Immunopathogenesis of Vulvo-Vaginal Candidiasis in Human Immunodeficiency Virus infected Women

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Submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy in Medical Microbiology and Infection Control

University of KwaZulu-Natal, South Africa

2014

As the candidate's supervisor I agree to the submission of this thesis.

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DECLARATION

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PRESENTATIONS at CONFERENCES and SCIENTFIC MEETINGS of DATA EMANATING from this THESIS

1. Teke Apalata 2012; Candida Vaginitis: Fungicidal Activity of Human Monocytes and Polymorphonuclear Leukocytes in a Synthetic Vagina-Simulative Medium. (poster)
    - 13th International Union against Sexually Transmitted Infections World Congress
    - 15 – 24 October 2012, Melbourne, Australia.
2. Teke Apalata 2012; Candida Vaginitis: Fungicidal Activity of Human Monocytes and Polymorphonuclear Leukocytes in a Synthetic Vagina-Simulative Medium. (oral presentation)
    - 5th WSU International Research Conference (5th WSU-IRC) held at the East London International Convention Centre, 22 – 24 August 2012, East London.
3. Teke Apalata 2012; Vulvo-Vaginal Candidiasis in the setting of HIV infection. (invited presentation)
    - 3rd Continental Congress of the International Society of Dermatology (ISD) and the Dermatology Society of South Africa (DSSA) and the 65th National Congress of the DSSA, 24 – 27 October 2012, ICC – Durban.
4. Apalata T, Longo-Mbenza B, Carr HW, Sturm AW, Moodley P 2013; Concentration of selected plasma cytokines in HIV infected and uninfected women with vulvo-vaginal candidiasis. (oral presentation)
    - College of Health Sciences Research Symposium - University of KwaZulu-Natal, 12-13 September 2013.

5. Apalata T, Longo-Mbenza B, Carr HW, Sturm AW, Moodley P 2013; Influence of HIV-1 related immunosuppression on symptomatic vulvo-vaginal candidiasis amongst women in KwaZulu-Natal, South Africa. (poster)
    - College of Health Sciences Research Symposium - University of KwaZulu-Natal, 12-13 September 2013.
6. Apalata T, Longo-Mbenza B, Carr HW, Sturm AW, Moodley, P 2013; Association between symptomatic vulvo-vaginal candidiasi and HIV RNA expression in plasma and genital secretions amongst women on HAART attending a primary healthcare clinic in KwaZulu-Natal, South Africa. (poster)
    - College of Health Sciences Research Symposium - University of KwaZulu-Natal, 12-13 September 2013.
7. Teke Apalata 2014; Concentration of selected plasma cytokines in HIV infected and uninfected women with vulvo-vaginal candidiasis. (oral presentation)
    - 2014 Keystone Symposium (HIV Pathogenesis: Virus versus Host) held at the Fairmont Banff Springs in Banff, Canada on March 9 – 14, 2014.

ACKNOWLEDGEMENTS

No journey is successfully completed alone. I would like to thank the following people for their support and involvement in the finalization of this thesis.

Prof. Prashini Moodley my supervisor for her expert advice, constructive criticism and guidance throughout my study. I don't know if I could have achieved this height in my career and life without your help.

Prof. A Willem Sturm, the man I met on June 8, 2003 at a time when I had lost all hope. All that I have achieved is to your credit. You had faith in a young man when everyone else doubted. Thank you Sir for shaping my career.

I am truly grateful to my mentors Prof. Benjamin Longo-Mbenza and Dr William H. Carr. Thank you for burning the midnight oil with me during my research and in the finalization of this thesis.

How does a person continue to say 'thank you' when there are still so many people to thank? This thesis represents the fruit of your labour too. A big thank you to all my colleagues in the Department of Medical Microbiology, Infection Prevention and Control - University of KwaZulu-Natal.

DEDICATION

To the loving memory of my father, Guillaume Apalata, who passed away under undescriptable circumstances – you cast a shining light along the path of my early journey in life. You are sorely missed.

To my boys, Joel and Benedict – I hated that I always missed your football games and failed to attend your school events because I was too busy working on my study and career. It's your love that gets me over the hurdles and sacrifices. I look forward to making up on the time we spent apart.

To my only daughter, Kayla – being your dad fills me with so much joy!

To the person without whose love and support this thesis would not have materialised, my wife Edith – you endured numerous difficulties by taking care of the kids alone. Marrying you is my life's most treasured achievement.

Last but not the least, to 'God Almighty', the source of my life and insipiration. My Lord 'Jesus Christ' inspires in me a reason for existing above the reality.

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ALS	Agglutinin-like sequence
ANOVA	Analysis of variance
APC	Antigen presenting cells
ART	Antiretroviral therapy
ATCC	American type culture collection
BD	Becton Dickinson
BV	Bacterial vaginosis
CCR5	Chemokine receptor type 5
CD	Cluster of differentiation
CDA	Canonical discriminant analysis
CDC	US centers for diseases control and prevention
CI	Confidence interval
CTL	Cytotoxic T-cell
CVF	Cervico-vaginal fluid
CXC4	C-x-c chemokine receptor type 4
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
G-CSF	Granulocyte colony-stimulating factor

GM-CSF	Granulocyte macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
GUS	Genital ulcer syndrome
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
IL	Interleukin
IFN-γ	Interferon gamma
IQR	Interquartile range
KZN	KwaZulu-Natal
LGTI	Lower genital tract infection
MBL	Mannose binding lectin
MCP-1	Monocyte chemotactic protein-1
MHC	Major Histocompatibility Complex
MIC	Minimum inhibitory concentration
MIP-1β	Macrophage inflammatory protein-1
MMWR	Morbidity and mortality weekly report
NK	Natural killer
NRTI	Nucleoside reverse-transcriptase inhibitor
NNRTI	Non-nucleoside reverse-transcriptase inhibitor

OPC	Oro-pharyngeal candidiasis
OR	Odds ratio
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PH	Potential hydrogen
PHC	Primary healthcare clinic
PI	Protease inhibitor
PMN	Polymorphonuclear
PRR	Pathogen recognition receptor
RANTES	Regulated on activation, normal T cell expressed and secreted
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute Medium
RT-PCR	Reverse transcription polymerase chain reaction
RR	Relative risk
RVVC	Recurrent vulvo-vaginal candidiasis
SAB	Sabouraud dextrose agar with chloramphenicol
SAP	Secreted aspartyl proteinase
SD	Standard deviation
SDA	Strand displacement amplification
SEM	Standard error of the mean

STI	Sexually transmitted infection
TGF-β	Transforming growth factor beta
TLR	Toll-like receptors
TH cell	T helper cell
TNF-α	Tumor necrosis factor
Treg cell	Regulatory T cell
UNAIDS	Joint United Nations Programme on HIV/AIDS
UKZN	University of KwaZulu-Natal
USA	United States of America
VDS	Vaginal discharge syndrome
VL	Viral load
VSM	Vagina-simulative medium
VVC	Vulvo-vaginal candidiasis
WHO	World Health Organization
WOS	White to opaque switching

PREFACE

Vulvovaginal candidiasis (VVC) is an important cause of lower genital tract infections in women. There are currently numerous clinical observations linking increased cases of symptomatic VVC to the progression of HIV epidemic. While the pathogenesis of other commonly encountered mucosal candidiasis (oral and oesophageal) in the context of HIV infection has been well studied, gaps in our knowledge remain regarding candida vaginitis.

With increasing degree of immunosuppression, symptomatic VVC in HIV infected women is frequent, severe, recurrent and less responsive to conventional anti-fungal therapy. The quality of life is greatly diminished for women who experience recurrent episodes of symptomatic VVC. Furthermore, the high prevalence of HIV infected women adds to the burden of healthcare.

In this study, we sought to further understand the pathogenesis of symptomatic VVC and the associated host defense mechanisms in HIV infected women.

The results of this study are presented as a collection of 5 papers, 4 of which are published in peer reviewed journals, 1 is still under review. The initial chapter of this thesis, 'Introduction and Background', is followed by the 5 manuscripts which are grouped into 3 consecutive result chapters as follows:

I. Impact of HIV on symptomatic VVC.
II. Impact of symptomatic VVC on HIV RNA levels in plasma and genital secretions.
III. Plasma and vaginal-associated immune responses in women with symptomatic VVC.

The final chapter, 'Discussion and Conclusions', rounds up the thesis.

CHAPTER 1 – Introduction and Background to the Study

1.1. Introduction

Vulvo-vaginitis is one of the most common problems leading women to seek advice in primary health clinics. Bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC) are responsible for the vast majority of cases of infectious vulvo-vaginitis (Sobel, 2007). About 75% of healthy women develop VVC at least once during their reproductive age (Ohmit et al., 2003, Fidel et al., 2004, Fidel, 2004). It is estimated that 5-10% of women develop recurrent VVC (Ohmit et al., 2003, Fidel et al., 2004, Fidel, 2004). The latter is defined as the occurrence of 4 or more episodes of VVC per year (Sobel, 1997). Symptomatic VVC is clinically characterized by an acute onset of vulvovaginal pruritis, irritation and/or soreness accompanied by the presence of vaginal erythema/ oedema, and/or discharge (Sherrard et al., 2011). *Candida albicans* has been reported as the cause of VVC in 85-95% of cases whilst *Candida glabrata* represents the most common cause of non-albicans candida vulvo-vaginitis (Sobel, 2007).

In HIV infected patients, whilst oro-oesophageal candidiasis is known to appear at any time during the course of progression of HIV infection, symptomatic VVC develops significantly later (Beltrame et al., 2006). It has been shown that oro-oesophageal candidiasis occurs as a result of the loss of mucosal and systemic cell-mediated immunity while animal studies (de Repentigny et al., 2004, Beltrame et al., 2006, Taylor et al., 2000, Fidel, 2006) and in vitro experiments indicate that systemic cell-mediated immunity does not play a protective role

against candida vulvo-vaginitis (Sobel et al., 2000, Taylor et al., 2000). Previous clinical studies have reported on the association between low CD4 counts (< 200 cells/mm^3) and symptomatic VVC (Duerr et al., 1997, Duerr et al., 2003). Although symptomatic VVC is not classified among AIDS-defining conditions, it has been reported to be frequent among HIV-infected women with CD4 counts < 200 cells/mm^3 (Duerr et al., 1997, Duerr et al., 2003).

Whilst the majority of cases of symptomatic VVC develop without predisposing factors, this condition remains a common problem in women with HIV infection (Fauci *et al*., 2008). Symptomatic VVC, one of the major opportunistic fungal infections in HIV positive individuals, is therefore a key challenge faced by clinicians worldwide, particularly in developing countries where the vast majority (95%) of approximately 34 million HIV infected people is found.

In South Africa, cross-sectional studies of women attending antenatal clinics and family planning clinics over the past 20 years showed a weighted average prevalence of 26% for symptomatic VVC. Among these studies, many have been conducted in KwaZulu-Natal (Coetzee and Johnson 2010: 219). In 1998, Ramjee *et al.* reported the prevalence of 36.1% and 45.7% for symptomatic VVC respectively among HIV negative and HIV positive sex workers in KwaZulu-Natal (Ramjee et al., 1998). In 2011, during a nested case control study within a prospective trial of 1 404 women (716 HIV positive compared with 688 HIV negative) over a 2-year period in KwaZulu-Natal, Sebitloane *et al.* reported on symptomatic VVC as being significantly more prevalent among HIV infected than uninfected women [39.2% versus 30%; RR = 1.31 (1.08 - 1.59); $P = 0.006$] (Sebitloane et al., 2011).

The Province of KwaZulu-Natal in South Africa where this study was conducted is an epicenter of HIV infection. There are anecdotal reports that HIV-infected women present in primary healthcare facilities with frequent, severe and recurrent forms of symptomatic VVC particularly during advanced stages of HIV infection.

Despite the fact that symptomatic VVC is associated with very low mortality (CDC MMWR 2009) and recurrent episodes of symptomatic VVC are not known as an AIDS-defining condition (Sobel, 1997), the disease is however known to cause sexual dysfunction with discord in relationships due to chronic vaginitis particularly in HIV-infected women (Sobel, 2006). Another challenge associated with recurrent episodes of symptomatic VVC in HIV positive women is the higher cost of managing these cases due to prolonged maintenance therapy (Sobel, 2006). In addition, the observed symptoms-pruritis (itching), discomfort, dyspareunia (pain during intercourse), and dysuria (pain or burning during micturition) contribute significantly to morbidity for those HIV-infected women.

The pathogenesis of symptomatic VVC during the course of HIV disease progression is not well understood. The present study sought to determine both epidemiological factors and host immune responses associated with symptomatic VVC in HIV-infected individuals. The effects of HIV-induced immunosuppression as well as the associated virologic correlates on the occurrence of symptomatic VVC were also measured.

1.2. Background to the study

1.2.1. Definitions, classification and predisposing factors

Vulvo-vaginal candidiasis is defined as an inflammatory response of the mucosal surface of the vagina caused by the presence of *Candida* spp. in the absence of other aetiological agents of infectious vulvo-vaginitis (Fischer, 2012, Achkar and Fries, 2010). VVC can be classified as uncomplicated in the presence of sporadic or infrequent occurrence of mild to moderate disease, often caused by *Candida albicans*, in immunocompetent women. However, complicated VVC includes severe clinical forms of VVC, VVC caused by non-*C.albicans* spp., VVC associated with concurrent conditions – pregnancy and uncontrolled diabetes mellitus, VVC associated with immunosuppression (i.e. HIV-induced), as well as recurrent VVC in immunocompetent women (Pappas et al., 2009, Workowski and Berman, 2006).

Recurrent VVC (RVVC) is defined as the occurrence of 4 or more episodes of VVC per year (Sobel, 1997). Recurrent episodes of VVC can be classified as primary RVVC and secondary RVVC. The latter is characterized by its association with identified predisposing factors (Table 1) (Cotch et al., 1998, Spinillo et al., 1999, Wilton et al., 2003, Peer et al., 1993, Rahman T, 1990, Foxman, 1990, Reed et al., 2003, Geiger AM, 1998, Geiger AM, 1995, Sobel, 1993, Sebitloane et al., 2011, Duerr et al., 2003, Duerr et al., 1997, Chaim et al., 1997, Calderon et al., 2003, Babula O, 2005, Nadeem SG, 2013, Ghosh, 2009, Sobel, 2007, Cassone et al., 2007).
Primary RVVC however is idiopathic and does not correlate with any of the reported predisposing factors (Cassone et al., 2007).

Table 1: Factors associated with secondary cases of recurrent vulvo-vaginal candidiasis

Host factots	pregnancy[1,2] uncontrolled diabetes mellitus[3,4] use of contraceptives and hormone replacement therapy[1,5,6] corticosteroids[1] antimicrobial drugs[7,8] HIV and AIDS[9-11] cancer chemotherapy[12-14] organ transplantation[12-14]
Genetic factors	ABO-Lewis non-secretor phenotype[15] gene polymorphisms of the innate immunity (polymorphism in codon 54 of the MBL gene, C to T substitution at position 589 of the IL-4 gene)[16,17]
Vaginal environmental factors	temperature ≥ 37˚C (normal vaginal temperature being 37˚C)[18] increased CO2/O2 ratio[18] pH of 4 - 4.5 (normal vaginal pH ranging from 3.8 to 4.2)[18] nitrogen starvation[19] presence of tyrosol as a quorum sensing molecule[19]
Microbial factors	non-albicans Candida sp.[1,14] Candida albicans with increased MICs to azoles[1,14]
Behavioral factors	increased sexual intercourse frequency and periodicity[12-14]

[1]Sobel, 2007; [2]Cotch et al., 1998; [3]Peer et al., 1993; [4]Rahman et al., 1990; [5]Foxman, 1990; [6]Reed et al., 2003
[7]Spinillo et al., 1999; [8]Wilton et al., 2003; [9]Sebitloane et al., 2011; [10]Duerr et al., 2003; [11]Duerr et al., 1997
[12]Geiger and Foxman 1998; [13]Geiger et al., 1995; [14]Sobel, 1993; [15]Chaim et al., 1997; [16]Babula et al., 2005
[17]Calderon et al., 2003; [18]Nadeen et al., 2013; [19]Ghosh, 2009.

The ambiguity about the definition of RVVC is when some patients do not have recurrent symptoms, but their disease is chronic, continuous and unremitting (Fischer, 2012).

1.2.2. Candida commensalism and virulence in human vaginal mucosa

Candida albicans often colonises the vagina without causing disease; it can cause symptomatic VVC by responding to a variety of environmental signals – i.e. changes in vaginal pH (pH of 4 to

4.5) lead to a switch from *C.albicans* blastospore phenotype into a filamentous form (hyphae or pseudohyphae), increasing its ability to cause vaginitis (Ghosh, 2009). However, it is well established that *C. albicans* is both a commensal and a pathogen that can exhibit yeast, hyphal, or pseudohyphal morphology. These morphological transitions promote colonization and invasion at different anatomical sites, including vaginal mucosa. Whilst the yeast form is associated with colonization and dissemination of Candida at the mucosal surface of the vagina, the hyphal form is however associated with mucosal adhesion, tissue invasion, and proteolytic activity (Whiteway and Bachewich, 2007). Hence, genes involved in these functions (i.e. ALS3, SAP4 to -6, HWP1, HYR1, and ECE1) are also differentially expressed.

The mitogen-activated protein (MAP) kinase, cyclic AMP, and pH-sensing Rim101 signal transduction pathways regulate cellular morphology and expression of hypha-associated genes (Whiteway and Bachewich, 2007).

The extracellular hydrolytic enzymes such as the secreted aspartyl proteinase (SAP) and phospholipase (PLB) gene products have been shown to directly contribute to C. albicans virulence (Monod and Borg-von, 2002, Monod and Borg-von Zepelin, 2002, Monod et al., 2002).

The *C.albicans* agglutinin-like sequence (ALS) family includes eight genes that encode large cell surface glycoproteins that play the adhesive function (Munro et al., 2005).

1.2.3. Pathogenesis of acute and recurrent vulvo-vaginal candidiasis

a) RVVC as a result of relapse rather than reinfection with Candida

RVVC is rarely a result of antifungal resistance, but a deficiency in the normal protective vaginal bacterial flora that permits unsuppressed growth and the proliferation and germination of colonising yeast microorganisms has been hypothesized by others (Sobel, 2007). Studies reported that strains isolated before and after treatment were identical in the vast majority of recurrences, and those microorganisms may persist within the vaginal lumen, generally in numbers too small to be detected by conventional vaginal cultures, only to re-emerge some weeks or months later (Sobel, 2007). Numerous studies on molecular typing of *C.albicans* from RVVC patients support relapse (Chong et al., 2003) rather than reinfection of candida vaginitis following successful anti-fungal therapy (Li *et al.* 2008).

It has been hypothesized that patients with RVVC lack important anti-candida immune factors in the vagina (Fidel et al., 1997, Fidel, 2004, Fidel et al., 2004). For example, in human reconstituted vaginal epithelial tissue, some enzymes of the secreted aspartic proteinase (Sap) family members (namely, Sap1 and Sap2) were shown to attack and derange the epithelial architecture, eliminating its physical and functional antifungal properties (Cassone et al., 2007, Schaller et al., 2003). In addition, the epithelial keratinocytes are known to produce a cascade of inflammatory cytokines (e.g. tumor necrosis factor alpha, interleukin-10 [IL-10], and gamma interferon) that would typically induce a regulated and protective immune response in the normal host. The lack of such response at the mucosal surface of the vagina may lead to recurrent

episodes of VVC (Romani and Puccetti, 2006, Schaller et al., 2003). Reduced vaginal levels of mannose-binding lectin (MBL) and nitric oxide and increased occurrence of polymorphism of the IL-4 (T-589) gene has been reported to correlate with high prevalence of RVVC in Latvian and Chinese women (Liu et al., 2006, Babula O, 2005).

During RVVC, Candida is therefore not completely eliminated by anti-fungal treatment, leading to recurrence through relapses (Fidel, 2004, Fidel et al., 2004).

In general terms, women with RVVC respond adequately to initial antifungal regimens. However, these regimens do not prevent further recurrence (Sobel et al., 2004). In South Africa, the standard treatment regimen for uncomplicated symptomatic VVC is made of 500 mg clotrimazole pessaries (single dose).

b) Role of IgE-mediated hypersensitivity reaction

Although the conventional theory has been that women with RVVC lack immunity to Candida, and that systemic immunity is not relevant and the problem is specific to the vagina, it is also suggested that RVVC is the result of an allergic reaction mediated by *Candida*-specific immunoglobulin (Ig) E. This hypothesis has been demonstrated by only limited number of studies (Fischer, 2012, Sobel et al., 2004, Calderon et al., 2003).

c) Role of HIV-related immune suppression and Th1/Th2 paradigm

There are reports linking acute VVC and recurrent episodes of symptomatic VVC to HIV related immunosuppression (Duerr et al., 2003, Duerr et al., 1997, Sebitloane et al., 2011).

Previously published studies hypothesized that systemic cell-mediated immunity might not play a protective role against candida vaginitis (de Repentigny et al., 2004, Fidel, 2006). Those studies based their arguments on two critical theories (a) the theory of compartmentalization of vaginal cell-mediated response from systemic cell-mediated immunity (Fidel, 2005): T lymphocytes found in the vaginal mucosa are phenotypically distinct from those in the peripheral blood and the proportion of γ/δ TCR_+ T cells in the vagina is significantly higher (15-50% γ/δ TCR_+ T cells) than α/β TCR_+ T cells compared to the periphery. In addition, after measuring HIV specific CD8+ T cells, it was reported that there was no correlation between plasma and cervico-vaginal responses (Gumbi et al., 2008); (b) the theory of immunoregulation (Taylor et al., 2000): despite reports on the presence of a protective candida-specific Th1-type immunity during a symptomatic VVC (Fidel et al., 2004), a strong down-regulatory cytokine, TGF-β, constitutively expressed by the vaginal mucosa was also noticed (Taylor et al., 2000).

In 2006, Beltrame *et al.* reported that candidal vaginal colonization, a precursor of vaginitis, develops when CD_{4+} T-lymphocyte counts fall to ≤ 100 cells /μL in the course of HIV infection (Beltrame et al., 2006). Prior to this report, Sobel *et al*. (2000) found out that higher HIV loads rather than lower CD_{4+} T-lymphocyte counts were associated with statistically significant increased odds for both persistent candidal vaginal colonization and symptomatic VVC. However, the study by Sobel *et al.* (2000) only found an association between plasma HIV viral

load and the proportion of *Candida* infections that were non-*C.albicans*, not the absolute prevalence of symptomatic VVC (Sobel et al., 2000).

d) The paradigm shift: Role of Th17 cells

Mucosal immune determinants associated with sensitivity and resistances to symptomatic VVC in HIV infected women have not been fully studied. Shacklett and Anton (2010) reported on the presence of Th17, a subset of CD4+ T cells as able to secrete IL-17 and IL-22. The latter induced the production of antimicrobial peptides able to curb microorganisms such as *C.albicans*, the cause of symptomatic VVC (Shacklett and Anton, 2010). Whether or not the action of Th17 cells is reduced due to CD4+ T cells depletion observed during advanced HIV infection, leading to recurrent VVC, requires further investigation.

e) Role of vaginal mucosa innate and adaptive immunity

Studies on protective immunity against candida vaginitis should focus on vaginal innate and adaptive mucosal immunity. Vaginal epithelial cells have been reported to inhibit candida growth in vitro (Barousse et al., 2001). However, their protective role in vivo remains unknown. Barousse *et al.* (2001) reported on the vaginal epithelial cells as able to provide an innate host resistance mechanism against candida colonization, and therefore, its decrease in such activity may contribute to symptomatic VVC.

In studies on primary RVVC, using an intra-vaginal live challenge model of *C.albicans*, Fidel *et al.* (2004) found out that RVVC could be associated with a strong but non-protective neutrophil response against *C.albicans*.

Various immunomodulators have been reported as associated with the anti-candida activity of neutrophils. Whilst cytokines such as tumor necrosis factor and IL-8 increase the activity of neutrophils, glucocorticoids and progesterone are inhibiting factors.

In vitro studies reported on the role played by lactoferrin, a natural peptide found in cervico-vaginal secretions, in modulating the functions of phagocytic cells such as adhesiveness of both neutrophils and macrophages (Novak *et al.* 2007). Okutomi *et al*. (1997) found that the growth inhibition effects of neutrophils on candida cells were enhanced in the presence of a physiological concentration of lactoferrin (Okutomi et al., 1997). A study by Saavedra *et al.* (1999), which examined the kinetic production of chemokines associated with the chemotaxis of macrophages (RANTES, MIP-1α, MCP-1) and neutrophils (MIP-2, IL-8) showed that higher levels of MCP-1 are able to control *C.albicans* titers in mice with candida vaginitis, suggestive of a strong role played by MCP-1 in the vaginal immune response to *C.albicans* (Saavedra et al., 1999).

f) Role of pattern recognition receptors

Pattern recognition of *C.albicans* is mediated by the recognition of mannans and β- glucans by Toll-like Receptors (TLR2 and TLR4) and lectin receptors such as dectin-1 (Ferwerda et al., 2009) and mannose-binding lectin (MBL) receptors on the surface of the cell (van Till et al., 2008).

The cell wall of *C.albicans* is composed of pathogen-associated molecular patterns (PAMPs), especially polysaccharides like chitin, 1,3-β-glucans and 1,6-β-glucans, and proteins that are heavily mannosylated with mannan side-chains (Jouault et al., 2003, Netea et al., 2006)McGreal et al., 2006, Brown and Gordon, 2001).

Pathogen recognition receptors (PRRs), such as the Toll-like receptors (TLRs) and C-type lectins (CLRs) on the surface of antigen presenting cells (APCs) are able to recognize PAMPs. Studies have shown that TLR2 recognizes phospholipomannans; TLR4 recognizes O-linked mannans; and macrophage mannose receptor (MMR) recognizes N-linked mannans (Jouault et al., 2003, Netea et al., 2006). Whilst the CLR Dectin-1 recognizes β-glucan, CLR Dectin-2 recognizes mannose residues (McGreal et al., 2006, Brown and Gordon, 2001).

Immune cell populations involved in recognition of *C. albicans* during the innate immune response include monocytes, macrophages and neutrophils (Gauglitz et al., 2012, Jouault et al., 2003). Dendritic cells are crucial for processing of and antigen presentation to T cells, and therefore to activation of specific immunity (Gauglitz et al., 2012, Jouault et al., 2003). This recognition of *C.albicans* by immune cells is done mainly through TLRs (Gauglitz et al., 2012, Jouault et al., 2003). The latter are involved in inflammatory responses induced by *C. albicans*, of which TLR2 and TLR4 are the most studied (Jouault et al., 2003). They are expressed by monocytes, macrophages, dendritic cells, neutrophils, CD4+ T cells and epithelial cells (Weindl et al., 2007, Netea et al., 2006). Studies have shown that the activation of TLR2 signal pathways in these antigen-presenting cells (APCs) by ligation of *C. albicans* cell-wall components such as phospholipomannan leads to production of cytokines that are able to induce a Th2 cellular response (Weis et al., 1998, Bellocchio et al., 2004, van de Veerdonk et al., 2008, Miyazato et

al., 2009). Hence, blocking TLR2 with a TLR2-specific antibody after stimulation of monocytes by *C. albicans* was shown to result in diminished release of Th2-associated cytokines (van de Veerdonk et al., 2008). In contrary, the activation of TLR4 signal pathways during candidiasis will result to the production of cytokines able to induce a Th1 cellular response. Mannans of *C. albicans* are recognized by TLR4 leading to the production of pro-inflammatory cytokines (IFN-γ, IL-12 and TNF-α) (Roeder et al., 2004).

HIV infects Langherhans cells mainly found in the epithelial layers (Challacombe and Naglik, 2006, Miller and Shattock, 2003), as well as T cells (Gupta et al., 2002), macrophages (Anderson et al., 2008), and dendritic cells in the subepithelial tissues (Kaul and Hirbod, 2010). Whether the reduction in concentrations of these immune cells leads to symptomatic VVC remains to be understood.

In addition, studies reported that CD8+ cytotoxic T-cells (CTL) were able to limit HIV replication in mucosal lymphoid tissues (Shacklett, 2009); however, CTL have been identified throughout the female reproductive tract of healthy women but during HIV infection, their activity is suppressed leading to higher HIV loads (Shacklett et al., 2009). Whether advanced HIV infection with higher viral loads changes the vaginal environment and stimulates *C.albicans* to switch into a filamentous form that causes symptomatic VVC is a matter of uncertainty. What is known is that following HIV infection cytokines and chemokines are produced by infected leukocytes (Barousse et al., 2004) that attract more target cells for HIV infection and allow further stimulation of the expression of HIV via toll-like receptors (Bergmeier and Lehner, 2006). It is not well understood however, whether the produced chemokines and cytokines

interfere with the activity of the PMN cells and macrophages and whether this interference facilitates the development of symptomatic VVC.

Technological advances improved our understanding about the role played by the cervicovaginal mucosa (Shacklett, 2008). It is well established that cervicovaginal immune cells respond to foreign micro-organisms independently of systemic immune responses (Gumbi et al., 2008). In addition, there is a continuous cross talk and feedback regulation between innate and adaptive responses at cervicovaginal level through functional activities of cytokines and chemokines (Fahey et al., 2005). Moreover, leukocyte trafficking into cervicovaginal tissues has been shown to be controlled by the production of cytokines and chemokines (Belay et al., 2002). Macrophages, dendritic cells, neutrophils, NK cells and langerhans cells also migrate into tissues through the secretion of chemokines and cytokines (Karupiah, 2003). During a specific infectious disease, predominantly expressed chemokines and a subgroup of cytokines generate a profile to be used for diagnostic purposes, and provide leads for studying the pathogenesis of such disease.

The present study was therefore essential in elucidating possible reasons for the differences observed in the rates of symptomatic VVC between HIV infected and uninfected women. It attempted to determine potential targets for an optimal immunotherapy able to curb enormous cases of recurrent VVC in the face of an alarming HIV epidemic.

1.3. Purpose of the study

The aim of this study was to assess the effects of HIV infection on the occurrence of symptomatic VVC and antifungal immune responses in the blood and lower genital tract of HIV-infected women with symptomatic VVC.

1.4. Specific Objectives and Hypotheses:

1. To determine the association between HIV-induced immunosuppression, virologic correlates and symptomatic VVC.

This objective tested the hypothesis that higher HIV loads (VL>10 000 RNA copies/ml) and lower CD4+ T-cell counts (< 200 cells /mm^3) are associated with symptomatic VVC in HIV-infected women.

2. To determine the association between symptomatic VVC and HIV RNA expression in plasma and genital tracts of HIV infected women on highly active anti-retroviral therapy (HAART).

This objective tested the hypothesis that HIV infected women with symptomatic VVC have significantly higher HIV loads in their plasma and lower genital tracts than HIV infected women without symptomatic VVC.

3. To determine independent predictors of symptomatic VVC in HIV infected women.

4. To determine plasma and genital concentration of 20 different cytokines/chemokines associated with symptomatic VVC in HIV infected and uninfected women.

This objective tested the hypothesis that significantly higher levels of systemic and genital anti-inflammatory cytokines (IL-10^{pos}, IL-4^{pos}, and TGF-β^{pos}) as compared to pro-inflammatory cytokines (IL-1β^{pos}, IL-2^{pos}, IL-8^{pos}, TNF-α^{pos}, IFN-γ^{pos}, MCP-1^{pos}) are associated with symptomatic VVC in HIV-infected women.

5. To assess the expression of Toll-like receptor (TLR)-2 and TLR4 on monocytes following stimulations by genital secretions of HIV infected and uninfected women presented with symptomatic VVC.

This objective tested the hypothesis that HIV infection alters TLR2 (but not TLR4) dependent responses to candida antigens by monocytes.

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CHAPTER 2

Impact of Human Immunodeficiency Virus on Symptomatic Vulvo-Vaginal Candidiasis

Synopsis:

This chapter comprises 2 manuscripts which are currently published. The articles deal with factors associated with symptomatic vulvo-vaginal candidiasis (VVC) and the determinants thereof in HIV-1 infected women. The hypothesized factors studied in this chapter were: age, contraceptive use, pregnancy, antibiotic use, presence or recent history of sexually transmitted infections, bacterial vaginosis and HIV serostatus. For HIV infected women, the impact of plasma HIV loads, genital HIV shedding, CD4+ T cell count and use of HAART on the occurrence of VVC was also determined.

Factors Associated with Symptomatic Vulvo-Vaginal Candidiasis: A Study among Women Attending a Primary Healthcare Clinic in KwaZulu-Natal, South Africa

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Annals of Medical & Health Sciences Research 2014; 4(3): 410 – 416.

Abstract

Background: Symptomatic vulvovaginal candidiasis (VVC) is one of the most common problems leading women to seek advice in primary healthcare facilities. **Aim:** The aim of this study is to describe the associations between some hypothesized factors and the presence of symptomatic VVC. **Subjects and Methods:** An analytical cross-sectional study was conducted. A total of 90 women diagnosed with symptomatic VVC and 108 women without symptomatic VVC were recruited when attending Umlazi D clinic, a primary health clinic in KwaZulu-Natal, South Africa between June 2011 and December 2011. Confirmed symptomatic VVC was determined by Gram stain and microbiological culture of vaginal swabs. For human immunodeficiency virus (HIV)-infected women, HIV ribonucleic acid load in plasma and genital fluid was determined by real-time-polymerase chain reaction (BioMerieux, Lyon, France). CD4 counts were obtained from patients' medical records. Data were analyzed using the statistical package for the social sciences (SPSS) version 21.0 (SPSS Inc.; Chicago, IL, USA). Multiple logistic regression models were used to exclude univariate confounders. All tests were two-sided and a $P < 0.05$ was considered to be significant. **Results:** A total of 90% (81/90) of patients with symptomatic VVC complained of vulval itching, soreness and vaginal discharge when compared to 75.9% (82/108) of patients without symptomatic VVC ($P < 0.01$).Whilst pregnancy was independently associated with symptomatic VVC ($P < 0.01$), the latter was inversely related to Nugent's scores ($P < 0.01$). When compared with HIV negative women, the odds for symptomatic VVC increased among women with HIV-associated immunocompromise (CD4 counts <200 cells/mm^3, $P < 0.001$), significantly shedding HIV in their genital tracts ($P = 0.04$), with plasma HIV load >1000 copies/mL ($P < 0.001$). There was a significant negative association between the use of highly active anti-retroviral therapy and the presence of

symptomatic VVC in HIV-infected women ($P < 0.01$). **Conclusion:** Although symptomatic VVC is not classified as acquired immunodeficiency syndrome-related condition, HIV-related immune compromised women and particularly those who are anti-retroviral therapy-naïve are likely to develop symptomatic VVC.

Keywords: Human immunodeficiency virus-related immune suppression, Vulvovaginal candidiasis, primary healthcare clinic.

Introduction

Vaginitis is one of the most common problems leading women to seek advice in gynecology and primary health clinics. Bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC) are responsible for the vast majority of cases of infectious vaginitis (1). Almost 75% of healthy women develop VVC at least once during their reproductive age (1). It is estimated that 5-10% of women develop recurrent VVC (1, 2). The latter is defined as the occurrence of 4 or more episodes of VVC per year (3). Symptomatic VVC is characterized by an acute onset of vulvovaginal pruritis, irritation and/or soreness accompanied by the presence of vaginal erythema/edema, and/or discharge (4). *Candida albicans* has been reported as the cause of VVC in 85-95% of cases whilst *Candida glabrata* represents the most common cause of non-albicans candida vaginitis (1).

Although *C. albicans* often colonises the vagina without causing disease, it can cause VVC by responding to a variety of environmental signals– i.e. the normal vaginal pH ranges from 3.8 to 4.2. Any changes in vaginal pH (>4.2) lead to a switch from *C. albicans* blastospore phenotype into a filamentous form (hyphae or pseudohyphae), increasing its ability to cause vaginitis (5). In vitro experiments showed that under acidic conditions, Candida cells can raise the pH from 4 to >7 resulting in autoinduction of the yeast-hyphal transition, a critical virulence trait (6). However, clinical studies have reported that in women with abnormal vaginal discharge, the detection of a pH of <4.5 is a good indicator of VVC and can help to differentiate it from bacterial vaginosis and trichomoniasis, both of which typically produce a pH of >4.5 (7). This is also in keeping with results from Moodley *et al.* (2002) who found out that yeast colonization

and symptomatic VVC were inversely related to Nugent's scores among women in KZN, South Africa (8). Numerous risk factors have been reported as being associated with VVC. Those risk factors for VVC include mainly pregnancy (1, 9, 10), use of broad spectrum antibiotics (1, 11), uncontrolled diabetes mellitus (1), use of contraceptives and hormone replacement therapy (1, 12, 13), use of corticosteroids (1), cancer chemotherapy (14, 15), organ transplantation (14, 15), tight-fitting clothing (1, 14, 15), synthetic underwear (14, 15), various dietary deficiencies or excesses (1, 13, 14), increase sexual activity (1, 14, 15), and vaginal douching (1, 12, 14, 15). Existing data pertaining to some of these factors on the risk of developing VVC are conflicting (1, 12).

Human immunodeficiency virus (HIV) infection has been also reported by others as a risk factor for developing VVC (16-18). Rates of vaginal colonization and symptomatic VVC were reported to increase with immune compromise, especially at CD4 counts below 200 cells/mm^3 (12, 17, 18). Some of the authors have suggested that women with low CD4 counts should be closely monitored for the development of symptomatic VVC (12, 17, 18). Although VVC is not known as an acquired immunodeficiency syndrome (AIDS)-defining condition, cases of VVC are often diagnosed among women with HIV-associated immunosuppression.

The province of KwaZulu-Natal in South Africa where this study was conducted is an epicenter of HIV infection. There are anecdotal reports that HIV-infected women present in primary health-care facilities with frequent, severe and recurrent forms of VVC during advanced stages of HIV infection. We aimed to describe the associations between some hypothesized risk factors

and the presence of symptomatic VVC among primary healthcare attendees in rural KwaZulu-Natal, South Africa.

Subjects and Methods

Study design and population

This is an analytical cross-sectional study. Study subjects were women who consecutively presented at Umlazi D clinic, a primary healthcare facility in KwaZulu-Natal, between June 2011 and December 2011 for signs and symptoms suggestive of lower genital tract infections (LGTIs), and were diagnosed or not with symptomatic VVC.

A standardized questionnaire was used to collect information regarding patients' demographics (age and race), presenting symptoms, history of sexually transmitted infections (vaginal discharge syndrome, genital ulcer syndrome or mixed infections) within the past 3 months and selected risk factors for symptomatic VVC including prior knowledge of HIV sero-status. Known HIV positive women were further asked whether or not they were receiving highly active anti-retroviral therapy (HAART) while confirmation of the use of HAART and CD4+ T lymphocytes count values were obtained from patients' medical records. This study questionnaire has been used by the STI research group of the Department of Medical Microbiology, UKZN for the past many years and has been tested for validity and reliability (19). Validity was established using a panel of experts in the Department and a field test that determined whether the questionnaire measured what it intended to, does it represented the appropriate content, was it appropriate for the study population and was the questionnaire

comprehensive enough to collect the needed information. Reliability was computed after a pilot field test to indicate the accuracy of the measuring questionnaire using the test-retest approach as numerous knowledge questions were part of the study questionnaire.

A physical examination was performed by the attending medical practitioner and signs of the genital tract infections were noted. Patients were recruited in the study only if they had signs and symptoms suggestive of non-ulcerative LGTIs/vaginal discharge syndrome whilst those with confirmed genital ulcer syndrome were excluded. All patients were treated using the standard of care treatment in South Africa for the presenting syndrome.

The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Ref. BE 224/11). Consent forms were signed by all participants and confidentiality was maintained throughout the study.

Specimen collection and process

Cervical and vaginal Probetec swabs (Becton Dickinson, Sparks, Maryland, USA) were obtained from patients with vaginal discharge syndrome. The first vaginal swab, which collected material from the posterior fornix, was used to make a smear onto a glass slide for Gram staining. The second vaginal swab (obtained from the anterior fornix) as well as the cervical swabs were stored in a dry container and kept in a cooler box with ice-pack awaiting transport to the laboratory.

A vaginal tampon (8 Ks, Tampax Regular® Compak) was inserted into the vagina, left *in situ* for 3 min. After removal, the tampon (from which vaginal fluid was expressed for the quantification of HIV loads) was immersed in 10 mL of phosphate buffered saline (PBS; Oxoid Limited

Basingstoke, Hampshire, United Kingdom) (pH = 6.9) in a sterile container. All samples were immediately stored at 4°C prior to transport to the laboratory.

Blood samples were collected by venipuncture into sterile vacutainers (Becton Dickinson) containing ethylenediaminetetraacetic acid for plasma samples and without anticoagulant (red cap tubes) for serum samples.

All specimens were transported within 4 h to the Infection Prevention and Control laboratory, Nelson R Mandela School of Medicine, UKZN.

Initial HIV test was performed in the clinic by a trained research nurse on blood using the HIV rapid test determine HIV-1/2/O (Abbott Laboratories, Abbott Park, IL, USA) following voluntary counseling and testing. The diagnosis of HIV negative with an appointment for another test 3 months later was given to the patient following a negative initial test. Positive samples were transported in the research laboratory and were subsequently retested by a medical technologist using a second HIV rapid test SmartCheck test (World Diagnostics Inc. USA). A diagnosis of HIV infection was reported to the participants if both rapid tests were positive. Samples that showed discordant results were further evaluated with a third rapid test Uni-Gold™ Recombigen® HIV (Trinity Biotech PLC, USA) and only two positive test results were interpreted as a positive diagnosis for HIV.

As part of the routine management of the patients in the clinic, all HIV-infected patients benefited directly from CD4+ T cell count measurements and CD4+ T cell counts used in this study were obtained from patients' medical records. However, for the purpose of this research,

HIV-1 ribonucleic acid was measured from the plasma and cell-free fraction of vaginal secretions using Nuclisens Easyq HIV-1 assay version 2.0 (BioMerieux, Lyon, France) with a lowest detection limit of 20 copies/mL.

Vaginal fluid was expressed from vaginal tampon using an autoclaved wooden tongue depressor and filtered through a 0.22 μm Costar Spin-X cellulose acetate filter membranes (Sigma). The filtered soluble fraction was aliquoted (in 1 mL cryotubes) and stored at −70°C until use for the quantification of HIV loads by Nuclisens Easyq HIV-1 assay.

Vaginal swab taken from the anterior fornix was directly plated onto Sabouraud Dextrose agar with chloramphenicol (BBL™ Becton Dickinson) and incubated at 29°C, 48 h to estimate the relative vaginal fungal burden. The numbers of yeast colonies were recorded as the number of colonies per plate (evidence level III, recommendation grade B) (4, 20, 21).

BV was diagnosed using the Nugent score, which ranges from 0 to 10. A score of 7-10 is consistent with BV (22). Microscopic slides for the diagnosis of BV were viewed consistently and independently by two different medical technologists, all blinded to the patients' clinical history. In the case of a discrepancy among the two readers, a third reader was assigned the task of viewing discrepant slides.

BD ProbeTec ET *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (CT/GC) amplified deoxyribonucleic acid (DNA) assay (Becton Dickinson Probetec Assays, Sparks, Maryland, USA) using strand displacement amplification technology for the direct, qualitative detection of *C. trachomatis* and *N. gonorrhoeae* DNA in endocervical swabs was performed.

DNA product for the detection of *Mycoplasma genitalium, Trichomonas vaginalis* and herpes simplex virus type 2 was extracted from a volume of 200 µl of genital swab eluate using specific QIAamp DNA mini kits (Qiagen Ltd, Chastsworth, CA) according to the manufacturer's protocols as previously described (19). Amplification was performed by in-house polymerase chain reaction (PCR) under specific thermal cycling conditions using the ThermoCycler instrument. The following DNA oligonucleotide primers (Roche Diagnostics, Basel, Switzerland) appropriate for PCR amplification were used – for *M. genitalium*: MgPa1 (5'-AGT TGA TGA AAC CTT AAC CCC TTG G-3') and MgPa3 (5'-CCG TTG AGG GGT TTT CCA TTT TTG C-3') (23); for *T. vaginalis*: TVK3 (5′AT TGT CGA ACA TTG GTC TTA CCC TC3′) and TVK7 (5′ TCT GTG CCG TCT TCA AGT ATG C3′) (24); for herpes simplex virus type 2: KS30 (5'-TTC AAG GCC ACC ATG TAC TAC AAA GAC GT-3') and KS31(5'-GCC GTA AAA CGG GGA CAT GTA CAC AAA GT-3') (25). The amplified PCR product was analyzed by electrophoresis in 2% of agarose gels stained with ethidium bromide under ultra violet (UV) light (254-366 nm); and the gel image was recorded by taking a Polaroid™ photograph. Identification of the PCR product was based on the appearance of a DNA band of the expected length. Sizing of the DNA bands was achieved by running the PCR products next to DNA markers.

Diagnostic criteria of symptomatic VVC and vaginal candida colonization

The diagnosis of symptomatic VVC was based on a combination of clinical and laboratory criteria (evidence level III, recommendation grade B) (4, 20, 21).

Symptoms suggestive of symptomatic VVC included vulval pruritus/itching, vulval soreness, superficial dyspareunia and/or non-offensive vaginal discharge. Signs included vulval erythema, vulval edema, fissures, excoriation, or thick curdy vaginal discharge.

In addition to the self-reported above symptoms and observation of signs suggestive of VVC in physical examination, cases of symptomatic VVC were confirmed if one of the following criteria was fulfilled (evidence level III, recommendation grade B) (4, 20, 21): (i) A positive Gram-stain preparation with budding yeasts, pseudohyphae, and/or hyphal forms; (ii) positive culture with either moderate (10-99 colonies per plate) or heavy candida growth (>100 colonies per plate).

Participants without symptomatic VVC were defined as: (i) Patients whose genital specimens had a negative microscopy result for yeasts, pseudohyphae and/or hyphal forms of candida together with negative culture; (ii) patients whose genital specimens had a negative microscopy result for yeasts, pseudohyphae, and/or hyphal forms of candida together with light candida growth (<10 colonies per plate). The latter was considered to indicate vaginal candida colonization rather than infection.

Statistical analyses

Data analysis was performed using the statistical package for the social sciences (SPSS)® statistical software version 21.0 (SPSS Inc; Chicago, IL, USA). Data were expressed as proportions (percentages) for the categorical variables. Student's *t*-test was performed to assess differences between two means and ANOVA between groups. Either Chi-square test with and

without trend or Fischer's exact test was used to test the degree of association of categorical variables. Multiple logistic regression models were used to evaluate the prediction capacity of each independent variable in the occurrence of the expected condition. Unadjusted odds ratios (ORs) were initially calculated to screen for inclusion in multivariate models; variables that exhibited at least moderate association ($P < 0.20$) with the outcome were considered for inclusion in the final models. Multivariate ORs (95% CI) were computed after adjusting for confounding univariate factors. All tests were two-sided and a $P < 0.05$ was considered to be significant.

Results

Univariate associations of women with symptomatic VVC and those without symptomatic VVC with selected hypothesized factors are depicted in Table 1. A total of 90% (81/90) of patients diagnosed with symptomatic VVC complained of vulval itching, soreness and vaginal discharge when compared to 75.9% (82/108) of controls ($P < 0.01$). Pregnancy was significantly ($P = 0.01$) associated with symptomatic VVC. Although women with symptomatic VVC reported of using the contraceptive products (of any type) more than women without VVC, this difference did not reach statistical significance ($P = 0.06$). Nugent score <7 ($P < 0.01$), plasma HIV load >1000 copies/mL ($P < 0.001$), genital HIV load >1000 copies/mL ($P = 0.04$), CD4 count <200 cells/mm^3 ($P < 0.001$) and absence of anti-retroviral therapy ($P < 0.01$) among HIV positive women were significantly associated with symptomatic VVC.

Table 1: Univariate associations of symptomatic vulvo-vaginal candidiasis and vaginal Candida colonization with selected host characteristics among 198 women attending a primary healthcare clinic between June - December 2011

Characteristics	Symptomatic vulvo-vaginal candidiasis n (%)	Vaginal Candida colonization n (%)	*P* value
Age categories (years)			0.99
18 - 24	47 (52.2)	56 (51.9)	
25 - 34	30 (33.3)	37 (34.3)	
≥ 35	13 (14.4)	15 (13.9)	
Presenting complains			< 0.01
Group 1 : vulval itching - vulval soreness- vaginal discharge	81 (90)	82 (75.9)	
Group 2 : low abdominal pain - dysuria - dyspareunia	9 (10)	26 (24.1)	
Contraceptive use	77 (85.6)	82 (75.9)	0.06
Pregnancy	16 (17.8)	7 (6.5)	0.01
History of antibiotic use within the past 3 months	53 (58.9)	60 (55.6)	0.64
History of sexually transmitted infections (STIs) within the past 3 months			0.49
vaginal discharge syndrome	32 (35.6)	46 (42.6)	
genital ulcer syndrome	11 (12.2)	9 (8.3)	
no defined sexually transmitted infections	47 (52.2)	53 (49.1)	
Concurently isolated STI pathogens			0.33
Trichomonas vaginalis	10 (11.1)	21 (19.4)	
Chlamydia trachomatis	3 (3.3)	8 (7.4)	
Neisseria gonorrhoeae	5 (5.6)	6 (5.6)	
Mycoplasma genitalium	1 (1.1)	2 (1.9)	
Herpes simplex virus type 2	5 (5.6)	3 (2.8)	
Polymicrobial infection	12 (13.3)	18 (16.7)	
No STI pathogen identified	54 (60)	50 (46.3)	
Vaginal flora (Nugent scores)			< 0.01
0 to 3	19 (21.1)	9 (8.3)	
4 to 6	11 (12.2)	8 (7.4)	
7 to 10	60 (66.7)	91 (84.3)	
Plasma HIV viral load (VL) categories			< 0.001
HIV negative	38 (42.2)	63 (58.3)	
HIV positive with plasma VL less than 1000 copies /mL	22 (24.4)	34 (31.5)	
HIV positive with plasma VL more than 1000 copies /mL	30 (33.3)	11 (10.2)	
Genital HIV viral load (VL) categories			0.04
HIV negative	38 (42.2)	63 (58.3)	
HIV positive with genital VL less than 1000 copies /mL	44 (48.9)	41 (38)	
HIV positive with genital VL more than 1000 copies /mL	8 (8.9)	4 (3.7)	
CD4+ T cell stages			< 0.001
HIV negative	38 (42.2)	63 (58.3)	
HIV positive with CD4 count ≥ 350 cells/ mm3	5 (5.6)	19 (17.6)	
HIV positive with CD4 count: 200 - 349 cells/ mm3	17 (18.9)	15 (13.9)	
HIV positive with CD4 count less than 200 cells/ mm3	30 (33.3)	11 (10.2)	
Therapy groups			< 0.01
HIV negative	38 (42.2)	63 (58.3)	
HIV positive anti-retroviral therapy (ART)-naïve	26 (28.9)	11 (10.2)	
HIV positive receiving highly active anti-retroviral therapy	26 (28.9)	34 (31.5)	

When controlled for the confounding effects of contraceptive use and the presence of concurrent pathogens causing sexually transmitted infections, pregnancy was 5-fold ($P < 0.01$) as likely to be associated with symptomatic VVC in a logistic multivariate analysis [Table 2]. Symptomatic VVC remained inversely related to Nugent's scores as depicted in Table 2. The combination of

vulval itching, soreness and vaginal discharge remained independently associated with symptomatic VVC ($P < 0.01$) as compared to other presenting symptoms.

Table 2: Independent determinants of symptomatic vulvo-vaginal candidiasis among 198 women attending a primary healthcare clinic between June - December 2011

	B Coefficient	Standard Error	Wald Chi-square	OR (95% CI)	*P* value
Independent variables					
Vaginal flora (Nugent scores)					
0 to 3	1.377	0.497	7.688	4 (1.5 - 10.5)	< 0.01
4 to 6	1.287	0.574	5.029	3.6 (1.2 - 11.2)	0.03
7 to 10			Referent	1	
Genital HIV viral load (VL) categories					
HIV positive with genital VL more than 1000 copies /mL	1.318	0.672	3.843	3.7 (1.03 - 14)	0.04
HIV positive with genital VL less than 1000 copies /mL	0.696	0.34	4.183	2 (1.03 - 3.9)	0.04
HIV negative			Referent	1	
Pregnancy					
Yes	1.646	0.567	8.435	5.2 (1.7 - 15.7)	< 0.01
No			Referent	1	
Presenting complains					
Group 1 : vulval itching - vulval soreness- vaginal discharge	1.622	0.512	10.029	5.1 (1.9 - 13.8)	< 0.01
Group 2: others			Referent	1	
Constant	-2.436	0.54	20.391		< 0.001

Adjusted for contraceptive use and concurrently isolated pathogens causing sexually transmitted infections

When compared to HIV negative women, the risk of symptomatic VVC increased with HIV genital shedding among HIV-infected women. The risk of symptomatic VVC was 2-fold ($P = 0.04$) and 4-fold ($P = 0.04$) higher when genital HIV load was respectively below and above 1000 copies/mL [Table 2]. In addition, as compared with HIV negative women, HIV-infected women with plasma HIV load above 1000 copies/mL were 8 times likely to develop symptomatic VVC ($P < 0.001$, OR = 7.6 [3.2-18.2]). Furthermore, HIV-infected women with CD4 count below 200 cells/mm^3 had 8-fold higher risk for symptomatic VVC as compared with HIV negative women ($P < 0.001$, OR = 7.7 [3.2-18.4]). Finally, a logistic multivariate analysis

showed that HIV positive women but anti-retroviral therapy (ART)-naïve had 5-fold ($P < 0.001$, OR = 4.5 [1.9-10.7]) higher risk for symptomatic VVC as compared to HIV negative women.

Discussion

Six factors clearly increased risk for symptomatic VVC in this study. Four factors were associated with HIV infection– increased HIV shedding in the vagina, plasma HIV load above 1000 copies/mL, CD4 count below 200 cells/mm^3 and absence of HAART. Previous studies have reported on the association between low CD4 counts particularly <200 cells/mm^3 and symptomatic VVC (17, 18). Although symptomatic VVC is not classified among AIDS-defining conditions, the present study provides an additional body of evidence that symptomatic VVC is frequent among HIV-infected women with CD4 counts <200 cells/mm^3. Data on the association between HIV loads and symptomatic VVC are very scanty. In 2003, Ohmit *et al*. found that odds of symptomatic VVC increased by >2-fold for women whose plasma HIV load was >1000 copies/mL. The authors found an increase of 11-14% for every Log_{10} increase in plasma HIV viral load (26). In addition, Sobel *et al*. (2000) reported that higher HIV loads rather than lower CD4+ T-lymphocyte counts were associated with statistically significant increased odds for both persistent candidal vaginal colonization and symptomatic VVC (27). However, the study by Sobel *et al.* (2000) only found an association between plasma HIV viral load and the proportion of *Candida* infections that were non-*C. albicans*, not the absolute prevalence of symptomatic VVC (27). The present study determined plasma HIV load >1000 copies/mL and genital HIV shedding (below and above 1000 copies/mL) as independently associated with increased odds for symptomatic VVC when both plasma and genital HIV loads were not included at the same time

within the same multivariate statistical model. It is therefore possible that the relationship between genital HIV shedding and VVC may be indirect because plasma viral load predicts genital shedding and therefore the relationship between VVC and genital tract shedding may be because plasma viral load is associated with both.

Plausible biological reasons why HIV viral loads can correlate with symptomatic VVC have not been clearly established.

From the findings of this study, it can be hypothesized that during advanced HIV infection (as measured by systemic CD4+ T cell levels) with subsequently observed higher HIV viral loads in plasma and in the vagina, HIV particles might change the vaginal environment by down regulating the activation of mucosal CD4+ T cells and the recruitment of other immune cells into vaginal tissues, hence promoting virulence of *Candida species* by switching from its non-pathogenic form into a filamentous form that causes symptomatic VVC. It can be further speculated that because ART-naïve HIV-infected women had 5-fold higher risk of developing symptomatic VVC as compared to HIV negative women, controlling the replication of HIV by using HAART could possibly restore local mucosal immune functions in the vagina, suggesting that high level of HIV load could suppress genital mucosal immune mechanisms independently of systemic cell-mediated immunity, leading to symptomatic VVC.

The present study found a significant negative association between the presence of BV and symptomatic VVC. The present study is in support of a previous study from KwaZulu-Natal in South Africa that reported BV as a predominant cause of vaginitis among the clinic's attendees. In 2002, Moodley *et al*., during a study in northern KwaZuluNatal found the prevalence of BV to

be 70% among their study population (8). In addition, this study found that symptomatic VVC was significantly associated with Nugent's score below 7. Symptomatic VVC has been reported as associated with normal vaginal pH (pH < 4.5) while BV is established when pH of the vaginal fluid becomes > 4.5. In addition, these findings are also in keeping with results from Moodley *et al.* (2002) who found out that yeast colonization and symptomatic VVC were inversely related to Nugent's scores (8).

Neutral pH has been long recognized as an inducer of hyphal morphogenesis, and cells in alkalinizing media shift to the hyphal form; thus, *C. albicans* effectively autoinduces morphogenesis under these conditions (6). The rise in pH in women with VVC is associated with the release of ammonia, a highly basic compound (6) whilst the rise of pH in cases of BV has been shown to be significantly associated with the absence of peroxide hydrogen (H2O2)-producing lactobacilli during a longitudinal study (28). Although in vitro, Candida cells raise the pH from 4 to >7 in order to create autoinduction of the yeast-hyphal transition (6), it can be hypothesized that in women with VVC this yeast-hyphal switch starts as early as when the pH is slightly above 4.2 but below 4.5 explaining at least partially why most of women with VVC have a pH <4.5 as compared to women with BV who have a pH >4.5 (7) .

Another factor identified in this study as associated with symptomatic VVC was pregnancy. This finding was consistent with what has been published by others (1, 29). Studies have shown that high concentrations of reproductive hormones can result in the increase of the glycogen content in the vaginal tissue, providing a carbon source for Candida cells (30, 31). In addition, oestrogen may enhance adherence of yeast to vaginal epithelial cells; and that a cytosol receptor or binding

system for female reproductive hormones has been documented in *C albicans*, resulting in enhanced mycelial formation (32).

Other traditionally reported risk factors such as contraceptive use and use of antimicrobial agents were not shown by this study to be independently associated with symptomatic VVC. Reports from the literature have shown conflicting data regarding these two risk factors for VVC (13, 29). In addition, we had a small sample size and relied on the history of antibiotic use to collect information that might not be totally accurate.

Conclusion

The study showed that HIV-related immune compromised women and particularly those who are ART-naïve are likely to develop symptomatic VVC. Limitation to this study is mainly its cross-sectional design and a small population size. Another limitation includes the lack of information regarding the types of contraceptive products used by the study participants. A future study after addressing these limitations will be necessary in order to ascertain our conclusions.

Acknowledgments

We would like to thank the nursing staff at Umlazi D clinic in KwaZulu-Natal and laboratory staff at the STD research section of the Department of Infection Prevention and Control for assisting in this project. The study was funded by Hasso Plattner Foundation (Grant to TA) and NIH grant NIH K01--TW007793 (Grant to WC).

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Determinants of Symptomatic Vulvo-Vaginal Candidiasis among Human Immuno-deficiency Virus Type 1 infected Women in Rural KwaZulu-Natal, South Africa

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Infectious Diseases in Obstetrics and Gynecology 2014; vol. 2014, Article ID 387070

Abstract

Introduction: The study sought to determine the association between HIV-induced immunosuppression, virologic correlates and symptomatic vulvo-vaginal candidiasis (VVC).

Methods: This is a retrospective cohort study, where HIV infected and uninfected women were studied with VVC being the primary outcome of interest. Ninety-seven HIV-infected and 101 HIV-uninfected primary health clinic's attendees were enrolled in KwaZulu-Natal, South Africa between June 2011 and December 2011. Confirmed symptomatic VVC was determined by Gram stain and microbiological culture of vaginal swabs. HIV RNA load in plasma and genital fluid was determined by RT-PCR with a detection limit of 20 copies/ mL. CD4 counts were obtained from medical records during clinic visits.

Results: Fifty-two of 97 (53.6%) HIV-infected women and 38/101 (37.6%) HIV-uninfected women were diagnosed with symptomatic VVC (p = 0.032). The relative risk value for symptomatic VVC amongst HIV-infected patients was 1.53 (95% CI: 1.04 – 2 P = 0.024).The risk of developing symptomatic VVC increased with immune compromise, especially at CD4+ T cell count below 200 cells/mm^3 (P < 0.0001) and plasma HIV RNA load above 10 000 copies/mL (P < 0.0001). Symptomatic VVC was associated with increased genital shedding of HIV (P = 0.002); and there was a linear correlation between plasma HIV viral load and genital HIV shedding (r = 0.540; R^2 = 0.292; P < 0.0001). Women on HAART had 4-fold reduced risk (P = 0.029) of developing symptomatic VVC as compared to ART-naïve women.

Conclusion: CD4 counts below 200 cells/ mm^3 and plasma HIV loads ≥10 000 copies/ mL were independently associated with symptomatic VVC.

Key words: Candida, vulvo-vaginitis, HIV-1, immune suppression, HAART.

Introduction

Symptomatic vulvo-vaginal candidiasis (VVC) is caused by a number of species belonging to the genus *Candida* which are commensal fungi of the gastrointestinal tract and vagina. *Candida albicans* has been reported as the cause of symptomatic VVC in 85-95% of cases whilst *C. glabrata* represents the most common cause of non-albicans candida vaginitis (1).

Whilst 75% of healthy women develop symptomatic VVC at least once during their reproductive age, it is estimated that 5-10% of women develop recurrent vulvo-vaginal candidiasis (RVVC) (1, 2). The latter is defined as the occurrence of four or more episodes of symptomatic VVC per year (3).

In HIV infected patients, whilst oro-oesophageal candidiasis is known to appear at any time during the course of progression of HIV infection, symptomatic VVC develops significantly later (4). It has been shown that oro-oesophageal candidiasis occurs as a result of the loss of mucosal and systemic cell-mediated immunity while animal studies (4-7), in vitro experiments and clinical trials indicate that systemic cell-mediated immunity does not play a protective role against candida vaginitis (7, 8).

Cross-sectional studies of women attending antenatal clinics and family planning clinics in South Africa over the past 20 years showed a weighted average prevalence of 26% for symptomatic VVC (9-13). Among these studies, many have been conducted in KwaZulu-Natal where there is a high prevalence of HIV in South Africa. Data from the national population-based survey conducted in 2008 estimated HIV prevalence in young people aged 15–24 years to be 15.3%

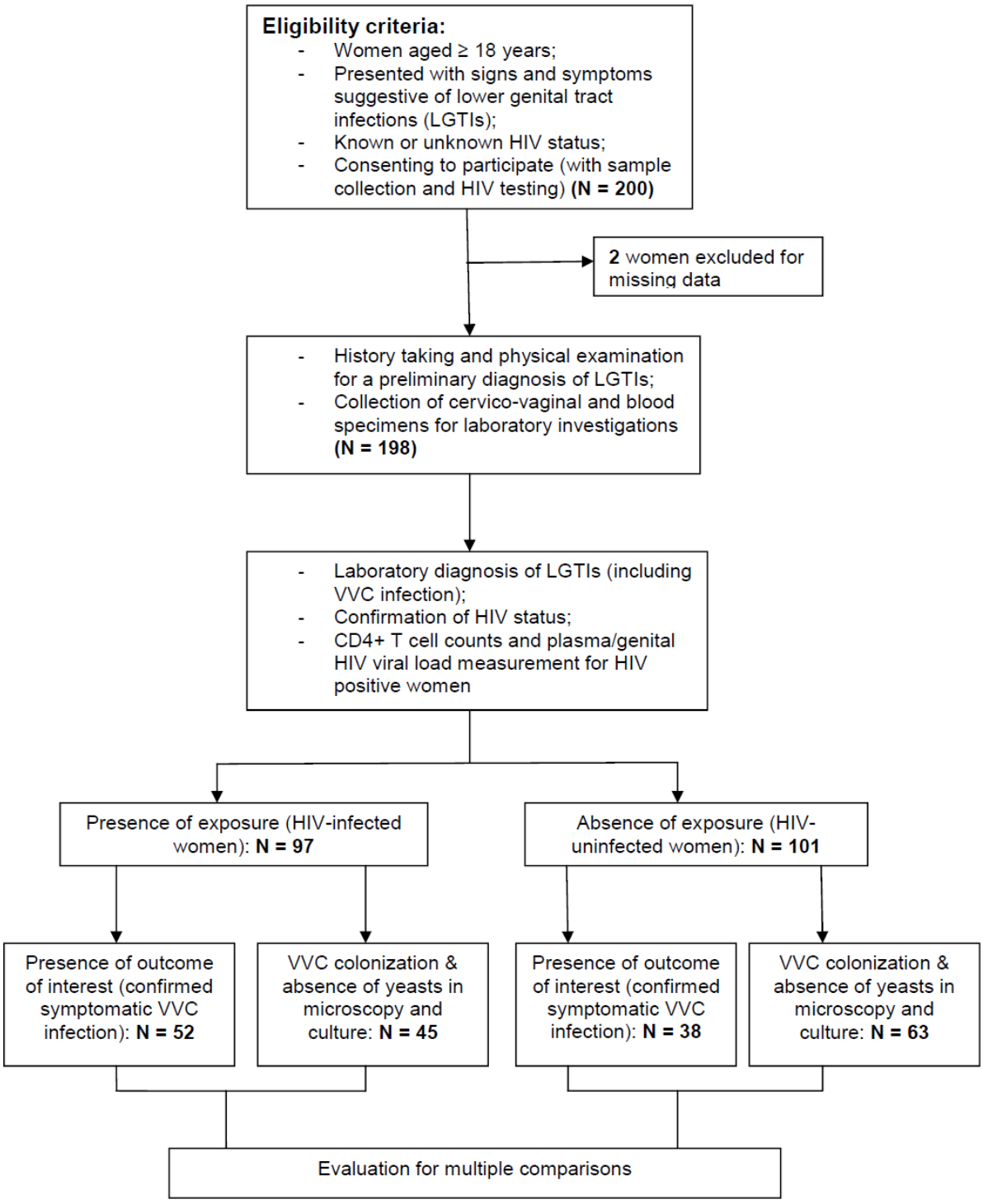
(95% CI 11.8–19.7) in the province of KwaZulu-Natal compared to the national estimate of 8.7% (95% CI 7.2–10.4) (13). Ramjee, Karim and Sturm (1998) reported the prevalence of 36.1% and 45.7% for symptomatic VVC respectively among HIV negative and HIV positive sex workers in KwaZulu-Natal (9). In 2011, during a nested control analysis from a prospective trial of 1 404 women (716 HIV infected compared with 688 HIV non-infected) over a 2-year period in KwaZulu-Natal, Sebitloane, Moodley and Esterhuizen reported on symptomatic VVC as being significantly more prevalent among HIV infected than uninfected women [39.2% versus 30%; RR = 1.31 (1.08 - 1.59); *P* = 0.006] (10).

The aim of this study was to determine predictors of symptomatic VVC in HIV infected women.

Materials and Methods

Patients and sampling strategy:

This is a retrospective cohort study design. Consecutive consenting female patients attending Umlazi D clinic, a primary healthcare facility in KwaZulu-Natal were recruited between June 2011 and December 2011. Presentation with lower genital tract signs and symptoms was one of the eligibility criteria as depicted in Flowchart 1.



Flowchart 1: Selection criteria and characterization of the study population by HIV groups

A standardized questionnaire was used to collect patient demographic information, history of STIs and treatment thereof over the past 3 months, condom utilization behaviour and prior knowledge of HIV sero-status. HIV positive women were further questioned about receiving HAART, and this was confirmed by a review of the medical records.

CD4 counts were obtained from patients' medical records and were not older than 3 months. Blood and genital secretions for measurement of HIV viral loads were collected from patients at recruitment.

Patients were invited to participate in the study if they had signs and symptoms of vaginal discharge syndrome. All patients were treated syndromically as per standard of care STI treatment in South Africa. The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Ref. BE 224/11). Consent forms were signed by all participants and confidentiality was maintained throughout the study.

Specimen collection and processing:

One cervical and two vaginal swabs (Probetec® swabs, Becton Dickinson, Sparks, Maryland, USA) were used. The two vaginal swabs collected material from the anterior and posterior fornix respectively. A smear was made on a glass slide using the latter, and the former as well as the cervical swab was placed in a sterile dry container. A vaginal tampon (8 Ks, Tampax Regular® Compak) was thereafter inserted into the vagina, and removed after 3 minutes. This was placed in a sterile container with 10ml of phosphate buffered saline (PBS; Oxoid Limited Basingstoke, Hampshire, UK) (pH = 6.9). Blood samples were collected into Ethylenediaminetetraacetic acid (EDTA) and non EDTA containing tubes.

All specimens were kept in a cooler box with ice awaiting transportation (within 4 hours) to the Infection Prevention and Control laboratory, Nelson R Mandela School of Medicine, UKZN.

The anterior fornix vaginal swab was used to inoculate Sabouraud Dextrose agar with chloramphenicol (BBL^{TM} Becton Dickinson) and incubated at 29^{o}C for 48 h. The relative vaginal fungal burden was estimated as previously described (14-16).

The smear was stained using Gram's Method and evaluated by two independent microscopists for bacterial vaginosis (BV) using Nugent's score (17). Discrepancies were evaluated by an independent medical microbiologist.

A vaginal swab that collected cells from the posterior fornix was used to prepare a smear onto a glass slide for Gram staining. Neutrophil counts were determined by counting a total number of PMN cells in randomly selected five different microscopic fields under oil immersion (X1000 magnification) as previously described with the following scores – score 1: 1-10 PMN / 5 fields; score 2: 11-20 PMN / 5 fields; score 3: >20 PMN / 5 fields.

The cervical swab was used for the detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* using a strand displacement amplification technology (Becton Dickinson Probetec Assays, Sparks, Maryland, USA) method as previously described (18). DNA of *Mycoplama genitalium, Trichomonas vaginalis* and herpes simplex virus type 2 was obtained using QIAamp DNA mini kits (Qiagen Ltd, Chatsworth, CA) (18). An in house PCR method for amplification was used as previously described (19-21). The amplified PCR product was analyzed by gel electrophoresis.

Vaginal fluid was expressed from vaginal tampon using an autoclaved wooden tongue depressor and filtered through a 0.22 μm Costar Spin-X cellulose acetate filter membranes (Sigma) and used for quantifying HIV viral load in genital secretions.

Diagnostic criteria of symptomatic vulvo-vaginal candidiasis and VVC colonization:

The diagnosis of symptomatic VVC was based on a combination of clinical and laboratory criteria (evidence level III, recommendation grade B) as described by the European (IUSTI/WHO) guideline on the management of vaginal discharge, 2011 (22).

Symptoms suggestive of symptomatic VVC included vulval pruritus/itching, vulval soreness, superficial dyspareunia, and/ or malodorous vaginal discharge. Signs included vulval erythema, vulval oedema, fissures, excoriation, or thick curdy vaginal discharge.

In addition to the self-reported above symptoms and observation of signs suggestive of VVC in physical examination, cases of symptomatic VVC were confirmed if one of the following criteria was fulfilled: (i) a positive Gram-stain preparation with budding yeasts, pseudohyphae, and/or hyphal forms; (ii) positive culture with either moderate (10–99 colonies per plate) or heavy candida growth (>100 colonies per plate).

Participants without symptomatic VVC were defined as: (i) patients whose genital specimens had a negative microscopy result for yeasts, pseudohyphae, and/or hyphal forms of candida together with negative culture; (ii) patients whose genital specimens had a negative microscopy result for yeasts, pseudohyphae, and/or hyphal forms of candida together with light candida

growth (< 10 colonies per plate). The latter was considered to indicate vaginal *candida* colonization rather than infection.

HIV testing and definitions of HIV-induced immunosuppression:

Initial HIV test was performed in the clinic by a trained research nurse on blood using the HIV rapid test Determine HIV-1/2/O (Abbott Laboratories, Abbott Park, IL) following voluntary counseling and testing. The diagnosis of HIV negative with an appointment for a further rapid testing 3 months later was given to the patient following a negative initial test. Positive samples were transported in the Infection Prevention and Control research laboratory at the Nelson R Mandela School of Medicine, UKZN and were subsequently retested by a medical technologist using a second HIV rapid test SmartCheck test (World Diagnostics Inc, USA). A diagnosis of HIV positive was reported to the participants only if both rapid tests were positive. Samples that showed discordant results were further evaluated with a third rapid test Uni-Gold™ Recombigen® HIV (Trinity Biotech PLC, USA), and only two positive test results were interpreted as a positive diagnosis for HIV.

As part of the routine management of the patients in the clinic, all HIV-infected patients benefited directly from CD4+ T cell count measurements, and CD4+ T cell counts used in this study were obtained from patients' medical records and were not older than 3 months. However, at the time patients were evaluated for this research, HIV-1 RNA was measured from the plasma and cell-free fraction of vaginal secretions using Nuclisens Easyq HIV-1 assay v2.0 (BioMerieux, Lyon, France) with a lowest detection limit of 20 copies/ mL.

Absolute values of CD4+ T cell counts (cells/mm^3) were used to determine the degree or severity of immunocompromise following the World Health Organization (WHO) immunological staging criteria: CD4 levels < 200/mm^3 (severe immunosuppression), CD4 levels 200 – 349/mm^3 (advanced immunosuppression), and CD4 levels 350 – 499/mm^3 (mild immunosuppression) (WHO, 2005). In addition, virological correlates (plasma HIV viral loads) were also measured since they are potentially useful markers of disease progression. For the purpose of this study, plasma viremia was classified as: < 20 copies/mL (below detectable level of the used test), 20 – 9999 copies/mL, and ≥ 10 000 copies/mL. We also log transformed HIV loads for improved symmetry and included them in multivariate logistic regression models as continuous values.

***Statistical analyses*:**

Data analysis was performed using SPSS® statistical software version 21.0 (SPSS Inc; Chicago, IL). Data were expressed as means ± standard deviation (SD) for the continuous variables and proportions (percentages) for the categorical variables. Student's *t-test* was performed to assess differences between two means and ANOVA between groups. When data were not normally distributed, the Mann-Whitney U test was used. Either Chi-square test with and without trend or Fischer's exact test was used to test the degree of association of categorical variables.

Multiple logistic regression models were used to evaluate the prediction capacity of each independent variable in the occurrence of the expected condition. Unadjusted odds ratios (ORs) were initially calculated to screen for inclusion in multivariate models; variables that exhibited at least moderate association ($P < 0.20$) with the outcome were considered for inclusion in the final models. Multivariate ORs (95% CI) were computed after adjusting for confounding univariate factors. All tests were two-sided and a P value of <0.05 was considered significant.

Results

Of the 200 patients enrolled with vaginal discharge syndrome (VDS), 2 were excluded for missing results. Of the 198 participants, 97 were HIV-infected and 101 HIV-uninfected. This was a simple coincidence that the first 200 women who met the eligibility criteria comprised of approximately equal numbers of HIV positive and HIV negative.

From the 97 HIV-infected women, 52 cases of symptomatic VVC were diagnosed (53.6%) while 38 cases of symptomatic VVC were diagnosed among the 101 HIV-uninfected women (37.6%) (P = 0.032). The relative risk value for symptomatic VVC amongst HIV-infected patients was 1.53 (95% CI: 1.04 - *2* P = 0.024). Table 1 depicts the baseline characteristics of the study population.

Table 1: Baseline characteristics of the study population (n = 198) who contributed symptomatic vulvo-vaginal candidiasis (VVC) and vaginal *Candida species* colonization data by human immunodeficiency virus (HIV) serostatus

Characteristics	HIV-infected n = 97	HIV-uninfected n = 101	*P* value
Age in years, median (range)			< 0.0001
21 (18 - 24)	34 (33)	69 (67)	
30 (25 - 34)	48 (71.6)	19 (28.4)	
40.2 (35 -46)	15 (53.6)	13 (46.4)	
Major presenting symptoms			0.008
Genital itching and soreness	87 (53.4)	76 (46.6)	
vaginal discharge and low abdominal pain	10 (28.6)	25 (71.4)	
History of antibiotic use within the past 3 months	64 (56.6)	49 (43.4)	0.013
Condom use	25 (64.1)	14 (35.9)	0.035
Pregnancy	4 (17.4)	19 (82.6)	< 0.001
History of sexually transmitted infections (STIs) within the past 3 months			0.093
vaginal discharge syndrome	42 (53.8)	36 (46.2)	
genital ulcer syndrome	13 (65)	7 (35)	
no defined sexually transmitted infection	42 (42)	58 (58)	
Currently isolated pathogens for STIs			0.085
Trichomonas vaginalis	15 (48.4)	16 (51.6)	0.683
Chlamydia trachomatis	7 (63.6)	4 (36.4)	0.228
Neisseria gonorrhoeae	6 (54.5)	5 (45.5)	0.516
Mycoplasma genitalium	2 (66.7)	1 (33.3)	0.456
Herpes simplex virus type 2	8 (100)	0 (0)	0.999
STI caused by more than 1 aetiology	13 (43.3)	17 (56.7)	0.93
No identified STI pathogen	46 (44.2)	58 (55.8)	1
Vaginal flora (Nugent's score)			0.819
0 to 3	14 (50)	14 (50)	
4 to 6	8 (42.1)	11 (57.9)	
7 to 10	75 (49.7)	76 (50.3)	
Absolute count of neutrophil cells in genital fluid			0.77
score 1	42 (43.3)	41 (40)	
score 2	23 (23.7)	21 (20.8)	
score 3	32 (32.9)	39 (38.6)	
Symptomatic vulvo-vaginal candidiasis			0.032
yes	52 (53.6)	38 (37.6)	
no (colonization or absence)	45 (41.7)	63 (58.3)	
CD4+ T cell count (cells/mm3)			
< 200	41 (42.3)	N/A	N/A
200 to 349	32 (33)	N/A	N/A
≥ 350	24 (24.7)	N/A	N/A
Plasma HIV viral load (copies/mL)			
≥ 10 000	22 (22.7)	N/A	N/A
20 to 9999	51 (52.6)	N/A	N/A
< 20	24 (24.7)	N/A	N/A
Genital HIV viral load (copies/mL)			
≥ 10 000	12 (12.4)	N/A	N/A
20 to 9999	21 (21.7)	N/A	N/A
< 20	64 (65.9)	N/A	N/A
Users of Highly Active Antiretroviral Therapy			
Yes	60 (61.9)	N/A	N/A
No	37 (38.1)	N/A	N/A

N/A = Not applicable

In HIV-infected women, univariate analysis (Table 2) showed that women's vaginal flora with Nugent's score < 7 ($P = 0.01$), plasma HIV viral loads ≥ 10 000 copies/mL ($P < 0.0001$), genital HIV viral loads ≥ 10 000 copies/mL ($P = 0.002$), CD4 levels < 200 cells/mm^3 ($P < 0.0001$), and absence of HAART ($P < 0.001$) were significantly associated with symptomatic VVC. There

was a linear correlation between plasma HIV viral load and genital HIV shedding (r = 0.540; R^2 = 0.110; *P* < 0.0001) (Figure 1).

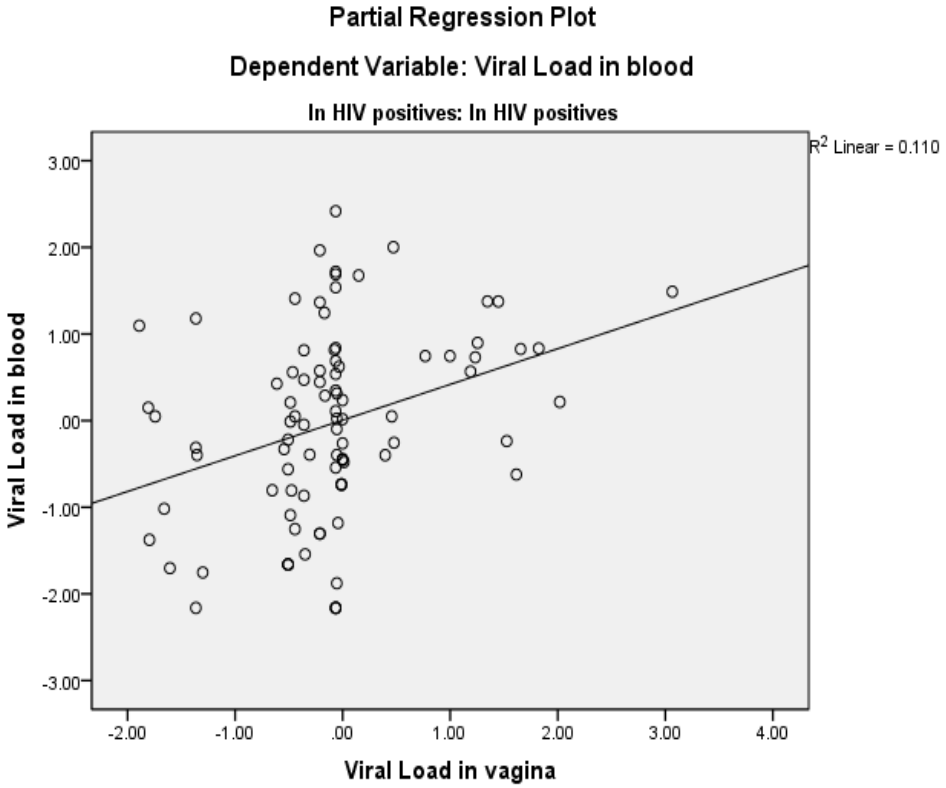


Figure 1 Correlation between Plasma and Genital HIV levels (Log10 transformed values of HIV loads in copies/mL)

Findings (Figures 2 and 3) have shown that HIV viral loads increased across progressive stages of HIV-induced immunocompromise: CD4 count < 200 cells/mm^3, between 200 – 349 cells/mm^3 and ≥350 cells/mm^3 significantly correlated with the mean plasma HIV load of 4.43 (4.1 – 4.9)Log_{10}, 2.24 (2 – 2.5)Log_{10} and 1.3Log_{10} respectively (*P* < 0.0001); and also with the mean genital HIV load of 2.51 (2.1 – 2.9)Log_{10}, 1.63 (1.4 – 1.9)Log_{10} and 1.66 (1.3 – 2)Log_{10} respectively (*P* < 0.001).

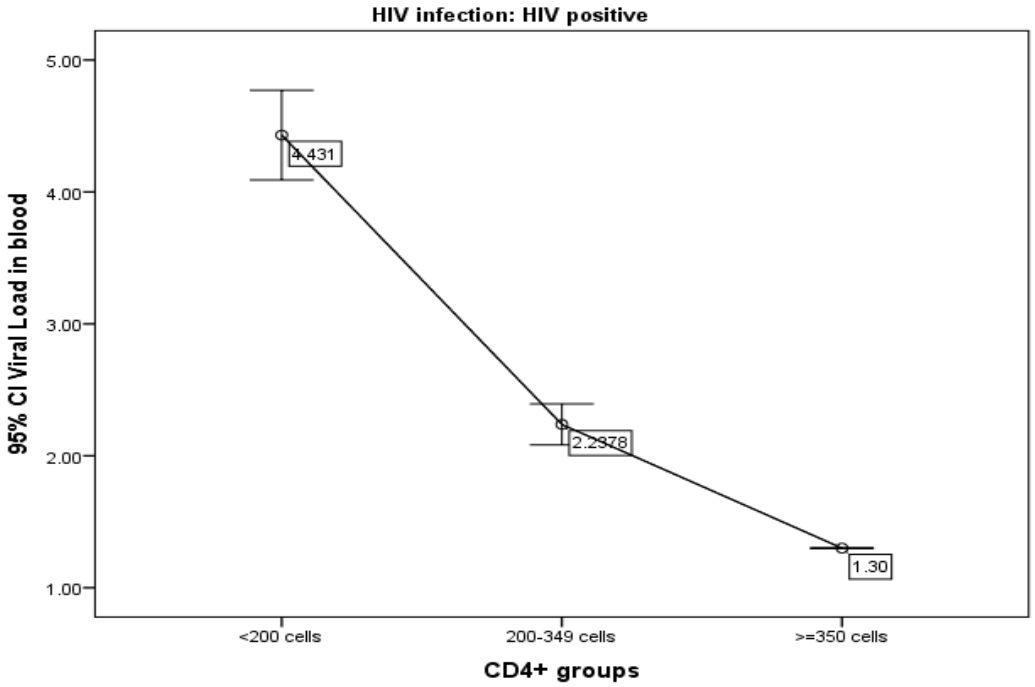


Figure 2 Negative association between CD4+ T cell stages and plasma Log_{10} HIV viral load values ($P < 0.0001$)

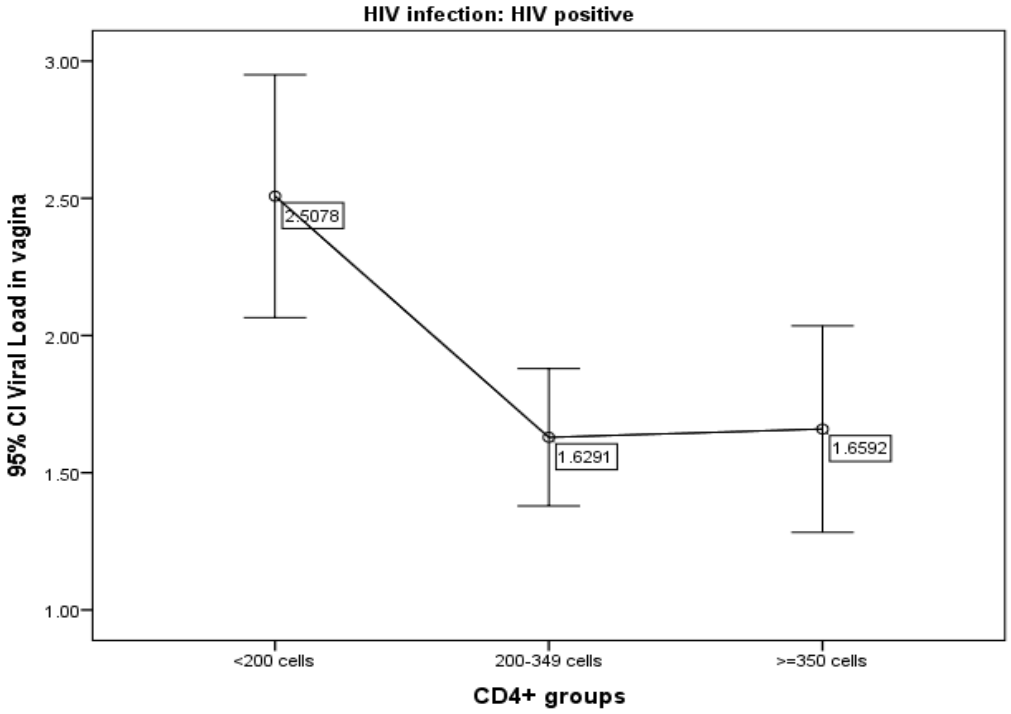


Figure 3 Negative association between CD4+ T cell stages and genital Log_{10} HIV viral load values ($P < 0.001$)

Plasma HIV viral load and genital HIV viral load significantly varied by HAART groups. Higher mean of Log_{10} HIV viral load values was shown in the plasma [3.41 (2.8 – 4) Log_{10}] of HIV-infected women anti-retroviral therapy (ART)-naïve as compared to patients on HAART [2.68 (2.34 – 3) Log_{10}] ($P = 0.014$). Similarly, higher mean of Log_{10} HIV viral load values was also observed in the genital tracts [2.61 (2.1 – 3.10) Log_{10}] of HIV-infected women anti-retroviral therapy (ART)-naïve as compared to patients receiving HAART [1.67 (1.4 – 1.9) Log_{10}] ($P < 0.0001$). [All genital and plasma Log10 transformed values of HIV loads were measured as mean± standard error of the mean (SEM) in copies/mL].

Bacterial vaginosis was frequent among HIV infected women in the absence of symptomatic VVC ($P = 0.01$) (Table 2). There was also a significant association between bacterial vaginosis and higher levels of HIV RNA in the plasma. Nugent scores between 0 – 3; 4 – 6; and 7 – 10 were associated with mean plasma HIV loads of $1.9Log_{10}$, $2.45Log_{10}$; and $3.20Log_{10}$ respectively (ANOVA, $P = 0.006$). However, higher levels of genital HIV RNA were not significantly associated with Nugent score categories (ANOVA, $P = 0.062$).

Table 2: Univariate associations between variables of interest and symptomatic vulvo-vaginal candidiasis among HIV-infected women (n = 97)

Variables of Interest	symptomatic VVC (n = 52) n (%)	VVC colonization (n = 45) n (%)	*P*-value
Age in years, median (range)			0.484
21 (18 - 24)	21 (40.4)	13 (28.9)	
30 (25 - 34)	24 (46.2)	24 (53.3)	
40.2 (35 - 46)	7 (13.5)	8 (17.8)	
Major Presenting complains			0.362
Vaginal discharge	4 (7.7)	6 (13.3)	
Genital itching and soreness	48 (92.3)	39 (86.7)	
History of antibiotic use within the past 3 months	36 (69.2)	28 (62.2)	0.467
No history of condom use	42 (80.8)	30 (66.7)	0.113
History of Sexually Transmitted Infections within the past 3 months			0.312
Vaginal Discharge Syndrome	18 (34.6)	24 (53.3)	
Genital Ulcer Syndrome	1 (1.9)	1 (2.2)	
Mixed Sexually Transmitted Infections	7 (13.5)	4 (4.1)	
No Defined Sexually Transmitted Infections	26 (50)	16 (36)	
Currently Isolated Pathogens for STIs			0.153
Trichomonas vaginalis	5 (9.6)	10 (22.2)	0.07
Chlamydia trachomatis	1 (1.9)	6 (13.3)	0.046
Neisseria gonorrhoeae	3 (5.8)	3 (6.7)	0.612
Mycoplasma genitalium	1 (1.9)	1 (2.2)	0.76
Herpes Simplex Virus Type-2	5 (9.6)	3 (6.7)	0.93
STI caused by more than 1 aetiology	9 (17.3)	4 (8.9)	0.583
No STI pathogen identified	28 (53.8)	18 (40)	1
Vaginal Flora (Nugent's Scores)			0.01
less than 7 (BV negative)	17 (32.7)	5 (11.1)	
≥ 7 (BV positive)	35 (67.3)	40 (88.9)	
Pregnancy	4 (7.7)	0 (0)	0.057
Plasma HIV viral load (VL) categories			< 0.0001
≥ 10000 copies	19 (86.4)	3 (13.6)	
20 - 9999 copies	28 (54.9)	23 (45.1)	
< 20 copies	5 (20.8)	19 (79.2)	
Genital HIV viral load (VL) categories			0.002
≥ 10000 copies	12 (100)	0 (0)	
20 - 9999 copies	12 (57.1)	9 (42.9)	
< 20 copies	28 (44.4)	35 (55.6)	
CD4+ T cell counts			<0.0001
< 200 cells	30 (57.7)	11 (24.4)	
200 - 349 cells	17 (32.7)	15 (33.3)	
≥ 350 cells	5 (9.6)	19 (42.2)	
Therapy Groups			<0.001
Patients on anti-retroviral therapy	26 (50)	34 (75.6)	
Patients not receiving anti-retroviral therapy	26 (50)	11 (24.4)	

Findings showed a negative but not significant (Spearman's Rho = -0.101; *P* = 0.323) correlation between Log_{10} levels of HIV RNA expression in plasma and absolute counts of neutrophils in the genital fluid of the study participants. However, there was a positive and significant correlation (Spearman's Rho = 0.234; R^2 = 0.028; Y = 1.83 + 5.54 - 3*X; *P* = 0.021) between Log_{10} levels of HIV RNA expression in the vagina and absolute counts of neutrophils in the genital fluid of the study participants. There was also a negative but significant association between CD4 count stages and absolute count of neutrophils in the genital fluid: HIV-infected women with CD4 count < 200 cells/mm^3 (n=41) had an average 59.6 ± 6.6 PMN cells/5 High Microscopic Fields (HMF) as compared to HIV-infected women with CD4 count > 200 cells/mm^3 (n = 56) who had an average 18.1 ± 13.8 PMN cells/5HMF (*P* < 0.0001).

Independent determinants of symptomatic VVC in HIV infected women are shown in Table 3 using multiple logistic regressions. The model showed that the risk of developing symptomatic VVC increased with the severity of HIV-induced immunosuppression. Adjusted *odds ratio* values were 9 (*P* = 0.015) and 60 (*P* < 0.0001) respectively during advanced (CD4+ T cells = 200 – 349/mm^3) and severe (CD4+ T cells < 200/mm^3) stages of HIV-induced immunosuppression. In addition, women not receiving HAART had 4-fold higher risk (*P* = 0.029) for symptomatic VVC as compared to women on HAART. Findings also showed that the risk of developing symptomatic VVC increased with the presence of higher levels of HIV RNA in the blood [approximately 100-fold higher for HIV positive women with plasma HIV RNA load ≥ 10 000 copies/mL (*P* < 0.0001)].

There was a significant negative association between the presence of bacterial vaginosis (Nugent score 7 – 10) and symptomatic VVC as depicted in Table 3.

Table 3: Independent determinants of symptomatic vulvo-vaginal candidiasis among HIV-infected women

Independent Variables	B Coefficient	Standard Error	Wald Chi-square	OR (95% CI)	*P*-value
CD4+ T cell Groups					
< 200 cells	4.1	0.971	17.847	60.3 (9 - 134.8)	< 0.0001
200 - 349 cells	2.186	0.901	5.886	8.9 (1.5 - 17.36)	0.015
≥ 350 cells			Referent	1	
Vaginal Flora (Nugent's Scores)					
less than 7 (BV negative)	3.143	0.954	10.844	23.2 (3.6 - 50.13)	< 0.001
≥ 7 (BV positive)			Referent	1	
Therapy Groups					
Patients not receiving anti-retroviral therapy	1.251	0.573	4.768	3.5 (1.1 - 10.7)	0.029
Patients on anti-retroviral therapy			Referent	1	
Constant	-3.13	0.904	11.981		< 0.001

Adjusted for previous history of sexually transmitted infections (STIs), presence of current STIs, history of condom use, pregnancy and presenting symptoms

In HIV negative women however, after adjusting for univariate factors that exhibited at least moderate association ($P < 0.20$) with symptomatic VVC (Table 4), only pregnancy was independently associated with symptomatic VVC (OR = 4.1 95% CI 1.3 – 10.5, $P = 0.011$).

Table 4: Univariate associations between variables of interest and symptomatic vulvo-vaginal candidiasis (VVC) among HIV-uninfected women (n = 101)

Variables of Interest	symptomatic VVC (n = 38) n (%)	VVC Colonization (n = 63) n (%)	*P*-value
Age in years, median (range)			0.705
21 (18 - 24)	26 (68.4)	43 (68.3)	
30 (25 - 34)	6 (15.8)	13 (20.6)	
40.2 (35 - 46)	6 (15.8)	7 (11.1)	
History of antibiotic use within the past 3 months	17 (44.7)	32 (50.8)	0.555
No history of condom use	35 (92.1)	52 (82.5)	0.178
History of Sexually Transmitted Infections within the past 3 months			0.504
Vaginal Discharge Syndrome	14 (36.8)	22 (34.9)	
Genital Ulcer Syndrome	0 (0)	2 (3.2)	
Mixed Sexually Transmitted Infections	3 (7.9)	2 (3.2)	
No Defined Sexually Transmitted Infections	21 (55.3)	37 (58.7)	
Currently Isolated Pathogens for STIs			0.377
Trichomonas vaginalis	5 (13.2)	11 (17.5)	0.333
Chlamydia trachomatis	2 (5.3)	2 (3.2)	0.841
Neisseria gonorrhoeae	2 (5.3)	3 (4.8)	0.835
Mycoplasma genitalium	0 (0)	1 (1.6)	1
Herpes Simplex Virus Type-2	0 (0)	0 (0)	0.053
STI caused by more than 1 aetiology	3 (7.9)	14 (22.2)	1
No STI pathogen identified	26 (68.4)	14 (22.2)	1
Vaginal Flora (Nugent's Scores)			0.08
less than 7 (BV negative)	13 (34.2)	12 (19)	
≥ 7 (BV positive)	25 (65.8)	51 (81)	
Pregnancy	12 (31.6)	7 (11.1)	0.011

BV = bacterial vaginosis

Discussion

Findings from this study confirmed previously published reports on symptomatic VVC as being significantly more prevalent among HIV infected than HIV uninfected women (10, 23). Whilst some studies have shown the association between low CD4+ T-lymphocyte counts and symptomatic VVC (4, 24, 25), results from this present study provide an additional body of

evidence that low CD4+ T-lymphocyte counts, particularly CD4+ T cells < 200/mm^3, are independently associated with symptomatic VVC.

In 2006, Beltrame *et al.* reported that candidal vaginal colonization, a precursor of vaginitis, develops when CD4+ T-lymphocyte counts fall to ≤ 100 cells /μL in the course of HIV infection (4). Prior to this report, Duer *et al.* (1997) published on the rates of candidal vaginal colonization and symptomatic VVC as being similar among nonimmunocompromised HIV-positive women and HIV-negative women (24). According to these authors, elevated rates of yeast colonization and vaginitis were not observed among their cohort of HIV-infected women before immune compromise. However, rates of vaginal colonization and symptomatic VVC were seen to increase with immune compromise, especially at CD4 counts below 200 cells/ mm^3 (24). In 2000, Shifrin *et al.* reported that HIV-positive women with a CD4 count less than 200 cells/mm3 had an increase of 8.2 times in the incidence of symptomatic vulvovaginal candidiasis. They suggested that women with low CD4 counts should be closely monitored for the development of symptomatic VVC prior to the development of symptoms independent of candida colonization status (26).

Others reported that the prevalence of symptomatic VVC, although higher among HIV-infected women, did not significantly differ by HIV serostatus of the participants at baseline (27). However, the authors found that during follow up visits, the rate of acquisition of symptomatic VVC was significantly higher among HIV-infected women as compared to HIV-uninfected women. Furthermore, whilst Ohmit *et al.* (2003) identified the level of HIV-associated immunodefiency (measured by CD4+ T cell counts) as not significantly associated with

increased odds for symptomatic VVC, the authors found however that odds of symptomatic VVC increased by > 2-fold for women whose plasma HIV load was > 1000 copies/mL. They found an increase of 11% to 14% for every Log_{10} increase in plasma HIV viral load (27).

Prior to this report, Sobel *et al*. (2001) found out that higher HIV loads rather than lower CD4+ T-lymphocyte counts were associated with statistically significant increased odds for both persistent candidal vaginal colonization and symptomatic VVC (8). However, the study by Sobel *et al.* (2001) only found an association between plasma HIV viral load and the proportion of *Candida* infections that were non-*C. albicans*, not the absolute prevalence of symptomatic VVC (8).

Data from the present study demonstrated that plasma HIV RNA levels were significantly associated with symptomatic VVC in HIV positive women. Compared with patients whose plasma HIV load was suppressed below the detectable level (< 20 copies/mL), the risk of developing symptomatic VVC increased with an increase of plasma HIV load. Plausible biological reasons why blood HIV viral loads can correlate with symptomatic VVC have been hypothesized by others. Following a cross-sectional study on oral candidiasis, Gottfredsson *et al.* (1999) hypothesized that high level of plasma HIV-1 can suppress local mucosal immune mechanisms independently of systemic cell-mediated immunity, leading to mucosal colonization with *Candida species* (28). The authors also speculated that controlling the replication of HIV-1 could possibly restore local mucosal immune functions.

Similarly, during an in vitro analysis, Gruber *et al.* (1998) reported that high plasma and therefore salivary or oral HIV-1 load might promote virulence of *C. albicans* at oral mucosal surfaces (29). In the present study, findings showed a significant linear correlation between plasma HIV viral load and genital HIV shedding. This study also found that HIV viral loads (in plasma and vagina) increased across progressive stages of HIV-induced immunocompromise by CD4+ T cell stages.

Prior studies demonstrated that HIV induces lymphoid activation despite CD4+ T cell depletion in patients with severe immunosuppression, leading to an increased production of MIP-1β, and other markers of T cell activation such as CD38 and HLA-DR(30, 31). This chronic immune activation is usually associated with HIV viral persistence and increased cases of opportunistic infections such as mucosal candidiasis. However, Ohmit et al (2003) did not establish a significant correlation between CD4+ lymphocyte count and the occurrence of VVC, excluding the hypothesis of compromised cellular immunodeficiency as the basis for VVC. The authors showed that HIV load independently influenced the odds of VVC. Similar findings were initially reported by Sobel et al. in 2001. It can therefore be hypothesized that during advanced HIV infection (as measured by low CD4+ T cell levels) with subsequently observed higher HIV viral loads, HIV particles might change the vaginal environment, hence promoting virulence of *Candida species* by switching from its non-pathogenic form into a filamentous form that causes symptomatic VVC. It can be further speculated that because ART-naïve HIV-infected women had 4-fold higher risk of developing symptomatic VVC as compared to women on HAART, controlling the replication of HIV by using HAART could possibly restore local mucosal immune functions in the vagina. This suggests that high levels of plasma HIV load could suppress genital mucosal immune mechanisms independently of systemic cell-mediated

immunity, leading to symptomatic VVC. Further studies are required to confirm this hypothesis. Although during our multivariate logistic regression models, genital HIV shedding was not identified as an independent determinant of symptomatic VVC, increased genital HIV load was however shown to correlate with symptomatic VVC only in univariate analysis. This could be explained by the fact that HIV load was only measured from the cell-free fraction of cervico-vaginal fluid (HIV RNA expression). HIV is more readily detected in the cell-associated component of genital secretions (HIV DNA) than in the cell-free component (32). It is possible that the cell-associated fraction of vaginal secretions could reflect better shedding of HIV infected cells from the vagina while the cell-free fraction may only represent HIV released locally by actively replicating cells. Whilst a univariate association was shown between genital HIV load and symptomatic VVC, there was a positive and significant correlation between Log_{10} levels of genital HIV RNA expression and absolute counts of neutrophils in the genital fluid. In addition, due to the inverse relationship between CD4 count and absolute neutrophil count shown in this present study, it can be postulated that this is the result of increased HIV shedding in the vagina with a concomitant inflammatory response in the setting of increasing immune suppression. Alternatively, lower CD4 counts in the genital tract would correlate with lower blood CD4 counts (33), hence suggest an immunocompromised state. Women who are more immunocompromised would be more likely to have VVC and may be several other STIs or BV and therefore have higher neutrophil counts in their genital tracts. Another possibility is also the fact that other authorities reported that Th17 cells rather than Th1/Th2 paradigm are key players in the defense and development of effector responses in HIV infected women with symptomatic VVC (34-38). The prototype cytokine for Th17 cell response, IL-17, coordinates tissue inflammation through the induction of proinflammatory cytokines and chemokines, such as IL-6

and IL-8. Whilst IL-6 is a multifunctional pleiotropic cytokine involved in the regulation of immune responses, acute-phase responses, hematopoiesis, and inflammation, IL-8 is a neutrophil-specific chemotactic factor classified as a member of the CXC chemokine family. The major effector function of IL-8 is the activation and recruitment of neutrophils at the site of infection. Another study is required to ascertain this hypothesis.

Based on data from a human Candida challenge model, Fidel *et al.* (2004) in more than one occasion hypothesized that following the interaction of *Candida* with vaginal epithelial cells, symptomatic VVC was associated with signals that promoted a non-protective inflammatory leukocyte response and concomitant clinical symptoms. They indicated that neutrophils contribute to the pathogenesis and local inflammation in symptomatic VVC (39, 40).

There was a significant negative association between the presence of BV and symptomatic VVC. A high prevalence of BV was diagnosed among both HIV positive and HIV negative women. The rate of BV has been always reported to be very high in the South African province of KwaZulu-Natal (41, 42). In 1996, Govender *et al.* during a study on BV and associated pregnancy outcomes among asymptomatic pregnant women attending antenatal clinic at King Edward VIII hospital in Durban/ KwaZulu-Natal, reported a prevalence of 52% for BV, and BV was the leading lower genital tract infection according to the authors (41). In 2002, Moodley, Connolly and Sturm during a study at Hlabisa Health District in northern KwaZuluNatal found the prevalence of BV to be 70% among their 598 enrolled women (42). BV is known as a syndrome characterized by imbalance of the vaginal ecosystem. It can be postulated that in the KwaZulu-Natal province of South Africa, the excessive use of topically applied substances in the

vagina with antimicrobial effects interferes with the lactobacilli (43, 44). The depletion of lactobacilli may limit the production of hydrogen peroxide. Vallone *et al.* (2012) postulated that low vaginal pH inhibits CD4 lymphocyte activation and reduces HIV target cells in the vagina while elevated vaginal pH may enhance HIV adherence to vaginal eukaryotic cells (45). In this present study, although BV was prevalent among both HIV positive and HIV negative, there was a significant association between BV and higher expression of HIV RNA in the plasma of HIV-infected women.

Our findings have also shown a significant association between the composition of the vaginal microbiota and the development of symptomatic VVC in HIV infected women. Using the Nugent score to assess the degree of alteration of the vaginal flora, symptomatic VVC was significantly associated with a score of < 7. These findings strongly support the hypothesis that symptomatic VVC is usually associated with a normal vaginal pH (pH < 4.5) while BV is established when pH of the vaginal fluid becomes > 4.5. In addition, these findings are also in keeping with results from Moodley, Connolly and Sturm (2002) who found out that Yeast colonization and symptomatic VVC were inversely related to Nugent's scores (42). In women with VVC yeast to hyphal morphogenesis switch starts as early as when the pH is slightly above 4.2 but below 4.5 explaining at least partially why most of women with VVC have a pH <4.5 as compared to women with BV who have a pH >4.5 (46) . While the rise in pH in women with VVC is associated with the release of ammonia (47), the rise of pH in cases of BV has been shown to be significantly associated with the absence of peroxide hydrogen (H2O2)-producing lactobacilli during a longitudinal study (48).

In addition to low Nugent score, another factor that was identified as being significantly associated with symptomatic VVC was pregnancy, particularly among HIV-uninfected women. This finding was consistent with what has been found in other studies (1, 49).

Although HIV-uninfected women with symptomatic VVC reported a history of antibiotic use within the past 3 months prior to the clinic visit more than women without symptomatic VVC, this difference was not statistically significant. The use of broad spectrum antibiotic has been extensively reported from the literature as a determinant of symptomatic VVC (1, 50, 51). The fact that patients were been asked if they received any antibiotic in the last 3 months may not provide an accurate information as some patients might forgot or could not differentiate antibiotics from other types of medicines that they received.

Although the presence of concurrent lower genital tract pathogens were not shown to be significantly associated with symptomatic VVC, there was a negative association between the presence of symptomatic VVC and vaginitis caused by *Trichomonas vaginalis*. Moodley, Connolly and Sturm reported that *T.vaginalis* was prevalent in patients with all levels of abnormal vaginal flora (bacterial vaginosis score of Gram stain >4) suggesting that this pathogen might contribute to the change in vaginal flora leading to BV (42). The authors further identified that the presence of yeasts on microscopy was inversely related to the level of ecologic disturbance. They argued that BV environment is not conducive to yeast multiplication, and yeast vaginitis therefore does not occur frequently in the presence of both BV and *T.vaginalis* (42).

Limitations

A small sample size needs to be underlined as a first limitation. The fact that CD4 counts obtained from the patient medical records might be at least 3 months old presents a limitation in the interpretation of our results, in that the degree of immune suppression may have been over or under estimated. However, in our study there was a significant negative association between CD4 counts and HIV viral loads in plasma ($p < 0.0001$). A further bias in the study is the fact that we used patients' records of ARV prescriptions and patient interviews as a proxy for ARV treatment adherence. The latter is often subjective. Measurement of drug levels is a more objective test of adherence. However this was not done in our study.

Although we assessed the history of previous vaginal discharge syndrome, this was not conclusive of symptomatic VVC, hence could not establish possible cases of recurrent VVC. Finally, we prevented colinearities in our logistic models by not including variables that were highly correlated, but the 95% confidence intervals around the odds ratios were slightly wide for certain variables. This might be due to potential variability observed during the measurement of CD4+ T cells and HIV loads. Despite these limitations, findings from this study add on the existing body of evidence on the correlation between HIV-induced immunocompromise and the presence of symptomatic VVC. Commencing HIV-infected women on HAART could prevent new incident cases of symptomatic VVC.

Acknowledgments

We are grateful to Hasso Plattner Foundation (Grant to TA) and National Research Foundation (Grant to PM) for funding this study. This study was also partially supported by NIH grant NIH K01--TW007793 (Grant to WC).

Conflicts of interest:

There are no conflicts of interest.

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CHAPTER 3

Impact of Symptomatic Vulvo-Vaginal Candidiasis on Human Immunodeficiency Virus Ribonucleic Acid Levels in Plasma and Genital Secretions

Synopsis:

The chapter comprises 1 article which is currently published in the Southern African Journal of HIV Medicine. It addresses the association between symptomatic vulvo-vaginal candidiasis (VVC) and HIV RNA levels in plasma and genital secretions of women receiving highly active antiretroviral therapy (HAART).

Association between Symptomatic Vulvo-Vaginal Candidiasis and HIV RNA Levels in Plasma and Genital Secretions amongst Women on HAART attending a Primary Healthcare Clinic in KwaZulu-Natal, South Africa

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Southern African Journal of HIV Medicine 2014; 15(2):57-64.

Abstract

Introduction: Genital tract inflammation plays a major role in HIV transmission. We aimed to determine the association between symptomatic vulvo-vaginal candidiasis (VVC) and HIV RNA levels in plasma and genital tracts of HIV infected women on HAART.

Methods: Women with VVC on HAART were recruited from a primary healthcare clinic in KwaZulu-Natal between June 2011 and December 2011. VVC was diagnosed clinically, supported by Gram stain and culture of genital secretions. HIV RNA load was determined by RT-PCR. CD4 counts were obtained from patients' medical records.

Results: Plasma HIV RNA was detected in 42 of 60 (70%) patients on HAART. The mean duration on HAART for these 42 patients was 4.2±1.6 months versus 10.7±1.4 months for the remaining 18 patients without detectable plasma HIV RNA ($P < 0.0001$). Of the 42 women with detectable plasma HIV RNA, 12 (28.6%) had detectable genital HIV RNA. Plasma HIV RNA levels ranged from 2.5±0.8 to 4.1±0.8 Log_{10} copies/mL. Genital HIV RNA levels ranged from 1.4 to 2.5±1.1 Log_{10} copies/mL. The adjusted Odds Ratios of plasma HIV RNA levels increased for patients < 35 years ($P = 0.039$) and in those with VVC ($P = 0.008$). Genital HIV detectability was significantly observed in patients with plasma HIV load ≥ 10,000 copies/mL ($P = 0.032$) and when genital absolute counts of neutrophils > 10 cells/5 HMF ($P = 0.007$).

Discussion: Given that the majority of women had just initiated HAART (high rate of detectable plasma HIV RNA) there is insufficient evidence to conclude that VVC was predictive of high plasma HIV RNA levels. It is more likely that this cohort of immunosuppressed women were rather prone to develop VVC. Plasma HIV loads and local genital inflammations were predictors of genital HIV detectability.

Key words: HIV RNA levels, lower genital tract, plasma, PMN cells, symptomatic VVC.

Introduction

Ulcerative and non-ulcerative lower genital tract infections (LGTIs) are thought to play a major role in early HIV pathogenesis and transmission. LGTIs and plasma HIV viral load have been reported to partially explain HIV shedding in the female genital tract (GT) (1). Some studies have suggested that changes in the normal microbial flora of the female genital tract contribute to viral shedding (1, 2).

Conditions associated with genital inflammation, particularly genital ulcers have been associated with HIV shedding in the genital tract (3, 4). Whilst the association between nonulcerative lower genital tract infections such as bacterial vaginosis (BV) and genital tract HIV shedding has been well established (5, 6), current studies have less well defined the relationship between VVC and HIV shedding. In addition to BV that is not an established sexually transmitted infection (STI), vaginitis caused by *Trichomonas vaginalis, a STI,* has been also shown to increase the risk of HIV acquisition among women (7, 8). Thus, these vaginal infections may contribute to increased risk of sexual and perinatal HIV transmission.

Data on the effect of highly active anti-retroviral therapy (HAART) on vaginal infections among HIV-infected women are limited. Factors such as changes in vaginal milieu mainly immunologic cell populations, cellular activation, and cytokine production, were been reported to alter susceptibility or response to genital infections (9-12). It remains however unclear whether chemokines and cytokines interfere with the activity of polymorpho-nuclear neutrophil (PMN) cells and macrophages in the mucosa or whether they facilitate the development of VVC.

In this study we aimed to determine the association between symptomatic VVC and HIV RNA levels in plasma and genital tracts of HIV infected women on HAART. We hypothesized that in HIV infected women, the presence of symptomatic VVC would impact local HIV shedding, but not systemic levels.

Methods

Study Population:

For this cross-sectional study, consecutive adult (18 years or above) women (all HIV infected) who attended Umlazi D clinic, a primary healthcare facility in KwaZulu-Natal between June 2011 and December 2011 were enrolled by informed consent. Current symptoms suggestive of lower genital tract infections (LGTIs) were obtained through a standard questionnaire. Patients on highly active antiretroviral therapy (HAART) were defined as those receiving treatment with ≥2 nucleoside reverse-transcriptase inhibitors (NRTIs) (emtricitabine, abacavir, stavudine, zidovudine, tenofovir or lamivudine) and ≥1 protease inhibitors (PIs) (lopinavir/ritonavir, atazanavir/ritonavir) or non-nucleoside reverse-transcriptase inhibitors (NNRTIs) (efavirenz or nevirapine).

A physical examination was performed by the attending medical practitioner and signs of LGTIs were noted. Sixty paired plasma and cervico-vaginal samples were collected from HIV-infected women receiving HAART. Patients aged < 18 years as well as those menstruating or had visible blood contamination of genital samples were excluded from the study.

The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Ref. BE 224/11). Consent forms were signed by all participants and confidentiality was maintained throughout the study.

Collection and process of cervico-vaginal fluid:

A vaginal tampon (8 Ks), Tampax Regular® (Compak) was inserted into the vagina, left in situ for 3 minutes, and then placed into a sterile container containing 10 mL of phosphate buffered saline (PBS; Oxoid Limited Basingstoke, UK) (pH = 6.9). All samples were stored at 4^{o}C for a period of 4 hours prior to transport to the laboratory. Vaginal fluid was expressed using an autoclaved wooden tongue depressor and filtered through a 0.22 μm Costar Spin-X cellulose acetate filter membranes (Sigma). The filtered soluble fraction was aliquoted (in 1 mL cryotubes) and stored at -70^{o}C until use.

Measurement of absolute polymorphonuclear neutrophil (PMN) cell counts from lower genital tract samples:

A vaginal swab (Becton Dickinson) was used to collect cells from the posterior fornix and to prepare a tissue smear onto a glass slide for Gram staining. Absolute counts of neutrophils were determined by counting a total number of PMN cells in randomly selected five different microscopic fields under oil immersion (X1000 magnification) as previously described with the following scores – score 1: 1-10 PMN / 5 fields; score 2: 11-20 PMN / 5 fields; score 3: >20 PMN / 5 fields (13-15).

Diagnostic criteria of symptomatic vulvo-vaginal candidiasis and VVC colonization:

The diagnosis of symptomatic VVC was based on a combination of clinical and laboratory criteria (evidence level III, recommendation grade B) (16-19).

Symptoms suggestive of VVC included vulval pruritus/itching, vulval soreness, superficial dyspareunia, and/ or non offensive vaginal discharge. Signs included vulval erythema, vulval oedema, fissures, excoriation, or thick curdy vaginal discharge.

Vaginal swab (Becton Dickinson) taken from the anterior fornix was directly plated onto Sabouraud Dextrose agar with chloramphenicol (BBL^{TM} Becton Dickinson) and incubated at 29˚C, 48 h to estimate the relative vaginal fungal burden. The numbers of yeast colonies were recorded as the number of colonies per plate (16-19). In addition to the self-reported above symptoms and observation of signs suggestive of VVC in physical examination, cases of symptomatic VVC were confirmed if one of the following criteria was fulfilled (evidence level III, recommendation grade B): (i) a positive Gram-stain preparation with budding yeasts, pseudohyphae, and/or hyphal forms; (ii) positive culture with either moderate (10–99 colonies per plate) or heavy candida growth (>100 colonies per plate).

Participants without symptomatic VVC were defined as: (i) patients whose genital specimens had a negative microscopy result for yeasts, pseudohyphae, and/or hyphal forms of candida together with negative culture; (ii) patients whose genital specimens had a negative microscopy result for yeasts, pseudohyphae, and/or hyphal forms of candida together with light candida growth (< 10 colonies per plate). The latter was considered to indicate vaginal *candida* colonization rather than infection.

Definitions of HIV-induced immunosuppression:

As part of the routine management and standard of care in the clinic, CD4+ T cell counts were measured from the blood of all HIV-infected patients; and CD4+ T cell counts used in this study were measured within the past 3 months and were obtained from patients' medical records. However, for the purpose of this research, HIV RNA was measured from the plasma and cell-free fraction of vaginal secretions using Nuclisens Easyq HIV-1 version 2.0 (BioMerieux, Lyon, France) with a lowest detection limit of 20 copies/ mL (20, 21).

Absolute values of CD4+ T cell counts (cells/mm^3) were used to determine the degree or severity of immunosuppression following the World Health Organization (WHO) immunological staging criteria: CD4 levels < 200/mm^3 (severe immunosuppression), CD4 levels 200 – 349/mm^3 (advanced immunosuppression), and CD4 levels 350 – 499/mm^3 (mild immunosuppression) (WHO, 2005). HIV viral load and CD4 counts were used as markers of disease progression. For the purpose of this study, plasma viremia was classified as: < 20 copies/mL (below detectable level of the used test), 20 – 9999 copies/mL, and ≥ 10 000 copies/mL. We also log transformed HIV loads for improved symmetry and included them in multivariate logistic regression models as continuous values.

Diagnosis of other lower genital tract infections (LGTIs):

For each study participant, clinical symptoms and signs suggestive of LGTIs were recorded and categorized as absent, mild, moderate or severe. The diagnosis of Bacterial vaginosis was diagnosed using Nugent's scores (14, 15). BD nucleic acid amplification tests (Becton Dickinson Probetec Assays, Sparks) were performed on all cervical swabs for the detection of *Chlamydia*

trachomatis and *Neisseria gonorrhoeae*. In addition PCR amplifications of DNA isolated from vaginal swabs were performed as described previously for the detection of *Mycoplasma genitalium, Trichomonas vaginalis* and Herpes Simples Virus-Type 2 (22, 23).

Statistical analyses:

Categorical variables were expressed as proportions (%) whilst quantitative (discrete ordinal) variables were presented as mean ± standard deviation. For univariate analyses, Chi-square test was used to compare proportions, whilst the R coefficient was computed to analyze the correlations between quantitative variables. Furthermore, univariate association between HIV RNA detectability (in plasma or vagina) and putative risk indicators was defined by Odds Ratios (OR) with 95% confidence intervals (CI). For multivariate analyses, linear multiple regression and logistic regression were used to identify independent determinants associated with HIV RNA detectability in the vagina or plasma. Multivariate Odds Ratios (95% CI) of HIV RNA genital/plasma detectability was computed after adjusting for confounding factors. Excess risks were measured using the risk difference method.

All tests were two-sided and a *P* value of <0.05 was considered significant. Data were analyzed using SPSS® statistical software version 21.0 (SPSS Inc; Chicago, IL).

Results

The median age of the 60 women (all HIV infected on HAART) enrolled in the study was 30 years ranging from 18 years to 46 years; all black Africans.

Plasma HIV RNA was detected in 42 of 60 (70%) patients on HAART. The mean duration on HAART for these 42 patients with detectable plasma HIV RNA (CD4+ T count < 350 cells/mm^3) was 4.2 ± 1.6 months versus 10.7 ± 1.4 months for the remaining 18 patients without detectable plasma HIV RNA (CD4+ T count ≥ 350 cells/mm3) ($P < 0.0001$).

Of the 42 women with detectable plasma HIV RNA, 12 (28.6%) had detectable genital HIV RNA. Only 1 patient had detectable genital HIV RNA in the absence of detectable plasma HIV RNA. Plasma HIV RNA levels ranged from 2.5±0.8 to 4.1±0.8 Log_{10} copies/mL. Genital HIV RNA levels ranged from 1.4 to 2.5±1.1 Log_{10} copies/mL (Table 1). Eight patients out of 12 (66.7%) with detectable genital HIV RNA had plasma HIV loads > 10 000 copies/ml while the remaining patients had plasma HIV loads between 20 – 9999 copies/ml. Figure 1 depicts the distribution of plasma HIV loads and genital HIV RNA detectability by duration of HAART.

Table 1 HIV RNA levels in Plasma and Genital Tract by HAART Duration among the Clinic's Attendees (n = 60)

HAART Duration (months)	Patient groups (n)	VL in plasma (Log10 copies/ml) (Mean ± SD)	VL in vagina (Log10 copies/ml) (Mean ± SD)
0 – 3	1A (n = 8/21)	4.1 ± 0.8	2.5 ± 1.1
	1B (n = 13/21)	3.3 ± 1.4	< 1.3 (not detectable)
4 – 9	2A (n = 4/21)	2.5 ± 0.9	1.6 ± 0.5
	2B (n = 17/21)	2.9 ± 1.1	< 1.3 (not detectable)
≥ 10	3A (n = 1/18)	< 1.3 (not detectable)	1.4
	3B (n = 17/18)	< 1.3 (not detectable)	< 1.3 (not detectable)
ANOVA *P*-value	$P < 0.0001$		

Group 1A and 2A = HIV RNA detectable in plasma and genital tract; 1B and 2B = HIV RNA detectable in plasma but not in genital tract; 3A = HIV RNA detectable in genital tract only; 3B = HIV RNA not detectable. ANOVA is comparing groups 1, 2 and 3 by HAART duration.

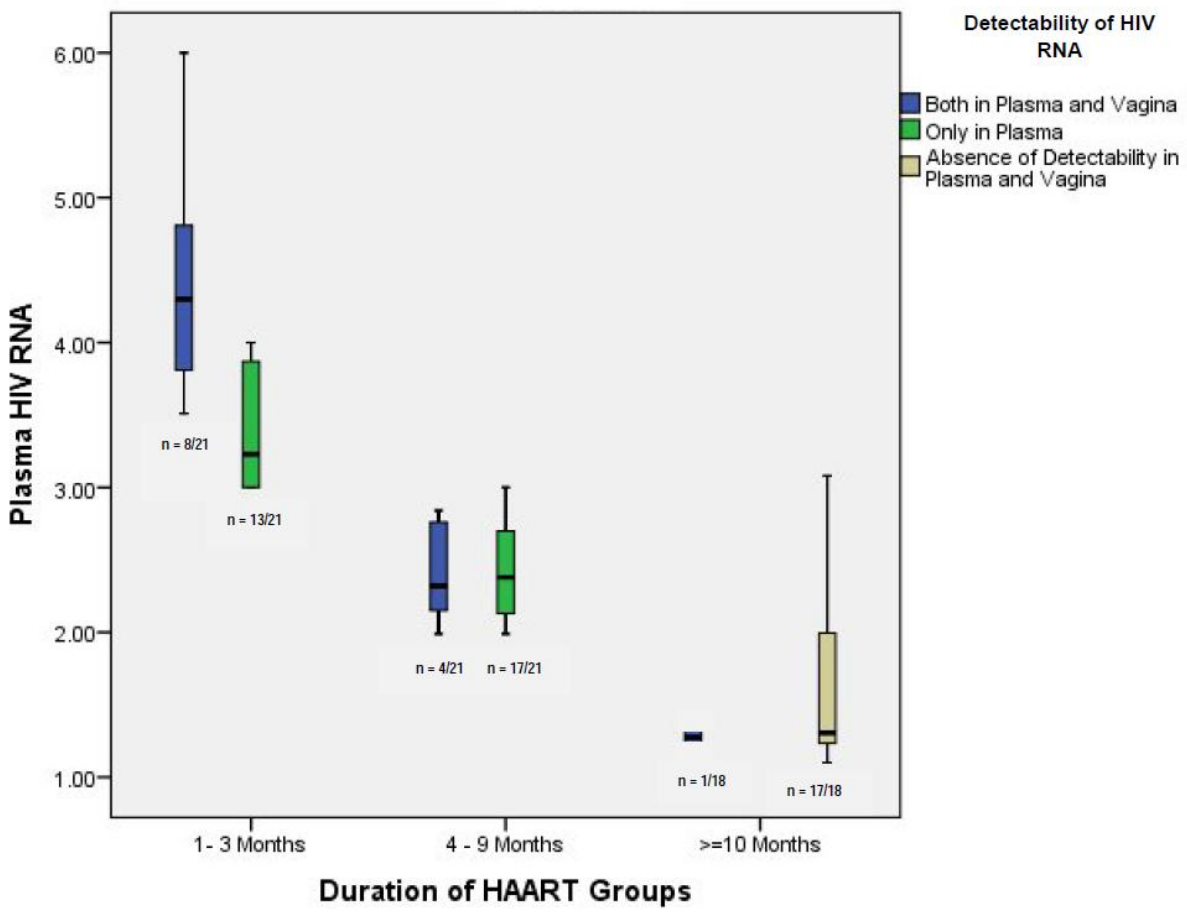

Figure 1 Distribution of Plasma HIV levels and Genital HIV detectability by HAART Duration (Patients on HAART for ≤ 3 months had higher plasma HIV loads with increased HIV shedding in their genital tracts as compared to those who received HAART for 4 to 9 months. Most of women who received HAART for ≥10 months are aviraemic except 1 patient)

All patients were on HAART regimen 1 comprised of a treatment with 2 nucleoside reverse-transcriptase inhibitors (NRTIs) and 1 non-nucleoside reverse-transcriptase inhibitor (NNRTI). There was no patient who received a regimen made of a protease inhibitor (PI).

Factors associated with plasma HIV RNA levels among HIV-infected women receiving HAART:

In univariate analysis (Table 2), there was a significant association between symptomatic VVC and plasma HIV RNA detectability (P = 0.006). Likewise, patients who complained with symptoms suggestive of symptomatic VVC (vulval itching, vulval soreness and vaginal discharge) had significant HIV RNA levels in their plasma (P = 0.039). In addition, a recent history (within the past 3 months) of STIs (P = 0.035) and patient's age below 35 years (P = 0.018) were also significantly associated with detectable HIV RNA in the plasma. Furthermore, the presence of both advanced (CD4+ T count = 200 – 349 cells/mm^3) and severe (CD4+ T count < 200 cells/mm^3) HIV-associated immunosupression was significantly associated with HIV RNA detectability in the plasma (P < 0.0001). Alternatively, no patient with CD4+ T cell count ≥ 350 cells/mm^3 had detectable plasma HIV RNA.

Table 2: Univariate analysis of factors associated with plasma HIV levels in HIV positive women receiving HAART (n = 60)

Associated factors	Detectable plasma HIV RNA n (%)	OR (95% CI)	*P* value
Age groups (years)			0.018
18 - 24	11 (84.6)		
25 - 34	25 (75.8)		
≥ 35	6 (42.9)		
Symptomatic vulvo-vaginal candidiasis		6.1 (1.5 - 24.1)	0.006
Yes	23 (88.5)		
No	19 (55.9)		
Presenting complains		5.7 (0.9 - 34.7)	0.039
Group 1 (vulval itching-vulval soreness-vaginal discharge)	40 (74.1)		
Group 2 (low abdominal pain-vaginal discharge)	2 (33.3)		
Recent history (past 3 months) of STIs			0.035
Vaginal discharge syndrome	26 (78.8)		
Genital ulcer syndrome	6 (85.7)		
No STI diagnosed	10 (50)		
CD4+ T count (cells/mm3)			< 0.0001
< 200	22 (100)		
200 - 349	20 (100)		
≥ 350	0 (0)		
Genital HIV load categories (copies/mL)			0.062
≥ 10,000	2 (100)		
20 - 9,999	10 (90)		
< 20	30 (65.2)		
Genital absolute neutrophil counts			0.095
> 20 cells per 5 high microscopic fields	12 (60)		
11 - 20 cells per 5 high microscopic fields	13 (92.9)		
1 - 10 cells per 5 high microscopic fields	17 (65.4)		

STIs = sexually transmitted infections; OR = Odds Ratios; CI = confidence interval; HAART = highly active anti-retroviral therapy.

In multivariate analysis (Table 3), logistic regression was used to identify independent determinants of HIV RNA levels in plasma after adjusting for univariate confounding factors (presenting complains and recent history of STIs). CD4+ T cell counts were not included in the logistic regression model because all patients with detectable plasma HIV RNA had either advanced or severe immunosupression.

Multivariate analysis showed that having a current episode of symptomatic VVC was independently (P = 0.008) associated with detectable HIV RNA in the plasma. It was also found that HIV infected women aged below 35 years were more likely to be associated with higher HIV RNA levels in the plasma than women aged ≥ 35 years (P = 0.039) (Table 3).

Table 3: Independent determinants of plasma HIV RNA levels in HIV infected women receiving HAART (n = 60)

	β-coefficient	Standard Error	Wald Chi-square	OR (95% CI)	*P* value
Independent variables					
Age groups (years)					0.039
18 - 24	2.328	1.043	4.978	10.3 (1.3 - 79.2)	0.026
25 - 34	1.765	0.789	5.001	5.8 (1.2 - 27.5)	0.025
≥ 35			Reference	1	
Symptomatic VVC					
Yes	2.082	0.79	6.943	8 (1.7 - 37.7)	0.008
No			Reference	1	
Constant	-1.236	0.719	2.954		0.086

Adjusted for presenting complains and recent history of sexually transmitted infections (STIs)

Factors associated with genital HIV RNA shedding among HIV-infected women receiving HAART:

Of the 42 HIV infected women receiving HAART and shown to have detectable plasma HIV RNA, 12 (28.6%) had detectable levels of HIV RNA in cell-free vaginal fluid.

Table 4 shows univariate factors associated with genital HIV RNA detectability among HIV positive women receiving HAART. Women with a recent history (less than 3 months duration) of vaginal discharge syndrome (P = 0.05) and genital ulcer syndrome (P = 0.038) were more likely to have detectable HIV RNA in their genital tracts (Table 4). However, data showed no association between GC, VVC or BV and genital HIV shedding.

Women with neutrophil cell count score > 1 (means >10 PMN cells/5 HMF) had 5-fold higher risk (OR = 5.3 95% CI 1.6 – 17.2; P = 0.004) of having detectable HIV RNA in their genital tracts than women with neutrophil cell count score 1 (means ≤ 10 PMN cells/5 HMF). A recent history (within the past 3 months) of use of broad spectrum antimicrobial agents (P = 0.048) was shown to be associated with detectable HIV shedding in univariate analysis (Table 4).

Table 4: Univariate analysis of factors associated with genital HIV RNA detectability in HIV infected women receiving HAART (n = 60)

Associated factors	Detectable genital HIV RNA n (%)	OR (95% CI)	*P*-value
Current sexually transmitted infections			
Trichomonas vaginalis		0.7 (0.3 - 2.1)	0.554
positive	6 (25)		
negative	18 (31.6)		
Chlamydia trachomatis		0.8 (0.2 - 2.9)	0.781
positive	4 (26.7)		
negative	20 (30.3)		
Neisseria gonorrhoeae		1.2 (0.3 - 5.3)	0.796
positive	3 (33.3)		
negative	21 (29.2)		
Mycoplasma genitalium		0.5 (0.1 - 4.1)	0.478
positive	1 (16.7)		
negative	23 (30.7)		
Herpes simplex virus type 2		0.7 (0.6 - 0.8)	0.183
positive	0 (0)		
negative	24 (31.2)		
Other lower genital tract infections			
Vulvo-vaginal candidiasis (VVC)		1.2 (0.5 - 3.2)	0.649
infection	13 (32.5)		
colonization	12 (27.9)		
Bacterial vaginosis (BV)		1.2 (0.4 - 4.3)	0.747
positive	21 (30.9)		
negative	4 (26.7)		
Recent history (past 3 months) of STIs			
vaginal discharge syndrome	14 (36.8)	3.2 (1 - 10.1)	0.05
genital ulcer syndrome	6 (46.2)	4.6 (1.1 - 19.7)	0.038
no STI	5 (15.6)	1	
Genital absolute neutrophil counts		5.3 (1.6 - 17.2)	0.004
score > 1	21 (42)		
score = 1	4 (12.1)		
Plasma HIV RNA levels			< 0.0001
≥ 10 000 copies/ml (≥ 4log10)	8 (100)		
20 - 9999 copies/ml (1.3log10 - 3.99log10)	4 (11.8)		
< 20 copies/ml (< 1.3log10)	1 (5.6)		
Antibiotic (ATB) usage		3.2 (1.01 - 10.6)	0.048
yes	21 (36.8)		
no	4 (15.4)		

HAART = highly active antiretroviral therapy; neutrophil count score 1 = 1-10 cells per 5 high microscopic field

In multivariate analysis, after adjusting for the presence of univariate confounding factors (use of broad-spectrum antibiotics and recent history of STIs), an increase in the vaginal absolute neutrophil counts (p = 0.007) and plasma HIV RNA levels (p = 0.032) remained independently associated with genital HIV shedding (Table 5; $p = 0.005$ Figure 2). Plasma viral load (20 – 9999 copies/ml) tended to positively correlate with genital HIV shedding, but this correlation did not reach statistical significance ($P = 0.166$). However, plasma HIV RNA levels ≥ 10,000 copies/mL

(P = 0.032) were independently associated with genital HIV RNA detectability to a detection limit of 20 copies/mL (Table 5).

Table 5: Independent determinants of HIV RNA detectability in genital tract of HIV infected women receiving HAART (n = 60)

	β-coefficient	Standard Error	Wald Chi-square	OR (95% CI)	*P* value
Independent variables					
Plasma HIV RNA (copies/ml)					
≥ 10 000	2.062	0.962	4.593	7.9 (1.2 - 51.8)	0.032
20 - 9999	1.168	0.843	1.918	3.2 (0.6 - 16.8)	0.166
< 20			Reference	1	
Vaginal PMN cells					
score > 1	1.758	0.65	7.308	5.8 (1.6 - 20.8)	0.007
score = 1			Reference	1	
Constant	-1.123	0.518	2.98		0.005

Adjusted for HAART, ATB usage and previous STIs

Furthermore, when using transformed Log10 HIV RNA values, correlations between plasma HIV RNA levels and genital PMN cell counts >10 cells/5HMF with detectability of genital HIV RNA shedding showed significant associations (Figure 3). Detectability and levels of mean Log_{10} HIV RNA in the vaginal fluid increased with the increase of plasma HIV RNA levels (Log 10) (r = 0.2, R^2 = 0.04; P = 0.032) (Figure 3a) and by increased absolute counts of genital PMN cells (r = 0.21, R^2 = 0.045; P = 0.007) (Figure 3b).

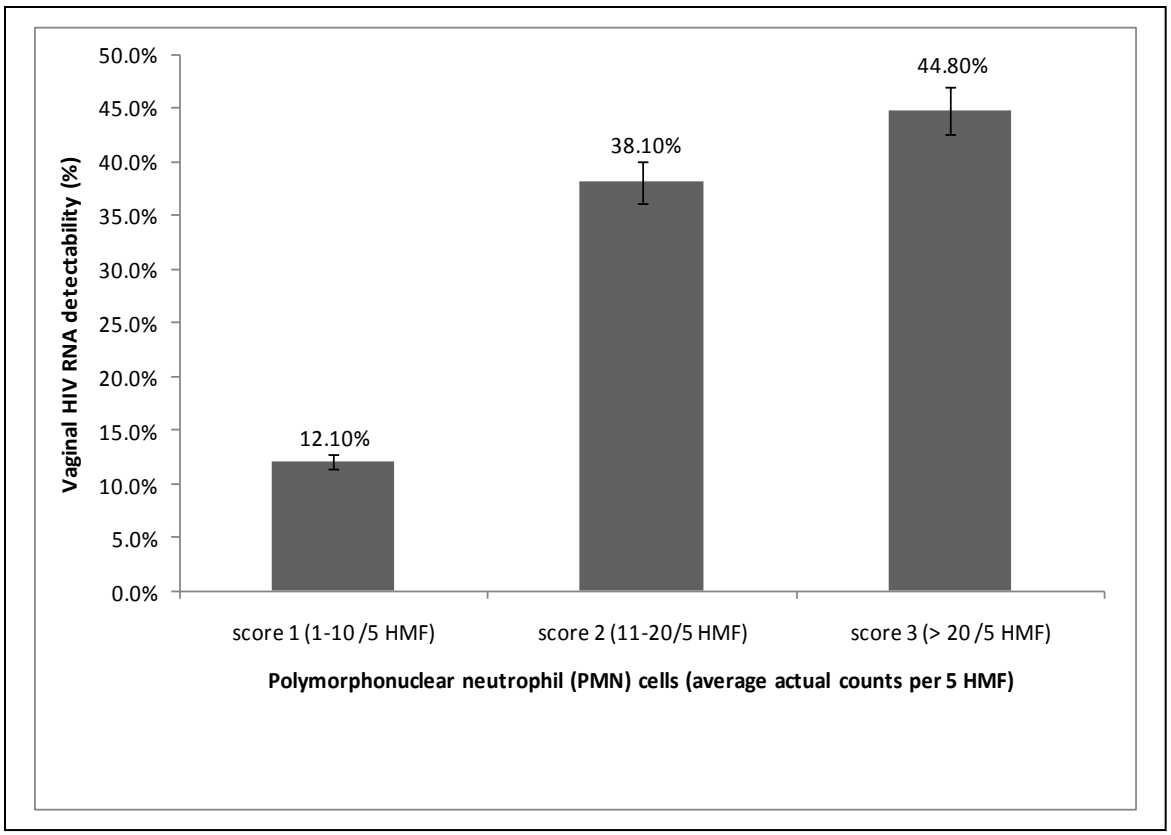

Figure 4 Distribution of Genital HIV Detectability by PMN cells (ANOVA p trend = 0.005)

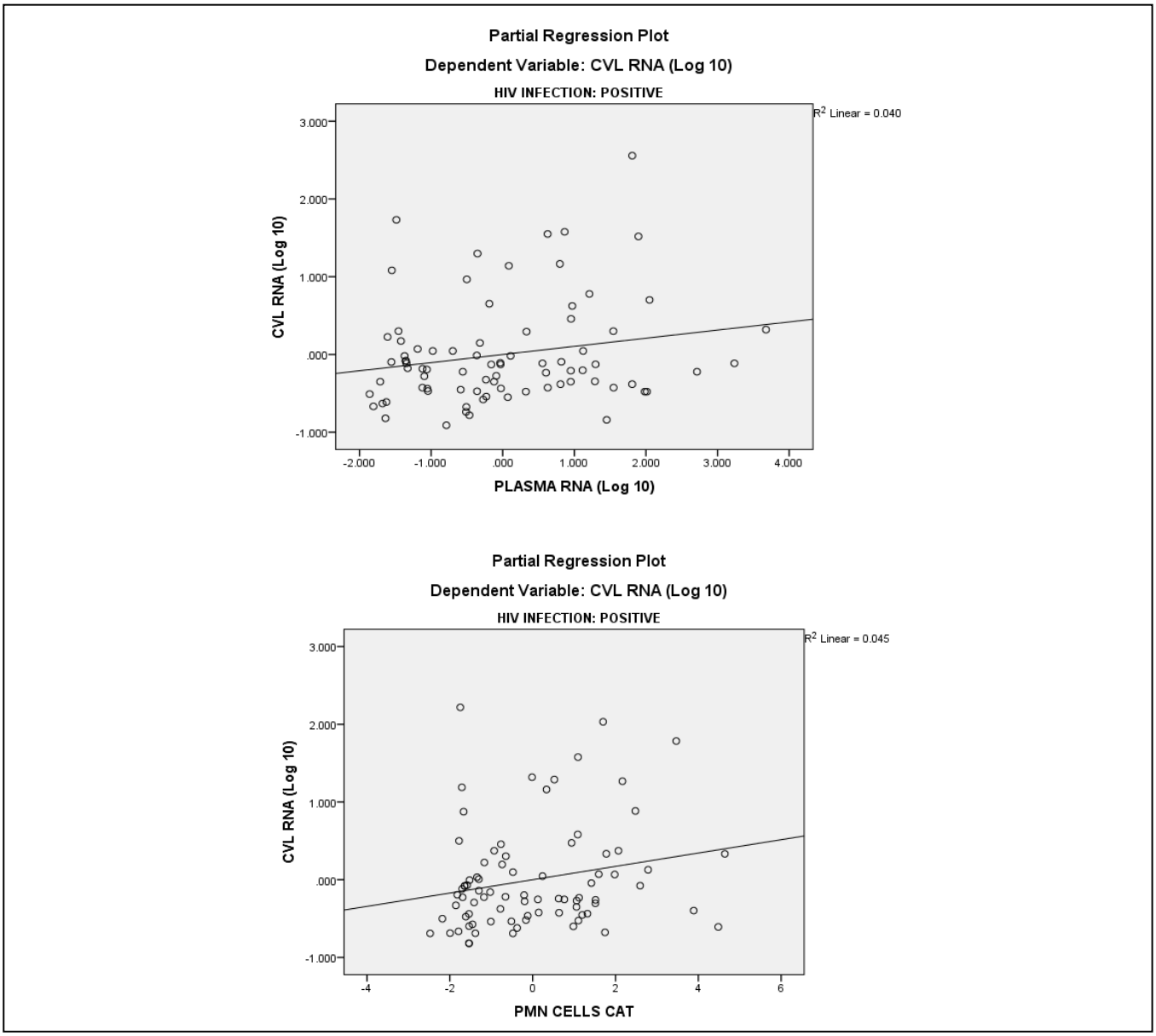

Figure 5 Variation of genital HIV levels as predicted by plasma HIV loads (r = 0.2, R^2 = 0.04; p = 0.032) (Fig. 3A) and genital PMN cells (r = 0.21, R^2 = 0.045; p = 0.007) (Fig. 3B)

Discussion

Inflammation of the genital tract during LGTIs particularly STIs and BV have been reported to predict higher plasma viral loads in HIV infected women (24). In this present study, given that the majority of women had just initiated HAART (high rate of detectable plasma HIV RNA and low CD4 counts), findings had insufficient evidence to demonstrate that VVC was predictive of high plasma HIV RNA levels. Although cause and effect is difficult to unravel in the current study design, it seems more likely that the observed higher level of immunosuppression among the study participants influenced the occurrence of VVC. In addition, plasma HIV loads and local genital inflammation were predictors of genital HIV shedding, not the presence of VVC.

Women aged below 35 years were more likely to have increased plasma HIV RNA levels than women aged ≥ 35 years. A previous study on the relationship between age and plasma viral load in HIV-infected individuals showed that older age (> 50 years) as opposed to younger age (18 – 39 years) was associated with lower levels of HIV-1 replication, independent of antiretroviral therapy usage, regimen adherence, and disease stage (25). One possible explanation of the relationship between the plasma viral load and age category would be immunological differences. It has been shown that older age was associated with an increase in peripheral CD4+CD8+ T lymphocytes, and that thymic involution was associated with this effect, leading to lower expression of plasma HIV-1 RNA (25).

There have been conflicting results about the association between HIV viral load (VL) in the female genital tract and HIV VL in the plasma. In this study 28.6% of the HIV seropositive

women on HAART shed HIV in their genital secretions. This finding is consistent with previous reports of HIV-1 DNA and / or RNA being detected in the genital tract of 28-60% of HIV-1 seropositive women (24, 26-28). These previous studies also demonstrated that genital HIV-1 was more likely to be detected from either the cell associated fraction of the cervico-vaginal fluid or whole genital fluid than cell-free vaginal fluid (24, 26-28). Although within the published range, our finding of a relatively low frequency of viremia in the genital tract of our study population may be attributed to the type of sample that was analyzed (measurement of HIV loads in cell-free fraction alone). Alternatively, the low viraemia observed in the genital tract could due to the use of vaginal tampon as the method for collecting samples. It is difficult to conclude that antiretroviral therapy (ART) significantly lowered HIV RNA levels in genital secretions given the fact that women had just initiated HAART. However, by dividing women according to HAART duration, only patients who received HAART for ≤3 months had HIV shedding in their genital tracts (Table 1 and Figure 1) as compared to women who received HAART for >3 months. In that sense, it can be concluded that ART significantly lowered HIV RNA in genital secretions.

The study found that women with a recent history (less than 3 months duration) of vaginal discharge syndrome and genital ulcer syndrome were more likely to shed HIV in their genital tracts than women without a recent history of such STIs. However, after testing for the commonest causes of lower genital tract infections, none of the tested aetiological agents of vaginal discharge syndrome (VDS) showed significant association with genital HIV shedding.

The fact that HIV VL was only measured from the cell-free fraction of genital secretions may account for the lack of an association between genital tract viremia and tested causes of VDS including symptomatic VVC.

Two independent factors were identified as being associated with HIV RNA shedding in the vagina: plasma HIV load and genital neutrophil counts. Among these factors, the association between higher levels of plasma HIV RNA and the ability to detect HIV in genital secretions had been previously identified (26, 29). Consistent with previous studies it was found that genital HIV RNA detectability strongly correlated with plasma HIV load but those correlations were weak, except when plasma HIV VL was ≥ 10 000 copies/mL. Others have suggested that compartmentalization of vaginal immune response from systemic immunity may account for such weak correlations (30, 31). This might also be explained by the fact that HIV load was only measured from the cell-free fraction of the genital secretions.

The present study also found that the count of neutrophils in the genital mucosa was another factor associated with genital HIV RNA shedding. Previous studies have shown the role of genital inflammation in HIV-1 shedding (7, 32). It has been reported that following HIV-1 infection cytokines and chemokines are produced by infected leukocytes that attract more target cells for HIV-1 infection and allow further stimulation of the expression of HIV-1 via toll-like receptors (7-9).

Here it was found that the number of neutrophils in the female genital tract was associated with HIV shedding. Of particular interest, this study did not show an association between symptomatic VVC and HIV shedding in the vagina. In addition to the fact that HIV was

quantified only in cell-free fraction of genital fluids, this lack of association could also be the result of strong recruitment of neutrophils in the vagina because various immunomodulators have been reported as associated with the anti-candida activity of neutrophils. Cytokines such as TNF-α and IL-8 increase the activity of neutrophils against *Candida albicans*. Gumbi *et al.* (2008) reported that women who were detectably shedding HIV-1 in the genital tract had significantly increased cervical levels of TNF-α, IL-1β, IL-6, and IL-8 compared to women who were not detectably shedding the virus (30). It remains unclear from our observations whether or not symptomatic VVC is more likely to occur among HIV infected women who are not detectably shedding the virus.

Another possible reason why symptomatic VVC was not associated with genital HIV shedding could be challenges associated with the diagnosis of VVC. Defining VVC clinically could be problematic even when microscopic tests and cultures of genital samples are used to support the clinical diagnosis of VVC. Whilst Gram stain has the sensitivity of 75% in supporting the clinical diagnosis of VVC, up to 30% of women with significant chronic symptoms suggestive of VVC may have negative cultures at presentation (33).

Limitations of this study include the use of its cross-sectional design and a small sample size. The impact of factors such as HAART regimen composition, adherence to HAART and effects of HIV disease stages were not explored in this study.

The majority of our patients had just initiated HAART. It is therefore difficult to say that they were not successfully treated. A plausible hypothesis why we did not have association between VVC and genital HIV shedding could have been the fact that HIV RNA was only measured from

the cell-free fraction of cervico-vaginal secretions. If both HIV cDNA and HIV RNA were measured, results could have been different. This might also explain why plasma HIV loads < 10 000 copies/ ml were not significantly associated with genital HIV shedding in multivariate logistic analysis.

Finally, findings present insufficient evidence on the true impact of VVC on HIV RNA levels in plasma and genital secretions.

Conclusions: (i) factors identified in this present study as being associated with HIV RNA loads in plasma and female genital secretions were consistent with previously published factors from the literature; (ii) of particular note, this study did not have enough evidence to demonstrate that symptomatic VVC was predictive of higher levels of HIV RNA in plasma and genital secretions. However, unlike STIs and BV, symptomatic VVC was less likely to occur among HIV infected women who were detectably shedding the virus in their lower genital tract possibly due to the observed strong recruitment of neutrophils that possess anti-candida activity; (iii) about 70% of the study population had detectable HIV RNA in their plasma and this finding was supported by the fact that all those patients had CD4+ T count < 350 cells/mm^3, a marker of systemic immunosuppression. It is high likely that the observed higher rate of HIV RNA detectability was purely the result of immunosuppression; and the associated high prevalence of symptomatic VVC could have been just a consequence of an overall immunosuppression. Further studies are required to ascertain the impact of VVC on HIV RNA levels in plasma and genital secretions.

Acknowledgments

We are grateful to Hasso Plattner Foundation (Grant to TA) and National Research Foundation (Grant to PM) for partially funding this study. This study was also partially supported by NIH grant NIH K01--TW007793 (Grant to WC).

Conflict of interest: none.

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CHAPTER 4

Plasma and Vaginal-associated Immune Responses in Women with Symptomatic Vulvo-Vaginal Candidiasis

Synopsis:

This chapter comprises 2 manuscripts, one of which is under review and the second has recently been published. It describes relationships between 20 cytokines and chemokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-1β, TNF-α, TGF-β1, TGF-β2 and TGF-β3) and their associations with symptomatic vulvo-vaginal candidiasis (VVC) in women, stratified by HIV status.

Concentrations of selected plasma and genital cytokines and chemokines in human immunodeficiency virus infected and uninfected women with symptomatic vulvo-vaginal candidiasis

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The American Journal of the Medical Sciences: **Under Review**

MAJ 13-847

Abstract

Background: The pathogenesis of symptomatic vulvo-vaginal candidiasis (VVC) in HIV infected women is not well understood. The study sought to measure the concentrations of 20 cytokines/chemokines associated with increased susceptibility to VVC, and to determine which of these cytokines /chemokines discriminate between HIV-infected and HIV-uninfected women.

Methods: Plasma and genital samples were obtained from 51 clinic attendees in KwaZulu-Natal between June 2011 and December 2011. A multifactorial design was employed and cytokine concentrations were measured by Luminex® Multiplex Immunoassays. A discriminant analysis (DA) was carried out and Receiver-Operating-Characteristic curves (ROC) were computed for every significant cytokine.

Results: Of the 51 participants, 16/26 HIV infected and 15/25 HIV uninfected women were diagnosed with VVC, but free from other lower genital tract infections.

DA identified 2 variables (MIP-1β and TGF-β3) in plasma [Box's M (5.49), p (.57) > α (.001); Wilk's Lambda = .116, p < .0001] and 1 variable (IL-13) in vaginal secretions [Box's M (2.063), p (.37) > α (.001); Wilk's Lambda = .677, p < .0001] as able to discriminate HIV+VVC+ group whilst TGF-β1 in plasma discriminated HIV+VVC- group. Genital cut-off levels ≥ 38.8 pg/ml for GM-CSF (p = .008) in HIV uninfected women, and ≥ 0.5 pg/ml for IL-13 (p < .0001) in HIV infected women perfectly discriminated those with VVC.

Conclusions: In HIV infected women, VVC was strongly predicted by: (i) circulatory MIP-1β, a marker of chronic immune activation observed during advanced HIV-induced

immunosuppression; (ii) circulatory TGF-β3, a key cytokine in Th17 cell differentiation in the presence of IL-6 as well as Treg cell induced cytokine with a strong down-regulatory and anti-inflammatory response in the presence of IL-10; (iii) vaginal IL-13, an allergic mediate Th2 cytokine.

Key words: HIV, VVC, cytokines, discriminant functions, ROC.

Introduction

In HIV infected patients, whilst oro-oesophageal candidiasis is known to appear at any time during the course of HIV progression, vulvo-vaginal candidiasis (VVC) develops significantly later (1). It has been shown that oro-oesophageal candidiasis occurs as a result of the loss of mucosal and systemic cell-mediated immunity (2, 3). In contrast, animal studies and in vitro experiments indicated that systemic cell-mediated immunity does not play a protective role against candida vaginitis (4). Despite reports on the presence of a protective candida-specific Th1-type immunity during a symptomatic VVC, a strong down-regulatory cytokine, TGF-β, constitutively expressed by the vaginal mucosa was also noticed (4, 5).

In 2010, Shacklett and Anton reported on the presence of Th17, a subset of CD4+ T cells as able to secrete IL-17 and IL-22. The latter induced the production of antimicrobial peptides able to inhibit microorganisms such as *Candida albicans*, the cause of VVC (6). It is not well elucidated whether or not the action of Th17 cells is reduced due to CD4+ T cells depletion observed during advanced HIV infection, leading to recurrent episodes of VVC.

A study by Saavedra *et al.* (1999), which examined the kinetic production of chemokines associated with the chemotaxis of macrophages (RANTES, MIP-1α, MCP-1) and neutrophils (MIP-2, IL-8) showed that higher levels of MCP-1 are able to control *C.albicans* titers in mice with candida vaginitis, suggestive of a strong role played by MCP-1 in the vaginal immune response to *C.albicans* (7).

Pattern recognition of *C.albicans* is mediated by the recognition of mannans and β-glucans by Toll-like Receptors (TLR2 and TLR4) and lectin receptors such as dectin-1(8) and mannose-binding lectin (MBL) receptors on the surface of the cell (9). The soluble MBL, a calcium-dependent serum protein that binds to the carbohydrates on the surface of the pathogen allowing binding followed by phagocytosis by MBL receptor- carrying cells like macrophages and dendritic cells (9). Binding to these receptors induces the production of cytokines, mainly IL-4 and IL-1β (8, 10). It is however unclear whether immune changes observed in HIV infected women interfere with the pattern recognition process of *C.albicans* by innate immune cells.

What is known is that following HIV infection cytokines and chemokines are produced by infected leukocytes that attract more target cells for HIV infection and allow further stimulation of the expression of HIV via toll-like receptors (11, 12). It is also not well understood, whether the produced chemokines and cytokines interfere with the activity of the PMN cells and macrophages and whether this interference facilitates the development of VVC.

The present study sought to quantify plasma cytokines/chemokines associated with increased susceptibility for vulvo-vaginal candidiasis, and to determine which cytokines /chemokines discriminate between HIV-infected and HIV-uninfected women with and without VVC.

Materials and Methods

Study Participants (Patients):

A total of 51 women (26 HIV-infected and 25 HIV-uninfected), who were recruited into a cross sectional study at the Umlazi D Clinic, a primary healthcare facility in KwaZulu-Natal, South Africa were retained as study participants and followed up between June 2011 and December 2011. These women were initially enrolled if they were black Africans, aged 18 years or above, presented with signs and symptoms suggestive of lower genital tract infections (LGTIs), and consenting to participate following an informed consent. Patients aged < 18 years as well as those menstruating or had visible blood contamination of genital samples were excluded from the study.

All participants were thereafter screened for the presence of LGTIs caused by *Chlamydia trachomatis, Neisseria gonorrhoeae, Mycoplama genitalium, Trichomonas vaginalis*, herpes simplex virus type 2 and bacterial vaginosis as described elsewhere (13). Patients were finally retained in the study only if they were free of the above aetiological agents causing LGTIs.

Of the selected 51 participants, 26 were HIV infected versus 25 HIV uninfected. A total of 16/26 HIV infected and 15/25 HIV uninfected women were diagnosed with symptomatic VVC but free of other causes of LGTIs.

Ethics approval was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Ref. BE 224/11).

Diagnostic criteria of symptomatic vulvo-vaginal candidiasis:

Vaginal swab (Becton Dickinson) taken from the anterior fornix was directly plated onto Sabouraud Dextrose agar with chloramphenicol (BBL^{TM} Becton Dickinson) and incubated at

29˚C, 48 h to estimate the relative vaginal fungal burden. The numbers of yeast colonies were recorded as the number of colonies per plate (14).

Cases of symptomatic VVC were defined if clinical and laboratory criteria described by the 2011 European (IUSTI/WHO) guideline on the management of vaginal discharge were fulfilled (evidence level III, recommendation grade B) (14, 15).

Participants without symptomatic VVC were defined as patients whose genital specimens had negative microscopy and culture results for Candida or had microscopy negative with < 10 colonies of Candida onto the plate (15).

Specimen Collection and Measurement of Cytokines/chemokines:

Blood samples were collected by venipuncture into sterile vacutainers (Becton Dickinson) containing ethylenediaminetetraacetic acid (K_2EDTA BD Vacutainer®) and non EDTA containing tubes.

A vaginal tampon (8 Ks), Tampax Regular® (Compak) was inserted into the vagina, left in situ for 3 minutes, and then placed into a sterile container containing 10 mL of phosphate buffered saline (PBS; Oxoid Limited Basingstoke, UK) (pH = 6.9). All samples were stored at 4^{o}C for a period of 4 hours prior to transport to the laboratory. Vaginal fluid was expressed using an autoclaved wooden tongue depressor and filtered through a 0.22 μm Costar Spin-X cellulose acetate filter membranes (Sigma).

Concentrations (in pg/ml) of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-1β, TNF-α, TGF-β1, TGF-β2 and TGF-β3 were measured by a multiplex microbead system (Invitrogen, UK) using a Luminex platform. Multiplex cytokine fluorescent bead-based immunoassays were performed using two different commercially available multi-plex luminex kits: Bio-plex pro human cytokine 17-plex assay and Bio-plex pro TGF-β 3-plex assay (Bio-Rad Laboratories, Inc., Parkwood).

The assay sensitivity or limit of detection (pg/mL) was: IL-1β (0.6), IL-2 (1.6), IL-4 (0.7), IL-5 (0.6), IL-6 (2.6), IL-7 (1.1), IL-8 (1.0), IL-10 (0.3), IL-12 (3.5), IL-13 (0.7), IL-17 (3.3), G-CSF (1.7), GM-CSF (2.2), IFN-γ (6.4), MCP-1 (1.1), MIP-1β (2.4), TNF-α (6), TGF-β1 (1.7), TGF-β2 (14.7) and TGF-β3 (2.8); and a 5 PL regression formula was used to calculate cytokine/chemokine concentrations from the standard curves (Bio-Plex Manager software, version 4). Cytokine/chemokine concentrations below the lower limit of detection were reported as the midpoint between the lowest concentrations measured for each cytokine and zero.

Statistical Analysis:

Data were expressed as means ± standard error of the mean (SEM) for the continuous variables and proportions (percentages) for the categorical variables. When data were normally distributed, analysis of the variance (ANOVA) was used to examine differences between groups. However, non-parametric Mann-Whitney U or Kruskal-Wallis tests were used when data were asymmetrically distributed. Multiple comparisons of means of cytokine/chemokine levels displaying significant differences in univariate analyses across the 4 study groups were

performed using Post Hoc Bonferroni pairwise tests considering a type I error rate of 0.05. This method aimed at testing the difference between each pair of means of cytokine/chemokine values while controlling the α-level error rate for each pairwise comparison.

Canonical discriminant analysis (DA) was used to identify linear combinations of canonical variates that would maximally discriminate group memberships.

The major underlying assumptions of DA were: (i) each predictor variable is normally distributed; (ii) there must be homogeneity of covariance across the groups; (iii) there must be at least two groups or categories, with each study case belonging to only one group so that the groups are mutually exclusive and collectively exhaustive; (iv) the groups or categories should be defined before collecting the data; (v) the predictor variable(s) used to separate the groups should discriminate quite clearly between the groups so that group or category overlap is clearly non-existent or minimal; (vi) group sizes of the dependents should not be grossly different and should be at least five times the number of independent variables.

The Box's Test of Equality of Covariance Matrices was used to check the assumption of homogeneity of covariance across the groups using $p < .001$ as a criterion.

Mahalanobis distances were used for supporting the classification of canonical variates into distinct groups and for comparing divergence among populations' group centroids in order to determine the degree of segregation with values of Wilk's Lambda closer to zero being the evidence for well separated groups. The Mahalanobis distance is a measure of the distance between a point P and a distribution D. It is a multi-dimensional generalization of the idea of

measuring how many standard deviations away P is from the mean of D. This distance is zero if P is at the mean of D, and grows as P moves away from the mean. Sometimes, the Mahalanobis distance corresponds to standard Euclidean distance in the transformed space. In the discriminant analysis that was performed, each variate that was more than 1.96 Mahalanobis distance units from the centroid group had a less than 5% chance of belonging to that group.

Receiver-Operating-Characteristic curves (ROC) were calculated for every genital cytokine and chemokine that displayed significant univariate differences across the groups, and expressed as area under the curve (AUC) with an asymptotic 95% confidence interval (CI).

A *p* value <0.05 was considered as significant. Data analysis was performed using SPSS® statistical software version 21.0 (SPSS Inc; Chicago, IL).

Results

Concentrations of plasma cytokines/chemokines associated with increased odds for symptomatic vulvovaginal candidiasis in HIV infected and uninfected women:

In univariate analysis, Table 1 depicts the mean concentrations of all plasma cytokines/chemokines that displayed significant differences across the 4 groups. Five potential predictor variables (IL-1β, MIP-1β, TGF-β1, TGF-β2 and TGF-β3) were identified by ANOVA as meeting the initial DA assumption of being normally distributed (Table 1). Higher mean levels of plasma IL-1β, TGF-β1 and TGF-β2 were significantly observed in HIV+VVC- group whilst higher mean levels of plasma MIP-1β and TGF-β3 were seen in HIV+VVC+ group.

After adjusting for multiple comparisons, those 5 potential predictor variables were confirmed to display significant differences across the study groups using post hoc Bonferonni pairwise tests (Figure 1): HIV+VVC+ group had a significantly lower mean level of IL-1β compared to the mean level of HIV+VVC- group ($p < 0.0001$) and HIV-VVC- group ($p = 0.023$), suggesting that lower concentrations of IL-1β were strongly associated with symptomatic VVC in HIV-infected women (Figure 1A).

When comparing the mean levels of MIP-1β, the HIV-VVC- group had a significantly lower mean concentration than the mean value of HIV+VVC- group ($p = 0.012$), and more importantly of HIV+VVC+ group ($p < 0.0001$) (Figure 1B).

The highest mean concentration of TGF-β1 was observed in HIV+VVC- group as compared to the mean level of every other group ($p < 0.0001$) (Figure 1C).

A significant difference in mean levels of TGF-β2 was observed when comparing HIV-VVC- group with HIV-VVC+ group ($p < 0.006$); and also between HIV-VVC- group with HIV+VVC- group ($p < 0.006$) (Figure 1D).

The mean level of TGF-β3 was significantly higher ($p = 0.013$) in HIV+VVC+ group and also in HIV-VVC+ group as compared to the mean level of every other remaining group, suggesting that higher levels of TGF-β3 were strongly associated with symptomatic VVC irrespective of HIV sero-status (Figure 1E).

Table 1: Comparisons of the plasma mean levels (in pg/ml) of cytokines and chemokines across the study groups by HIV/VVC status

		Study Groups				
		HIV- and VVC- Mean ± SEM	**HIV- and VVC+** Mean ± SEM	**HIV+ and VVC-** Mean ± SEM	**HIV+ and VVC+** Mean ± SEM	**ANOVA** p value (unadjusted)
cytokine types	**Cytokine limit of detection**					
Pro-inflammatory	(pg/mL)					
IL-1β	0.6	4.01 ± 0.70	3.04 ± 0.60	5.71 ± 0.01	2.07 ± 0.21	<0.0001
MIP-1β	2.4	68.11 ± 1.17	128.86 ± 17.07	158.24 ± 0.01	182.20 ± 22.85	<0.0001
G-CSF*	1.7	0.85 ± 0.00	3.43 ± 1.81	4.44 ± 0.002	2.16 ± 1.35	0.038*
GM-CSF*	2.2	48.12 ± 0.76	30.04 ± 9.27	1.10 ± 0.00	88.18 ± 58.75	0.018*
IFN-γ*	6.4	28.02 ± 9.33	96.66 ± 73.35	3.20 ± 0.00	10.60 ± 4.35	0.032*
IL-2*	1.6	3.07 ± 1.02	18.93 ± 13.06	0.8 ± 0.00	0.8 ± 0.00	0.003*
IL-8**	1	6.05 ± 1.092	7.16 ± 0.94	9.79 ± 0.001	7.07 ± 0.91	0.015**
TNF-α**	6	27.31 ± 7.38	24.23 ± 17.07	6.86 ± 0.001	3.28 ± 2.31	< 0.0001**
MCP-1**	1.1	101.15 ± 2.05	100.33 ± 25.11	70.88 ± 0.001	75.92 ± 11.11	0.075**
IL-6*	2.6	19.24 ± 4.18	33.53 ± 20	5.64 ± 0.001	6.76 ± 0.99	0.007*
IL-7	1.1	15 ± 1.96	53.07 ± 39.57	16.99 ± 0.001	16.33 ± 1.78	0.502
IL-12	3.5	10.14 ± 3.37	84.98 ± 70.64	3.38 ± 0.001	13.36 ± 5.96	0.383
IL-17	3.3	1.65 ± 0.00	3.22 ± 0.81	1.65 ± 0.00	1.65 ± 0.00	0.374
Anti-inflammatory						
IL-4	0.7	0.84 ± 0.22	1.03 ± 0.26	1.03 ± 0.001	0.92 ± 0.19	0.909
IL-5	0.6	2.12 ± 0.50	7.16 ± 4.04	2.45 ± 0.001	2.79 ± 0.62	0.327
IL-13	0.7	2.43 ± 0.15	27.18 ± 22.83	2.37 ± 0.001	3.27 ± 0.51	0.38
IL-10*	0.3	6.10 ± 0.32	74.66 ± 46.85	2.06 ± 0.003	7.81 ± 4.04	<0.0001*
TGF-β1	1.7	13.77 ± 4.58	37.85 ± 17.47	187.59 ± 0.01	33 ± 16.83	<0.0001
TGF-β2	14.7	40.33 ± 9.62	59.44 ± 8.29	86.71 ± 0.01	69.79 ± 9.06	0.008
TGF-β3	2.8	1.4 ± 0.00	4.30 ± 1.19	1.4 ± 0.00	4.55 ± 1.64	0.013

SEM: standard error of the mean (in pg/ml)
VVC+: presence of vulvo-vaginal candidiasis infection; VVC- = absence of vulvo-vaginal candidiasis infection
HIV: human immune deficiency virus
IL = interleukin; MIP = macrophage inflammatory protein; TGF = transforming growth factor
IFN = interferon; G-CSF = granulocyte colony-stimulating factor
GM-CSF: granulocyte-macrophage colony-stimulating factor
*Mann-Whitney U non-parametric tests; **Kruskal-Wallis non-parametric tests

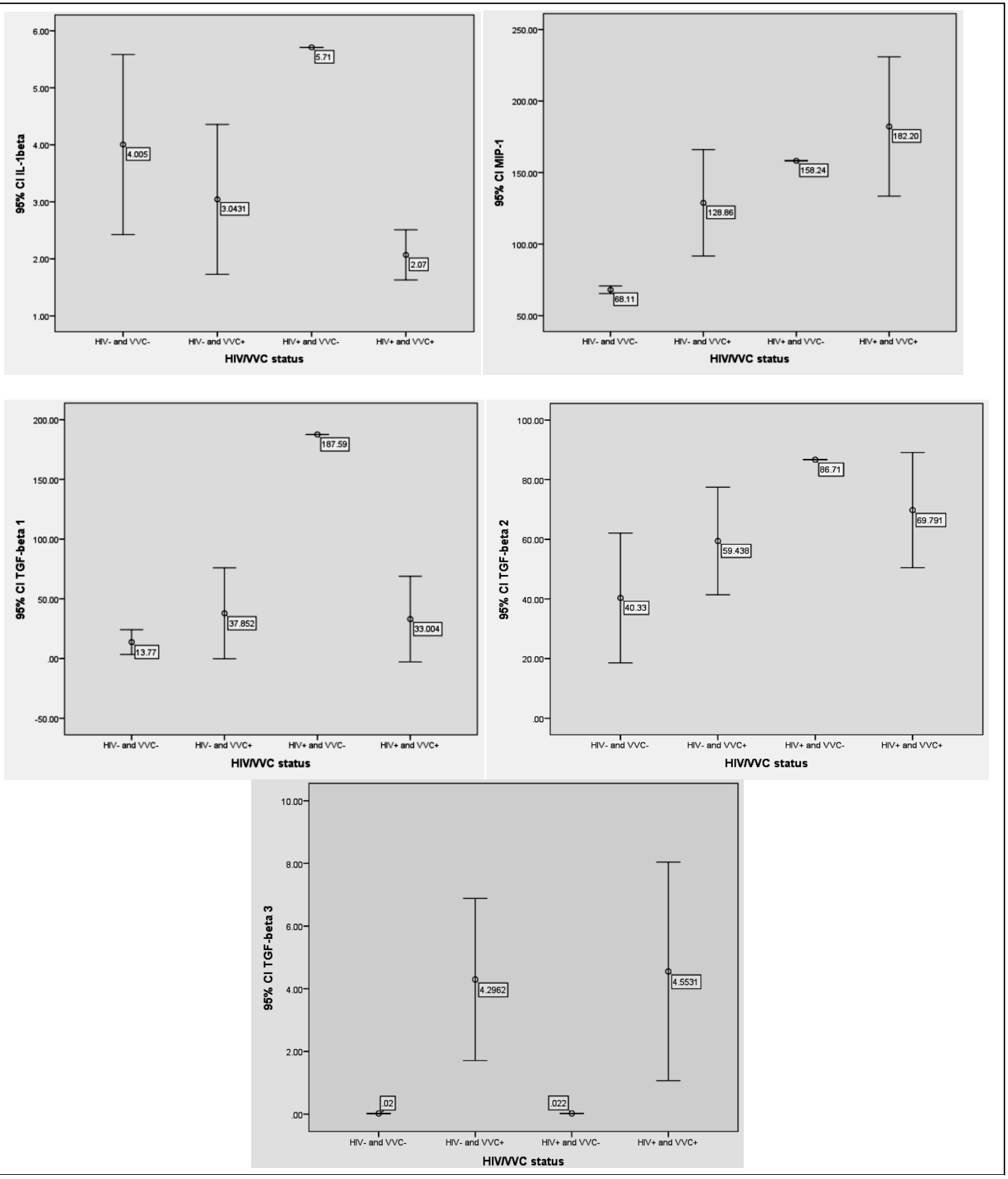

Figure 1 Multiple Comparisons of the mean concentrations of IL-1β, MIP-1β, TGF-β1, TGF-β2 and TGF-β3 using Bonferroni Post hoc tests

Canonical discriminant analysis (CDA) was carried out with normally distributed variables that showed significant univariate associations. CDA identified 3 canonical functions and 3 significant variates (MIP-1β, TGF-β1 and TGF-β3) able to discriminate between the 4 study groups.

The obtained 3 discriminant functions had Eigen values of 3.516; 0.864; and 0.022 with canonical correlations of 0.882; 0.681; and 0.147 respectively. The larger the Eigen value represents more shared variance in the linear combination of variables. Of the 3 identified canonical functions, the first 2 functions contributed a total of 99.5% of the total variance, which easily stratifies into the 4 study groups (HIV-VVC-; HIV-VVC+; HIV+VVC; and HIV+VVC+).

The multivariate Box M test was used and confirmed that variables followed a multivariate normal distribution, and the variance-covariance matrices were equal (homogeneity of variances/covariances) across the 4 study groups [Box's M (5.49), p (.57) > α (.001)]. The Wilks' Lambda value was significant: 0.116; $\chi 2 = 95.78$; $p < .0001$ (Table 2).

Table 2: Wilk's Lambda Values

Test of Functions	Wilk's Lambda	Chi-square Test	df	P-Value
1 through 3	0.116	95.78	9	<0.0001
2 through 3	0.525	28.694	4	<0.0001
3	0.978	0.978	1	0.323

Functions' coefficients were computed and used in deciding which variable predicted group membership. Comparing the values between groups, the higher coefficient means the predictor variable attributes more for that group (Table 3). TGF-β1 strongly predicted HIV+VVC- group while TGF-β3 and MIP-1β predicted HIV+VVC+ group as shown in Table 3.

Table 3: Classification results: functions' coefficients

	Predicted Group Membership			
	HIV-VVC-	HIV-VVC+	HIV+VVC-	HIV+VVC+
Predictor variables				
TGF-β1	-0.003	-0.016	0.084	-0.028
TGF-β3	0.135	0.598	-0.345	0.792
MIP-1β	0.022	0.051	0.027	0.072
(constant)	-2.113	-5.683	-11.41	-9.249

Standardized canonical discriminant function coefficients were used for predicting functions defining group centroids (Figure 2). The Canonical group means (also called group centroids) are the mean for each group's canonical observation scores. The larger the difference between the canonical groups implies the better the predictive power of the canonical discriminant function in classifying observations.

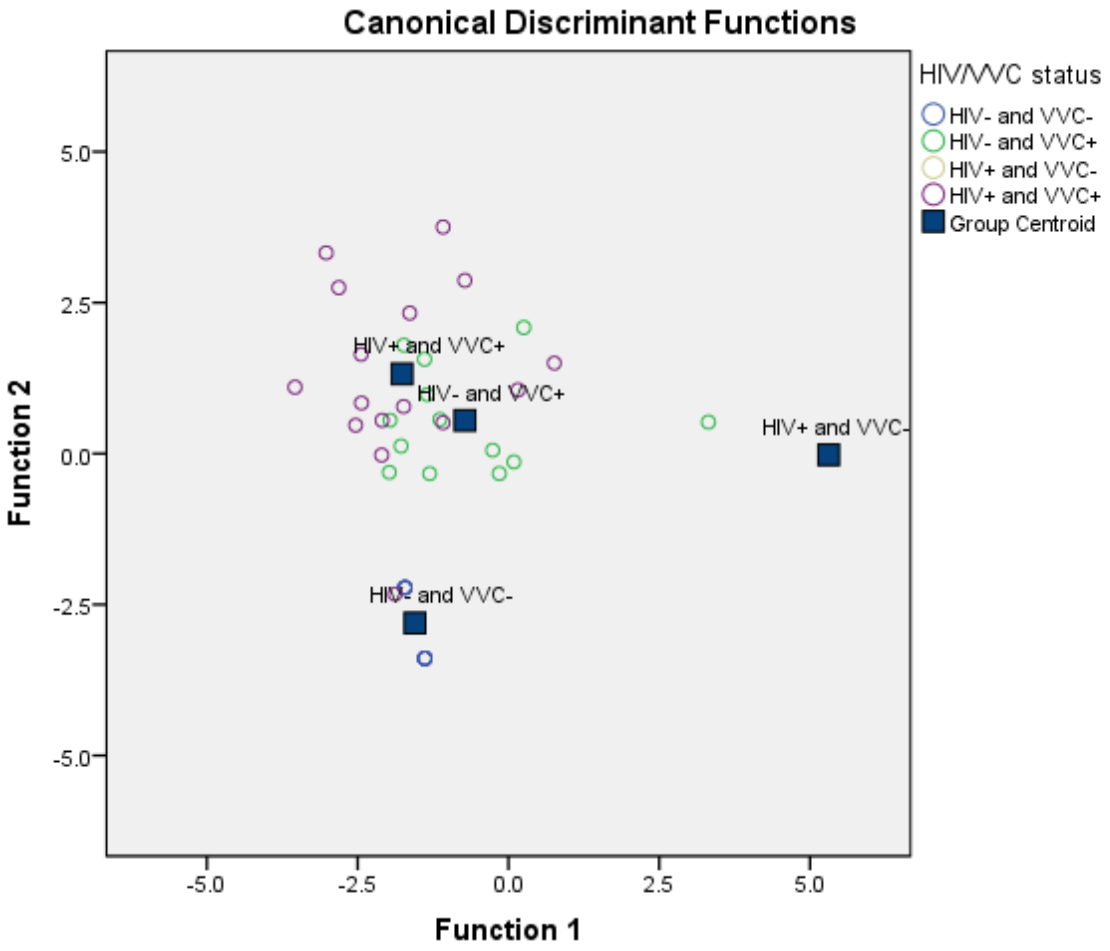

Figure 2 Canonical Discriminant Functions displaying Centroid Groups: 4 study groups (HIV-VVC-; HIV-VVC+; HIV+VVC; and HIV+VVC+) discriminated by 2 CDA functions made of 3 significant variates (MIP-1β, TGF-β1 and TGF-β3) which contributed 99.5% of the total variance.

Predictor variables maximizing Mahalanobis distances between two closest groups are depicted in Table 4.

Table 4: Predictor variables displaying maximum Mahalanobis distances between the two closest groups (HIV-VVC+ and HIV+VVC+)

		Mahalanobis distances (D squared)		
		Exact F		
Predictor variables	**SD value**	**df1**	**df2**	**P-value**
MIP-1β	0.93	1	45	0.34
TGF-β3	3.271	2	44	0.047
TGF-β1	2.703	3	43	0.05

In Table 3, it was found that MIP-1ß and TGF-ß3 significantly predicted the HIV+VVC+ group while TGF-ß1 predicted the HIV-VVC+ group. Taking into account that each variate that was more than 1.96 Mahalanobis distance units away from a specific centroid group had a less than 5% chance of belonging to that group, from the Table 4 above, it can be concluded that MIP-1ß was 0.93 Mahalanobis distance units away from HIV+VVC+ group, TGF-ß3 was 3.271 Mahalanobis distance units away from HIV-VVC+ group and TGF-ß1 was 2.703 Mahalanobis distance units away from HIV+VVC+ group.

Concentrations of genital cytokines/chemokines associated with increased odds for symptomatic vulvovaginal candidiasis in HIV infected and uninfected women:

In univariate analysis, of the 20 cytokines/chemokines measured from cervico-vaginal secretions, mean levels of IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-12, IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, MCP-1, TNF-α were normally distributed and showed significant differences across the groups using ANOVA (Table 5).

After adjusting for multiple comparisons using post hoc Bonferroni pairwise tests, mean concentrations of IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-12, IL-13, IL-17, G-CSF, and MCP-1 were confirmed to display significant differences across the study groups. With the exception of the proinflammatory chemokine IL-8 that had higher mean genital levels in HIV-VVC+ group, all other pro-inflammatory (IL-6, G-CSF and MCP-1), Th1 (IL2 and IL12), Th2 (IL4, IL5, and IL13), and Th17 (IL17) cytokines displayed higher mean levels in HIV+VVC+ group.

Table 5: Comparisons of cervico-vaginal secretions' mean levels (in pg/ml) of cytokines distribution across the study groups

		Study Groups				
		HIV- and VVC- Mean ± SEM	HIV- and VVC+ Mean ± SEM	HIV+ and VVC- Mean ± SEM	HIV+ and VVC+ Mean ± SEM	ANOVA P-value
cytokine types	**Cytokine lower detection limit (pg/mL)**					
Pro-inflammatory						
G-CSF	1.7	3.82±1.3	14.075±3.42	0.85±0.00	21.894±5.62	0.002
GM-CSF	2.2	42.24±2.55	57.81±3.59	42.34±0.001	57.98±5.34	0.007
IFN-γ	6.4	7.148±3.63	29.206±8.62	3.91±0.001	55.62±20.035	0.037
IL-2	1.6	0.8±0.00	4.795±1.81	0.80±0.00	6.77±2.12	0.022
IL-8	1	26.486±8.156	494.553±148.95	10.84±0.001	320.67±97.65	0.004
TNF-α	6	13.106±6.48	44.079±10.04	14.07±0.001	56.32±11.09	0.003
MCP-1	1.1	2.35±0.34	8.03±2.23	2.32±0.001	13.21±4.27	0.037
IL-1β**	0.6	127.61± 59.79	1975.77±779.89	143.11±0.001	2181.43±803.23	0.001**
IL-7	1.1	0.55±0.00	0.963±0.31	0.57±0.00	1.076±0.3	0.012
MIP-1β	2.4	8.86±2.39	12.23±3.43	8.13±0.001	16.67±3.95	0.235
IL-12	3.5	1.916±0.58	3.127±0.71	1.75±0.00	4.15±0.82	0.001
IL-17	3.3	5.799±2.27	19.003±4.34	1.95±0.001	20.53±4.33	0.002
IL-6	2.6	1.911±0.548	5.582±1.35	1.38±0.001	11.583±3.74	0.017
Anti-inflammatory						
IL-4	0.7	0.59±0.18	2.062±0.44	0.62±0.001	2.27±0.46	0.003
IL-5	0.6	0.257±0.09	0.394±0.057	0.30±0.00	0.524±0.08	0.004
IL-13	0.7	0.83±0.22	1.56±0.22	0.47±0.001	1.78±0.26	<0.0001
TGF-β1**	1.7	4.72±1.92	4.16±1.6	6.88±0.001	1.588±1.09	0.024**
TGF-β2**	14.7	13.034±1.16	11.96±1.21	8.39±0.001	12.52±2.09	0.012**
TGF-β3**	2.8	2.51±0.44	8.56±6.003	1.42±0.001	2.32±0.25	0.001**
IL-10	0.3	0.902±0.078	0.978±0.09	0.9±0.001	1.249±0.17	0.135

SEM: standard error of the mean (in pg/ml)
VVC+: presence of vulvo-vaginal candidiasis infection; VVC- = absence of vulvo-vaginal candidiasis infection
HIV: human immune deficiency virus; MIP = macrophage inflammatory protein; TGF = transforming growth factor
IL = interleukin; IFN = interferon; G-CSF = granulocyte colony-stimulating factor
GM-CSF: granulocyte-macrophage colony-stimulating factor; TNF = tumor necrosis factor
**Kruskal-Wallis non-parametric tests

Furthermore, after performing the canonical discriminant analysis from all normally distributed variables that displayed significant univariate associations, only one canonical function composed of one cytokine (IL-13), an immune-allergic mediated anti-inflammatory cytokine, was shown to significantly predict a case classification into HIV+VVC+ group.

One discriminant function had Eigen value of 0.48 with a canonical correlation of 0.57. This function contributed 100% of the total variance. Box's M (2.063), p (.37) > α (.001); Wilk's Lambda = .677, $\chi 2 = 17.76$; $p < .0001$.

In order to identify cytokines/chemokines able to predict case classification into other groups, Receiver-Operating-Characteristic curves (ROC) were calculated for every cytokine and chemokine that displayed significant univariate differences across the groups, and expressed as area under the curve (AUC) with an asymptotic 95% confidence interval (CI).

ROC identified that in HIV uninfected women, at an optimal cut-off level ≥ 38.8 pg/ml, genital GM-CSF perfectly discriminated women with and without symptomatic VVC (AUC = 0.831 95% CI 0.660 – 1.000; 92.3% sensitivity and 70% specificity, p = 0.008) (Figure 3A). However in HIV infected women, at an optimal cut-off level ≥ 0.5 pg/ml, genital IL-13 perfectly discriminated women with and without symptomatic VVC (AUC = 0.938 95% CI 0.819 – 1.000; 93.8% sensitivity and 100% specificity, p < 0.0001) (Figure 3B).

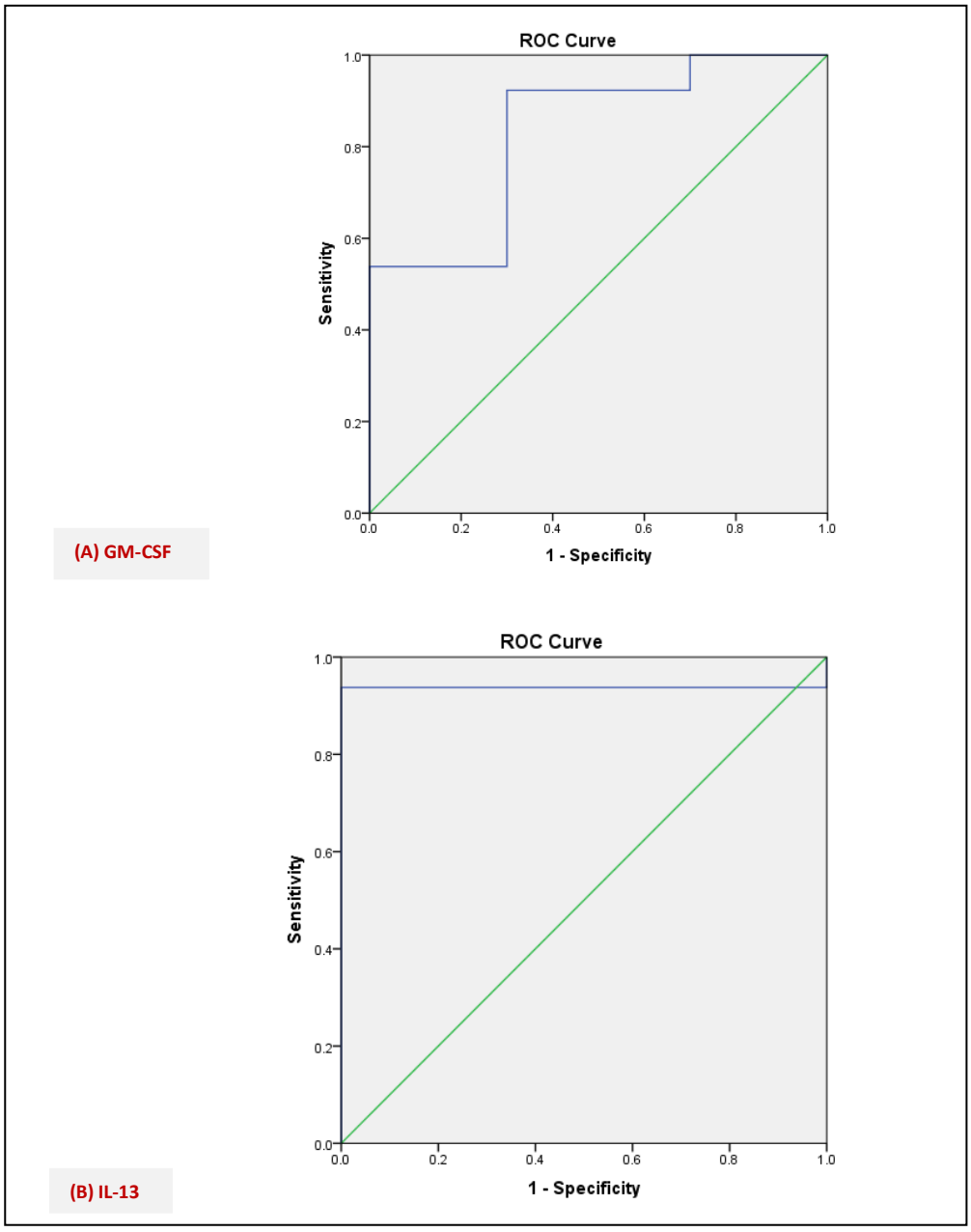

Figure 3 Receiver- Operating-Characteristic curves for GM-CSF (AUC = 0.831 95% CI 0.660 – 1.000; 92.3% sensitivity and 70% specificity, p = 0.008) and IL-13 (AUC = 0.938 95% CI 0.819 – 1.000; 93.8% sensitivity and 100% specificity, p < 0.0001)

Discussion

This study showed that increased systemic levels of MIP-1β and TGF-β3 were strong predictors for HIV+VVC+ group while TGF-β1 strongly predicted HIV+VVC- group. At genital level, an increased IL-13 level was predictive of HIV+VVC+ group.

HIV infected women had higher levels of plasma MIP-1β when co-infected with VVC as compared to women without VVC. MIP-1β, a cc family member of chemokines, is produced by stimulated CD4 and CD8 T cells, NK cells, and monocytes (16). HIV induces lymphoid activation despite CD4+ T cell depletion, reflected by increased production of chemokines (i.e. MIP-1β), and other markers of T cell activation such as CD38 and HLA-DR (17).

This chronic immune activation is usually associated with HIV viral persistence and increased cases of opportunistic infections such as mucosal candidiasis. In vitro, this chemokine is known to suppress replication of macrophage-tropic (but not lymphotropic) HIV strains as a result of a competitive inhibition of HIV attachment to CCR5 (18, 19). As HIV infection progresses to AIDS, a change in coreceptor usage is observed. Macrophage-tropic HIV strains (which use CCR5) are replaced by lymphotropic strains (which prefer CXCR4) (18, 19).

VVC occurs in advanced HIV infection as indicated by the presence of higher concentrations of MIP-1β. Previous studies have shown that persons with AIDS coinfected with either candidiasis or cryptococcosis had paucity of inflammatory cells not as a result of a defective release of MIP-1β but by a failure of the released chemokine to attract leukocytes (20).This could be due, at least

in part, to the quantitative defect in circulating CD4+ T cells and the qualitative defects in phagocyte function.

While the production of transforming growth factor β (TGF-β) has been documented in mice infected with *C. albicans* and is known to suppress different phagocyte functions(21), only circulating TGF-β3 was found to have a concentration significantly higher in HIV+VVC+ group as opposed to TGF-β1 and TGF-β2 levels. Although TGF-β is generally thought to influence the differentiation of naive $CD4^{+}$T cells toward the Th2 profile, it has been also shown to inhibit Th2 differentiation by inhibiting GATA-3 expression at transcriptional level (22). This might explain why TGF-β1 was a predictor of HIV+VVC- group among our study population.

Circulating TGF-β3 levels were also significantly higher in HIV-VVC+ group in addition to HIV+VVC+ group, suggesting that this T reg cell induced cytokine was associated with symptomatic VVC irrespective of HIV serostatus. TGF-β3 has been reported as an important inhibitory cytokine able to down-regulate activated monocytes and macrophages, thus suppressing IFN-γ-induced production of nitric oxide, which would favor the development of candidiasis (23). TGF-β3 is known to strongly influence the differentiation of naive CD4+ T cells toward the Th2 responses (24).

Although not shown to be a predictor by discriminant analysis, lower concentration of circulating IL-1β, a protective proinflammatory cytokine, was strongly associated with HIV+VVC+ group when performing Bonferroni multiple comparison tests. Data from this study provide additional evidence that IL-1β play an important role in host defense against *C.albicans*. Different

mechanisms through which IL-1β confers protection have been reported. First, studies have shown that IL-1β can enhance granulopoiesis and influx of neutrophils to the site of infection (25, 26). Exogenous administration of IL-1β has been reported to induce peripheral blood granulocytosis (25, 26). Moreover, neutrophil recruitment in response to heat-killed *C.albicans* resulted from the effect of endogenous IL-1β observed in the early phase of neutrophil influx (25, 26).

Additionally, down-regulation of IL-1β has been reported to affect the phagocytic function of neutrophils (25, 26). When using a mouse model of candidiasis, others have demonstrated that IL-1β-deficient neutrophils had impaired superoxide production (25, 26). Furthermore, they found that IL-1β induces the production of IL-8, another proinflammatory cytokine known to stimulate neutrophil function; IL-1β-deficient mice had impaired PMN function and reduced anticandidal defense mechanism (25, 26).

At the vaginal mucosal level, the immune response as measured by genital cytokine/chemokine levels was completely different from the systemic response. This is in favour of a previously hypothesized "theory of compartmentalization" of vaginal cell-mediated response from systemic cell-mediated immunity (4, 27): T lymphocytes found in the vaginal mucosa are phenotypically distinct from those in the peripheral blood and the proportion of γ/δ TCR+ T cells in the vagina is significantly higher (15-50% γ/δ TCR+ T cells) than α/β TCR+ T cells compared to the periphery. In addition, after measuring HIV specific CD8+ T cells, it was reported that there was no correlation between plasma and cervico-vaginal responses (28).

Canonical discriminant functions and ROC curves identified genital IL-13 concentration as predictive of HIV+VVC+ group. HIV-infected women had significantly higher mean levels of IL-13 from their genital secretions as compared to HIV uninfected women, suggesting the possibility of an allergic reaction during *Candida* spp. induced vaginitis.

This is in support of the hypothesis that an allergic reaction mediated by *Candida*-specific immunoglobulin (Ig)E might explain the presence of frequent and severe genital symptoms in HIV infected women diagnosed with symptomatic VVC (29).

Factors such as chemical contact, atopy, local allergy, or hypersensitivity reactions have been reported as able to alter the vaginal milieu and facilitate transformation from asymptomatic colonisation to symptomatic vaginitis (30). It has been recently postulated that symptomatic VVC might not be an opportunistic infection or a result of an immunodeficiency but a hypersensitivity response to a commensal organism, Candida spp (29).

In addition to IL-13, other local Th2 cytokines shown to display significant levels in HIV+VVC+ by Bonferroni multiple comparison tests in this study and known to cause strong allergic disorders were IL-4 and IL-5.

IL-4 is a cytokine that induces differentiation of naive helper T cells (Th0 cells) to Th2 cells. It has been suggested that basophils may be the effector cell (31). IL-4 is closely related and has functions similar to IL-13, and thus an overproduction of IL-4 is associated with allergies (31). IL-4 is a key regulator in humoral and adaptive immunity (31). It induces B-cell class switching to IgE, and up-regulates MHC class II production (31).

In addition to IL-4, IL-5 is produced by Th2 cells and mast cells (31). IL-5 is the key cytokine in eosinophil production, activation and localization. It is associated with several allergic disorders (31).

All main Th2-type cytokines (IL4, IL5 and IL13) which are associated with the promotion of IgE (severe allergies) and eosinophils response were significantly associated with symptomatic VVC in HIV-infected women suggestive of a local hypersensitivity response to Candida antigens (31).

Th17 helper cells mediate genital host immunity (via production of IL17) was also shown to be significantly associated with symptomatic VVC in HIV infected women. Th17 is triggered by IL6 and TGF-β. The latter was not significantly observed in the vaginal secretions. The main Th17 effecter cells are neutrophils and IL-17 CD4+ T cells. If Th17 cells lose their stability they become highly proinflammatory but might also cause complement-mediated hypersensitivity reaction (32).

An effective phagocytic response absolutely depends on a balance between pro-and anti-inflammatory cytokines and on T-helper cells. Cytokine imbalances between Th1/Th2 groups have been reported in patients with candidiasis, co-infected with HIV (33). In this present study, local proinflammatory cytokines found to be significantly associated with symptomatic VVC in HIV infected women were G-CSF, MCP-1 and Th1 response (IL2 and IL12).

Studies have shown that G-CSF plays a key role in the regulation of granulopoesis (34, 35), and mice-deficient G-CSF genes have shown to possess only 20-30% of circulating neutrophils (34, 35). MCP-1 is known to attract monocytes/macrophages at the site of infection (31).

IL2 is produced primarily by activated CD4+ T cells and its presence plays an important role in the long-term survival of activated helper T cells and CD8+ cytotoxic T cells. IL12 plays an important role in the mediation of the cytotoxic activity of NK cells and CD8+ cytotoxic T cells. It participates in the differentiation of naïve T cells in Th1 cells (31).

Taken together, after adjusting for multiple comparisons using post hoc Bonferroni pairwise tests, mean concentrations of genital IL-6, IL-8, IL-10, IL-17 and GM-CSF were significantly higher in HIV infected women co-infected with VVC. In the presence of the observed higher levels of TGF-β3 and IL-6, naïve CD+ T cells differentiate to Th17 cells (21, 36-39). The latter produce IL-6, IL-8 and IL-17 as effector cytokines. IL-17 coordinates tissue inflammation through the induction of proinflammatory cytokines and chemokines, such as IL-6 and IL-8. The major effector function of IL-8 is the activation and recruitment of neutrophils and GM-CSF to the site of infection. TGF-β favors the production of higher levels of IL-10 as also shown in our study. It has been demonstrated that in the presence of TGF-β and IL-10, CD4+ T cells will differentiate to regulatory T cells (Treg cells) resulting in the production of more IL-10.

Although some Th1 cytokines (IL-2 and IL-12) were significantly higher among HIV infected women with VVC, IFN-γ, "a prototype Th1 cytokine" was shown to be very low. Therefore, data from this study strongly support the hypothesis that Th17 cells rather than Th1/Th2 paradigm were key players in the defense and development of effector responses in HIV infected women

with symptomatic VVC. In addition, the hypothesis that VVC in HIV infected women can be a result of either *Candida*-specific IgE production or histamine-induced prostaglandin E2 as demonstrated by higher levels of IL-4, IL-5 and IL-13 in this study might be relevant to only a group of women with some predisposing factors.

In HIV uninfected women however, genital granulocyte/ macrophage colony-stimulating factor (GM-CSF) levels strongly predicted HIV-VVC+ group. GM-CSF plays a critical role in the regulation of granulopoesis (34, 35), and an over expression of GM-CSF as observed in the genital secretions of HIV uninfected women in our study population could have been a response to an active VVC infection as it is unequivocally known that neutrophils predominate the first phase of a host's response to *Candida albicans* (34, 35). In addition, IL8, a chemokine responsible for the attraction of neutrophils was also significantly associated with HIV-VVC+ group. IL8 is produced by activated macrophages in response to toll-like receptor agonists (31).

Conclusions and Limitations

In HIV infected women, VVC was strongly predicted by: (i) circulatory MIP-1β, a marker of chronic immune activation observed during advanced HIV-induced immunosuppression; (ii) circulatory TGF-β3, a key factor for Th17 differentiation at the presence of IL-6 but can also differentiate to Treg cells and becomes a strong down-regulatory and anti-inflammatory response at the presence of IL-10; (iii) vaginal IL-13, an allergic mediate Th2 cytokine.

The limitation of this study is the cross sectional design. Another limitation is the fact that a discriminant function analysis that was performed did not take into account CD4 counts of the study participants. The two closest centroid groups were HIV+VVC+ and HIV-VVC+. It is possible that HIV+VVC+ women on HAART with CD4 count > 350 cells/mm3 could have an immune response relatively comparable to HIV-VVC+ group, thus bringing group centroids for the two study groups more closer. IgE levels in the genital tract of women were not measured in order to backup the theory of hypersentivity. **Conflict of interest:** none.

Acknowledgments:

We express our gratitude to the laboratory staff – Immunology Department, National Health Laboratory Services at the University of Pretoria for their assistance in the use of Luminex.

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Expression of Toll-like Receptor (TLR)-2 and TLR4 in Monocytes following Stimulations by Genital Secretions of HIV infected and uninfected Women with Symptomatic Vulvo-Vaginal Candidiasis

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International Journal of Medicine and Medical Sciences 2014; 6(5): 134-139.

Abstract

Background: Vulvo-vaginal candidiasis (VVC) is a common condition in HIV-infected women. Toll-like receptor (TLR) 2 and TLR4 are key pattern-recognition receptors of the innate immune system in sensing *Candida albicans*. The aim of this study was to assess the expression of TLR2 and TLR4 signaling pathways in HIV-infected and uninfected women with VVC.

Methods: Cervico-vaginal fluids (CVF) were obtained from 7 HIV infected and 11 HIV uninfected clinic attendees in KwaZulu-Natal between June 2011 and December 2011. VVC was diagnosed clinically and confirmed by Gram stain and culture of genital samples. Monocytes were isolated from a healthy adult volunteer, pre-incubated with anti-TLR2, anti-TLR4 and a combination of anti-TLR2/anti-TLR4 monoclonal antibodies. Monocytes were then stimulated by CVF. Levels of cytokines were measured by Luminex® Multiplex Immunoassays.

Results: Compared with baseline concentrations, stimulation with CVF of HIV+VVC+ women post-TLR2 blockage decreased IL-10 production by 44.76% while decreasing the production of IL6 below its detection limit. Using paired T tests, there was no significant difference in the reduction of the concentrations of IL-10 (P = 0.3) when comparing stimulation by CVF of HIV+VVC+ versus stimulation by CVF of HIV-VVC+ patients. No significant changes in cytokine/chemokine levels were observed post-TLR4 blockage after stimulation of monocytes by CVF of VVC+ women irrespective of their HIV serostatus. There was a linear correlation between genital HIV RNA loads and mean level production of IL-6 (r = 0.722; R^2 = 0.679; P = 0.067) as well as IL-8 (r = 0.910; R^2 = 0.833; P = 0.004) post-TLR2 blockage.

Conclusion: Findings suggest potential roles of TLR2 (but not TLR4) in the pathogenesis of VVC among HIV-infected women.

Key words: symptomatic VVC, HIV, TLR2 and TLR4.

Introduction

In HIV infected women, symptomatic vulvo-vaginal candidiasis (VVC) is seen to be frequent and less effectively responsive to conventional anti-fungal therapy (1, 2). The pathogenesis of VVC in HIV infected women is not well understood. *Candida albicans* has been reported as the cause of VVC in 85-95% of cases (3). The cell wall of *Candida albicans* is composed of pathogen-associated molecular patterns (PAMPs), especially polysaccharides like chitin, 1,3-β-glucans and 1,6-β-glucans, and proteins that are heavily mannosylated with mannan side-chains.

Pathogen recognition receptors (PRRs), such as the Toll-like receptors (TLRs) and C-type lectins (CLRs) on the surface of antigen presenting cells (APCs) are able to recognize PAMPs. Studies have shown that TLR2 recognizes phospholipomannans; TLR4 recognizes O-linked mannans; and macrophage mannose receptor (MMR) recognizes N-linked mannans (4). Whilst the CLR Dectin-1 recognizes β-glucan, CLR Dectin-2 recognizes mannose residues (5, 6).

Immune cell populations involved in recognition of *C. albicans* during the innate immune response include monocytes, macrophages and neutrophils (7-10). Dendritic cells are crucial for processing of and antigen presentation to T cells, and therefore to activation of specific immunity (7-10). This recognition of *C.albicans* by immune cells is done mainly through TLRs (7). The latter are involved in inflammatory responses induced by *C. albicans*, of which TLR2 and TLR4 are the most studied (4). They are expressed by monocytes, macrophages, dendritic cells, neutrophils, CD4+ T cells and epithelial cells (11). Studies have shown that the activation of TLR2 signal pathways in these antigen-presenting cells (APCs) by ligation of *C. albicans* cell-

wall components such as phospholipomannan leads to the production of cytokines that are able to induce a Th2 cellular response (11-14). Hence, blocking TLR2 with a TLR2-specific antibody before stimulation of monocytes by *C. albicans* was shown to result in diminished release of Th2-associated cytokines (15). In contrary, the activation of TLR4 signal pathways during candidiasis will result to the production of cytokines able to induce a Th1 cellular response. Mannans of *C. albicans* are recognized by TLR4 leading to the production of pro-inflammatory cytokines (16).

It is however unclear whether immune changes observed at vaginal mucosal surfaces of HIV infected women interfere with the pattern recognition process of *C.albicans* by innate immune cells. Hence, the aim of this study was to assess the expression of TLR2 and TLR4 on monocytes following stimulations by genital secretions of HIV infected and uninfected women presented with symptomatic VVC. We hypothesized that HIV infection alters TLR2 (but not TLR4) dependent responses to Candida antigens by monocytes resulting in symptomatic VVC. The underlying assumption for this hypothesis is that the activation of TLR2 signal pathways leads to the production of cytokines that are able to induce a Th2 cellular response that might favor the occurrence of VVC.

Materials and Methods

Study participants:

A total of 18 women (7 HIV-infected and 11 HIV-uninfected), aged ≥18 years, all black Africans, attending Umlazi D clinic, a primary healthcare facility in KwaZulu-Natal between

June 2011 and December 2011 were consecutively enrolled by informed consent. Patients aged < 18 years as well as those menstruating or had visible blood contamination of genital samples were excluded. All patients presented initially with signs and symptoms suggestive of lower genital tract infections (LGTIs), and were thereafter screened for the presence of LGTIs caused by *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplama genitalium*, *Trichomonas vaginalis*, herpes simplex virus type 2 and bacterial vaginosis as described elsewhere(17, 18). The selected 18 participants were retained in the study because they were free from the above aetiological agents causing LGTIs.

The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Ref. BE 224/11).

Diagnostic criteria of symptomatic vulvo-vaginal candidiasis:

Vaginal swab (Becton Dickinson) taken from the anterior fornix was directly plated onto Sabouraud Dextrose agar with chloramphenicol (BBL™ Becton Dickinson) and incubated at 29˚C, 48 h to estimate the relative vaginal fungal burden. The numbers of yeast colonies were recorded as the number of colonies per plate (19).

Cases of symptomatic VVC were defined according to clinical and laboratory criteria as described by the 2011 European (IUSTI/WHO) guideline on the management of vaginal discharge (evidence level III, recommendation grade B) (19).

Isolation of monocytes from peripheral human blood

Using Histopaque® 1077 and Histopaque® 1119 (Sigma-Aldrich®) per manufacturer's instructions, we isolated neutrophils and monocytes from fresh human blood collected from a

healthy donor (neutrophils were used for other experiments not discussed here) (20). After centrifugation and different wash steps, the peripheral blood mononuclear cells (PBMCs) appeared as a dense white band above the Histopaque® 1077 and granulocyte layer. This was removed with a 5 ml plastic pipette. Monocytes were separated from lymphocytes on the basis of their differential adherence to plastic (20). The cells were finally resuspended into 2mL RPMI-1640 medium supplemented with D-glutamate (HiMedia Laboratories, Mumbai, India). The cell count was done using the dye exclusion test. Briefly, a total of 90µl of isotonic phosphate buffered saline (PBS; Oxoid Limited Basingstoke, UK) (pH = 6.9) and 10µl of monocytes was mixed and added to 100µl of Trypan Blue Solution, 0.4% (Gibco®). The number of cells was counted with a haemocytometer under an inverted microscope, and adjusted to $1x10^6$ cells /mL.

Collection of and stimulation of monocytes with cervico-vaginal fluids (CVF)

A vaginal tampon (8 Ks), Tampax Regular® (Compak) was inserted into the vagina, left in situ for 3 minutes, and then placed into a sterile container containing 10 mL of phosphate buffered saline (PBS; Oxoid Limited Basingstoke, UK) (pH = 6.9). Vaginal fluid was expressed using an autoclaved wooden tongue depressor and filtered through a 0.22 µm Costar Spin-X cellulose acetate filter membranes (Sigma).

In testing the roles of TLR 2 and TLR 4, monocytes were pre-incubated (1h at 37°C) separately with anti-TLR2 (Abcam®) and anti-TLR4 (Abcam®) specific monoclonal antibodies before stimulation with CVF or sterile normal saline (negative control) into 96 wells tissue culture plates. A total of 500µl of $1x10^6$ monocytes/ mL were pre-incubated with anti-TLR2 (50µl). Another 500µl of $1x10^6$ monocytes/ mL were pre-incubated with anti-TLR 4 (50µl). Another

500µl of $1x10^6$ monocytes/ mL were pre-incubated with a mixture of 50µl of anti-TLR 2 and 50µl of anti-TLR 4 antibodies. We also used 500µl of $1x10^6$ monocytes/ mL pre-incubated with 50µl of sterile normal saline (no anti-TLR antibodies) as controls.

At the end of the pre-incubation period, 50µl of $1x10^6$ monocytes/ mL were mixed with 50µl of CVF obtained from HIV infected and HIV uninfected women diagnosed with symptomatic VVC. The mixture was incubated into 96 wells plate @ 37˚C and supernatant was collected after 4h and 24h following stimulation in order to measure cytokines and chemokines.

Measurement of Cytokines/chemokines

Concentrations (in pg/ml) of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-1β, TNF-α, TGF-β1, TGF-β2 and TGF-β3 were measured by a multiplex microbead system (Invitrogen, UK) using a Luminex platform. Multiplex cytokine fluorescent bead-based immunoassays were performed using two different commercially available multi-plex luminex kits: Bio-plex pro human cytokine 17-plex assay and Bio-plex pro TGF-β 3-plex assay (Bio-Rad Laboratories, Inc., Parkwood).

The assay sensitivity or limit of detection (pg/mL) was: IL-1β (0.6), IL-2 (1.6), IL-4 (0.7), IL-5 (0.6), IL-6 (2.6), IL-7 (1.1), IL-8 (1.0), IL-10 (0.3), IL-12 (3.5), IL-13 (0.7), IL-17 (3.3), G-CSF (1.7), GM-CSF (2.2), IFN-γ (6.4), MCP-1 (1.1), MIP-1β (2.4), TNF-α (6), TGF-β1 (1.7), TGF-β2 (14.7) and TGF-β3 (2.8); and a 5 PL regression formula was used to calculate cytokine/chemokine concentrations from the standard curves (Bio-Plex Manager software, version 4). Cytokine/chemokine concentrations below the lower limit of detection were reported as the midpoint between the lowest concentrations measured for each cytokine and zero.

Statistical analysis

Data were expressed as means ± standard error of the mean (SEM) for the continuous variables and proportions (percentages) for the categorical variables. When data were normally distributed, analysis of the variance (ANOVA) was used to examine differences between groups. However, non-parametric Mann-Whitney U or Kruskal-Wallis tests were used when data were asymmetrically distributed. Multiple comparisons of means of cytokine/chemokine levels displaying significant differences in univariate analyses across the study groups were performed using Post Hoc Bonferroni pairwise tests considering a type I error rate of 0.05. For normally distributed variables, Paired T tests were used to determine if two sets of variables were significantly different from each other.

Data were analysed using SPSS® statistical software version 21.0 (SPSS Inc; Chicago, IL). All tests were two sided and a *p* value <0.05 was considered as significant.

Results

Of the 18 participants, symptomatic VVC was diagnosed from 7/7 (100%) of HIV infected and 6/11 (54.5%) of HIV uninfected women.

Following blockage of TLR2 with an anti-TLR2 monoclonal antibody, monocytes were stimulated with CVF of HIV-VVC-, HIV-VVC+, and HIV+VVC+ women. Table 1 depicts the mean concentrations of 20 cytokines/chemokines measured with adjusted p-values displaying significant differences across the 3 study groups.

Bonferroni multiple comparison tests were performed for variables that showed significant univariate associations (Table 1). Of the 5 cytokines that showed significant differences across the study groups, 1 anti-inflammatory (IL-10) and 1 pro-inflammatory (IL-6) cytokines were confirmed by Bonferroni tests. The mean level of IL-6 was significantly higher in HIV+VVC+ group as compared to HIV-VVC+ group (P = 0.03) according to Bonferroni test results. In addition, there were significantly higher mean levels of IL-10 (P = 0.003) in HIV+VVC+ group as compared to HIV-VVC- group.

Table 1: Comparison of cytokine/chemokine mean levels across the groups post-TLR2 blockage

Variables	Cytokine detection limit (pg/ml)	Study Groups			ANOVA
		HIV- and VVC- (n = 5) Mean±SEM	HIV- and VVC+ (n = 6) Mean±SEM	HIV+ and VVC+ (n = 7) Mean±SEM	*P*-value
Anti-inflammatory					
IL-10	0.3	0.17 ± 0.001	0.42 ± 0.12	0.69 ± 0.12	0.001
IL-13*	0.7	0.50 ± 0.001	0.60 ± 0.001	0.66 ± 0.04	0.019
IL-4*	0.7	0.61 ± 0.001	0.463 ± 0.043	0.498 ± 0.05	0.085
IL-5*	0.6	0.37 ± 0.001	0.408 ± 0.038	0.348 ± 0.026	0.318
TGF-β1	1.7	187.33±0.001	192.3875±8.49	197.068±21.65	0.903
TGF-β2**	14.7	60 ± 0.001	47.37 ± 4.23	51.6 ± 1.69	0.021
TGF-β3	2.8	2.74±0.001	4.993±0.55	4.974±1.29	0.205
Pro-inflammatory					
IL-1β	0.6	4.23±0.001	1.25±0.31	3.494±1.63	0.217
MIP-1β	2.4	14.45±0.001	12.7075±4.51	18.818±3.73	0.45
G-CSF*	1.7	0.02±0.001	0.02±0.001	0.362±0.342	0.489
GM-CSF	2.2	33.53±0.001	31.44±2.21	28.654±5.34	0.663
IFN-γ*	6.4	3.22±0.001	3.22±0.001	3.438±0.22	0.489
IL-2*	1.6	0.82±0.001	0.82±0.001	0.82±0.001	1
IL-8	1	5.66±0.001	3.29±1.41	12.266±5.95	0.302
TNF-α*	6	3.02±0.001	3.02±0.001	3.02±0.001	1
IL-12*	3.5	1.77±0.001	1.77±0.001	1.77±0.001	1
IL-17*	3.3	1.67±0.001	1.67±0.001	1.67±0.001	1
IL-7*	1.1	0.57±0.001	0.57±0.001	0.57±0.001	1
IL-6*	2.6	2.65 ± 0.001	2.56 ± 0.08	2.08 ± 0.16	0.021
MCP-1**	1.1	1.05 ± 0.001	0.93 ± 0.004	1.01 ± 0.12	0.028

SEM = standard error of the mean; **Kruskal-Wallis non-parametric tests
VVC+: presence of vulvo-vaginal candidiasis infection; VVC- = absence of vulvo-vaginal candidiasis infection
HIV: human immune deficiency virus; TLR2 = toll like receptor 2
IL = interleukin; TGF = transforming growth factor; MCP-1 = monocyte chemoattractant protein 1
*Cytokine value below detectable level but a mid number between 0 and lower detectable limit of the cytokine was given

However, compared with baseline concentrations, stimulation with CVF of HIV+VVC+ women post-TLR2 blockage decreased IL-10 production by 44.76% while decreasing the production of

IL-6 below its detectable limit (Figure 1). Moreover, stimulation with CVF of HIV-VVC+ women also decreased IL-10 production by 57.05% but equally decreased the concentration of IL-6 below its detectable limit (Figure 2). Using paired T test, there was no significant difference in the reduction of the concentrations of IL-10 (P = 0.3) when comparing stimulation by CVF of HIV+VVC+ versus stimulation by CVF of HIV-VVC+ patients.

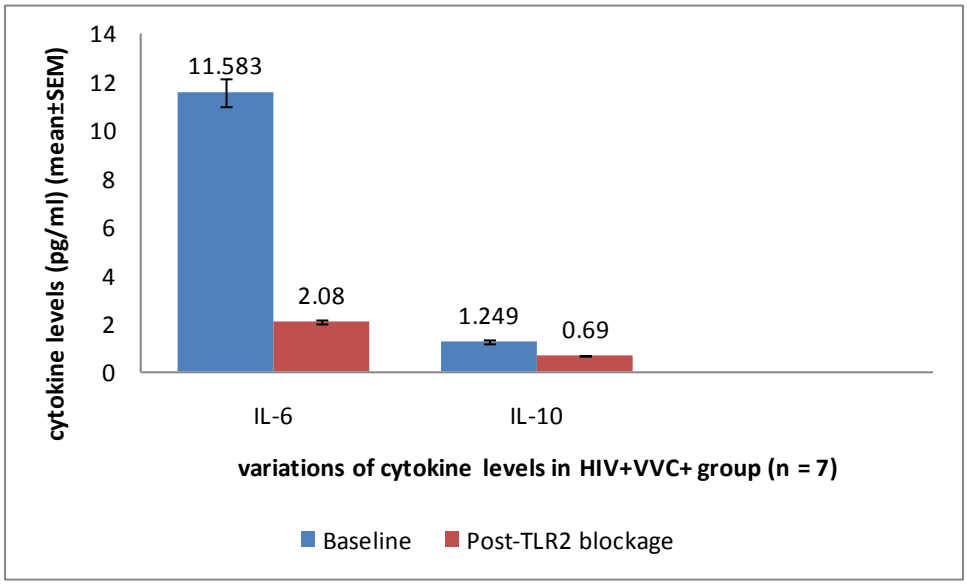

Figure 1 Variations of Cytokine Levels in HIV infected Women

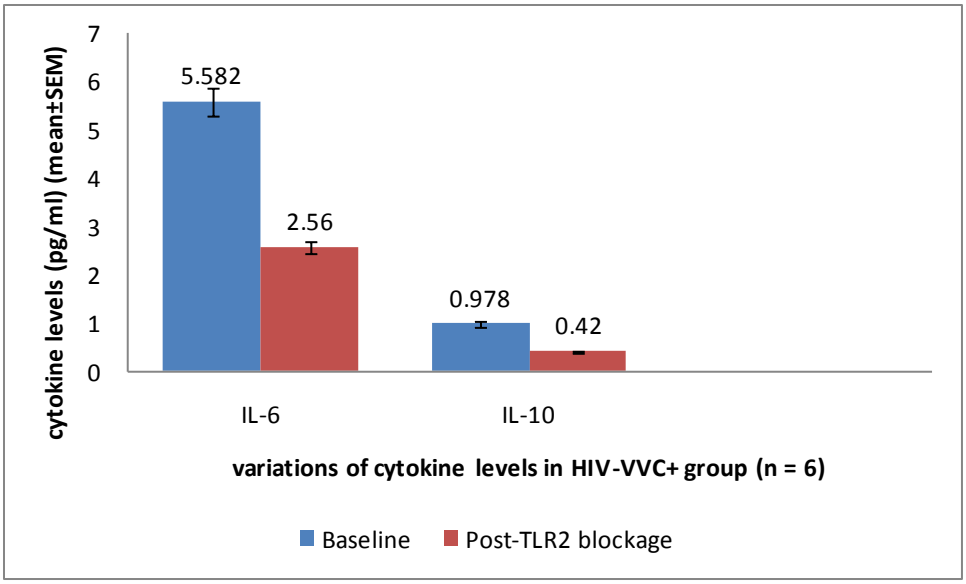

Figure 2 Variations of Cytokine Levels in HIV uninfected Women

Stimulation post-TLR4 blockage by CVF of HIV-VVC+ and CVF of HIV+VVC+ women did not show significant differences of the mean concentrations of all tested cytokines across the study groups.

After blocking TLR2 and TLR4 simultaneously with specific monoclonal antibodies, IL-10 levels decreased when monocytes were stimulated with CVF of HIV+VVC+ as depicted in Figure 3.

Figure 3 also shows that after stimulation with saline (negative control) only IL-10 was detected in HIV+VVC+ group. However, its concentration decreased from the baseline value of 0.69±0.12 pg/mL to 0.3 pg/mL.

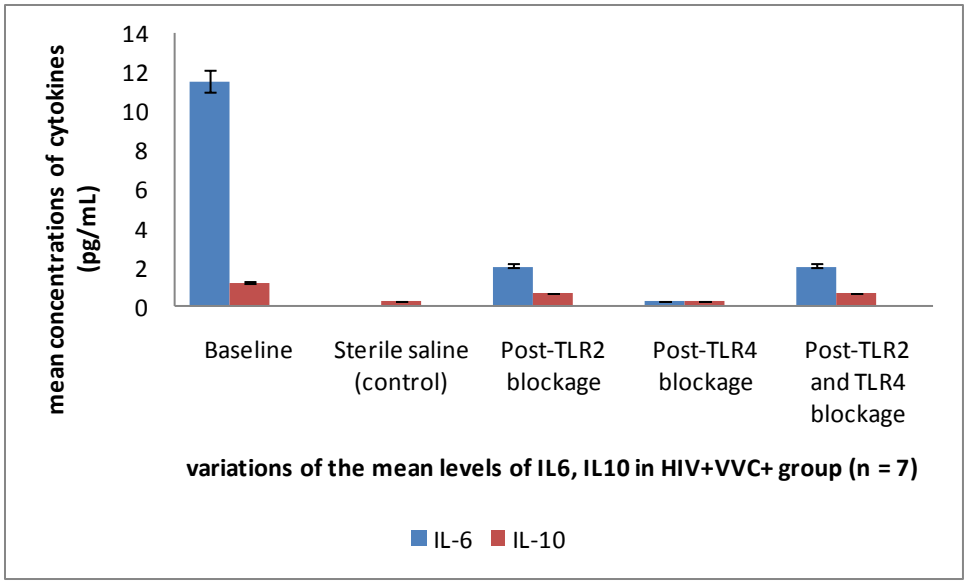

Figure 3 Post-TLR2 and TLR4 blockage Cytokine Levels in HIV infected Women

Comparisons between baseline levels of IL6 and IL10 and levels after TLR2 blockage are depicted in Figures 4 and 5 below.

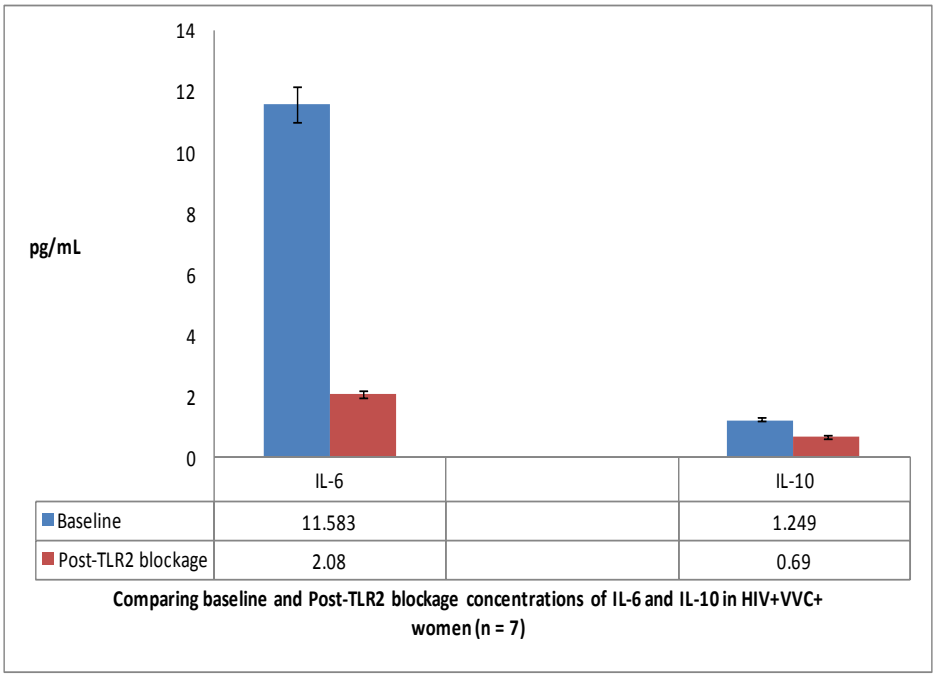

Figure 4 Baseline versus Post-TLR2 blockage Cytokine Levels in HIV infected Women

Figure 4 shows that IL-6 decreased from 11.58 pg/mL at baseline to below detectable cutoff after TLR2 blockage. IL-10 decreased by 44.76% from the baseline to the post-TLR2 blockage.

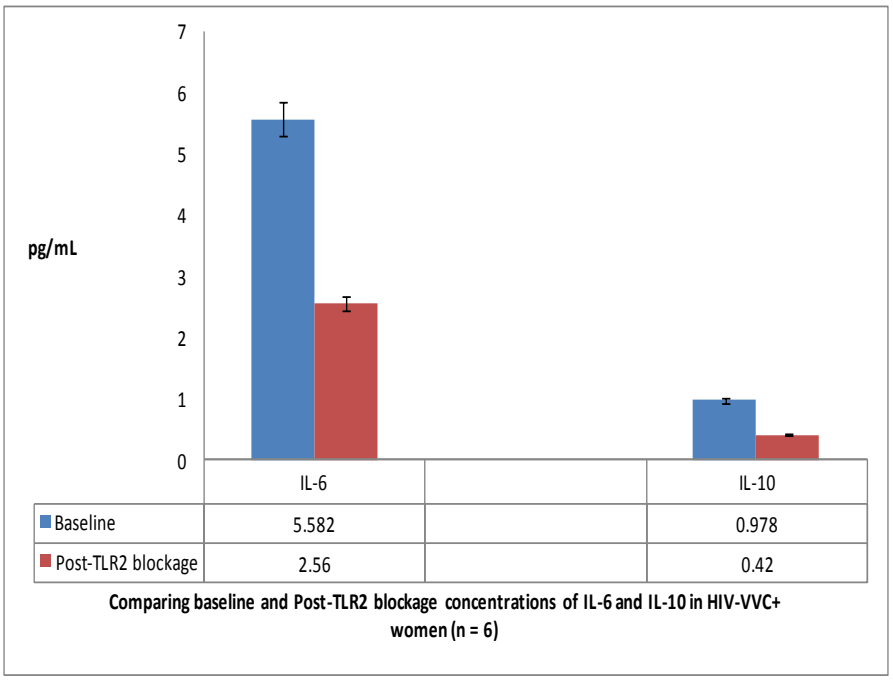

Figure 5 Baseline versus Post-TLR2 blockage Cytokine Levels in HIV uninfected Women

In HIV uninfected women (Figure 5), whilst IL-6 decreased to below detectable values as compared to baseline, IL-10 however decreased by 57.05% from the baseline after blocking of TLR2 by anti-TLR2 monoclonal antibody.

Findings also showed a linear correlation between genital HIV RNA loads and mean level production of IL-6 ($r = 0.722$; $R^2 = 0.679$; $P = 0.067$) as well as IL-8 ($r = 0.910$; $R^2 = 0.833$; $P = 0.004$) post-TLR2 blockage as shown in the Figures 6 and 7 below.

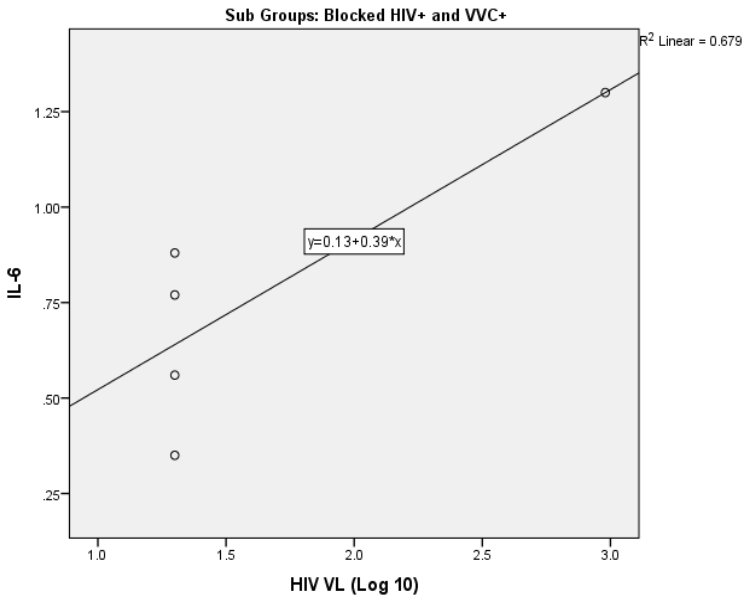

Figure 6 Correlation between IL-6 and genital HIV loads Post-TLR2 blockage Cytokine Levels in HIV+VVC+ women ($r = 0.722$; $R^2 = 0.679$; $P = 0.067$)

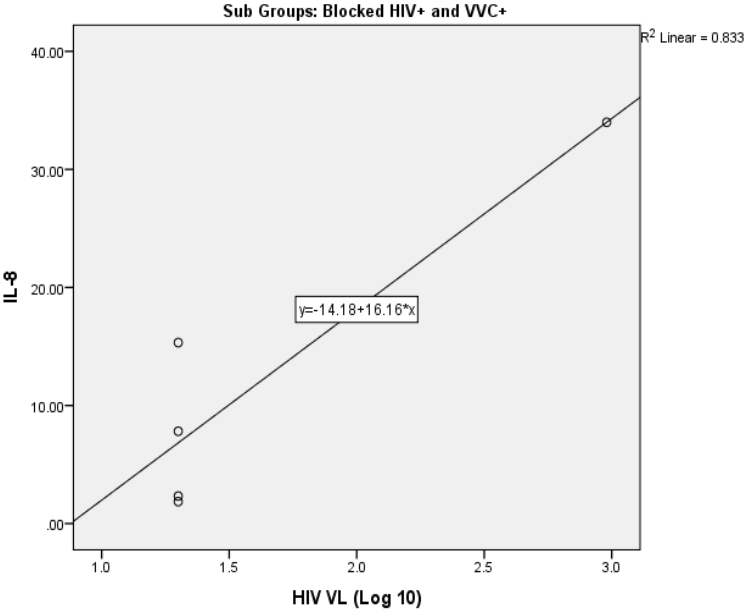

Figure 6 Correlation between IL-8 and genital HIV loads Post-TLR2 blockage Cytokine Levels in HIV+VVC+ women ($r = 0.910$; $R^2 = 0.833$; $P = 0.004$)

Discussion

Findings from this study have shown that blocking TLR2 with TLR2-specific antibody followed by stimulation of monocytes with CVF of HIV-infected women who were also co-infected with symptomatic VVC resulted in 44.76% decrease in the concentrations of IL-10 whilst stimulation with CVF of HIV-VVC+ women showed a reduction of 57.05% of IL-10 levels. However, irrespective of their HIV serostatus, all VVC+ women had decreased levels of pro-inflammatory IL-6 below detectable values after TLR2 blockage on monocytes.

Studies in mice have shown that the development of protective anticandidal Th1 responses requires the concerted actions of several pro-inflammatory cytokines in the relative absence of inhibitory Th2 cytokines, such as IL-4 and IL-10, which inhibit development of Th1 responses (21). In the present study, whilst we found decreased mean levels of a proiflammatory cytokine (IL6) below lower detectable limits, there was also a simultaneous decrease but still quantifiable production of anti-inflammatory cytokine (IL-10) post-TLR2 blockage. All Th1 cytokines (IL-2, IL-12 and IFNγ) as well as Th2 cytokines (IL-4, IL-5 and IL-13) were undetectable post-TLR2 blockage among all patients. These findings do not support our underlying hypothesis that in HIV infected women, the activation of TLR2 signal pathways leads to the production of cytokines that are able to induce a Th2 cellular response that might favor the occurrence of VVC. Due to the fact that all women had high detectable levels of TGF-ß cytokines post-TLR2 and despite decreased in their levels, detectable amounts of IL10 were still measured mainly after stimulation of monocytes by CVF of HIV+VVC+ women, it can be hypothesized that the likely mechanism of VVC occurrence was the differentiation of naïve CD4+ T cells to regulatory T cells (T reg cells). It has been shown that in the presence of TGF-β and IL-10, CD4+ T cells will

differentiate to regulatory T cells (Treg cells) resulting in the continuous production of IL-10. However, in the presence TGF-β and IL-6, naïve CD+ T cells differentiate to Th17 cells (22-26). The latter produce IL-6, IL-8 and IL-17 as effector cytokines. The fact that we had IL-6 levels below detectable levels post-TLR2 blockage in the presence of TGF-ß supports the absence of Th17 differentiation and explains why IL-17 was also undetectable. However, IL-8 was shown to be high in HIV+VVC+ post-TLR2 blockage and was in correlation with genital HIV loads.

In general, activation of TLR2 signal pathways during candidiasis should lead to the production of Th2 cytokines, thus blocking TLR2 with a TLR2-specific monoclonal antibody followed by stimulation of monocytes by *C. albicans* should result in diminished release of these Th2 cytokines. Netea et al (2004) showed that $TLR2^{-/-}$ mice are more resistant to disseminated *Candida* infection, and this is associated with increased chemotaxis and enhanced candidacidal capacity of $TLR2^{-/-}$ monocytes/macrophages. Whilst the production of pro-inflammatory cytokines can be normal, levels of anti-inflammatory cytokines are severely impaired in the $TLR2^{-/-}$ mice. The authors found that this was accompanied by a substantial decrease in the CD4+CD25+ regulatory T (Treg) cell population in $TLR2^{-/-}$ mice (8). Furthermore, in vitro studies confirmed that enhanced survival of Treg cells was induced by TLR2 agonists; *C. albicans* induces immunosuppression through TLR2-derived signals that mediate increased anti-inflammatory cytokine (i.e. IL-10) production and survival of Treg cells, playing a critical role in the pathogenesis of symptomatic VVC.

Conclusion:

The present study demonstrated that in HIV-infected individuals, there might be a decrease of the TLR2-mediated responses during stimulation of monocytes by *Candida* spp. leading to a decreased production of anti-inflammatory cytokines. However, although decreased, observed detectable levels of IL-10 in the presence of high levels of TGF-ß post-TLR2 blockage suggest an underlying role played by Th17/Treg cell populations in the occurrence of VVC during HIV infection.

Limitations:

The limitation of this study is mainly the small sample size that might not allow generalization of our findings. Monocytes used in the study were separated from lymphocytes on the basis of their differential adherence to plastic. Although this procedure plays also a role in the purification of monocytes achieved after Ficoll-Hypaque purification of peripheral blood mononuclear cells (PBMC), we acknowledge that the procedure has 2 major disadvantages – residual contamination with lymphocytes and a transient activation from monocytes. A further study is warranted using a large number of specimens as well as a more appropriate method in monocyte purification is warranted in order to generalize findings from this present study.

Another limitation is the fact that we did not have HIV+VVC- patients to compare with. In order to truly demonstrate that Candida present in CVF from HIV+VVC+ women is able to stimulate monocytes through TLR2, the production of cytokines with and without TLR2 blocking should have been compared with cytokine production after incubation with CVF from HIV+VVC-

patients. Finally, genital secretions collected from women with and without VVC might contain high concentrations of other cytokines. These secretions were then added to monocyte after stimulation. It is difficult to conclude whether cytokines measured were or not just those originally contained within the genital fluid without measuring mRNA in monocytes.

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CHAPTER 5 - Discussion and Conclusions

Although symptomatic vulvo-vaginal candidiasis (VVC) is not classified as an AIDS-defining condition, women with HIV-associated immunosuppression, particularly those who are ART-naïve, are more likely to develop symptomatic VVC. However, findings from this study showed that more than one pathogenic mechanism can explain the occurrence of symptomatic VVC in HIV infected women:

(i) HIV-induced immunosuppression:

Level of HIV-induced immunosuppression, as measured by CD4+ T lymphocytes category, was significantly related to increased odds of symptomatic vulvo-vaginal candidiasis (VVC) only among women with severe immunosuppression (CD4 levels <200/mm3). When compared with HIV negative women, odds of VVC increased by >7-fold for women whose CD4 levels were <200/mm3 and there was no increased risk for other immunosuppressed women whose CD4 levels were between 200 – 500/mm^3. However, when considering only HIV positive women and comparing risk of developing VVC among the 3 WHO stages of HIV-induced immunosuppression (WHO, 2005), odds of VVC increased by 9-fold for women with advanced immunosuppression (CD4 levels 200–349/mm3) and by 60-fold for women with severe immunosuppression (CD4 levels <200/mm3) with HIV loads not included in the multivariate model. There was no increased risk observed for women with mild immunosuppression (CD4 levels 350–499/mm3). Furthermore, among HIV-seropositive women, odds of VVC increased 4-fold and 8-fold respectively for women whose genital and plasma HIV load was >1000 copies/mL when CD4 levels were not included in the multivariate model; but odds highly increased by almost 100-fold for HIV positive women with plasma HIV load ≥10 000

copies/mL. Use of HAART was significantly associated with reduced odds of VVC, regardless of whether HIV load or CD4 count was included in the multivariate model.

There were limitations in this study that might prevent generalization of the above findings: (1) a cross-sectional study design with a small sample size; (2) although multivariate regression analyses were used to control numerous confounding factors, stratification of continuous variables (i.e. CD4+ T cells and HIV loads), particularly when the sample size is small, may lead to residual confounders that might require further adjustment-based methods that were not considered in this study; (3) to prevent the effect of multicollinearity among variables that showed strong biological correlations (CD4+ T cells, HIV loads and use of HAART), CD4+ T cell levels and plasma/genital HIV viral loads were not included in the same multivariate models regardless of whether the use of HAART was included in these models.

Taken together, as compared with HIV negative women, the hypothesis that VVC is due to HIV-induced immunosuppression might be relevant to only a group of patients, particularly those with severe immunosuppression. This hypothesis is further supported in this study by the fact that the cohort of HIV infected women with severe immunosuppression had higher levels of plasma MIP-1β when co-infected with VVC as compared to women without VVC. It is well established that HIV induces lymphoid activation despite CD4+ T cell depletion in patients with severe immunosuppression, reflected by increased production of MIP-1β, and other markers of T cell activation such as CD38 and HLA-DR (Trumpfheller et al., 1998, Paiardini and Muller-Trutwin, 2013). This chronic immune activation is usually associated with HIV viral persistence and increased cases of opportunistic infections such as mucosal candidiasis. However, controversies exist in the literature regarding the role played by HIV-induced immunosuppression in the occurrence of VVC (Ohmit et al., 2003). During a longitudinal study by Ohmit et al (2003) with

a large cohort of HIV seropositive women, findings did not demonstrate a significant correlation between CD4+ lymphocyte count and the occurrence of VVC (Ohmit et al., 2003). The authors did not also found that the use of HAART prevented the occurrence of VVC; hence suggested that VVC may be less likely related to cellular immunodeficiency (Ohmit et al., 2003). However, in agreement with our findings, the Ohmit et al showed that HIV load independently influenced the odds of VVC because higher HIV loads were found to be significantly associated with increased odds of VVC (Ohmit et al., 2003). Prior to this report, Sobel et al. (2000) found out that higher HIV loads rather than lower CD4+ T-lymphocyte counts were associated with statistically significant increased odds for both persistent candidal vaginal colonization and symptomatic VVC (Sobel et al., 2000).

We can therefore conclude that if severe cellular immunodeciency induced by HIV does not directly influence the occurrence of VVC, the corresponding persistent and higher HIV load does. In the present study, we found a significant linear correlation between plasma HIV viral load and genital HIV shedding. I can be hypothesized that during HIV-induced severe immunosuppression with subsequently observed higher HIV loads, HIV particles might change the vaginal environment, hence promoting virulence of *Candida species* by switching from its nonpathogenic form into a filamentous form that causes symptomatic VVC. A further study is required to ascertain this hypothesis.

(ii) Th17 cells rather than Th1/Th2 paradigm were key players in the defense and development of effector responses in HIV infected women with symptomatic VVC:

Using discriminant analysis, it was found that mean levels of circulatory TGF-β3 were significantly higher in women with VVC regardless of their HIV serostatus. In addition, after

adjusting for multiple comparisons using post hoc Bonferroni pairwise tests, mean concentrations of genital IL-6, IL-8, IL-10, IL-17 and GM-CSF were significantly higher in HIV infected women co-infected with VVC. Collectively, it has been demonstrated that in the presence TGF-β3 and IL-6, naïve CD+ T cells differentiate to Th17 cells (Yano et al., 2012, Littman and Rudensky, 2010, Hernandez-Santos and Gaffen, 2012, Cypowyj et al., 2012, Bixler and Mattapallil, 2013). Th17 cells produce IL-6, IL-8 and IL-17 as effector cytokines (Yano et al., 2012, Littman and Rudensky, 2010, Hernandez-Santos and Gaffen, 2012, Cypowyj et al., 2012, Bixler and Mattapallil, 2013).

Although initial studies in human subjects pointed out that TGF-β was not a key factor for Th17 differentiation, more recent findings indicate that the presence of TGF-β is essential for the generation of functional Th17 cells (Yano et al., 2012, Littman and Rudensky, 2010, Hernandez-Santos and Gaffen, 2012, Cypowyj et al., 2012, Bixler and Mattapallil, 2013). Other key cytokines involved in Th17 cell differentiation and expansion are IL-21 and IL-23 but these cytokines were not measured in this present study (Yano et al., 2012, Littman and Rudensky, 2010, Hernandez-Santos and Gaffen, 2012, Cypowyj et al., 2012, Bixler and Mattapallil, 2013).

The prototype cytokine for Th17 cell response, IL-17, coordinates tissue inflammation through the induction of proinflammatory cytokines and chemokines, such as IL-6 and IL-8 (Yano et al., 2012, Littman and Rudensky, 2010, Hernandez-Santos and Gaffen, 2012, Cypowyj et al., 2012, Bixler and Mattapallil, 2013). IL-6 is a multifunctional pleiotropic cytokine involved in the regulation of immune responses, acute-phase responses, hematopoiesis, and inflammation (Kishimoto, 2010). IL-8 is a neutrophil-specific chemotactic factor classified as a member of the CXC chemokine family (Lin et al., 2004). The major effector function of IL-8 is the activation and recruitment of neutrophils, NK cells, T cells, basophils, and GM-CSF to the site of infection

(Lin et al., 2004). It has been demonstrated that the generation of classical Th17 cells in the presence of TGF-β favors the production of higher levels of IL-10 as also shown in our study. It has been demonstrated that in the presence of TGF-β and IL-10, CD4+ T cells will differentiate to regulatory T cells (Treg cells) resulting in the production of more IL-10 (Yano et al., 2012, Littman and Rudensky, 2010, Hernandez-Santos and Gaffen, 2012, Cypowyj et al., 2012, Bixler and Mattapallil, 2013). Therefore, IL-10 produced by Treg cells or directly by the more regulatory classical Th17 cells blocks Th17-mediated inflammation, suggesting the existence of regulatory mechanisms to avoid excessive tissue damage (Yano et al., 2012, Littman and Rudensky, 2010, Hernandez-Santos and Gaffen, 2012, Cypowyj et al., 2012, Bixler and Mattapallil, 2013).

In this study, although some Th1 cytokines (IL-2 and IL-12) were significantly higher among HIV infected women with VVC, IFN-γ, "a prototype Th1 cytokine" was shown to be very low. In general, infection of myeloid cells typically elicits production of IL-12, which induces differentiation of IFN-γ-producing Th1 cells (Liu et al., 2005). In 2007, Steinman reported on a central paradox that the signature Th1 effector cytokine, IFNγ, was significantly less important in various disease settings than was IL-12, the key Th1 inductive cytokine (Steinman, 2007, Hernandez-Santos and Gaffen, 2012). This significant overhaul in the prevailing paradigm of CD4-mediated immunity became evident with the discovery of Th17 cells, which reconciled these discrepancies (Hernandez-Santos and Gaffen, 2012). Of particular importance is the finding that the p40 subunit of IL-12 is shared with IL-23 and the IL-12Rβ1 is a subunit of the IL-23R (Ghilardi and Ouyang, 2007). Thus, mice lacking either IL-12p40 or IL-12Rβ1 are deficient not only in IL-12 (hence, lacking Th1 cells), but also IL-23 (hence, lacking Th17 cells) (Ghilardi and Ouyang, 2007).

In conclusion, there is Th1/Th2 paradigm shift in the pathogenesis of VVC among HIV infected women (Yano et al., 2012, Littman and Rudensky, 2010, Hernandez-Santos and Gaffen, 2012, Cypowyj et al., 2012, Bixler and Mattapallil, 2013). Of major importance was the observed significant level of TGF-β3, a member of TGF-β family of cytokines. It is well established that the immunoregulatory properties of these cytokines can explain the normal absence of cell-medicated immunity against candida in the vagina (LeBlanc et al., 2006, Kosonen et al., 2006, Romani, 2000). In addition, Th17 cells rather than Th1/Th2 paradigm were demonstrated to be key players in the defense and development of effector responses in HIV infected women with symptomatic VVC (Yano et al., 2012, Littman and Rudensky, 2010, Hernandez-Santos and Gaffen, 2012, Cypowyj et al., 2012, Bixler and Mattapallil, 2013). Studies have shown that during the course of HIV infection, there are progressive decline of Th17 cells as well as a decrease of Th17/Treg ratio (Bixler and Mattapallil, 2013). This unbalance seems to be more important in advanced HIV disease (Bixler and Mattapallil, 2013). Most of these studies however have been assessing the loss of Th17 cells in the gastro-intestinal mucosa of HIV infected patients (Bixler and Mattapallil, 2013). The role of Th17 cells during *Candida* infection in different other anatomical sites remains largely unknown. If there is no loss of Th17 cells (at the mucosal surface of the vagina) in HIV infected women with VVC, then an inflammatory but non-protective response induced by Th17 cells during *Candida* infection following a strong recruitment of PMN cells can explain the presence of symptoms in patients with VVC. Based on data from a human life challenge model, Fidel Jr. et al. hypothesized that following the interaction of *Candida* with vaginal epithelial cells, symptomatic VVC was associated with signals that promoted a non-protective inflammatory leukocyte response and concomitant

clinical symptoms (Fidel et al., 2004). They indicated that neutrophils contribute to the pathogenesis and local inflammation in symptomatic VVC (Fidel Jr. et al, 2004).

(iii) A role of IgE-mediated hypersensitivity responses:

Findings from this study showed that Th2-type cytokines (IL4, IL5 and IL13) were significantly increased in VVC women co-infected with HIV. Previous analyses of Th2 cytokines in clinical studies showed only a small amount of IL-4, IL-5 and IL-13 in vaginal fluid with no effect on fungal burden (Barousse et al., 2004). However, controversies were raised by Babula, *et al*. who showed increased vaginal IL-4 with evidence of a polymorphism in IL-4 in women with RVVC, possibly resulting in reduced production of anticandidal compounds such as nitric oxide and mannose-binding lectin (MBL) (Babula et al., 2005). A similar controversy from the literature was from a study that showed that increased IL-4 and IL-10 were associated with higher fungal burden and severity in pathology (Belay et al., 2002, Fischer, 2012). A proposed hypothesis linking IL-4, IL-5 and IL-13 to symptomatic VVC and also susceptibility to RVVC was raised and suggested the possibility of an allergic reaction triggered either by *Candida*-specific IgE production or through histamine-induced prostaglandin E2, resulting in the suppression of Th1-type CMI (Belay et al., 2002, Fischer, 2012). It has been established that IL-4, IL-5 and IL-13 are associated with the stimulation of IgE and eosinophil response (Belay et al., 2002, Fischer, 2012). The latter 2 were previously shown to be significantly associated with symptomatic VVC in HIV-infected women suggestive of a local hypersensitivity response to Candida antigens (Belay et al., 2002, Fischer, 2012).

Therefore, in addition to the above pathogenic mechanisms discussed, the role played by hypersensitivity cannot be neglected. Our cohort of HIV-infected women with symptomatic VVC had significantly higher levels of IL-4, IL-5 and IL-13 in their genital secretions, suggestive of a strong hypersensitivity response. In any case, the hypothesis that VVC in HIV infected women can be a result of either *Candida*-specific IgE production or histamine-induced prostaglandin E2 might be relevant to only a group of women with some predisposing factors. A further study in which genital IgE levels in HIV infected women with symptomatic VVC is measured may lend support to our results.

(iv) TLR2-mediated responses:

Compared with baseline concentrations, stimulation with CVF of HIV+VVC+ women post-TLR2 blockage on monocytes decreased IL-6 below its detectable limit but IL-10 production by 44.7%.

As attested by the 2011 Nobel Prize in Medicine or Physiology, the concept of "pattern recognition" by the innate immune system fundamentally changed the opinion of pathologists and scientists on how microorganisms are recognized by host immune cells at the molecular level. There is sufficient evidence from the literature that PRR engagement by C. albicans in antigen presenting cells (APCs) results in secretion of specific cytokines including IL-6 (Gow et al., 2012, Netea et al., 2008, Romani, 2011). Infection by Candida strongly influences the differentiation of Th17 cells and IL-6 is known to promote skewing of activated CD4+ T cells into the Th17 lineage, which express IL-17 (Yano et al., 2012, Littman and Rudensky, 2010, Hernandez-Santos and Gaffen, 2012, Cypowyj et al., 2012, Bixler and Mattapallil, 2013).

TLR2 has also been shown to suppress inflammatory responses to Candida via production of IL-10 and enhanced Treg cell survival (Netea et al., 2004). Accordingly, TLR2−/− mice are more resistant to disseminated candidiasis than wild type, supporting a detrimental rather than protective role for this receptor (Netea et al., 2004). Previous studies have also demonstrated that the generation of classical Th17 cells in the presence of TGF-β (and IL-6) favors the production of higher levels of IL-10-associated Treg response (Yano et al., 2012, Littman and Rudensky, 2010, Hernandez-Santos and Gaffen, 2012, Cypowyj et al., 2012, Bixler and Mattapallil, 2013).

In conclusion, the present study demonstrated that in HIV-infected individuals, there might be a decrease of the TLR2-mediated responses during stimulation of monocytes by *Candida* spp. leading to a decreased production of anti-inflammatory cytokines. However, although decreased, observed detectable levels of IL-10 in the presence of high levels of TGF-ß post-TLR2 blockage suggest an underlying role played by Th17/Treg cell populations in the occurrence of VVC during HIV infection.

Limitations in this study included: (i) the lack of CVF samples from HIV+/VVC- patients for comparison of cytokine production with HIV+/VVC+ patients after TLR2 blockage on monocytes; (ii) very small sample size for these experiments.

The interplay between infections and the immune system is intricate and relationships between factors not always explainable. It may be argued that this is a case of "cause and effect". However, the occurrence of symptomatic VVC with increasing immunosuppression in our population is of concern and different angles have to be explored in an effort to address this major public health problem.

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APPENDIX: RAW DATA

Table 1 PLASMA CYTOKINE RESULTS

Patient ID	HIV status	VVC	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
D016S	1	2	5.71	0.02	1.03	2.45	5.64	16.99	9.79	2.06
D027S	1	1	3.4	0.02	0.84	5.34	8.64	32.88	2.31	0.02
D031S	2	1	2.97	0.02	0.41	3.04	6.49	21.46	9.79	8.09
D033S	2	1	2.97	3.28	0.84	1.85	12.51	14.23	2.77	9.3
D036S	2	1	1.91	0.02	0.41	5.63	5	12.98	2.31	4.46
D186S	1	1	2.14	0.02	0.84	2.15	7.78	9.13	7.9	1.38
D043S	2	1	1.73	0.02	0.17	3.33	4.78	17.29	6.5	4.8
D047S	1	1	1.5	0.02	0.41	8.15	3.08	10.43	6.96	6.18
D051S	2	1	2.14	88.95	0.63	11.17	85.71	17.89	11.2	27.25
D054S	1	1	2.08	0.02	0.94	2.15	4.36	16.99	12.39	9.99
D057S	2	1	4.57	0.02	2.3	4.77	1.81	15.46	6.73	4.12
D058S	1	1	2.14	0.02	1.23	1.85	2.65	12.35	7.9	2.4
D068S	1	1	1.56	0.02	0.73	0.93	10.36	20.87	11.2	6.18
D069S	2	1	1.45	0.02	1.41	0.61	4.36	21.76	10.49	4.46
D071S	1	1	1.45	0.02	0.17	0.93	3.08	22.05	5.09	2.4
D086S	1	1	1.56	0.02	0.17	1.24	5.21	25.53	4.16	67
D089S	2	1	5.08	0.02	3.3	1.24	18.98	12.35	13.57	1.38
D093S	2	2	1.91	6.12	0.17	0.61	6.71	9.13	2.77	5.15
D099S	2	1	1.67	0.83	0.17	1.85	13.37	7.8	6.03	20.96
D116S	1	1	1.56	0.02	0.02	0.93	4.78	11.08	3.23	0.53
D128S	1	1	4.45	0.02	2.47	8.42	9.93	12.35	4.16	12.42
D134S	1	1	1.45	0.02	0.41	1.54	9.71	13.92	4.63	3.09
D140S	1	1	1.56	0.02	0.63	2.6	4.36	6.44	4.63	2.74
D141S	1	1	1.45	0.02	1.03	0.61	7.14	20.28	5.56	5.49

D143S	2	1	2.02	0.02	0.73	1.85	4.78	2.85	4.63	3.26
D149S	1	1	1.96	0.02	1.32	2.74	2.65	14.85	10.26	1.38
D158S	2	2	6.1	0.02	1.51	3.62	31.77	20.87	9.32	7.05
D163S	2	1	9.24	152.8	1.78	54.69	262.15	527.61	8.37	325.58
D166S	2	1	2.02	0.02	0.41	1.24	9.5	7.8	6.5	554.49
D177S	2	1	1.79	0.02	0.84	1.85	6.49	10.43	4.16	2.4
D197S	1	1	2.37	0.02	2.64	4.2	17.68	22.34	15.47	2.4
D200S	1	1	2.49	0.02	0.94	0.93	6.71	9.78	7.2	1.38
D016S	1	2	5.71	0.02	1.03	2.45	5.64	16.99	9.79	2.06
D016S	1	2	5.71	0.02	1.03	2.45	5.64	16.99	9.79	2.06
D016S	1	2	5.71	0.02	1.03	2.45	5.64	16.99	9.79	2.06
D016S	1	2	5.71	0.02	1.03	2.45	5.64	16.99	9.79	2.06
D016S	1	2	5.71	0.02	1.03	2.45	5.64	16.99	9.79	2.06
D016S	1	2	5.71	0.02	1.03	2.45	5.64	16.99	9.79	2.06
D016S	1	2	5.71	0.02	1.03	2.45	5.64	16.99	9.79	2.06
D016S	1	2	5.71	0.02	1.03	2.45	5.64	16.99	9.79	2.06
D016S	1	2	5.71	0.02	1.03	2.45	5.64	16.99	9.79	2.06
D093S	2	2	1.91	6.12	0.17	0.61	6.71	9.13	2.77	5.15
D093S	2	2	1.91	6.12	0.17	0.61	6.71	9.13	2.77	5.15
D093S	2	2	1.91	6.12	0.17	0.61	6.71	9.13	2.77	5.15
D093S	2	2	1.91	6.12	0.17	0.61	6.71	9.13	2.77	5.15
D158S	2	2	6.1	0.02	1.51	3.62	31.77	20.87	9.32	7.05
D158S	2	2	6.1	0.02	1.51	3.62	31.77	20.87	9.32	7.05
D158S	2	2	6.1	0.02	1.51	3.62	31.77	20.87	9.32	7.05
D158S	2	2	6.1	0.02	1.51	3.62	31.77	20.87	9.32	7.05

Patient ID	HIV status	VVC	IL-12	IL-13	IL-17	G-CSF	GM-CSF	IFN-γ	MCP-1	MIP-1β	TNF-α	TGF-β1	TGF-β2	TGF-β3
D016S	1	2	3.38	2.37	0.02	4.44	0.02	0.02	70.88	158.24	6.86	187.59	86.71	0.02
D027S	1	1	8.64	5.85	0.02	0.02	78.04	10.32	27.15	131.12	0.02	0.02	47.94	0.02
D031S	2	1	3.38	3.12	0.02	0.02	38.17	34.64	220.27	195.73	0.02	0.02	37.18	0.02
D033S	2	1	10.34	9.54	0.02	18.73	6.15	36.87	137.38	102.2	6.86	0.02	29.94	0.02
D036S	2	1	5.17	11.5	0.02	9.13	20.04	0.02	89.45	179.39	3.39	59.87	62.14	0.02
D186S	1	1	1.51	1.36	0.02	0.02	46.61	4.43	137.74	195.17	0.02	0.02	69.2	0.02
D043S	2	1	1.51	3.12	0.02	0.02	11.32	0.02	60.77	82.52	0.02	103.43	100.62	6.76
D047S	1	1	59.25	3.12	0.02	0.02	30.29	15.64	35.95	154.24	0.02	0.02	62.14	10.97
D051S	2	1	66.83	7.45	7.81	0.02	0.02	68.15	24.71	161.99	39.76	0.02	47.94	6.76
D054S	1	1	8.64	3.87	0.02	6.9	39.72	0.02	121.9	170.34	0.02	47.1	55.06	0.02
D057S	2	1	10.34	3.99	0.02	1.46	0.02	68.15	29.84	55.82	11.75	169.79	100.62	6.76
D058S	1	1	1.51	2.12	0.02	0.02	26.25	10.32	50.41	349.03	0.02	175.73	72.71	6.76
D068S	1	1	0.02	3.12	0.02	0.02	35.05	0.02	164.97	137.6	0.02	222.88	189.52	21.55
D069S	2	1	6.92	2.62	0.02	0.02	55.6	0.02	48.48	209.23	0.02	151.88	117.89	8.86
D071S	1	1	1.51	0.85	0.02	0.02	55.6	0.02	40.03	182.45	0.02	0.02	104.08	0.02
D086S	1	1	13.7	6.34	0.02	0.02	52.62	0.02	43.81	47.61	0.02	27.52	79.72	10.97
D089S	2	1	6.92	2.12	0.02	0.02	28.28	68.15	45.63	234.62	28.36	0.02	26.29	0.02
D093S	2	2	0.02	1.99	0.02	0.02	45.85	0.02	107.28	64.6	5.16	0.02	11.46	0.02
D099S	2	1	10.34	3.12	0.02	0.02	76.62	0.02	132.31	80.01	0.02	0.02	40.78	6.76
D116S	1	1	0.02	4.73	0.02	0.02	0.02	0.02	54.9	143.04	0.02	0.02	55.06	0.02
D128S	1	1	84.07	8.19	3.57	6.9	4.14	47.68	38.32	91.96	32.68	0.02	44.37	6.76
D134S	1	1	0.02	1.87	0.02	0.02	22.96	0.02	118.38	97.98	0.02	40.64	62.14	0.02
D140S	1	1	1.51	2.12	0.03	0.02	22.13	0.02	50.54	312.34	0.02	0.02	62.14	2.58
D141S	1	1	0.02	1.61	0.03	0.02	965.5	0.02	125.78	76.06	0.02	0.02	62.14	13.08

D143S	2	1	0.02	1.87	0.03	0.02	33.87	4.43	54.26	64.45	0.02	6.95	40.78	10.97
D149S	1	1	6.92	2.24	7.81	0.02	31.89	4.43	87.72	257.23	0.02	0.02	22.63	0.02
D158S	2	2	20.26	2.87	0.02	0.02	50.38	56.02	95.01	71.62	49.46	27.52	69.2	0.02
D163S	2	1	929.84	300.91	7.81	15.11	109.95	971.68	338.33	95.85	224.73	0.02	37.18	0.02
D166S	2	1	53.15	2.12	0.02	0.02	10.41	4.43	31.84	144.48	0.02	0.02	69.2	0.02
D177S	2	1	0.02	1.87	0.02	0.02	0.02	0.02	91.06	68.86	0.02	0.02	62.14	8.86
D197S	1	1	18.63	3.24	0.02	20.46	0.02	56.02	33.3	251.75	19.48	14	65.67	0.02
D200S	1	1	7.78	1.61	0.02	0.02	0.02	20.65	83.87	317.3	0.02	0.02	62.14	0.02
D016S	1	2	3.38	2.37	0.02	4.44	0.02	0.02	70.88	158.24	6.86	187.59	86.71	0.02
D016S	1	2	3.38	2.37	0.02	4.44	0.02	0.02	70.88	158.24	6.86	187.59	86.71	0.02
D016S	1	2	3.38	2.37	0.02	4.44	0.02	0.02	70.88	158.24	6.86	187.59	86.71	0.02
D016S	1	2	3.38	2.37	0.02	4.44	0.02	0.02	70.88	158.24	6.86	187.59	86.71	0.02
D016S	1	2	3.38	2.37	0.02	4.44	0.02	0.02	70.88	158.24	6.86	187.59	86.71	0.02
D016S	1	2	3.38	2.37	0.02	4.44	0.02	0.02	70.88	158.24	6.86	187.59	86.71	0.02
D016S	1	2	3.38	2.37	0.02	4.44	0.02	0.02	70.88	158.24	6.86	187.59	86.71	0.02
D016S	1	2	3.38	2.37	0.02	4.44	0.02	0.02	70.88	158.24	6.86	187.59	86.71	0.02
D016S	1	2	3.38	2.37	0.02	4.44	0.02	0.02	70.88	158.24	6.86	187.59	86.71	0.02
D093S	2	2	0.02	1.99	0.02	0.02	45.85	0.02	107.28	64.6	5.16	0.02	11.46	0.02
D093S	2	2	0.02	1.99	0.02	0.02	45.85	0.02	107.28	64.6	5.16	0.02	11.46	0.02
D093S	2	2	0.02	1.99	0.02	0.02	45.85	0.02	107.28	64.6	5.16	0.02	11.46	0.02
D093S	2	2	0.02	1.99	0.02	0.02	45.85	0.02	107.28	64.6	5.16	0.02	11.46	0.02
D158S	2	2	20.26	2.87	0.02	0.02	50.38	56.02	95.01	71.62	49.46	27.52	69.2	0.02
D158S	2	2	20.26	2.87	0.02	0.02	50.38	56.02	95.01	71.62	49.46	27.52	69.2	0.02
D158S	2	2	20.26	2.87	0.02	0.02	50.38	56.02	95.01	71.62	49.46	27.52	69.2	0.02
D158S	2	2	20.26	2.87	0.02	0.02	50.38	56.02	95.01	71.62	49.46	27.52	69.2	0.02

Table 2 LOWER GENITAL TRACT CYTOKINE RESULTS

Patient ID	HIV status	VVC	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
D016VF	1	2	143.11	0.02	0.62	0.15	0.98	0.02	10.84	0.9
D027VF	1	1	684.49	2.44	1.9	0.31	6.37	0.31	117.65	1.29
D031VF	2	1	1512.78	3.61	2.01	0.39	4.96	0.02	100.19	1.24
D033VF	2	1	437.89	0.02	1.4	0.23	2.86	0.52	103.84	0.9
D036VF	2	1	3996.81	8.61	3.63	0.54	7.89	1.26	469.25	1.24
D186VF	1	1	591.4	3.86	1.38	0.35	3.56	0.71	278.52	1.11
D043VF	2	1	382.66	0.39	1.32	0.23	3.99	0.2	831.06	0.51
D047VF	1	1	149.01	0.02	0.74	0.46	2.7	1.44	166.98	1.81
D051VF	2	1	306.42	0.02	1.14	0.54	3.45	0.99	1500	1.11
D054VF	1	1	2542.95	12.24	3.3	1.26	11.15	1.78	494.11	1.89
D057VF	2	1	1097.81	5.67	2.65	0.46	6.26	1.08	1500	0.94
D058VF	1	1	78.15	0.02	0.66	0.31	2.16	1.26	178.8	1.11
D068VF	1	1	1962.38	6.55	2.55	0.46	5.29	0.02	213.95	0.02
D069VF	2	1	420.1	4.52	1.38	0.31	4.31	1.7	169.2	0.77
D071VF	1	1	8500	20.07	5.28	0.61	15.35	1.26	1500	1.2
D086VF	1	1	64.65	0.02	0.4	0.07	0.56	0.02	0.46	0.86
D089VF	2	1	73.3	0.02	0.4	0.15	0.77	0.02	9.87	1.11
D093VF	2	2	0.86	0.02	0.21	0.11	0.24	0.02	0.02	1.16
D099VF	2	1	1537.06	4.35	1.74	0.39	4.64	0.71	299.94	1.2
D116VF	1	1	714.94	3.95	2.01	0.61	38.36	1.7	702.51	1.29
D128VF	1	1	179.22	0.02	1.1	0.72	7.24	0.9	48.06	0.94
D134VF	1	1	976.86	6.9	2.11	0.39	5.61	0.71	99.06	0.6
D140VF	1	1	8500	18.81	4.81	0.69	15.63	0.99	140.03	1.72
D141VF	1	1	215.88	0.02	1.14	0.42	5.61	1.08	222.37	0.64

D143VF	2	1	4.16	0.02	0.26	0.02	0.14	0.02	0.12	0.94
D149VF	1	1	1231.63	6.11	2.07	0.31	5.83	0.02	4.28	1.37
D158VF	2	2	401.24	2.91	1.44	0.69	4.21	0.61	29.31	0.86
D163VF	2	1	7131.36	13.31	3.42	0.65	9.68	0.31	45.82	0.86
D166VF	2	1	8500	21.78	6.07	0.76	19.35	4.17	932.74	1.63
D177VF	2	1	284.69	0.02	1.38	0.46	4.26	1.52	467.16	0.26
D197VF	1	1	8500	27.28	6.59	1.19	56.35	5	800.85	3.02
D200VF	1	1	11.31	0.02	0.35	0.23	3.56	0.02	163.04	1.11
D002VF	2	2	22.99	0.02	0.26	0.02	1.84	0.02	58.95	0.6
D093VF	2	2	0.86	0.02	0.21	0.11	0.24	0.02	0.02	1.16
D093VF	2	2	0.86	0.02	0.21	0.11	0.24	0.02	0.02	1.16
D093VF	2	2	0.86	0.02	0.21	0.11	0.24	0.02	0.02	1.16
D158VF	2	2	401.24	2.91	1.44	0.69	4.21	0.61	29.31	0.86
D158VF	2	2	401.24	2.91	1.44	0.69	4.21	0.61	29.31	0.86
D002VF	2	2	22.99	0.02	0.26	0.02	1.84	0.02	58.95	0.6
D002VF	2	2	22.99	0.02	0.26	0.02	1.84	0.02	58.95	0.6
D016VF	1	2	143.11	0.02	0.62	0.15	0.98	0.02	10.84	0.9
D016VF	1	2	143.11	0.02	0.62	0.15	0.98	0.02	10.84	0.9
D016VF	1	2	143.11	0.02	0.62	0.15	0.98	0.02	10.84	0.9
D016VF	1	2	143.11	0.02	0.62	0.15	0.98	0.02	10.84	0.9
D016VF	1	2	143.11	0.02	0.62	0.15	0.98	0.02	10.84	0.9
D016VF	1	2	143.11	0.02	0.62	0.15	0.98	0.02	10.84	0.9
D016VF	1	2	143.11	0.02	0.62	0.15	0.98	0.02	10.84	0.9
D016VF	1	2	143.11	0.02	0.62	0.15	0.98	0.02	10.84	0.9
D016VF	1	2	143.11	0.02	0.62	0.15	0.98	0.02	10.84	0.9

Patient ID	HIV status	VVC	IL-12	IL-13	IL-17	G-CSF	GM-CSF	IFN-γ	MCP-1	MIP-1β	TNF-α	TGF-β1	TGF-β2	TGF-β3
D016VF	1	2	0.02	0.47	1.95	0.02	42.34	3.91	2.32	8.13	14.07	6.88	8.39	0.02
D027VF	1	1	3.42	1.43	17.86	13.19	44.59	28.4	5.56	14.22	55.3	0.02	11.09	0.02
D031VF	2	1	1.73	1.09	16.97	11.85	65.67	25.18	4.08	3.87	45.22	0.02	5.66	0.65
D033VF	2	1	3.01	1.06	21.34	7.54	52.36	14	2.69	5.79	21.66	14.97	15.54	2.74
D036VF	2	1	4.86	1.52	29.3	22.29	64.45	33.77	5.01	20.91	56.18	6.88	8.39	2.74
D186VF	1	1	2.59	1.4	12.01	10.12	54.08	20.94	7.93	10.72	42.81	0.02	10.19	0.13
D043VF	2	1	3.01	1.25	15.46	8.3	50.47	18.03	8.79	25.81	21.34	0.02	20.8	2.74
D047VF	1	1	3.42	0.84	6.88	7.54	46.27	10.31	3.26	4.98	21.02	0.02	10.19	0.13
D051VF	2	1	2.59	1.71	15.46	11.85	48.1	19.01	18	9.79	32.11	10.16	11.09	0.65
D054VF	1	1	6.27	2.69	31.67	32.24	70.54	59.64	14.43	18.09	91.28	10.16	40.6	2.74
D057VF	2	1	4.45	1.77	18.45	15.12	65.06	31.1	23.13	4.98	57.35	13.38	11.99	1.69
D058VF	1	1	2.16	0.97	5.75	3.3	40.65	8.66	2.32	9.58	12.37	0.02	8.39	1.69
D068VF	1	1	2.16	1.52	17.86	15.12	70.92	43.25	11.08	13.29	61.15	0.02	2.87	1.69
D069VF	2	1	2.59	2.26	7.6	9.77	57.82	19.49	3.12	10.13	36.43	0.02	8.39	0.65
D071VF	1	1	6.07	3.61	49.8	83.89	91.01	89.61	20	21.46	123.1	14.97	19.93	0.13
D086VF	1	1	0.02	0.34	0.02	0.02	40.41	2.58	1.54	2.01	4.87	0.02	14.65	0.02
D089VF	2	1	0.02	0.4	0.02	0.36	41.05	0.02	1.51	2.65	1.72	0.02	17.3	79.47
D093VF	2	2	0.02	0.4	2.43	0.02	39.43	0.02	1.16	1.6	0.02	11.78	17.3	2.74
D099VF	2	1	1.73	1.46	16.67	13.19	62.76	22.84	3.76	18.44	51.18	0.02	11.99	0.02
D116VF	1	1	5.06	2.48	26.34	15.75	37.31	107.89	16.65	29.73	52.65	0.02	10.19	1.69
D128VF	1	1	4.25	1.28	11.36	9.04	39.6	19.01	3.83	68.26	22.63	0.02	11.99	1.69
D134VF	1	1	3.42	1.86	18.16	16.68	63.37	38.99	4.25	8.31	57.65	0.02	6.57	0.65
D140VF	1	1	7.47	3	41.81	33.01	91.16	76.04	8.42	12.1	129.31	0.02	11.99	1.17
D141VF	1	1	4.66	1.31	12.33	9.04	42.18	18.03	4.32	11.05	29.3	0.02	6.57	0.02

D143VF	2	1	0.02	0.28	0.02	0.02	38.13	0.02	1.08	1.74	0.02	0.02	7.48	0.65
D149VF	1	1	1.73	1.4	16.97	12.86	59.68	34.65	3.76	4.98	53.83	0.02	11.99	2.74
D158VF	2	2	3.84	1.83	16.07	9.41	53.53	23.78	2.8	18.91	42.81	0.02	10.19	0.02
D163VF	2	1	3.84	1.89	31.14	24.26	71.75	54.82	6.89	6.08	84.28	0.02	8.39	0.02
D166VF	2	1	10.21	3.3	61.01	47.96	84.5	121.42	23.38	44.7	136.55	8.53	14.65	4.86
D177VF	2	1	2.59	2.26	13.6	10.47	49.36	19.98	2.9	4.14	28.99	0.02	13.77	14.46
D197VF	1	1	13.67	3.79	59.63	63.41	100.83	331.82	36.15	28.04	142.95	0.02	11.99	0.13
D200VF	1	1	0.02	0.53	0.02	25.09	35.01	0.02	67.87	9.96	0.85	0.02	11.09	0.02
D002VF	2	2	0.02	0.4	0.02	3.3	34.68	0.02	3.48	8.49	0.85	0.02	10.19	0.02
D093VF	2	2	0.02	0.4	2.43	0.02	39.43	0.02	1.16	1.6	0.02	11.78	17.3	2.74
D093VF	2	2	0.02	0.4	2.43	0.02	39.43	0.02	1.16	1.6	0.02	11.78	17.3	2.74
D093VF	2	2	0.02	0.4	2.43	0.02	39.43	0.02	1.16	1.6	0.02	11.78	17.3	2.74
D158VF	2	2	3.84	1.83	16.07	9.41	53.53	23.78	2.8	18.91	42.81	0.02	10.19	0.02
D158VF	2	2	3.84	1.83	16.07	9.41	53.53	23.78	2.8	18.91	42.81	0.02	10.19	0.02
D002VF	2	2	0.02	0.4	0.02	3.3	34.68	0.02	3.48	8.49	0.85	0.02	10.19	0.02
D002VF	2	2	0.02	0.4	0.02	3.3	34.68	0.02	3.48	8.49	0.85	0.02	10.19	0.02
D016VF	1	2	0.02	0.47	1.95	0.02	42.34	3.91	2.32	8.13	14.07	6.88	8.39	0.02
D016VF	1	2	0.02	0.47	1.95	0.02	42.34	3.91	2.32	8.13	14.07	6.88	8.39	0.02
D016VF	1	2	0.02	0.47	1.95	0.02	42.34	3.91	2.32	8.13	14.07	6.88	8.39	0.02
D016VF	1	2	0.02	0.47	1.95	0.02	42.34	3.91	2.32	8.13	14.07	6.88	8.39	0.02
D016VF	1	2	0.02	0.47	1.95	0.02	42.34	3.91	2.32	8.13	14.07	6.88	8.39	0.02
D016VF	1	2	0.02	0.47	1.95	0.02	42.34	3.91	2.32	8.13	14.07	6.88	8.39	0.02
D016VF	1	2	0.02	0.47	1.95	0.02	42.34	3.91	2.32	8.13	14.07	6.88	8.39	0.02
D016VF	1	2	0.02	0.47	1.95	0.02	42.34	3.91	2.32	8.13	14.07	6.88	8.39	0.02
D016VF	1	2	0.02	0.47	1.95	0.02	42.34	3.91	2.32	8.13	14.07	6.88	8.39	0.02

Table 3 CYTOKINE LEVELS POST-TLR BLOCKAGE

Patient ID	HIV status	VVC	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	
1TLR2(D128)		1	1	1.73	0.02	0.02	0.02	0.56	0.02	7.82	0.43
2TLR2(D149)		1	1	9.12	0.02	0.16	0.15	0.35	0.02	1.86	0.6
3TLR2(D116)		1	1	5.23	0.02	0.04	0.02	1.3	0.02	33.99	0.51
4TLR2(D200)		1	1	0.62	0.02	0.26	0.02	0.88	0.02	15.33	0.9
5TLR2(D086)		1	1	0.77	0.02	0.26	0.03	0.77	0.02	2.33	1.03
6TLR2(D069)		2	1	1.41	0.02	0.21	0.07	0.4	0.02	1.74	0.86
7TLR2(D158)		2	2	4.23	0.02	0.26	0.07	0.35	0.02	5.66	0.02
8TLR2(D165)		2	1	2.05	0.02	0.16	0.19	0.24	0.02	3.04	0.77
9TLR2(D181)		2	1	0.93	0.02	0.04	0.02	0.03	0.02	7.34	0.34
10TLR2(CONTROL)		2	1	0.61	0.02	0.04	0.15	0.35	0.02	1.04	0.77
31NOTLR(D128)		1	1	2.01	0.02	0.02	0.19	0.56	0.02	5.54	0.69
32NOTLR(D149)		1	1	7.6	0.02	0.02	0.15	0.02	0.02	1.39	0.6
36NOTLR(D069)		2	1	0.77	0.02	0.02	0.02	0.24	0.02	0.41	0.47
37NOTLR(D158)		2	2	4.02	0.02	0.02	0.03	0.02	0.02	3.75	0.51
40NOTLR(CONTROL)		2	1	0.33	0.02	0.02	0.07	0.14	0.02	1.74	0.34
7TLR2(D158)		2	2	4.23	0.02	0.26	0.07	0.35	0.02	5.66	0.02
7TLR2(D158)		2	2	4.23	0.02	0.26	0.07	0.35	0.02	5.66	0.02
7TLR2(D158)		2	2	4.23	0.02	0.26	0.07	0.35	0.02	5.66	0.02

Patient ID	HIV status	VVC	IL-12	IL-13	IL-17	G-CSF	GM-CSF	IFN-γ	MCP-1	MIP-1β	TNF-α	TGF-β1	TGF-β2	TGF-β3
1TLR2(D128)	1	1	0.02	0.4	0.02	0.02	30.19	0.02	1.05	30.98	0.02	230.56	49.91	3.8
2TLR2(D149)	1	1	0.02	0.37	0.02	1.73	9.54	1.11	1.12	9.83	0.02	242.62	65.85	8.05
3TLR2(D116)	1	1	0.02	0.34	0.02	0.02	26.47	0.02	1.12	12.39	0.02	214.41	55.8	5.39
4TLR2(D200)	1	1	0.02	0.28	0.02	0.02	37.64	0.02	5.87	21.77	0.02	173.7	51.6	6.98
5TLR2(D086)	1	1	0.02	0.15	0.02	0.02	39.43	0.02	1.12	19.12	0.02	124.05	51.6	0.65
6TLR2(D069)	2	1	0.02	0.21	0.02	0.02	37.97	0.02	1.12	4.41	0.02	203.6	47.38	4.86
7TLR2(D158)	2	2	0.02	0.15	0.02	0.02	33.53	0.02	0.97	14.45	0.02	187.33	51.6	2.74
8TLR2(D165)	2	1	0.02	0.31	0.02	0.02	28.5	0.02	0.85	13.45	0.02	172.33	41.45	4.86
9TLR2(D181)	2	1	0.02	0.21	0.02	0.02	28.93	0.02	0.89	25.02	0.02	184.61	49.07	3.8
10TLR2(CON	2	1	0.02	0.28	0.02	0.02	30.36	0.02	0.97	7.95	0.02	209.01	50.76	6.45
31NOTLR(D1	1	1	0.02	0.08	0.02	0.02	25.44	1.11	1.12	24.4	0.02	237.27	49.91	6.98
32NOTLR(D1	1	1	0.02	0.21	0.02	0.02	27.32	0.02	0.89	4.88	0.02	190.05	53.28	9.65
36NOTLR(DC	2	1	0.02	0.15	0.02	0.02	34.52	0.02	0.97	1.74	0.02	136.56	43.14	4.86
37NOTLR(D1	2	2	0.02	0.21	0.02	0.02	29.35	0.02	1.05	9.36	0.02	206.31	60	5.92
40NOTLR(CC	2	1	0.02	0.15	0.02	0.02	20.04	0.02	0.89	8.75	0.02	253.32	51.6	5.92
7TLR2(D158)	2	2	0.02	0.15	0.02	0.02	33.53	0.02	0.97	14.45	0.02	187.33	51.6	2.74
7TLR2(D158)	2	2	0.02	0.15	0.02	0.02	33.53	0.02	0.97	14.45	0.02	187.33	51.6	2.74
7TLR2(D158)	2	2	0.02	0.15	0.02	0.02	33.53	0.02	0.97	14.45	0.02	187.33	51.6	2.74