated into a plastic bulb pipette (Bebell *et al.*, 2008). Samples were centrifuged and supernatant stored at -80°C. CVL samples were not collected from menstruating participants, and sampling was postponed to the following visit. Blood was collected by venipuncture into acetate citrate dextran (ACD) vacutainer tubes. Plasma was isolated and aliquoted into cryovials and stored at -80°C. After thawing, CVLs were pre-filtered by centrifugation using 0.2 µm cellulose acetate filters (Sigma, U.S.A.). The concentrations of 42 cytokines were measured in CVL samples and 13 cytokines in plasma samples using Luminex Multiplex Flow Cytometric assays. Concentrations of 30/42 cytokines were measured using Human Cytokine LINCO*plex* Premixed kits (LINCO Research, MO, U.S.A.) according to the manufacturer's protocol. The lower limit of detection of these kits ranged between 0.25 and 27.65 pg/ml for each of the 30 cytokines measured. CVL cytokines measured using Human Cytokine kits included EGF, eotaxin/CCL11, FGF-2, fms-like tyrosine kinase-3 Ligand (FLT3L), fractalkine/CX_3CL1, G-CSF, growth related oncogene (GRO) family (CXCL1-CXCL3), IFN-α, IL-1α, IL-1Ra, IL-3, IL-8/CXCL8, IL-9, IL-12p40, IL-15, IL-17, IP-10/CXCL10, MCP-1/CCL2, MCP-3/CCL7, macrophage-derived chemokine (MDC)/CCL22, MIP-1α/CCL3, MIP-1β/CCL4, PDGF-AA, PDGF-AB/BB, RANTES/CCL5, soluble CD40 ligand (sCD40L), soluble IL-2 receptor α (sIL-2Rα), TGF-α, TNF-β, and VEGF. The concentrations of 13/42 cytokines were measured in CVL and plasma using High Sensitivity Human Cytokine LINCO*plex* Premixed kits: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, GM-CSF, IFN-γ and TNF-α. The lower limit of detection of these kits ranged between 0.01 and 0.48 pg/ml for each of the 13 cytokines measured. Data was collected using a Bio-Plex™ Suspension Array Reader (Bio-Rad Laboratories Inc®) and a 5 PL regression formula was used to calculate cytokine concentrations from the standard curves. Data was analysed using BIO-plex manager software (version 4; Bio-Rad Laboratories Inc®). Cytokine concentrations that were below the lower limit of detection of the assay were reported as the mid-point between the lowest concentration measured for each cytokine and zero. Previous studies have shown variation in the measurements of particular cytokines between separate Luminex assays (de Jager *et al.*, 2003). The concentrations of IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF-α, IP-10, MCP-1 and RANTES were also measured in CVL from the participants of this study using cytometric bead array (CBA; BD™). CBA results were compared to measurements obtained using Luminex (Appendix C). Furthermore, cytokine concentrations were measured in plasma and CVL samples using separate Luminex kits in experiments performed on different days in order to evaluate the reproducibility, reliability and sensitivity of Luminex measurements (Appendix C). As it was shown in this and previous studies that there is variability in measurements of some cytokines between separate Luminex assays, cytokine concentrations were only directly compared in this dissertation if the samples in each comparison group had been evaluated alongside each other in the same assay. In the case of large sample sizes, samples from each comparison group were equally distributed across all plates so that the variation between kits would contribute the same degree of variation to each comparison group.

2.3.4 Statistical analyses

Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, U.S.A.) and STATA™ (StataCorp, Texas, U.S.A.). Distribution of all variables was assessed by Shapiro-Wilk and Shapiro-Francia tests. Mann Whitney U test was used for unmatched comparisons. Spearman Rank tests were used for correlations. Logistic regression was used to assess associations between cytokine concentrations and STIs and BV, while adjusting for co-infections. Cox survival analysis was used to determine the relationships between cytokine concentrations and risk of HIV-1 infection. Participants lost to follow-up were censored at the last contact visit, while all other participants were censored at the time of the last clinic visit.

Exploratory factor analysis (EFA) and principal component analysis (PCA) were used to reduce the number of variables and the complexity of the dataset, while retaining much of the information in the dataset. Factor analysis is performed by analyzing the pattern of correlations between variables: Variables that are highly correlated are likely to be influenced by the same factor. This analysis is based on the Common Factor Model, which proposes that each variable is influenced by both underlying common factors and by unique factors (deCoster, 1998). For this study, the principal factor extraction method was used (communalities set to the squared multiple-correlation coefficients), with either an unrotated factor-loading matrix or a Promax Oblique rotation. For any given set of correlations or number of factors, there are an infinite number of ways to define the factors and still account for the covariance in cytokine concentrations. Different factor rotations are used to find a solution that has the simplest interpretation. Promax Oblique rotation was used because this rotation allows for correlations between factors. The Kaiser Criterion, which states that the number of factors/components generated is equal to the number of the eigenvalues of the correlation matrix that are greater than 1.00, was used to determine the number of factors or components (DeCoster, 1998). Following determination of the number of factors/components, factors/components were extracted using STATA™. Cytokines were grouped into factors/components according to the size of each factor loading value or component. Each factor loading or component is a standardized regression coefficient which indicates the relationship between each cytokine and each factor. Estimates/factor scores, which represent values of each of the underlying factors and include the common variance of each cytokine included in each factor or functional group, were generated for further analysis. Whereas factor analysis was used to identify common underlying factors that were responsible for groups of observed responses (cytokine concentrations), PCA was used to define simple linear combinations of the cytokines that were assessed in this study. Each component generated by PCA thus consists of both the common and unique variance of the included cytokines. Cytokine concentrations were log-transformed for regression analysis, Cox survival analysis, factor analyses and PCA. P-values <0.05 were considered significant. P-values were adjusted using a False discovery rate (FDR) step-down procedure in order to reduce false positive results when multiple comparisons were made (Columb and Sagadai, 2006).

2.4 Results

A total of 230 HIV-uninfected women at high risk of HIV-1 infection were included in this study and followed for up to 24 months. The median age of women at enrolment was 36 years (range 18 - 58). Most women had multiple partners (56.8%; 130/229), while 7.4% (17/229) were single and 35.8% (82/229) had a stable partner or were married. However, most women (94.7%) reported having had more than one casual sexual partner within the last 3 months and 78.8% were self-reported female sex workers (Mlisana *et al* , unpublished data)

The prevalence of both BV and STIs in this cohort was high at enrolment (Table 2.1, data provided by Dr. Koleka Mlisana; University of KwaZulu-Natal). It was found that 53% (122/230) of women had BV and 33% (76/230) of women had one or more active STI (excluding BV or HSV-2 serology) While only 8 women had detectable HSV-2 in their genital tracts by PCR, 200 women in the cohort tested positive for HSV-2 IgG antibodies (87.0%). Only 3 women had detectable *T. pallidum* antibodies and 3 women had *M. genitalium*, therefore syphilis and *M. genitalium* were excluded from further analysis.

Table 2.1 Prevalence of BV and active STIs at enrolment into the study

N (%)	BV	Trich	Gono	Chlamydia	HSV-2	Syphilis	Myco	HSV-2 Ab
87 (37.8%)								75
78 (33.9%)	75							66
13 (5.7%)		13						11
2 (0.9%)			2					2
2 (0.9%)				2				2
1 (0.4%)					1			1
22 (9.5%)	22	22						20
6 (2.6%)	6		6					6
2 (0.9%)	2			2				1
3 (1.3%)	3				3			3
2 (0.9%)	2					2		2
3 (1.3%)	3						3	1
2 (0.9%)		2	2					1
1 (0.4%)	1	1	1					1
3 (1.3%)	3	3		3				2
3 (1.3%)	3	3			3			3
1 (0.4%)	1	1				1		1
1 (0.4%)			1	1	1			1
1 (0.4%)	1	1	1	1				1
230	122 (53%)	46 (20%)	13 (5.7%)	9 (3.9%)	8 (3.5%)	3 (1.3%)	3 (1.3%)	200 (87.0%)

1 Infection 2 Infections 3 Infections 4 Infections

Trich: *Trichomonas vaginalis*; Gono: *Neisseria gonorrhoeae*; Chlamydia: *Chlamydia trachomatis*; HSV-2: Herpes simplex virus type 2; Myco: *Mycoplasma genitalium*; Ab: Antibody.

Thirty-five women (15.2%) had visible vaginal or cervical discharge. None of the women had genital ulceration or visible cervical or vaginal inflammation at enrolment into the study

2.4.1 Genital inflammatory cytokine concentrations were elevated in women who had STIs

The concentrations of 42 cytokines in CVL samples from women who had BV and/or one or more STIs were compared to those of women who tested negative for all of the assessed STIs and BV (Figure 2.1, Appendix C). It was found that women with BV without sexually transmitted co-infections (n=75) had up-regulated CVL concentrations of pro-inflammatory cytokines (IL-1α, IL-1β and TNF-β) relative to women who did not have BV or STIs (Figure 2.2) However, women who had BV also had lower CVL concentrations of several chemokines (IP-10, GRO, MDC and MIP-1α), hematopoietic cytokines (IL-7 and GM-CSF) and IFN-γ relative to women with no STIs or BV.

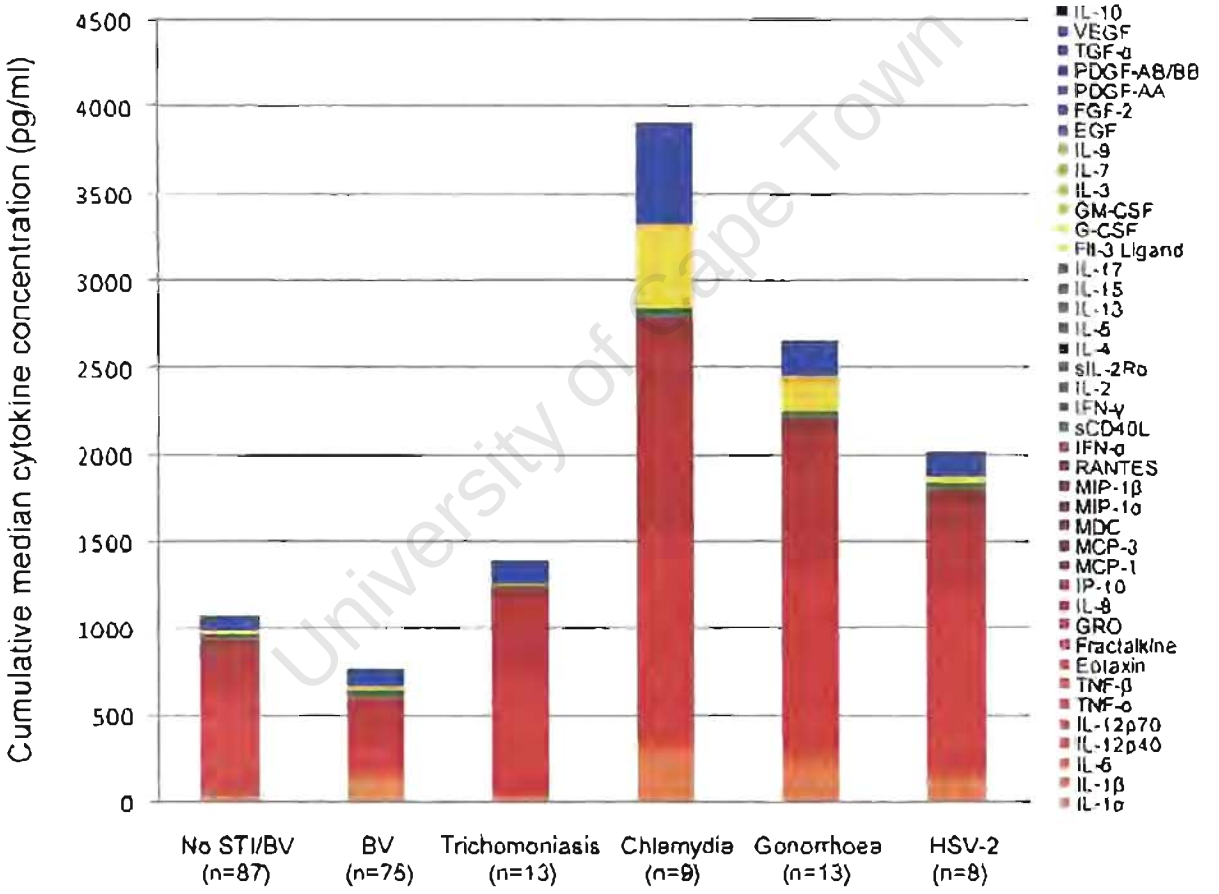


Figure 2.1 Cumulative median cytokine concentrations in the genital tracts of women who had STIs or BV The median concentrations of each of the 42 cytokines, except IL-1Ra, that were measured in CVL samples from women who had no STIs or BV and women who had either BV, trichomoniasis, chlamydia, gonorrhoea or active HSV-2 are represented in each stacked bar. Pro-inflammatory cytokines and chemokines are shown in oranges and reds. Adaptive immune mediators are green, hematopoietic cytokines are yellow, growth factors are blue and immunoregulatory IL-10 is black. IL-1Ra was excluded because, although this cytokine did not differ between each group, it is present in CVL samples at very high concentrations and its inclusion prevents visualization of the other cytokines.

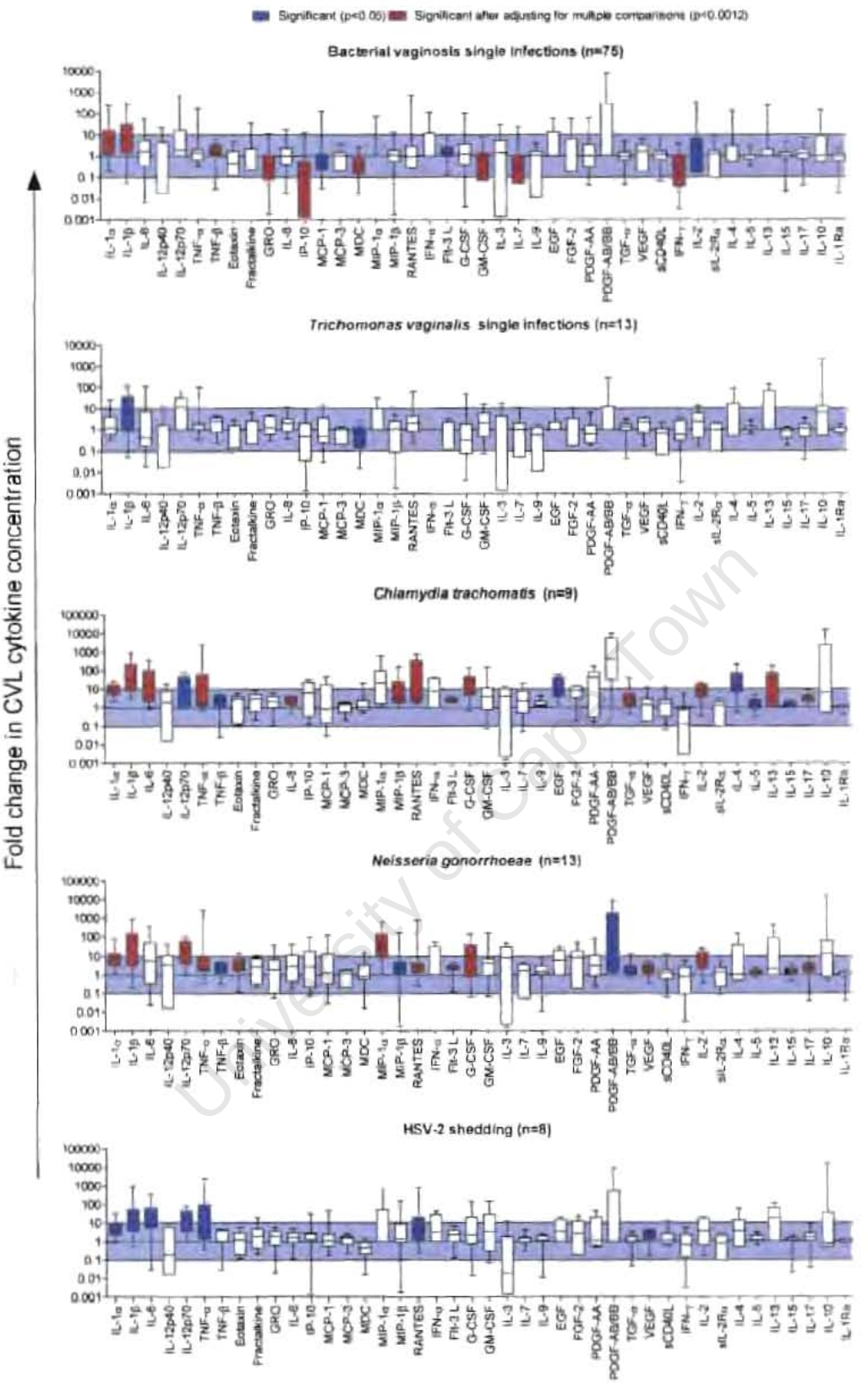


Figure 2.2 Fold change in cytokine concentrations in the genital tracts of women who had STIs or BV. Each bar indicates the fold change in CVL cytokine concentrations in women who had BV, trichomoniasis, chlamydia, gonorrhoea or HSV-2 relative to the median concentrations in women who did not have STIs/BV. Most women who had chlamydia, gonorrhoea or HSV-2 had co-infections. Cytokines that were significantly altered relative to women who did not have STIs or BV before adjusting for multiple comparisons are indicated by blue bars ($p<0.05$). Cytokines that were up- or down-regulated after adjustment are shown by red bars ($p<0.0012$).

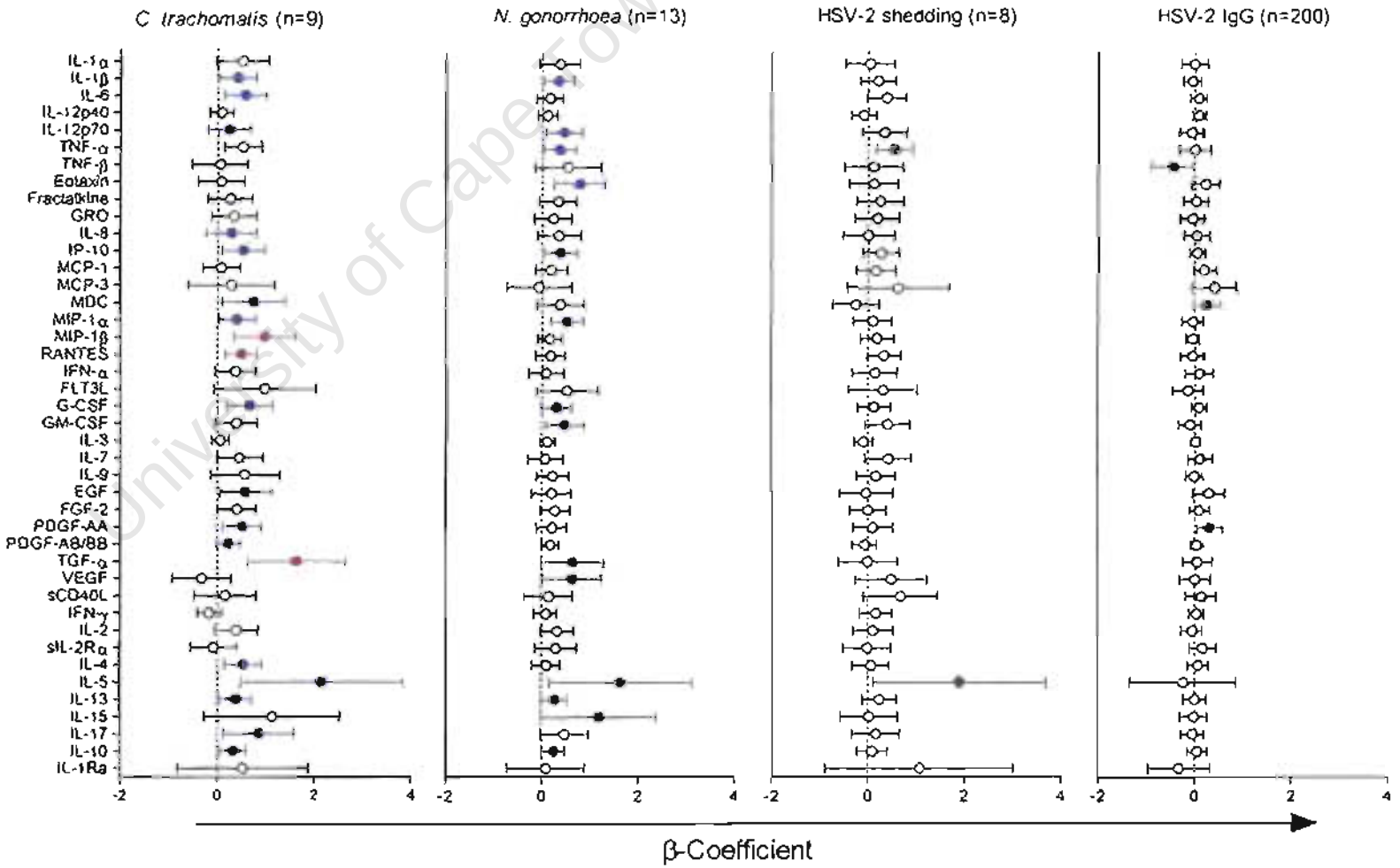


Figure 2.3 Associations between genital cytokine concentrations and STIs after adjusting for co-infections. Logistic regression was used to assess the relationships between log-transformed cervicovaginal cytokine concentrations and STIs, after adjusting for co-infections and BV. Dots indicate the β-coefficients of each regression equation. Error bars indicate 95% confidence intervals. A positive β-coefficient indicates that women with the STI of interest are more likely to have up-regulated genital cytokine concentrations relative to women who have no STIs/BV. A negative β-coeffficient indicates that women with each STI are more likely to have down-regulated genital cytokine concentrations relative to women who have no STIs/BV. Associations that were significant after adjusting for co-infections and BV are shown in blue ($p<0.05$). Associations that were significant after further adjusting for multiple comparisons are shown in red.

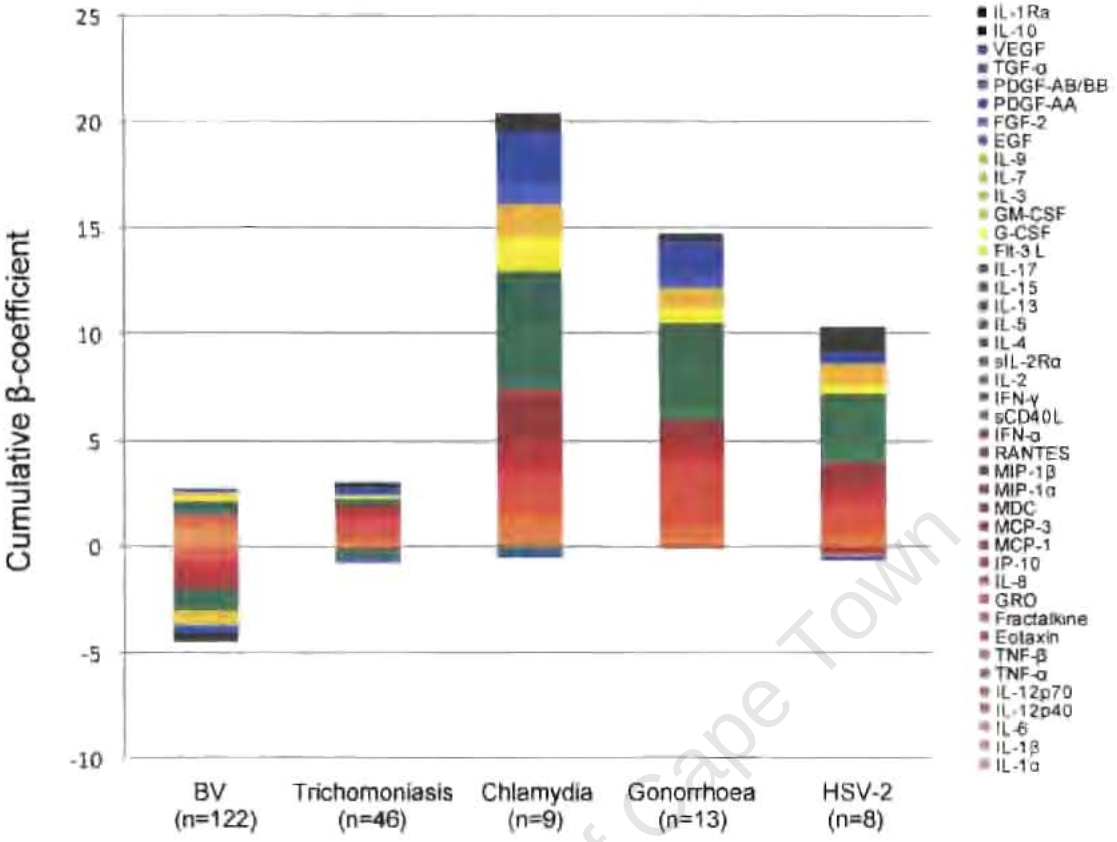


Figure 2.4 Cumulative β-coefficients indicating the relationships between cytokines and each STI or BV, while controlling for co-infections. β-coefficients were calculated using logistic regression. The β-coefficient for each of the 42 cytokines is represented in each stacked bar for each STI/BV. A positive β-coefficient indicates that higher concentrations of the cytokine are associated with BV or an STI, while a negative β-coefficient indicates that the cytokine concentration is more likely to be lower relative to women who did not have STIs or BV. BV: Bacterial vaginosis; HSV-2: Herpes simplex virus type 2.

Surprisingly, it was found that women who had trichomoniasis did not have elevated cytokine concentrations relative to women who did not have STIs or BV (n=13; Figure 2.2). IFN-α, which was the only cytokine that differed significantly in CVL from women with trichomoniasis compared to women with no STIs/BV, was down-regulated. Women who had chlamydia (n=9) or gonorrhoea (n=13) had up-regulated cervicovaginal inflammatory responses compared to women who did not have STIs or BV. In a univariate analysis, IL-1α, IL-1β, IL-6, TNF-α, IL-8, MIP-1β, RANTES, G-CSF, FLT3L, TGF-α, IL-2, IL-13 and IL-17 were up-regulated in CVL samples from women who had chlamydia (Figure 2.2). Because of the high prevalence of co-infections in this group of women, it was necessary to adjust for co-infections using logistic regression. After adjusting for co-infections and multiple comparisons, elevated concentrations of MIP-1β, RANTES and TGF-α were significantly associated with women being infected with chlamydia (Figures 2.3 and 2.4).

Similarly, IL-1α, IL-1β, IL-12p70, TNF-α, eotaxin, MIP-1α, RANTES, G-CSF, FLT3L, VEGF, IL-2, IL-5, IL-15 and IL-17 were up-regulated in CVL from women with gonococcal infections relative to women who did not have an STI or BV (Figure 2.2). After adjusting for co-infections and multiple comparisons, none of these associations remained significant (Figure 2.3). However, high levels of inflammation were observed in women who had gonorrhoea (Figure 2.4) and the loss of significance following adjustment for co-infections and multiple comparisons was most likely due to the small number of women who had this infection in this study, the frequency of co-infections in these women and the number of cytokines measured for which it was necessary to adjust all p-values.

Women who had detectable HSV-2 in their genital secretions (HSV-2 PCR positive) tended to have elevated CVL concentrations of several pro-inflammatory cytokines, however none were significant after adjusting for multiple comparisons and/or co-infections (Figures 2.3 and 2.4). Although these women were actively shedding HSV-2, none had visible genital ulceration. It was further found that genital cytokine concentrations did not differ between women who had positive and negative HSV-2 serology (Figure 2.3).

2.4.2 Clustering of cytokines using factor analysis in order to reduce the complexity of the dataset

The genital concentrations of most of the cytokines included in this study were collinear (correlated with one another), therefore multivariate regression analysis could not be used to identify clusters of cytokines that were associated with each infection. Exploratory factor analysis (EFA) was thus used to reduce the complexity of the dataset by decreasing the number of variables and identifying common underlying factors that are responsible for groups of observed responses. Estimates were produced using the principal-factor method with an unrotated factor-loading matrix. Eigenvalues were used to determine the number of factors generated. Six of the calculated eigenvalues were greater than 1.00, therefore each of the 42 cytokines loaded onto 1 of 6 factors. Because most cytokines correlated with one another, 27/42 (64.3%) were found to load onto the same factor following EFA with an unrotated factor-loading matrix (Figure 2.5). Factor 1 therefore included 27/42 cytokines: IL-6, IL-12p70, IL-12p40, TNF-β, eotaxin, fractalkine, GRO, IL-8, MIP-1α, MIP-1β, IFN-α, FLT3L, G-CSF, GM-CSF, IL-3, IL-9, EGF, FGF-2, PDGF-AA, TGF-α, VEGF, IL-2, sIL-2Rα, IL-4, IL-13, IL-15, IL-17. Factor 2 included 6/42 cytokines: IL-1β, TNF-α, IL-7, IFN-γ, IL-5 and IL-10. Factor 3 included 4/42 chemokines: IP-10, MCP-1, MCP-3 and MDC. IL-1Ra and IL-1α were the only cytokines that loaded onto Factor 4 and Factor 5, respectively. Factor 6 included 3/42 cytokines: RANTES, PDGF-AB/BB and sCD40L. Although IFN-γ loaded onto Factor 2, this was the only cytokine that was inversely associated with Factor 1. TNF-β, VEGF, IFN-γ, IL-1Ra and sCD40L were largely influenced by underlying unique factors (uniqueness >0.50), indicating that these cytokines do not correlate strongly with the other cytokines that were measured in this study. The relationships between the estimates of the underlying Factors 1-3 and Factor 6 and each STI and BV, while adjusting for co-infections, were determined using logistic regression (Table 2.2). While none of these Factors were associated with trichomoniasis

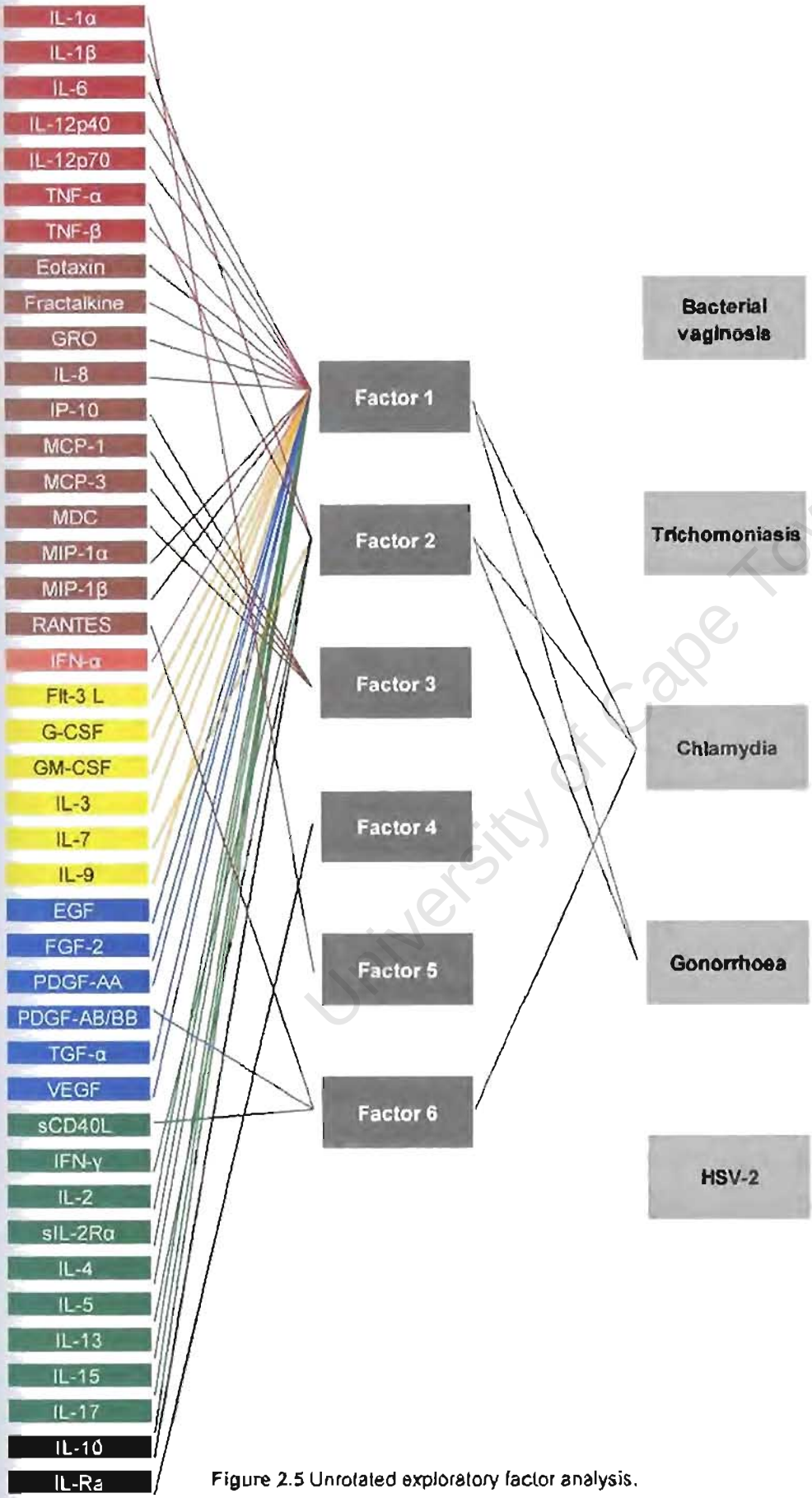


Figure 2.5 Unrotated exploratory factor analysis.

or HSV-2 shedding after adjusting for co-infections and multiple comparisons, Factor 3 (including 4 chemokines) was inversely associated with BV, all factors generated (including Factor 3) were positively associated with chlamydia and Factors 1 and 2 with gonorrhoea.

Using a Promax Oblique rotation (Figure 2.6), which allows for correlations between factors, cytokines were grouped as follows: Factor 1 (TNF-β, MIP-1β, FLT3L, IL-9, TGF-α, IL-15, IL-17); Factor 2 (IL-12p40, eotaxin, fractalkine, MCP-1, MIP-1α, IFN-α, IL-3, EGF, FGF-2, PDGF-AB/BB); Factor 3 (IL-6, IL-12p70, TNF-α, RANTES, GM-CSF, IL-7, PDGF-AA, VEGF, IL-2, IL-4, IL-5, IL-13, IL-10); Factor 4 (GRO, IP-10, MCP-3, MDC, G-CSF, sCD40L, IL-1Ra); Factor 5 (IFN-γ, sIL-2Rα); Factor 6 (IL-1α, IL-1β, IL-8). After adjusting for co-infections and multiple comparisons using logistic regression, Factor 4 was inversely associated with BV while Factor 6 was directly associated with BV. Factor 3 was directly associated with chlamydia, indicating that the estimates of this factor were higher in women who had chlamydia and that the cytokines in this cluster were mostly up-regulated in CVL from these women compared to women with no STIs/BV.

It was observed that factors that included mostly chemokines were inversely associated with BV, while those including mostly pro-inflammatory cytokines were directly associated. Therefore, women who had BV in this study had up-regulated pro-inflammatory cytokine concentrations and down-regulated chemokine concentrations in their genital tracts. Women who had chlamydia had broad, non-specific up-regulation of many of the cytokines assessed, regardless of functional grouping. Similar groupings of cytokines were obtained using principal component analysis (data not shown).

The above exploratory analyses grouped cytokines according to their correlations with one another, however, in most cases, these groupings were not indicative of the functions of the cytokines that were evaluated. In order to achieve a better understanding of the relationships between functional clusters of cytokines and STIs or BV, confirmatory factor analysis (CFA) was used to load the cytokines in each functional group (pro-inflammatory, chemokine, hematopoietic, growth factor, adaptive and regulatory) onto 1 factor and to generate the estimates of each group (Figure 2.7). IL-1α, IL-1β, IL-6, IL-12p40, IL-12p70, TNF-α and TNF-β were included in the pro-inflammatory cytokine group. Chemokines included eotaxin, fractalkine, GRO, IL-8, IP-10, MCP-1, MCP-3, MDC, MIP-1α, MIP-1β, RANTES. The hematopoietic cytokine cluster included FLT3L, G-CSF, GM-CSF, IL-3, IL-7 and IL-9. Growth factors included EGF, FGF-2, PDGF-AA, PDGF-AB/BB, TGF-α and VEGF. Adaptive immune mediators included IFN-γ, IL-2, IL-4, IL-5, IL-13, IL-15, IL-17, sCD40L and sIL-2Rα. IL-10 and IL-1Ra were included in the immunoregulatory group. Logistic regression was used to assess the relationships between the estimates of each functional factor and STIs, while adjusting for co-infections (Table 2.2). The estimates of the pro-inflammatory cytokine cluster were positively associated with BV, while the chemokine cluster was inversely associated. The estimates of all functional groups of cytokines that were included in this study were positively associated with chlamydia after adjusting for co-infections and multiple comparisons, while all functional groups, except immunoregulatory cytokines, were positively associated with gonorrhoea. Therefore, similar to

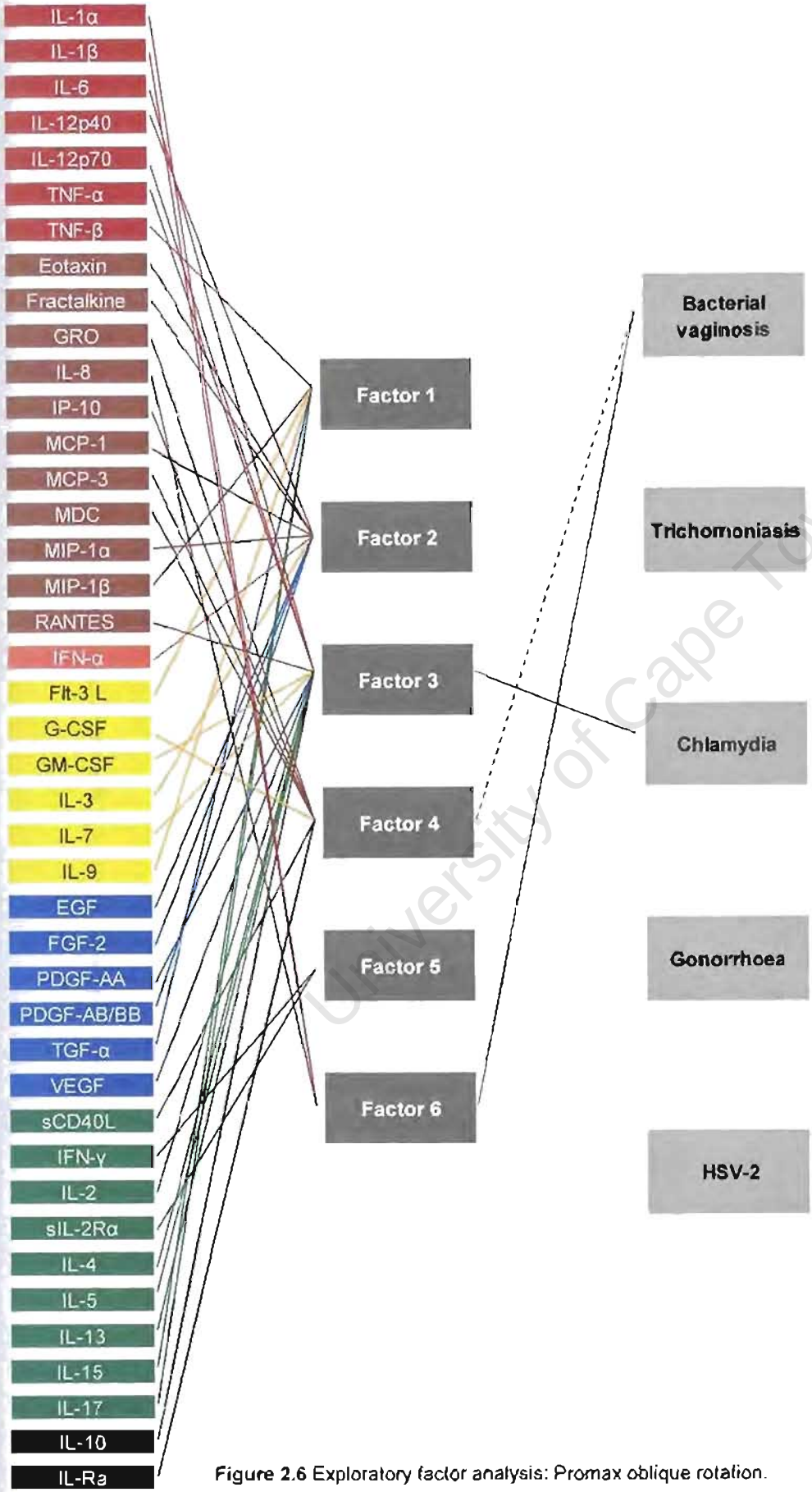


Figure 2.6 Exploratory factor analysis: Promax oblique rotation.

Table 2.2 Associations between factor scores and each STI and BV, following adjustment for co-infection

	BV (n=122)			*T. vaginalis* (n=46)			*C. trachomatis* (n=9)			*N. gonorrhoeae* (n=13)			HSV-2 (n=8)			Risk of HIV-1 infection		
Analysis	β	95% CI	Adj. p-value[a]	β	95% CI	Adj. p-value[a]	β	95% CI	Adj. p-value[a]	β	95% CI	Adj. p-value[a]	β	95% CI	Adj. p-value[a]	HR	95% CI	Adj. p-value[a]
Unrotated																		
Factor 1	-0.01	(-0.3 - 0.3)	...	0.09	(-0.3 - 0.5)	...	**1.22**	**(0.3 - 2.1)**	**0.009**	**0.98**	**(0.3 - 1.7)**	**0.024**	0.33	(-0.5 - 1.1)	...	1.17	(0.8 - 1.8)	...
Factor 2	-0.02	(-0.3 - 0.3)	...	0.15	(-0.2 - 0.5)	...	**0.89**	**(0.3 - 1.5)**	**0.010**	**0.66**	**(0.1 - 1.2)**	**0.034**	**0.70**	**(0.0 - 1.4)**	**0.064**	1.26	(0.9 - 1.9)	...
Factor 3	**-0.84**	**(-1.2 - -0.5)**	**<0.004**	0.38	(-0.1 - 0.8)	...	**1.07**	**(0.1 - 2.1)**	**0.037**	0.81	(-0.0 - 1.6)	...	0.26	(-0.7 - 1.2)	...	1.19	(0.7 - 1.9)	...
Factor 6	0.04	(-0.4 - 0.5)	...	-0.15	(-0.7 - 0.4)		**1.27**	**(0.4 - 2.1)**	**0.012**	0.54	(-0.2 - 1.3)	...	0.84	(-0.1 - 1.7)	...	1.00	(0.6 - 1.8)	...
Promax Oblique rotation																		
Factor 1	0.26	(0.0 - 0.6)	...	0.08	(-0.3 - 0.5)	...	**2.66**	**(0.4 - 4.9)**	**0.063**	1.03	(-0.1 - 2.1)	...	0.32	(-0.6 - 1.3)	...	0.90	(0.6 - 1.4)	...
Factor 2	-0.17	(-0.5 - 0.1)	...	-0.03	(-0.4 - 0.3)	...	0.67	(0.0 - 1.4)	...	**0.67**	**(0.1 - 1.3)**	**...**	0.00	(-0.8 - 0.8)	...	1.12	(0.7 - 1.7)	...
Factor 3	-0.14	(-0.4 - 0.2)	...	0.15	(-0.2 - 0.5)	...	**1.02**	**(0.3 - 1.7)**	**0.024**	**0.73**	**(0.2 - 1.3)**	**0.078**	**0.75**	**(0.0 - 1.5)**	**0.252**	1.29	(0.9 - 1.9)	...
Factor 4	**-0.69**	**(-1.0 - -0.4)**	**<0.006**	0.38	(0.0 - 0.8)	...	**1.20**	**(0.9 - 2.2)**	**0.063**	**0.81**	**(0.0 - 1.6)**	**...**	0.37	(-0.5 - 1.2)	...	1.43	(0.90- 2.3)	...
Factor 5	-0.63	(-1.3 - 0.0)	...	0.11	(-0.7 - 0.9)	...	-0.57	(-2.3 - 1.2)	...	-0.36	(-1.8 - 1.1)	...	0.63	(-1.2 - 2.4)	...	1.36	(0.5 - 3.7)	...
Factor 6	**0.84**	**(0.5 -1.2)**	**<0.006**	**0.55**	**(0.1 - 1.0)**	**0.084**	**1.13**	**(0.1 - 2.2)**	**...**	**0.81**	**(0.0 - 1.6)**	**...**	0.27	(-0.7 - 1.2)	...	**1.85**	**(1.1 - 3.0)**	**0.072**
Functional grouping																		
Pro-inflammatory	**0.59**	**(0.3 - 0.9)**	**0.003**	0.26	(-0.1 - 0.7)	...	**1.14**	**(0.3 - 2.0)**	**0.018**	**0.91**	**(0.2 - 1.6)**	**0.027**	**0.91**	**(0.0 - 1.8)**	**0.234**	**1.53**	**(1.0 - 2.3)**	**0.234**
Chemokine	**-0.73**	**(-1.1- -0.4)**	**<0.006**	0.33	(-0.1 - 0.7)	...	**1.24**	**(0.3 - 2.2)**	**0.013**	**1.18**	**(0.4 - 2.0)**	**0.018**	0.45	(-0.4 - 1.3)	...	1.31	(0.8 - 2.1)	...
Hematopoeitic	0.14	(-0.2 - 0.5)	...	0.03	(-0.4 - 0.4)	...	**1.80**	**(0.5 - 3.1)**	**0.018**	**1.08**	**(0.2 - 2.0)**	**0.030**	0.52	(-0.5 - 1.5)	...	1.13	(0.7 - 1.8)	...
Grow th Factor	-0.14	(-0.5 - 0.2)	...	-0.02	(-0.4 - 0.4)	...	**1.42**	**(0.4- 2.5)**	**0.021**	**0.83**	**(-0.1 - 1.6)**	**0.030**	-0.03	(-0.9 - 0.8)	...	0.96	(0.6 - 1.5)	...
Adaptive	0.17	(-0.1 - 0.5)	...	0.16	(-0.2 - 0.6)	...	**1.41**	**(0.5 - 2.4)**	**0.024**	**0.93**	**(0.2 - 1.7)**	**0.024**	0.39	(-0.5 - 1.3)	...	1.15	(0.7 - 1.8)	...
Regulatory	-1.05	(-2.1 - 0.0)	...	0.85	(-0.4 - 2.1)	...	**3.34**	**(0.5 - 6.2)**	**0.021**	2.04	(-0.2 - 4.3)	...	1.64	(-1.2 - 4.4)	...	3.23	(0.7 - 15.4)	...

[a]P-value adjusted for multiple comparisons using FDR step-down procedure and for co-infections using logistic regression. Associations that were significant before adjusting for multiple comparisons are in bold. Associations that were significant after adjustment are underlined. P-values for non-significant effects were omitted. β: β-coefficient, CI: Confidence interval, HR: Hazard ratio.

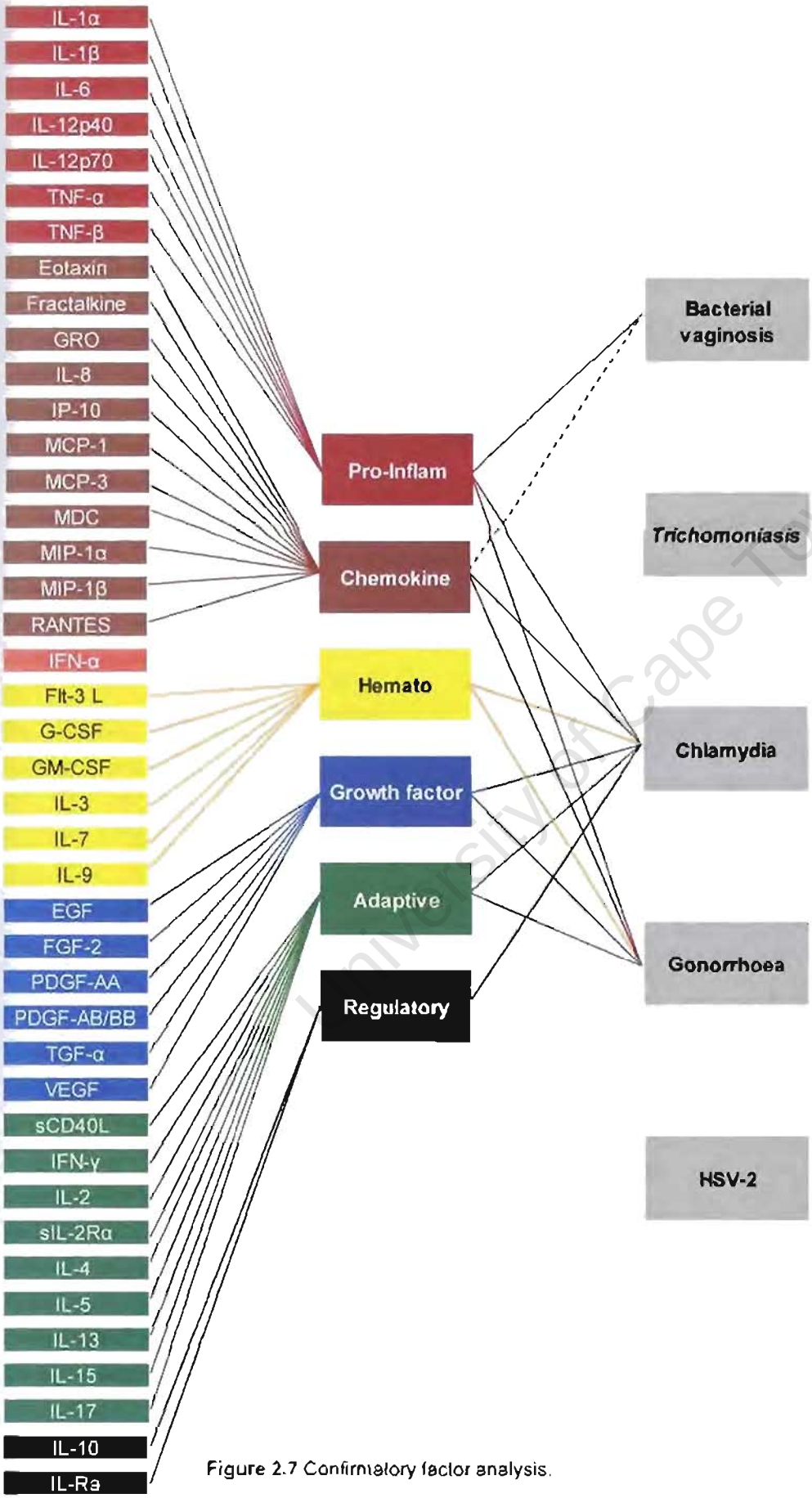


Figure 2.7 Confirmatory factor analysis.

the results of EFA, it was found that women who had chlamydia or gonorrhoea had broad, non-specific inflammatory responses in their genital tracts, whereas women who had BV had a mixed inflammatory profile, with up-regulated pro-inflammatory cytokines but down-regulated chemokines. Trichomoniasis and HSV-2 shedding were rarely significantly associated with any of the groups generated. As the cervicovaginal concentrations of most of the cytokines that were assessed in this study were highly correlated with one another, grouping using EFA produced clusters that included cytokines which were often functionally distinct. It was thus concluded that grouping of cytokines into defined functional groups using CFA was the most relevant of these analyses.

2.4.3 Women who had asymptomatic STIs had elevated genital tract inflammation

It was found that only a small proportion of women who had one or more laboratory-diagnosed active STI had clinical symptoms [12/66 (18.2%); Mlisana *et al.*, unpublished data]. Two of three STI syndromic management interventions (Grosskurth *et al*, 1995; Kamali *et al*, 2003; Gregson *et al* ,2007) have failed to reduce the risk of HIV-1 infection, suggesting that asymptomatic STIs, which would be left untreated in a syndromic management setting, may increase susceptibility to HIV-1 infection. Therefore genital inflammation was assessed in women who had STIs but no clinical

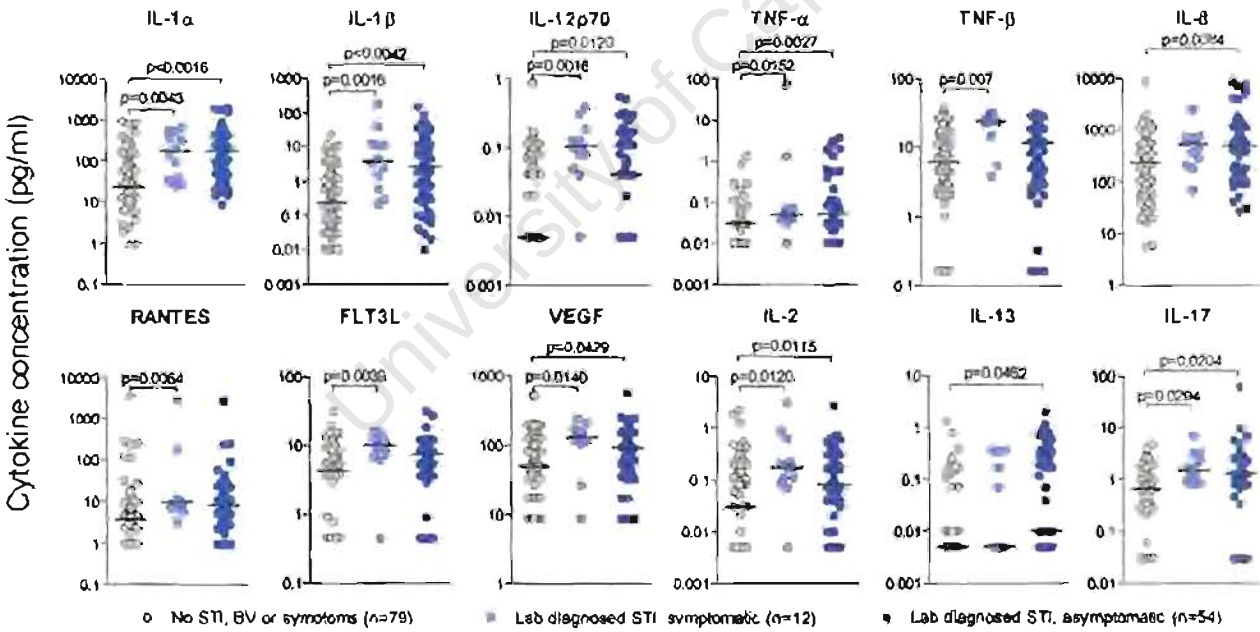


Figure 2.8 Asymptomatic STIs were associated with genital inflammation. CVL cytokine concentrations in (i) women with no STI, BV or clinical symptoms (grey dots), (ii) women with one or more active STI (excluding BV and HSV-2 serology) and genital discharge (light blue dots) and (iii) women with one or more active STI, but no clinical symptoms (dark blue dots). Lines indicate the median cytokine concentration in each group. Only cytokines that differed significantly between groups are shown. P-values were adjusted for multiple comparisons using an FDR step-down procedure.

symptoms (discharge, ulceration or visible inflammation), in order to determine whether women with asymptomatic STIs have subclinical genital inflammation which may place them at increased risk of HIV-1 infection. CVL cytokine concentrations were compared in i) women who tested negative for all assessed STIs, BV and had no symptoms, ii) women who had vaginal discharge and tested positive for one or more STIs (excluding BV and HSV-2 serology) and iii) women who had no clinical symptoms but tested positive for one or more of the above STIs. It was found that 10/42 cytokines assessed (IL-1α, IL-1β, IL-12p70, TNF-α, TNF-β, RANTES, FLT3L, VEGF, IL-2 and IL-17) were significantly elevated in women who had one or more STI and symptoms relative to women who had no STI, after adjusting for multiple comparisons (Figure 2.8). Of these cytokines, 7 (and additionally IL-8 and IL-13) were elevated in CVL from women who had an active STI, but had no clinical symptoms. Furthermore, cytokine concentrations did not differ between women who had asymptomatic STIs compared to symptomatic STIs. Therefore, women who have asymptomatic STIs may have subclinical inflammation that may increase their risk of HIV-1 infection.

In addition to STIs and BV, vaginal discharge may be caused by other factors, including *Candida albicans*, physiological factors such as age and hormonal changes, allergic reaction or irritation (Mitchell, 2004). Therefore, within the group of women who did not have an STI or BV, cytokine concentrations were compared in CVL from women who had discharge to women who did not have discharge. It was found that women who had discharge, but no STI or BV, had elevated IL-4, MCP-3, MDC and TGF-α concentrations (p=0.03, p=0.03, p=0.005, p=0.03, respectively) and lower IL-10 and IFN-γ concentrations (p=0.04 and p=0.03, respectively) in their genital tracts compared to women who did not have discharge, an STI or BV. However, these differences were not significant after adjusting for multiple comparisons. Therefore, vaginal discharge that was not caused by any of the STIs or BV for which the study participants were screened was not associated with substantial changes in cytokine production in the genital tract.

2.4.4 STIs and BV were not the only causes of inflammation in the female genital tract

Although the median concentrations of several cytokines were higher in the genital tracts of women who had STIs compared to women who had no STIs or BV, it was found that there was a large degree of variation in cytokine concentrations among women who had no STIs or BV. Furthermore, some women who had none of the STIs that were evaluated or BV had similar or higher inflammatory cytokine concentrations compared to women who had chlamydia or gonorrhoea (Figure 2.9). Therefore, STIs and BV were not the only cause of cervicovaginal inflammation in this cohort of women. Previous studies have suggested that certain behavioural or demographic factors may be associated with increased inflammation in the female genital tract. High-risk sexual activity or even "normal" sex acts may cause microabrasions in the genital tract, resulting in an inflammatory response (Norvell *et al.*, 1984; Guimaraes *et al.*, 1997; de Jong and Geijtenbeek, 2008). Antiseptic douching, exposure to proteins in seminal plasma, hormone contraceptive use and hormone cycling are also associated with changes in the genital inflammatory environment (Norvell *et al.*, 1984;

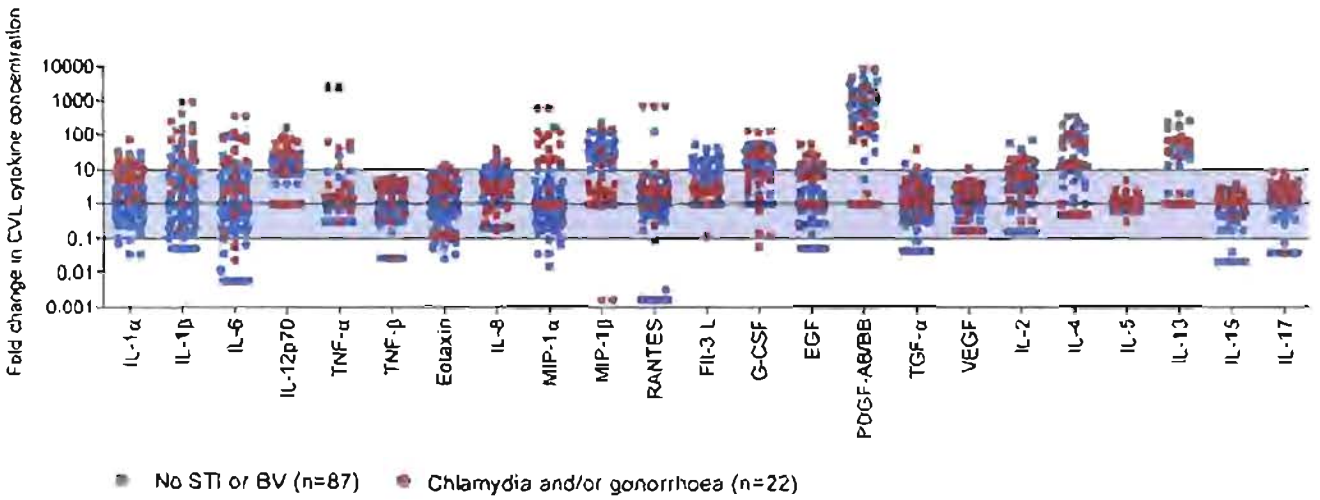


Figure 2.9 STIs and BV were not the only causes of genital inflammation. The fold change in cervicovaginal cytokine concentrations in women who did not have an STI or BV (blue dots) and women who had either chlamydia or gonorrhoea (red dots) relative to the median concentrations in women who did not have an STI or BV is shown.

Scholes *et al.*, 1993; Guimaraes *et al.*, 1997; Hunt *et al.*, 1997; Akoum *et al.*, 2000; Prakash *et al*, 2001 b, Ghanem *et al.*, 2005; Berlier *et al.*, 2006; Sharkey and Robertson, 2007; de Jong and Geijtenbeek, 2008).

In this study, associations between CVL cytokine concentrations and the age of study participants were investigated. It was found that IL-1β, IL-6, TNF-β, GRO, IL-8, GM-CSF, IL-5 and IL-1Ra were higher in younger women before adjusting for multiple comparisons, with IL-6 (rho=-0.212, adjusted p=0.025) and IL-1Ra (rho=-0.229, adjusted p=0.021) remaining significantly associated after adjustment. In contrast, EGF, IFN-α and PDGF-AA were positively associated with age, being highest in older women, however these associations were not significant after adjusting for multiple comparisons. Upon exclusion of all women who had an STI or BV, only TNF-β and PDGF-AA were correlated with age in the remaining women (n=87) and these associations were again not significant following adjustment for multiple comparisons. Therefore, higher levels of genital inflammation that were found in younger women may be due to their risk profile and thus increased prevalence of STIs and BV in these women compared to older women. In support, it was found that chlamydia infections were more common in younger women compared to older women in this cohort (β-coefficient=-2.78, p=0.016; Mlisana *et al.*, unpublished).

As a number of the women who were included in this study were potentially perimenopausal (n=38), cytokine concentrations were compared in women who were 45 years and older to women who were younger than 45 years. It was found that several pro-inflammatory cytokines (IL-1β, IL-6, IL-12p70, TNF-β), chemokine GRO, hematopoietic cytokines (G-CSF, IL-7, FLT3L), adaptive immune mediators (IL-2, IL-4, IL-5) and immunoregulatory IL-1Ra were lower in the genital tracts of women who were 45

years and older, although none were significant after adjusting for multiple comparisons. In order to account for the changes in cytokine concentrations that may occur in perimenopausal women, logistic regression analyses were used to examine the relationships between each cytokine functional group and each STI, while adjusting for other STIs and whether a women was likely to be perimenopausal or not. It was found that each association between each STI and cytokine functional factor that is shown in Table 2.2 was upheld after adjusting for other STIs, the age of study participants and multiple comparisons, except the association between gonorrhoea and adaptive immune mediators. Therefore, the relationship between cytokine functional clusters and STIs was found to be independent of whether or not a woman was likely to be perimenopausal.

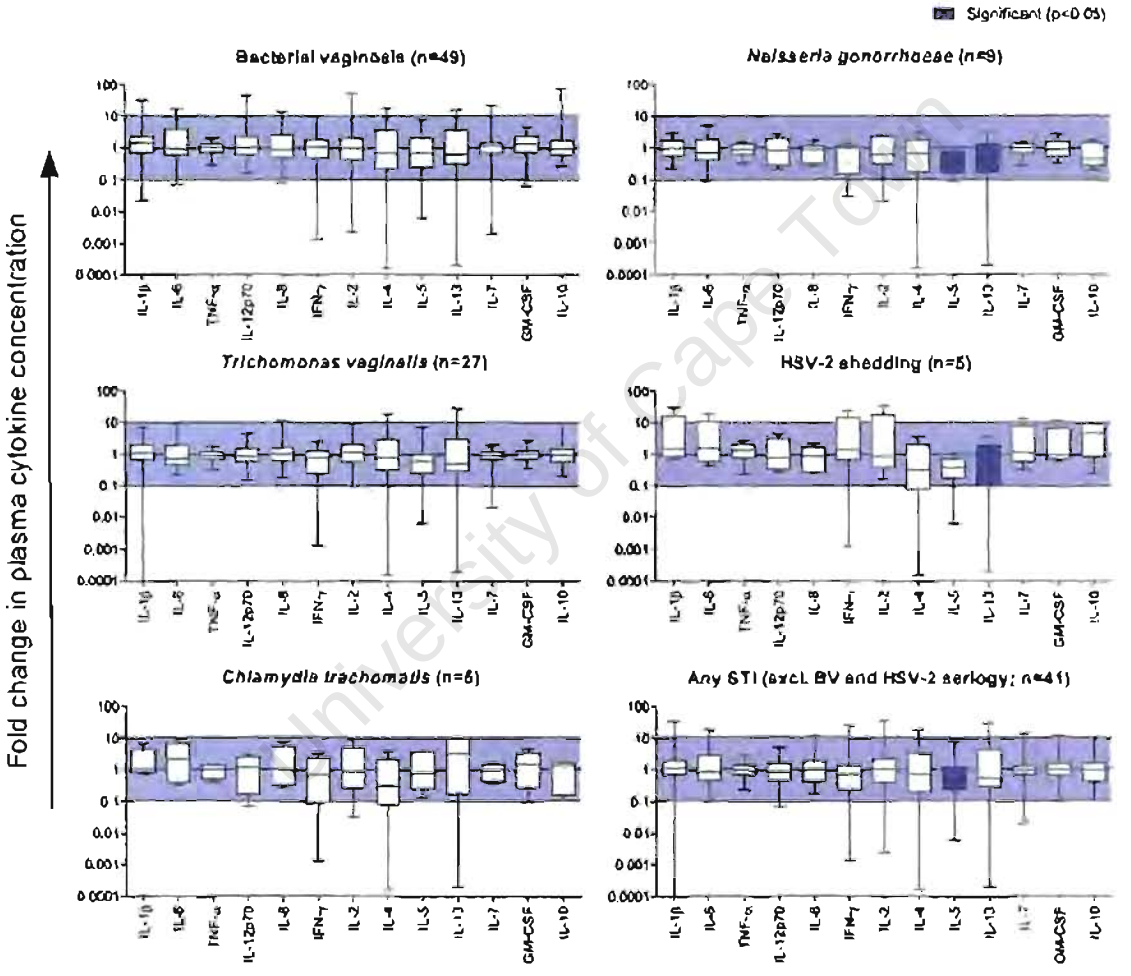


Figure 2.10 Local genital infections were not associated with systemic inflammation. Fold change in plasma cytokine concentrations in women who had bacterial vaginosis (BV), trichomoniasis, chlamydia, gonorrhoea, herpes simplex virus type 2 (HSV-2) and women with one or more of any of these infections (excluding BV) compared to the median cytokine concentrations in women who had no STIs or BV. Lines indicate the median fold change in cytokine concentrations in each group, whiskers indicate range. Blue bars were significant before adjusting for multiple comparisons ($p<0.05$). No differences in cytokine concentrations were significant after adjusting for multiple comparisons.

2.4.5 Localized genital infections were not associated with inflammation in blood

The concentrations of 13 cytokines were measured in plasma samples from 140 women who participated in this study in order to determine whether genital infections influence the systemic inflammatory environment. None of the inflammatory cytokines assessed differed significantly in plasma from women who had BV or STIs relative to women who did not have STIs or BV (Figure 2.10). Only concentrations of IL-5 and IL-13 in plasma samples from women who had active HSV-2 (p=0.039 and p=0.024, respectively) and IL-5 in women who had gonorrhoea (p=0.011) tended to be down-regulated relative to women who did not have an STI or BV. However, perhaps due to the small number of women with each of these STIs, these changes were not significant after adjusting for multiple comparisons. Women who had positive HSV-2 serology had lower concentrations of IL-4 in plasma compared to women who had negative serology (p=0.039), although this association was not upheld after adjustment for multiple comparisons (Figure 2.11). None of the 13 cytokines measured in plasma samples correlated with those measured in CVL samples (n=140), indicating that the inflammatory cytokine network in the genital tract is likely to be independent of circulating cytokine concentrations.

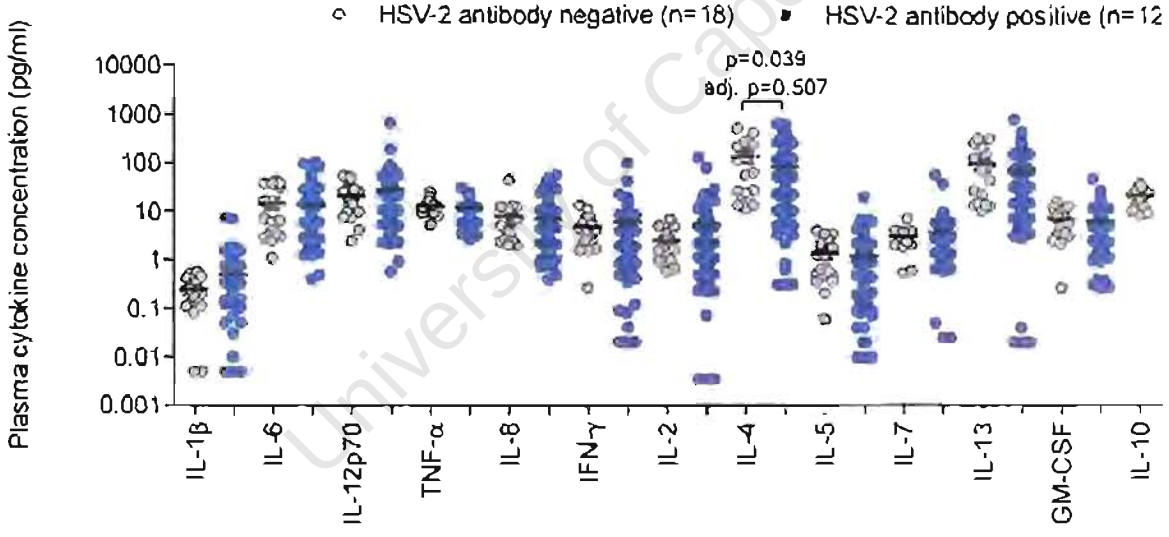


Figure 2.11 Women who were HSV-2 antibody positive did not have elevated plasma cytokine concentrations Cytokine concentrations in plasma from women who were HSV-2 IgG negative (grey dots; n=18) and HSV-2 IgG positive (blue dots; n=120) are shown. Lines indicate the median cytokine concentrations in each group. No differences in cytokine concentrations were significant after adjusting for multiple comparisons.

2.4.6 Women who later became HIV-1-infected had pre-infection genital inflammation

Of the 230 women included in this study, 24 became HIV-1-infected during the 24 month follow-up period (van Loggerenberg *et al.*, 2008). Cox survival analysis was used to investigate the relationship between inflammatory cytokine concentrations at enrolment and risk of HIV-1 infection. It was found that several cytokines were associated with risk of HIV-1 infection, although these associations were not significant after adjustment for multiple comparisons. Elevated concentrations of IL-1β, IL-6, IL-8 and sCD40L were associated with increased risk of HIV-1 infection [HR: 1.25 (95% CI. 1.05 – 1.50).

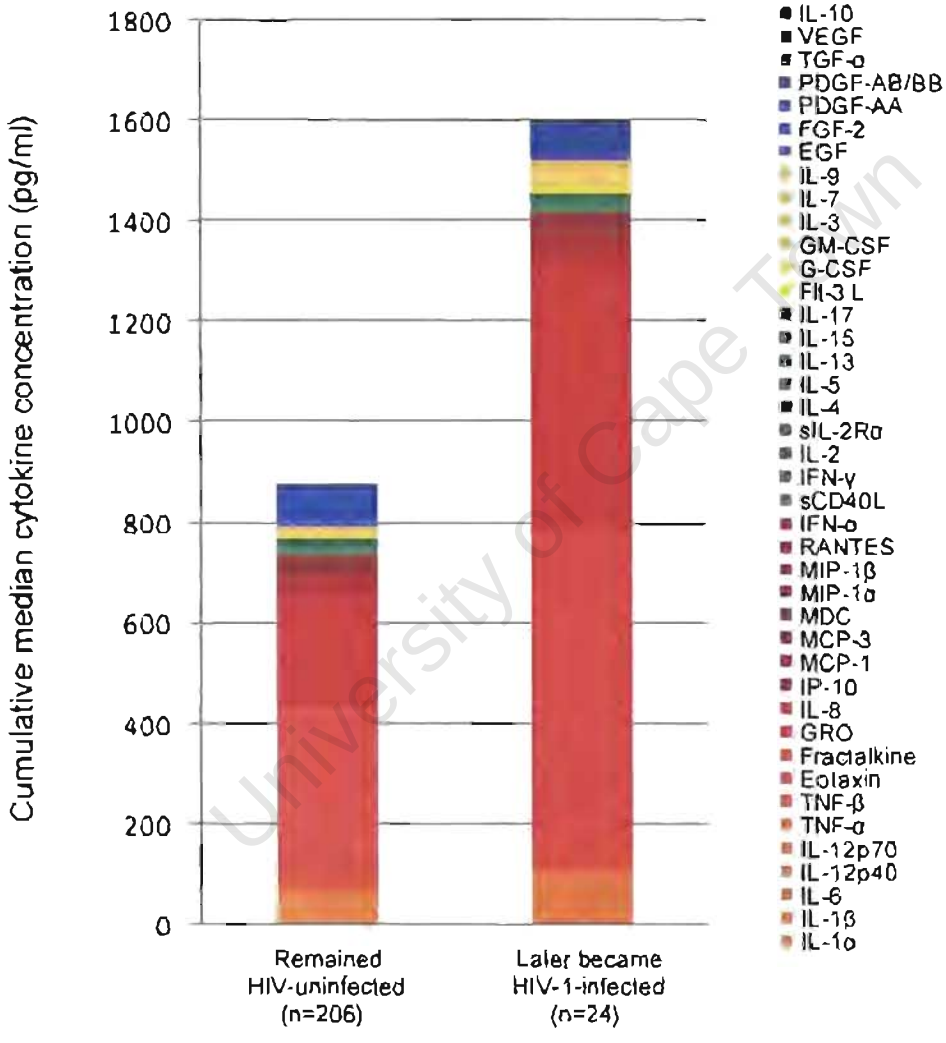


Figure 2.12 Cumulative median cytokine concentrations in the genital tracts of women who remained HIV-uninfected compared to women who later became infected. The median concentrations of each of the 42 cytokines, excluding IL-1Ra, that were measured in CVL samples from women who remained HIV-uninfected (n=206) and pre-infection CVL samples from women who later became HIV-1-infected (n=24) are shown in each stacked bar. Pro-inflammatory cytokines and chemokines are shown in oranges, reds and brown. Adaptive immune mediators are green, hematopoietic cytokines are yellow, growth factors are blue and immunoregulatory IL-10 is black. IL-1Ra was excluded because this cytokine is present in CVL samples at very high concentrations and its inclusion prevents visualization of the other cytokines.

1.28 (1.07 – 1.54), 1.39 (1.04 – 1.87) and 1.45 (1.02 – 2.07), respectively, per 1 $\log_{10}$ pg/ml increase in cytokine concentration]. Furthermore, the pro-inflammatory cytokine cluster was associated with increased susceptibility to HIV-1 infection [HR: 1.53 (95% CI: 1.02 - 2.28); Table 2.2]. Figure 2.12 shows the cumulative median cytokine concentrations measured in CVL from women who remained HIV-uninfected compared to women who later became HIV-1-infected. This analysis was however confounded by a number of factors, including (i) the long period of time between cytokine measurements and HIV-1 incidence (median 43 weeks, range 2 – 98), (ii) the relatively small proportion of women who became HIV-1-infected during the study period and (iii) the fact that women were treated for STIs during the follow-up period. Although samples for cytokine measurements were only collected at enrolment into the study, each woman was screened for STIs 6-monthly. It was found that the average number of STIs per visit was associated with risk of HIV-1 infection (Mlisana *et al.*, unpublished; Figure 2.13A). In order to achieve an estimate of the average level of genital inflammation that was present in each woman over time, each STI and BV were given scores according to the overall level of inflammation that was found in women who had these infections at enrolment into the study. This analysis included 215 women who remained HIV-uninfected and 25 women who became HIV-1-infected during follow-up (an additional 10 women whose cytokine concentrations were not assessed were included). STIs and BV were ranked according to the level of associated inflammation: BV or trichomoniasis (1), HSV-2 shedding (2) and chlamydia or gonorrhoea

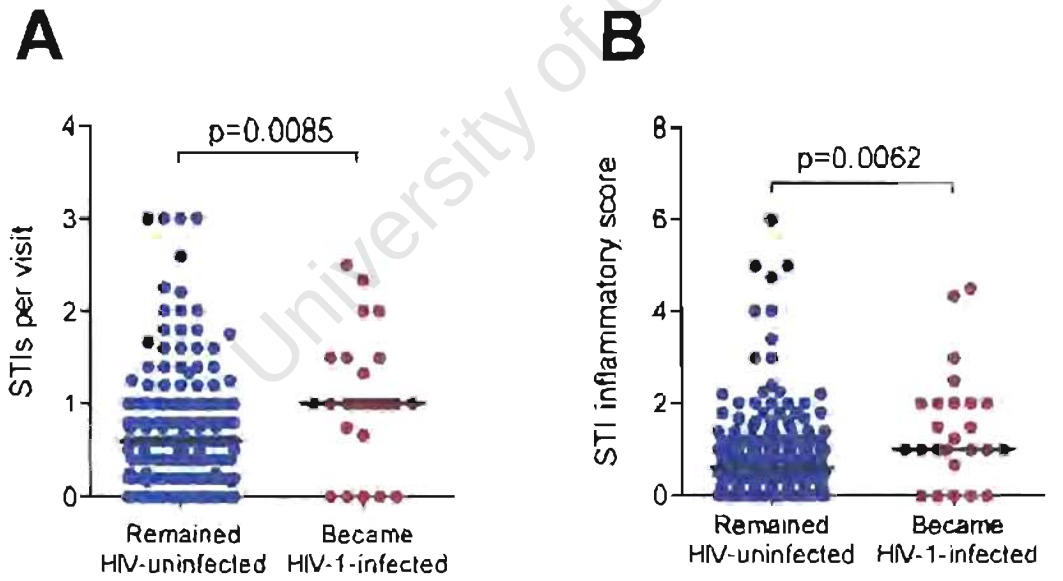


Figure 2.13 Recurrence of inflammatory STIs in women who later became HIV-1-infected. STI recurrence (average number of STIs diagnosed per visit) and STI inflammatory scores in women who remained HIV-uninfected (blue dots; n=215) compared to women who later became HIV-1-infected (red dots; n=25). Lines indicate the median number of STIs per visit (A) or STI inflammatory score (B) in each group. Women who later became HIV-1-infected had higher numbers of STIs and higher STI inflammatory scores per visit relative to women who remained HIV-uninfected.

(3). An "STI inflammatory score" per visit was then calculated for each woman according to the number of STIs or BV that she had at each visit. It was found that women who became HIV-1-infected had higher STI inflammatory scores compared to women who remained HIV-uninfected Figure 2.13B).

2.5 Discussion

HIV-uninfected women who had STIs, in particular those who had chlamydia or gonorrhoea, were found to have up-regulated cervicovaginal concentrations of many of the cytokines that were measured in this study. Pro-inflammatory cytokines and chemokines (IL-1α, IL-1β, IL-6, TNF-α, IL-8, MIP-1β and RANTES), hematopoietic G-CSF and FLT3L, growth factor TGF-α and adaptive immune mediators (IL-2, IL-13 and IL-17) were elevated in CVL from women who had chlamydia relative to women who did not have STIs or BV. Similarly, women who had gonorrhoea had higher cervicovaginal concentrations of pro-inflammatory cytokines (IL-1α, IL-1β, IL-12p70 and TNF-α), chemokines (eotaxin, MIP-1α and RANTES), G-CSF and FLT3L, growth factor VEGF and cytokines that function in adaptive immunity (IL-2, IL-5, IL-15 and IL-17) than women who did not have STIs or BV.

Binding of microbial products to TLRs, which are expressed on neutrophils, phagocytes, epithelial and endothelial cells, results in pro-inflammatory cytokine and chemokine induction and subsequent recruitment of additional immune cells (Dinarello *et al.*, 1997; Connolly *et al.*, 2005; Wira *et al.*, 2005; Abbas and Lichtman, 2007; Mirmonsef *et al.*, 2011). In addition, APCs present phagocytosed microbial proteins to T cells in peripheral lymph nodes, which in turn results in up-regulated chemokine receptor expression by these T cells and their migration to sites of inflammation (Banchereau and Steinman, 1998; Weninger *et al.*, 2002). Nkwanyana *et al.* (2009) demonstrated that concentrations of pro-inflammatory cytokines and chemokines (IL-1β, IL-6, IL-12p70, TNF-α and IL-8) measured in cervical cytobrush supernatant samples were positively correlated with numbers of neutrophils, T cells, B cells, monocytes and Langerhans cells in cervical samples. T cells are the principal source of many of the adaptive immune cytokines that were measured in this study (IFN-γ, IL-2, IL-4, IL-5, IL-13 and IL-17; Abbas and Lichtman, 2007). Therefore, recruitment and activation of T cells by pro-inflammatory cytokines and chemokines would likely result in increased production of adaptive immune cytokines in the genital tract. It was found that both Th1 cytokines (IFN-γ and IL-2), which promote cellular immunity, and Th2 cytokines (IL-5 and IL-13), which stimulate antibody responses (Abbas and Lichtman, 2007), were up-regulated in the genital tracts of women who had bacterial STIs. Nkwanyana *et al.* (2009) further demonstrated that elevated concentrations of regulatory IL-10 were associated with greater numbers of neutrophils and B cells in cervical cytobrush samples. In this study, genital IL-10 concentrations correlated positively with pro-inflammatory cytokine concentrations. The findings of this study are consistent with those of previous studies that IL-10 is up-regulated during inflammatory responses and may be induced by pro-inflammatory cytokines in order to reduce inflammation (Couper *et al.*, 2008). Pro-inflammatory cytokines stimulate

growth factor production, which function in tissue repair, lymphangiogenesis and angiogenesis (Tammela *et al.*, 2005; Rafii *et al.*, 2003; Cursiefen *et al.*, 2004; Ristimaki *et al.*, 1998; Ferrara, 2004). Therefore, up-regulation of pro-inflammatory cytokines is likely to be followed by increased growth factor secretion and one would thus expect to find growth factor up-regulation at sites of inflammation, as observed in the genital tracts of study participants who had bacterial STIs and robust inflammatory responses.

The cervicovaginal concentrations of the majority of the assessed cytokines correlated with one another, regardless of their functional classification, suggesting that most of the cytokines measured in this study are indeed components of an inflammatory cascade. Furthermore, in an unrotated EFA, 27/42 cytokines loaded onto the same factor and the estimates of most of the 6 factors generated correlated with one another. The primary goal of EFA in this study was to identify a common underlying factor or cluster of cytokines that was reflective of inflammation caused by STIs and predictive of susceptibility to HIV-1 infection. It was found that, of the clusters of cytokines that were investigated, up-regulation of the pro-inflammatory cytokine cluster (IL-1α, IL-1β, IL-6, IL-12p40, IL-12p70, TNF-α and TNF-β) was perhaps best indicative of the presence of an STI and increased risk of HIV-1 infection. This cluster was positively associated with BV, chlamydia and gonorrhoea after adjusting for multiple comparisons and with HSV-2 shedding and risk of HIV-1 infection before adjustment. Importantly, measurement of these cytokines may be useful as biomarkers of STIs and susceptibility to HIV-1 infection. Of the cytokines that were measured in this study, it was found that IL-1β was the best biomarker for BV and each of the STIs that were assessed. This cytokine was elevated in CVL from women who had BV, chlamydia or gonorrhoea after adjusting for multiple comparisons and from women who had trichomoniasis and active HSV-2 before adjustment. An increase of 1 $\log_{10}$ pg/ml IL-1β in CVL was associated with a 25% increased risk of HIV-1 infection, although this was not significant after adjusting for multiple comparisons.

In addition to BV and the STIs that were assessed in this study, several behavioural and demographic factors that were not evaluated in this study are thought to be associated with increased inflammation in the female genital tract. High-risk sexual activity or even "normal" sex acts may cause microabrasions in the genital tract which increase inflammation (Norvell *et al.*, 1984; Guimaraes *et al.*, 1997; de Jong and Geijtenbeek, 2008). Hygiene practices such as antiseptic douching, proteins in seminal plasma, lubricants, hormone contraceptive use and hormone cycling are associated with genital inflammation (Scholes *et al.*, 1993; Hunt *et al.*, 1997; Akoum *et al.*, 2000; Prakash *et al.*, 2001b; Fichorova *et al.*, 2001b; Ghanem *et al.*, 2005; Berlier *et al.*, 2006; Sharkey and Robertson, 2007). Several studies have also suggested that demographic characteristics of women, such as ethnicity, may influence the inflammatory environment in the female genital tract (Gonzalez *et al.*, 2005; Ryckman *et al.*, 2008; Zabaleta *et al.*, 2008; Field *et al.*, 2009; Liu *et al.*, 2010). In this study it was found that some women who did not have any of the STIs that were assessed or BV had similar levels of inflammation compared to women who had STIs that were associated with particularly robust inflammatory responses, chlamydia and gonorrhoea. The causes of this inflammation may be other

STIs, such as human papillomavirus (HPV; Crowley-Nowick *et al.*, 2000), or infections that were not assessed in this study or behavioural factors. Interestingly, it was found that younger women had higher cervicovaginal concentrations of several cytokines compared to older women, with pro-inflammatory IL-6 and anti-inflammatory IL-1Ra remaining significantly associated with age after adjusting for multiple comparisons. These associations were, however, not observed in women who did not have an STI or BV (n=87), suggesting that the relationship between age and genital cytokine concentrations may be due to an increased prevalence of STIs and BV in younger women, possibly due to high-risk behavior.

The primary functions of hematopoietic cytokines, growth factors and adaptive immune mediators are not in inflammation, however many have secondary pro-inflammatory functions. For example, G-CSF and GM-CSF, which play important roles in differentiation of granulocytes from stem cell progenitors, also stimulate pro-inflammatory cytokine production by immune cells and are chemoattractant for leukocytes (Wira *et al.*, 2005; Gomez-Cambronero *et al.*, 2003). Hematopoietic IL-7, which is important for T cell production, differentiation and survival, also induces NK-κB and pro-inflammatory cytokine expression (Alderson *et al.*, 1991; Chene *et al.*, 1999; Damås *et al.*, 2003). IFN-γ, which promotes cellular immunity during adaptive immune responses, may too induce pro-inflammatory cytokine production and promote leukocyte recruitment from circulation (Abbas and Lichtman, 2007). IL-17, which is produced by Th17 cells and plays an important role in defence against bacterial infections, stimulates pro-inflammatory cytokine production by macrophages (Abbas and Lichtman, 2007). VEGF is a growth factor that induces angiogenesis and lymphangiogenesis, which facilitates leukocyte trafficking (Tammela *et al.*, 2005; Rafii *et al.*, 2003; Cursiefen *et al.*, 2004). Therefore, these cytokines are induced during the inflammatory process and, in addition to their other immune functions, may play a role in promoting further inflammation.

Interestingly, while women with BV had up-regulated pro-inflammatory cytokine concentrations (IL-1α, IL-1β and TNF-β), it was found that several chemokines (IP-10, GRO, MDC and MIP-1α), hematopoietic cytokines (IL-7 and GM-CSF) and IFN-γ were down-regulated relative to women who did not have STIs or BV. Similarly, Ryckman *et al.* (2008) reported that, while cervicovaginal IL-1α was elevated in women with BV, IP-10 and MCP-1 were down-regulated, compared to women who did not have BV. Because most of these cytokines regulate the expression of others and are part of an inflammatory cascade, it is counterintuitive that one arm, the pro-inflammatory cytokines, is up-regulated in the genital tracts of women with BV, while chemokines and other cytokines that may participate in inflammation are down-regulated. A better understanding of this relationship may be achieved by profiling inflammatory transcriptional networks in immune and non-immune cells sampled from the genital tracts of women who have BV in future studies.

Women who had trichomoniasis did not have increased levels of inflammation relative to women who did not have STIs or BV in this study. Similarly, in a previous study it was found that, although women who had chlamydia or gonorrhoea had increased numbers of endocervical CD4+ T cells relative to

women with no STIs, this was not observed in women who had trichomoniasis (Levine *et al.*, 1998). Despite these findings, trichomoniasis has previously been associated with increased risk of HIV-1 infection, suggesting that other mechanisms may be responsible (Levine *et al.*, 1998). This may be mediated by punctuate mucosal haemorrhages that are caused by this infection (Fouts and Kraus, 1980), degradation of the anti-HIV-1 molecule SLPI (Draper *et al.*, 1889), and increased susceptibility to BV and other infections (Moodley *et al.*, 2002). Although women who were actively shedding HSV-2 tended to have up-regulated cytokine concentrations compared to women who did not have STIs or BV, none were significant after adjusting for multiple comparisons. However, this is most likely due to the small number of women who had active HSV-2 in this study (n=8).

It was found that localized genital infections were not associated with increased inflammatory cytokine concentrations in plasma. There was however a trend toward down-regulation of Th2 cytokines, IL-5 and IL-13, in plasma from women who were shedding HSV-2 or had gonorrhoea. Women who were shedding HSV-2 also tended to have higher plasma concentrations of pro-inflammatory IL-1β, IL-6 and IFN-γ, although this was not statistically significant. Previous studies have shown alterations in blood HIV-1-specific immune responses in individuals who have gonorrhoea or chronic HSV-2. In this study, detection of HSV-2 IgG in blood was not associated with changes in genital or plasma cytokine concentrations. It was found that none of the 13 cytokines that were measured in both plasma and CVL correlated between compartments, suggesting that cytokine expression networks and inflammation is compartmentalized in the genital tract.

Importantly, genital tract inflammatory cytokine concentrations were similar in women who had symptomatic and asymptomatic laboratory-diagnosed STIs (excluding BV and HSV-2 serology), and were elevated in these women relative to women who did not have STIs or BV. Previous studies have suggested that higher concentrations of inflammatory cytokines in the genital tract may facilitate HIV-1 transmission by directly up-regulating HIV-1 replication and by recruiting and activating immune cell targets for HIV-1 infection (Swingler *et al.*, 1999; Nkwanyana *et al.*, 2009; Li *et al.*, 2009). Inflammatory cytokines have also been associated with disruption of tight junctions between epithelial cells (Madara and Stafford, 1989; Schmitz *et al.*, 1996; Nazli *et al.*, 2010). In this study, weak associations between elevated concentrations of IL-1β, IL-6, IL-8 and sCD40L in the genital tract and increased risk of HIV-1 infection were found. This analysis was however confounded by the long period of time between cytokine measurements and HIV-1 infection, the relatively small number of women who became HIV-1-infected (n=24/230; van Loggerenberg *et al.*, 2008) and the fact that women were treated for STIs during the period between sample collection and HIV-1 infection. Therefore, recurrence of STIs/BV and the relative degree of genital inflammation associated with each STI/BV (STI inflammatory score) were used as indicators of sustained inflammation. It was found that women who later became HIV-1-infected had more frequent recurrence of STIs and higher inflammatory scores per visit relative to women who remained HIV-uninfected. Therefore, women who became HIV-1-infected in this study were more likely to have sustained genital inflammation over time. Previous studies have found that STIs and BV are often recurrent and women who are treated

for STIs may be re-infected by untreated partners (Sweet, 1993; Wawer *et al.*, 1999). The relationship between STI recurrence/STI inflammatory scores and HIV-1 infection may however be indirect. Recurrence of STIs may indicate high-risk behaviour (frequent sexual intercourse with multiple partners) and rates of HIV-1 infection in women with recurrent STIs may be higher as a result of more frequent exposure to HIV-1, rather than a direct association between inflammatory STIs and HIV-1.

The findings of this study suggest that even women who have no clinical symptoms, but have subclinical STIs, may be at increased risk of HIV-1 infection and that underlying inflammation may be an important mechanism for HIV-1 transmission in these women. In support, while the presence of any laboratory-diagnosed STI (excluding BV and HSV-2 serology) was associated with a 3.7-fold increased risk of HIV-1 infection in this cohort, vaginal discharge was not (Mlisana *et al.*, unpublished data). Therefore, these findings suggest a plausible explanation for the failure of two of three syndromic management interventions to reduce HIV-1 incidence (Grosskurth *et al.*, 1995; Kamali *et al.*, 2003; Gregson *et al.*, 2007). In a syndromic management setting, 81.8% of the women in this cohort who were asymptomatic but had active laboratory-evident STIs, but in many cases, large amounts of subclinical inflammation, would have been left untreated. Additionally, some women who tested negative for BV and each of the assessed STIs were found to have comparable levels of genital inflammation to women with particularly inflammatory STIs, chlamydia or gonorrhoea. Future studies should thus aim to assess other potential causes of this inflammation (including behavioural and demographic factors and other infections) and the possible role in risk of HIV-1 infection. Not only does this have important implications for HIV-1 prevention strategies, but also for prevention of complications that are associated with untreated inflammatory STIs, including pelvic inflammatory disease, ectopic pregnancy and tubal factor infertility (Moodley *et al.*, 2000; Wiesenfeld *et al.*, 2002). The findings of this study emphasize the need for better STI management strategies in countries with high incidence of HIV-1, such as South Africa, and suggest that laboratory diagnosis of STIs or measurement of cytokine biomarkers of inflammation, such as IL-1β, may reduce occurrence of both reproductive complications and HIV-1 incidence.

CHAPTER 3

Chapter 3

Genital tract inflammation during early HIV-1 infection predicts higher plasma viral load set-point in women

3.1 Summary

The biggest challenge in HIV-1 prevention in Africa is the high HIV-1 burden in young women. In macaques, pro-inflammatory cytokine production in the genital tract is necessary for target cell recruitment and establishment of SIV infection following vaginal inoculation. The purpose of this study was to assess if genital inflammation during early HIV-1 infection predisposes to rapid disease progression in women. Inflammatory cytokine concentrations were measured in cervicovaginal lavage (CVL) from 49 women 6, 17, 30 and 55 weeks post-HIV-1-infection and 22 of these women pre-infection using Luminex Multiplex Flow Cytometric Assays. Associations between genital inflammation and viral load set-point and blood CD4 counts 12 months post-infection were investigated. It was found that elevated genital cytokine concentrations 6 weeks (GM-CSF, IL-1β, IL-12p70 and IL-15) and 17 weeks (GM-CSF, IL-1β, IL-10, IL-6, RANTES, MIP-1β and IL-2) post-HIV-1-infection were associated with higher viral load set-points and, to a lesser extent, with CD4 depletion (GM-CSF). CVL cytokine concentrations during early infection did not differ relative to pre-infection, but were elevated in women who had vaginal discharge, detectable HIV-1 RNA in their genital tracts and lower blood CD4 counts. Genital inflammation during early HIV-1 infection was associated with higher viral load set-point and CD4 depletion, which are markers of rapid disease progression. Strategies aimed at reducing genital inflammation during early HIV-1 infection may slow disease progression.

3.2 Introduction

The genital mucosa is the initial site of viral replication following vaginal transmission of HIV-1 and SIV (Pope and Haase, 2001; Miller *et al.*, 2005). In rhesus macaques, vaginal inoculation with SIV is followed by local viral expansion in a small founder population of cells and subsequent seeding of the systemic compartment is dependent on pro-inflammatory cytokine production and recruitment of CD4+ T cell targets for SIV infection (Li *et al.*, 2009; Haase, 2011). These pro-inflammatory cytokines are produced in the genital tract shortly following vaginal infection and correlate with viral replication. In most cases, genital tract cytokine levels return to baseline as peak plasma SIV viremia declines (Abel *et al.*, 2005). HIV-1 infection may likewise be accompanied by an early and transient inflammatory cytokine cascade in the genital tract that is associated with viral replication in this compartment. During chronic HIV-1 infection, women with detectable HIV-1 RNA in their genital tracts have higher levels of several pro-inflammatory cytokines (Gumbi *et al.*, 2008). HIV-1 has been shown to directly induce inflammatory cytokine production via TLR 7 and 8 activation (Meier *et al.*, 2007). Elevated concentrations of inflammatory cytokines in turn may promote HIV-1 replication by recruiting and activating target cells for HIV-1 infection (Swingler *et al.*, 1999; Nkwanyana *et al.*, 2009; Li *et al.*, 2009), and directly up-regulating HIV-1 replication by NF-κB activation (Osborne *et al.*, 1989).

A number of studies have shown that genital tract pro-inflammatory cytokines are up-regulated in women with early or chronic HIV-1 infection relative to HIV-uninfected women (Belec *et al.*, 1995; Crowley-Nowick *et al.*, 2000; Zara *et al.*, 2004; Bebell *et al.*, 2008; Guha and Chatterjee, 2009; Nkwanyana *et al.*, 2009). However this up-regulation may be attributed to the high frequency of sexually transmitted co-infections or BV in these individuals rather than HIV-1 itself (Fennema *et al.*, 1995). For example, BV was associated with increased concentrations of pro-inflammatory IL-1β, while chronic HIV-1 infection was not (Mitchell *et al.*, 2008). HIV-1 shedding, which is associated with STIs (Johnson and Lewis, 2009), may induce further inflammatory cytokine production.

In macaques, treatment with cytokines such as IL-12p70 and IL-15 during acute SIV infection is associated with altered disease course (Ansari *et al.*, 2002; Mueller *et al.*, 2008; Okoye *et al.*, 2009). Several studies have suggested that cytokine responses in the genital tract during the early stages of SIV/HIV-1 infection may likewise be associated with disease progression. In macaques, induction of inflammatory cytokines and immune cell influx into the genital tract prior to vaginal SIV inoculation was associated with increased plasma viral load set-points (Wang *et al.*, 2005). This suggests that pre-existing genital inflammation in humans may similarly influence long-term HIV-1 disease progression. Zara *et al.* (2004) demonstrated that up-regulation of IL-1β in the genital tract of HIV-1-infected women was associated with increased plasma viral loads. Additionally, it was recently reported that elevated levels of IL-1β, IL-6 and IL-8 in CVL correlated with lower blood CD4+ T cell counts during early HIV-1 infection (Bebell *et al.*, 2008).

In this study, the relationship between genital tract inflammatory cytokine concentrations during the first year of HIV-1 infection and markers of HIV-1 disease progression, plasma viral load set-point and blood CD4+ T cell counts 12 months post-infection, were investigated.

3.3 Materials and methods

3.3.1 Description of study participants

Forty-nine South African women recently infected with HIV-1 Subtype C were recruited from HIV negative cohorts which were screened either monthly or 3 monthly for HIV-1 infection as part of the CAPRISA 002 Acute Infection Study (van Loggerenberg *et al.*, 2008). Each woman provided informed consent and then attended regular evaluations (weekly for 1 month following HIV-1 infection, fortnightly for 2 months, monthly for 9 months and quarterly thereafter) of HIV-1 disease status. Time of infection was defined as the mid-point between the last HIV-1 antibody negative test and the first HIV-1 antibody positive test, or as 14 days prior to a positive RNA PCR assay on the same date as a negative HIV-1 EIA. This study was approved by the University of KwaZulu-Natal and University of Cape Town Ethics Committees.

3.3.2 Cytokine Measurements

CVL samples for cytokine measurements were collected from 49 women at several time-points during HIV-1 infection. Of these women, 39 were assessed during early HIV-1 infection (median 6 weeks post-infection, range 1-13), 32 at 17 weeks post-infection (range 14-23), 39 at 30 weeks post-infection (range 24-36) and 40 at 55 weeks post-infection (range 50-62). Twenty-two women were assessed pre-infection as matched negative controls (median 36 weeks pre-infection, range 2-92). Eighteen of the 22 women who were assessed pre-infection had matching 6 week post-infection CVL samples available. CVL samples were collected from each woman, as previously described (Bebell *et al.*, 2008). Sterile normal saline (10ml) was used to repeatedly bathe the cervix and allowed to pool in the posterior fornix, where it was then aspirated into a plastic bulb pipette. Samples were centrifuged and the supernatant stored at -80°C. CVL samples were not collected from menstruating participants, and sampling was postponed to the following week. Prior to cytokine measurements, CVL samples were filtered by centrifugation using 0.2 µm cellulose acetate filters (Sigma, U.S.A). The concentrations of 20 cytokines were measured in CVL samples using Luminex Multiplex Flow Cytometric Assays. Concentrations of IL-1α, IL-1β, IL-2, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-15, eotaxin, fractalkine, G-CSF, GM-CSF, MCP-1, MIP-1α, MIP-1β, RANTES, sCD40L and TNF-α were measured in pre-infection and 6 week post-infection samples using High Sensitivity and Human Cytokine LINCO*plex* Premixed kits (LINCO Research, MO, U.S.A.) according to the manufacturer's protocol. These cytokines were also measured in 17, 30 and 55 week post-infection CVL using Milliplex^{TM} High Sensitivity and Human Cytokine kits (Millipore Corporation, MA, U.S.A). The lower limit of detection of these kits ranged between 0.05 and 18.33 pg/ml for each of the 20 cytokines measured. Data was

collected using a Bio-Plex™ Suspension Array Reader (Bio-Rad Laboratories Inc®) and a 5 PL regression formula was used to calculate cytokine concentrations from the standard curves. Data was analysed using BIO-plex manager software (version 4; Bio-Rad Laboratories Inc®). IFN-α and MIP-3α were measured in pre-infection and 6 week post-infection CVL using Human IFN-α Multi-Subtype Colorimetric Sandwich ELISA and Human CCL20/MIP-3α Quantikine Colorimetric Sandwich ELISA (PBL Biomedical Laboratories; R&D Systems, Inc.). The lower limits of detection of these kits were 12.5 pg/ml and 0.47 pg/ml for IFN-α and MIP-3α, respectively. Cytokine levels that were below the lower limit of detection of the assay were reported as the mid-point between the lowest concentration measured for each cytokine and zero. Cytokines were selected for this study based associations between cytokines and STIs that were identified in Chapter 2, the findings of previous studies (Bebell *et al.*, 2008), and the reliability and reproducibility of their measurement using Luminex.

3.3.3 Clinical characteristics

Blood for viral load and CD4+ T cell measurements was collected at all visits from study participants by venipuncture into ACD vacutainer tubes. Absolute blood CD4+ T cell counts (cells/μl) were measured using a FACSCalibur flow cytometer. Plasma HIV-1 RNA concentrations (copies/ml) were quantified using the COBAS AMPLICOR™ HIV-1 Monitor v1.5 or COBAS Ampliprep/COBAS TaqMan 48 Analyser (Roche Diagnostics, Branchburg, New Jersey, U.S.A.). Viral loads in CVL were determined using Nuclisens Easyq HIV-1 version 1.2. The detection limit of the assay was 50 copies/ml.

Viral load set-point after 3 months post-infection has previously been shown to be predictive of time to AIDS (Lyles *et al.*, 2000). In this study, viral load set-point, which was defined as the average viral load measurement of 3 consecutive visits between medians of 47 and 56 weeks post-infection (range 37-69), was used to assess disease progression. Additionally, associations between cytokines and i) average of CD4+ T cell measurements at the same 3 consecutive visits and ii) CD4+ T cell loss between pre-infection and 12 months post-infection were investigated.

3.3.4 Screening for STIs and BV

Participants included in the study were screened and treated for STIs prior to HIV-1 infection (median 36 weeks pre-infection), at the visit immediately following an HIV-1 positive test (median 6 weeks post-infection) and 6 monthly thereafter. Gynaecological exams were performed with collection of cervical swabs. *C. trachomatis, N. gonorrhoeae* and HSV-2 were assessed by PCR and *T. vaginalis* by Diamond's culture and PCR. Agents of BV were assessed by Gram stain.

3.3.5 Statistical analyses

Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, U.S.A.) and STATA™ version 10 (StataCorp, Texas, U.S.A.). Distribution of all variables was assessed by Shapiro-Wilk and Shapiro-Francia tests. Chi-squared test was used to compare proportions of women with STIs. Mann Whitney U test was used for unmatched comparisons. Wilcoxon Signed Rank test was used to compare cytokine levels in women matched pre- and post-infection. Spearman Rank test was used for correlations. For regression analyses, cytokines, viral load and CD4 count measurements were log-transformed and cytokines were standardized to allow for direct comparison of β-coefficients. Linear regression was used to evaluate the relationships between cytokine levels and i) CD4+ T cell counts 12 months post-infection and ii) viral load set-point 12 months post-infection. Multivariate regression was used to adjust for blood CD4 counts and plasma viral loads measured at the same visits as cytokine concentrations. Mixed-effects logistic regression was used to assess the change in HIV-1 shedding over time. Regression analyses were used to assess the relationships between disease progression and cytokine concentrations and, although CD4+ T cell counts and plasma viral load set-points were used as dependent variables, causation was not inferred. P-values <0.05 were considered significant. P-values were adjusted using a FDR step-down procedure in order to reduce false positive results when multiple comparisons were made (Columb and Sagadai, 2006).

3.4 Results

Forty-nine women recently infected with HIV-1 were recruited and followed longitudinally. Their median age was 24 years (range 18-59). Most women (98%) were unmarried and 20% reported having more than one partner. A total of 32% reported using injectable hormonal contraceptives. During early HIV-1 infection (6 weeks post-infection), the median CD4+ T cell count in these women was 524 cells/μl (Table 3.1). Their median CD4+ T cell loss during the first 12 months of HIV-1 infection was 486 cells/μl, which represents a 50% decline in CD4+ T cell numbers relative to pre-infection. The median early infection plasma viral load in this group of women was 56 500 copies/ml while the median set-point viral load was 39 783 copies/ml (Table 3.1). Before becoming infected with HIV-1, the prevalence of STIs in this cohort was high, with 36.3% of women diagnosed and treated for active *C. trachomatis, N. gonorrhoeae, T. vaginalis,* or HSV-2 and 68.2% of these women having BV (Table 3.1). During early HIV-1 infection, 38.6% of women tested positive for one or more active STIs and 77.4% had BV.

3.4.1 Cytokine concentrations in the genital tracts of women recently infected with HIV-1 were not significantly elevated compared to pre-infection

The concentrations of 22 cytokines in CVL from women prior to HIV-1 infection (median 36 weeks pre-infection) were compared to those of the same women during early HIV-1 infection (median 6

Table 3.1 Clinical characteristics of study participants

CD4+ T cell counts (cells/µl)	**Median (IQR); cells/µl**	**N**
Pre-infection CD4+ T cell count	975 (860-1149)	22
Six week post-infection CD4+ T cell count	524 (379-685)	48
CD4+ T cell count set-point (average of 3 visits overlying 12 months post-infection)	408 (339-551)	46
CD4+ T cell loss (pre-infection minus 12 month post-infection CD4+ T cell count)	486 (254-653)	20
Plasma viral loads (copies/ml)	**Median (IQR); copies/ml**	**N**
Six week post-infection plasma viral load	56500 (14200-370500)	49
Plasma viral load set-point (ave of 3 visits overlying 12 months post-infection)	39783 (7248-102208)	46
Sexually transmitted infections	**Pre-infection; N/total (%)**	**Early infection; N/total (%)**
Prevalence of active STIs (women with lab diagnosed STI[a])	8/22 (36.3)	17/44 (38.6)
Bacterial vaginosis (women gram stain positive for BV)	15/22 (68.2)	34/44 (77.4)
Vaginal discharge (women with visible discharge)	3/22 (13.7)	8/49 (16.3)
Genital ulcer [women with visible genital ulcer(s)]	0/22 (0)	6/49 (12.2)
Multiple HIV-1 variant transmission	**Dual; N/total (%)**	**Heterogenous; N/total (%)**
Participants with multiple transmitted variants	4/45 (8.9)	7/45 (15.6)

[a]*C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis* or HSV-2. STI: Sexually transmitted infection. IQR: Interquartlie range.

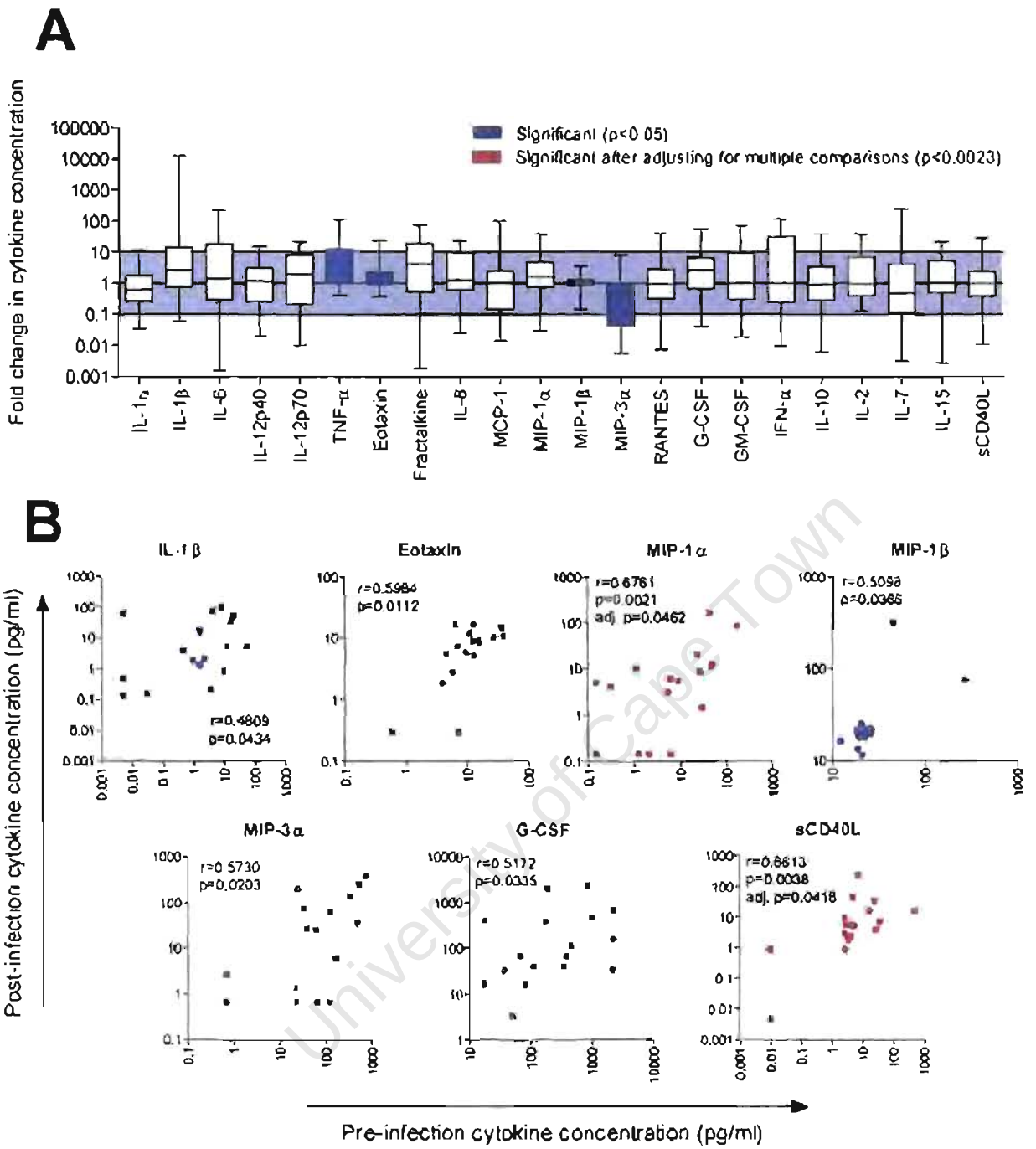


Figure 3.1 Comparison of CVL cytokine concentrations in women (n=18) before infection (median 36 weeks pre-infection) and during early HIV-1 infection (median 6 weeks post-infection). A) Fold changes in cytokine concentrations following HIV-1 infection are shown as box-and-whisker plots, error bars indicate the range. TNF-α, and eotaxin concentrations were elevated in women with early HIV-1 infection relative to concentrations pre-infection, while MIP-3α concentrations were lower in women with early infection. These changes were not significant after adjusting for multiple comparisons and are indicated by blue bars (p<0.05). B) Spearman correlations between cytokine concentrations measured in pre- and post-infection CVL. MIP-1β was no longer correlated between time-points following exclusion of outliers. Blue dots indicate cytokines that correlated significantly before adjusting for multiple comparisons (p<0.05). Red dots indicate cytokines which remained significantly correlated after adjustment (p<0.0023).

weeks post-infection) for whom samples at both time-points were available (n=18/49). In matched samples, TNF-α and eotaxin concentrations were higher in CVLs from early HIV-1 infection compared to pre-infection, whereas MIP-3α concentrations were lower ($p<0.05$; Figure 3.1A). However, these changes were not significant after adjustment for multiple comparisons. Furthermore, in an unmatched analysis, including all women who had either pre-infection (n=22) or 6 week post-infection samples available (n=39), only MIP-3α concentrations were lower in post-infection CVLs, before adjusting for multiple comparisons. These data indicate that, in contrast to previous reports which have compared genital tract cytokine concentrations in unmatched women (Belec *et al.*, 1995; Crowley-Nowick *et al.*, 2000; Zara *et al.*, 2004; Bebell *et al.*, 2008; Guha and Chatterjee, 2009; Nkwanyana *et al.*, 2009), inflammatory cytokines in the genital tracts of the women in this study were not significantly altered during early HIV-1 infection relative to matched pre-infection samples.

Interestingly, the concentrations of 7/22 cytokines measured in CVLs were found to correlate pre- and post-HIV-1 infection (Figure 3.1B; IL-1β, eotaxin, MIP-1α, MIP-1β, MIP-3α, G-CSF, and sCD40L). MIP-1α and sCD40L remained significantly correlated after adjusting for multiple comparisons. Furthermore, following evaluation of cytokine concentrations in longitudinal samples (at 17, 30 and 55 weeks post-infection), it was found that more than half of the cytokines assessed (11/20) correlated between at least 3 of the 5 time-points (IL-1α, IL-β, IL-6, IL-12p40, IL-8, MIP-1α, MIP-1β, RANTES, G-CSF, GM-CSF and sCD40L; Table 3.2). IL-1α, IL-1β, IL-6, IL-8, MIP-1α, G-CSF and GM-CSF concentrations in CVL correlated across ≥ 3 time-points after adjusting for multiple comparisons. These findings suggest that the relative degrees of genital tract inflammation in individual women remained relatively constant pre- and post-infection, as well as during the first 12 months of HIV-1 infection.

3.4.2 Genital tract inflammation during early HIV-1 infection and multi-variant HIV-1 transmission

Abrahams *et al.* (2009) have reported that in this cohort of recently infected women, 22% were infected with more than one HIV-1 genetic variant. In a previous study, it was found that multi-variant transmission was associated with the presence of inflammatory STIs (Haaland *et al.*, 2009). Therefore, in order to investigate the relationship between inflammatory cytokine concentrations in the genital tract and transmission of multiple HIV-1 variants, cytokine concentrations at 6 weeks post-infection were compared in women with multiple (n=10) or single (n=25) transmitted variants in this cohort. No differences were found between the concentrations of any of the genital tract inflammatory or adaptive cytokines assessed (Figure 3.2), suggesting that genital inflammation was not associated with the break in HIV-1 transmission bottleneck in this cohort.

Table 3.2 Spearman Rank correlations between cytokines measured at different time-points

Cytokine	-36/6 wks (n=18)	-36/17 wks (n=15)	-36/30 wks (n=19)	-36/55 wks (n=20)	6/17 wks(n=25)	6/30 wks (n=31)	6/55 wks (n=32)	17/30 wks (n=24)	17/55 wks (n=27)	30/55 wks (n=33)
	Rho (p-value)	Rho (p-value)	Rho (p-value)	Rho (p-value)	Rho (p-value)	Rho (p-value)	Rho (p-value)	Rho (p-value)	Rho (p-value)	Rho (p-value)
IL-1α	0.296 (0.233)	0.393 (0.148)	0.091 (0.710)	0.211 (0.387)	**0.524 (0.007)**	**0.429 (0.016)**	**0.575 (0.0007)**	0.395 (0.056)	**0.484 (0.011)**	**0.694 (<0.0001)**
IL-1β	**0.481 (0.043)**	0.454 (0.089)	-0.011 (0.966)	0.096 (0.686)	**0.495 (0.012)**	**0.357 (0.049)**	**0.439 (0.012)**	0.265 (0.210)	**0.640 (0.0003)**	**0.551 (0.0009)**
IL-6	0.232 (0.354)	0.349 (0.203)	-0.107 (0.663)	0.264 (0.261)	**0.405 (0.045)**	**0.581 (0.0006)**	**0.377 (0.033)**	0.373 (0.073)	**0.539 (0.004)**	0.281 (0.114)
IL-12p40	0.423 (0.081)	-0.151 (0.591)	**0.575 (0.010)**	-0.274 (0.256)	0.207 (0.321)	**0.361 (0.046)**	0.012 (0.947)	-0.095 (0.659)	-0.191 (0.341)	-0.201 (0.270)
IL-12p70	-0.075 (0.769)	-0.211 (0.450)	0.394 (0.095)	-0.028 (0.908)	0.377 (0.064)	0.182 (0.328)	0.077 (0.675)	0.214 (0.316)	0.126 (0.533)	-0.211 (0.239)
TNF-α	0.219 (0.382)	-0.274 (0.323)	0.071 (0.773)	0.316 (0.175)	0.330 (0.107)	0.267 (0.147)	0.322 (0.072)	0.402 (0.052)	**0.421 (0.029)**	0.145 (0.420)
Eotaxin	**0.598 (0.011)**	-0.041 (0.889)	0.163 (0.505)	-0.010 (0.970)	0.326 (0.112)	0.190 (0.306)	0.145 (0.437)	0.348 (0.095)	0.224 (0.261)	0.027 (0.886)
Fractalkine	0.276 (0.268)	0.008 (0.976)	0.320 (0.182)	-0.018 (0.941)	0.087 (0.681)	0.245 (0.185)	0.075 (0.688)	**0.492 (0.015)**	0.103 (0.611)	0.135 (0.463)
IL-8	0.327 (0.186)	**0.768 (0.0008)**	0.270 (0.263)	0.325 (0.175)	**0.476 (0.016)**	0.082 (0.663)	**0.574 (0.0007)**	0.356 (0.088)	**0.567 (0.002)**	0.268 (0.138)
MCP-1	0.265 (0.288)	-0.229 (0.412)	0.001 (0.996)	-0.166 (0.496)	0.177 (0.396)	0.217 (0.241)	**0.404 (0.024)**	0.166 (0.440)	0.138 (0.494)	0.264 (0.145)
MIP-1α	**0.676 (0.002)**	0.102 (0.718)	0.427 (0.069)	-0.121 (0.623)	0.169 (0.420)	**0.407 (0.023)**	0.051 (0.785)	**0.663 (0.0004)**	**0.384 (0.048)**	-0.002 (0.990)
MIP-1β	**0.510 (0.037)**	0.004 (0.989)	0.116 (0.646)	0.178 (0.467)	-0.028 (0.894)	-0.027 (0.885)	0.170 (0.360)	**0.473 (0.020)**	**0.437 (0.023)**	0.053 (0.775)
MIP-3α	**0.573 (0.020)**	..	..	..	..	..	..	..	..	..
RANTES	0.319 (0.228)	0.260 (0.350)	0.221 (0.362)	-0.018 (0.942)	0.244 (0.250)	**0.481 (0.008)**	**0.426 (0.019)**	0.165 (0.441)	**0.388 (0.046)**	0.334 (0.062)
G-CSF	**0.517 (0.034)**	0.012 (0.970)	0.165 (0.512)	0.331 (0.166)	0.333 (0.104)	**0.480 (0.006)**	0.279 (0.129)	0.347 (0.096)	**0.534 (0.004)**	0.085 (0.643)
GM-CSF	0.458 (0.056)	-0.074 (0.792)	0.339 (0.155)	0.037 (0.876)	**0.690 (0.0001)**	**0.563 (0.001)**	0.204 (0.262)	**0.472 (0.020)**	0.217 (0.277)	0.076 (0.674)
IFN-α	-0.405 (0.095)	..	..	..	..	..	..	..	..	..
IL-10	0.275 (0.269)	0.275 (0.275)	0.034 (0.889)	0.208 (0.379)	0.388 (0.056)	0.124 (0.506)	0.152 (0.406)	**0.447 (0.028)**	0.270 (0.173)	0.107 (0.554)
IL-2	0.113 (0.654)	0.006 (0.982)	0.027 (0.913)	0.330 (0.155)	**0.666 (0.0003)**	0.185 (0.318)	0.217 (0.232)	0.035 (0.870)	0.101 (0.617)	-0.135 (0.455)
IL-7	0.246 (0.326)	-0.077 (0.785)	0.143 (0.559)	-0.300 (0.199)	1.667 (0.426)	0.218 (0.239)	-0.030 (0.873)	0.109 (0.611)	**0.445 (0.020)**	0.010 (0.958)
IL-15	0.448 (0.072)	0.236 (0.416)	0.373 (0.116)	-0.139 (0.582)	0.251 (0.226)	0.184 (0.323)	-0.126 (0.500)	0.228 (0.283)	-0.094 (0.642)	0.033 (0.857)
sCD40L	**0.661 (0.004)**	0.266 (0.338)	0.390 (0.110)	-0.050 (0.840)	0.031 (0.884)	**0.423 (0.018)**	0.100 (0.593)	0.145 (0.500)	0.229 (0.251)	-0.105 (0.568)

Spearman rho and p-values in bold were significant (p<0.05) while those underlined were significant after adjusting multiple comparisons.

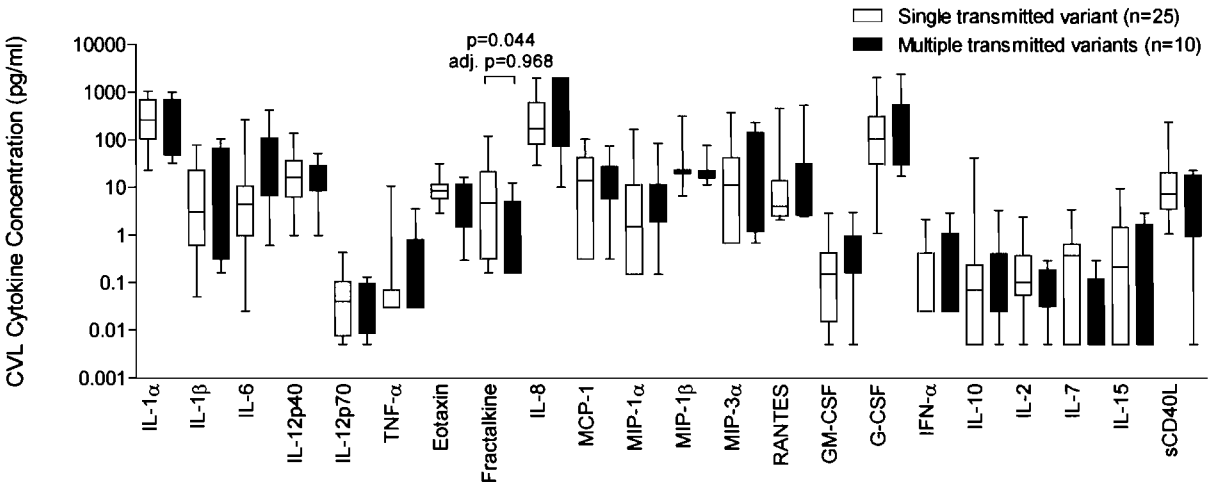


Figure 3.2 Comparison of cytokine concentrations in 6-week post-infection cervicovaginal lavage (CVL) from women who were found to have been infected by single (n=25) or multiple (n=10) HIV-1 genetic variants. Mann-Whitney U test was used to compare groups. No differences in cytokine concentrations between these two groups were significant after adjusting for multiple comparisons.

3.4.3 Genital tract inflammation during early HIV-1 infection was associated with STIs

In Chapter 2 it was found that women who had STIs, particularly those with chlamydia or gonorrhoea infections, had up-regulated inflammatory cytokine concentrations in their genital tracts compared to women who did not have an STI or BV. In order to investigate the relationship between STIs and genital inflammation during early HIV-1 infection, cytokine concentrations in CVL from women 6 weeks post-infection who tested positive for ≥1 STI or who had visible symptoms of STIs (cervicovaginal discharge or genital ulceration) were compared to those of women who did not have an STI or BV (Figure 3.3). Similar to the results reported in Chapter 2, women who had ≥1 active STI (HSV-2, *C. trachomatis*, *N. gonorrhoeae* or *T. vaginalis;* n=14) tended to have elevated pro-inflammatory cytokine concentrations, although the sample size was smaller in this study. The medians of IL-1α, IL-1β, IL-12p70, TNF-α, eotaxin and G-CSF, and T cell homeostatic IL-2 and IL-15 in the genital tracts of women with active infections were up to 151-fold higher than the median concentrations in women who had no active STI. These changes were not significant after adjusting for multiple comparisons. Abnormal vaginal discharge was associated with elevated concentrations of several pro-inflammatory cytokines which were significant after adjusting for multiple comparisons. The median concentrations of IL-1α, IL-1β, IL-12p40, eotaxin, MIP-1α and IL-15 were 2- to 318-fold higher in CVL from women with discharge relative to women who had no active STI or BV. Although vaginal discharge may be caused by other factors in addition to STIs [including BV, *Candida albicans*, allergic reaction, irritation, or physiological changes (Mitchell, 2004)], in Chapter 2 it was found that women who had discharge but no STI/BV had only marginally altered cytokine concentrations in their genital tracts. Therefore STIs were likely the major cause of inflammation

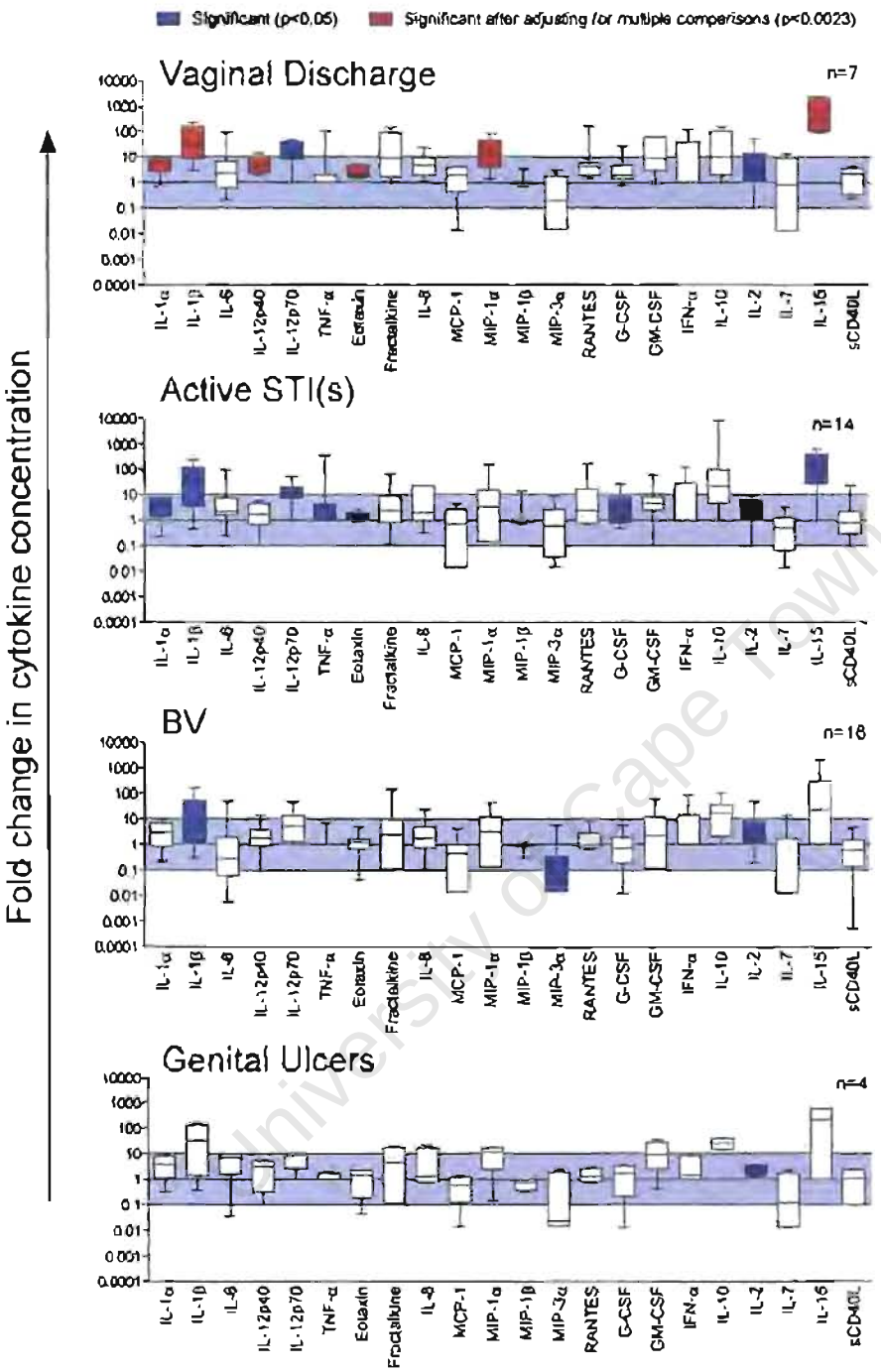


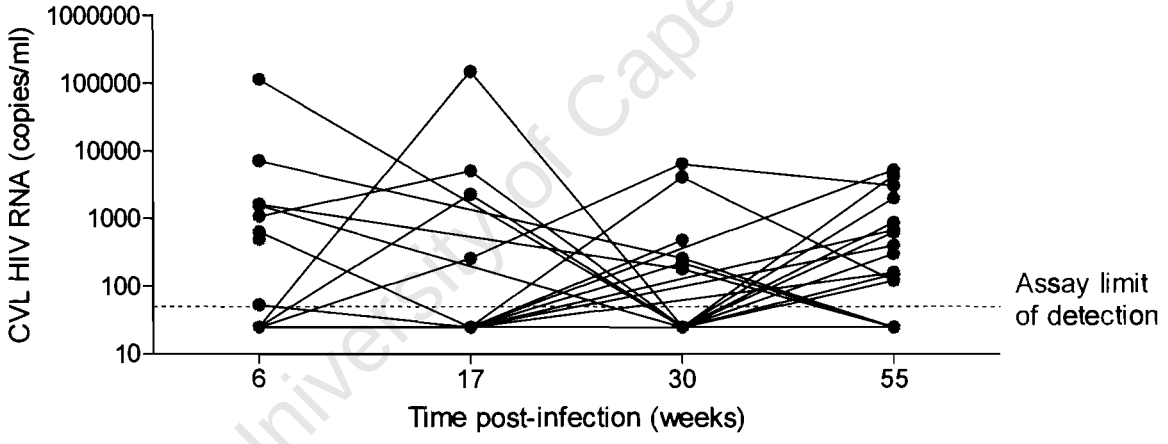
Figure 3.3 Fold change in cytokine concentrations in cervicovaginal lavage (CVL) from women who had a sexually transmitted infection (STI), bacterial vaginosis (BV) or STI symptom compared to the median concentrations in women who did not have an STI. Cytokine concentrations were measured in CVL samples from HIV-1-infected women (median 6 weeks post-infection) who had no STI (n=7), women who had visible vaginal discharge (n=7) or genital ulceration (n=3), women who had one or more laboratory-diagnosed active STIs (*Chlamydia trachomatis*, *Neisseria gonorrhoea*, *Trichomonas vaginalis* or herpes simplex virus type 2 (HSV-2); n=14] and women who had BV (n=16). Fold changes in cytokine concentrations are shown as box and whisker plots, error bars indicate the range.

Figure 3.3 continued. Blue bars indicate cytokines that were significantly changed before adjusting for multiple comparisons ($p<0.05$). Red bars indicate cytokines which remained significantly changed after adjustment ($p<0.0023$).

in women who had discharge in this study. This supports the findings of Mitchell *et al.* (2008) that STIs/BV, not HIV-1, are the major drivers of genital tract inflammation.

3.4.4 Genital tract inflammation during HIV-1 infection was associated with HIV-1 shedding

HIV-1 viral loads were measured in CVL samples collected at 6, 17, 30 and 55 weeks post-infection as an indicator of HIV-1 shedding (Figure 3.4A). CVL viral loads and proportions of women with detectable HIV-1 RNA did not differ significantly between time-points (Figure 3.4B). Additionally, CVL viral loads did not correlate between time-points, indicating that different women were shedding HIV-1 at different times.



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Women shedding [N/total (%)]	8/39 (21)	4/32 (13)	6/38 (16)	13/38 (34)
e^{β} (95% CI)	1.00	-0.64 (-0.21 - 0.81)	-0.40 (-1.71 - 0.91)	0.85 (-0.33 - 2.04)

Figure 3.4 HIV-1 RNA concentrations in CVL at 6, 17, 30 and 55 weeks post-infection. **A)** HIV-1 viral loads in cervicovaginal lavage (CVL) from each woman are indicated by grey dots. Lines connect RNA concentrations in CVL from the same women at different time-points. No significant Spearman correlations were found between HIV-1 RNA levels at each time-point, indicating that different women were shedding at each time-point. **B)** The proportion of women who had detectable HIV-1 RNA at each time-point is shown. Mixed-effects logistic regression was used to determine whether the proportion of women shedding HIV-1 at 17, 30 and 55 weeks post-infection differed relative to 6 weeks post-infection. No significant differences were found.

Table 3.3 Spearman Rank correlations between CVL HIV-1 RNA concentrations and blood CD4 counts, plasma viral loads and CVL cytokine concentrations

	6 wks post-infection		17 wks post-infection		30 wks post-infection		55 wks post-infection	
	Rho (p-value)		Rho (p-value)		Rho (p-value)		Rho (p-value)	
Blood CD4+ T cell count	**-0.485**	**(0.002)**	-0.305	(0.090)	**-0.428**	**(0.007)**	-0.047	(0.782)
Plasma viral load	**0.430**	**(0.006)**	**0.533**	**(0.002)**	0.314	(0.055)	**0.371**	**(0.022)**
IL-1α	0.236	(0.147)	0.154	(0.401)	0.260	(0.114)	**0.337**	**(0.039)***
IL-1β	**0.355**	**(0.026)***	**0.478**	**(0.006)****	0.207	(0.212)	**0.513**	**(0.001)****
IL-6	**0.427**	**(0.007)***	**0.446**	**(0.011)****	0.261	(0.113)	**0.338**	**(0.038)***
IL-12p40	0.159	(0.333)	0.211	(0.247)	0.294	(0.073)	0.175	(0.293)
IL-12p70	**0.325**	**(0.044)**	0.204	(0.263)	0.177	(0.287)	0.027	(0.873)
TNF-α	**0.388**	**(0.015)***	**0.382**	**(0.031)***	**0.415**	**(0.010)***	0.309	(0.059)
Eotaxin	0.216	(0.186)	0.227	(0.211)	-0.038	(0.821)	**0.473**	**(0.003)****
Fractalkine	0.163	(0.321)	**0.405**	**(0.022)***	-0.186	(0.264)	**0.331**	**(0.042)***
IL-8	**0.323**	**(0.045)**	0.279	(0.122)	**0.393**	**(0.015)***	**0.437**	**(0.006)****
MCP-1	**0.419**	**(0.008)***	**0.487**	**(0.005)****	0.099	(0.555)	**0.432**	**(0.007)****
MIP-1α	0.155	(0.345)	**0.385**	**(0.030)***	0.059	(0.726)	0.128	(0.445)
MIP-1β	0.186	(0.258)	**0.570**	**(0.001)****	0.087	(0.604)	0.314	(0.055)
RANTES	**0.490**	**(0.002)***	**0.531**	**(0.002)****	0.267	(0.106)	**0.549**	**(0.0004)****
G-CSF	0.310	(0.055)	**0.457**	**(0.009)****	0.198	(0.234)	**0.379**	**(0.019)***
GM-CSF	0.090	(0.585)	**0.537**	**(0.002)****	0.315	(0.054)	0.077	(0.645)
MIP-3α	0.226	(0.207)	ND		ND		ND	
IFN-α	0.212	(0.201)	ND		ND		ND	
IL-10	**0.378**	**(0.018)***	0.247	(0.173)	0.038	(0.821)	-0.160	(0.336)
IL-2	**0.385**	**(0.015)***	**0.534**	**(0.002)****	0.137	(0.413)	-0.134	(0.421)
IL-7	0.260	(0.110)	0.119	(0.517)	0.126	(0.450)	-0.057	(0.736)
IL-15	**0.382**	**(0.016)***	0.220	(0.226)	**0.375**	**(0.020)***	-0.013	(0.939)
sCD40L	**0.331**	**(0.040)***	0.309	(0.085)	0.218	(0.188)	0.123	(0.462)

Significant associations are shown in bold ($p<0.05$). Associations that were significant after adjusting for multiple comparisons are underlined. *Cytokine concentration significantly higher in women who had detectable HIV-1 RNA in their genital tracts compared to women who had undetectable HIV-1 RNA concentrations ($p<0.05$). **Cytokine concentration significantly higher in women who had detectable HIV-1 RNA after adjusting for multiple comparisons. ND: Not done.

Cytokine concentrations in CVL correlated with CVL viral loads at all time-points and women who had detectable HIV-1 RNA in their genital tracts had higher cytokine concentrations compared to women who were not shedding HIV-1 (Table 3.3). Pre-infection cytokine concentrations did not differ between women who were shedding and women who were not shedding HIV-1 6 weeks post-infection. Additionally, lower

blood CD4 counts and/or higher plasma viral loads were associated with CVL viral loads at each time-point. Women with detectable HIV-1 RNA in their genital tracts 6 weeks post-infection were not more likely to have sexually transmitted co-infections compared to women who were not shedding HIV-1. A total of 11/31 (35.5%) of women who were not shedding HIV-1 had ≥1 active STI, while 3/7 (42.9%) of women who were shedding HIV-1 had ≥1 active STI (p=1.000). There was furthermore no significant difference between BV or vaginal discharge in women shedding compared to women who were not shedding (p=1.000 and p=1.000, respectively) and genital ulceration was only observed in women who were not shedding.

3.4.5 Elevated genital tract cytokine concentrations during early HIV-1 infection correlated with lower blood CD4+ T cell counts

It has previously been reported that genital tract IL-1β, IL-6 and IL-8 concentrations during early HIV-1 infection were associated with lower blood CD4+ T cell counts at the same time-points (Bebell *et al.*, 2008). In this study, it was similarly found that higher concentrations of genital tract IL-6 (rho=-0.455, adj. p=0.030), TNF-α (rho=-0.498, adj. p=0.017), RANTES (rho=-0.460, adj. p=0.026) and IL-10 (rho=-0.503, adj. p=0.027) correlated with lower blood CD4 counts during early HIV-1 infection after adjusting for multiple comparisons (Figure 3.5). Only weak associations between 17, 30 and 55 week cytokine concentrations and concurrent CD4 counts were found and these were not significant after adjusting for multiple comparisons, indicating that the relationship was restricted to early HIV-1 infection.

Despite finding a significant correlation between HIV-1 shedding and both plasma viral load and genital inflammation, few associations were found between genital tract cytokine concentrations and plasma viral loads measured at the same time-points (only IL-2 at 17 weeks post-infection correlated with concurrent plasma viral loads after adjusting for multiple comparisons). This suggests that changes in inflammatory cytokine production in the genital tract during early HIV-1 infection are largely independent of systemic viral load.

3.4.6 Genital tract inflammation during early HIV-1 infection was associated with CD4+ T cell depletion during the first year of infection

In order to determine whether cytokine concentrations in the genital tract at various stages during HIV-1 infection were associated with disease progression, the relationships between cytokines and average CD4 counts measured at 3 consecutive visits overlying 12 months post-infection were investigated using linear regression. The size of each β-coefficient generated indicates the amount of change in $\log_{10}$ cells/μl 12 month CD4 count that is associated with a 1 standard deviation increase in $\log_{10}$ pg/ml cytokine concentration (effect size). A negative β-coefficient indicates an inverse relationship, while a positive β-

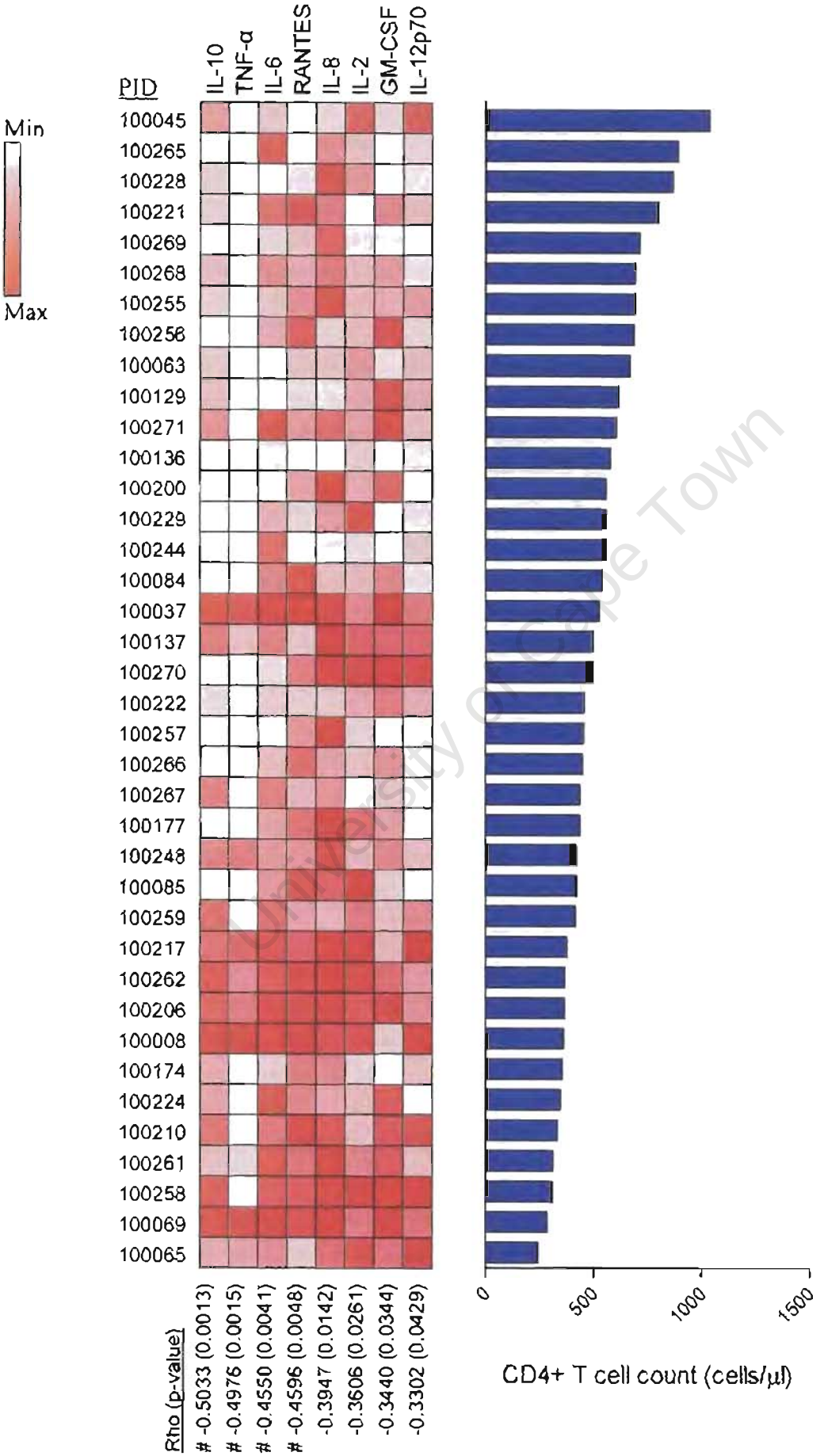


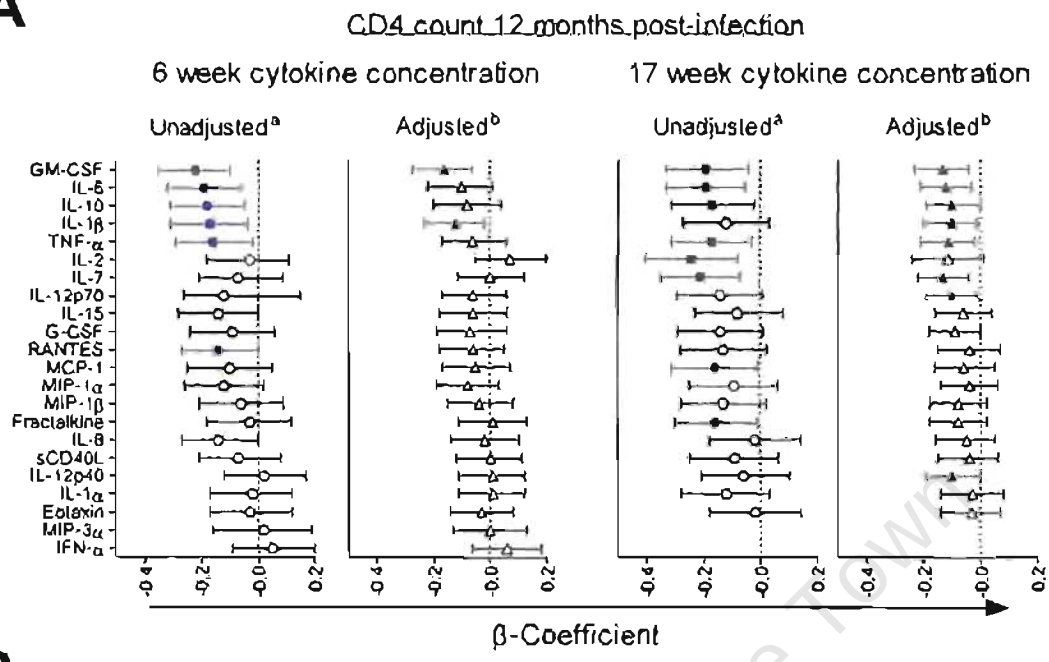
Figure 3.5 CVL inflammatory cytokine concentrations were associated with concurrent blood CD4 counts in women with early HIV-1 infection (6 weeks post-infection). Only cytokines that correlated significantly with CD4 counts before adjustment for multiple comparisons are represented (p-values and Spearman rho values below heat map). Women are ranked according to early infection blood CD4 counts. Relative early infection CVL cytokine concentrations of study participants are shown as a heat map, with each row representing the cytokine concentrations in an individual woman and falling alongside her CD4 count. For each particular cytokine, the concentrations found in this group of women were ranked and assigned an appropriate colour ranging from white (lowest concentration) to red (highest concentration). Repeated values were assigned the same rank and hence colour. PID: Patient Identity Number. # IL-10, TNF-α, IL-6, and RANTES remained significantly correlated with CD4 count following adjustment for multiple comparisons.

coefficient indicates a direct relationship. Although higher concentrations of several inflammatory and T cell homeostatic cytokines in the genital tract at multiple time-points were associated with lower blood CD4 counts 12 months post-infection before adjusting for multiple comparisons, only GM-CSF concentrations 6 weeks post-infection remained significantly associated after adjustment (Figure 3.6A). However, the effect sizes of several cytokines approached those of standardized early infection blood CD4 counts, which were strongly associated with 12 month CD4 counts [β=0.26 (95% CI, 0.17-0.36) and β=0.30 (95% CI, 0.23-0.39) for CD4 counts at 6 and 17 weeks post-infection, respectively]. No associations between cytokine concentrations at 30 and 55 weeks post-infection and 12 month CD4 counts were significant after adjusting for multiple comparisons (data not shown). No significant relationship between pre-infection cytokine concentrations and CD4 counts 12 months post-infection was found, although relatively few CVL samples (22/49) from pre-infection time-points were available for this analysis.

Blood CD4 counts and plasma viral loads during early HIV-1 infection were also associated with 12 month post-infection CD4 counts [β=0.70 (95% CI, 0.46-0.95) and β=-0.07 (95% CI, -0.12- -0.02), respectively]. HIV-1 disease status could predispose individuals to higher incidence of STIs (Ghys *et al.*, 1995) and shedding of HIV-1 in the genital tract (Ghys *et al.*, 1997), thereby exacerbating genital tract inflammation which would therefore be indirectly rather than directly associated with disease progression. Multivariate regression was thus used to adjust for both CD4 counts and viral loads measured at the same time-points as cytokine concentrations. Again, there was a strong trend toward an association between higher cytokine concentrations and lower 12 month CD4 counts (Figure 3.6A).

Finally, associations between pre-infection (n=22) and 6 week post-infection (n=18) cytokine levels and blood CD4+ T cell loss during the first 12 months of HIV-1 infection were investigated in a subset of women for whom pre-infection CD4 count measurements were available. CD4 depletion during the first year of infection was associated with higher pre-infection cervicovaginal concentrations of RANTES (rho=0.464, p=0.03) and 6 week post-infection concentrations of IL-1β (rho=0.532, p=0.02) and GM-CSF (rho=0.622, p=0.006). However, these associations were not upheld after adjusting for multiple

A



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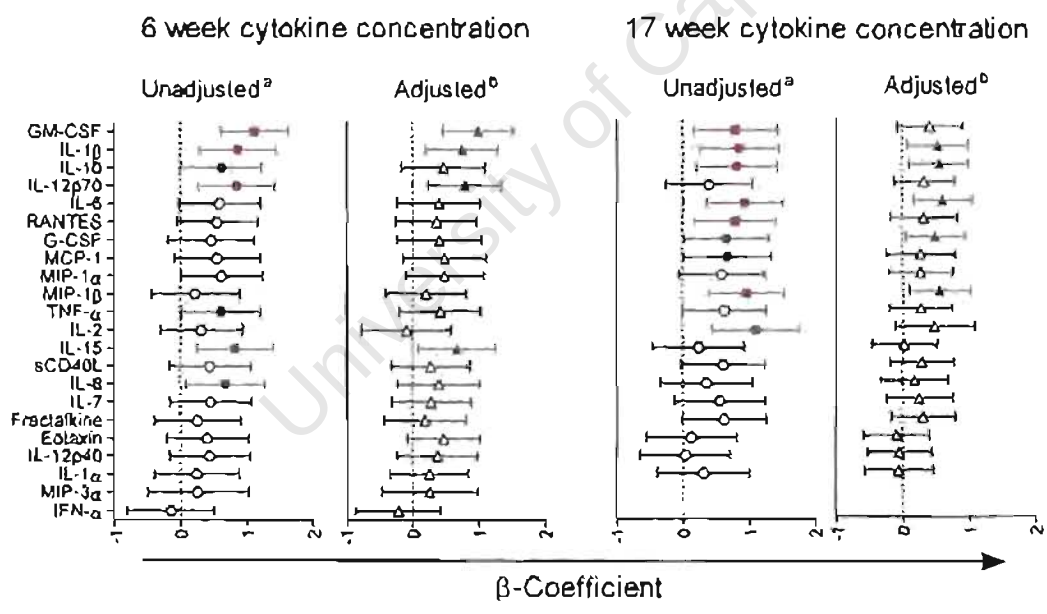

- Associations between CVL cytokine concentrations and CD4 counts/ viral load set-point 12 months post-infection
- Significant ($p<0.05$)
- Significant after adjusting for multiple comparisons
- Adjusted for blood CD4 counts and plasma viral loads at the same visits as cytokine measurements
- Significant ($p<0.05$)
- Significant after adjusting for multiple comparisons

Figure 3.6 The relationships between cervicovaginal lavage (CVL) cytokine concentrations and HIV-1 disease progression. Linear regression was used to determine associations between cytokine concentrations in CVL from women 6 (n=37) and 17 (n=31) weeks post-infection and average blood CD4 counts of 3 consecutive visits overlying the 12 month post-infection time-point **(A)** and viral load set-points **(B)**. Viral loads, CD4 count measurements and cytokine concentrations were log-transformed, cytokine concentrations were standardized to allow for direct comparison of β-coefficients. [a]β-coefficients that were generated by univariate regression are indicated by circles and show the relationship between each cytokine and 12 month CD4 counts or viral load set-points. Error bars indicate 95% confidence intervals. Blood CD4 counts and plasma viral loads at visits matching those of cytokine measurements were included as predictor variables in multivariate regression analyses. [b]β-coefficients that indicate the relationships between cytokine concentrations and 12 month CD4 counts or viral load set-points, following adjustment for CD4 counts and viral loads at the same visits as cytokine measurements, are represented by triangles. Cytokines are ranked according to the strength of their associations with 12 month CD4 counts or viral load set-point. Significant associations are shown in blue ($p<0.05$). Associations that were significant after adjusting for multiple comparisons are shown in red.

comparisons which may be due to the relatively small samples sizes available for this analysis (Figure 3.7).

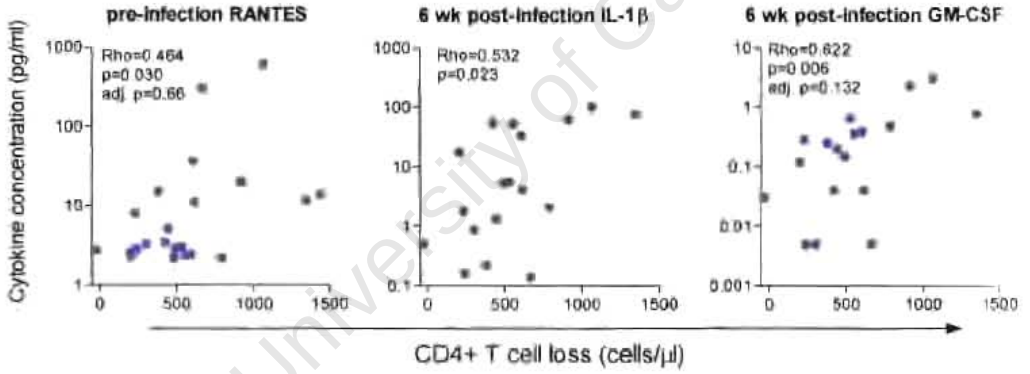

Figure 3.7 Genital tract inflammation 6 weeks post-infection was associated with greater CD4+ T cell loss during the first 12 months of HIV-1 infection. Spearman correlations between pre-infection (n=22) and 6 week post-infection (n=16) CVL cytokine concentrations and CD4+ T cell loss were determined. Only significant associations are shown ($p<0.05$). Elevated pre-infection RANTES and 6 week post-infection IL-1β and GM-CSF concentrations correlated with greater CD4+ T cell loss before adjustment for multiple comparisons.

3.4.7 Genital tract inflammation during early HIV-1 infection was associated with higher viral load set-point (12 months post-infection)

In order to further investigate the relationship between genital tract cytokine concentrations and HIV-1 disease progression, the relationships between inflammatory cytokine concentrations and viral load set-

point 12 months post-infection were investigated using linear regression. Several inflammatory and T cell homeostatic cytokines that were measured during early HIV-1 infection (6 and 17 weeks post-infection) were associated with higher viral load set-point (Figure 3.6B), even though these cytokines were not associated with plasma viral loads measured at the same time-points as cytokine measurements. No associations between cytokine concentrations at 30 and 55 weeks post-infection and viral load set-point were significant after adjusting for multiple comparisons (data not shown). Additionally, although cytokine concentrations did not differ between pre-infection and early infection time-points, no significant associations between pre-infection cytokine concentrations and viral load set-point were found. Elevated GM-CSF, IL-1β, IL-12p70 and IL-15 concentrations at 6 weeks post-infection and GM-CSF, IL-1β, IL-10, IL-6, RANTES, MIP-1β and IL-2 concentrations at 17 weeks post-infection were associated with higher viral load set-point. GM-CSF at 6 weeks post-infection had the largest effect size, with a 1 standard deviation increase in the log-transformed concentration of this cytokine associated with a 1.12 $\log_{10}$ copies/ml increase in viral load set-point. Multivariate regression was used to adjust for both CD4 counts and viral loads measured at the same time-points as cytokine concentrations. GM-CSF concentrations 6 weeks post-infection remained significantly associated with higher viral load set-point, after adjusting for 6 week post-infection CD4 counts and viral loads, suggesting that the relationship between this inflammatory cytokine and disease progression is at least partly independent of early infection systemic disease state.

Cervicovaginal GM-CSF concentration 6 weeks post-infection was the strongest cytokine correlate of HIV-1 disease progression, predicting 35% of the variation in viral load set-point (adj. R^2=0.347), 26% of the variation in CD4 count 12 months post-infection (adj. R^2=0.260) and 21% of CD4 loss during the first 12 months of infection (adj. R^2=0.211). In comparison, blood CD4 count 6 weeks post-infection was predictive of 42% of the variation in 12 month CD4 counts, but only 2% of the variation in viral load set-point and 4% of CD4 loss (adj. R^2=0.424, 0.023 and 0.035, respectively). Plasma viral load 6 weeks post-infection was less predictive of viral load set-point (adj. R^2=0.256), 12 month CD4 count (adj. R^2=0.114) and CD4 loss (adj. R^2=0.026) than GM-CSF concentrations.

3.5 Discussion

Previous studies have shown that women with early and chronic HIV-1 infection have elevated inflammatory cytokine concentrations in their genital tracts relative to unmatched HIV-1 negative women (Belec *et al.*, 1995; Crowley-Nowick *et al.*, 2000; Zara *et al.*, 2004; Bebell *et al.*, 2008; Guha and Chatterjee, 2009; Nkwanyana *et al.*, 2009). This study is the first to compare cytokine concentrations in cervicovaginal samples from the same women pre-infection and during early HIV-1 infection. It was found that inflammatory cytokines pre-infection were correlated with those post-infection and in fact were not significantly elevated shortly following HIV-1 infection (median 6 weeks post-infection). However, genital

tract cytokine concentrations were higher in women who had active STIs or vaginal discharge and in women who were shedding HIV-1 in their genital secretions. Additionally, cytokine concentrations were elevated in women who had lower blood CD4+ T cell counts 6 weeks post-infection (Bebell *et al.*, 2008). Elevated cervicovaginal cytokines during early HIV-1 infection (6 and 17 weeks post-infection) were associated with higher plasma viral load set-point, which is predictive of time to AIDS (Lyles *et al.*, 2000). Additionally, higher GM-CSF concentrations 6 weeks post-infection were associated with lower CD4 counts 12 months post-infection.

A previous study has demonstrated an association between the use of injectable hormone contraception and risk of HIV-1 infection (Heffron *et al.*, 2012), Although it has been shown that progesterone-based injectable hormone contraceptive use is associated with increased numbers of inflammatory cells in cervicovaginal fluid (Ghanem *et al.*, 2005), others have shown that progesterone withdrawal during the menstrual cycle is associated with increased concentrations of IL-8 and MCP-1 and recruitment and activation of monocytes and neutrophils (Critchley et al., 2001). In this study, no evidence for increased inflammation was found in women using injectable contraception. Although it was not possible to adjust for STIs because of the relatively small sample size and the prevalence of STIs in this study, it was found that IL-1α concentrations were significantly lower in women using contraception (data not shown). When cervicovaginal cytokine concentrations were compared in the same women pre-infection and at several time-points during the first 12 months of HIV-1 infection, some women in this study maintained relatively higher levels of genital tract inflammation over time, while others had consistently low cytokine concentrations. The prevalence of each STI did not differ between pre- and post-HIV-1-infection, thus the cause of sustained inflammation may be STI recurrence. Similar to the findings of this study, Mitchell *et al.* (2008) demonstrated that inflammatory cytokines were not elevated in the genital tract of chronically HIV-1-infected women relative to uninfected women, but were rather associated with BV. The higher frequency of STIs in HIV-1-infected compared to uninfected women (Fennema *et al.*, 1995) may account for previous findings that genital tract inflammatory cytokines are elevated in women with early HIV-1 infection. It is possible that elevated genital inflammatory cytokine responses, similar to those reported shortly after SIV infection of macaques (Abel *et al.*, 2005), may have subsided by the earliest time of sample collection in this study (6 weeks). Alternatively, genital inflammatory cytokine concentrations may increase over time during HIV-1 infection and may thus be higher in women with chronic infection relative to early infection (Bélec *et al.*, 1995). This study demonstrates the importance of comparing cytokine levels in women matched pre- and post-infection and controlling for STIs when evaluating HIV-1-specific changes in genital tract cytokine levels.

HIV-1 RNA concentrations in the genital tract correlated not only with higher levels of cervicovaginal inflammation, but were also associated with lower blood CD4 counts and with higher plasma viral loads at the same time-points. While these findings may suggest that high systemic viral loads drive HIV-1

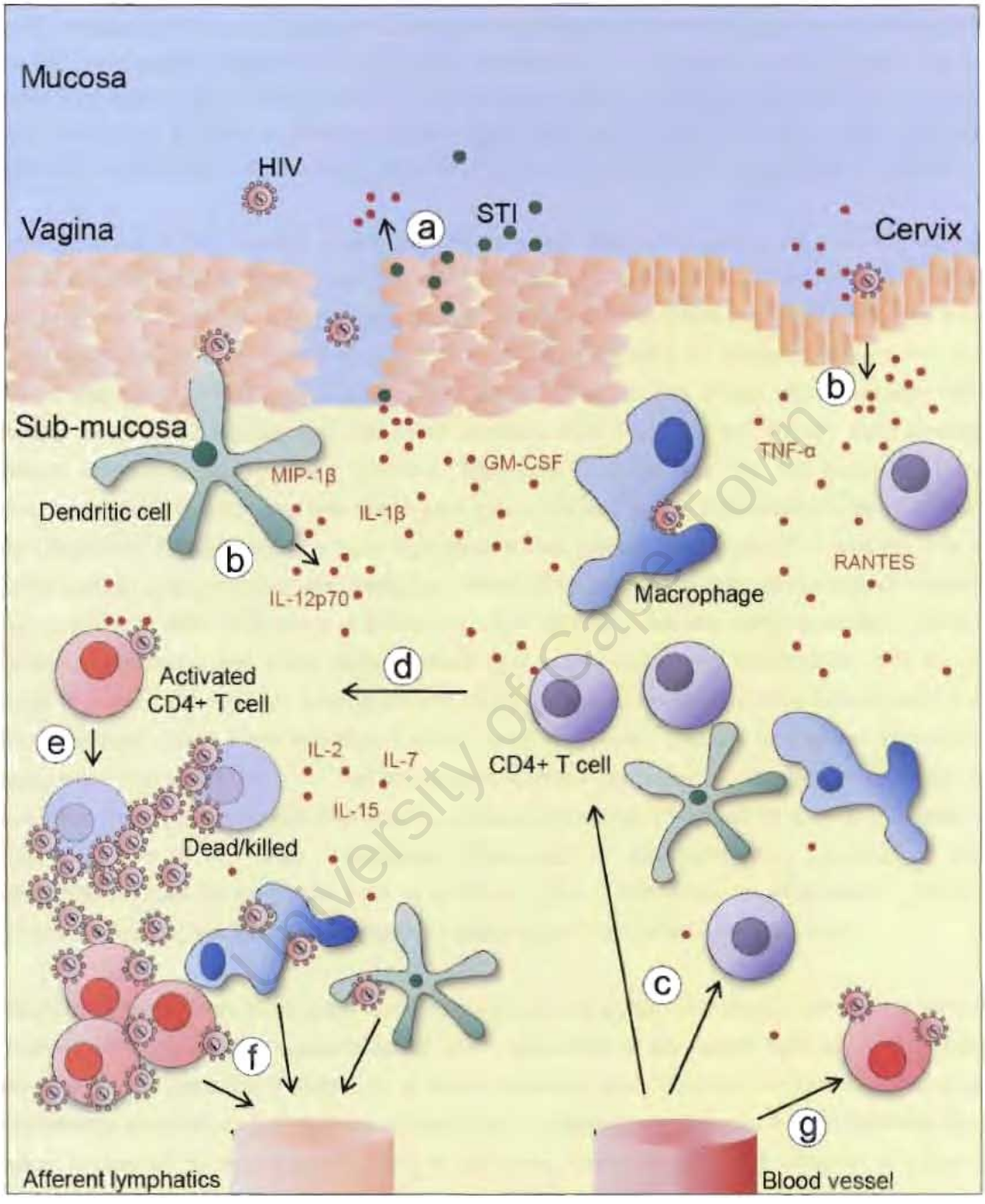

Figure 3.8 Proposed events in the female genital tract during early HIV-1 infection. **a)** STIs engage toll-like receptors, resulting in inflammatory cytokine secretion by epithelial and immune cells. **b)** Immune and other cells produce inflammatory cytokines in response to HIV-1 infection. **c)** High concentrations of inflammatory cytokines in the genital tract recruit large numbers of immune cells, including CD4+ T cells, from circulation. **d)** CD4+ T cells are activated by inflammatory cytokines. **e)** Large numbers of activated target cells allows for rapid viral expansion. CD4+ T cell

Figure 3.8 continued. depletion results in up-regulation of T cell homeostatic cytokine production, which in turn induce inflammatory cytokine production. **f)** Dendritic cells capture HIV-1 particles or are infected, activated CD4+ T cells and macrophages are infected and these cells transport HIV-1 to the lymph nodes via afferent lymphatics. Further dissemination into the blood will occur via the efferent lymphatics. Rapid viral replication in the genital tract results in seeding of the systemic compartment with large amounts of virus. **g)** HIV-1-infected CD4+ T cells enter the genital tract from circulation, contributing to viral expansion, HIV-1 shedding and CD4 depletion in this compartment.

shedding, which in turn induces genital tract inflammation, which thus correlates with long term HIV-1 disease progression, shedding during early HIV-1 infection was not associated with 12 month CD4 counts or plasma viral load set-point. Additionally, genital tract inflammation during early HIV-1 infection was only weakly associated with concurrent plasma viral load before adjusting for multiple comparisons, but was strongly associated with plasma viral loads at subsequent time-points. It was also found that, although cytokine levels in the genital tract correlated positively over time, different women were shedding at different time-points during HIV-1 infection. Therefore shedding was not the cause of sustained cervicovaginal inflammation that was observed in this cohort and genital inflammation may rather facilitate HIV-1 shedding in individuals who have high plasma viral loads by recruiting HIV-1-infected cells to the genital mucosa and promoting viral replication. These findings suggest that cervicovaginal inflammation was not primarily driven by plasma viral load and HIV-1 shedding, but was rather associated with STIs. A significant relationship was found between blood CD4 counts and genital inflammation, with lower CD4 counts 6 weeks post-infection associated with higher CVL inflammatory cytokine concentrations at the same time-point. CD4+ T cell depletion in blood during early HIV-1 infection may reflect depletion in the genital tract. This would result in T cell homeostatic cytokine production in the genital tract, which in turn have been found to induce pro-inflammatory cytokine production (Alderson *et al.*, 1991; Damas *et al.*, 2003; Catalfamo *et al.*, 2008). Alternatively, high levels of pro-inflammatory cytokines in mucosal compartments, such as the genital tract, may recruit CD4+ T cells under an inflammatory gradient and fuel viral replication, resulting in CD4 depletion locally and in the systemic compartment.

Therefore, STIs are likely to be major contributors to cervicovaginal inflammation detected in early HIV-1 infection, with inflammation exacerbated by HIV-1 replication in the genital tract and the homeostatic response to CD4 depletion (Figure 3.8). A recent macaque study demonstrated the importance of pro-inflammatory cytokine production in the genital tract in establishment of productive SIV infection following vaginal inoculation (Li *et al.*, 2009). Wang *et al.* (2005) further showed that induction of inflammatory cytokine responses and immune cell influx into the genital tract of macaques prior to vaginal inoculation with SIV was associated with increased plasma viral load set-point following infection, suggesting that genital tract cytokine concentrations at the time of infection may play a role in disease progression. This study is the first to investigate associations between genital tract inflammation and HIV-1 disease progression in humans. It was found that, similar to macaque studies, higher levels of genital tract pro-inflammatory and T cell homeostatic cytokines during early HIV-1 infection were associated with more

rapid HIV-1 disease progression. Additionally, the findings of this study suggest that the relationship between genital tract cytokine levels during early infection and disease progression are partly independent of blood CD4 counts and plasma viral loads measured at the same time-points as cytokine concentrations. In this study, pre-infection genital cytokine concentrations correlated with those measured during the early and chronic stages of HIV-1 infection. Although the earliest time-point included in this study (6 weeks post-infection) was past the window period during which viral dissemination to blood occurs, inflammation in the genital tract at 6 weeks post-infection is likely to reflect inflammation at the time of infection. Although pre-infection genital inflammation may be associated with HIV-1 disease progression, this could not be conclusively investigated here as the number of women for whom pre-infection samples were available was small (22/49). Cytokine concentrations measured 6 weeks post-infection (range 1 - 13) may however be a closer representation of the level of genital inflammation present at the time of HIV-1 infection than pre-infection cytokine concentrations that were measured 36 weeks prior to infection (range 2 - 92). Higher levels of inflammatory cytokines in the genital tract at the time of HIV-1 transmission may favor disease progression by recruiting and activating CD4+ T cell targets for HIV-1 infection and by directly up-regulating viral replication (Osborne *et al.*, 1989; Swingler *et al.*, 1999; Nkwanyana *et al.*, 2009). This may lead to rapid viral expansion in the genital tract, depletion of CD4+ T cells and seeding of the systemic compartment with higher concentrations of virus. Once infection has been established in the systemic compartment, high plasma viral loads may increase HIV-1 shedding and low blood CD4 counts may contribute to CD4 depletion in the genital tract, resulting in a further increase in local inflammation. Genital tract inflammation may facilitate ongoing HIV-1 replication in this compartment, continued seeding of the systemic compartment and further blood CD4 depletion during early HIV-1 infection, leading to more rapid HIV-1 disease progression. The findings of this study suggest that the inflammatory environment in the genital tracts of women who become infected with HIV-1 through sexual transmission may influence disease outcome and that strategies to reduce genital inflammation may slow disease progression.

CHAPTER 4

Chapter 4

Plasma cytokine levels during early HIV-1 infection predict HIV-1 disease progression

4.1 Summary

In Chapter 3 it was found that elevated pro-inflammatory and T cell homeostatic cytokine concentrations in the female genital tract during early HIV-1 infection were associated with markers of rapid disease progression. In the systemic compartment, previous studies have shown that both blood T cell activation during early HIV-1 infection and soluble markers of immune activation measurable in plasma during chronic infection are predictive of HIV-1 disease progression. Although the acute phase of HIV-1 infection is associated with increased plasma pro-inflammatory cytokine production, the relationship between plasma cytokine concentrations and HIV-1 pathogenesis is unknown. The aim of this study was to identify cytokine biomarkers measurable in plasma during early HIV-1 infection that predict HIV-1 disease progression. This study included forty South African women who became infected with HIV-1 and were followed longitudinally from the time of infection. The concentrations of 30 cytokines in plasma from women with early HIV-1 infection (median 6 weeks post-infection) were measured and associations between cytokine levels and both plasma viral load set-point 12 months post-infection and time taken for CD4 counts to fall below 350 cells/μl were determined using multivariate and Cox proportional-hazards regression. It was found that the concentrations of 5 plasma cytokines, IL-12p40, IL-12p70, IFN-γ, IL-7 and IL-15, in women with early infection predicted 66% of the variation in plasma viral load set-point 12 months post infection. IL-12p40, IL-12p70 and IFN-γ were significantly associated with lower viral load whereas IL-7 and IL-15 were associated with higher viral load. Plasma concentrations of IL-12p40 and GM-CSF during early infection were associated with maintenance of CD4 counts above 350 cells/μl while IL-1α, eotaxin and IL-7 were associated with more rapid CD4 loss. A small panel of plasma cytokines during early HIV-1 infection was predictive of long-term HIV-1 disease prognosis in this group of South African women.

4.2 Introduction

Early systemic immune events during HIV-1 infection are associated with the rate of subsequent disease progression (Deeks *et al.*, 2004). Peak viremia is accompanied by systemic immune activation and CD4+ T cell depletion, particularly from the gastrointestinal tract (Brenchley *et al.*, 2004; Mehandru *et al.*, 2004; Stacey *et al.*, 2009). Although viral load subsequently declines, immune activation in blood persists, viral replication continues and CD4+ T cells are progressively lost (Deeks *et al.*, 2004; Hazenberg *et al.*, 2003). Immune activation during HIV-1 infection involves activation and proliferation of most immune cells, including T cells, B cells, NK cells and macrophages (Lane *et al.*, 1983; Alter *et al.*, 2004; Brenchley *et al.*, 2006; Catalfamo *et al.*, 2008). It also includes increased production of pro-inflammatory cytokines (Stacey *et al.*, 2009; Norris *et al.*, 2006; Bebell *et al.*, 2008). In the blood of acutely-infected individuals, an intense pro-inflammatory cytokine "storm" is followed by immunoregulatory cytokine production (Stacey *et al.*, 2009). Pro-inflammatory cytokines enhance HIV-1 replication and CD4+ T cell loss by directly promoting proviral transcription, by recruiting and activating CD4+ T cell targets for HIV-1 infection, and by activation-induced apoptosis of bystander T cells (Osborne *et al.*, 1989; Lin *et al.*, 1997; Swingler *et al.*, 1999).

While T cell activation in blood during early HIV-1 infection has been found to be associated with subsequent disease progression (Deeks *et al.*, 2004), the relationship between plasma cytokine production during early infection and disease prognosis has not been investigated. In Chapter 3, it was shown that elevated pro-inflammatory and T cell homeostatic cytokine concentrations in the genital tract during early HIV-1 infection were associated with higher plasma viral load set-point. Plasma biomarkers that can be used to predict the rate of HIV-1 disease progression could be useful in the clinical management of infected individuals, and for the evaluation of candidate HIV-1 vaccines or microbicides aimed at reducing the rate of disease progression, rather than preventing infection (Mascola and Nabel, 2001; Goujard *et al.*, 2006). Blood CD4 counts and viral load measurements during primary, chronic and advanced HIV-1 infection are strongly predictive of subsequent disease progression (Goujard *et al.*, 2006; Mellors *et al.*, 1997; de Wolf *et al.*, 1997; Lepri *et al.*, 1998). However, it has been argued that coupling CD4 counts and viral load measurements with estimates of T cell proliferation and activation during acute and/or chronic HIV-1 infection could provide significantly increased predictive power (Deeks *et al.*, 2004; Hazenberg *et al.*, 2003; Liu *et al.*, 1997; Giorgi *et al.*, 1999). Soluble markers of immune activation, such as TNF-α receptor II p75, neopterin and β_2-microglobulin, are more easily measurable in plasma samples than cellular activation, and have been found to predict HIV-1 disease progression with comparable efficiency to CD4 counts and viral load measurements (Mellors *et al.*, 1997; Fahey *et al.*, 1998; Zangerle *et al.*, 1998). Although the benefits of being able to predict, and possibly modify, disease course during early HIV-1 infection would be

substantial, the predictive value of biomarkers of immune activation has largely been investigated during chronic HIV-1 infection.

In this study, associations between plasma cytokine concentrations during early infection and established markers of long-term HIV-1 disease progression, including blood CD4+ T cell loss and plasma viral load set-points, were investigated. Two models were generated based on the easily measurable concentrations of a small number of plasma cytokines that can be used during early infection to predict HIV-1 disease progression.

4.3 Materials and methods

4.3.1 Description of study participants

Consenting women, recently infected with HIV-1 Subtype C, were recruited from HIV-1 negative cohorts which were screened either monthly or 3 monthly for HIV-1 infection as part of the CAPRISA 002 Acute Infection Study (van Loggerenberg *et al.*, 2008; also described in Chapter 3). Time of infection was defined as the mid-point between the last HIV-1 antibody negative test and the first HIV-1 antibody positive test, or as 14 days prior to a positive RNA PCR assay on the same day as a negative HIV-1 EIA. Plasma samples from 40 women at a median of 6 weeks post-infection (range 1-12), and from 14/40 of these women 25.5 weeks pre-infection (range 2-66) were available for analysis and were included in this study. This study was approved by the University of KwaZulu-Natal and the University of Cape Town Ethics Committees.

4.3.2 Markers of HIV-1 disease progression

Absolute blood CD4+ T cell counts (cells/μl) were measured using a FACSCalibur flow cytometer at regular intervals during HIV-1 infection (weekly for a month following HIV-1 infection, fortnightly for 2 months, monthly for 9 months and quarterly thereafter). Plasma HIV-1 RNA concentrations (copies/ml) were quantified using the COBAS AMPLICOR™ HIV-1 Monitor v1.5 or COBAS Ampliprep/COBAS TaqMan 48 Analyser (Roche Diagnostics, Branchburg, New Jersey, U.S.A.). Viral load and CD4 count set-points were defined as the average CD4+ T cell or viral load measurements of 3 consecutive visits between medians of 47 and 55 weeks post-infection (range: 37-69 weeks) overlying the 12 month post-infection time-point.

4.3.3 Measurement of plasma cytokines

Thirty cytokines were measured in plasma from HIV-1-infected and uninfected women using Luminex Multiplex Flow Cytometric assays. High Sensitivity Human Cytokine LINCO*plex* kits (LINCO Research, MO, U.S.A.) were used to measure the concentrations of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, GM-CSF, IFN-γ and TNF-α, while Human Cytokine LINCO*plex* kits were used to measured the concentrations of IL-1α, IL-1Ra, IL-8, IL-12p40, IL-15, IL-17, EGF, eotaxin, fractalkine, G-CSF, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, sCD40L, TGF-α and VEGF. The sensitivity of these kits ranged between 0.01 and 27 pg/ml for each of the cytokines measured. Plasma cytokine concentrations were only assessed using High Sensitivity kits for 4/40 of the study participants due to sample availability. All samples were assayed concurrently, on the same plates, in order to avoid intra-assay variability. Each sample was assayed twice using separate High Sensitivity kits, and the average cytokine concentrations of the two assays were used for all analyses. Data was collected using a Bio-Plex™ Suspension Array Reader (Bio-Rad Laboratories Inc®). Cytokine concentrations below the lower limits of detection were reported as the midpoint between the lowest concentration for each cytokine measured and zero.

4.3.4 Statistical analyses

Univariate analyses were performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, U.S.A.). Mann-Whitney U and Wilcoxon Signed Rank tests were used for unmatched and matched comparisons, respectively. Spearman Rank tests were used to test for correlations. P-values <0.05 were considered significant. P-values were adjusted using a FDR step-down procedure (Columb and Sagadai, 2006) in order to reduce false positive results when multiple comparisons were made.

Multivariate analyses were performed using STATA™ version 10 (StataCorp, Texas, U.S.A.). A multivariate regression model was used to determine the cytokines that best predicted 12 month viral load set-points. Log-transformed viral loads and cytokine concentrations were used, except for IL-12p40 which was not normalized following log transformation and was therefore included as a categorical variable [response/detectable (1) versus no response/undetectable (0)]. Using univariate regression as a starting point, cytokines that were significantly associated with viral load set-point, while controlling for each of the cytokines already included, were added to the model in a stepwise manner. Likelihood ratio tests were used to compare nested models. Due to the relatively small size of the study group, the sample of individuals used to develop the model included all women who were followed for at least 12 months post-infection and who had complete cytokine datasets (n=31). Model performance evaluation was conducted by repeatedly and randomly sampling subsets (n=10) consisting of three-quarters of the developmental sample and reapplying the model. The validity of the

assumptions underlying the model was evaluated and outliers and influential data points were determined using an analysis of residuals (Appendix A). Predicted viral load set-points were calculated for each study participant using the standardized β-coefficients of each of the cytokines included in the model and the observed concentrations or response of each cytokine in the following regression equation:

$$Log_{10}\lambda_i = \beta_0 + \beta_1 X_{1i} + \beta_2 X_{2i} + \ldots + \beta_\rho X_\rho i$$

λ_i is the predicted viral load set-point of the *i*th patient whose log-transformed cytokine concentrations or response were $X_{1i}, X_{2i}, \ldots X_{\rho i}$.

A Cox proportional-hazards model was used to determine the cytokines significantly associated with the time taken for study participant CD4 counts to fall below 350 cells/μl for two or more consecutive visits (survival time). Log-transformed cytokine concentrations were used, except in the case of IL-12p40. Following univariate analysis, cytokines which were significantly associated with survival time, while controlling for each of the cytokines already included, were added to the model in a stepwise manner. The likelihood ratio test was used to compare nested models and non-nested models were compared using Aikaike's Information Criterion (AIC), with the lowest AIC indicating the best model in terms of fit. The sample for model development included all women for whom complete cytokine datasets were obtained (n=35). Model performance evaluation was conducted by repeatedly sampling three-quarter subsets (n=10) of the developmental sample and reapplying the model. The validity of the assumptions underlying the model was evaluated and outliers and influential data points were determined by an analysis of residuals (Appendix B).

Risk scores were calculated for each participant using the β-coefficients of the Cox proportional-hazards model (Lee *et al.*, 1987) and observed concentrations or response of cytokines included in the model according to the following equation:

$$\lambda_i = \beta_1 X_{1i} + \beta_2 X_{2i} + \ldots + \beta_\rho X_{\rho i}$$

λ_i is the risk score of the *i*th patient whose log transformed cytokine concentrations or response are $X_{1i}, X_{2i}, \ldots X_{\rho i}$. The study group was divided into 3 groups based on risk scores as follows: low risk (0-15); medium risk (15-20); high risk (20-25).

4.4 Results

Forty black women from Durban, South Africa, recently infected with HIV-1 were recruited into this study (a subset of the 49 women included in Chapter 3). In this group of women, the median age was 25 years (range 18-59), 97.5% were unmarried and 20% reported having more than one partner. The median early infection (6 weeks post-infection) CD4 count and viral load in this group of women were 477 cells/μl and 76 200 copies/ml, respectively (Table 4.1). The median CD4 count and viral load set-point of women who were followed for at least 12 months post-infection were 415 cells/μl and 39 783 copies/ml, respectively.

Table 4.1 Clinical characteristics of study participants

Clinical Characteristics	Median (IQR)	N
CD4+ T cell counts:		
Early infection CD4+ T cell count (cells/μl)	477 (385-676)	40
CD4+ T cell count set-point (ave. of 3 visits overlying 12 months post-infection; cells/μl)	415 (314-607)	36
Plasma viral load:		
Early infection plasma viral load (copies/ml)	76 200 (117 775-339 250)	40
Plasma viral load set-point (ave. of 3 visits overlying 12 months post-infection; copies/ml)	39 783 (6 613-104 825)	36

Blood CD4+ T cell counts and plasma viral loads were determined for each woman (n=40) during early HIV-1 infection. CD4+ T cell count and viral load set-points were defined as the average CD4+ T cell or viral load measurements of 3 consecutive visits overlying the 12 month post-infection time-point for each of the 36 women who were followed for at least 12 months post-infection.

4.4.1 Plasma inflammatory cytokine concentrations were elevated during early HIV-1 infection and were associated with peak viremia

Cytokine concentrations in plasma from HIV-uninfected women (median of 25.5 weeks pre-infection; n=14) were compared with those of the same women recently infected with HIV-1 (median of 6 weeks post-infection; Figure 4.1 and Table 4.2). Plasma concentrations of several pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, TNF-α, IL-8, fractalkine and IP-10), anti-inflammatory IL-10, and T cell homeostatic cytokines (IL-2 and IL-7) were increased during early HIV-1 infection compared to matched pre-infection samples. After adjusting for multiple comparisons, IL-1α, IL-1β, TNF-α, IP-10 and IL-10 remained significantly elevated (Figure 4.1). Most cytokines (24/30) tended to be elevated during early infection (Table 4.2). No associations between plasma cytokine concentrations and STIs were found by logistic regression analysis. However, due to the small number of women who did not have an STI or BV (n=2) and the prevalence of multiple infections in this group, a more detailed analysis in a larger cohort of women would be required to confirm these findings.

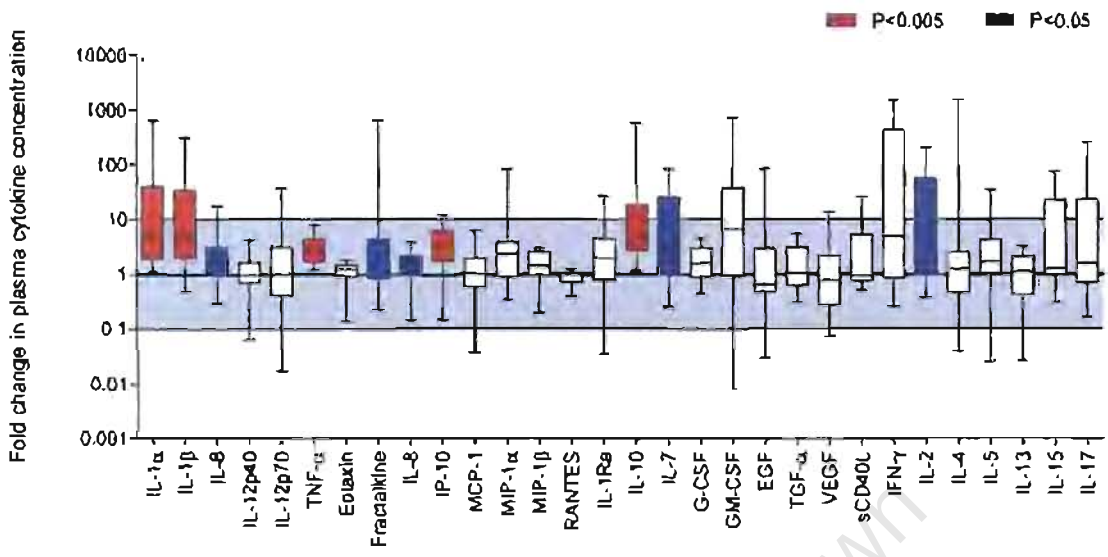

Figure 4.1. Comparison of plasma cytokine concentrations in women (n=14) before infection (median 25.5 weeks pre-infection) and during early HIV-1 infection (median 6 weeks post-infection). Wilcoxon Signed Ranks test was used for matched comparisons. Fold up-regulation in plasma cytokine concentrations following infection is shown. P-values <0.005 remained significant following adjustment for multiple comparisons (red bars). Blue bars (p<0.05) indicate cytokines that were significantly up-regulated before adjustment for multiple comparisons. IQR: Interquartile range.

It was found that IP-10, TNF-α, IL-1α, IL-1β, IFN-γ, and IL-10 correlated with the magnitude of viral load at the same time-point (Figure 4.2). After adjustment for multiple comparisons, IP-10 (adjusted p=0.012) and TNF-α (adjusted p=0.0165) concentrations remained significantly associated with viral load.

4.4.2 Plasma cytokine concentrations during early HIV-1 infection predicted viral load set-point

Univariate regression analysis was used to determine the relationship between plasma cytokine concentrations during early HIV-1 infection and viral load set-point (Table 4.3). As IL-12p70 was most strongly associated with set-point in the univariate analysis, this cytokine was used as a starting point to develop a multivariate model that included the cytokines that together most strongly predicted viral load set-point. Five cytokines were incorporated into this model, each of which was significantly associated with viral load set-point while controlling for the other cytokines included (p<0.005; Figure 4.3A). A positive IL-12p40 response (detection in CVL) and higher concentrations of IL-12p70 and IFN-γ were associated with lower viral load set-point, while higher concentrations of IL-7 and IL-15 were associated with higher set-point. The model was a good fit (F-statistic p<0.0001) and, together, the

Table 4.2 Comparison of plasma cytokine concentrations in women (n=14) before infection and during early HIV-1 infection

Function	Cytokine	Median cytokine concentration (IQR; pg/ml)		Median cytokine concentration change (IQR; pg/ml)	P-value
		HIV-1 negative	HIV-1 positive		
Inflammatory	IL-1α	65.31 (0.32-211.9)	225.60 (130.1-668.3)	119.60 (55.12-515.5)	0.0002**
	IL-1β	0.27 (0.03-0.96)	1.42 (0.79-2.08)	0.98 (0.3-1.58)	0.0012**
	IL-6	2.44 (1.42-6.15)	4.52 (2.33-10.93)	1.30 (0.08-6.75)	0.0168*
	IL-12p40	8.23 (1.97-90.99)	1.97 (1.97-96.87)	0.00 (-6.26-13.63)	0.6406
	IL-12p70	0.05 (0.01-0.63)	0.03 (0.01-0.46)	0.00 (-0.17-0.18)	0.8311
	TNF-α	2.42 (1.66-3.41)	6.60 (4.86-8.30)	3.77 (2.58-5.77)	0.0001**
Chemokines	Eotaxin	42.77 (28.99-75.68)	58.85 (37.61-84.5)	10.58 (-3.6-20.59)	0.1909
	Fractalkine	34.63 (18.21-61.44)	60.52 (51.06-91.56)	32.26 (-6.69-62.63)	0.0295*
	IL-8	1.87 (1.07-3.73)	2.61 (1.36-5.21)	0.83 (0.11-1.62)	0.0295*
	IP-10	132.40 (81.38-223.5)	555.40 (188.2-1412)	220.30 (46.73-718)	0.0046**
	MCP-1	54.50 (24.6-130)	57.34 (31.72-144.7)	1.52 (-48.28-52.73)	0.7148
	MIP-1α	22.83 (10.26-47.7)	30.64 (23.54-41.01)	15.89 (-3.94-28.59)	0.0574
	MIP-1β	21.36 (13.02-42.87)	28.07 (18.63-40.65)	9.46 (-0.41-15.69)	0.1763
	RANTES	484.60 (436.8-1104)	467.10 (428.3-519.9)	0.00 (-222.7-28.74)	0.6221
Anti-inflammatory	IL-1Ra	85.47 (45.84-335.5)	134.80 (72.96-309.4)	23.62 (-49.23-120.2)	0.391
	IL-10	3.21 (1.66-9.17)	18.16 (10.78-35.46)	12.17 (6.34-24.2)	0.0001**
Growth Factors	TGF-α	4.47 (1.83-11.92)	3.99 (2.74-9.58)	0.09 (-1.81-4.97)	0.5879
	EGF	18.78 (10.97-54.13)	14.29 (11.4-51.85)	-6.80 (-14.36-17.54)	0.5016
	VEGF	18.88 (9.38-33.7)	14.49 (6.97-45.3)	-2.79 (-14.04-9.06)	0.6257
Hematopoietic	IL-7	0.11 (0.05-1.04)	1.07 (0.59-2.68)	0.95 (-0.003-1.78)	0.0398*
	G-CSF	48.42 (25.94-108.4)	77.04 (42.64-123.4)	8.77 (-10.34-72.04)	0.1531
	GM-CSF	0.05 (0.01-0.35)	0.38 (0.01-1.03)	0.22 (-0.03-0.95)	0.083
Adaptive	sCD40L	5050.00 (2067-12601)	6713.00 (4225-10386)	-199.9 (-2012-4694)	0.5879
	IFN-γ	0.63 (0.01-4.87)	3.76 (2.26-9.23)	2.87 (-0.55-6.63)	0.1272
	IL-2	2.21 (0.46-9.86)	10.87 (5.87-16.66)	7.74 (1.44-14.64)	0.0203*
	IL-4	33.68 (4.36-67.15)	30.42 (0.86-137.60)	2.11 (-9.42-83.79)	0.3575
	IL-5	0.17 (0.05-0.38)	0.28 (0.10-0.53)	0.09 (-0.01-0.2)	0.2166
	IL-13	8.31 (0.87-23.11)	6.65 (0.13-35.86)	0.13 (-2-7.42)	0.4697
	IL-15	0.04 (0.04-2.5)	2.01 (0.04-3.32)	0.29 (0-2.32)	0.1289
	IL-17	3.81 (0.34-5.64)	4.30 (2.49-5.9)	1.40 (-1.14-3.47)	0.5186

*Did not remain significant following false discovery rate adjustment for multiple comparisons ** Remained significant following adjustment for multiple comparisons. P-values <0.05 were considered significant and highlighted. IQR: Interquartile range

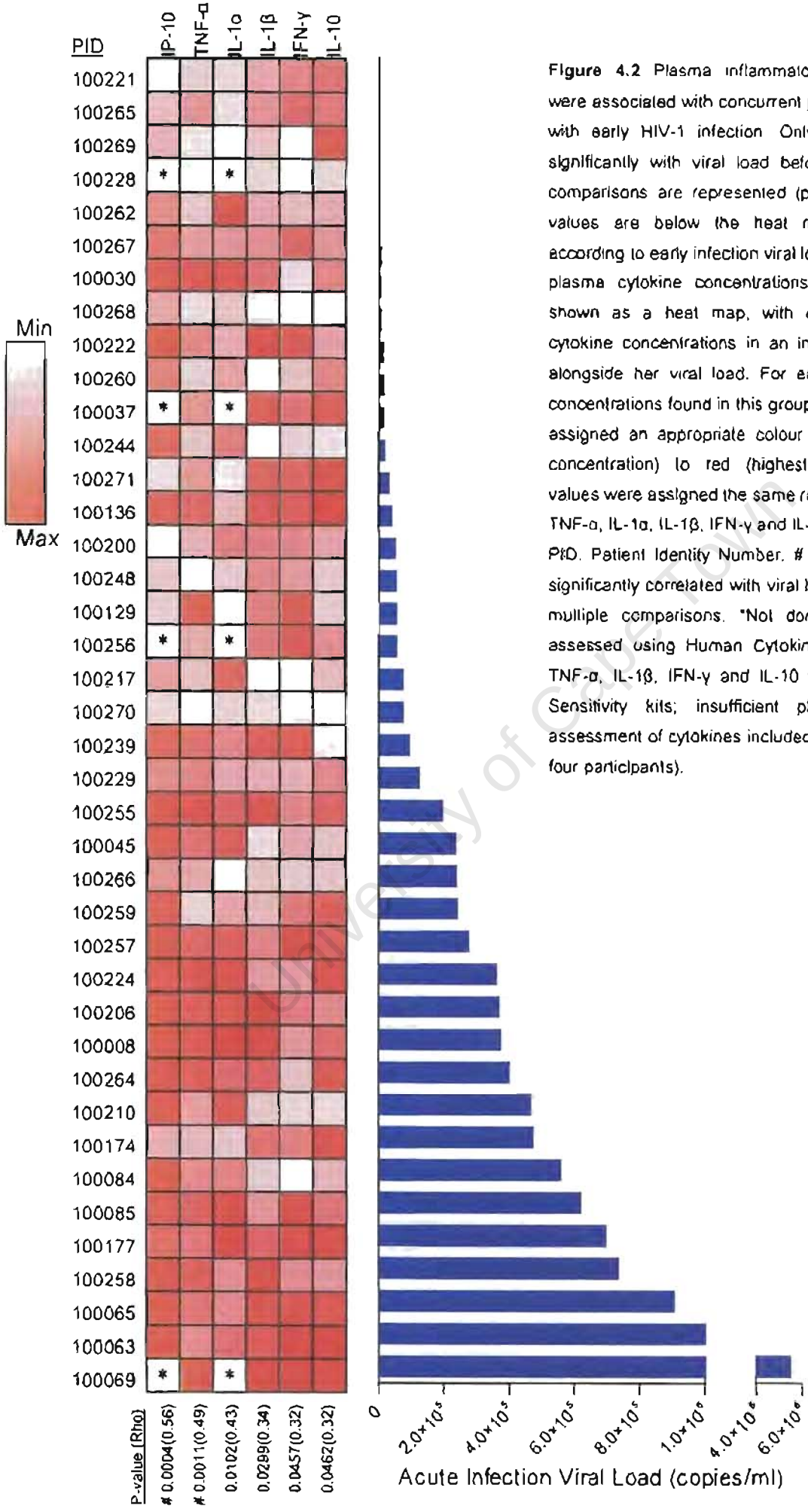

Figure 4.2 Plasma inflammatory cytokine concentrations were associated with concurrent plasma viral loads in women with early HIV-1 infection. Only cytokines that correlated significantly with viral load before adjustment for multiple comparisons are represented (p-values and Spearman rho values are below the heat map). Women are ranked according to early infection viral loads. Relative early infection plasma cytokine concentrations of study participants are shown as a heat map, with each row representing the cytokine concentrations in an individual woman and falling alongside her viral load. For each particular cytokine, the concentrations found in this group of women were ranked and assigned an appropriate colour ranging from white (lowest concentration) to red (highest concentration). Repeated values were assigned the same rank and hence colour. IP-10, TNF-α, IL-1α, IL-1β, IFN-γ and IL-10 correlated with viral load. PID, Patient Identity Number. # IP-10 and TNF-α remained significantly correlated with viral load following adjustment for multiple comparisons. *Not done (IP-10 and IL-1α were assessed using Human Cytokine LINCO*plex* kits whereas TNF-α, IL-1β, IFN-γ and IL-10 were measured using High Sensitivity kits; insufficient plasma was available for assessment of cytokines included on Human Cytokine kits for four participants).

concentrations of these 5 cytokines in plasma during early infection predicted 66% (adjusted R^2=0.6577) of the variation in viral set-point at 12 months post-infection.

Table 4.3 Univariate regression analysis of the associations between cytokine levels during early HIV-1 infection and viral load set-point

Function	Cytokine	β-coefficient	Std Error	P-value	95% Confidence Interval	
Inflammatory	IL-1α	0.27	0.17	0.138	-0.09	0.62
	IL-1β	0.54	0.35	0.130	-0.17	1.24
	IL-6	0.10	0.17	0.588	-0.26	0.45
	IL-12p40	-0.25	0.18	0.173	-0.61	0.11
	IL-12p70	**-0.38**	**0.14**	**0.011**	**-0.67**	**-0.09**
	TNF-α	0.45	0.52	0.392	-0.60	1.50
Chemokines	IL-8	0.57	0.38	0.149	-0.21	1.35
	RANTES	0.87	1.33	0.520	-1.86	3.60
	MIP-1α	0.22	0.28	0.430	-0.35	0.79
	MIP-1β	-0.36	0.38	0.342	-1.13	0.41
	MCP-1	0.06	0.33	0.856	-0.62	0.74
	Fractalkine	-0.10	0.23	0.672	-0.56	0.36
	IP-10	0.01	0.31	0.981	-0.62	0.64
	Eotaxin	0.47	0.56	0.408	-0.67	1.61
Anti-inflammatory	IL-1Ra	0.26	0.28	0.360	-0.31	0.84
	IL-10	-0.08	0.45	0.860	-1.00	0.84
Growth Factors	TGF-α	0.21	0.32	0.518	-0.44	0.85
	EGF	0.26	0.23	0.266	-0.21	0.73
	VEGF	-0.01	0.24	0.964	-0.49	0.47
Hematopoeitic	**IL-7**	**0.43**	**0.20**	**0.042**	**0.02**	**0.84**
	G-CSF	-0.53	0.40	0.190	-1.35	0.28
	GM-CSF	0.19	0.14	0.191	-0.10	0.48
Adaptive	sCD40L	0.18	0.39	0.636	-0.60	0.97
	IFN-γ	0.12	0.13	0.361	-0.15	0.39
	IL-2	**0.56**	**0.26**	**0.037**	**0.04**	**1.09**
	IL-4	0.09	0.12	0.456	-0.15	0.34
	IL-5	-0.10	0.19	0.621	-0.48	0.29
	IL-13	0.06	0.13	0.657	-0.20	0.32
	IL-15	0.19	0.13	0.146	-0.07	0.45
	IL-17	0.15	0.17	0.384	-0.20	0.50

The validity of the assumptions underlying the model was evaluated and outliers and influential data points were determined using an analysis of residuals (Appendix A). Exclusion of potential outliers from the dataset and reapplication of the model did not substantially influence the strength of the model. As the time from infection of sampling varied widely (1-12 weeks post-infection), this was included as a variable in the model. However, incorporation of time from infection did not significantly influence the

A

Number of observations = 31
Adjusted R^2 = 0.6577
Prob > F < 0.0001

Cytokine	β-coefficient	Std Error	P value	95% CI		Std β-coefficient
Per positive response						
IL-12p40	-1.27	0.39	0.003	2.07	0.47	-0.36
Per 1 log_{10} pg/ml increase						
IL-12p70	-0.51	0.10	<0.001	0.72	0.31	-0.61
IFN-γ	-0.38	0.09	<0.001	0.57	0.19	-0.49
IL-7	0.73	0.15	<0.001	0.42	1.06	0.63
IL-15	0.32	0.08	<0.001	0.16	0.48	0.45

B

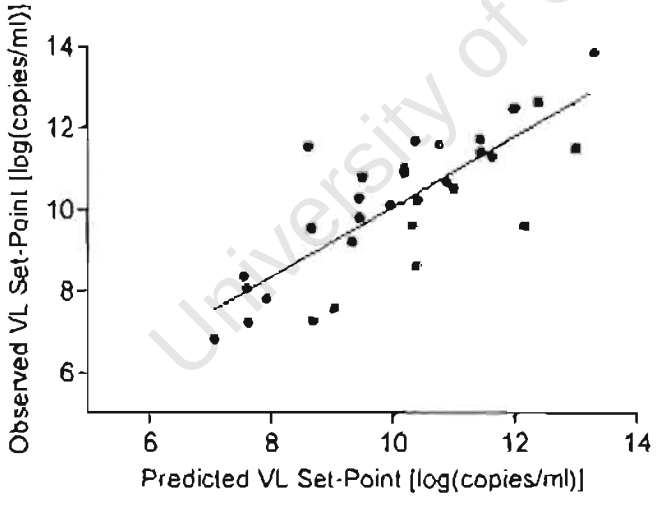

Figure 4.3 Early infection IL-12p40, IL-12p70, IFN-γ, IL-7 and IL-15 concentrations were predictive of viral load set-point. A) Each cytokine was significantly associated with viral load set-point (p-values <0.005). The model fitted the data significantly (p>F<0.0001), and together the 5 cytokines predicted 65.77% (Adjusted R^2 = 0.6577) of the variation in set-point. B) Set-point viral loads (VL) as predicted by the model correlated with observed set-point viral loads.

strength of the model (adjusted R^2=0.6435), nor was it significantly associated with viral load set-point (p=0.996; Appendix A). Thus the model presented does not include this variable (Figure 4.3A). It was furthermore found that the concentrations of the cytokines included in the model did not demonstrate any association with timing in relation to the estimated date of infection. Upon evaluation of model performance by reapplication of the model to 10 randomly-chosen three-quarter subsets of the study group, the influence of each variable on viral load set-point remained statistically significant and the directionality of the relationships between the variables and set-point remained constant, indicating that the model estimates are stable (Appendix A).

Predicted viral load set-points were calculated for each study participant using the standardized β-coefficients of each of the cytokines included in the model (Figure 4.3A) and the observed concentrations or response of each cytokine according to the following equation:

Log_{10} (VL set-point) = 10.07 + (-0.61 x $\log_{10}$ IL-12p70) + (0.63 x $\log_{10}$ IL-7) + (-0.49 x $\log_{10}$ IFN-γ) + (0.45 x $\log_{10}$ IL-15) + (-0.36 x IL-12p40 response)

As expected, predicted viral load set-points correlated well with observed set-points (Figure 4.3B). The model including IL-12p40, IL-12p70, IFN-γ, IL-7 and IL-15 concentrations during early infection fitted the viral set-point data of this cohort better (R^2=0.6577; p<0.0001) than either early infection viral load (R^2=0.0941; p=0.03) or CD4 counts (R^2=0.0734; p=0.0577) or the combination of both (R^2=0.0800; p=0.0917).

The substantially better R^2 value of the cytokine model is due in part to this model having been formulated with this cohort. Although the significance of the relationship between these cytokines and viral load set-point was strongly upheld upon reapplication of the model to three-quarter subsets of the study group, assessment of the true predictive power of this model would require application of the model to an entirely different, larger dataset.

4.4.3 Plasma cytokine concentrations during early HIV-1 infection predicted time taken for CD4 counts to fall below 350 cells/μl

Next, a subset of cytokines which were most significantly associated with the time taken for participant CD4 counts to fall below 350 cells/μl was determined using a Cox proportional-hazards model. Using univariate survival analysis as a starting point (Table 4.4), cytokines that were significantly associated with survival time were added to the model in a stepwise manner. Plasma concentrations of IL-1α, eotaxin and IL-7 were significantly associated with increased risk of CD4 loss, while GM-CSF and IL-12p40 were associated with reduced risk (p<0.05; Figure 4.4A). The model was a good fit (χ^2

p<0.0001) and exclusion of potential outliers from the dataset and reapplication of the model did not substantially influence the strength of the model (Appendix B). Time (post- infection) of sampling was

Table 4.4 Univariate Cox survival analysis of the associations between cytokine levels during early infection and time to CD4 below 350 cells/ul

Function	Cytokine	Hazard Ratio	Std Error	P-value	95% Confidence Interval	
Inflammatory	**IL-1α**	**1.74**	**0.39**	**0.014**	**1.12**	**2.71**
	IL-1β	1.72	0.53	0.078	0.94	3.14
	IL-6	1.04	0.13	0.777	0.80	1.34
	IL-12p40	0.76	0.13	0.112	0.55	1.06
	IL-12p70	0.85	0.10	0.166	0.68	1.07
	TNF-α	2.61	1.33	0.060	0.96	7.11
Chemokines	IL-8	1.30	0.35	0.334	0.77	2.19
	RANTES	1.33	1.10	0.729	0.26	6.70
	MIP-1α	1.19	0.30	0.491	0.72	1.96
	MIP-1β	1.09	0.35	0.781	0.59	2.04
	MCP-1	**1.93**	**0.53**	**0.017**	**1.13**	**3.30**
	Fractalkine	1.06	0.20	0.759	0.74	1.52
	IP-10	1.46	0.38	0.140	0.88	2.42
	Eotaxin	**2.59**	**0.99**	**0.013**	**1.22**	**5.48**
Anti-inflammatory	IL-1Ra	1.12	0.24	0.586	0.74	1.70
	IL-10	**2.11**	**0.66**	**0.018**	**1.14**	**3.89**
Growth Factors	TGF-α	0.96	0.23	0.849	0.60	1.53
	EGF	0.83	0.15	0.311	0.58	1.19
	VEGF	0.94	0.17	0.754	0.66	1.35
Hematopoeitic	IL-7	1.19	0.19	0.278	0.87	1.64
	G-CSF	0.81	0.26	0.520	0.43	1.53
	GM-CSF	1.05	0.13	0.702	0.83	1.32
Adaptive	sCD40L	1.16	0.33	0.613	0.66	2.04
	IFN-γ	1.25	0.17	0.090	0.97	1.63
	IL-2	1.73	0.50	0.057	0.98	3.05
	IL-4	0.95	0.09	0.597	0.80	1.14
	IL-5	0.94	0.13	0.622	0.72	1.22
	IL-13	0.94	0.09	0.477	0.78	1.12
Innate	IL-15	0.99	0.10	0.894	0.81	1.20
	IL-17	1.17	0.20	0.378	0.83	1.64

A

Number of observations = 35
LR χ^2 = 32.79
Prob > χ^2 < 0.0001

Cytokine	Hazard Ratio	Std Error	P value	95% CI		β-coefficient
Per positive response						
IL-12p40	0.08	0.07	0.005	0.01	0.46	-2.58
Per 1 $\log_{10}$ pg/ml increase						
GM-CSF	0.44	0.11	0.001	0.27	0.73	-0.81
IL-1α	9.49	6.03	<0.001	2.73	32.97	2.25
Eotaxin	3.71	2.00	0.015	1.29	10.67	1.31
IL-7	2.06	0.61	0.016	1.15	3.69	0.72

B

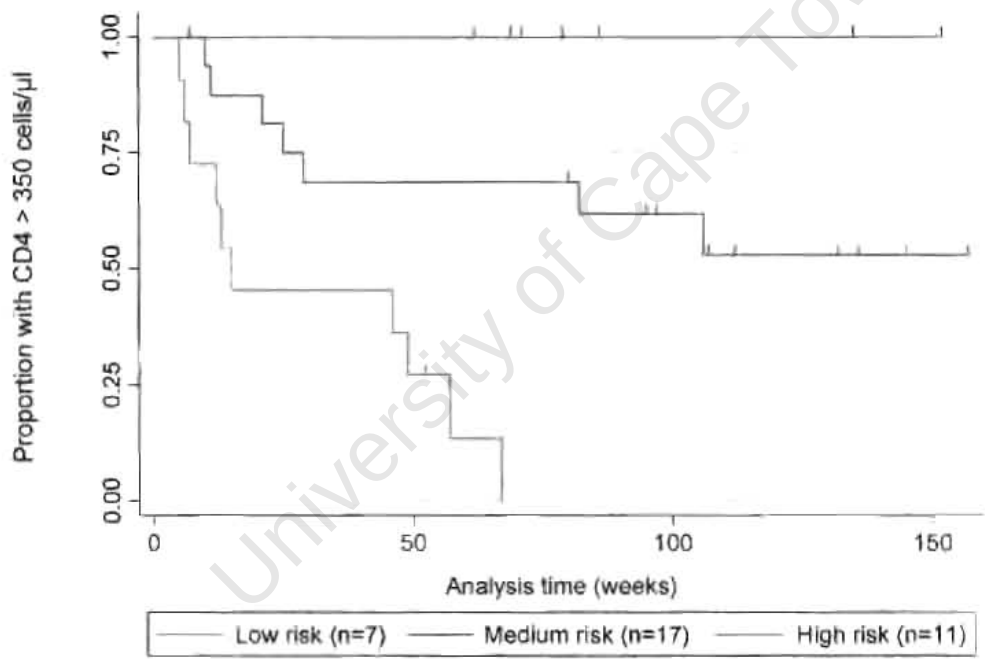

Figure 4.4 Early infection IL-12p40, GM-CSF, IL-1α, Eotaxin and IL-7 concentrations were associated with the time taken for the study participant CD4 counts to fall below 350 cells/µl. **A)** Each cytokine was significantly associated with survival time (p values <0.05) and the model fitted the data well ($p>\chi^2<0.0001$). **B)** Kaplan Meier survival estimates of women grouped according to risk score. Risk scores were calculated for each participant using the β coefficients of each cytokine included in the Cox proportional-hazards model, and women were divided into low (0-15), medium (15-20) and high (20-25) risk groups based on risk scores. Women in the high risk group experienced rapid CD4 loss, while women in the low risk group maintained CD4 counts above 350 cells/µl for the duration of follow-up. Each dash indicates a time point at which an individual woman left the study (censored event)

included in the model, however this variable was not significantly associated with survival time (p=0.357), while the relationships between each of the cytokines and survival time remained significant (Appendix B). Reapplication of the model to 10 randomly-chosen three-quarter subsets of the study group revealed that model performance was good, with the directionality of the relationships between each of the variables and survival time remaining constant (Appendix B).

Risk scores were calculated for each participant using the β-coefficients of the Cox proportional-hazards model (Lee *et al.*, 1987; Figure 4.4A) and observed early infection plasma concentrations or response of each of the cytokines included in the model according to the following equation:

Risk score = $1.31(\log_{10}\text{eotaxin}) + 2.25(\log_{10}\text{IL-1}\alpha) + 0.72(\log_{10}\text{IL-7}) - 2.58(\text{IL-12p40 response}) - 0.81(\log_{10}\text{GM-CSF})$

HIV-1-infected participants were divided into low, medium or high risk groups according to their risk scores. Women in the high risk group (n=11) experienced rapid CD4+ T cell loss to below 350 cells/μl during the study period (median time to event: 15 weeks post-infection; Figure 4.4B), with the exception of a single woman who left the study at 52 weeks post-infection. Women in the low risk group (n=7) maintained CD4 counts above 350 cells/μl during the study (median follow-up: 76 weeks post-infection). The Cox proportional-hazards model including early infection IL-1α, eotaxin, IL-7, GM-CSF and IL-12p40 was a better model in terms of fit (AIC: 85.09) when compared to models including only early infection CD4 count (AIC: 113.25), or viral load (AIC: 124.81), or the combination of both (AIC: 112.05).

4.5 Discussion

Systemic immune activation during HIV-1 infection has been identified as a major contributor to HIV-1 disease progression, and is the product of inflammatory responses to HIV-encoded TLR ligands, microbial translocation and the homeostatic response to CD4+ T cell depletion (Deeks *et al.*, 2004; Brenchley *et al.*, 2006; Catalfamo *et al.*, 2008; Meier *et al.*, 2007; Centlivre *et al.*, 2007). Here two models are proposed, based on a restricted set of plasma cytokines measured during early HIV-1 infection, which are useful for the prediction of viral load set-point and CD4 decline. It was found that the concentrations of IL-7, IL-12p40, IL-12p70, IFN-γ and IL-15 during early infection better predicted viral load set-point than early infection viral load, early infection CD4 counts, or the combination of both. Further, plasma concentrations of IL-7, IL-12p40, IL-1α, eotaxin and GM-CSF during early infection were strongly predictive of CD4 loss.

HIV-1 viral loads in plasma and systemic CD4 counts are widely accepted predictors of HIV-1 disease progression (de Wolf *et al.*, 1997; Mellors *et al.*, 1997; Lepri *et al.*, 1998; Goujard *et al.*, 2006). In addition, T cell proliferative capacity and activation states during early and chronic HIV-1 infection are predictive of disease progression (Liu *et al.*, 1997; Giorgi *et al.*, 1999; Hazenberg *et al.*, 2003; Deeks *et al.*, 2004). Concentrations of soluble biomarkers such as TNF-RII, neopterin and β_2-microglobulin during chronic infection have also been shown to predict progression to AIDS and/or CD4 decline with a degree of accuracy comparable to that of CD4 counts and viral load measurements (Mellors *et al.*, 1997; Fahey *et al.*, 1998; Zangerle *et al.*, 1998). While biomarkers of HIV-1 disease progression identified during chronic infection are useful for determining rates of progression to AIDS, the ability of these markers to predict clinical course at earlier infection stages has not been tested.

It has previously been shown that early HIV-1 infection is accompanied by a robust plasma pro-inflammatory cytokine response (Stacey *et al.*, 2009). In this Chapter it was found that plasma immunoregulatory IL-10 and pro-inflammatory IL-1α, IL-1β, TNF-α and IP-10 were elevated in women with early HIV-1 infection relative to pre-infection. Up-regulated pro-inflammatory cytokines, IP-10 and TNF-α, were significantly associated with higher HIV-1 viral load, suggesting that the observed inflammatory cytokine "storm" during early infection is induced, at least in part, in response to the presence of HIV-1 replication and products.

It was found that higher concentrations of IL-12p70, IL-12p40 and IFN-γ were associated with lower viral set-point, while IL-12p40 and GM-CSF were associated with prolonged maintenance of CD4 counts above 350 cells/μl. Production of these cytokines is partly regulated by a positive feedback loop, with IFN-γ and GM-CSF promoting IL-12p70 production and IL-12p70 in turn stimulating IFN-γ and GM-CSF secretion (Gazzinelli *et al.*, 1993; Kubin *et al.*, 1994; Flesch *et al.*, 1995). IL-12p70 and IFN-γ promote Th1 differentiation, favouring cell-mediated immunity and inhibiting Th2 responses (Manetti *et al.*, 1993; Manetti *et al.*, 1994; Boehm *et al.*, 1997). IFN-γ has previously been identified as a correlate of better disease prognosis in HIV-1 infection, and was positively associated with CD8+ T cell and activated NK cell counts (Ullum *et al.*, 1997; Bailer *et al.*, 1999). In SIV-infected macaques, IL-12p70 treatment during acute infection was associated with decreased viral loads, increased CD8+ NK and T cells, reduced naïve CD4+ T cells expressing homing markers, retention of HIV-specific CTL and prolonged survival (Ansari *et al.*, 2002). IFN-γ, GM-CSF and IL-12p40 are also principal macrophage-inducing cytokines, promoting their production, recruitment and/or activation (Nathan *et al.*, 1983; Metcalf, 1986; Gomez-Cambronero *et al.*, 2003; Jana *et al.*, 2003; Wira *et al.*, 2005; Cooper and Khader, 2007).

Although higher concentrations of GM-CSF and IL-12p70 in plasma during early HIV-1 infection were associated with slow disease progression, elevated concentrations in the genital tract correlated with

markers of rapid disease progression (Chapter 3). These findings likely reflect the compartmentalized nature of the female genital tract and differences in cell populations and phenotypes, gene expression patterns, and thus cytokine secretion profiles in the blood compared to the genital mucosa (Horton *et al.*, 2009). In Chapter 2, it was found that inflammatory cytokine concentrations in plasma did not correlate with cervicovaginal cytokine concentrations and STIs and BV, which cause inflammation in the female genital tract, were not associated with systemic inflammatory responses. Additionally, as IL-12p70 and GM-CSF have both pro-inflammatory functions and promote cellular immunity and macrophage activation (Metcalf, 1986; Jana *et al.*, 2003; Wira *et al.*, 2005; Abbas and Lichtman, 2007), their primary functions may be distinct in different compartments. It was found that GM-CSF and IL-12p70 in plasma were weakly correlated with pro-inflammatory cytokine levels in plasma, while in the genital tract these cytokines exhibited a high degree of correlation with other pro-inflammatory mediators. Therefore, in the genital tract, GM-CSF and IL-12p70 may be components of a nonspecific inflammatory reaction, whereas in blood, these cytokines may be markers of effective cytotoxic T lymphocyte (CTL) responses and macrophage activation which may control HIV-1 replication. The compartmentalized nature of the relationships between these cytokines and HIV-1 disease progression may represent an interesting research question for investigation in future studies.

In this study, elevated IL-7 and IL-15 concentrations were associated with higher viral load set-point, while IL-7, IL-1α and eotaxin were associated with greater CD4 loss. Eotaxin and IL-1α are chemotactic for T cells, potentially recruiting targets for HIV-1 infection (Miossec and Yu, 1984; Gerber *et al.*, 1997). IL-1α has additionally been found to strongly induce NF-κB activation, which binds to HIV LTR sequences and in so doing may directly up-regulate HIV-1 replication (Osborne *et al.*, 1989; Niu *et al.*, 2004). IL-7 and IL-15 are the principal regulators of CD4+ and CD8+ T cell homeostasis (Fry and Mackall, 2005; Picker *et al.*, 2006). Picker and colleagues have proposed a model whereby the balance between CD4+ T_{CM} and T_{EM} dictates the rate of HIV-1 disease progression (Okoye *et al.*, 2007). T_{EM} home to effector sites and serve as the primary targets for HIV-1 infection and destruction, but it appears to be their longer-lived T_{CM} precursors, which replenish these populations and decay more gradually, that determine the tempo of disease progression. Higher IL-7 levels are associated with lymphopenic states during HIV-1 infection (Napolitano *et al.*, 2001; Catalfamo *et al.*, 2008). IL-7 selectively induces proliferation of naïve T cells and T_{CM} cells and it has been proposed that, at high levels, IL-7 may disrupt the normal naïve/memory differentiation pathway by inducing memory-like characteristics on naïve cells. Exhaustion or excessive differentiation could reduce the longevity of the T_{CM} cell population, its ability to self-renew, expand and differentiate upon antigen stimulation (Picker *et al.*, 2006). Other studies have shown that, although circulating IL-7 levels are persistently elevated during HIV-1 infection, IL-7 signalling is impaired in both naïve and memory CD4+ T cells and may result in the loss of CD4+ T cell homeostasis (Juffroy et al., 2010; Landires et al., 2011).

IL-15 can induce antigen-independent proliferation and differentiation of T_{EM} from T_{CM} (Picker *et al.*, 2006; Geginat *et al.*, 2001). Thus, elevated IL-15 levels during early infection may accelerate the loss of T_{EM}, thereby depleting T_{CM} more rapidly. Additionally, increased IL-15 levels during acute SIV infection led to an up-regulation of CD4 expression on memory CD4+ T cells which increased in their susceptibility to SIV infection (Eberly *et al.*, 2009). Burger *et al.* (2009) have recently demonstrated that a greater destruction of the CD8+ T_{CM} compartment and accumulation of CD8+ T_{EM} correlated with a higher viral set-point. This may lead to exhaustion of CD8+ resources required for the control of HIV-1 and other infections.

IL-15 has also been implicated in polyclonal B cell activation in HIV-1 infection (Kacani *et al.*, 1999). Polyclonal B cell activation and differentiation, together with the destruction of germinal centres, has recently been described in acute HIV-1 infection (Levesque *et al.*, 2009) and likely results in the characteristically 'delayed' antibody response to HIV-1. Early dysregulation of the B cell response due to elevated IL-15 levels may lead to reduced viral control, as reflected by higher set-point viral loads. Thus, memory CD4+ T cell dysfunction and depletion, CD8+ T cell exhaustion and B cell dysfunction may partly be driven by elevated levels of IL-7 and IL-15 from the earliest stages of infection, setting the course for accelerated disease progression.

It was found that anti-inflammatory IL-10 was significantly elevated during early HIV-1 infection, was correlated directly with early infection viral loads before adjustment for multiple comparisons, and was associated with greater risk of CD4+ T cell loss in a univariate Cox survival analysis. Although IL-10 reduces HIV-1 replication in macrophages (Akridge *et al.*, 1994), this cytokine may contribute to HIV-1 persistence by suppressing effector T cell responses (Brooks *et al.*, 2006; Ejrnaes *et al.*, 2006; D'Andrea *et al.*, 1993). In support, it has been demonstrated that serum IL-10 levels increase with disease progression in HIV-1-infected individuals (Stylianou *et al.*, 1999). Additionally, the frequency of regulatory T cells in blood, an important source of IL-10 (Couper *et al.*, 2008), were shown to correlate inversely with the magnitude of SIV-specific CTL responses during acute SIV infection, and may contribute to viral persistence (Estes *et al.*, 2006).

In conclusion, the potential to use plasma cytokine concentrations during early HIV-1 infection to predict subsequent disease progression was demonstrated in this study. Two clusters of cytokines were more strongly predictive of viral load set-point and CD4+ T cell loss than either early infection CD4 counts, viral loads or both combined. The identification of cytokine biomarkers which are (1) indicative of early immune activation, (2) predictive of subsequent HIV-1 disease prognosis, and (3) can be measured directly in plasma samples from individuals with early HIV-1 infection, may inform approaches for evaluating the ability of therapeutic HIV-1 vaccines and microbicides to control HIV-1 infection.

CHAPTER 5

Chapter 5

Role of genital tract inflammatory cytokines in susceptibility to HIV-1 infection in women who became infected despite using the CAPRISA 004 1% Tenofovir microbicide gel

5.1 Summary

Tenofovir (1% TFV) gel is a vaginal microbicide that has recently been shown to reduce male-to-female sexual transmission of HIV-1 by 39%. In contrast, some earlier microbicides enhanced HIV-1 transmission by disrupting the vaginal epithelial barrier and/or inducing inflammatory cytokine responses and recruiting target cells for HIV-1 infection. In this study the impact of TFV gel use on cervicovaginal lavage (CVL) cytokine concentrations in trial participants, and signatures of increased susceptibility to HIV-1 infection in women who became infected, were investigated. HIV-uninfected South African women (n=889) were enrolled in the CAPRISA 004 trial and randomized to use either placebo or 1% TFV gel. Luminex was used to measure the concentrations of 12 cytokines in pre-infection CVL samples from 62 women who later became HIV-1-infected and from 104 women who remained uninfected. It was found that CVL cytokine concentrations did not differ between TFV and placebo gel users. However, elevated cervicovaginal concentrations of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, GM-CSF) and immunoregulatory IL-10 were associated with increased risk of HIV-1 infection, irrespective of gel use. The level of genital inflammation in individual women was similar at two separate time-points assessed in this study (8-104 weeks apart). These findings suggest that elevated levels of genital inflammation, which are sustained over time, may facilitate breakthrough HIV-1 infections, even in women using 1% TFV gel. Increasing the efficacy of the TFV microbicide gel may either require better management of factors associated with inflammation or augmentation of the next generation microbicides with agents to control inflammation.

5.2 Introduction

In sub-Saharan Africa, where 68% of all HIV-1-infected individuals reside, most new HIV-1 infections occur by sexual transmission to women (UNAIDS, 2010). Topically-applied vaginal microbicide gels have been tested as a strategy for preventing sexual HIV-1 transmission to women, although the development of a safe and efficacious microbicide has proved to be challenging (Voelker, 2006). TFV (1%) gel was the first anti-retroviral drug-containing microbicide formulation to be tested and to show protection against male-to-female sexual transmission of HIV-1 in a large-scale Phase IIb clinical trial (Abdool Karim *et al.*, 2010a). TFV gel reduced the risk of HIV-1 infection by 39% overall, and by 54% in high gel adherers (gel adherence >80%).

Earlier candidate microbicides, N-9 and CS, were found to induce genital inflammatory cytokine responses and were associated with increased risk of HIV-1 infection (Mesquita *et al.*, 2009; Fichorova *et al.*, 2001b; Schwartz *et al.*, 2006). Although a direct association between inflammation and susceptibility to HIV-1 infection has not yet been demonstrated, several studies have suggested that there is likely to be a relationship. *In vitro* studies have shown that inflammatory cytokines may directly increase HIV-1 replication by activating NF-κB which binds to the HIV-1 enhancer, long terminal repeat, and up-regulates transcription (Osborne *et al.*, 1989; Poli *et al.*, 1990). Inflammatory cytokines are also associated with immune cell recruitment, differentiation and activation at the genital mucosa and with disruption of tight junctions between cervicovaginal epithelial cells and increased permeability of this barrier (Wira *et al.*, 2005; Charo *et al.*, 2006; Nkwanyana *et al.*, 2009; Nazli *et al.*, 2010). Non-ulcerative STIs and BV, which cause genital inflammation, are associated with increased risk of HIV-1 infection (Levine *et al.*, 1998; Fichorova *et al.*, 2001a; Yudin *et al.*, 2003; Reddy *et al.*, 2004; Novak *et al.*, 2007; Rebbapragada and Kaul, 2007). In Chapter 2 a weak relationship between elevated pro-inflammatory cytokine concentrations and risk of HIV-1 infection was found, despite the long period of time between sample collection and HIV-1 incidence (median 43 weeks) and the fact that women were treated for laboratory-diagnosed STIs and BV during the study. In rhesus macaques, establishment of a productive SIV infection following vaginal exposure was shown to be dependent on pro-inflammatory chemokine production and recruitment of CD4+ T cell targets for SIV infection (Li *et al.*, 2009; Haase, 2011). Furthermore, inhibition of inflammatory responses using a topically-applied anti-inflammatory agent (glycerol-monolaurate) was found to reduce chemokine concentrations in the genital tract and prevent SIV infection (Li *et al.*, 2009).

In a pre-clinical study, TFV gel treatment of cervical epithelial cells in culture did not result in up-regulation of inflammatory cytokine production (Mesquita *et al.*, 2009). Additionally, early small-scale safety trials demonstrated that daily TFV application for 2 weeks was safe, well-tolerated and did not influence the mucosal inflammatory environment (Mayer *et al.*, 2006; Keller *et al.*, 2010). The aim of this study was to determine if 1% TFV gel affected inflammatory cytokine production, thus providing additional safety data

on TFV gel use in women participating in the CAPRISA 004 TFV microbicide trial. In addition, the role of genital tract inflammation in susceptibility to HIV-1 infection was investigated in those women who became infected despite using TFV gel.

5.3 Materials and methods

5.3.1 Description of study participants

A total of 889 HIV-uninfected South African women were enrolled in the CAPRISA Phase IIb, two-arm, double-blind, 1% TFV trial through the rural CAPRISA Vulindlela and the urban eThekwini clinics (Abdool Karim *et al.*, 2010a). Women were randomized to use either placebo or 1% TFV gel. Women were enrolled and followed between May 2007 and March 2010. HIV-1 testing was conducted monthly using 2 rapid HIV-1 antibody tests [Abbott determine™ (Abbott Laboratories, Illinois, U.S.A.) and Uni-Gold™ (Trinity Biotech, Ireland)] and confirmed with 2 independent RNA-PCR assays at least 1 week apart. Pelvic exams were performed at each visit and the presence of any genital abnormalities was recorded. Of the women initially enrolled in the trial, 98 became infected with HIV-1. A total of 62/98 women who became infected with HIV-1 and 104 women who remained HIV-uninfected were included in this study. These women included 75 TFV gel users and 91 placebo users.

5.3.2 Cytokine measurements

Multiplex Flow Cytometric Assays were used to measure the concentrations of 12 cytokines (IL-1α, IL-1β, IL-6, IL-7, IL-8, IL-10, GM-CSF, IP-10, MCP-1, MIP-1α, MIP-1β and TNF-α) in pre-infection (median 18 weeks pre-infection, range 2-70) CVL from women who later became HIV-1 infected, and in CVL from women who remained HIV-uninfected during the follow-up period. Cytokines were selected for this study based on associations found between these cytokines and STIs and HIV-1 disease progression, as discussed in Chapters 2 and 3. CVL samples were collected from each woman, essentially as previously described (Bebell *et al.*, 2008) with the following modifications: Three millilitres of sterile normal saline (instead of 10ml used in Chapters 2 and 3) was used to repeatedly bathe the cervix and allowed to pool in the posterior fornix, where it was then aspirated into a plastic bulb pipette. Samples were centrifuged and the supernatant stored at -80°C. CVL samples were not collected from menstruating participants, and sampling was postponed to the following week. Prior to cytokine measurements, CVL samples were filtered by centrifugation using 0.2 µm cellulose acetate filters (Sigma, U.S.A.). Concentrations of cytokines were measured in CVL using Milliplex™ High Sensitivity and Human Cytokine kits according to the manufacturer's protocol (Millipore Corporation, MA, U.S.A.). The sensitivity of these kits ranged between 0.05 and 6.6 pg/ml for each of the 12 cytokines measured. Data were collected using a Bio-Plex™ Suspension Array Reader (Bio-Rad Laboratories Inc®) and a 5 PL regression formula was used to

calculate cytokine concentrations from the standard curves. Data were analysed using BIO-plex manager software (version 4; Bio-Rad Laboratories Inc®). Cytokine levels that were below the lower limit of detection of the assay were reported as the mid-point between the lowest concentration measured for each cytokine and zero. Samples were assayed in duplicate using separate Milliplex kits in order to confirm the reliability and reproducibility of detected concentrations. Cytokines were selected for this study based associations between cytokines and STIs and risk of HIV-1 infection that were identified in Chapter 2, associations between cytokines and HIV-1 disease progression that were found in Chapter 3 and the reliability and reproducibility of their measurement using Luminex.

5.3.3 Statistical analyses

Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, U.S.A.) and STATA™ version 10 (StataCorp, Texas, U.S.A.). Distribution of all variables was assessed by Shapiro-Wilk and Shapiro-Francia tests. Mann Whitney U test was used for unmatched comparisons. Logistic regression was used to determine associations between cytokine concentrations and risk of HIV-1 infection while controlling for TFV or placebo gel use and age of study participants. Cytokine concentrations and age were log-transformed. Multivariate linear regression and logistic regression were used to determine the relationships between genital inflammatory cytokine concentrations and demographic and clinical characteristics. Cytokines that were not normally-distributed following log-transformation (TNF-α, MIP-1α, MIP-1β and IL-7) were converted to categorical variables and logistic regression was used to determine the relationships between each of these cytokines and each demographic/clinical characteristic. Spearman Rank test and Wilcoxon Signed Rank test were used to evaluate whether each woman was similarly ranked at each time-point according to her CVL cytokine concentrations and whether cytokine concentrations in each woman differed between time-points. P-values were adjusted using an FDR step-down procedure in order to reduce false positive results when multiple comparisons were made (Columb and Sagadai, 2006). Adjusted p-values <0.05 were considered significant.

5.4 Results

Sixty-two women who became HIV-1-infected and 104 women who remained HIV-uninfected (Table 5.1) while participating in the CAPRISA 004 1% TFV gel double blinded placebo controlled phase IIb clinical microbicide trial were evaluated in this study (Abdool Karim *et al.*, 2010a). A total of 26/62 women who became HIV-1-infected (41.9%) and 49/104 women who remained HIV-uninfected (47.1%) were using 1% TFV gel. The median age of study participants was 23 years (range 18-39). Women who later became HIV-1-infected were younger (median 22 years, range 18-36) than women who remained uninfected (median 24 years, range 18-39; p=0.01). Cervicitis, candidiasis and genital ulceration were

uncommon in the women who were included in this study. Vaginal discharge was however observed in 15.0% of women who later became HIV-1-infected and 8.8% of uninfected women (p=0.30).

Table 5.1 Demographic, behavioural and clinical characteristics of study participants.

	Pre-infection	HIV-uninfected
N	62	104
TFV gel users [N/total (%)]	26/62 (41.9)	49/104 (47.1)
Demographic and behavioral [Median (range)]		
Age	22 (18-36)	24 (18-39)*
Returned used applicators per month	8 (0-20)	8 (0-50)
Reported sexual intercourse per month	5 (0-20)	6 (0-85)
Genital abnormalities [N/total (%)]		
Cervicitis	3/60[a] (5.0)	1/102[a] (1.0)
Vaginal candidiasis	2/60 (3.3)	0/102 (0)
Vaginal discharge	9/60 (15.0)	9/102 (8.8)
Genital ulceration	1/60 (1.7)	1/102 (1.0)
Contraception [N/total (%)]		
Depo-Provera	44/60[a] (73.3)	59/102[a] (57.8)
Nuristerate	9/60 (15.0)	17/102 (16.7)
Oral Contraception	7/60 (11.7)	20/102 (19.6)
Condom	46/60 (76.7)	79/102 (77.5)

*p<0.05. [a]Four participants included in this study did not have gynaecological or contraceptive data for the visits at which CVL samples were collected.

5.4.1 Women who were using 1% TFV gel did not have elevated genital tract cytokine concentrations compared to women in the placebo arm

Earlier candidate microbicides were found to induce genital inflammatory cytokine responses and were associated with increased risk of HIV-1 infection (Mesquita *et al.*, 2009; Fichorova *et al.*, 2001b; Schwartz *et al.*, 2006). In order to evaluate whether the use of 1% TFV gel increased genital inflammation, cytokine concentrations were compared in CVLs from 75 women who were using TFV gel to those of 91 placebo-users, each of whom had been applying gel for at least 3 months. It was found that none of the inflammatory cytokines measured were elevated in the genital tracts of women using TFV gel compared to placebo users (Figure 5.1). Although this analysis included pre-infection CVL from 62 women who later became HIV-1-infected (26 TFV and 36 placebo users) and CVL from 104 women who remained HIV-uninfected (49 TFV and 55 placebo users), no significant differences were found between cytokine concentrations in TFV compared to placebo users within the pre-infection or the HIV-uninfected groups (data not shown).

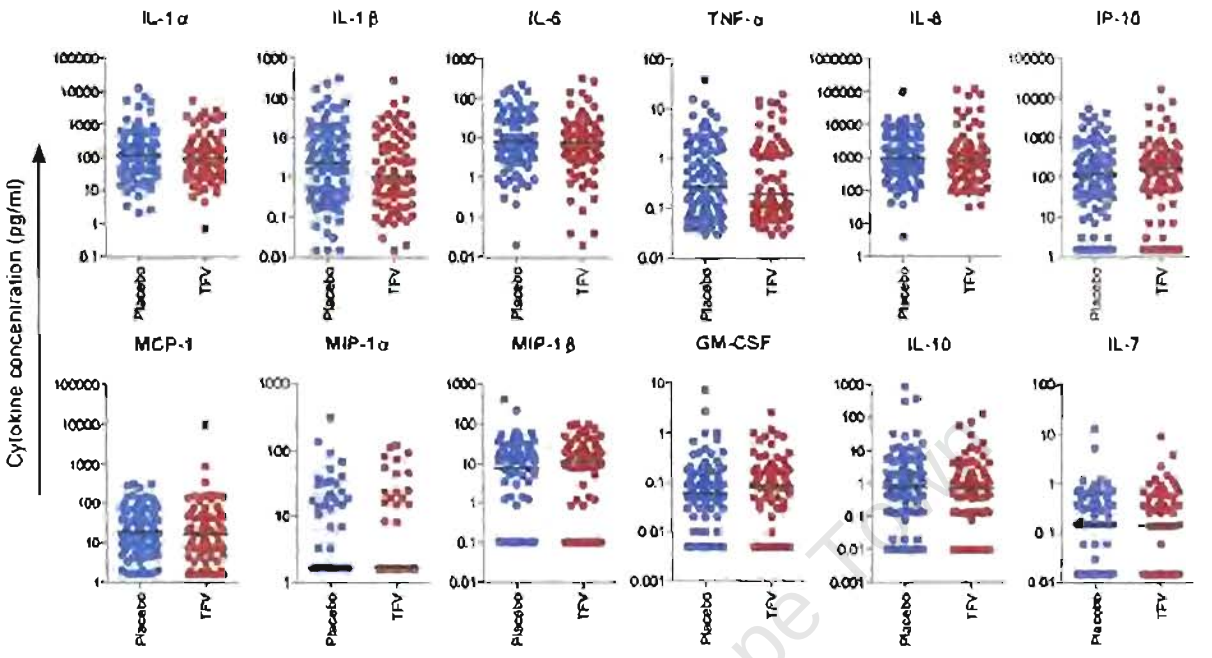

Figure 5.1 Cytokine concentrations in CVL samples from HIV-uninfected placebo and TFV gel users. CVL cytokine concentrations in women who were using placebo gel (n=91) are indicated by blue dots, while red dots indicate those of TFV gel users (n=75).

5.4.2 Women with elevated genital tract inflammatory cytokine concentrations were at increased risk of HIV-1 infection

Inflammatory cytokine concentrations were compared in pre-infection CVLs from 62 women who later became HIV-1-infected (sampled at a median of 18 weeks pre-infection, range 2-70) and from 104 women who remained HIV-uninfected. Figure 5.2 shows the relative cytokine concentrations measured in CVLs from each woman. Women were clustered according to their genital cytokine concentrations. It was found that women who later became HIV-1-infected had up-regulated pre-infection cytokine concentrations and tended to cluster separately from women who remained uninfected. Elevated cervicovaginal concentrations of 10/12 cytokines measured were associated with increased risk of HIV-1-infection, after adjusting for multiple comparisons [Figure 5.3; MIP-1α, IL-8, MIP-1β, IL-1α, GM-CSF, MCP-1, IL-6, IP-10, IL-1β and IL-10]. It was found that 1 $\log_{10}$ pg/ml increases in the concentrations of these cytokines were associated with 14 - 68% increased risk of HIV-1-infection [odds ratios (OR) 1.14 -

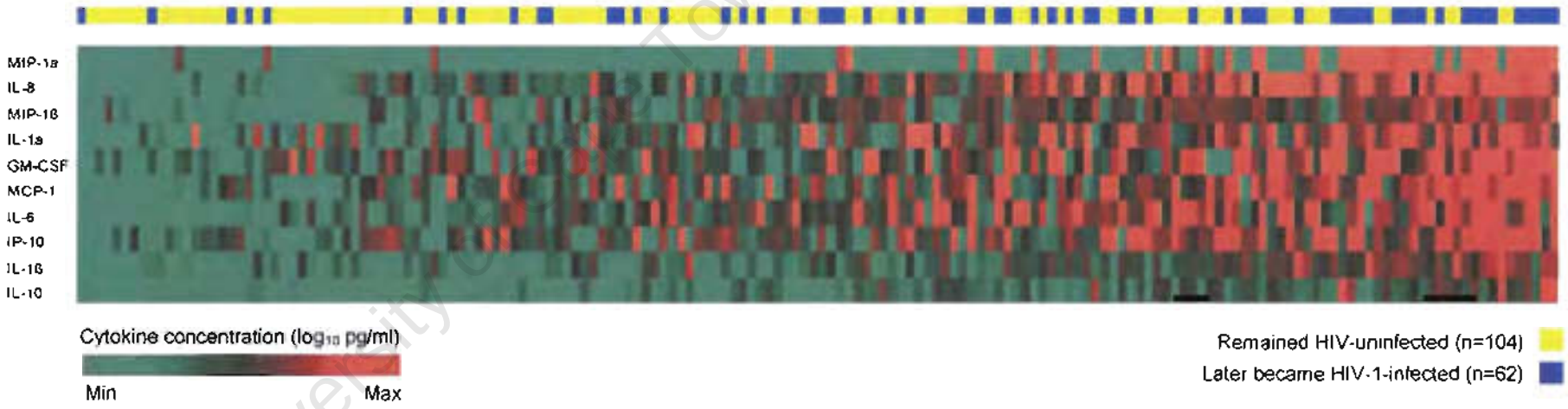

Figure 5.2 Cytokine concentrations in CVL from women who remained HIV-uninfected and pre-infection CVL from women who later became HIV-1-infected. Cytokine concentrations (log_{10} pg/ml) are shown as a heat map, with green indicating low concentrations and red indicating high concentrations. Women who later became HIV-1-infected (n=62; indicated by blue blocks) had up-regulated pre-infection CVL cytokine concentrations and tended to cluster with one another, while women who remained HIV-uninfected had lower cytokine concentrations and clustered together (n=104; indicated by yellow blocks).

1.68). Furthermore, detection of either MIP-1α (>3.3 pg/ml CVL) or MIP-1β (>0.21 pg/ml) in the genital tract was associated with a 321% and 367% increased risk of HIV-1 infection, respectively [OR (95% confidence interval, CI): 4.21 (2.0 - 8.7) and 4.67 (2.1 - 10.5), respectively]. The relationships between inflammatory cytokine concentrations and risk of HIV-1 infection were found to be independent of TFV or placebo gel use (Figure 5.3). As women in the HIV-uninfected group tended to be older than women who later became HIV-1-infected, a logistic regression analysis was conducted in order to confirm the relationship between inflammatory cytokine concentrations and increased risk of HIV-1 infection, while adjusting for age. Elevated concentrations of MIP-1α, IL-8, MIP-1β, IL-1α, GM-CSF and IL-1β remained significantly associated with increased risk of HIV-1 infection, after adjusting for gel use, age and multiple comparisons (Figure 5.3).

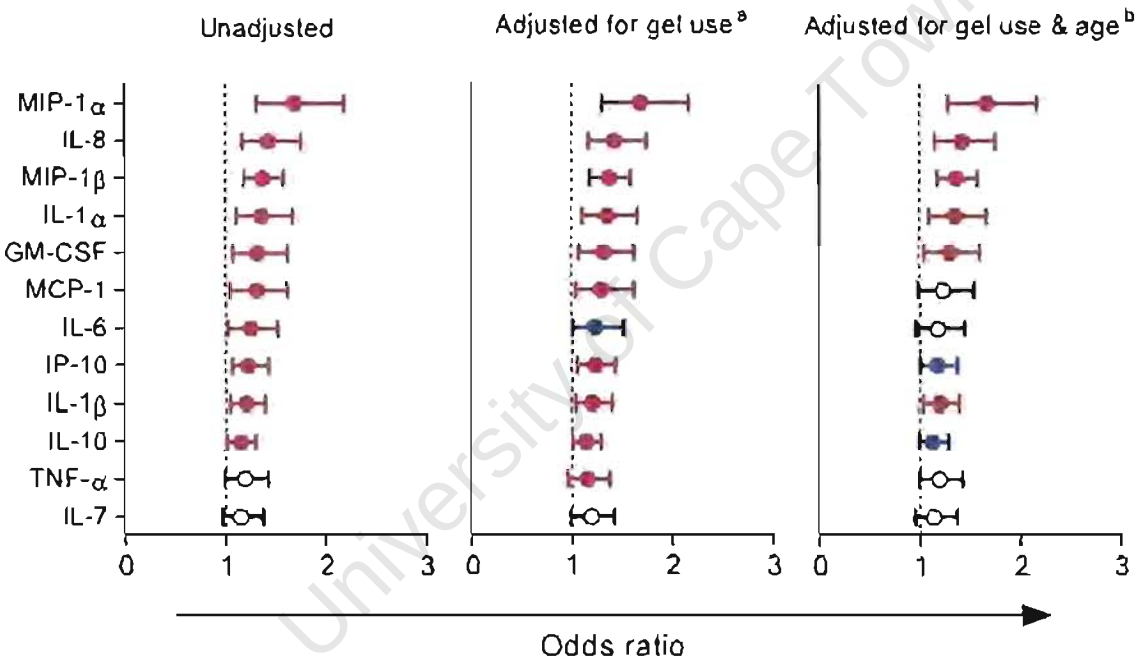

Figure 5.3 Relationships between CVL cytokine concentrations and risk of HIV-1 infection. Logistic regression was used to determine associations between CVL cytokine concentrations and risk of HIV-1 infection. This analysis included cytokine concentrations measured in pre-infection CVL from 62 women who later became HIV-1-infected and CVL from 104 women who remained uninfected. [a]Odds ratios (OR) adjusted for TFV or placebo gel use. [b]OR adjusted for TFV or placebo gel use and age of study participants. Error bars indicate 95% confidence intervals. Cytokines in blue were significantly ($p<0.05$) associated with risk of HIV-1 infection. Cytokines in red were significantly associated after adjusting for multiple comparisons

5.4.3 Genital tract cytokine concentrations were associated with vaginal discharge and age of study participants

In Chapters 2 and 3 it was found that cervicovaginal cytokine concentrations were associated with STIs, BV, vaginal discharge and the age of study participants. Therefore, in order to determine the possible causes of inflammation in the women who were included in this study, associations between genital cytokine concentrations and age, cervicitis, vaginal candidiasis, vaginal discharge, genital ulceration, hormone contraceptive choice and condom use were evaluated. Similar to the findings discussed in Chapter 2, it was found that IL-1α and IL-1β concentrations were elevated in the genital tracts of women who had vaginal discharge compared to women who did not have discharge, although this was not significant after adjusting for multiple comparisons (Figure 5.4A). Higher cervicovaginal concentrations of IL-6, IP-10 and MCP-1 were significantly associated with younger age in univariate regression analyses, after adjusting for multiple comparisons (Figure 5.4B). These findings were confirmed in multivariate linear and logistic regression analyses, including TFV or placebo gel use in addition to each of the demographic and clinical factors that were evaluated (Figure 5.4C). As few women had cervicitis, vaginal candidiasis or genital ulceration (n=4, n=3 and n=2, respectively), the relationships between each of these manifestations and genital cytokine concentrations could not be assessed by univariate analysis. However, these factors were included in each multivariate regression analysis in order to control for their potential influence on cytokine concentrations. It was found that IL-6, IP-10 and MCP-1 remained inversely associated with age [β coefficient (95% CI): -1.9 (-3.4 - -0.5), -2.6 (-4.5 - -0.7) and -1.6 (-2.9 - -0.3), respectively] and IL-1α and IL-1β concentrations remained associated with vaginal discharge [1.1 (0.2 - 2.0) and 1.5 (0.3 - 2.6), respectively] after adjusting for other all other demographic and clinical factors assessed. No associations between hormone contraceptive or condom use and genital cytokine concentrations were observed in the univariate analysis, however GM-CSF was up-regulated in the genital tracts of women using oral contraception and IP-10 was down-regulated in women using condoms after adjusting for each of the other possible causes of genital inflammation that were evaluated in this study.

5.4.4 Genital tract inflammatory cytokine concentrations in each woman remained relatively constant over time

The concentrations of each cytokine were compared longitudinally for a subset of women included in the study in order to determine whether genital inflammation was sustained over time in women who became HIV-1-infected compared to those that remained uninfected. Two pre-infection time-points from 21/62 women who later became infected and 2 time-points from 36/104 women who remained uninfected were included in this analysis. The median time between the 2 sampling time-points was 48 weeks (range 8 –

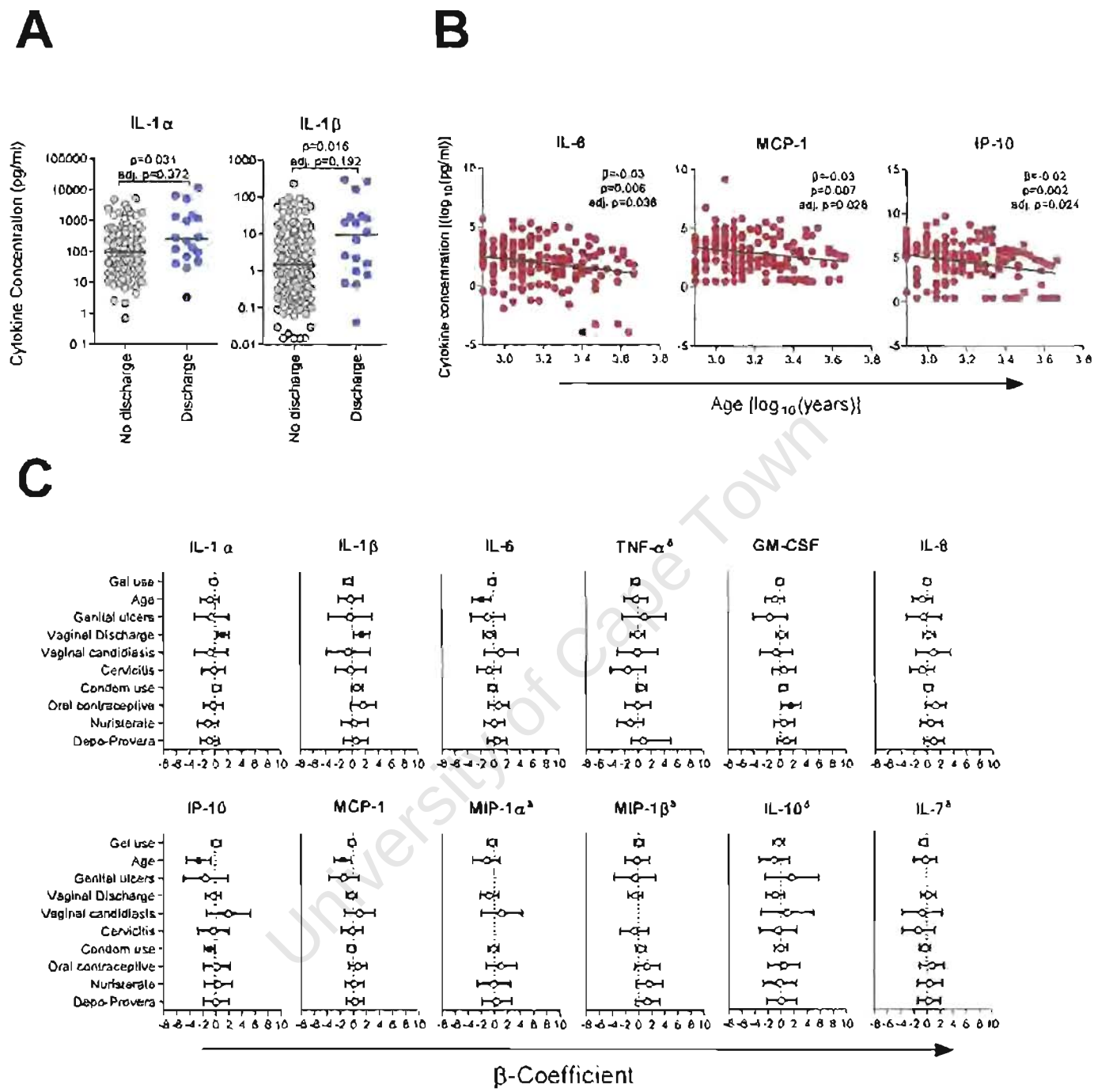

Figure 5.4 Cervicovaginal cytokine concentrations were associated with vaginal discharge and age of study participants. Univariate analyses (Mann Whitney U test or linear regression) were used to assess the relationships between cytokine concentrations and Depo-Provera, Nuristerate or oral contraceptive use, condom use, vaginal discharge and age of women (n=162) participating in this study. Significant associations between cytokine concentrations and A) vaginal discharge and B) age of women are shown. C) Multivariate linear regression and logistic regression were used to confirm these relationships, while controlling for each of the other demographic or clinical characteristics assessed in this study (Depo-Provera, Nuristerate or oral contraceptive use, condom use,

Figure 5.4 continued. cervicitis, vaginal candidiasis, vaginal discharge and genital ulceration) and TFV or placebo gel use. [$]Cytokines were not normally-distributed following log-transformation and were converted to categorical variables for logistic regression analysis. All women who had genital ulceration had undetectable IL-7 and MIP-1α concentrations in their genital tracts and women who had cervicitis had undetectable MIP-1α concentrations. Therefore these variables were excluded from these analyses. β-coefficients are indicated by circles, error bars indicate 95% confidence intervals. Significant associations are coloured blue ($p<0.05$).

104). It was found that the concentrations of 7 of the 12 cytokines measured at each time-point were correlated (Figure 5.5). Additionally, none of the cytokines assessed differed significantly between the two time-points. Therefore, genital tract inflammation in individual women ranked similarly at both time-points assessed, indicating that women with elevated inflammation at one time point were the same women with elevated inflammation at the subsequent time point.

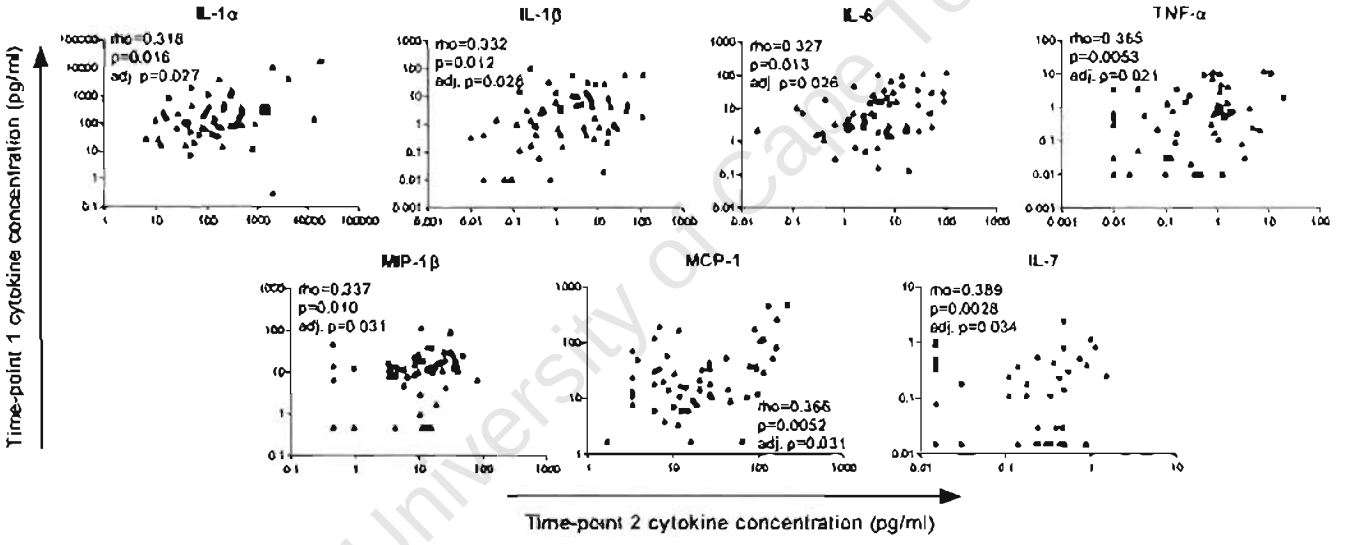

Figure 5.5 Spearman correlations between genital tract cytokine concentrations in the same women at 2 different time-points [median 48 weeks apart (range 8 – 104)]. Cytokine concentrations were measured in pre-infection CVL samples from 21 women who later became HIV-1-infected and CVL from 36 women who remained HIV-uninfected. Only cytokines that correlated significantly between time-points after adjusting for multiple comparisons are shown.

5.5 Discussion

This study has demonstrated that an important risk factor for breakthrough infections in women who became infected with HIV-1 in the TFV microbicide trial was persistently elevated inflammatory cytokines in their genital tracts. Higher cervicovaginal concentrations of several pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β and GM-CSF) and anti-inflammatory IL-10 were associated with increased risk of HIV-1 infection. Following adjustment for TFV gel use, age and multiple comparisons, six of the nine pro-inflammatory cytokine concentrations (IL-1α, IL-1β, IL-8, MIP-1α, MIP-1β and GM-CSF) remained significantly associated with susceptibility to HIV-1 infection. Furthermore, the level of genital inflammation in individual women was similar at two separate time-points assessed in this study (8-104 weeks apart), with 7/12 of the cytokines measured correlating between these time-points. This indicates that each woman was similarly ranked according to her genital cytokine concentrations at each time-point, suggesting that some women had consistently low cytokine concentrations in their genital tracts while others had consistently high cytokine concentrations.

It is well established that STIs and BV are associated with elevated inflammatory cytokine production in the genital tract (Levine *et al.*, 1998; Fichorova *et al.*, 2001a; Yudin *et al.*, 2003; Reddy *et al.*, 2004; Novak *et al.*, 2007; Rebbapragada and Kaul, 2007). Other possible correlates of inflammation include hygiene practices such as antiseptic douching, exposure to seminal proteins and lubricants (Scholes *et al.*, 1993; Fichorova *et al.*, 2001b; Sharkey and Robertson, 2007; Berlier *et al.*, 2006). Hormone cycling and hormone contraceptive use are also associated with changes in the genital inflammatory response, as are microabrasions caused by sexual activity (Hunt *et al.*, 1997; Ghanem *et al.*, 2005; Akoum *et al.*, 2000; Critchley *et al.*, 2001; Bengtsson *et al.*, 2004; Ghanem *et al.*, 2005; Guimaraes *et al.*, 1997; de Jong and Geijtenbeek, 2008). Additionally, demographic or genetic factors, such as race or cytokine gene copy number, may play a role (Gonzalez *et al.*, 2005; Field *et al.*, 2009; Liu *et al.*, 2010; Ryckman *et al.*, 2008; Zabaleta *et al.*, 2008). In Chapter 2 it was found that cervicovaginal inflammation was associated with BV, STIs (both symptomatic and asymptomatic) and the age of the women in the study. Although STIs were diagnosed syndromically and laboratory testing was not conducted in the 1% TFV microbicide study, associations between inflammatory cytokine concentrations and symptoms of STIs, as well as vaginal candidiasis, hormonal contraceptive use, condom use and age were explored. Similar to the findings in Chapter 2, it was found that women who had vaginal discharge had higher genital concentrations of IL-1α and IL-1β, indicating that STIs may be partly responsible for genital inflammation in the women participating in this study. Additionally, as found in Chapter 2, younger women had higher cytokine concentrations in their genital tracts compared to older women. IL-6 was again identified as a correlate of age, as well as IP-10 and MCP-1, with each of these cytokines highest in younger women. Despite these associations, the relationship between inflammatory cytokine concentrations and risk of HIV-1 infection was found to be independent of the age of study participants. However, the finding that younger women

have higher levels of genital inflammation offers a further explanation, in addition to behavioral factors, for the high rates of HIV-1 infection that are seen in young South African women (UNAIDS, 2010). The finding that genital inflammation in individual women was sustained over time suggests that certain women have persistent inflammation that may be a result of untreated subclinical STIs, recurrence of previously treated infections or BV, genetic factors, vaginal hygeine practices, such as repeated use of antiseptics or soaps and/or frequent exposure to seminal plasma proteins or lubricants. Irrespective of the cause of inflammation in this study, it is clear that increased cervicovaginal cytokine concentrations predisposed women to HIV-1 infection, even in women who were using the topically-applied potent antiretroviral TFV. Genital tract inflammation may thus substantially increase the level of protection needed to prevent HIV-1 infection using microbicide gel formulations.

Elevated inflammatory cytokine concentrations in the female genital tract may facilitate HIV-1 infection by recruiting, activating and inducing differentiation of HIV-1 target cells (Wira *et al.*, 2005; Charo *et al.*, 2006; Nkwanyana *et al.*, 2009, Li *et al.*, 2009). Furthermore, it has previously been shown that pro-inflammatory cytokines, including IL-1α/β, IL-6, IL-7 and TNF-α, can up-regulate HIV-1 replication through NF-κB activation (Chene *et al.*, 1999; Osborne *et al.*, 1989; Poli *et al.*, 1990; Niu *et al.*, 2004). Pro-inflammatory cytokines may also disrupt tight junctions between cervicovaginal epithelial cells, increasing the permeability of this barrier (Madara and Stafford, 1989; Schmitz *et al.*, 1996; Nazli *et al.*, 2010). It is interesting that the CCR5-binding chemokines, MIP-1α and MIP-1β, were identified as strong correlates of susceptibility to HIV-1 infection in this study, whereas previous studies have proposed that these chemokines may protect against HIV-1 infection by competitively inhibiting HIV-1 binding to CCR5 (Cocchi *et al.*, 1995; Gonzalez *et al.*, 2005; Hirbod *et al.*, 2006). The results of this study suggest the opposite may be true and that higher concentrations of MIP-1α and MIP-1β in the genital tract may facilitate HIV-1 infection, perhaps by specifically recruiting CCR5+ target cells for HIV-1 replication (Swingler *et al.*, 1999; Dufour *et al.*, 2002; Wira *et al.*, 2005; Li *et al.*, 2009). In non-human primates, vaginal infection with SIV was shortly followed by accumulation of DCs in the endocervical epithelium, production of MIP-1α and MIP-1β, and influx of CD4+ T cells. In the transformation zone and vaginal mucosa, SIV-infected cells were concentrated in areas containing IL-8-producing cells (Li *et al.*, 2009). IL-8, which was also associated with increased HIV-1 transmission in cervical explant tissue in a previous study (Narimatsu *et al.*, 2005), similarly correlated with susceptibility to HIV-1 infection in this study. Establishment of SIV infection following vaginal exposure was shown to be dependent on the production of these and other pro-inflammatory chemokines in order to recruit CD4+ T cell targets that are needed for SIV replication (Li *et al.*, 2009; Haase, 2011). Furthermore, inhibition of inflammatory responses using a topically-applied anti-inflammatory agent (glycerol-monolaurate) was found to prevent SIV infection (Li *et al.*, 2009).

This study has demonstrated an important relationship between cervicovaginal inflammation in women, which was sustained over time, and increased susceptibility to HIV-1 infection. The finding that this relationship was independent of TFV or placebo gel use suggests that even women who were using TFV gel, but had genital inflammation, were at increased risk of HIV-1 infection. Therefore, reducing genital inflammation may substantially decrease the risk of HIV-1 infection and further increase the efficacy of the TFV microbicide.

Subsequent to this study, the TFV vaginal gel arm of the VOICE trial was discontinued following an interim analysis that found that daily application of TFV gel was ineffective at preventing HIV-1 infection. The reasons for discrepant results between the VOICE trial and CAPRISA 004 trial are not known and a third trial is currently underway in South Africa to evaluate the effectiveness of TFV gel as a microcide using the CAPRISA 004 regime.

CHAPTER 6

Chapter 6
Discussion

6.1 Genital inflammation, susceptibility to HIV-1 infection and disease progression

In this dissertation, the relationships between cervicovaginal cytokine concentrations and HIV-1 acquisition were investigated. In Chapter 5 it was found that women who later became HIV-1-infected had higher pre-infection cervicovaginal inflammatory cytokine concentrations that were sustained over time, whereas women who remained uninfected had consistently lower cytokine concentrations. Further, it was found that even women who were using the 1% TFV microbicide were at increased risk of HIV-1 infection if they had genital inflammation. TFV gel use was found to confer 54% protection in women who were considered high adherers to the study dosing strategy (Abdool Karim *et al.*, 2010a). The results of this dissertation offer an explanation for the finding that TFV gel was not fully protective and suggest that cervicovaginal inflammation may substantially increase the level of protection needed to prevent HIV-1 infection using TFV gel or other antiretroviral microbicides.

Genital inflammatory cytokine concentrations were found to be elevated in HIV-uninfected women who had STIs, with chlamydia and gonorrhoea being particularly inflammatory infections (Chapter 2). Women who had BV had a mixed inflammatory profile in their genital tracts, with up-regulated pro-inflammatory cytokine concentrations but down-regulated chemokines. In South Africa, where STIs are currently managed syndromically based on clinical signs and symptoms, almost 50% of women who have STIs are asymptomatic (Wilkinson *et al.*, 1999; World Health Organization, 2003). In Chapter 2, it was found that the level of genital inflammation was similar in women who had asymptomatic STIs compared to those who had symptomatic infections and elevated in both of these groups compared to women who did not have an STI or BV. In addition to increasing the risk of HIV-1 acquisition, persistent inflammation may cause reproductive complications, including pelvic inflammatory disease, ectopic pregnancy and tubal factor infertility (Moodley and Sturm, 2000). The results of Chapter 2 show that, in a syndromic management setting where asymptomatic STIs would be left untreated, many women may have sustained subclinical cervicovaginal inflammation which would increase their risk of reproductive complications and HIV-1 acquisition.

In addition to STIs, other factors may influence genital inflammation, including hygiene practices such as antiseptic douching, exposure to seminal proteins and lubricants, hormone cycling, hormone contraceptive use, microabrasions caused by sexual activity and demographic or genetic factors (Scholes *et al.*, 1993; Guimaraes *et al.*, 1997; Hunt et al, 1997; Akoum *et al.*, 2000; Fichorova *et al.*, 2001b;

Bengtsson *et al.*, 2004; Ghanem *et al.*, 2005; Gonzalez *et al.*, 2005; Berlier *et al.*, 2006; Sharkey and Robertson, 2007; Zabaleta *et al.*, 2008). In Chapter 2, it was found that some of the women who did not have BV or any of the common STIs for which they were screened (chlamydia, gonorrhoea, trichomoniasis, HSV-2, *M. genitalium* and syphilis) had levels of cervicovaginal inflammation that were comparable to women who had highly inflammatory STIs, gonorrhoea and chlamydia. Inflammatory cytokine concentrations were found to be elevated in the genital tracts of women who had vaginal discharge, which is associated with other factors in addition to STIs and BV, including *C. albicans*, physiological factors such as age and hormone changes, allergic reaction or irritation (Mitchell, 2004). However, in the subset of women with no concurrent STI/BV, those with vaginal discharge had only marginal changes in genital cytokine concentrations, compared to those with no discharge, which were not significant after adjusting for multiple comparisons. This suggests that other factors which may cause vaginal discharge may not necessarily be associated with substantial changes in cervicovaginal inflammatory cytokine concentrations. Associations between genital inflammation and age, condom use and hormone contraception were also investigated. In Chapter 2 it was found that younger women had higher levels of pro-inflammatory IL-6 and anti-inflammatory IL-1Ra in their genital tracts compared to older women, while in Chapter 5, chemokines IP-10 and MCP-1 were identified as correlates of age, in addition to IL-6. Previous studies have shown that changes in oestrogen and progestin concentrations that are associated with hormone cycling and cytological changes at the cervix that occur during adolescence may influence inflammation in the female genital tract (Hunt *et al.*, 1997; Akoum *et al.*, 2000; Prakash *et al.*, 2001; Ghanem *et al.*, 2005; Zara *et al.*, 2008). Alternatively, cervicovaginal inflammation in younger women may be the result of high-risk behaviour and increased incidence of STIs compared to older women. In Chapter 2, it was found that there was no association between inflammation and age in an analysis including only women who did not have an STI or BV. The relationship between age and cervicovaginal inflammation warrants further investigation in future studies as this may offer an explanation, in addition to behavioral factors, for the high rates of HIV-1 infection that are seen in young South African women (UNAIDS, 2010).

Although associations between genital inflammation and the use of combined oral contraception, Depo-Povera and Nur-Isterate were evaluated in Chapter 5, only a small number of women were not using hormone contraception and the stage of the menstrual cycle at which samples were collected or the elapsed time since each women had received injectable contraception were unknown. Despite these confounding factors, it was found that GM-CSF was up-regulated in the genital tracts of women who were using combined oral contraception, after adjusting for all other possible correlates of inflammation that were assessed in this study (age, vaginal discharge, cervicitis, candidiasis, genital ulceration, condom use and injectable contraceptive use).

In addition to potentially influencing HIV-1 acquisition, several previous studies have suggested that inflammation in the female genital tract at the time of HIV-1 transmission and during HIV-1 infection may influence disease progression. In macaques, induction of pro-inflammatory cytokine production in the genital tract prior to vaginal infection with SIV was found to be associated with higher viral load set-points (Wang *et al.*, 2005). In humans, higher concentrations of inflammatory cytokines IL-1β, IL-6 and IL-8 in genital secretions during acute HIV-1 infection correlated with lower blood CD4+ T cell counts at the same time-point and IL-1β levels during chronic HIV-1 infection were associated with higher plasma and cervical HIV-1 viral loads (Zara *et al.*, 2004; Bebell *et al.*, 2008).

In Chapter 3, it was found that higher concentrations of pro-inflammatory (IL-1β, IL-12p70, GM-CSF, IL-6, RANTES MIP-1β), anti-inflammatory (IL-10) and T cell homeostatic (IL-2 and IL-15) cytokines in the genital tracts of women with early HIV-1 infection were associated with higher viral load set-points, while GM-CSF was associated with greater CD4+ T cell depletion in blood during the first 12 months of infection. During early HIV-1 infection, women who had elevated cervicovaginal inflammatory cytokine concentrations had vaginal discharge, detectable HIV-1 RNA in their genital tracts and lower blood CD4+ T cell counts at the same time point. Therefore, genital inflammation during early HIV-1 infection may be partly caused by STIs and BV, which were found to be the primary causes of vaginal discharge in Chapter 2. Higher levels of genital inflammation may facilitate HIV-1 shedding by recruiting and activating HIV-1-infected immune cells and directly promoting HIV-1 replication (Osborne *et al.*, 1989; Swingler *et al.*, 1999; Gumbi *et al.*, 2008; Li *et al.*, 2009; Nkwanyana *et al.*, 2009). HIV-1 replication in the genital tract would likely induce further pro-inflammatory cytokine production via TLR 7 and 8 activation (Meier *et al.*, 2007).

CD4+ T cell depletion in blood during early HIV-1 infection may reflect depletion in the genital tract. This would result in T cell homeostatic cytokine production in the genital tract, which in turn induce pro-inflammatory cytokine production (Alderson *et al.*, 1991; Damas *et al.*, 2003; Catalfamo *et al.*, 2008). Alternatively, high levels of pro-inflammatory cytokines in mucosal compartments, such as the genital tract, may recruit CD4+ T cells under an inflammatory gradient and fuel viral replication, resulting in CD4 depletion locally and in the systemic compartment.

It was further shown in Chapter 3 that genital inflammation shortly following HIV-1 infection was at least partly pre-existing and was not a consequence of HIV-1 infection. When inflammatory cytokine concentrations were compared in CVLs from the same women pre-infection and during early HIV-1 infection, cytokine concentrations pre-infection were correlated with those post-infection and were not significantly elevated following HIV-1 infection. Additionally, when cervicovaginal cytokine concentrations were compared in the same women at several time-points during the first 12 months of HIV-1 infection, some women in this study had consistently high levels of genital inflammation over time, while others had

consistently low inflammatory cytokine concentrations. Sustained genital inflammation may be the result of STI recurrence or persistence because the prevalence of STIs and occurrence of vaginal discharge did not differ between pre- and post-HIV-1-infection. Although the results of this study and previous macaque studies (Wang *et al.*, 2005; Li *et al.*, 2009) suggest that genital inflammation at the time of HIV-1 acquisition may influence subsequent disease progression, the number of pre-infection samples that were available in this study was too small to conclusively evaluate the relationship between pre-infection cytokine concentrations and markers of HIV-1 disease progression. Future longitudinal studies that aim to investigate pre-infection genital inflammation and subsequent SIV/HIV-1 disease progression in macaques or humans will provide valuable insight into this relationship.

As genital inflammation was found to be associated with increased risk of HIV-1 infection and may influence disease progression, better management of the causes of inflammation, particularly in high-risk women, may be essential for prevention of HIV-1 infection and may improve disease prognosis. Development of strategies to effectively reduce genital inflammation in high-risk women will require a better understanding of all possible causes. In addition to STIs and BV, other factors that may influence the inflammatory environment in the genital tract that were not evaluated in this study include hygiene practices such as antiseptic douching, exposure to seminal proteins and lubricants, hormone cycling, microabrasions caused by sexual activity and demographic or genetic factors (Scholes *et al.*, 1993; Guimaraes *et al.*, 1997; Hunt et al, 1997; Akoum *et al.*, 2000; Fichorova *et al.*, 2001b; Bengtsson *et al.*, 2004; Ghanem *et al.*, 2005; Gonzalez *et al.*, 2005; Berlier *et al.*, 2006; Sharkey and Robertson, 2007; Zabaleta *et al.*, 2008). A comprehensive evaluation of each of these possible causes of inflammation in a single study comprising a large cohort of HIV-uninfected women would be very informative, although some factors may be difficult to investigate and evaluation of others would be dependent on the accuracy of self-reporting.

In addition to assessing the causes of inflammation, further investigation into the mechanisms underlying the relationships between elevated cytokine concentrations in the female genital tract and (1) increased risk of HIV-1 infection and (2) disease progression is required. Higher levels of inflammatory cytokines in the genital tract may facilitate establishment of HIV-1 infection by increasing the permeability of the epithelial barrier (Madara and Stafford, 1989; Schmitz *et al.*, 1996; Nazli *et al.*, 2010). Additionally, elevated cervicovaginal inflammatory cytokine concentrations at the time of HIV-1 transmission may fuel viral replication by recruiting greater numbers of activated target cells for HIV-1 replication and by activating the HIV-1 enhancer NF-κB (Osborne *et al.*, 1989; Swingler *et al.*, 1999; Li *et al.*, 2009; Nkwanyana *et al.*, 2009). As a result, the systemic compartment, lymph nodes and gastrointestinal tract may be seeded with higher concentrations of virus earlier during infection, followed by a period of rapid viral replication before the initiation of adaptive immune responses. This may cause greater CD4+ T cell loss during early infection, as a result of direct viral infection and activation-induced apoptosis and

excessive differentiation and proliferation of bystander CD4+ T cells (Hellerstein and McCune, 1997; Lin *et al.*, 1997; Liu *et al.,* 1997; Brenchley *et al.*, 2003; Hazenberg *et al.,* 2003; Picker *et al.*, 2006; Okoye *et al.*, 2007; Burgers *et al.*, 2009). The influence of the key cytokines that were identified in Chapters 3 and 5 on immune cell recruitment and activation, HIV-1 replication and trans-epithelial resistance should be investigated in human cervicovaginal and animal models. Additionally, it would likely be informative to utilize rhesus macaques to evaluate the impact of induction or suppression of genital inflammatory responses prior to vaginal SIV infection on the timing of initiation of viral replication and degree of CD4+ T cell depletion in blood, lymph nodes and mucosal compartments shortly following infection.

Alternatively, high-risk women who have frequent sexual intercourse with multiple partners and are thus at greater risk of HIV-1 infection would be more likely to have genital inflammation as a result of STIs, microabrasions in the genital mucosa and/or exposure to seminal plasma from different partners compared to women who are in stable monogamous relationships. Therefore, it is possible that the relationship between genital inflammation and risk of HIV-1 infection may be indirect and future studies should be designed to address this question.

Once a better understanding of the causes of cervicovaginal inflammation and the association with susceptibility to HIV-1 infection and disease progression has been achieved, strategies to reduce inflammation in the female genital tract should be further investigated. In macaques, application of a topically-applied anti-inflammatory agent (glycerol-monolaurate) has been found to down-regulate pro-inflammatory chemokine concentrations in the genital tract and prevent SIV infection (Li *et al.*, 2009). However, as this study included a small number of macaques (n=10) and evaluated a restricted panel of cytokines (IFN-α, IFN-β, IL-8, MIP-1α MIP-1β, MIP-3α), it would be important to confirm these findings in future studies with larger sample sizes. In women, addition of an anti-inflammatory agent to the 1% TFV microbicide could substantially improve the efficacy of this product. Intravaginal application of the steroidal anti-inflammatory hydrocortisone as a suppository or cream is used to treat vaginitis and cervicitis (Sobel, 2000; Anderson *et al.*, 2002; Sobel, 2003). However, many existing broad spectrum anti-inflammatory agents that are primarily used systemically are associated with mild to severe side-effects that may result in increased, rather than reduced, susceptibility to HIV-1 infection if applied topically in the female genital tract. Non-steroidal anti-inflammatory agents cause gastrointestinal ulceration by disrupting the mucus layer and causing vasoconstriction which results in local tissue hypoxia and epithelial necrosis (Whittle, 1977; Larkai *et al.*, 1987; Schoen and Vender, 1989; Jones *et al.*, 2008). Additionally, anti-inflammatory agents are also associated with increased susceptibility to infections as they inhibit the innate immune response (Patton *et al.*, 1997; Dinarello, 2010). Therefore, regular application of a topical anti-inflammatory may disrupt the naturally protective mucus and epithelial barrier in the female genital tract and may lead to an outgrowth or persistence of existing STIs or *C. albicans* and possibly increased susceptibility to other STIs and HIV-1.

Antagonists for TLR 7 and 8 that recognize HIV-1 ligands are being explored as mechanisms to inhibit inflammatory cytokine production in response to HIV-1 and thus reduce recruitment of additional immune cells that aid the establishment of HIV-1 infection (Fraietta *et al.*, 2010; Meier *et al.*, 2007). However this approach would not address pre-existing inflammation that is caused by factors other than HIV-1 that may induce genital inflammatory responses by binding to other TLRs and pattern recognition receptors. Identification of an appropriate target for modulation of the inflammatory pathway would require a better understanding of both factors that may influence the level of inflammation and the key players in the inflammatory cascade and transcriptional network in the female genital tract.

An alternative to suppressing the genital inflammatory response in high-risk women would be to identify and treat the causes. In Mwanza, Tanzania, large-scale implementation of syndromic management of STIs successfully reduced HIV-1 incidence (Grosskurth *et al.*, 1995). However, other strategies aimed at reducing HIV-1 infection by treating STIs, including mass treatment of bacterial STIs, HSV-2 suppressive therapy and two other syndromic management interventions, did not significantly impact HIV-1 incidence (Wawer *et al.*, 1999; Gray *et al.*, 2001; Kamali *et al.*, 2003; Kaul *et al.*, 2004; Gregson *et al.*, 2007; Celum *et al.*, 2008; Watson-Jones *et al.*, 2008). These findings highlight the difficulties in implementing large-scale STI management and further suggest that laboratory testing in order to identify asymptomatic STIs and the causative agents of symptomatic infections, followed by targeted treatment, may have a more substantial effect on rates of HIV-1 infection. Since lactobacilli are the predominant bacterial commensal in the female genital tract and colonization with lactobacilli is associated with anti-viral properties (due to low vaginal pH associated with lactic acid production), it has been suggested that the use of exogenous lactobacilli may improve vaginal health and increase resistance to BV and STIs (Antonio *et al.*, 2003; Senok *et al.*, 2009). Efforts are also underway to further augment this natural anti-viral property of lactobacilli by bioengineering recombinant organisms that produce antiviral proteins (Chang *et al.*, 2003; Rao *et al.*, 2005; Liu *et al.*, 2007). This strategy may provide protection against HIV-1 infection on multiple levels, improving vaginal health, increasing resistance to colonization by STIs and organisms associated with BV and by supplying large amounts of antiviral proteins that are constitutively secreted by the recombinant lactobacilli. However, although treatment of STIs and improvement of vaginal health may substantially reduce genital inflammation, these efforts may not address other potential causes of genital inflammation which may be more difficult to address, such as genetic, demographic and behavioural factors.

The results of Chapter 5 show that women who had genital inflammation, even those who were applying the highly active antiretroviral microbicide, 1% TFV, were at increased risk of HIV-1 infection. Therefore, an antiretroviral microbicide alone may not be sufficient to provide a high level of protection against HIV-1 infection in women who have genital inflammation. It remains to be seen whether genital inflammation will

also influence the efficacy of candidate HIV-1 vaccines that are aimed at protecting against sexual HIV-1 transmission. Not only are women who have inflammation in their genital tracts at increased risk of HIV-1 infection, but they may also experience more rapid disease progression should they become infected. The findings of this study emphasize the need for better STI management strategies, particularly in high-risk populations such as South Africa, as well as efforts to improve vaginal flora and discourage hygiene practises and high-risk behaviour that may influence the genital inflammatory environment.

6.2 Plasma cytokine concentrations during early HIV-1 infection were predictive of HIV-1 disease progression

HIV-1 infection is accompanied by widespread activation of the immune system, involving increased pro-inflammatory cytokine production and activation and proliferation of most immune cells (Lane *et al.*, 1983; Alter *et al.*, 2004; Brenchley *et al.*, 2006; Norris *et al.*, 2006; Bebell *et al.*, 2008; Catalfamo *et al.*, 2008; Stacey *et al.*, 2009). In addition to blood CD4+ T cell counts and viral loads, systemic T cell activation during early HIV-1 infection is associated with more rapid disease progression (de Wolf *et al.*, 1997; Mellors *et al.*, 1997; Lepri *et al.*, 1998; Deeks *et al.*, 2004; Goujard *et al.*, 2006). Systemic biomarkers of immune activation that can be used to predict the rate of HIV-1 disease progression could be useful for clinical management of HIV-1-infected individuals, and for evaluation of candidate HIV-1 vaccines or microbicides aimed at reducing the rate of disease progression, rather than preventing infection (Mascola and Nabel, 2001; Goujard *et al.*, 2006). Soluble markers of immune activation, which are more easily measurable in plasma than markers of cellular activation, have been shown to predict HIV-1 disease progression with comparable efficiency to CD4+ T cell counts and viral load measurements (Mellors *et al.*, 1997; Fahey *et al.*, 1998; Zangerle *et al.*, 1998). In Chapter 4, the potential to use plasma cytokine concentrations during early HIV-1 infection as biomarkers to predict subsequent disease progression was investigated. Two clusters of five cytokines each were constructed and were more strongly predictive of viral load set-point and CD4+ T cell loss than either early infection CD4 counts, viral loads or both combined. Plasma concentrations of IL-7, IL-12p40, IL-12p70, IFN-γ and IL-15 during early infection were found to be predictive of viral load set-point, while concentrations of IL-7, IL-12p40, IL-1α, eotaxin and GM-CSF were predictive of CD4 loss. Elevated concentrations of cytokines which promote cell-mediated immune responses (IL-12p70 and IFN-γ) and macrophage activation (GM-CSF, IL-12p40 and IFN-γ) were found to be associated with slow disease progression, while elevated concentrations of pro-inflammatory cytokines (IL-1α and eotaxin) and T cell homeostatic cytokines (IL-7 and IL-15) predicted rapid disease progression. Plasma concentrations of several inflammatory cytokines and immunoregulatory IL-10 were found to be up-regulated during early HIV-1 infection compared to pre-infection. Elevated pro-inflammatory cytokines were associated with higher plasma HIV-1 viral load during early infection, suggesting that inflammatory cytokine production in the systemic compartment during early infection is induced, at least in part, in response to the presence of HIV-1 replication and products.

6.3 Conclusion

In this dissertation it was found that elevated plasma concentrations of cytokines which promote cell-mediated immune responses during early HIV-1 infection were associated with slow disease progression, while elevated pro-inflammatory and T cell homeostatic cytokines were associated with poor disease prognosis. Furthermore, the potential to use plasma cytokine concentrations during early HIV-1 infection, which are easily measurable, to predict subsequent disease progression was demonstrated. Therefore, early HIV-1 infection plasma cytokine concentrations may be useful biomarkers for evaluating the ability of therapeutic HIV-1 vaccines and microbicides to control HIV-1 infection.

Additionally, it was demonstrated in this dissertation that inflammation in the female genital tract likely plays an important role in facilitating HIV-1 infection and may further influence the rate of subsequent HIV-1 disease progression. Importantly, it was found that the relationship between elevated cervicovaginal pro-inflammatory cytokine concentrations and risk of HIV-1 infection was independent of TFV microbicide gel use. Genital inflammation may thus have facilitated breakthrough HIV-1 infections in women who were using this antiretroviral microbicide and may also substantially reduce the efficacy of other microbicide formulations and vaccines. Although other factors may play a role, STIs and BV were found to be major causes of cervicovaginal inflammation in this and previous studies. Implementation of better STI management and strategies to improve vaginal health, particularly in high risk settings, may considerably reduce genital inflammation, HIV-1 incidence and rates of disease progression.

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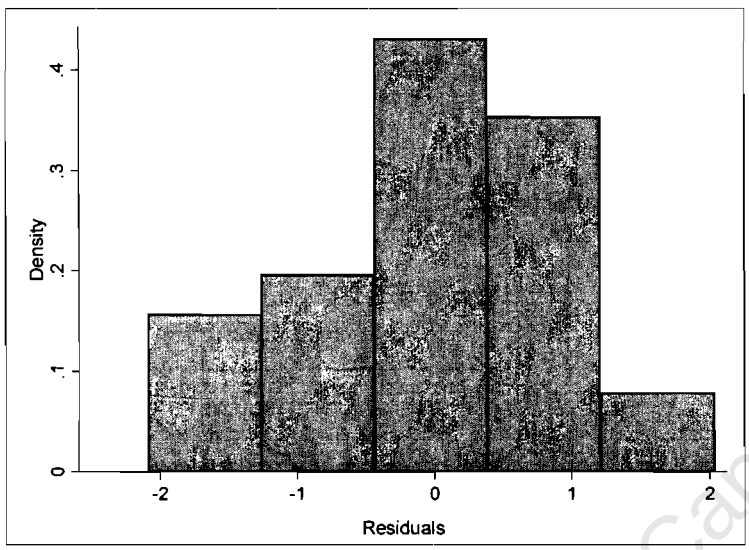
Appendix A

Multivariate regression: Cytokine model to predict viral load set-point (Chapter 4)

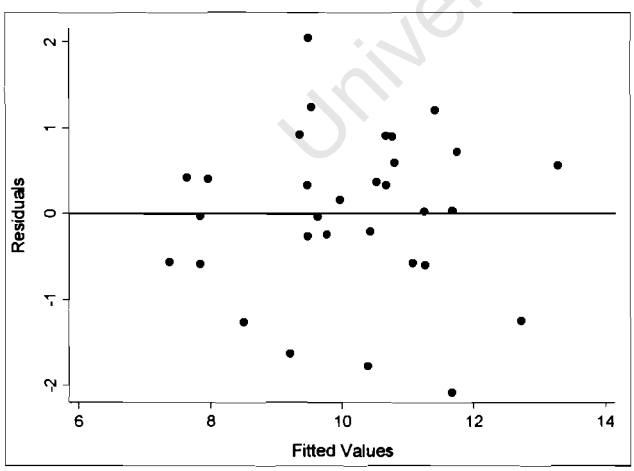
Model checking procedure

1) Assessment of the assumptions underlying the model (Figure A1)



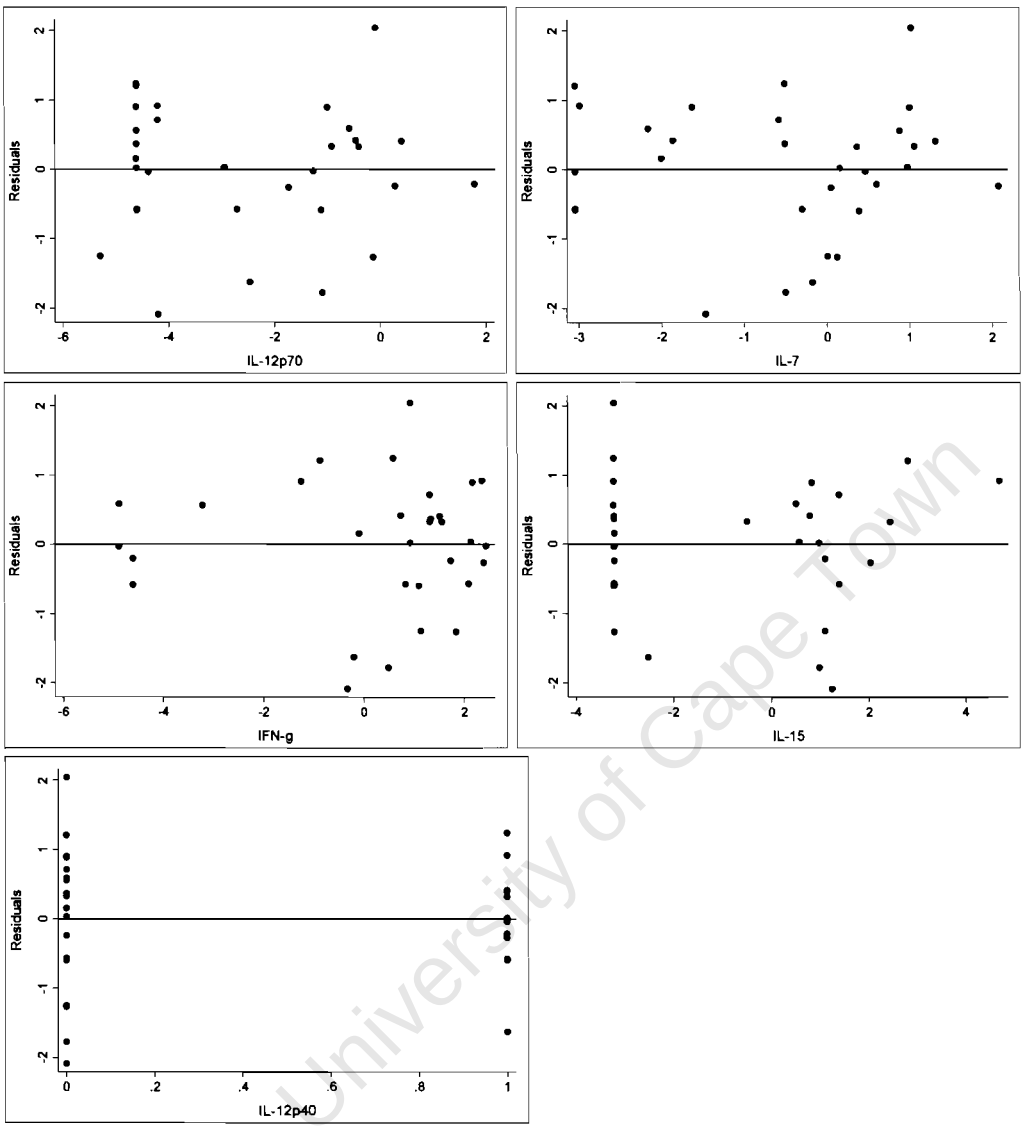
Residuals were used to test the assumptions underlying the model. Residuals are the differences between the actual value of the dependent variable and the value of the variable that was predicted by the model. This histogram shows that the residuals are normally distributed, therefore the assumption that the residuals are normally distributed is justified.

2) Evaluation of homogeneity of variance, residuals versus fitted values (Figure A2)



The purpose of this analysis was to assess whether the variance of the residuals was equal across all levels of the predicted value (fitted values). This figure shows that the data points are randomly distributed in a horizontal band, therefore the model is adequate.

3) Evaluation of homogeneity of variance, residuals versus predictor variables (Figure A3 A-E)



The purpose of this analysis was to assess whether the variance of the residuals was equal across all levels of the predictor variables (cytokines). It was found that IFN-γ and IL-15 may better fit the model as categorical variables (i.e. response versus no response) because the residuals were not evenly distributed for all levels of these cytokines. However this weakened the strength of the model and increased the P-values of these cytokines (although the P-values remained significant).

Inclusion of IL-15 as a categorical variable (Table A1):

```
Number of obs =      31
F(  5,    25) =    8.20
Prob > F      =  0.0001
R-squared     =  0.6213
Adj R-squared =  0.5455
```

VL Set-Point	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
IL-12p70	-.5158225	.117037	-4.41	0.000	-.7568647	-.2747803
IL-7	.6372519	.1738368	3.67	0.001	.2792284	.9952755
IFN-γ	-.3213381	.10606	-3.03	0.006	-.5397728	-.1029033
IL-15	1.068859	.4327877	2.47	0.021	.1775163	1.960202
IL-12p40	-1.162984	.4459473	-2.61	0.015	-2.081429	-.244538
Constant	9.110068	.4677729	19.48	0.000	8.146672	10.07346

Inclusion of IFN-γ as a categorical variable (Table A2):

```
Number of obs =      31
F(  5,    25) =    7.96
Prob > F      =  0.0001
R-squared     =  0.6143
Adj R-squared =  0.5371
```

VL Set-Point	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
IL-12p70	-.450157	.116056	-3.88	0.001	-.6891788	-.2111351
IL-7	.660758	.1809058	3.65	0.001	.2881756	1.03334
IFN-γ	-1.249447	.5312145	-2.35	0.027	-2.343503	-.1553899
IL-15	.2928574	.0918969	3.19	0.004	.1035922	.4821226
IL-12p40	-1.106907	.4519617	-2.45	0.022	-2.037739	-.1760744
Constant	10.8707	.584147	18.61	0.000	9.667627	12.07377

4) Identification of outliers and influential observations (Table A3) by examination of

i) Studentised Residuals

ii) Cooks Distance

iii) dfits

PID	Studentised Residuals	Cooks Distance	Dfits
100008	-0.6	0.01	-0.27
100030	0.93	0.02	0.36
100045	0.45	0.01	0.21
100065	0.93	0.02	0.36
100084	0.69	0.04	0.49
100085	-0.03	0	-0.01
100136	-1.38	0.06	-0.62
100174	0.37	0	0.14
100177	1.11	0.11	0.8
100200	-1.35	0.06	-0.59
100206	0.73	0.01	0.25
100210	1.36	0.09	0.74
100217	-0.26	0.01	-0.23
100221	0.34	0	0.11
100228	1.36	0.07	0.66
100229	0.03	0	0.01
100239	-2.33	0.09	-0.81
100244	0.02	0	0.01
100248	-0.62	0.01	-0.25
100256	-0.28	0	-0.11
100257	2.32	0.13	0.84
100261	-1.93	0.07	-0.68
100262	0.26	0	0.06
100264	0.43	0.01	0.22
100265	-1.75	0.06	-0.61
100266	0.34	0	0.15
100267	-0.04	0	-0.03
100268	-0.68	0.03	-0.45
100269	0.66	0.04	0.46
100270	-0.26	0	-0.13
100271	-0.68	0.04	-0.51

Conclusion: PID 100239 and 100257 were identified as potential outliers, however exclusion of these participants from the dataset did not substantially influence the model.

Exclusion of participant number 100257 (Table A4):

```
Number of obs =      30
F(  5,    24) =   11.93
Prob > F      =  0.0000
R-squared     =  0.7131
Adj R-squared =  0.6533

------------------------------------------------------------------------------
VL Set-Point |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
IL-12p70     |  -.5132693   .1034971    -4.96   0.000    -.7268767   -.2996618
IL-7         |   .7405126   .1566156     4.73   0.000      .417274    1.063751
IFN-γ        |  -.3753753   .0968627    -3.88   0.001     -.57529   -.1754605
IL-15        |   .3239237   .0819641     3.95   0.001     .1547581    .4930892
IL-12p40     |  -1.244509   .4046333    -3.08   0.005    -2.079631   -.4093871
Constant     |   10.07345   .3790146    26.58   0.000     9.291201    10.8557
------------------------------------------------------------------------------
```

Exclusion of participant number 100239 (Table A5):

```
Number of obs =      30
F(  5,    24) =   11.55
Prob > F      =  0.0000
R-squared     =  0.7064
Adj R-squared =  0.6452

------------------------------------------------------------------------------
VL Set-Point |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
IL-12p70     |  -.5139312   .1039341    -4.94   0.000    -.7284406   -.2994218
IL-7         |   .7382468   .1592268     4.64   0.000     .4096189    1.066875
IFN-γ        |  -.3794839    .095991    -3.95   0.001    -.5775997   -.1813681
IL-15        |   .3197612   .0817519     3.91   0.001     .1510335    .4884889
IL-12p40     |  -1.265557   .4004842    -3.16   0.004    -2.092116   -.4389984
Constant     |    10.0656   .3839646    26.21   0.000     9.273133    10.85806
------------------------------------------------------------------------------
```

5) Evaluation of model performance by repeated reapplication of the model to randomly-chosen three-quarter subsets of the study group (Tables A6-9)

```
Number of obs =      24
F(  5,    18) =   11.68
Prob > F      =  0.0000
R-squared     =  0.7644
Adj R-squared =  0.6989

------------------------------------------------------------------------------
VL Set-Point |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
IL-12p70     |  -.5123082    .113833    -4.50   0.000    -.7514625   -.2731538
IL-7         |   .7840502   .1728024     4.54   0.000     .4210058    1.147095
IFN-γ        |  -.4293284   .1043839    -4.11   0.001    -.6486309   -.2100259
IL-15        |   .2857644   .1031438     2.77   0.013     .0690673    .5024615
IL-12p40     |   -1.55049   .4715529    -3.29   0.004    -2.541186   -.5597942
Constant     |   10.17256   .4139958    24.57   0.000     9.302785    11.04233
------------------------------------------------------------------------------
```

```
Number of obs =      24
F(  5,    18) =    8.08
Prob > F      =  0.0004
R-squared     =  0.6918
Adj R-squared =  0.6062

------------------------------------------------------------------------------
VL Set-Point |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
IL-12p70     |  -.5182453   .1304991    -3.97   0.001    -.7924139   -.2440768
IL-7         |   .7823971    .190715     4.10   0.001     .3817198    1.183074
IFN-γ        |  -.3895189   .1120131    -3.48   0.003    -.6248497    -.154188
IL-15        |   .3280968   .1004631     3.27   0.004     .1170316     .539162
IL-12p40     |  -1.148303   .4981025    -2.31   0.033    -2.194778   -.1018287
Constant     |   10.01416   .4585021    21.84   0.000     9.050879    10.97743
------------------------------------------------------------------------------

Number of obs =      24
F(  5,    18) =   10.30
Prob > F      =  0.0001
R-squared     =  0.7411
Adj R-squared =  0.6691

------------------------------------------------------------------------------
VL Set-Point |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
IL-12p70     |   -.528411   .1133207    -4.66   0.000    -.7664889   -.2903331
IL-7         |   .7123439   .1997651     3.57   0.002      .292653    1.132035
IFN-γ        |   -.396381   .1116387    -3.55   0.002    -.6309251   -.1618369
IL-15        |     .29355   .0988004     2.97   0.008     .0859781    .5011219
IL-12p40     |  -1.459014   .4492832    -3.25   0.004    -2.402923   -.5151045
Constant     |   10.10387   .4117282    24.54   0.000     9.238865    10.96888
------------------------------------------------------------------------------

Number of obs =      25
F(  5,    19) =    6.99
Prob > F      =  0.0007
R-squared     =  0.6479
Adj R-squared =  0.5552

------------------------------------------------------------------------------
VL Set-Point |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
IL-12p70     |  -.4531332   .1212835    -3.74   0.001    -.7069823    -.199284
IL-7         |   .7569352   .1879483     4.03   0.001     .3635548    1.150316
IFN-γ        |  -.3195459   .1169405    -2.73   0.013    -.5643051   -.0747867
IL-15        |   .2911915   .0955998     3.05   0.007     .0910989    .4912842
IL-12p40     |  -1.041887   .4605358    -2.26   0.036      -2.0058   -.0779747
Constant     |   9.990396   .4207481    23.74   0.000      9.10976    10.87103
------------------------------------------------------------------------------
```

Conclusion: The influence of each variable on viral load set-point remained statistically significant upon exclusion of various one-quarter subsets of the dataset, and the directionality of the relationships between the variables and set-point remained constant.

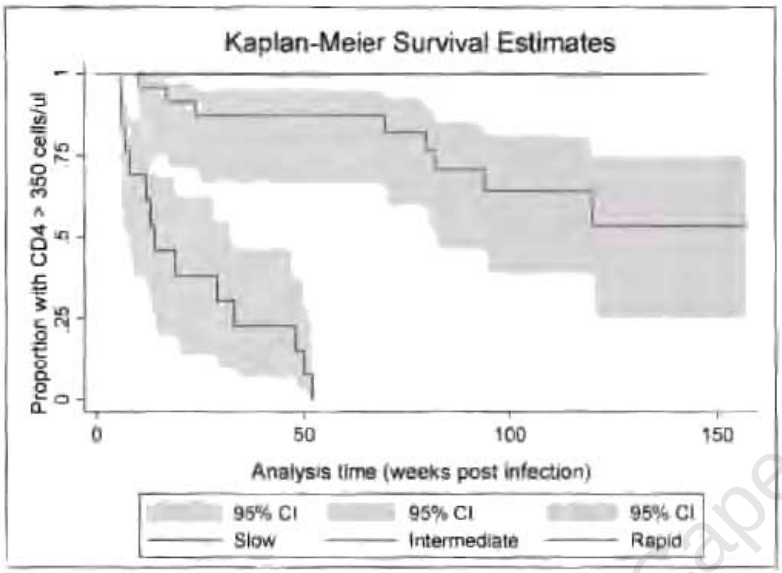
6) Inclusion of time since infection as a variable in the model (Table A10)

```
Number of obs =      31
F(  6,    24) =   10.02
Prob > F      =  0.0000
R-squared     =  0.7148
Adj R-squared =  0.6435
Root MSE      =   1.048

------------------------------------------------------------------------------
 VL_12mthlog |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
IL-12p40     |   -1.26709   .4017045    -3.15   0.004    -2.096168    -.438013
IL-12p70     |  -.5143144   .1062259    -4.84   0.000    -.7335539   -.2950749
IFN-g        |  -.3794393   .0971746    -3.90   0.001    -.5799978   -.1788809
IL-7         |   .7393788   .1632796     4.53   0.000     .4023863    1.076371
IL-15        |   .3200834   .0819479     3.91   0.001     .1509512    .4892155
Sample time  |  -.0004059   .0877248    -0.00   0.996    -.1814609    .1806492
       _cons |   10.06988   .6161591    16.34   0.000     8.798191    11.34157
------------------------------------------------------------------------------

Likelihood-ratio test                                 LR chi2(1)  =      0.00
(Assumption: b nested in a)                           Prob > chi2 =    0.9958

-----------------------------------------------------------------------------
       Model |    Obs    ll(null)   ll(model)     df          AIC         BIC
-------------+---------------------------------------------------------------
Excl. time   |     31   -60.91949   -41.47452      6     94.94905     103.553
Incl. time   |     31   -60.91949   -41.47451      7     96.94902    106.9869
-----------------------------------------------------------------------------
```

Appendix B

Cox proportional hazards model: Time to CD4 count below 350 cells/μl (Chapter 4)

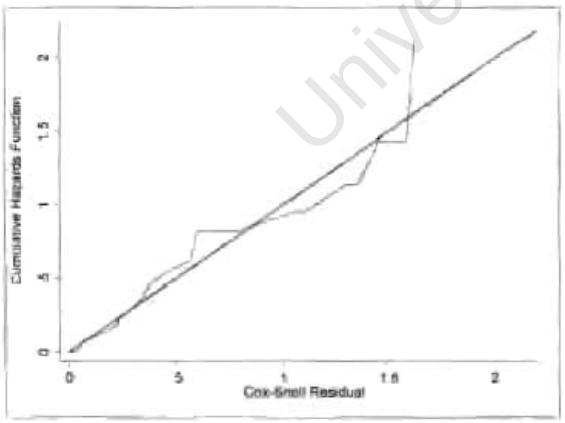
Clinical significance of using time taken for CD4 counts to drop below 350 cells/μl as the dependent variable (Figure B1)



Conclusion: Survival curves and 95% confidence bands of individuals classified as slow, intermediate or rapid progressors later during infection are clearly separated. Hazard Ratio: 19.78 (95% CI 5.46-71.66).

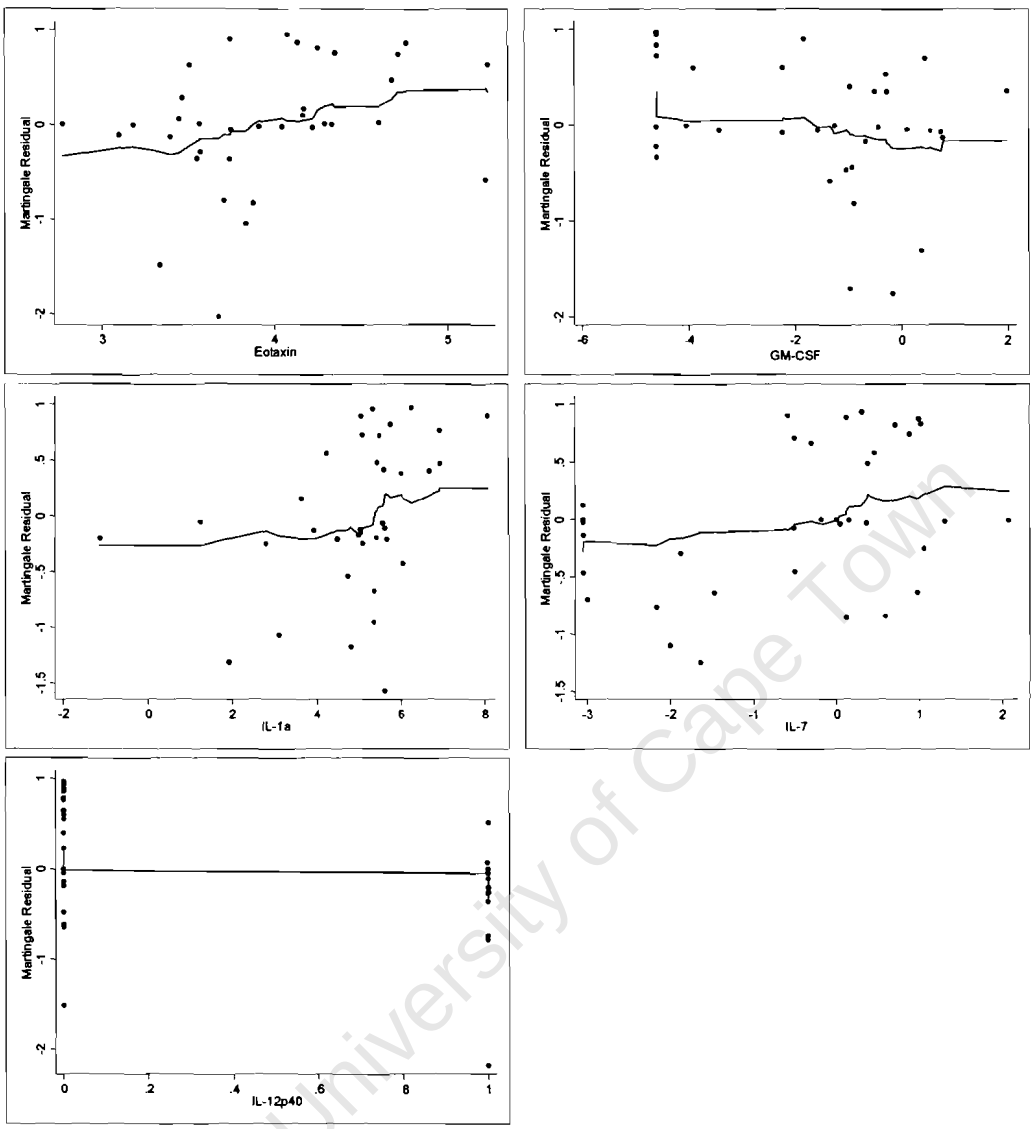
Model checking procedure

1) Assessment of overall model fit using Cox-Snell residuals (Figure B2)



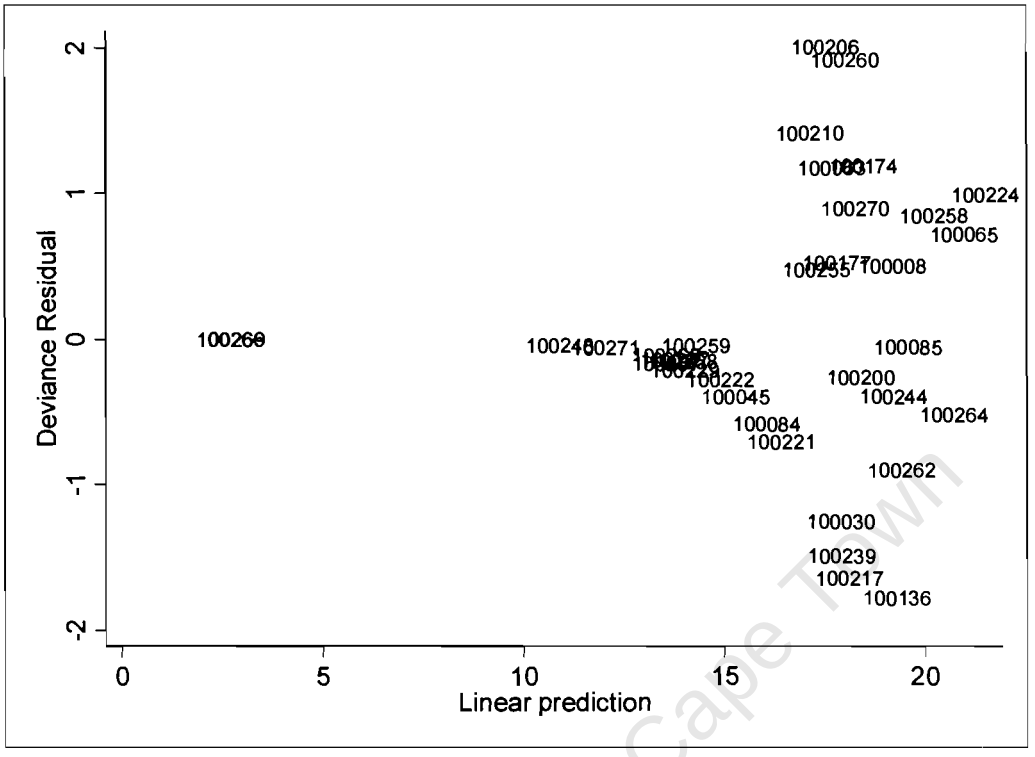
Conclusion: Line does not deviate substantially from reference line, thus the Cox model provides a reasonably good fit for the data.

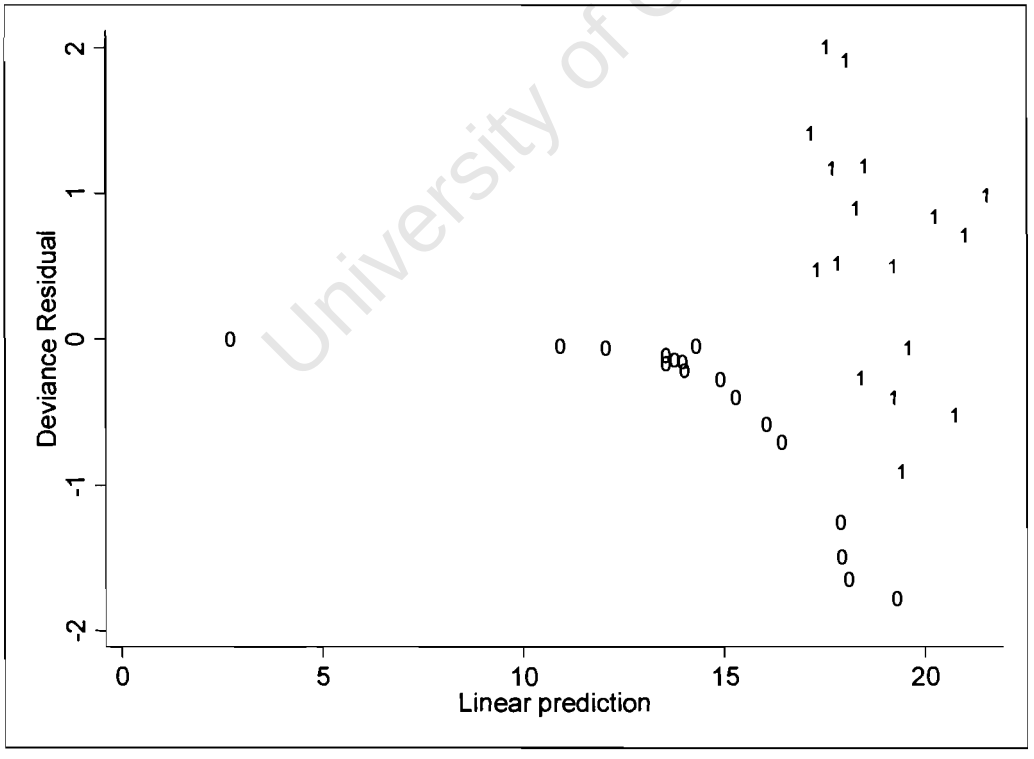
2) Assessment of functional form of covariates using martingale residuals (Figure B3 A-E)



Conclusion: Each plot is roughly linear and not horizontal, indicating that the variables should be included as is.

3) Identification of outliers using deviance residuals (Figure B4 A and B)





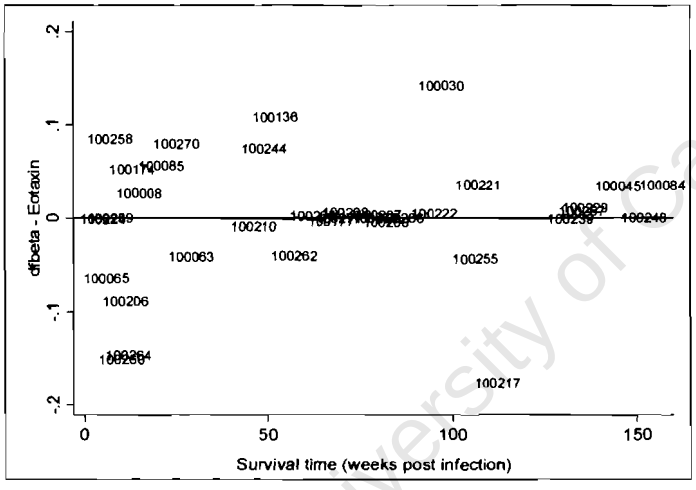
Conclusion: PID 100269 and 100266 were identified as potential outliers. Upon exclusion of these participants from the dataset and reapplication of the model, it was found that the model did not change substantially (see Table B1).

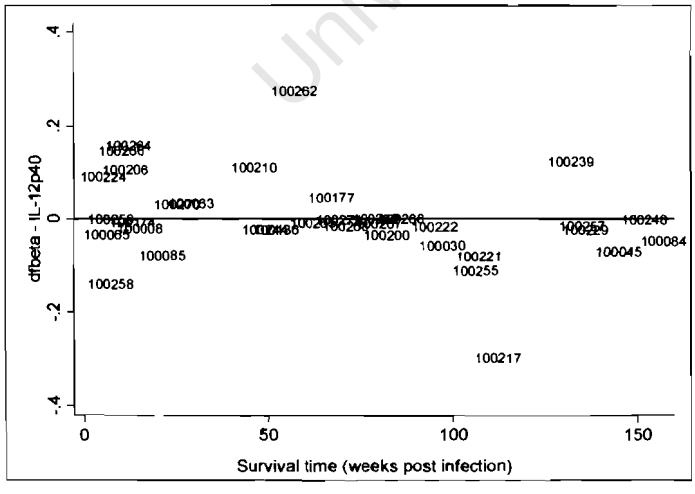
Exclusion of participant number 100269 and 100266 (Table B1):

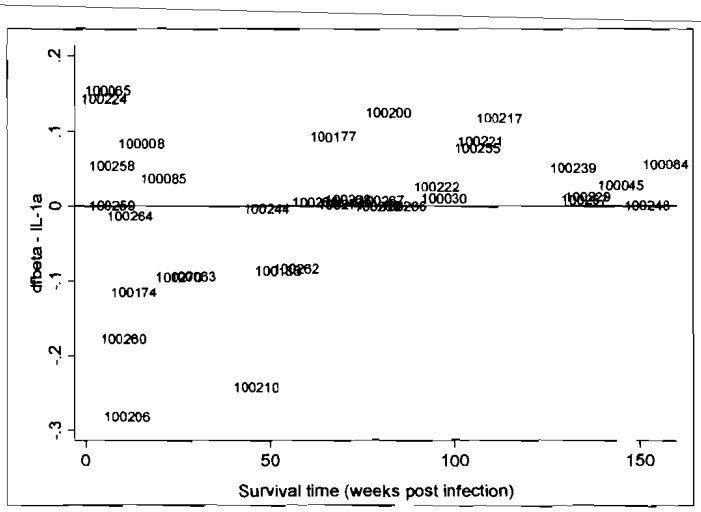
```
No. of subjects =           33                     Number of obs    =        33
No. of failures =           17
Time at risk    =         2170
                                                   LR chi2(5)       =     30.23
Log likelihood  =   -37.545269                     Prob > chi2      =    0.0000
```

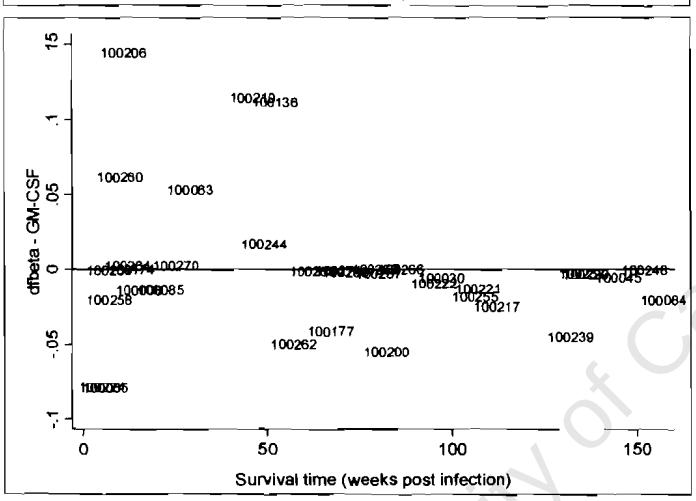
_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
Eotaxin	3.713615	1.999993	2.44	0.015	1.292339	10.6713
IL-12p40	.0757344	.0696266	-2.81	0.005	.0124951	.4590371
IL-1α	9.488842	6.029308	3.54	0.000	2.731184	32.9667
GM-CSF	.4435924	.11334	-3.18	0.001	.2688426	.731931
IL-7	2.05592	.6130975	2.42	0.016	1.145958	3.688448

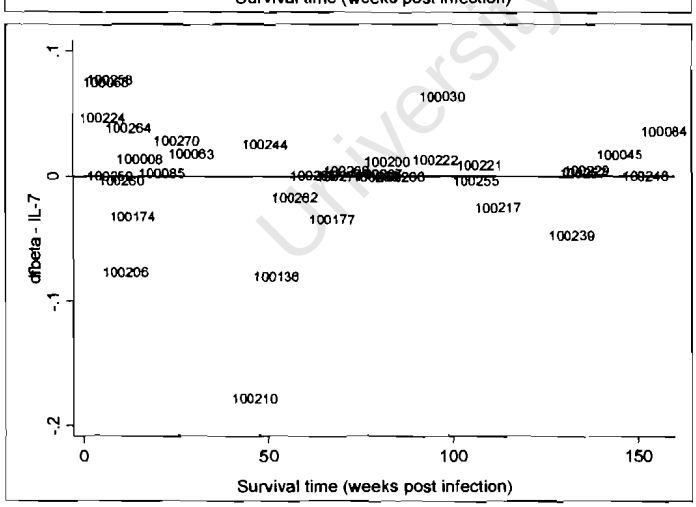
4) Identification of influential observations using ERS residuals (Figure B5 A-E)











Conclusion: PID 100206, 100210 and 100217 were identified as potentially influential observations. These participants were each excluded from the dataset in order to evaluate the degree of their influence. It was found that removal of each of these participants increased the strength of the model, although not greatly (see Tables B2-4).

Exclusion of participant number 100206 (Table B2):

```
No. of subjects =           34                     Number of obs    =        34
No. of failures =           16
Time at risk    =         2324
                                                   LR chi2(5)       =     34.81
Log likelihood  =   -33.007542                     Prob > chi2      =    0.0000
```

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
Eotaxin	4.207515	2.469052	2.45	0.014	1.332061	13.29007
IL-12p40	.064587	.0647809	-2.73	0.006	.0090446	.4612142
IL-1α	13.31017	9.545602	3.61	0.000	3.263854	54.27958
GM-CSF	.3745102	.1058815	-3.47	0.001	.215185	.6518014
IL-7	2.252387	.7249281	2.52	0.012	1.19863	4.232542

Exclusion of participant number 100210 (Table B3):

```
No. of subjects =           34                     Number of obs    =        34
No. of failures =           16
Time at risk    =         2289
                                                   LR chi2(5)       =     34.05
Log likelihood  =   -33.390763                     Prob > chi2      =    0.0000
```

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
Eotaxin	3.866486	2.260313	2.31	0.021	1.229452	12.15966
IL-12p40	.0622243	.0644376	-2.68	0.007	.0081749	.4736301
IL-1α	13.16909	9.503184	3.57	0.000	3.201143	54.17596
GM-CSF	.3815463	.1094529	-3.36	0.001	.2174532	.6694661
IL-7	2.610039	.9459265	2.65	0.008	1.282791	5.310534

Exclusion of participant number 100217 (Table B4):

```
No. of subjects =           34                     Number of obs    =        34
No. of failures =           17
Time at risk    =         2223
                                                   LR chi2(5)       =     35.95
Log likelihood  =    -35.17986                     Prob > chi2      =    0.0000
```

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
Eotaxin	6.082938	3.693009	2.97	0.003	1.85072	19.99337
IL-12p40	.1419868	.1246483	-2.22	0.026	.0254091	.7934262
IL-1α	7.453458	4.582138	3.27	0.001	2.233919	24.86842
GM-CSF	.4657017	.12188	-2.92	0.003	.2788287	.7778181
IL-7	2.21576	.6307492	2.79	0.005	1.268281	3.871061

5) Evaluation of the proportional-hazards assumption using Schoenfeld residuals (Table B5)

Time: Rank(t)

	rho	chi2	df	Prob>chi2
Eotaxin	-0.03696	0.01	1	0.9062
IL-12p40	-0.06334	0.04	1	0.8513
IL-1α	-0.06274	0.08	1	0.7743
GM-CSF	0.17316	0.71	1	0.3978
IL-7	-0.39388	3.43	1	0.0642
global test		5.80	5	0.3257

Conclusion: Each of the slopes do not differ significantly from zero, therefore the log hazard ratio function is constant over time. The P value for IL-7 is fairly small, although not significant, indicating that the effect of this cytokine may vary slightly over time.

6) Evaluation of model performance by repeated reapplication of the model to randomly-chosen three-quarter subsets of the study group (Tables B6-9)

No. of subjects = 26 Number of obs = 26
No. of failures = 15
Time at risk = 1380
LR chi2(5) = 25.60
Log likelihood = -28.588881 Prob > chi2 = 0.0001

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
Eotaxin	5.739329	4.014744	2.50	0.012	1.456912	22.6094
IL-12p40	.1090675	.1503802	-1.61	0.108	.0073127	1.626732
IL-1α	9.107016	7.322133	2.75	0.006	1.883655	44.03021
GM-CSF	.4227638	.1319363	-2.76	0.006	.2293266	.7793655
IL-7	2.894015	1.447346	2.12	0.034	1.085927	7.712607

No. of subjects = 28 Number of obs = 28
No. of failures = 14
Time at risk = 1839
LR chi2(5) = 26.13
Log likelihood = -28.938417 Prob > chi2 = 0.0001

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
Eotaxin	3.17031	1.937801	1.89	0.059	.956794	10.50473
IL-12p40	.0918364	.0996992	-2.20	0.028	.010938	.7710697
IL-1α	7.336424	4.631664	3.16	0.002	2.128629	25.28534
GM-CSF	.4842639	.1258778	-2.79	0.005	.2909537	.8060097
IL-7	1.885006	.6933787	1.72	0.085	.9166584	3.876304

```
No. of subjects =           26                     Number of obs    =        26
No. of failures =           12
Time at risk    =         1677
                                                   LR chi2(5)       =     18.65
Log likelihood  =   -24.967442                     Prob > chi2      =    0.0022
```

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
Eotaxin	3.289419	3.836502	1.02	0.307	.3344639	32.35111
IL-12p40	.0971798	.1594298	-1.42	0.155	.0039005	2.421183
IL-1α	7.107517	5.058979	2.76	0.006	1.76137	28.68039
GM-CSF	.5074762	.1336907	-2.57	0.010	.302812	.8504684
IL-7	1.718986	.5133742	1.81	0.070	.9573293	3.08662

```
No. of subjects =           27                     Number of obs    =        27
No. of failures =           14
Time at risk    =         1534
                                                   LR chi2(5)       =     24.55
Log likelihood  =   -27.939789                     Prob > chi2      =    0.0002
```

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
Eotaxin	3.591013	2.205703	2.08	0.037	1.077419	11.96877
IL-12p40	.0748074	.0770861	-2.52	0.012	.0099269	.5637351
IL-1α	10.50482	8.192444	3.02	0.003	2.278022	48.44168
GM-CSF	.4501839	.1349285	-2.66	0.008	.2501895	.8100485
IL-7	1.829348	.5892068	1.88	0.061	.9730533	3.43919

Conclusion: Although statistical significance was occasionally lost upon exclusion of one-quarter of the dataset, the directionality of the relationships between each of the variables and CD4 loss was upheld.

7) Inclusion of time since infection as a variable in the model (Table B10)

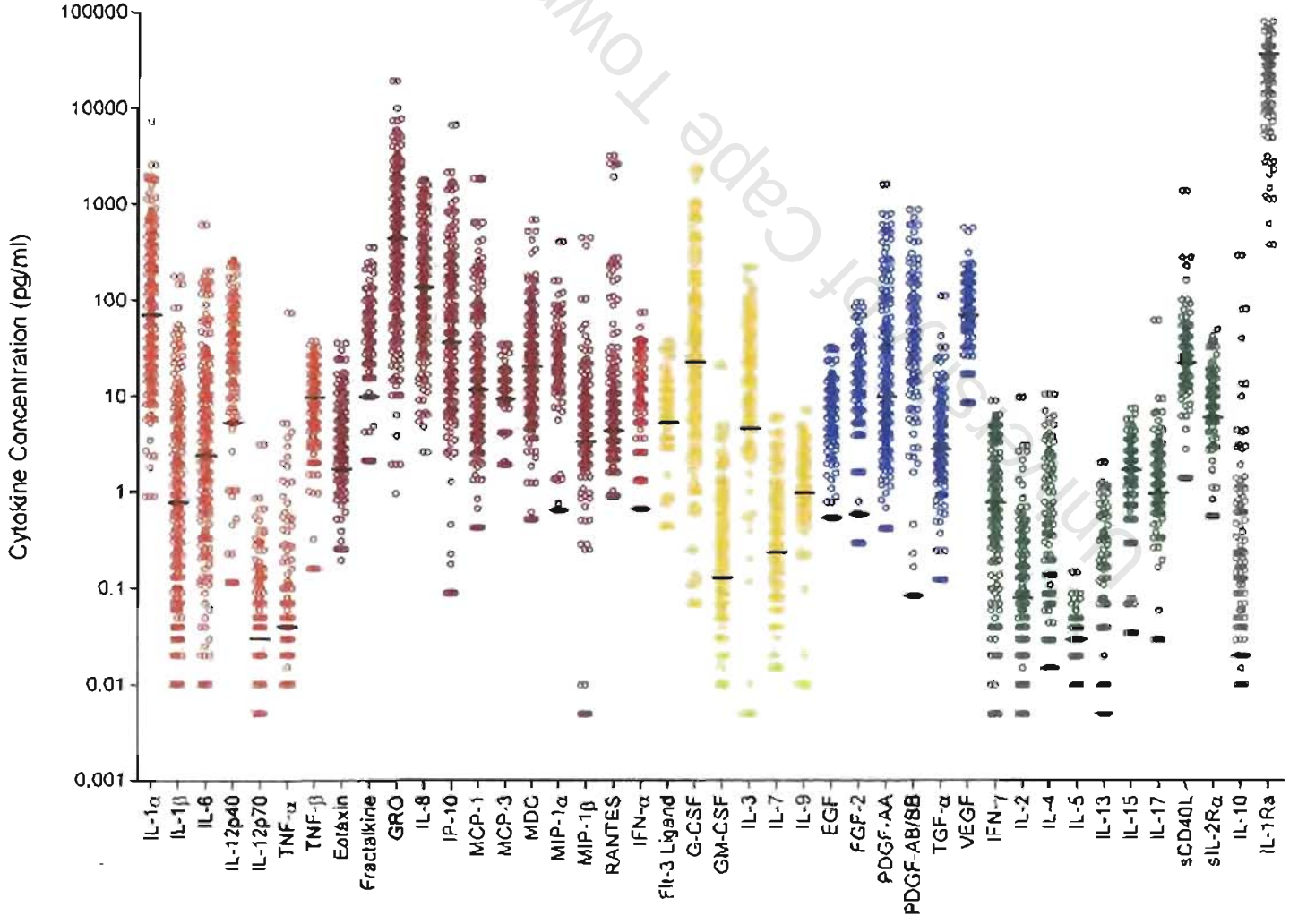
```
Number of obs   =        35
LR chi2(6)      =     33.67
Prob > chi2     =    0.0000
```

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
IL-12p40	.0435063	.0507066	-2.69	0.007	.0044308	.4271961
IL-7	1.99977	.5709175	2.43	0.015	1.142797	3.499379
IL-1a	10.2031	6.801889	3.48	0.000	2.762356	37.68642
Eotaxin	6.371027	5.365939	2.20	0.028	1.222624	33.19909
GM-CSF	.4461328	.1144586	-3.15	0.002	.2698251	.7376426
Sample time	.8740257	.1276596	-0.92	0.357	.6564449	1.163724

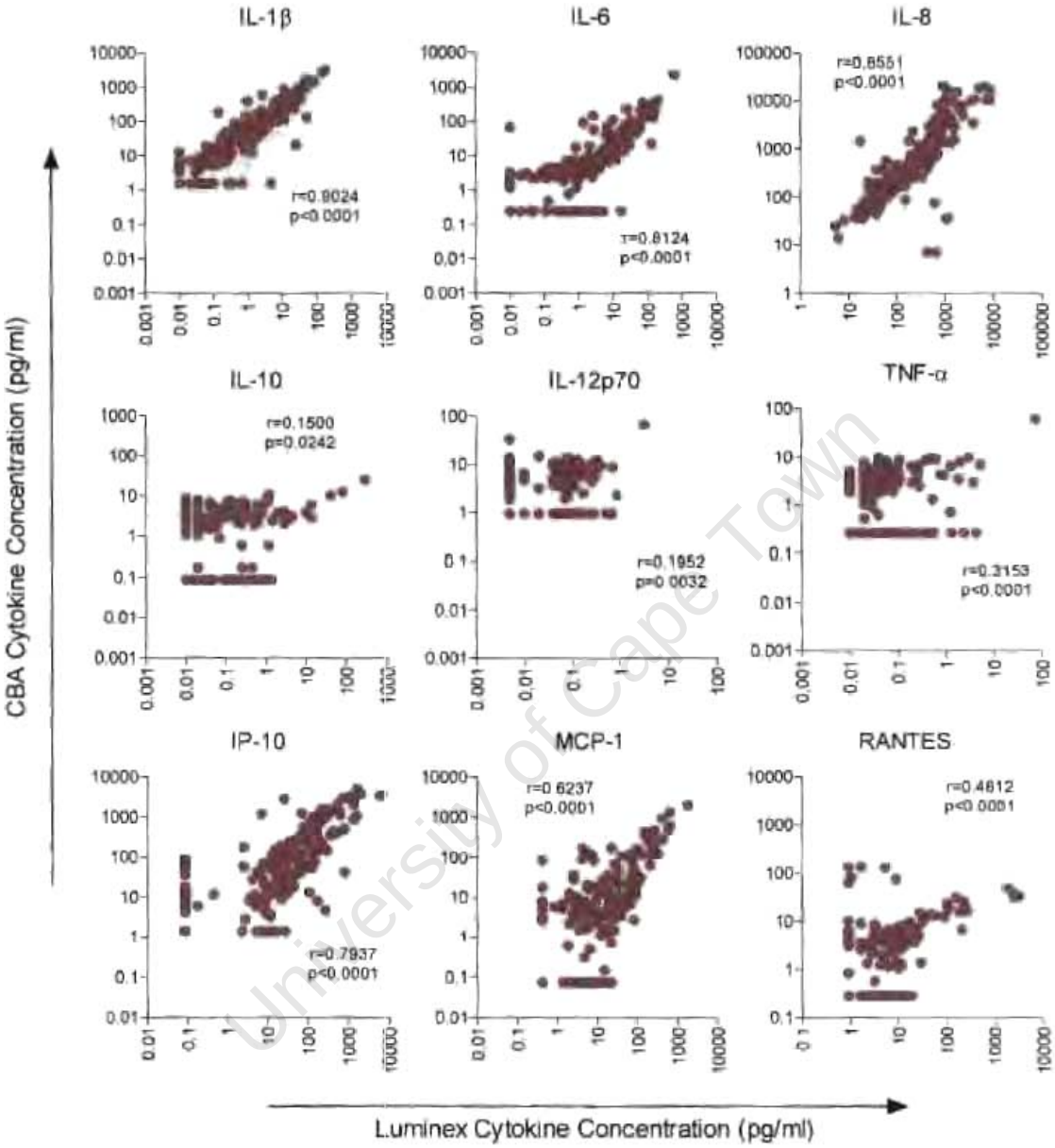
Model	Obs	ll(null)	ll(model)	df	AIC	BIC
Incl. time	35	-53.93858	-37.10183	6	86.20366	95.53574
Excl. time	35	-53.93858	-37.54527	5	85.09054	92.86728

Validation of Luminex Multiplex Flow Cytometric assays

1) Absolute concentrations of cytokines measured in 10 ml cervicovaginal lavage (CVL)



2) Comparison between cytokine concentrations measured in CVL using cytometric bead array (CBA) and Luminex (Chapter 2)



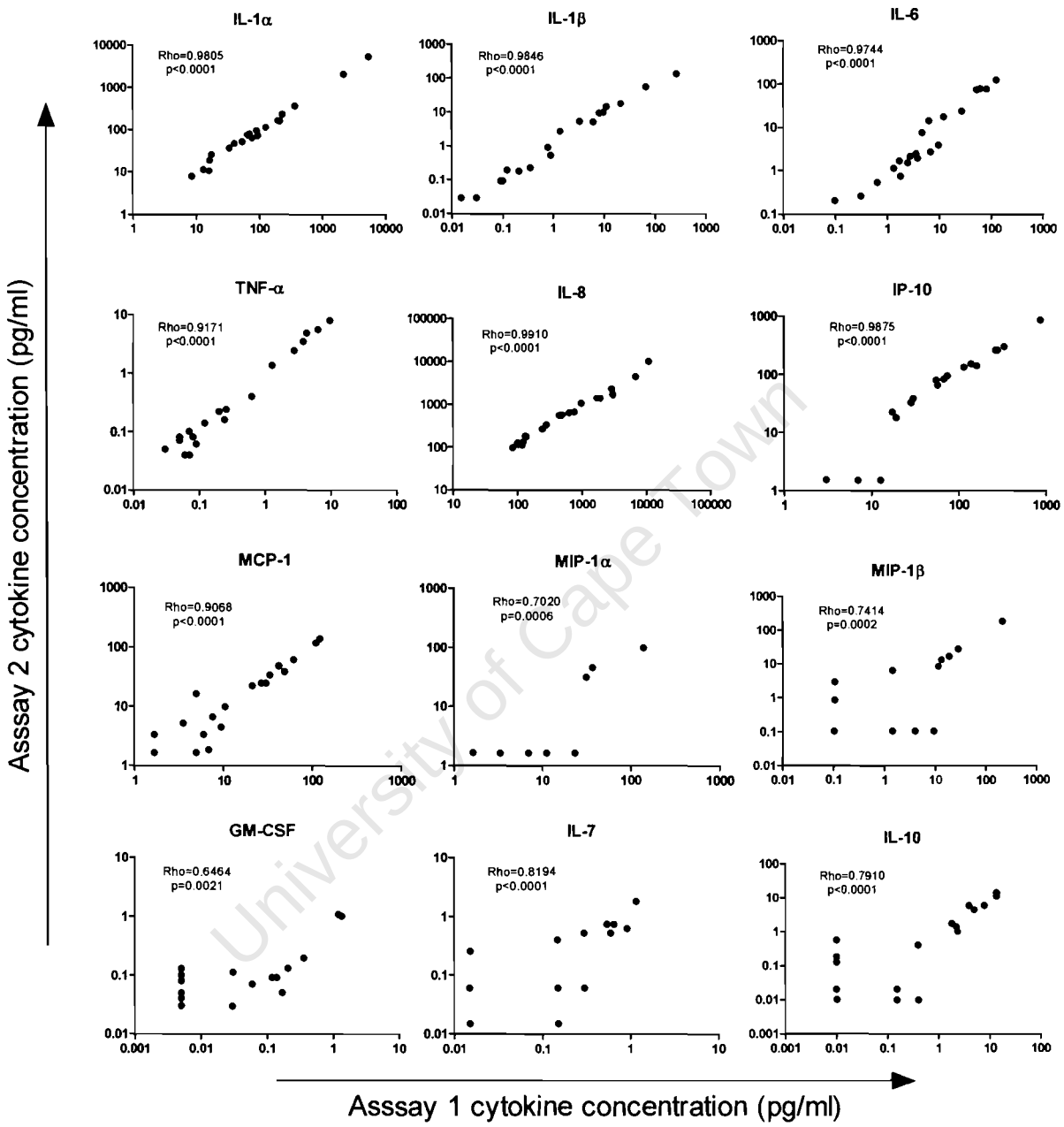
CVL samples from 230 HIV-uninfected women were measured using BD™ CBA Human Inflammation/Chemokine kits and using MILLIPLEX™ Human Cytokine/Chemokine kits (High Sensitivity Premixed 13 plex kits and 30 plex kits). The two methods produced results that correlated significantly for each of the 9 cytokines and chemokines that were measured. However, several cytokines (IL-10, IL-12p70, TNF-α and RANTES) had low Spearman Rho scores, indicating varying sensitivity of these assays and poor reproducibility between assays for these cytokines. Overall, it was found that Luminex was more sensitive and detected low concentrations of the cytokines that were assessed.

3) Comparison between cytokine concentrations measured in plasma in separate Luminex assays (Chapter 4)

Cytokine	Median (IQR)		Spearman rho	P-value
	Assay 1	Assay 2		
IL-1β	1.24 (0.43-2.00)	1.08 (0.24-1.37)	0.8168	<0.0001
IL-2	11.79 (4.50-23.56)	4.42 (2.15-9.33)	0.7426	<0.0001
IL-4	11.06 (1.00-143.30)	12.51 (0.08-59.27)	0.9578	<0.0001
IL-5	0.36 (0.10-0.86)	0.16 (0.05-0.47)	0.9057	<0.0001
IL-6	3.26 (0.58-11.55)	2.66 (0.57-8.15)	0.9616	<0.0001
IL-7	1.46 (0.22-3.03)	0.54 (0.05-1.16)	0.8300	<0.0001
IL-8	2.54 (1.58-5.30)	1.95 (1.33-4.16)	0.9343	<0.0001
IL-10	17.84 (8.76-24.68)	15.35 (8.96-20.98)	0.9104	<0.0001
IL-12p70	0.17 (0.01-1.05)	0.08 (0.015-0.43)	0.7477	<0.0001
IL-13	1.84 (0.10-31.54)	3.57 (0.14-23.36)	0.9596	<0.0001
IFN-γ	3.52 (0.66-8.48)	1.29 (0.18-5.24)	0.7048	<0.0001
GM-CSF	0.37 (0.015-1.09)	0.28 (0.005-0.56)	0.9329	<0.0001
TNF-α	5.68 (4.22-7.66)	6.06 (4.03-7.97)	0.8810	<0.0001

Cytokine concentrations in the same plasma samples were measured using two separate LINCOplex High Sensitivity kits on two different days. The concentrations of each of the cytokines assessed was found to correlate well between assays (Rho >0.7, P-value <0.0001).

4) Comparison between cytokine concentrations measured in cervicovaginal lavage in separate Luminex assays (Chapter 5)



Cytokine concentrations in the same CVL samples were measured using two separate MILLIPLEX™ kits on two different days. The concentrations of each of the cytokines assessed was found to correlate well between assays (Rho >0.6, P-value <0.0001). However, it was found that the reliability of measurements was reduced at lower concentrations for some cytokines. Therefore, these cytokines were also evaluated as dichotomous variables in order to confirm the findings reported in Chapter 5.

Conclusions

Due to the variability in cytokine measurements between separate Luminex assays, cytokine measurements were only directly compared in this dissertation if the samples in each comparison group had been assayed using the same kit. In the case of large sample sizes, samples from each comparison group were equally distributed across all plates so that the variation between kits would contribute the same amount of variation to each comparison group.