The Impact of Semen Exposure on Cytokine Response and Bacterial Vaginosis in the Female Genital Tract

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PREFACE

The experimental work described in this thesis was conducted at the Centre for the AIDS Programme of Research in South Africa (CAPRISA), Doris Duke Medical Research Institute, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa from April 2017 to November 2018, under the supervision of Dr Sinaye Ngcapu.

This work has not been submitted in any form for any degree or diploma to any tertiary institution, where use has been made of the work of others, it is duly acknowledged in the text.

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PLAGIARISM DECLARATION

I, **Khanyisile Mngomezulu** declare as follows:

(i) The research reported in this thesis, except otherwise indicated, is my original work.

(ii) This thesis has not been submitted for any degree or examination at any other university.

(iii) This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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b) Where their exact words have been used, their writing has been placed inside quotation marks and referenced.

Khanyisile Mngomezulu [redacted] Date: 22 November 2018

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ETHICS DECLARATION

This study was approved by the Biomedical Research Ethics Committee of the University of Kwa-Zulu Natal (BE316/17).

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
BV	Bacterial vaginosis
CCR6	CC chemokine receptor 6
CD4	Cluster of Differentiation 4
CIA	Chromatographic Immunoassay
CI	Confidence Interval
CTACK	Cutaneous T-cell attracting chemokine
CVL	Cervicovaginal lavage
ELISA	Enzyme-linked immunosorbent assay
FGT	Female genital tract
FGF-basic	Basic fibroblast growth factor
FSW	Female sex workers
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GRO-α	Growth regulated
H_2O_2	Hydrogen peroxide
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HSV-2	Herpes simplex virus - 2
IFN	Interferon-gamma
IL	Interleukin
IP-10	Interferon gamma-induced protein
IQR	Interquartile range
LIF	Leukemia inhibitory factor
M-CSF	Macrophage colony-stimulating factor
MCP	Monocyte chemotactic protein
MIF	Macrophage migration inhibitory factor
MIG	Monokine induced by gamma- interferon
MIP-1α	Macrophage inflammatory protein- alpha
MIP-1β	Macrophage inflammatory protein- beta
NGF-β	Nerve growth factor
PCA	Principal Component analysis
PCR	Polymerase Chain Reaction

PDGF-ββ	Platelet derived growth factor
PGE2	Prostaglandin E2
PrEP	Pre-exposure prophylaxis
PSA	Prostate specific antigen
RANTES	Regulated upon activation normal T cell expressed and presumably secreted
RIE	Rocket immune electrophoresis
RR	Relative risk
RT	Room temperature
SCF	Stem cell factor
SCGF-β	Stem Cell Growth Factor-beta
SDF-1α	Stromal cell-derived factors 1- alpha
SE	Standard error
SLP-1	Secretory leukocyte protease
STI	Sexually transmitted infections
TGF-β	Transforming growth factor
T regs	T regulatory cells
Th17	T helper 17
TNF-α	Tumour necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
VEGF	Vascular endothelial growth factor

PRESENTATIONS AND PUBLICATIONS

1. **Khanyisile Mngomezulu**, Farzana Osman, Cheryl Baxter, Andile Mtshali, Lenine Liebenberg, Nigel Garrett, Sinaye Ngcapu. Impact of semen exposure on cytokine response and bacterial vaginosis in the female genital tract. Keystone Symposia on Molecular and Cellular Biology (Reproductive Microbiome Meeting in Cape Town), December 11-15, 2018, Cape Town, South Africa. (Poster presentation).
2. **Khanyisile Mngomezulu**, Veron Ramsuran, Cheryl Baxter, Sinaye Ngcapu. PSA – an essential tool for assessing recent unprotected sex and confounders in HIV prevention studies of the female genital mucosa.: Manuscript submitted in Frontiers (submission number- 419712)

ABSTRACT

Background: Diverse microbial communities and inflammatory cytokine responses in the lower female genital tract (FGT) are closely associated with increased human immunodeficiency virus (HIV-1) risk, possibly through increasing mucosal HIV target cell frequency and T-cell activation. The presence of semen in the vagina during unprotected sex has been associated with short-term activation of mucosal immunity. Here, we investigated the extent to which partner semen impacts on cytokine and microbial profiles measured in 248 HIV-uninfected women at high risk for HIV infection.

Methods: We assessed the semen exposure in SoftCup supernatants by quantifying prostate specific antigen (PSA) levels using enzyme-linked immunosorbent assay (ELISA). Luminex was used to measure 48 cytokines in SoftCup supernatants and the vaginal swabs were used for diagnosis of bacterial vaginosis by Nugent score.

Results: PSA, which denotes semen exposure within 48 hours prior to sampling, was detected in 19% (43/248) of SoftCup supernatants. Of the 43 PSA positive women, 70% (30/43) had self-reported condom use at their last sex act and 84% (36/43) had non-Lactobacillus dominant microbiota (Nugent score >7). In addition, PSA was significantly associated with prevalent bacterial vaginosis (Relative Risk (RR), 2.609; 95% Confidence Interval (CI), 1.104 - 6.165; p = 0.029), after adjusting for potential confounders such as age, STIs, current contraceptive use and condom use. Furthermore, women with detectable PSA had high median concentrations of Macrophage inflammatory protein- beta (MIP-1β) (p=0.047) compared to those without PSA.

Conclusion: These findings suggest that the presence of semen has a potential to alter the inflammatory response and microbial communities of the FGT, which may facilitate recruitment of HIV susceptible cells, resulting in increased susceptibility to HIV-1 infection.

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CHAPTER 1: INTRODUCTION

Despite efforts to improve the formatting and phrasing of sexual behaviour questionnaires, over-reporting of condom use and safe sexual practices have been identified as shortcomings in several clinical prevention studies (Turner and Miller, 1997, Zenilman et al., 1995, Minnis et al., 2009). Over-reporting of condom use may lead to inaccurate estimates of the effectiveness of interventions, such as vaginal microbicides to reduce risk of HIV and other sexually transmitted infections (STIs).

Several studies have demonstrated that the presence of semen in the vagina during unprotected sex is associated with inflammatory response and short-term activation of mucosal immunity (Robertson, 2005, Robertson et al., 2009, Sharkey et al., 2012). In addition to spermatozoa, seminal fluid contains potent anti-inflammatory cytokines (Transforming growth factor-beta (TGF-β), Interleukin (IL)-10, Prostaglandin E2 (PGE2), and pro-inflammatory cytokines [IL-8, secretory leukocyte protease (SLP)-1], all with the capacity to alter the immune environment of the vaginal mucosa (Sharkey et al., 2007, Denison et al., 1999). Seminal fluid also contains signaling molecules that increased expression of IL-1 beta (IL-1β), IL-6 and leukemia inhibitory factor (LIF) by endometrial epithelial cells *in vitro* (Gutsche et al., 2003, Sharkey et al., 2012). Furthermore, *in vitro* studies utilising endometrial epithelial cells demonstrated that human seminal plasma reduced the secretions of innate antiviral factors (e.g. secretory leukocyte protease inhibitor), while inducing a cascade of inflammatory cytokines and chemokines (Granulocyte macrophage colony-stimulating factor (GM-CSF), IL-1 alpha (IL-1α), IL-1β, Growth regulating alpha (GROα), Macrophage inflammatory protein- alpha (MIP-1α), MIP-1β, MIP-3 alpha (MIP-3α)) as well as the chemokine ligand for CC chemokine receptor 6 (CCR6) receptor expressed by cluster of differentiation 4 (CD4+) T helper (Th)-17 cells and Langerhans cells (Berlier et al., 2006, Sharkey et al., 2012). Expression of these cytokines is known to trigger the recruitment and activation of susceptible cells (Kachkache et al., 1991, McMaster et al., 1992, Prakash et al., 2003, Sharkey et al., 2012), suggesting that semen can increase a woman's susceptibility to STIs, including HIV.

Another contributing factor responsible for semen-induced immunity and inflammatory responses is the presence of microbial communities within semen, which has the potential to alter the composition of the vaginal microbiota (Mändar et al., 2018, Mändar et al., 2015, Hou et al., 2013). After unprotected sexual intercourse, the seminal microbial communities have been associated with a significant decrease in the relative abundance of the naturally occurring *Lactobacillus* species and an increased relative abundance of diverse bacterial species linked to bacterial vaginosis (BV) (Hou et al., 2013, Mändar et al., 2015, Cherpes et al., 2008). Recently, studies examining the vaginal microbiota by sequencing the 16S rRNA bacterial gene showed diverse vaginal microbiota in young women elevated inflammation, which subsequently led to increased HIV risk by inducing the mucosal HIV target cell frequency and activation (Anahtar et al., 2015, Gosmann et al., 2017). The diversity of microbial assemblages have also been shown to increase HIV risk by weakening the mucosal epithelial barrier function and reducing protective factors such as antimicrobial agents (Nunn et al., 2014).

Given the impact unprotected sexual intercourse has on vaginal immune response and microbiome, an objective assessment of semen exposure is needed to accurately interpret mucosal immunity and microbiota data from vaginal fluids within women enrolled in HIV prevention trials for vaccines, microbicides, and pre-exposure prophylaxis (PrEP). Therefore, researchers have focused on identifying more robust methods to determine sexual and semen exposure, further reducing the reliance on self-reporting in studies investigating immunological factors in the female genital tract, risk of infection or probability of pregnancy (Mauck et al., 2007, Walsh et al., 2003). Two semen biomarkers; prostate-specific antigen (PSA) and the Y-chromosome DNA, have been used to indicate the presence of semen within the FGT (Chomont et al., 2001, Bahamondes et al., 2008). Y-chromosome DNA is detectable for up to 2 weeks post sexual intercourse, using a polymerase chain reaction (PCR) based assay (Zenilman et al., 2005, Penrose et al., 2014), while the PSA protein has a short half-life of 48 hours within the vaginal tract. The PSA, which can be found in high concentrations in vaginal fluids obtained from self-collected swabs post recent semen exposure (Gallo et al., 2006, Hobbs et al., 2009, Mauck, 2009), is more frequently used as a surrogate indicator for unprotected sexual intercourse than the Y-chromosome DNA (Gallo et al., 2013, Jamshidi et al., 2013, Jespers et al., 2017). However, very few studies have used PSA to control for the potential confounding effect of semen in the female genital tract (Jespers et al., 2014, Aho et al., 2010). Most studies use self-reported frequency of sex, the number of partners and condom use to control for confounding (Ravel et al., 2011, Anahtar et al., 2015).

CHAPTER 2:
LITERATURE REVIEW

2.1 PSA as a surrogate indicator for unprotected sexual intercourse

PSA (also known as human tissue kallikrein-3), is a 33kDa glycoprotein secreted in large amounts (0.2–5 mg/mL) by the epithelial cells covering the acini and ducts of prostate gland of males (Sensabaugh, 1978, Lilja et al., 1987). It liquefies the seminal coagulum and promotes the release of motile spermatozoa via degradation of fibronectin and seminogen I and II (Lilja et al., 1987). Studies demonstrated that PSA is not confined to males but levels about 1000-fold lower than those produced by the prostate gland are also found in females (Mannello et al., 1997, Melegos et al., 1996, Diamandis and Yu, 1997). PSA is one of the major proteins of seminal fluid that can be detected at concentrations of ≥1ng/ml within the female genital tract up to 48 hours after unprotected sexual intercourse (Gallo et al., 2013, Jamshidi et al., 2013). Currently, there are several commercially available PSA kits, including quantitative ELISA, rocket immune-electrophoresis (RIE) and chromatographic immunoassay (CIA), with varying specificities and sensitivities (Walsh et al., 2012).

2.2 PSA as a validation marker for self-reported condom use

Several studies have shown that self-reported condom use may be biased towards over- or under-reporting due to participants perceiving some topics as sensitive or the perceived fear of being non-compliant with barrier method use recommended during counselling sessions with study staff, inability to recall experience (including distortion and reconstruction), and unknown condom leakage (Anglewicz et al., 2013, Brener et al., 2003).

To circumvent this bias, PSA has been used to improve the validity of unprotected sexual measurement (Walsh et al., 1999, Thomsen et al., 2007). Table 2.1 shows a summary of previous studies that have investigated the relationship between self-reported condom use and PSA use as a biomarker of recent semen exposure. Findings demonstrated that PSA was detected in the vaginal fluids of women who reported consistent (100%) condom use (Mose et al., 2013, Aho et al., 2010). Similarly, a significant degree of discordance was observed between self-reports and PSA positivity in the vaginal fluids of women reporting no sexual activity or condom-protected vaginal sexual acts within 48 hours prior sample collection (Gallo et al., 2007, Minnis et al., 2009, Woolf-King et al., 2017). A randomised controlled study in Zimbabwe, demonstrated that regardless of the interview approach, self-report was a poor predictor of recent sexual activity and condom use. In this study, 48% (94/196) of women were PSA positive, of which 12% (23/94) had reported no recent sexual activity while 36% (71/94) reported condom protected sexual intercourse (Minnis et al., 2009). Similarly, a study among HIV discordant couples from Kenya showed that 10% (10/98) of women who reported 100% condom-protected vaginal sex in the previous 4 weeks tested positive for PSA (Mose et al., 2013). In another study among female sex workers (FSW), PSA was detected in 35.8% (77/215) of women who self-reported condom use at their last sexual activity (Aho et al., 2010). In contrast, there was no correlation between positive PSA results and condom use in the vaginal fluids of women who reported condom failures (Walsh et al., 2012, Walsh et al., 1999). The contrasting findings with PSA testing may be due to PSA's rapid half-life of 48 hours (Macaluso et al., 1999), low amount of detectable PSA, use of different PSA assays between the studies or use of vaginal products such as spermicides and lubricants (Snead et al., 2013). A more sensitive Y-chromosome PCR assay, which detects DNA, could be used in conjunction with the PSA assay. However, it would be costly to run both assays in large size cohorts and Y chromosome may also not be a reliable biomarker of recent semen exposure, since the Y-chromosome DNA can be detected up to 10 days after unprotected sexual intercourse (Zenilman et al., 2005).

2.3 Effect of PSA on genital inflammation

The mucosal epithelium of the lower female reproductive tract provides the first line of defence against pathogen entry and mediates the initial host immune response against invading pathogens such as STIs and HIV (Kaushic, 2011, Wira et al., 2005b). The vagina and cervix are common sites for transmission of the virus because semen containing HIV would come into contact with these sites (Hladik and Hope, 2009). The surface area of the lower reproductive tract exposed during sexual intercourse in women is greater than the reproductive tissue of men, which may increase the surface area exposed, time in contact with infectious fluids post-coitus, and exposure of intraepithelial HIV target cells to pathogens (Kaushic, 2011, Wira et al., 2005a, Wira et al., 2005b). Seminal fluid contains potent anti-inflammatory (TGF-β, IL-10, and PGE2), pro-inflammatory cytokines (IL-8, SLP1) and bacteria, all with the capacity to alter the immune environment of the vaginal mucosa (Figure 2.1) (Mändar et al., 2015, Hou et al., 2013, Mändar et al., 2018, Sharkey et al., 2007, Denison et al., 1999). Therefore, objective assessment of semen exposure is important to assist in the accurate interpretation of data in studies of the immunological environment in the female genital tract.

Table 2. 1: Summary of studies exploring the relationship between self-reported sexual activity and PSA detection

Study	N, population	PSA detection
Mose *et al*., 2013	125 HIV discordant couples, 124 were tested for PSA	10% of 98 women who reported 100% use of condoms in previous month tested positive for PSA
Minnis *et al*., 2009	910 sexually active, HIV uninfected 18-49 years old	Among those with PSA detected, 48% reported no unprotected coitus in the past 2 days
Gallo *et al*., 2006	332 female sex workers	Study found an important discordance between self-reported recent condom use and the presence of PSA in FSW, with PSA being present in 39% of FSW who reported protected sexual intercourse only in the preceding 48 h & 21% reporting no sex
Aho *et al*., 2010	223 female sex workers	Found PSA in 70 of the 196 FSW (38.4%) who reported no unprotected intercourse in the past 48 h
Jespers *et al*., 2014	430 women from Kenya, South Africa, Rwanda	The detection of PSA in the cervicovaginal lavages of all these women was a better predictor of BV than self-reported sexual behaviour, which then shows that self-reported sexual behaviour is often inaccurate
McCoy *et al*., 2014	195 Zimbabwean women, HIV uninfected	Of the 195 women tested positive for PSA, 94 women misreported sexual behaviour, reporting no sex or only condom-protected sex in the previous 2 days
Zia *et al*., 2017	73 HIV-infected & 24 HIV uninfected Malawian women on DMPA and LVG	Tested 539 vaginal swabs from 97 women, of these women 55 were PSA-positive and 54 had reported unprotected coitus, while among the PSA positive samples, 62% (65/105) of these women reported no unprotected sex

PSA=Prostate specific antigen, FSW=Female sex workers; DMPA= Depot Medroxyprogesterone Acetate*; LVG=* Levonorgestrel

To eliminate the hidden effects of semen on immunological data, several immunological studies measure the presence of semen contamination using PSA in vaginal swab supernatants. PSA results can be used to adjust for the confounding effect of semen and/or stratify participants according to those with presence or absence of PSA to delineate the effect of semen contamination. In one study, recent sexual intercourse, as measured by the presence of PSA in vaginal fluids, was associated with significantly higher levels of pro-inflammatory cytokines (IL-6, IL-12 p70 , and IFN-γ-induced protein 10 (IP-10)) (Jespers et al., 2017). These findings are in agreement with previous studies that reported a significant association between the presence of PSA and levels of IL-6 and IP-10 in the vaginal fluids of women (Francis et al., 2016, Kyongo et al., 2012). Similar semen-induced expression of female genital tract pro-inflammatory cytokines and chemokines such as IL-6, IL-8, monocyte chemoattractant protein

(MCP)-1, and GM-CSF by endometrial epithelial cells *in vitro* have been reported (Kyongo et al., 2012, Francis et al., 2016). Expression of these cytokines is known to trigger the recruitment and activation of macrophages, dendritic cells, T lymphocytes and granulocytes (Prakash et al., 2003, Sharkey et al., 2007). Although inflammation is thought to promote conducive environments for conception and pregnancy (Robertson, 2005, Schuberth et al., 2008), genital inflammation has also been associated with an increased risk of infections through disruption of the epithelial barrier and/or recruitment of susceptible target cells to a site of viral infection (Lawn et al., 2001, Masson et al., 2015). While non-human primate studies did not demonstrate significant effect on recruitment of target cells and transmission following *in vitro*/*in vivo* semen exposure (Miller et al., 1994, Neildez et al., 1998, Allen et al., 2015), the semen-mediated effects emphasize a need to assess the camouflaged effects of recent semen exposure on the immune environment in the female genital tract.

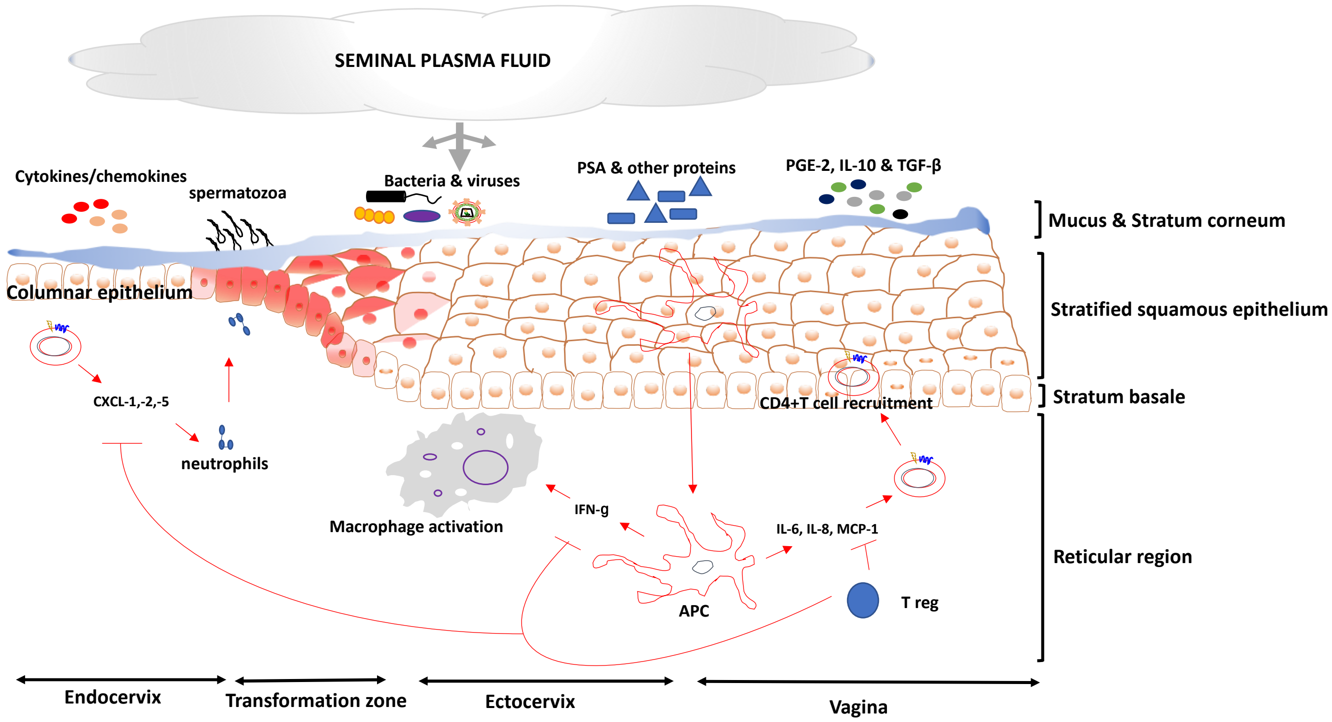

Figure 2.1: Impact of semen on female genital tract microenvironment. In addition to spermatozoa, seminal fluid contains potent anti-inflammatory cytokines, soluble proteins, bacteria, viruses and pro-inflammatory cytokines, all with the capacity to alter the immune environment of the vaginal mucosa. It has been shown that presence of semen in the female genital tract induced inflammatory response which resulted in activation of tissue residence macrophages, neutrophils and T cells. Mucosal T regulatory cells (T regs) have been shown to prevent the semen-induced inflammatory response in the female genital tract. Figure modified from (Rametse et al., 2014) (Viral immunology).

2.4 Effect of PSA on genital microbial community

The female genital tract contains many microbial species, with lactic-acid producing *Lactobacillus* species typically dominating the genital mucosa of reproductive-age women (Aroutcheva et al., 2001, Selle and Klaenhammer, 2013). The genital mucosal surface not dominated by *Lactobacillus* species may facilitate transmission of STIs, including HIV, as well as increase the risk of urogenital disease, miscarriages, preterm births and sepsis in pregnant women (van de Wijgert et al., 2008, Srinivasan et al., 2012). *Lactobacillus* species (*L. crispatus*, *L. gasseri* and *L. jensenii*), which are well established as healthy vaginal commensal organisms, play a role in inhibiting the colonization and survival of reproductive tract pathogens, as they produce lactic acid, hydrogen peroxide (H_2O_2) and bacteriocins (Buve et al., 2014, Hayes et al., 2010). The absence of lactic acid producing *Lactobacillus* species may lead to BV, a common vaginal dysbiosis that has been associated with increased risk of HIV acquisition in observational studies (Buve et al., 2014, van de Wijgert et al., 2014).

Semen can serve as a medium for the transmission of bacterial communities between men and women (Hou et al., 2013, Gallo et al., 2011). It is expected that vaginal microbiota would be affected by the seminal communities transferred into the FGT during unprotected sexual intercourse. Although data is inconsistent, several studies have demonstrated that new or multiple sexual partners and frequent unprotected sexual intercourse have been significantly associated with an increased risk of BV (Cherpes et al., 2008, Fethers et al., 2008, Schwebke et al., 1999), while others did not show similar findings (Baeten et al., 2009, Newton et al., 2001, Eschenbach et al., 2001). Unprotected sexual intercourse has been associated with an increase in the BV-related microbiota, with a significant reduction in *Lactobacillus* species (Brotman et al., 2010, Gajer et al., 2012). Similarly, unprotected sexual intercourse has been associated with significant decrease in relative abundance of *Lactobacillus crispatus* in couples (Mändar et al., 2015). Recent semen exposure, as measured by the presence of PSA in the vaginal fluid, has been associated with significant decrease in the abundance of *Lactobacillus* species (Jespers et al., 2017, Jespers et al., 2015) and increased BV recurrence (Turner et al., 2016). The association between semen exposure and changes in the vaginal microbiota confirms the need to frequently screen vaginal fluids for the presence of semen and to adjust for recent semen exposure, even if the study participant did not report sexual intercourse.

2.5 Study Aims, Objectives & Hypothesis

2.5.1 Aims:

- To determine the extent to which partner semen contamination impacts on cytokine profiles measured in SoftCup supernatants from sexually active women.
- To evaluate the relationship between presence of PSA and incident and recurrent STIs and BV.

Objectives:

- To detect presence of semen in SoftCup supernatants using PSA ELISA.
- To determine the concordance between self-reporting of consistent condom use and the presence of PSA.
- To measure the impact of semen contamination on cytokine profiles.
- Correlate the detection of PSA with incident and recurrent STIs and BV in women on antimicrobial therapy.

Hypothesis:

SoftCup supernatants from sexually active women will be contaminated with trace amounts of semen that will alter cytokine levels in women enrolled in CAPRISA 083 cohort. Furthermore, recent unprotected sexual intercourse will be associated with recurrence of BV and STIs, regardless of treatment exposure.

CHAPTER 3:

Impact of semen exposure on cytokine response and bacterial vaginosis in the female genital tract

3.1 Introduction

Despite improvements in study questionnaire format, self-reported condom use is still a drawback that may lead to bias estimates of the influence of prevention strategies on risk for sexual transmitted infections, including HIV (Ghanem et al., 2007). To circumvent this, PSA has been applied in biomedical prevention strategies as biomarker of semen exposure (Macaluso et al., 1999, Aho et al., 2010).

Semen exposure through unprotected sexual intercourse as well as inconsistent and incorrect condom use has been associated with increased recruitment of mucosal immune cells and a change in the vaginal microbiota with impairment in H_2O_2 producing lactobacilli colonization (Jespers et al., 2017, Eschenbach et al., 2001, Jespers et al., 2015, O'Hanlon et al., 2010). Both high levels of female genital tract inflammatory cytokines and altered vaginal bacterial communities have been associated with elevated genital inflammation and increased HIV risk, likely by increasing mucosal HIV target cell frequency and T cell activation (Anahtar et al., 2015, Gosmann et al., 2017, Masson et al., 2015). Furthermore, semen effect on the mucosal micro-environment may impact both physiological and patho-physiological events at the FGT. This includes tissue remodelling, response to foreign antigens in seminal fluid and bacterial and viral infections such as HIV. Thus, an objective assessment of how semen contamination impacts the immunological environment as well as the vaginal microbiota is important.

Taking this into consideration, these recent findings highlight the importance of understanding the concordance between self-reporting of consistent condom use and the presence of semen biomarkers, as measured by PSA. In addition, it is also clear that an extensive understanding of the impact of semen exposure on FGT cytokine milieu and microbial communities is warranted. In this study, we determined the concordance between self-reporting of consistent condom use and the presence of semen biomarkers. We also evaluated the extent to which partner semen contamination impacts on cytokine profiles, STIs and BV measured in SoftCup supernatant samples from sexually active women. Furthermore, we proposed to investigate the relationship between semen exposure and incident and recurrent STIs and BV.

3.2 Methods and Materials

3.2.1 Study design, participants and specimen collection

SoftCup supernatant, genital swabs, cytobrushes and cervicovaginal fluids were collected at baseline, week 6 and month 3 from 248 women undergoing STI management in the CAPRISA 083 study. CAPRISA 083 is a prospective study aimed at reducing STIs in women by enhancing the STI management package offered for targeted laboratory-diagnosed STI care, ensuring that the individual is cured and by reducing the risk of reinfection using expedited partner therapy. Participant demographics and clinical data were collected at enrolment through structured questionnaire. At enrolment, vulvovaginal swabs were collected from the posterior fornices and lateral vaginal walls, followed by placing menstrual cup (SoftCup®, EuroFemPro, Netherlands) for one hour to collect genital secretions for both microbial and immunological assays. At each study visit, HIV rapid testing, Herpes Simplex Virus type 2 (HSV-2) and Human papilloma virus (HPV) were done using real time PCR and conversional PCR to control for the impact of common viral causes of female genital tract inflammation. Study participants were eligible for enrolment if they were female, aged 18-40 years and HIV-1 uninfected. Participants were not eligible for enrolment if they were HIV-1 infected, on their menstrual cycle at sample collection, pregnant, women who disclose any form of sex work, and women who have had antibiotic treatment within the last 7 days. Point of care STI screening was performed using GeneXpert (Cepheid, North America) assays for *Chlamydia trachomatis (C. trachomatis*) and *Neisseria gonorrhoea (N. gonorrhoea)*. *Trichomonas vaginalis* (*T .vaginalis*) assessment was conducted using the wet prep and results were confirmed with PCR. Women infected with *C. trachomatis*, *N. gonorrhoea* and *T. vaginalis* were treated with 1g azithromycin, 250mg ceftriaxone IMI and 2g metronidazole, respectively. BV was determined by Nugent score (score of < 3 was regarded as normal vaginal flora, 4-6 as intermediate flora and 7-10 as BV). Women who were diagnosed with intermediate flora, BV and *T. vaginalis* were offered a single dose of oral metronidazole 2g.

3.2.2 ELISA to detect prostate specific antigen (PSA)

Human tissue kallikrein 3 (R&D Systems, Quantikine ELISA, Inc., Minneapolis, USA), commonly known as PSA, was measured in SoftCup supernatants using ELISA. Briefly, 50 µl of SoftCup supernatant was used for PSA detection, with upper limit of detection of 60 ng/ml and a threshold positivity of 0.94 ng/ml, as per manufacturer’s protocol. Every plate included PSA standards (provided in the kit) and negative control containing sterile PCR-grade water and reaction mix. The average absorbance values for each set of reference standards, negative control, positive control and the samples were measured at 450 nm wavelength using the VersaMax™ absorbance microplate reader (Molecular Devices, Inc., Sunnyvale, USA). For a detailed method refer to (Appendix A, 1.1).

3.2.3 Cytokines measurements

At baseline, concentration levels of 48 cytokines were detected in SoftCup supernatants and expressed in log_{10} (pg/ml) from CAP083 female participants. The cytokine panel included chemokines, pro-inflammatory cytokines, adaptive, growth factors and anti-inflammatory: IL-1β, IL-1Rα, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-12p40, IL-16, IL-18, IL-1A, IL-2RA, IL-3, IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF-basic), cutaneous T-cell attracting chemokine (CTACK), Eotaxin, granulocyte colony-stimulating factor (G-CSF), GM–CSF,GRO-α, hepatocyte growth factor (HGF), IFN-γ, IFN-α2, IP-10, LIF, MCP-1, MCP-3, macrophage colony-stimulating factor (M-CSF), monokine induced by gamma- interferon (MIG), Macrophage migration inhibitory factor (MIF), MIP–1α, MIP-1β, nerve growth factor-beta (NGF-β), platelet derived growth factor (PDGF-ββ), regulated upon activation normal T cell expressed and presumably secreted (RANTES),stem cell factor (SCF), stem cell growth factor-beta (SCGF-β), stromal cell-derived factors 1- alpha (SDF-1α), tumour necrosis factor alpha (TNF–α), TNF-beta (TNF-β), TNF-related apoptosis-inducing ligand (TRAIL), and vascular endothelial growth factor (VEGF) were measured using the Bio-Plex Pro Human Cytokine kits Group I (27-Plex Panel) and Group II (21-Plex Panel) in a Bio-Plex Reader™200 system (Bio-Rad Laboratories, USA). Assays were performed according to the manufacturer's protocol. SoftCup supernatants were thawed overnight on ice and filtered by centrifugation using 0.2 μm cellulose acetate filters (Sigma, USA). Bio-Plex manager software (version 5.0; Bio-Rad Laboratories Inc®, USA) was also used to analyse the data and all analyte concentrations were extrapolated from the standard curves using a 5 PL regression equation. Analyte concentrations that were below the lower limit of detection of the assay were reported as the mid-point between zero and the lowest concentration measured for each analyte. For a detailed method refer to (Appendix A, 1.2).

3.2.4 Statistical analysis

Descriptive statistics were summarized using medians and interquartile ranges for continuous variables and proportions for categorical variables. The Fisher's exact test was used to compare proportions between groups, whilst the Wilcoxon rank sums test was used to compare two medians. To measure the impact of semen exposure on cytokine concentrations, linear mixed models were fitted to log-transformed cytokine concentrations. Multivariable models adjusted for age, STI, BV, current contraception use and condom use. Statistical analyses were conducted using GraphPad Prism 7.05 (GraphPad Software, USA) and SAS version 9.3 (SAS Institute Inc., Cary).

3.3 Results

3.3.1 Clinical and socio-behavioural characteristics of the study participants

Overall, the median age of the women was 23 years (interquartile range (IQR) 21-27 years), with majority (73%, 177/244) completing secondary education. About 73% (182/248) of the women reported using condoms with a partner to prevent STIs while 65% (161/248) reported using a condom occasionally. Only 35.9% (89/248) of study participants reported the use of any form of contraception to prevent unplanned pregnancies. Of the 35.9% women who reported contraception use, 58% (52/89) were using progesterone based injectables (Table 3.1).

Of the 248 women enrolled in this study, only 43 (19%) women tested positive for PSA in SoftCup supernatants by ELISA. About 69% (30/43) of the women who reported condom use with their partner tested positive for PSA, suggesting that condom use was likely over-reported or they engaged in unprotected sexual intercourse 48 hours before sample collection. PSA was detected in 30% (13/66) of SoftCup supernatants from women who reported no condom use with a partner. Although not significant, PSA was more frequently detected in women using progesterone based injectables compared to other forms of contraceptive users (oral-contraceptive pill, subdermal implant and condoms).

We examined the relationship between PSA and prevalent BV or STIs. Of the 248 women who were screened for BV, 31% (76/248) had a normal vaginal flora as indicated by Nugent score of < 3 (dominated by *Lactobacillus spp*.), 35% (87/248) had intermediate BV (Nugent score 4-6, with a diversity of bacteria) and 34% (85/248) had BV (Nugent score >7, with a diversity of anaerobic bacteria). Women in whom PSA was detected had slightly higher BV prevalence than PSA-negative women (Table 3.1). Women with intermediate BV were more likely to have PSA detected (49%, 21/43; p=0.038), while women with any form of STIs were less likely to have PSA in their genital secretions (20%, 10/50) (Table 3.1). At baseline, the majority 14% (35/248) of women were infected with *C. trachomatis*, followed by *N. gonorrhoeae* (4%, 11/248) and *T. vaginalis* (4%, 9/248). 23% (8/35) of women with *C. trachomatis* and 11% (1/9) in those with *N. gonorrhoeae* or T. *vaginalis* 9% (1/11), respectively.

Twenty-eight percent (61/228) of women cleared both STI (n=43) and BV (n=18) after treatment at baseline, with an exception of 54 women who continued to have persistent STI (n=2) or BV (n=52). Of these women 28% (15/54) tested positive for PSA. Persistence of STIs and BV might have been due to that the participants may have not completed treatment or engaged in unprotected sex with STI/BV infected partner or may have been infected with drug resistant isolate (e.g. *C. trachomatis*, *Gardnerella vaginalis etc.*). The small number of participants with recurrent STI or BV at follow-up (week 6, n=76 and month 3, n=74) limited our analysis and could not determine the relationship between semen exposure and incident and recurrent STI or BV.

Table 3.1: Baseline participant demographics according to presence of PSA in genital secretions

Variable	Level	Overall N=248	PSA+ N=43	PSA- N=205	P value
		% (n/N) or Median (IQR)			
Age	Median (IQR)	23 (21 - 27)	23 (21 - 27)	23 (21- 26)	0.291
Highest level of education	Primary Education	0.4 (1/244)	0	0.5 (1/201)	0.651
	Secondary Education	72.5 (177/244)	76.7 (33)	71.6 (144/201)	
	Tertiary Education	27.0 (66/244)	23.3 (10)	27.9 (56/201)	
Do you use condoms with your partner(s) to protect yourself from STIs?	Yes	73.4 (182)	69.8 (30)	74.1 (152)	0.572
	No	26.6 (66)	30.2 (13)	25.9 (53)	
How often do you use condoms?	Always	4.0 (10)	0	4.9 (10)	0.290
	Sometimes	64.9 (161)	62.8 (27)	65.4 (134)	
	Never	31.0 (77)	37.2 (16)	29.8 (61)	
Are you using contraception or practicing any form of birth control?	Yes	35.9 (89)	27.9 (12)	37.6 (77)	0.294
	No	64.1 (159)	72.1 (31)	62.4 (128)	
Contraception use	Condom only	7.9 (7/89)	0	9.1 (7/77)	0.590
	Oral-contraceptive pill	11.2 (10/89)	8.3 (1/12)	11.7 (9/77)	
	Progesterone injections	58.4 (52/89)	83.3 (10/12)	54.5 (42/77)	
	Subdermal Implant	20.2 (18/89)	8.3 (1/12)	22.1 (17/77)	
	Intra-uterine device (IUD)	2.2 (2/89)	0	2.6 (2/77)	
Bacterial vaginosis:	Normal	30.6 (76)	16.3 (7)	33.7 (69)	**0.038***
	Intermediate	35.1 (87)	48.8 (21)	32.2 (66)	
	BV	34.3 (85)	34.9 (15)	34.1 (70)	
Sexual transmitted infections					
T. vaginalis	Positive	3.6 (9)	2.3 (1)	3.9 (8)	1.000
	Negative	96.4 (239)	97.7 (42)	96.1 (197)	
C. trachomatis	Positive	14.1 (35)	18.6 (8)	13.2 (27)	0.342
	Negative	85.9 (213)	81.4 (35)	86.8 (178)	

N. gonorrhoeae	Positive	4.4 (11)	2.3 (1)	4.9 (10)	0.695
	Negative	95.6 (237)	97.7 (42)	95.1 (195)	
Any STI (*C. trachomatis*, *N. gonorrhoeae* or *T. vaginalis*)	Positive	20.2 (50)	23.3 (10)	19.5 (40)	0.539
	Negative	79.8 (198)	76.7 (33)	80.5 (165)	

** P < 0.05*, *PSA=prostate specific antigen, C. trachomatis-Chlamydia Trachomatis, N. gonorrhoeae -Neisseria gonorrhoeae, T. vaginalis-Trichomonas vaginalis, BV-bacterial vaginosis, STIs-sexually transmitted infections, IQR- interquartile range. Descriptive statistics are reported as medians and IQRs (continuous data) or percentages (categorical data). Numbers were not the same in some groups due to missing data. PSA concentrations greater than 1.0 ng/mL were considered as providing evidence of semen exposure within the past 2 day.*

3.3.2 Recent unprotected sex and relative risk for BV or STI

We next assessed the relative risk of acquiring BV or STIs in women whom PSA was detected using a logistic regression model. After adjusting for potential confounders such as age, STIs, current contraceptive use and condom use, PSA was significantly associated with prevalent BV (RR, 2.607; 95% CI, 1.086 - 6.258; p=0.032) (Table 3.2).

Table 3.2: Associations between recent unprotected sex and BV

				95% Confidence Interval		
Characteristic	**Level**	**Relative Risk**	**Standard error**	**Lower**	**Upper**	**P value**
PSA	**Negative**	Ref				
	Positive (unadjusted)	2.609	1.145	1.104	6.165	0.029
	Positive (adjusted)*	2.607	1.165	1.086	6.258	0.032

*P<0.05, * multivariate analysis (Adjusted for age, STIs, condom use and contraceptive use)*

In contrast, we observed no significant association between recent unprotected sex, as measured by PSA, and relative risk of acquiring STIs (RR, 1.074; 95% CI, 2.419 – 0.476; p = 0.864) (Table 3.3).

Table 3.3: Associations between recent unprotected sex and STIs

				95% Confidence Interval		
Characteristic	**Level**	**Relative Risk**	**Standard error**	**Lower**	**Upper**	**P value**
PSA	**Negative**	Ref				
	Positive (unadjusted)	1.250	0.502	0.569	2.747	0.579
	Positive (adjusted)*	1.074	0.445	0.476	2.419	0.864

*P<0.05, *multivariate analysis (Adjusted for age, condom use, current contraceptive use, BV)*

3.3.3 Cytokine expression profiles in women with and without PSA

The concentrations of 48 cytokines were assessed in the SoftCup supernatants of each participant at baseline. Unsupervised hierarchical clustering of cytokines identified no overt differences of cytokine expression profiles in women with or without PSA in their genital fluid (Figure 3.1a). Principal component analysis (PCA) confirmed this finding, with no notable differences in principal component distribution of cytokines observed in women who PSA was detected versus those without PSA (Figure 3.1b).

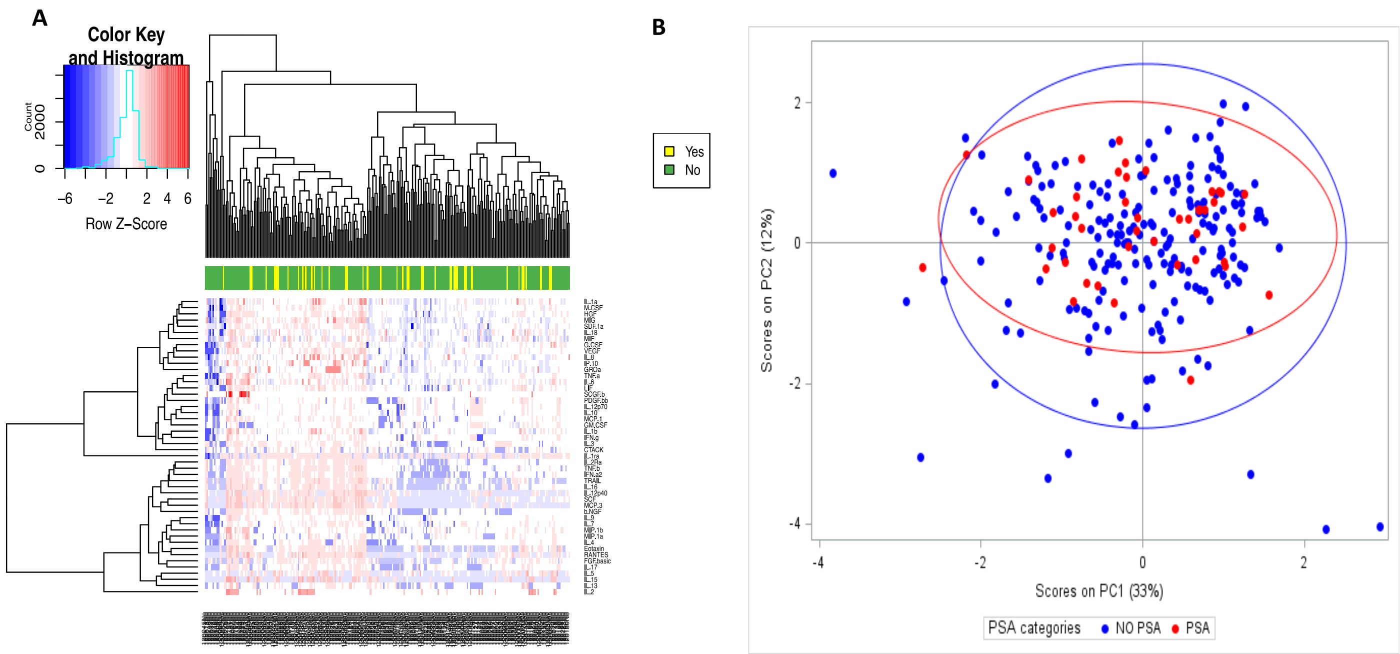

Figure 3.1: Cytokine concentrations in women with PSA versus those without PSA. (A): Hierarchical clustering depicting cytokine expression profiles. Red and blue colours represent the standardized cytokine concentration values above and below zero, respectively. Yellow bars indicate women who tested positive for PSA while green indicate those without detectable PSA. The vertical axes represent the individual cytokines (right) and clusters (left); while horizontal axes represent participant identities. Cytokine values were scaled and centred for dendogram plotting. **(B)**: Principal component analysis of cytokines in women who PSA was detected versus those without PSA. Red dots (n=43) indicate women who tested positive for PSA while blue dots (n=205) indicate those without detectable PSA.

3.3.4 Effect of semen altered interferon gamma in SoftCup supernatants of women with BV

Next, we investigated the impact of semen exposure in SoftCup supernatants cytokine concentrations from women with BV. There was no significant difference found in women with BV and tested PSA positive compared to those with BV and without PSA (Figure 3.2). To circumvent the potential bias BV may have on cytokines (Masson et al., 2015), we removed those that were BV positive and analysed only those without BV. Women without BV and who had recent unprotected sex had decreased concentrations of IFN-γ (p=0.014), compared to women who tested negative for both BV and PSA (Figure 3.2). The association remained strong even after adjusting for age, current contraceptive use, STIs and condom use.

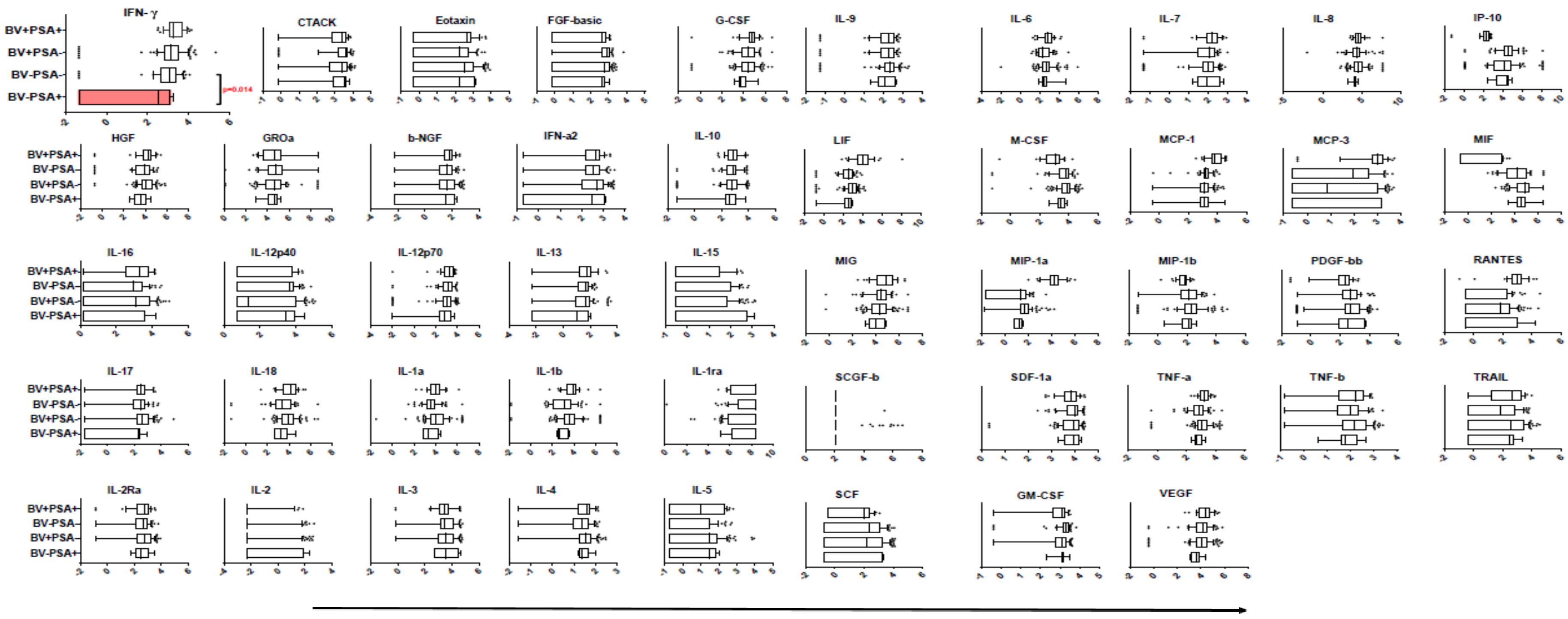

Figure 3.2: **Impact of semen exposure on SoftCup supernatant cytokine concentrations in women who tested for BV and PSA.** These were stratified into two comparative groups such as (i) BV positive and PSA positive versus BV positive and PSA negative and (ii) BV negative and PSA positive versus BV negative and PSA negative. Box-and-whisker plot range between the 25th – 75th percentiles, lines indicate medians, whiskers indicate 10-90th percentiles, dots indicate outliers. A significant difference is shown by p<0.05.

3.3.5 Impact of PSA on innate factors in the female genital tract

We also assessed the impact of PSA on cytokine milieu of women with STIs. The concentrations of soluble factors MIP-1α (p=0.047) were higher in women with STIs and had recent unprotected sex compared to women with STIs and tested negative for PSA. However, this did not remain significant after adjusting for confounders such as age, condom use, BV and current contraceptive use (Figure 3.3).

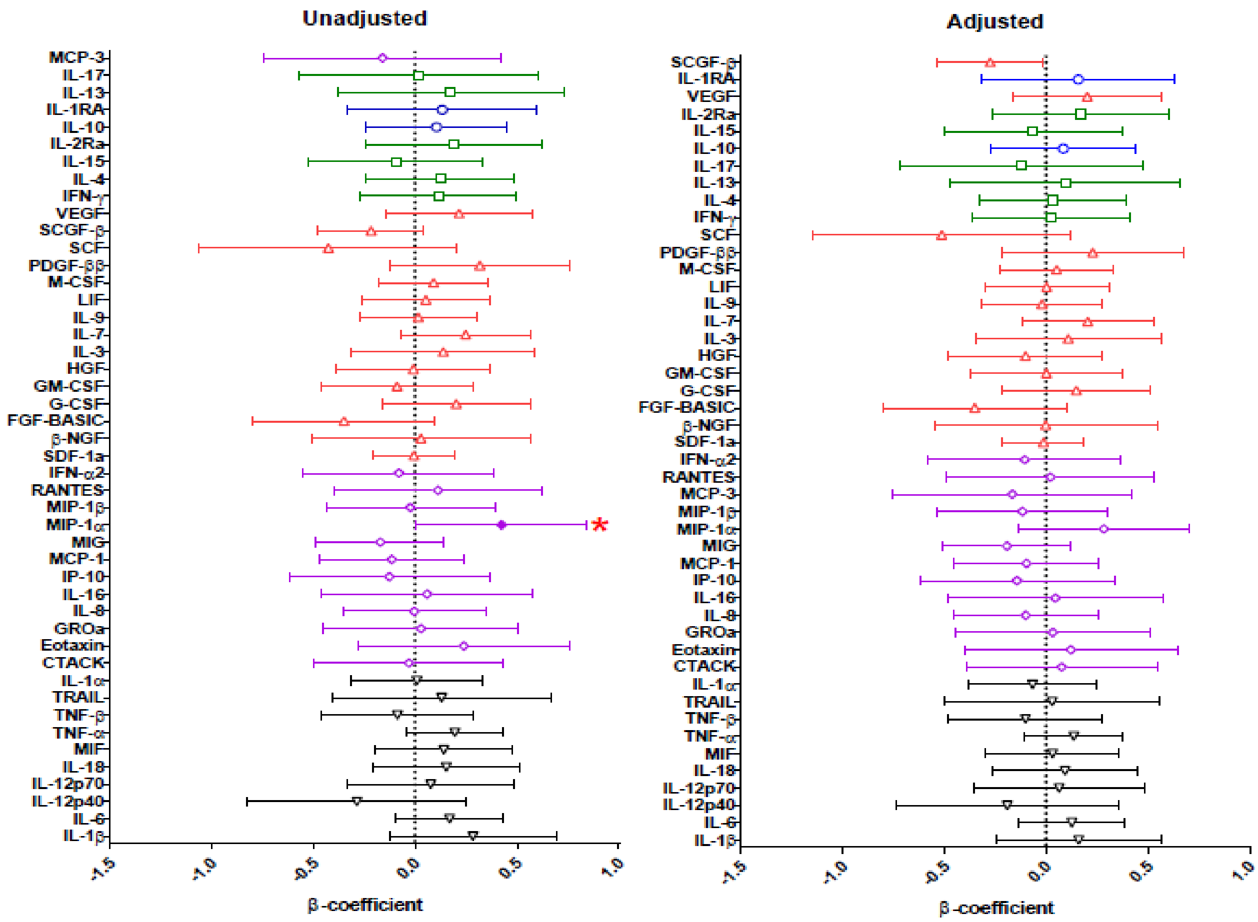

Figure 3.3: **Linear regression model was used to evaluate the relationship between cytokine concentrations in SoftCup supernatants and PSA from 248 HIV uninfected women.** The cytokine concentrations were log-transformed and the cytokine concentrations were compared to PSA (whether positive or negative). The error bars indicate 95% confidence intervals. A significant association is shown by a shade circle and red asterisk (p<0.05). Unadjusted is for the univariate analysis and adjusted is for the multivariate analysis. *Cytokine functions: pro-inflammatory – black inverted pyramid, chemokines – purple diamond, growth factors – red triangle, adaptive - green squares and anti-inflammatory cytokines – blue circles.*

Furthermore, we excluded women with STIs to remove the biased effects STIs have on cytokines (Masson et al., 2016, Masson et al., 2014). The concentrations of MIP-1α were significantly increased in PSA positive and STI negative women versus PSA negative and STI negative women (p=0.030), but no significant associations were observed after adjusting for age, condom use, BV and current contraceptives use (Table 3.4). In addition, IL-1β (p=0.075); IL-6 (p=0.052) and TNF-α (p=0.056) concentrations tended to be increased in women who were PSA positive without STIs, although not after adjusting for multiple comparisons, except for IL-6 (p=0.094).

Table 3.4*:* Effect of semen of on cytokine/chemokine profiles in female genital tract secretions

Functional groups	Cytokines	PSA positive STI negative		PSA negative STI negative		Univariate		#Multivariate	
		Median (pg/ml)	IQR	Median (pg/ml)	IQR	β coefficient (SE)	P value	βcoefficient (SE)	P value
Pro-Inflammatory	IL-1α	3.865	3.340 - 4.335	3.835	3.323 - 4.338	0.048 (0.174)	0.787	-0.016 (0.175)	0.929
	IL-1β	3.851	2.897 - 4.202	3.401	2.763 - 3.855	0.422 (0.235)	**0.075**	0.267 (0.234)	0.255
	IL-6	2.828	2.548 -3.328	2.520	2.066 - 2.917	0.297 (0.152)	**0.052**	0.259 (0.155)	**0.094**
	IL-12p40	3.259	0.705 - 3.792	3.345	0.705 - 3.847	-0.219 (0.306)	0.474	-0.078 (0.306)	0.800
	IL-12p70	3.300	2.674 - 3.610	3.068	2.619 - 3.487	0.062 (0.250)	0.805	0.054 (0.256)	0.833
	IL-18	3.887	3.061 - 4.626	3.900	3.018 -4.306	0.173 (0.221)	0.435	0.089 (0.220)	0.687
	MIF	4.702	4.134 - 5.171	4.739	3.728 - 5.201	0.107 (0.194)	0.582	-0.015 (0.192)	0.937
	TNF-α	3.222	2.793 - 3.508	2.943	2.676 - 3.290	0.266 (0.138)	**0.056**	0.207 (0.140)	0.142
	TNF-β	2.205	1.597 - 2.568	2.059	1.559 - 2.552	-0.075(0.218)	0.729	-0.081 (0.220)	0.715
	TRAIL	2.472	-0.367 - 3.149	2.339	-0.367 - 3.095	0.272 (0.309)	0.381	0.213(0.309)	0.493
Chemokines	CTACK	3.451	2.870 - 3.632	3.478	2.862 - 3.668	-0.112 (0.275)	0.685	0.038 (0.273)	0.891
	EOTAXIN	2.640	-0.352 - 2.898	2.432	-0.352 - 2.892	0.354 (0.299)	0.237	0.224 (0.302)	0.459
	GRO-a	4.658	3.792 - 5.065	4.609	3.775 - 5.312	0.257 (0.295)	0.386	0.257 (0.291)	0.377
	IL-8	4.466	4.103 - 4.854	4.465	4.042 - 4.931	0.142 (0.204)	0.487	0.032 (0.205)	0.876
	IL-16	3.223	0.158 - 3.760	2.975	0.158 - 3.569	0.189 (0.295)	0.524	0.161 (0.304)	0.597
	IP-10	4.177	3.440 - 4.607	4.252	3.289 - 4.895	0.088 (0.288)	0.761	0.099 (0.282)	0.723
	MCP-1	3.038	2.809 - 3.236	3.102	2.870 - 3.291	-0.041 (0.210)	0.847	0.002 (0.209)	0.994
	MCP-3	-0.629	-0.629 - (2.892)	-0.629	-0.629 - (2.829)	-0.087 (0.335)	0.795	-0.057 (0.339)	0.866
	MIG	4.270	3.826 - 4.660	4.310	3.699 - 4.786	-0.126 (0.188)	0.500	-0.134 (0.189)	0.480
	MIP-1α	1.682	1.436 - 1.911	1.544	1.180 - 1.920	0.536 (0.245)	**0.030**	0.383 (0.243)	0.116
	MIP-1β	2.204	1.851 - 2.593	2.230	1.700 - 2.591	0.223 (0.239)	0.353	0.112 (0.241)	0.644
	RANTES	1.897	-0.523 - 2.395	1.737	-0.523 - 2.366	0.287 (0.290)	0.324	0.201 (0.295)	0.496

	IFN-α2	2.634	2.163 - 2.857	2.550	1.883 - 2.851	-0.127 (0.273)	0.643	-0.131 (0.276)	0.636
Growth Factors	β-NGF	1.794	1.354 - 1.997	1.611	1.076 - 2.070	-0.073 (0.318)	0.818	-0.097 (0.327)	0.767
	FGF-Basic	2.656	-0.171 - 2.840	2.800	-0.171 - 2.991	-0.331 (0.262)	0.207	-0.314 (0.263)	0.234
	G-CSF	4.729	4.246 - 5.005	4.403	3.728 - 4.964	0.243 (0.209)	0.245	0.218 (0.214)	0.310
	GM-CSF	3.093	2.719 - 3.198	3.188	2.936 - 3.341	-0.079 (0.196)	0.688	-0.023 (0.199)	0.906
	HGF	4.162	3.654 - 4.524	4.035	3.487 - 4.470	0.193 (0.224)	0.390	0.095 (0.227)	0.676
	IL-3	3.475	3.032 - 3.767	3.409	3.011 - 4.061	0.139 (0.263)	0.595	0.132 (0.266)	0.620
	IL-7	2.201	1.951 - 2.530	2.046	1.748 - 2.324	0.237 (0.192)	0.219	0.196 (0.197)	0.316
	IL-9	2.317	2.050 - 2.546	2.309	2.013 - 2.546	0.126 (0.170)	0.461	0.089 (0.173)	0.604
	LIF	2.979	2.401 - 3.334	2.788	2.417 - 3.221	0.046 (0.188)	0.809	-0.019 (0.185)	0.918
	M-CSF	3.767	3.452 - 4.240	3.803	3.459 - 4.061	0.106 (0.168)	0.531	0.067 (0.173)	0.700
	PDGF-ββ	2.956	2.514 - 3.316	2.769	2.232 - 3.241	0.386 (0.271)	0.156	0.311 (0.2744)	0.258
	SCF	-0.757	-0.757-(3.010)	1.914	-0.757 - 2.922	-0.306 (0.368)	0.407	-0.357 (0.370)	0.337
	SCGF-β	2.073	2.073 - 2.073	2.073	2.073 - 2.073	-0.112 (0.104)	0.286	-0.148 (0.106)	0.167
	SDF-1α	3.84	3.537 - 4.085	3.875	3.499 - 4.139	0.053 (0.102)	0.602	0.053 (0.103)	0.611
	VEGF	4.313	3.763 - 4.672	4.127	3.686 - 4.584	0.243 (0.215)	0.259	0.234 (0.220)	0.289
Adaptive	IFN-γ	3.227	2.947 - 3.572	3.109	2.729 - 3.495	0.119 (0.229)	0.606	-0.001 (0.232)	0.998
	IL-2†	-2.301	-2.301 - (-2.301)	-2.301	-2.301 - (2.301)	-	-	-	-
	IL-4	1.510	1.221 - 1.703	1.443	1.140 - 1.731	0.270 (0.222)	0.224	0.176 (0.225)	0.434
	IL-5†	1.035	-0.745 - (2.044)	1.035	-0.745 - (1.717)	-	-	-	-
	IL-13	1.631	1.068 - 1.989	1.647	1.061 - 1.952	0.079 (0.334)	0.813	-0.001 (0.341)	0.999
	IL-15	-0.561	-0.5607-(1.631)	-0.561	-0.561 - (1.925)	-0.132 (0.251)	0.600	-0.114 (0.253)	0.654
	IL-17	2.392	2.120 - 2.713	2.481	2.027 - 2.898	0.039 (0.341)	0.909	-0.105 (0.345)	0.761
	IL-2RA	2.752	2.062 -3.081	2.65	2.062 - 3.061	0.280 (0.259)	0.280	0.305 (0.263)	0.248
Anti-Inflammatory	IL-10	2.859	2.450 - 3.154	2.822	2.430 - 3.155	0.143 (0.211)	0.499	0.119 (0.215)	0.581
	IL-1RA	8.425	6.115 - 8.425	8.425	6.423 - 8.425	0.002 (0.264)	0.523	0.002 (0.271)	0.993

SE=standard error, IQR = Interquartile range, PSA = prostate specific antigen. #Multivariate analysis adjusted for age, STIs, BV, current contraceptive use and condom use. †Variables with at least a third of concentrations that were undetectable were dichotomised and a logistic regression model was fitted to estimate the effect of PSA on detectability of these cytokine. Bold indicates significance $p<0.05$.

3.4 DISCUSSION

This study observed high levels of discordance between participant's self-report of consistent condom use and PSA positivity. This is particularly not surprising especially in large observational reproductive and sexual health research studies. Two-thirds of women who reported condom use with their partners to protect themselves from STIs tested positive for PSA. A positive PSA ELISA in women who reported 100% condom use likely indicates biased self-reporting of condom use or incorrect condom use (unprotected exposure to semen) by male partner during sexual act 48 hours prior sampling. Several studies have reported high rates (up to 38%) of breakage, leakage, slipping off, reuse, and the late application or early removal of condoms in young people (Crosby et al., 2005, Visser and Smith, 2000). Other than possible false positive results, inconsistencies between self-reported condom use and PSA positivity in SoftCup supernatants may also be due to participants perceiving some topics as sensitive or the perceived fear of being non-compliant with barrier method use recommended during counselling sessions with study staff. Furthermore, use of hormonal contraception may contribute to inconsistencies between self-reported condom use and PSA positivity, as contraceptive users are less likely to use a condom (McCoy et al., 2014). PSA was also detected in women who had reported never using condoms during coitus and this was an expected result for these participants.

Semen has been shown to serve as a medium for the transmission of bacterial communities between unprotected sexual partners (Hou et al., 2013, Gallo et al., 2011), resulting in changes in the vaginal microbial communities. Our study found that recent semen exposure (as measured by PSA positivity) was associated with BV prevalence. These findings are consistent with a study that found a microbial shift after unprotected sexual intercourse, resulting in a decreased abundance of *Lactobacillus spp.* and overgrowth of anaerobic BV-associated bacteria such as *Gardnerella vaginalis, Prevotella, Atopobium vaginae* (Brotman et al., 2010, Hou et al., 2013, Jespers et al., 2014). In addition, another study showed a significant association between BV, being a sex worker and recent semen exposure amongst female sex workers recruited from three different African countries (Jespers et al., 2014). It is plausible to assume that the microbial changes brought by the presence of semen exposure are short lived and may be due to the alkaline pH found in semen. *Lactobacillus spp.* thrive in acidic environment with high glycogen content while they struggle in environments with pH greater than 4.5 (Ravel et al., 2011, Petrova et al., 2015). In contrast, bacterial species such as *Gardnerella vaginalis, Prevotella, Atopobium vaginae* dominate in high pH environments (Onderdonk et al., 2016, Srinivasan et al., 2012, van de Wijgert et al., 2014). Despite this strong link between recent semen exposure and women with intermediate flora, no differences in prevalence of STIs (including *C. trachomatis, N. gonorrhoea, and T. vaginalis*) were found.

Previous studies have demonstrated a semen induced inflammatory response by endometrial epithelial cells *in vitro* (Robertson, 2007, Robertson, 2005, Robertson et al., 2009). In agreement with previous studies, this study found that SoftCup supernatants of women with BV and semen present (as measured by PSA positivity) had reduced concentrations of inflammatory IFN-γ, while an increased expression of the MIP-1α was observed in women with STI and semen present (Jespers et al., 2017). Furthermore, there was a trend for increased pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in PSA positive women without any STI compared to PSA negative/STI negative women. Several studies showed that high levels of pro-inflammatory cytokines such as IL-6, IFN-γ, MIP-1α were closely associated with elevated female genital tract inflammation and increased HIV risk, likely by increasing mucosal HIV target cell frequency and T cell activation (Masson et al., 2015, Anahtar et al., 2015, Francis et al., 2016, Kyongo et al., 2012). The associations between cytokines and recent semen exposure should be interpreted conservatively as none of these associations were significant after adjusting for multiple comparisons and sample sizes for these analyses were relatively small. In addition, seminal fluids might dilute mucosal secretions and may result in reduced concentrations of some cytokines in secretions.

This study had several limitations. Firstly, there was a relatively small sample size at the follow-up visits (week 6 = 76 and month 3 = 74) due to lost to follow up. Secondly, the present study did not include Y chromosome data, which is indicative of unprotected sexual act with 15 days. Furthermore, there was a lack of information from the participants on self-reported timing and use of condoms in the recent sex act. Several studies have reported BV and/or STIs recurrence even after successful treatment (Bradshaw et al., 2006, Eschenbach et al., 2001) and this recurrence has been attributed to biofilm (produced by microbes such as *Gardnerella vaginalis*) or reinfection from "BV/STI boyfriends", an untreated sexual partner with BV and/or STIs (Manhart et al., 2013). The present study could not evaluate the relationship between the presence of recent semen exposure and incident and recurrent STIs and BV. This was attributed to small samples size of those who cleared BV/STI and had recurrence. The impact of recent semen exposure on FGT cytokines was also assessed cross-sectionally instead of longitudinally, where analytes are investigated in the same women prior and post coitus. Furthermore, cytokine levels are higher in mucosal secretions from younger women compared to older women, yet this study did not age-match participants for subsequent cytokine analyses. All limitations mentioned above are being addressed in further studies.

3.5 Conclusion and future consideration

This study found a significantly high level of discordance between self-report of condom use and presence of semen (PSA positivity). The findings of this study suggest that the presence of semen has a potential to alter the inflammatory response and microbial communities of the FGT, which may facilitate recruitment of HIV susceptible cells, resulting in increased susceptibility to HIV-1 infection. Thus, the detection of recent semen exposure by measuring the presence of PSA in the vaginal fluids is a potentially important tool to reduce the biases inherent to self-reporting of condom use in participants of HIV prevention trials. In addition, the assessment or validation of self-reported condom use is essential in biomedical prevention studies aimed at identifying modifiable behavioural and biological factors that increase women's vulnerability to infection.

In future, PSA could be used to reflect sexual risk behaviour and provide an objective interpretation of data in studies of the female mucosal microenvironment as semen exposure may substantially modify the microbial and immune environment within the female genital mucosa. Detection of PSA in the vaginal fluids provides a unique opportunity to be considered as a tool to verify recent semen exposure (from an infected male partner) in future studies assessing HIV infection risk by detecting HIV DNA in vagina fluids from HIV-uninfected women or those primarily interested in measuring incidence rates of STIs. Another opportunity for investigation is the potential for longitudinal analysis to evaluate the relationship between the presence of semen biomarkers and recurrent STIs and/or BV. Considering the accuracy and sensitivity of PSA assays and the impact of recent semen exposure on mucosal microenvironment, clinical trials should consider measuring PSA to objectively measure sexual risky behaviours and to accurately assess the immune response in the female genital mucosa.

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APPENDICES

APPENDIX A: ELISA to detect PSA

1.1 ELISA to detect prostate specific antigen (PSA)

The *ELISA* was used to detect the human tissue kallikrein 3 (R&D Systems, Inc., Minneapolis, USA), commonly known as prostate specific antigen in SoftCup supernatant and this was conducted according to the manufacturer's protocol.

1.1.1 Reagent Preparation

All the reagents were brought to room temperature before use.

List of reagents supplied by manufacturer (R&D systems, Inc. USA and Canada)

- Human KLK3/PSA microplate
- Human KLK3/PSA conjugate
- Human KLK3/PSA standard
- Assay Diluent RD1W
- Calibrator Diluent RD5-19
- Wash Buffer Concentrate
- Colour Reagent A
- Colour Reagent B
- Stop solution
- Plate sealers

Other supplies used include:

- VersaMax™ ELISA Microplate Reader
- BioTek microplate washer
- Pipettes, pipette tips
- Deionized water
- Automated microplate washer
- 500 ml graduated cylinder and test tubes for dilution standards.

Figure A1.1: Illustration showing preparation of wash buffer

1.1.2 Preparation of 500 ml wash buffer

The 500 ml wash buffer was prepared by adding 20 ml of the wash buffer concentrate to 480 ml of deionized water and mixed well by shaking the bottle (Figure A1.1).

1.1.3 Preparation of Human KLK3/PSA Standards

Firstly, the lyophilized standard concentrate was reconstituted with 1000 µl of deionized water. The powder was dissolved completely by shaking and vortexing, and was left to stand for 15 minutes with gentle shaking.

1.1.3.1 Preparation of serial dilutions

Seven Facs tubes (BD) were labelled from 0 – 8 (60 ng/ml, 30 ng/ml, 15 ng/ml, 7.5 ng/ml, 3.75 ng/ml, 1.88 ng/ml and 0.94 ng/ml) for the different concentrations. To 60 ng/ml tube, 900 ul of Calibrator Diluent RD5-19 (used for cell culture supernatant samples) was added and to the remaining tubes 500 µl was added. The prepared standard stock solution was used to make serial dilutions (Figure A1.2). Hundred microliters (100 µl) of the stock solution was pipetted into the 60 ng/ml tube, the solution was then mixed well, vortexed and 500 µl transferred into the 30 ng/ml tube. Each tube was mixed thoroughly before the next transfer. The serial dilution was conducted till the 0.94 ng/ml tube. Into the tube labelled zero, 500 µl of deionized water was added and this tube served as a blank.

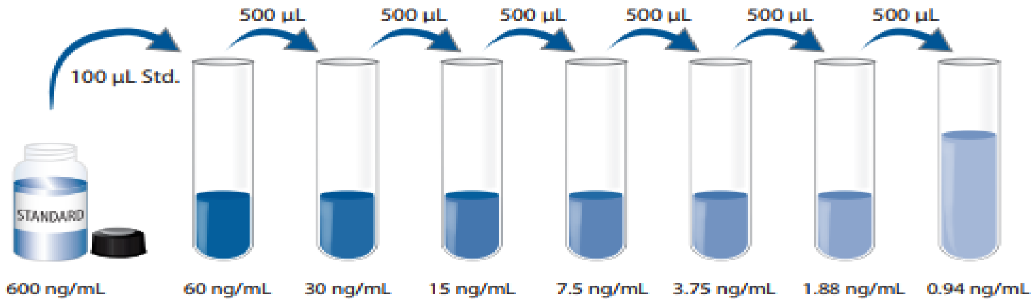

Figure A1.2: Representative diagram of preparation of standards (Adapted from manufacturers protocol, R&D systems, Inc., USA and Canada)

1.1.4 PSA ELISA assay procedure

Briefly, the plate seal was removed and 100 µl of Assay Diluent RDW1 (used for cell culture samples) was pipetted into each well. Thereafter, 50 µl of standards and samples were added to all the designated wells and the plate was sealed with the cover strips and then incubated for 2 hours at room temperature (RT). After incubation, the plate was placed in an automated plate washer and each well was aspirated and washed using the wash buffer. Each well was washed four times, then after washing, 200 µl of Human KLK3/PSA conjugate was added to each well and incubated for 2 hours at RT. After the incubation period was over, the plate was placed in the automated plate washer, the liquid was aspirated and each well washed four times using wash buffer. Next, a volume of 200 µl of substrate solution was added to each well, the plate was then covered with foil to protect from exposure to light and then incubated for 30 minutes at RT. After incubation, 50 µl of stop solution was pipetted to each well and a colour change was observed. The colour in the wells changed from blue to yellow upon addition of the stop solution. If in some wells the colour was not uniform, the plate was gently tapped to ensure thorough mixing. Figure A1.3 shows the PSA ELISA plate with standards, samples and positive wells in which the KLK3/PSA conjugate acted on the substrate to produce an initial blue colour and upon addition of the stop solution, a yellow-green colour developed. The plate was then placed on the VersaMax™ absorbance microplate reader (Molecular Devices, Inc., Sunnyvale, CA, USA) for measurement of PSA protein from the samples for 30 minutes. The machine was set at wavelength of 450 nm with wavelength corrections set to 540 nm or 570 nm. Figure A1.4 shows the readings obtained from the microplate reader after 30 minutes.

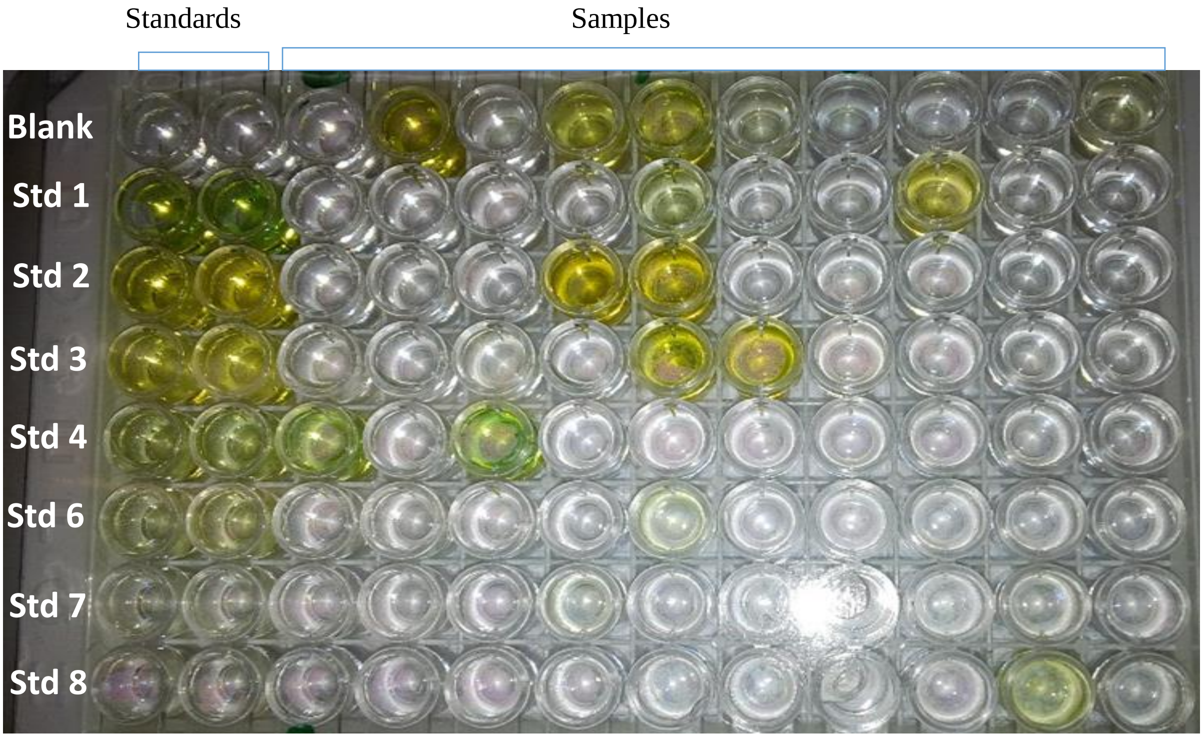

Figure A1.3: PSA ELISA plate. In the positive wells, the KLK3/PSA conjugate acted on the substrate to produce an initial blue colour and upon addition of the stop solution, a yellow-green colour developed.

Plate01

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.055 0.039	0.056 0.040	0.055 0.039	3.536 0.125	0.066 0.039	0.757 0.052	2.359 0.081	0.170 0.041	0.058 0.042	0.056 0.040	0.055 0.039	0.210 0.043
B	2.802 0.116	2.562 0.125	0.053 0.038	0.059 0.041	0.063 0.048	0.055 0.039	0.383 0.051	0.054 0.040	0.053 0.039	0.683 0.047	0.054 0.039	0.055 0.038
C	1.842 0.070	2.082 0.068	0.066 0.048	0.070 0.042	0.057 0.041	3.703 0.141	3.756 0.141	0.054 0.039	0.061 0.041	0.056 0.040	0.055 0.039	0.057 0.039
D	1.010 0.057	1.130 0.058	0.081 0.041	0.058 0.041	0.144 0.044	0.073 0.042	4.000 0.161	3.982 0.153	0.113 0.039	0.056 0.040	0.053 0.037	0.090 0.072
E	0.402 0.055	0.463 0.063	0.926 0.084	0.055 0.041	2.550 0.159	0.057 0.042	0.056 0.041	0.057 0.040	0.060 0.045	0.058 0.043	0.057 0.043	0.056 0.041
F	0.284 0.068	0.330 0.049	0.055 0.040	0.078 0.045	0.065 0.050	0.058 0.041	0.280 0.056	0.060 0.041	0.054 0.038	0.057 0.042	0.056 0.042	0.057 0.040
G	0.127 0.044	0.136 0.047	0.051 0.039	0.054 0.040	0.051 0.038	0.154 0.046	0.057 0.041	0.057 0.043	0.059 0.040	0.053 0.039	0.101 0.042	0.059 0.043
H	0.100 0.043	0.103 0.043	0.051 0.037	0.053 0.039	0.065 0.041	0.059 0.041	0.055 0.039	0.061 0.042	0.058 0.042	0.055 0.039	0.509 0.055	0.094 0.042

Figure A1.4: Plate layout with PSA reading (results above 0.1 ng/ml were regarded as positive).

1.2 Female genital tract cytokine concentration measurements

The concentrations of 48 cytokines were measured in SoftCup supernatants from women enrolled in the CAP083 study. The cytokine panel included pro-inflammatory cytokines, chemokines, growth factors, adaptive and anti-inflammatory: Interleukin (IL)-1β, IL-1Rα, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-12p40, IL-16, IL-18, IL-1A, IL-2RA, IL-3, IL-13, IL-15, IL-17, basic FGF, CTACK, Eotaxin, G-CSF, GM–CSF, GRO-α, HGF, IFN-γ, IFN-α2, IP-10, LIF, MCP-1, MCP-3, M-CSF, MIG, MIF, MIP–1α, MIP-1β, β-NGF, PDGF-ββ, RANTES, SCF, SCGF-β, SDF-1α, TNF-α, TNF-β, TRAIL, and VEGF were measured using the Bio-Plex Pro Human Cytokine Group I (27-Plex Panel) and Group II (21-Plex Panel) Bio-Plex Reader™200 system (Bio-Rad Laboratories,USA). The cytokines were grouped according to their immune characteristics as shown Table A1.1.

Table A1.1: Showing cytokines grouped according to their general immune characteristics

Pro-inflammatory	Chemokines	Growth Factors	Adaptive	Anti-inflammatory
IL-1α	CTACK	β-NGF	IFN-γ	IL-10
IL-1β	EOTAXIN	FGF-BASIC	IL-2	IL-1RA
IL-6	GRO-α	G-CSF	IL-4	
IL-12p40	IL-8	GM-CSF	IL-5	
IL-12p70	IL-16	HGF	IL-13	
IL-18	IP-10	IL-3	IL-15	
MIF	MCP-1	IL-9	IL-17	
TNF-α	MCP-3	LIF	IL-2RA	
TNF-β	MIG	M-CSF		

TRAIL	MIP-1α	PDGF-ββ
	MIP-1β	SCF
	RANTES	SCGF-β
	IFN-α2	SDF-1α
		VEGF
		IL-7

1.2.1 Cytokine measurement assay

1.2.1.1 List of reagents supplied by manufacturer (Bio-Rad Laboratories, Inc., USA)

- Standard diluent
- Sample diluent
- Assay buffer
- Detection antibody diluent
- Streptavidin-PE
- Filter plater and/or flat bottom plate (96 well)
- Sealing tape
- Instruction manual
- Coupled magnetic beads
- Detection antibodies

Other supplies recommended:

- Bio-Plex® 200 system
- Bio-Plex Pro wash station
- Microtiter plate shaker,
- Vortex
- Reagent reservoirs

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S9	S9	5	5		21	29	37	45	53
					120003_26-Jul-16_1030	120003_26-Jul-16_1030	120010_18-May-16_1000	120014_19-May-16_1000	120017_23-May-16_1000	120020_06-Jul-16_1020	120023_19-Aug-16_1030	120027_26-May-16_1000
B	S2	S2	S10	S10	6	6	14	22	30	38	46	54
					120004_13-May-16_1000	120004_13-May-16_1000	120010_12-Jul-16_1020	120014_30-Jun-16_1020	120017_19-Jul-16_1020	120020_31-Aug-16_1030	120024_26-May-16_1000	120028_31-May-16_1000
C	S3	S3	Blank	Blank	7	7	15	23	31	39	47	55
					120005_13-May-16_1000	120005_13-May-16_1000	120010_15-Aug-16_1030	120015_23-May-16_1000	120017_16-Aug-16_1030	120021_24-May-16_1000	120024_15-Jul-16_1020	120028_21-Jul-16_1020
D	S4	S4	Cntrl	Cntrl	8	8	16	24	32	40	48	56
					120006_17-May-16_1000	120006_17-May-16_1000	120011_19-May-16_1000	120015_19-Jul-16_1020	120018_24-May-16_1000	120022_25-May-16_1000	120024_19-Aug-16_1030	120028_06-Oct-16_1030
E	S5	S5	1	1	9	9	17	25	33	41	49	57
			120001_12-May-16_1000	120001_12-May-16_1000	120007_17-May-16_1000	120007_17-May-16_1000	120012_19-May-16_1000	120015_23-Aug-16_1030	120019_24-May-16_1000	120022_07-Jul-16_1020	120025_26-May-16_1000	120029_31-May-16_1000
F	S6	S6	2	2	10	10	18	26	34	42	50	58
			120002_12-May-16_1000	120002_12-May-16_1000	120008_17-May-16_1000	120008_17-May-16_1000	120013_19-May-16_1000	120016_23-May-16_1000	120019_06-Jul-16_1020	120022_17-Aug-16_1030	120026_26-May-16_1000	120030_31-May-16_1000
G	S7	S7	3	3	11	11	19	27	35	43	51	59
			120003_13-May-16_1000	120003_13-May-16_1000	120008_17-Aug-16_1030	120008_17-Aug-16_1030	120013_01-Jul-16_1020	120016_04-Jul-16_1020	120019_18-Aug-16_1030	120023_25-May-16_1000	120026_11-Jul-16_1020	120030_20-Jul-16_1020
H	S8	S8	4	4	12	12	20	28	36	44	52	60
			120003_22-Jun-16_1020	120003_22-Jun-16_1020	120009_18-May-16_1000	120009_18-May-16_1000	120013_12-Aug-16_1030	120016_22-Aug-16_1030	120020_25-May-16_1000	120023_19-Jul-16_1020	120026_24-Aug-16_1030	120030_26-Aug-16_1030

Figure A1.5: Representation of cytokine assay plate layout with standards, blanks, controls and samples.

1.2.2 Cytokine assay procedure

To analyse the concentrations of the 48 cytokines, luminex was conducted according to the manufacturer's protocol (Bio-Plex Pro Human Cytokine Group I (27-Plex Panel) and Group II (21-Plex Panel), Bio-Rad Laboratories, Inc., USA). For sample preparation, prior to assay setup: 50 ul of SoftCup supernatants was added to 300 ul of PBS, spun down and filtered using a spin column. The standards and quality control were included in the kit. All buffers, standards, coupled beads and samples were brought to room temperature prior to use. To briefly explain the assay, Figure A1.5 shows the plate layout that was designed. The lyophilized standards was reconstituted with 500 µl diluent and mixed gently by vortexing for 3 seconds then incubated on ice for 30 minutes. Following reconstitution, the standards were serially diluted to 1:4 dilutions in assay buffer and used as a reference for the quantification of the analytes. The coupled beads were diluted to a 1× concentration assay buffer and vortexed for 30 seconds and then 50 µl of the assay buffer was added to each well. The plate was first washed twice with 100 µl of wash buffer Bio-Plex Reader™200 system (Bio-Rad Laboratories, USA). Following washing, 50 µl of standards, controls and samples were added into their designated wells and incubated for 30 minutes at RT while on the shaker (the plate was covered with aluminium foil for protection from light). After incubation, the plate was washed three times with 100 µl of wash buffer, then 25 µl of detection antibodies were added to each well and the plate was sealed and incubated at RT for 30 minutes. Following incubation, the plate was taken to the plate washer and washed three times with wash buffer. After washing, a volume of 50 µl of streptavidin-PE was added to each well with 10 minutes incubation at RT on a shaker. Following incubation, the beads were re-suspended in 125 µl of assay buffer and this was added to each well. The samples were then quantified using the Bio-Plex 200 system (Bio-Rad, Inc., USA).

APPENDIX B: Raw data

Table B1: Univariate linear model for cytokines and PSA (with STI positives)

			95% CI		
Cytokine	**β coefficient**	**Std error**	**Upper**	**Lower**	**P value**
b-NGF	0.03	0.27	0.56	-0.51	0.917
CTACK	-0.03	0.24	0.43	-0.50	0.897
Eotaxin	0.24	0.26	0.76	-0.28	0.367
FGF-basic	-0.35	0.23	0.10	-0.80	0.126
G-CSF	0.20	0.19	0.57	-0.17	0.280
GM-CSF	-0.09	0.19	0.28	-0.46	0.639
GROa	0.03	0.24	0.51	-0.45	0.905
HGF	-0.01	0.19	0.37	-0.39	0.964
IFN-a2	-0.08	0.24	0.39	-0.55	0.738
IFN-g	0.11	0.19	0.50	-0.27	0.561
IL-10	0.11	0.18	0.45	-0.24	0.552
IL-12p70	0.08	0.21	0.49	-0.33	0.710
IL-12p40	-0.29	0.27	0.25	-0.82	0.296
IL-13	0.17	0.28	0.73	-0.38	0.539
IL-15	-0.10	0.22	0.34	-0.53	0.663
IL-16	0.06	0.26	0.58	-0.46	0.823
IL-17	0.02	0.30	0.60	-0.57	0.959
IL-18	0.15	0.18	0.51	-0.21	0.401
IL-1a	0.01	0.16	0.33	-0.32	0.961
IL-1b	0.28	0.21	0.70	-0.13	0.177
IL-1ra	0.13	0.24	0.60	-0.33	0.574
IL-2	-0.17	0.26	0.35	-0.68	0.527
IL-2Ra	0.19	0.22	0.62	-0.24	0.382
IL-3	0.14	0.23	0.59	-0.31	0.546
IL-4	0.12	0.18	0.48	-0.24	0.506
IL-5	-0.05	0.23	0.40	-0.50	0.831
IL-6	0.17	0.13	0.43	-0.09	0.204
IL-7	0.25	0.16	0.57	-0.07	0.128

IL-8	-0.004	0.18	0.35	-0.36	0.981
IL-9	0.02	0.15	0.31	-0.27	0.911
IP-10	-0.13	0.25	0.36	-0.62	0.610
LIF	0.05	0.16	0.37	-0.26	0.745
M-CSF	0.09	0.14	0.36	-0.18	0.516
MCP-1	-0.12	0.18	0.24	-0.47	0.524
MCP-3	-0.16	0.30	0.42	-0.74	0.589
MIF	0.14	0.17	0.48	-0.20	0.413
MIG	-0.17	0.16	0.14	-0.49	0.285
MIP-1a	0.42	0.21	0.84	0.01	0.047
MIP-1b	-0.02	0.21	0.39	-0.44	0.912
PDGF-bb	0.32	0.22	0.76	-0.12	0.158
RANTES	0.11	0.26	0.62	-0.40	0.667
SCF	-0.43	0.32	0.21	-1.06	0.186
SCGF-b	-0.22	0.13	0.04	-0.48	0.101
SDF-1a	-0.01	0.10	0.19	-0.20	0.949
TNF-a	0.19	0.12	0.43	-0.05	0.111
TNF-b	-0.09	0.19	0.29	-0.46	0.644
TRAIL	0.13	0.27	0.67	-0.41	0.634
VEGF	0.22	0.18	0.58	-0.14	0.239

Univariate logistic regression (STI participants included)

Cytokine	**Relative Risk**	**Standard Error**	**95% CI**		**P value**
			Lower	**Upper**	
IL-2	0.761	0.363	0.299	1.938	0.567
IL-5	0.831	0.280	0.429	1.609	0.583

Table B2: Multivariate linear model for cytokines and PSA (Adjusted for Age, STI, BV, Current contraception use and condom use)

			95% CI		
Cytokine	**β coefficient**	**Standard Error**	**Lower**	**Upper**	**P value**
b-NGF	-0.002	0.278	-0.549	0.546	0.995
CTACK	0.077	0.239	-0.393	0.547	0.747

Eotaxin	0.121	0.266	-0.403	0.645	0.650
FGF-basic	-0.350	0.230	-0.804	0.103	0.129
G-CSF	0.149	0.187	-0.219	0.517	0.426
GM-CSF	0.002	0.188	-0.369	0.373	0.991
GROa	0.033	0.241	-0.442	0.509	0.891
HGF	-0.102	0.193	-0.483	0.279	0.599
IFN-a2	-0.106	0.242	-0.583	0.371	0.662
IFN-g	0.022	0.197	-0.367	0.410	0.912
IL-10	0.084	0.181	-0.272	0.440	0.643
IL-12p70	0.064	0.212	-0.354	0.483	0.763
IL-12p40	-0.191	0.276	-0.735	0.353	0.490
IL-13	0.095	0.288	-0.474	0.663	0.743
IL-15	-0.067	0.221	-0.502	0.367	0.760
IL-16	0.045	0.268	-0.483	0.573	0.867
IL-17	-0.125	0.303	-0.721	0.472	0.681
IL-18	0.093	0.180	-0.263	0.448	0.608
IL-1a	-0.067	0.161	-0.384	0.250	0.678
IL-1b	0.160	0.207	-0.249	0.569	0.441
IL-1ra	0.158	0.241	-0.317	0.634	0.512
IL-2Ra	0.168	0.221	-0.267	0.603	0.447
IL-3	0.109	0.232	-0.348	0.566	0.639
IL-4	0.031	0.182	-0.327	0.390	0.863
IL-6	0.126	0.133	-0.136	0.388	0.343
IL-7	0.205	0.165	-0.120	0.530	0.214
IL-8	-0.101	0.182	-0.459	0.257	0.579
IL-9	-0.020	0.150	-0.316	0.276	0.894
IP-10	-0.143	0.244	-0.623	0.337	0.558
LIF	0.003	0.156	-0.304	0.309	0.986
M-CSF	0.051	0.141	-0.226	0.328	0.715
MCP-1	-0.096	0.181	-0.452	0.259	0.594
MCP-3	-0.166	0.300	-0.757	0.424	0.579
MIF	0.032	0.167	-0.298	0.361	0.851
MIG	-0.194	0.159	-0.507	0.120	0.225
MIP-1a	0.284	0.212	-0.133	0.701	0.181
MIP-1b	-0.116	0.212	-0.534	0.302	0.585

PDGF-bb	0.229	0.226	-0.215	0.674	0.311
RANTES	0.021	0.261	-0.493	0.535	0.936
SCF	-0.513	0.320	-1.144	0.118	0.111
SCGF-b	-0.275	0.131	-0.534	-0.016	0.038
SDF-1a	-0.013	0.102	-0.214	0.187	0.896
TNF-a	0.136	0.122	-0.105	0.377	0.266
TNF-b	-0.101	0.191	-0.479	0.276	0.596
TRAIL	0.030	0.270	-0.502	0.561	0.912
VEGF	0.202	0.187	-0.167	0.571	0.282

Multivariate logistic regression (STI participants included)

Adjusted for age, STI, BV, current contraception use and condom use

Cytokine	**Relative Risk**	**Standard Error**	**95% CI**		**P value**
			Lower	**Upper**	
IL_2	0.713	0.354	0.269	1.88	0.495
IL-5	0.723	0.253	0.364	1.434	0.353

Table B3: Univariate linear model for cytokines and PSA (No STI)

			95% CI		
Cytokine	**β Coefficient**	**Standard Error**	**Lower**	**Upper**	**P value**
b-NGF	-0.073	0.318	-0.699	0.553	0.818
CTACK	-0.112	0.275	-0.655	0.431	0.685
Eotaxin	0.354	0.299	-0.235	0.943	0.237
FGF-basic	-0.331	0.262	-0.847	0.184	0.207
G-CSF	0.243	0.208	-0.168	0.655	0.245
GM-CSF	-0.079	0.196	-0.466	0.308	0.688
GROa	0.257	0.295	-0.326	0.839	0.386
HGF	0.193	0.224	-0.249	0.635	0.390
IFN-a2	-0.126	0.273	-0.664	0.411	0.643
IFN-g	0.119	0.229	-0.333	0.570	0.606
IL-10	0.143	0.211	-0.273	0.558	0.499
IL-12p70	0.062	0.250	-0.432	0.555	0.805

IL-12p40	-0.220	0.306	-0.823	0.384	0.474
IL-13	0.079	0.335	-0.581	0.739	0.813
IL-15	-0.132	0.251	-0.628	0.364	0.600
IL-16	0.188	0.295	-0.394	0.771	0.524
IL-17	0.039	0.341	-0.634	0.712	0.909
IL-18	0.173	0.221	-0.263	0.609	0.435
IL-1a	0.047	0.174	-0.295	0.389	0.787
IL-1b	0.422	0.235	-0.042	0.886	0.075
IL-1ra	0.002	0.264	-0.518	0.523	0.993
IL-2Ra	0.280	0.259	-0.230	0.790	0.280
IL-3	0.140	0.263	-0.378	0.658	0.595
IL-4	0.270	0.222	-0.167	0.707	0.224
IL-6	0.297	0.152	-0.002	0.597	0.052
IL-7	0.237	0.192	-0.142	0.616	0.219
IL-8	0.142	0.204	-0.261	0.545	0.487
IL-9	0.126	0.170	-0.210	0.462	0.461
IP-10	0.088	0.288	-0.481	0.656	0.761
LIF	0.045	0.188	-0.325	0.416	0.809
M-CSF	0.106	0.168	-0.226	0.438	0.531
MCP-1	-0.041	0.210	-0.456	0.374	0.847
MCP-3	-0.087	0.334	-0.747	0.573	0.795
MIF	0.107	0.195	-0.277	0.492	0.582
MIG	-0.127	0.188	-0.497	0.243	0.500
MIP-1a	0.536	0.245	0.053	1.019	0.030
MIP-1b	0.223	0.240	-0.249	0.695	0.353
PDGF-bb	0.386	0.271	-0.148	0.920	0.156
RANTES	0.287	0.290	-0.285	0.859	0.324
SCF	-0.306	0.368	-1.031	0.420	0.407
SCGF-b	-0.112	0.104	-0.318	0.094	0.286
SDF-1a	0.053	0.102	-0.148	0.255	0.602
TNF-a	0.266	0.138	-0.007	0.538	0.056
TNF-b	-0.075	0.218	-0.505	0.354	0.729
TRAIL	0.272	0.309	-0.338	0.882	0.381
VEGF	0.243	0.215	-0.181	0.668	0.259

Univariate logistic regression (STI participants excluded)

Cytokine	Relative Risk	Standard Error	95% CI		P value
			Lower	Upper	
IL-2	1.042	0.516	0.395	2.752	0.934
IL-5	0.930	0.355	0.440	1.964	0.849

Table B4: Multivariate linear model for cytokines and PSA (Adjusted for Age, STI, BV, Current contraception use and condom use

			95% CI		
Cytokine	**β Coefficient**	**Standard Error**	**Lower**	**Upper**	**P value**
b-NGF	-0.097	0.327	-0.743	0.549	0.767
CTACK	0.038	0.273	-0.501	0.576	0.891
Eotaxin	0.224	0.302	-0.372	0.821	0.459
FGF-basic	-0.314	0.263	-0.832	0.205	0.234
G-CSF	0.218	0.214	-0.204	0.639	0.310
GM-CSF	-0.023	0.199	-0.416	0.369	0.906
GROa	0.257	0.291	-0.316	0.831	0.377
HGF	0.095	0.227	-0.353	0.543	0.676
IFN-a2	-0.131	0.276	-0.675	0.413	0.636
IFN-g	-0.000	0.232	-0.458	0.457	0.998
IL-10	0.119	0.215	-0.305	0.544	0.581
IL-12p70	0.054	0.257	-0.452	0.561	0.833
IL-12p40	-0.078	0.306	-0.680	0.525	0.800
IL-13	-0.001	0.341	-0.672	0.671	0.999
IL-15	-0.114	0.253	-0.612	0.385	0.654
IL-16	0.161	0.304	-0.439	0.761	0.597
IL-17	-0.105	0.345	-0.786	0.576	0.761
IL-18	0.089	0.220	-0.346	0.523	0.687
IL-1a	-0.016	0.175	-0.360	0.329	0.929
IL-1b	0.267	0.234	-0.194	0.729	0.255
IL-2Ra	0.305	0.263	-0.214	0.823	0.248
IL-3	0.132	0.266	-0.392	0.656	0.620
IL-4	0.176	0.225	-0.267	0.620	0.434

IL-6	0.260	0.154	-0.045	0.564	0.094
IL-7	0.196	0.195	-0.188	0.580	0.316
IL-8	0.032	0.205	-0.372	0.436	0.876
IL-9	0.090	0.173	-0.251	0.430	0.604
IP-10	0.100	0.282	-0.456	0.655	0.723
LIF	-0.019	0.185	-0.384	0.346	0.918
M-CSF	0.067	0.172	-0.274	0.407	0.700
MCP-1	0.002	0.210	-0.412	0.415	0.994
MCP-3	-0.057	0.339	-0.725	0.611	0.866
MIF	-0.015	0.192	-0.394	0.364	0.937
MIG	-0.134	0.189	-0.507	0.239	0.480
MIP-1a	0.383	0.243	-0.096	0.862	0.116
MIP-1b	0.112	0.241	-0.363	0.587	0.644
PDGF-bb	0.311	0.274	-0.230	0.851	0.258
RANTES	0.201	0.295	-0.381	0.784	0.496
SCF	-0.357	0.370	-1.087	0.374	0.337
SDF-1a	0.052	0.103	-0.150	0.255	0.611
TNF-a	0.207	0.140	-0.070	0.483	0.142
TNF-b	-0.080	0.220	-0.515	0.354	0.715
TRAIL	0.213	0.310	-0.398	0.824	0.493
VEGF	0.234	0.220	-0.200	0.669	0.289

Multivariate logistic regression (STI participants excluded)

Adjusted for age, BV, current contraception use and condom use

Cytokine	**Relative Risk**	**Standard Error**	**95% CI**		**P value**
			Lower	**Upper**	
IL-2	0.992	0.522	0.354	2.782	0.988
IL-5	0.794	0.314	0.365	1.723	0.559