SEXUALLY TRANSMITTED INFECTIONS, BACTERIAL VAGINOSIS AND GENITAL INFLAMMATION AS RISK FACTORS OF HIV ACQUISITION IN ADOLESCENT GIRLS AND YOUNG WOMEN IN SOUTH AFRICA

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Pagarism Declaration

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Abstract

Background: In South Africa, young women are at increased risk of HIV infection, predominantly through heterosexual contact. Socio-behavioural and biological factors most likely play an important role at increasing risk. BV, STIs and genital yeast infections are known to influence HIV risk, although few studies have been conducted in African adolescent girls and young women (AGYW). Understanding the role of behavioural and biological risk factors in this key population is essential to inform on new prevention strategies.

Aims: (1) To define the prevalence of sexual risk behaviour, bacterial vaginosis (BV), sexually transmitted infections (STIs), and genital fungal infections in South African AGYW; (2) to assess the relationship between symptoms and an etiological STI diagnosis; (3) to evaluate the influence of electronic and paper-based data collection methods on reported demographics and sexual risk behavior; (4) to examine the impact of non-infectious and infectious causes on genital inflammatory cytokine profiles; and (5) to investigate the use of cytokine biomarkers to identify young women with asymptomatic vaginal dysbiosis and STIs.

Approach: To address these aims, the multi-center Women's Initiative in Sexual Health (WISH) study was conducted as a longitudinal observational trial that included HIV-negative, South African AGYW between the ages of 16-22 (EDCTP Strategic Primer 2013-2015), of which 149 from Masiphumelele, Cape Town were enrolled longitudinally for three visits and the 149 from Soweto, Johannesburg were seen cross-sectionally. Genital samples were collected to test for STIs (including *Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis, Mycoplasma genitalium,* herpes simplex virus (HSV)-1 and -2*, Haemophilus ducreyi, Treponema pallidum* and *Lymphogranuloma venerum*), BV (Nugent scoring) and yeast infections, to assess vaginal pH, and to measure prostate specific antigen (PSA) as a marker for the presence of semen. Multi-locus sequence typing (MLST) was performed on samples positive for *C. trachomatis*. In addition, 44 inflammatory, adaptive, regulatory cytokines, chemokines and growth factors were measured in genital secretions by

Luminex®. Concentrations of sex hormones (estrogen, progesterone and luteinizing hormone), HSV-serology and cotinine (smoking) were measured in blood plasma. A HIV test was done at screening and each visit. Only HIV negative women were enrolled in the study

Results: The prevalence of BV (Nugent 7-10) in South African AGYW were similarly high in Masiphumelele and Soweto (48% and 45%, respectively), as was intermediate microbiota (12% and 16%, respectively). In addition, 40% of South African AGYW were infected with any STI. *C. trachomatis* and *N. gonorrhoeae* were more prevalent in Masiphumelele compared to Soweto (42% vs. 18% for *C. trachomatis* and 11% vs. 5% for *N. gonorrhoeae*, respectively), while the prevalence of the other STIs tested for, including *T. vaginalis*, *M. genitalium* and HSV-2, was similar at both sites. Two-thirds (67%) of AGYW had HPV infection while 10% had a fungal infection.

The indicators of risky behavior (including partner's HIV status being unknown [57%], having unprotected vaginal sex in the last three months [40%], and being in HIV discordant relationships [20%]) were high in this population, and tended to be more prevalent in Soweto than Masiphumelele. It was striking that only 24% of women with a diagnosed STI or BV were symptomatic, underlining the inadequacy of syndromic management in this population. The most sensitive symptom in this cohort was dysuria, which had 50% sensitivity in diagnosing a STI, and the least sensitive was abnormal vaginal discharge with a sensitivity of 22%. Having multiple, concurrent conditions (multiple STIs or a STI and BV) did not increase the symptom frequency or severity.

In Chapter 3, two methods were compared to obtain demographic and sexual risk behaviour data – an electronic tablet device and a paper-based questionnaire. This was done to explore the presence of reporting bias and to improve the frequency of it in the cohort. There were no obvious differences seen in the reporting of risk between the two arms. It was striking that geographical differences in risk reporting were observed, with AGYW from Soweto reporting a higher frequency of HIV

discordant relationships and intergenerational relationships than women from Masiphumelele, independently of the data collection method used.

As genital inflammatory cytokines are known to influence HIV risk, physiological and pathological factors that may influence the genital cytokine profiles of AGYW were examined in Chapter 4 and 5. A change of vaginal pH appeared to play a crucial role, as it was associated with a significant increase in half (22/44) of the cytokines measured in AGYW with no STIs, no yeast infections and a Nugent score ≤3. Further, in these adolescents that one would consider healthy, a pronounced effect was seen with hormonal contraception choice, with Net-En significantly increasing the median concentrations of 31/44 cytokines, and DMPA upregulating 27/44 cytokine concentrations. Recent sex (being PSA positive) did not influence genital inflammation.

Next, the impact of BV, viral STIs (HPV and HSV-2) and yeast infections on genital inflammation in adolescents was investigated. BV was the most inflammatory condition seen with 34/44 cytokines being upregulated. Interestingly, BV was also associated with downregulation of chemokines at both sites, including GRO-α, IP-10 and MIG. BV alone was more inflammatory than coinfection of BV with *C. trachomatis*, BV with another STI, or BV, *C. trachomatis* and another STI. In this cohort, HPV and HSV-2 shedding did not influence genital inflammation.

In Chapter 5, the influence of bacterial and parasitic STIs (including *C. trachomatis, N. gonorrhoeae, T. vaginalis* and *M. genitalium*) on the genital inflammatory profile was explored. *C. trachomatis* and *T. vaginalis* were equally inflammatory, with 23/44 cytokines being elevated. *M. genitalium* infections had a very little impact with only GM-CSF being significantly downregulated in Masiphumelele, while *N. gonorrhoea* infection did not influence the genital inflammatory milieu.

Geographical variation in genital cytokine responses were observed in adolescents infected with *T. vaginalis*, which was moderately inflammatory in AGYW from Masiphumelele but not in AGYW from Soweto. With *C. trachomatis* infection,

however, there was a more pronounced inflammatory response seen in adolescents from Soweto (where 23/44 genital cytokines were significantly increased) than those from Masiphumelele (no cytokines were changed), compared to adolescents with no STIs, no yeast infections and a Nugent score ≤3. Using MLST, further differences were associated with *C. trachomatis* infection: sequence type (ST) 3 was the most inflammatory *C. trachomatis* ST detected with 14/44 cytokines upregulated; ST137 caused no significant changes in cytokine markers, and ST100b was the least inflammatory with four cytokines being down regulated.

Exploring the relationship between years of sexual experience and *C. trachomatis* infection showed low levels of inflammation in women with only with <2 year of sexual experience (only MIG was upregulated), while *C. trachomatis* infection in AGYW with ≥2 years sexual experience was associated with an increase in 19/44 cytokines.

Finally, in Chapter 6 the cytokine biomarkers IL-1α, IL-1β and IP-10 were evaluated in the classification of asymptomatic STIs, BV and intermediate microbiota. These biomarkers correctly classified 76% of women using their Softcup® samples, 76% of women using lateral vaginal wall swabs and 73% using vulvovaginal swab. The addition of pH to the biomarkers model improved the classification (82%) and sensitivity (87%) of results, but reduced specificity (64%). The validation of these biomarkers could lead to the development of a point-of-care test that could be used to triage women into risk categories independently of symptoms.

Conclusion: The results show a high prevalence of risky behavior, STIs and BV in this vulnerable population, with poor correlation between clinical symptoms and diagnosis of STIs or BV, with most cases in adolescents being asymptomatic. Injectable hormonal contraceptive use led to increased genital inflammation in adolescents. Despite the lack of symptoms, most cases of BV and STIs were associated with significantly elevated concentrations of genital cytokines in the genital mucosa of these at-risk adolescents compared to their uninfected counterparts, with variation in levels of inflammation seen by geographic location, type of infection, and years of sexual experience. These results supported the use of biomarkers as a potential

method to identify at risk women. Overall, these findings highlight the urgent need to include adolescents in the design and testing of new HIV prevention strategies and the importance of active identification and management of any infection or dysbiosis in this key at risk population.

List of Abbreviations

α	Alpha
β	Beta
γ	Gamma
^{o}C	Degree Celsius
ACD	Acid Citrate Dextran
AGYW	Adolescent girls and young women
AMP	Antimicrobial peptide
aRR	Adjusted risk ratio
AUC	Area under the curve
AUROC	Area under the receiver operating characteristic curve
aOR	Adjusted odds ratio
BV	Bacterial vaginosis
BVAB	BV-associated bacteria
cAMP	Cyclic adenosine monophosphate
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
CHIVSTI	Centre for HIV and STIs
CI	Confidence interval
CMV	Cytomegalovirus
COP	Combined oral contraception
CT	*C. trachomatis*
CVL	Cervicovaginal lavage
CXCR	C-X-C chemokine receptor
DC	Dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DMPA	Depot medroxyprogesterone acetate
DNA	Deoxyribonucleic acid
DTHF	Desmond Tutu HIV Youth Center
E2	Oestrogen
EPT	Expedited partner therapy
FGT	Female genital tract
Flt3L	FMS-like tyrosine kinase 3 ligand
FSH	Follicle stimulating hormone
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GGD	Geneeskundige en Gezondheids Dienst
GI	Gastro-intestinal
gp	Glycoprotein
GRO	Growth-regulated oncogene
GML	Glycerol monolaurate
GSK	GlaxoSmithKline
H_2O_2	Hydrogen peroxide
HBD	Human β-defensin

HC	Hormonal contraceptive
hCG	Human Chorionic Gonadotropin
HIV	Human immunodeficiency virus
HLA	Human Leukocyte Antigen
HLADR	Human Leukocyte Antigen – DR isotype
HPG	Hypothalamic–pituitary–gonadal
hr	high-risk
HR	Hazard ratio
HREC	Human Research Ethics Committee
HSP	Heat shock protein
HSV	Herpes Simplex Virus
HPV	Human Papilloma Virus
ICAM	Intracellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP	Interferon-inducible protein
IQR	Interquartile range
LC	Langerhans cell
LH	Luteinising hormone
LPS	Lipopolysaccharide
LR	low-risk
µg	Microgram
µL	Microliter
µM	Micromolar
MCP	Monocyte chemoattractant protein
MDC	Macrophage-derived chemokine
MG	*M. genitalium*
MIF	Macrophage migration inhibitory factor
MIG	Monokine induced by gamma interferon
min	Minutes
MIP	Macrophage inflammatory protein
mL	Milliliter
mM	Millimolar
MLST	Multi-Locus Sequence Typing
MM	Matrix metallopeptidases
MOMP	Major outer membrane protein
M-PCR	Multiplex polymerase chain reaction
n/a	Not available
NAAT	Nucleic acid amplification tests
Net-EN	Norethisterone enanthate
NF-κB	Nuclear factor-κB
NICD	National Institute for Communicable Diseases
NK	Natural killer cell
ng	Nanogram
NG	*N. gonorrhea*
NHLS	National Health Laboratory Service

NPV	Negative predictive value
OR	Odds Ratio
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
pG	Picogram
PHRU	Perinatal HIV Research Unit
PID	Pelvic inflammatory disease
POC	Point-of-care
PPV	Positive predictive value
PrEP	Pre-exposure antiretroviral prophylaxis
PRR	Pattern recognition receptors
PSA	Prostate specific antigen
RA	Receptor antagonist
RANTES	Regulated upon Activation, Normal T cell Expressed, and Secreted
ROC	Receiver operating characteristic
RR	Risk ratio
SA	South Africa
sec	Seconds
SIV	Simian immunodeficiency virus
SLPI	Secretory leukocyte protease inhibitor
SSA	Sub-Saharan Africa
SST	Serum Separating Tube
ST	Sequence type
STI	Sexually transmitted infection
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TV	*T. vaginalis*
UCT	University of Cape Town
UK	United Kingdom
US	United States
VCAM	Vascular cell adhesion molecule
VIP	Vaginal insertion practice
vs.	Versus
VVC	Vulvo-vaginal candidiasis
WHO	World Health Organization
WISH	Women's Initiative in Sexual Health

Chapter 1

Literature review

1.1 The feminine face of the HIV epidemic in South Africa

Since the beginning of the HIV epidemic, >70 million people have been infected and approximately 35 million people have died of HIV. Globally, at the end of 2015 36.7 million people were living with HIV. The burden of the epidemic varies considerably between countries and regions with Sub-Saharan Africa (SSA) the most severely affected, where ~4.4% of adults are living with HIV.[1] Nearly 70% of people living with HIV reside in SSA, a region with only 13% of the world's population, emphasizing the disproportional burden of the HIV epidemic in SSA.[1] Further, while new cases are reported in all regions of the world, approximately two-thirds of the new cases are in SSA, with almost half (46%) of all new cases occurring in Eastern and Southern Africa (UNAIDS 2016). Within the Sub-Saharan region and in the world, South Africa (SA) has the highest number of people living with HIV, with a national prevalence of 19.2% (UNAIDS 2016).

Internationally and in SSA, women are disproportionally affected by HIV, which is the leading cause of death among women of reproductive age (UNAIDS 2016). Inequalities in terms of gender, access to health care, and sexual violence put women in a vulnerable position with regards to HIV acquisition. In addition, it is argued that women are biologically more susceptible to HIV (section 1.5.2). Of note, young people between the ages 15 and 24 account for approximately a third of new HIV infections (UNAIDS 2016), and in SSA, young women between these ages account for 25% of all new HIV infections, even though they only represent 17% of the adult population (UNAIDS 2016). Thus, there is a disproportionally high burden of HIV risk and disease in adolescents, and especially young women.

Most infections in SSA are transmitted heterosexually, although risk varies depending on country and communities. Effective prevention strategies include the promotion of behavioral changes, barrier protection, regular and free HIV testing, risk reduction for injecting drug users, and male circumcision (UNAIDS 2016b). Additionally, providing treatment to HIV positive individuals significantly reduces the risk of transmission to their negative partners (Giannou *et al*. 2015). The WHO-recommended pre-exposure antiretroviral

[1] Online source, Available at: http://www.who.int/gho/hiv/en/, Accessed 02 April 2018

prophylaxis (PrEP) is also an effective HIV prevention strategy in individuals at high risk for HIV infection (World Health Organization 2015).

1.2 The female genital tract (FGT) and HIV infection

The FGT can be divided into the upper and lower genital tract (Kaushic *et al*. 2010). The upper tract consists of the endocervix, the endometrium and the fallopian tubes, all of which are lined by simple columnar epithelium (C. R. Wira et al. 2005). The lower tract comprises the introitus that is covered by keratinized stratified squamous epithelium, and the vaginal mucosa and ectocervix that are covered with non-keratinized stratified squamous epithelia (Pudney *et al*. 2005). The FGT is an immunologically unique site, as it can tolerate genetically dissimilar spermatozoa and products of conception in the upper reproductive tract, while at the same time respond quickly to diverse pathogens without compromising fetal survival (Quayle 2002).

The junction between the ectocervix (lower genital tract) and endocervix (upper genital tract) is where the columnar cells of the endocervix are replaced by a stratified squamous cell layer by metaplasia, and this area is called squamocolumnar junction or transformation zone (Coombs *et al*. 2003). This junction can be found on the ectocervix at various stages of life: during menarche, adolescence and after a viable pregnancy, when it is then termed cervical ectopy (D L Jacobson *et al*. 1999; Coombs *et al*. 2003; section 1.5.2.3). This squamocolumnar junction begins to recede into the endocervical canal during the reproductive years and, by the onset of menopause, is often completely receded (Coombs *et al*. 2003).

Based on cohorts in SSA, the average probability of male-female HIV-1 transmission per unprotected sexual act has been estimated to be 0.0001–0.0023 (1/1000) during non-acute HIV-1 infection (Gray & Wawer 2012; Wawer *et al*. 2005; Gray *et al*. 2001). Most HIV infections occur during unprotect sexual intercourse (Arien *et al*. 2011), with the risk of transmission being higher during anal (0.65% to 1.7%; Boily *et al*. 2009) than vaginal intercourse (0.03% to 0.5%; Arien *et al*. 2011). However, the duration of HIV-1 shedding, viral load, frequency of coital acts, and other factors influence infectiousness and/or susceptibility, and may increase the risk of HIV transmission (Shattock & Moore 2003). Epidemiological studies strongly

indicate that transmission is linked to HIV viral load (Quinn *et al*. 2000), which in turn is linked to the disease stage, as viral load is highest during acute infection and late-stage AIDS. Moreover, the presence of other sexually transmitted infections (STIs) have a significant effect on both, viral shedding and the risk of acquiring HIV (Castro & Alcaide 2016; Ward & Rönn 2010; section 1.4).

During sexual intercourse, HIV transmission from male to female is more efficient than *vice versa*, making women disproportionately more susceptible to HIV infection than men (UNAIDS 2016). Male circumcision provides significant protection against HIV infection (Szabo & Short 2000). As a consequence, 76% of young people (aged 15–24 years) living with HIV are female, with vaginal intercourse being the most common route of infection (UNAIDS 2016). The stratified squamous epithelium of the vagina and ectocervix, the cervical transformation zone and the columnar epithelium of the endocervix are thought to be the primary sites of infection, where HIV in semen deposited during unprotected sex interacts with the mucosal surfaces of the lower FGT for the first time (Tebit *et al*. 2012; Shen *et al*. 2012). More recently, however, Stieh *et al.* (2014) showed that infection can occur throughout the FGT (including the uterus and ovaries), as shown in a rhesus macaque vaginal transmission model using Simian immunodeficiency virus (SIV; Stieh *et al*. 2014). Although it has been assumed that cell-free virions are predominantly responsible for transmission, several authors have argued that seminal plasma and cervico-vaginal secretions also contain HIV-infected cells, which could facilitate HIV transmission (Tebit *et al*. 2012; Barreto-de-Souza *et al*. 2014). Seminal plasma contains up to 10^5 lymphocytes cells/mL, including HIV-susceptible macrophages and CD4+ T-cells (Anderson *et al*. 2010; Politch *et al*. 2014; Camus *et al*. 2016; Olivier *et al*. 2014), which is important when considering studies that suggest that HIV transcytosis across epithelia is more efficient if the viruses bud via viral synapses, rather than infecting via cell-free virions (Bobardt *et al*. 2007; Hubner *et al*. 2007).

During sexual transmission, HIV-1 virions have to cross the vaginal epithelium. Various potential mechanisms for HIV-1 transmission across mucosal epithelia have been proposed, such as (i) direct infection of epithelial cells, (ii) transcytosis through epithelial cells (iii) epithelial transmigration of infected donor cells, (iv) uptake by epithelial Langerhans cells; or (v) circumvention of the epithelial barrier through physical breaches (reviewed by Shattock &

Moore 2003). Within hours of infection, HIV crosses the mucosal barrier to establish a small founder population of infected cells (Miller *et al*. 2005; Deleage *et al*. 2016). In the FGT, virus transmission is thought to involve early capture by epidermal Langerhans cells (LCs) in the vagina and endo-cervical stratified epithelia, which is facilitated by the close proximity of these LCs to the mucosal surface (De Witte *et al*. 2007). LCs are often defined by the expression of CD1a and/or langerin, a C- type lectin receptor (Van De Ven et al. 2011). LCs bind the envelope gp120 of HIV through their C-type lectin, called langerin, which might lead to the degradation of HIV when the viral load is low (De Witte *et al*. 2007). At higher viral loads, the internalized virus will be transferred to T-cells, causing a productive infection of these cells within the mucosae or in draining lymph nodes, which is followed by migration of the LCs to secondary lymphoid organs (Hladik and McElrath 2008). Seminal cells containing HIV particles may become trapped in the mucus covering the epithelial surface (Maher et al. 2005). Upon contact with the epithelium, these cells can release free viruses captured by tissue-resident DCs, including Langerhans cells (LCs), and ultimately become internalised in the endocytic compartment (Maher et al. 2005; Miller et al. 2005). These cells may become activated and migrate into the basal compartments where they can transfer the virus to CD4+ T cells by *trans*-infection (Shey et al. 2015; Cavrois et al. 2007).

Recently, it has been shown that Th17 cells are preferentially infected with SIV following vaginal infection of macaques (Stieh *et al*. 2016). Successful transfer of virus particles across epithelial barriers could also result in HIV-1 uptake by migratory dendritic cells (by DC-SIGN or another mannose C-type lectin receptor), and subsequent dissemination to draining lymph nodes and/or localized mucosal HIV-1 infection site, leading to recruitment of additional susceptible cells (Miller *et al*. 2005). This population expands locally during the first week of infection, generating a viral pool that establishes a self-propagating systemic infection in the secondary lymphoid organs (Miller *et al*. 2005), including the gastro-intestinal (GI) mucosa where CD4+ T-cells are abundant (Brenchley & Douek 2008). As virus access to more susceptible target cells increases during the second week of infection, a significant increase in replication in the lymphatic tissues and the GI tract occurs (Kilby 2001). The peak of virus replication in blood and tissue happens during the second week after infection, before declining to stable levels around four weeks post infection (Haase 2010).

1.2.1 Protection from FGT pathogens through physiological barriers

The endocervix, the vaginal walls, and ectocervix contain numerous epithelial cell layers that form a physical barrier to infection. They act as the first line of defense and prevent the passage of pathogens. The combined surface area of the ectocervix and vaginal wall is fifteen times larger than the endocervix and provides multiple potential entry sites for pathogens, especially with the minor breaches that occur during sex (Hladik and McElrath 2008). Although the endocervix is covered by only a single layer of columnar epithelial cells, the tight junctions between these cells help to prevent infection (Kaushic *et al*. 2010).

1.2.2 Protection from FGT pathogens through innate immunity

Vaginal mucus and the glycocaylx (a pericellular matrix of glycoproteins and glycolipids) that coats or surrounds the cell membranes of epithelial cells in the FGT create an inhospitable environment for pathogens by trapping bacterial and viral pathogens, exerting antimicrobial activities, and providing an environment rich in trapped immunoglobulins targeting those pathogens (Eggert-Kruse *et al*. 2000; Blaskewicz *et al*. 2011; Franklin & Kutteh 1999; Kaushic *et al*. 2010). In addition, epithelial cells secrete innate proteins with anti-pathogenic properties. These include a secretory leukocyte protease inhibitor (SLPI), which has bactericidal activity against both Gram-positive and Gram-negative bacteria (King *et al*. 2000); lactoferrin, which can be either bacteriostatic or bactericidal (Sinha *et al*. 2013; Gifford *et al*. 2005), antimicrobial peptides like human β-defensins (HBDs) (Mitchell *et al*. 2013), trappin-2⁄elafin (Drannik *et al*. 2012; Drannik *et al*. 2013), and surfactant protein A that facilitates phagocytosis by opsonizing bacteria (MacNeill *et al*. 2004).

If the epithelial barrier of the FGT is compromised, pathogens will encounter the next layer of defense, the innate immune system, which includes specialized immune cells, such as macrophages, natural killer (NK) and dendritic cells (DC) that can take up and process pathogens (C. R. Wira et al. 2005). Pattern recognition receptors (PRRs) are found on the surface of various cells of the immune system, which are used to identify pathogen-associated molecular patterns (PAMPs) expressed by foreign pathogens (Medzhitov & Janeway 2000; C. R. Wira et al. 2005). Toll-like receptors (TLRs) are a type of PRRs that are found on neutrophils, macrophages, DCs, NK cells and epithelial cells (C. R. Wira et al. 2005). PAMPs act via TLRs to

induce the production of antimicrobial factors, cytokines and chemokines to recruit adaptive immune cells and link the innate to the adaptive immune system (Tomas 1999; Akira & Kiyoshi 2004; C. R. Wira, Fahey, *et al*. 2005; Medzhitov & Janeway 2000).

1.2.3 Protection from FGT pathogens through FGT commensals

The vagina and ectocervix contain a diverse arrange of commensal bacterial communities that help prevent infections. Various *Lactobacillus* spp. are associated with a healthy FGT, with the most frequent and abundant being *L. crispatus*, *L. gasseri*, *L. jensenii*, and *L. vaginalis* (Ravel *et al*. 2011). The role of *L. iners* is unclear as it has been associated with increased risk of *C. trachomatis* infection (Houdt et al. 2018) but has been associated with a healthy FGT (Petrova et al. 2017). *Lactobacillus* spp. have been shown to reach levels of up to 10^7 to 10^8 bacilli per gram of vaginal secretions (Sobel 1999). The key protective role of *Lactobacillus* spp. are usually attributed to their ability to lower environmental pH that is less accommodating of various pathogens, secondary to lactic acid and hydrogen peroxide (H_2O_2) production, the production of bacteriocins and other bacteriocidal compounds, and the competitive exclusion of other bacterial species in this biological niche (Eschenbach *et al*. 1989; O'Hanlon *et al*. 2013; Stoyancheva *et al*. 2014; Ma *et al*. 2013). The impact of the vaginal microbiota on genital health is described in more detail in section 1.4.7.

1.3 Anatomical changes in the FGT during puberty

Puberty is the period of biologic transition from childhood to adulthood, and is marked by increasing levels of gonadal hormones, the appearance of secondary sex characteristics and the achievement of reproductive functionality (Sawyer *et al*. 2018). In adolescent females, the pubertal changes are induced by the rise in oestrogen concentrations, which marks the onset of puberty (Ankarberg-Lindgren *et al*. 2014). Pubertal changes mainly occur in the organs of the female reproductive system, which cause anatomic changes of the FGT and result in reproductive maturity. In addition to oestrogen, the levels of adrenal and ovarian androgens also increase during puberty, promoting the appearance of secondary sex characteristics (Colvin & Abdullatif 2013).

1.3.1 Changes in the ovary during puberty

The first organ that undergoes structural changes during puberty are the ovaries, which then initiates physical pubertal changes through the secretion of sex steroid hormones (Blakemore *et al*. 2010). In very early puberty, follicle stimulating hormone (FSH) concentrations are proportionally higher than luteinizing hormone (LH) concentrations. FSH increases oestrogen production, promotes granulosa cell growth and proliferation in the ovaries - causing an increase in follicle numbers and circulating oestrogen concentrations (Rosenfield 2008). As puberty progresses, LH levels increase, inducing circulating androgens, luteinization and follicular maturation (formation of the corpus luteum from a mature ovarian follicle; François *et al*. 2017). Later during puberty, the balanced levels of FSH, estradiol and progesterone cause an increase in LH and secondarily ovulation, along with controlled follicle development. During this transitional period, follicular growth continues, resulting in a multi-follicular phenotype (Rosenfield 2008), which resolves once the hypothalamic–pituitary–gonadal (HPG) axis has fully matured and menarche has occurred.

1.3.2 Changes in the uterus, endometrium and vaginal epithelium during puberty

Like other reproductive organs, the uterus only develops a mature morphology under sex steroid stimulation (Colvin & Abdullatif 2013). The growth rate of the uterus greatly increases with the beginning of puberty and with maturation of the HPG axis. Along with changes in size and shape, the uterus also requires sex steroid stimulation to establish and maintain reproductive capability (Porcu 2004; Bridges *et al*. 1996).

Oestrogen induces proliferation of endometrial epithelium and stroma, and activates endo-cervical glandular mucous secretion (Chung *et al*. 2015). During puberty, the vaginal epithelium begins to thicken in response to oestrogen stimulation with progressive cellular proliferation and growth, which results in the formation of intermediate and superficial layers of cells (Colvin & Abdullatif 2013). Oestrogen-stimulated growth of the vaginal epithelium also increases glycogen concentrations in the superficial cells, which is then metabolized by beneficial *Lactobacillus* spp. that inhabit the vagina, increasing the numbers of *Lactobacillus* spp. and secondarily acidifying the vaginal environment (pH <4.5; Mirmonsef *et al*. 2016).

1.4 Prevalence of STIs and bacterial vaginosis (BV) in adolescents

Although HIV has been the major focus of STI research over the past few decades, over one million people acquire other STIs every day, with around 500 million people infected annually with the most common ones, including chlamydia, gonorrhoea, trichomonas and syphilis (World Health Organization 2013). The WHO estimated in a report from 2013 that 498.9 million people were infected with any one of these common STIs, including 105.7 million cases of *Chlamydia trachomatis*, 106.1 million cases of *Neisseria gonorrhoeae*, 10.6 million cases of syphilis, and four million cases of *Trichomonas vaginalis*. In addition, rates of STIs are increasing annually (World Health Organization 2013).

STIs constitute a huge health and economic burden for developing countries: STIs account for 17% of the economic losses in women aged 15-14 years due to poor health (Mayaud & Mabey 2004). STIs impose an enormous burden of morbidity and mortality in SSA, both directly through their impact on reproductive and child health, and indirectly through their role in facilitating the sexual transmission of HIV infection. As such, Johnson *et al.* (2009) estimated that gonorrhoea, chancroid and syphilis have contributed significantly to HIV transmission in the early stages of the epidemic, accounting for 12%, 8% and 5%, respectively, of heterosexual transmission in 1990. In 2009, the World Bank estimated that STIs, excluding HIV, are the second most common cause of healthy life years lost by women in the 15–44 age group in Africa, responsible for about 17% of the disease burden (World Health Organization 2009).

The most recent WHO estimates for the global burden of STIs report that the total incidence for curable STIs for the WHO Africa region was 92.6 million in 2008 (21.1 million cases of *C. trachomatis*, 8.3 million cases of *N. gonorrhoeae*, 3.4 million cases of syphilis and 59.7 million cases of *T. vaginalis*; World Health Organization 2012). Not all people infected with STIs are symptomatic, and therefore do not seek treatment. In South African women, an earlier study reported that 48% of those infected with *T. vaginalis*, *N. gonorrhoeae*, *C. trachomatis* or *T. pallidum* were asymptomatic (Wilkinson *et al*. 1999). Of the 50% that were symptomatic, only 1.7% would seek care. More recently, Mlisana *et al*. (2012) reported that only 12.3% of women who had a laboratory-diagnosed, discharge-causing STI had clinically evident discharge. This has crucial implications for STI management, as in South Africa the current

treatment guidelines are based on STI syndromes (South African Department of Health 2015; section 1.6.1).

1.4.1 *C. trachomatis*

C. trachomatis (CT) is the most commonly reported bacterial STI worldwide (Newman et al. 2015; Braxton et al. 2015b). During 2014, almost 1.5 million new cases of CT were reported in the United States (US; Braxton *et al*. 2014), with a prevalence of 11.2% reported among young women in 2016 in KwaZulu-Natal, SA (Francis *et al*. 2018). Adolescents are believed to represent at least one third of cases of CT infections worldwide (Chinsembu 2009).

CT infection is, however, predominately asymptomatic, with approximately 70% cases in women (Walsh *et al*. 2000; Peipert 2003) and 40% in men being clinically silent (Buimer *et al*. 1996; van den Brule *et al*. 2002). Due to its asymptomatic nature, many CT infections remain undiagnosed and untreated. Despite increased surveillance and screening programs over the last 20 years, rates of CT infections continue to rise (Brunham & Rappuoli 2013). However, it has been suggested that women can develop protective immunity to CT, as it has been observed that the rate of incident infections decreases over time, and reinfections are associated with lower bacterial loads (Batteiger *et al*. 2010).

The clinical presentation of CT varies. In women, the infection is initially cervical and may extend to the urethra, resulting in urethritis with recognizable symptoms like pyuria, dysuria and post-coitial bleeding (Peipert 2003). If untreated, CT can migrate to the upper reproductive tract, resulting in serious reproductive problems including ectopic pregnancy and infertility (Menon *et al*. 2015). CT infection in men results in urethritis and occasionally epididymitis (Mackern-Oberti *et al*. 2013). Infection during pregnancy can be associated with pre-term delivery and a range of adverse birth outcomes, such as low birth weight, post-partum endometritis and neonatal death (Peipert 2003). Infants who acquire infections during delivery can present with inclusion conjunctivitis or pneumonia (Peipert 2003). CT is responsible for more than 1.3 million cases of blinding trachoma annually in endemic areas, such as SSA, the Middle East, and Asia (Resnikoff *et al*. 2004). CT has been identified as a risk factor for both the acquisition and transmission of HIV in women (Buckner *et al*. 2016; Fleming

& Wasserheit 1999) and men, in whom risk can increase eight-fold after repeated rectal infection (Bernstein *et al*. 2010).

Screening and laboratory diagnosis of CT can be done using urine samples or vaginal swabs via nucleic acid amplification tests. If the cervix is sampled, nucleic acid hybridization, enzyme immunoassay antigen detection, or culture can be used besides nucleic acid amplification tests to diagnose CT (reviewed by Chernesky 2005).

At the present time, the CDC guidelines favor treatment of CT with a single 1 g dose of azithromycin over a seven-day course of doxycycline, which can be administered as either 100 mg of doxycycline orally twice daily, or as a single daily dose of a formulation that can be administered as 200 mg orally once daily, which is more expensive[2]. The treatment of sexual contacts is increasingly managed by expedited partner therapy (EPT), which is the clinical practice of providing prescriptions or medications to the patient to take to his/her partner(s) without the health care provider first examining the partner[3].

Currently there is no licensed vaccine against genital CT but evidence from animal models, human epidemiological studies, and early clinical trials suggest that a CT vaccine is feasible (Poston *et al*. 2017). The first phase one trial using neutralizing antibodies directed to the VD4 of the major outer membrane protein (MOMP-VD4 neutralizing antibodies) is currently ongoing (Poston *et al*. 2017).

1.4.1.1 Genetic variability in CT influences genital inflammation

CT infection has been associated with increased concentrations of several cytokines and chemokines in cervical secretions, including interleukin (IL)-1α, IL-1β, IL-6, IL-12(p70), Tumor necrosis factor (TNF)-α, IL-8, macrophage inflammatory protein (MIP)-1β, RANTES (Regulated upon Activation, Normal T cell Expressed, and Secreted), granulocyte colony stimulating factor (G-CSF), FMS-like tyrosine kinase 3 ligand (Flt3L), transforming growth factor 3 (TGF)-α, IL-2, IL-4, IL-5, IL-13, IL-15, and IL-17 (Masson *et al*. 2014). CT-infected epithelial cells recruit activated immune cells to the mucosa by secreting the chemokines IL-8 or MIP-2 (Darville &

[2] Online Source: https://www.cdc.gov/std/tg2015/qa/chlamydia-qa.htm, Accessed 27 Aug 2018
[3] Online Source: https://www.cdc.gov/std/ept/default.html, Accessed 27 Aug 2018

Hiltke 2010), indicating that genital inflammation may contribute to the pathogenesis associated with CT infection.

Genetic diversity of CT may influence the type and extent of genital inflammatory changes that occur. CT has historically been classified into serovars based on serology, but more recently DNA sequence differences in the major outer membrane protein (MOMP), encoded by ompA (Stephens *et al*. 1988; Batteiger *et al*. 1986), have allowed classification into genovars with better resolution. There is evidence that specific CT serovars may influence genital inflammation and development of adaptive immunity (Dean *et al*. 1995; Srivastava *et al*. 2008; Workowski *et al*. 2014), although pathogen or host factors influencing differential CT pathogenesis are not well understood. As serovars may oversimplify the classification of CT, genotypic approaches like Multi-Locus Sequence Typing (MLST) of the regions ompA and several other highly variable regions provides greater accuracy and resolution in identifying CT isolates, and is more likely to reveal associations between different CT isolates and genital inflammation. A recent high resolution MLST study in the Limpopo region of South Africa identified 26 CT sequence types, of which 18 (69%) were novel to the high resolution-MLST database (Versteeg *et al*. 2015).

1.4.1.2 Virulence determinants of CT

Inflammation during CT infection plays an important role in influencing clinical outcome, with interferon (IFN)-γ responses and CD4+ T-cells thought to be key components in resolving CT infection (Farris *et al*. 2010; Menon *et al*. 2015), while dampened inflammatory responses have been associated with poor clearance and bacterial persistence (Yang *et al*. 1996; Igietseme *et al*. 2000). There is some evidence from *in vitro* studies that primary CT infections are highly inflammatory compared to persistent infections (Schrader *et al*. 2007). However, potent inflammatory responses to CT may contribute to immune-mediated pathology, resulting in pelvic inflammatory disease (PID), infertility and scarring during repeat infections (Beatty *et al*. 1994).

CT encodes a number of virulence factors to enable its survival *in vivo*, including a membrane attack complex/perforin protein (which participates in lytic pore formation), phospholipase D (which generates phosphatidic acid as an intracellular signaling species), type III secretion

effectors (which enables CT to inject bacterial effector proteins directly into the host cell cytoplasm, bypassing the extracellular milieu), and stress response proteins (Elwell *et al*. 2016; Bonner *et al*. 2015). Of particular interest are virulence factors, including ChlaDub1 and CP0236, that inhibit host immune responses by blocking NF-κB signalling (Elwell *et al*. 2016). Additionally, most CT strains contain plasmids, which may encode contain a transcriptional regulator of virulence-associated genes (Song *et al*. 2013; Carlson *et al*. 2008). Apart from virulence factors, CT contains a number of antigens; including heat shock protein 60 (hsp60), MOMP, and chlamydial lipopolysaccharides (LPS; Gerard *et al*. 2004; Caldwell & Schachter 1982), which are all capable of eliciting host immunity and may influence bacterial persistence (Koehler et al. 1997; Ingalls et al. 1995; Gérard et al. 2004). Although there are some reports that MOMP diversity influences inflammation (Dean *et al*. 2000; Miyairi *et al*. 2006; Koehler *et al*. 1997), there have been no consistent findings on serovar or genovar-specific virulence (Lysen *et al*. 2004; Byrne 2010).

1.4.2 *N. gonorrhoeae*

N. gonorrhoea (NG) is a gram-negative diplococcus that primarily infects epithelial cells of the endocervix (Weissenbacher 2014). An accurate estimate of the global burden of NG is difficult to obtain, due to poor reporting and inadequate diagnostic capabilities in the developing world. The most recent estimates by the WHO found that there were 106 million cases of NG in 2008, a 21% increase from 2005 (World Health Organization 2012). In the US, NG was the second most prevalent STI with 350 062 cases reported in 2014 (Braxton et al. 2015b). These actual numbers of cases are most likely higher due to underreporting and the high prevalence of asymptomatic infections (Satterwhite *et al*. 2013). The African region had the highest incidence rates with 49.7 new infections per 1000 women and 60.3 per 1000 men (World Health Organization 2012), although the prevalence in young women in KwaZulu-Natal, SA, was lower at 18 per 1000 women in 2016 (Francis *et al*. 2018).

Common symptoms and signs of NG infection in women include odorless or mucopurulent discharge, post-coital bleeding, dyspareunia, cervicitis, and a friable cervix (Miller 2006). However, many infected women are asymptomatic (Workowski & Bolan 2015). In men, the primary symptom is urethritis and occasionally epididymitis (Miller 2006). While the male urethral infection can prompt health-seeking behavior because it is more likely to be

symptomatic, this often does not happen soon enough to interrupt transmission (Workowski & Bolan 2015).

In a study conducted in SA, NG has been shown to increase risk of HIV infection in women by almost five-fold (Mlisana *et al*. 2012). In HIV-infected Malawian men, urethritis was associated with an eight-fold increase in HIV-1 viral load in semen, with the highest levels seen with NG infection (Cohen *et al*. 1997). HIV-1 concentrations in semen were lowered by two-thirds after NG treatment (Cohen *et al*. 1997). In a second study in men, NG was associated with a 3.2-fold increased risk of HIV shedding (Moss *et al*. 1995), and a concurrent infection with both NG and HIV was associated with an increase in plasma levels of HIV-1 RNA (Anzala *et al*. 2000; Nkengasong *et al*. 2001). A Neisseria meningitides vaccine has been show to confer a modest protection against NG infection (Régnier & Huels 2014).

In the FGT, NG adheres to epithelial cells and interact with immune cells (including macrophages, DCs, T-cells and neutrophils), inducing the local production of inflammatory mediators and activation of a Th17-specific responses (CD4 T-cells that produce IL-17) at the mucosa (Criss & Seifert 2013; Masson, Salkin-, et al. 2015; Masson et al. 2014), and recruitment and activation of neutrophils (Stevens & Criss 2017). NG is thought to skew the immune response towards a dominant Th17-type response (rather than Th1 [CD4 T-cells producing cytokines like IFN-γ and IL-2] and Th2-responses [CD4 T-cells producing cytokines like IL-4 IL-5, IL-6, IL-10, and IL-13]) by inducing the local production of TGF-β and IL-10, which in turn enhances inflammation rather than enhancing the development of protective immunity (Stevens & Criss 2017). *In vitro* studies using endocervical epithelial cells further showed that NG infection induced expression of cytokines IL-8 and IL-6, the intracellular adhesion molecule (ICAM)-1, and CD66c (which is also involved in cell adhesion, growth, neutrophil activation, and signaling), and piliated gonococci in particular triggered strong IL-1 responses (Fichorova *et al*. 2001). It has recently also been described that NG can activate myosin II (responsible for cell movement) in human cervical tissue, leading to disruption of tight junctions, exfoliation of endocervical cells, and penetration of NG deeper into the epithelium (Wang *et al*. 2017), possibly explaining the increased risk of HIV shedding and infection.

The CDC recommends a dual treatment for NG, with a single dose of 250mg of intramuscular ceftriaxone and 1g of oral azithromycin.[4] In addition, EPT has been shown to reduce the rates of persistent and recurrent NG infection (Golden *et al*. 2005) but is difficult to implement as treatment includes an injection.[4] However, due to the rapidly evolving antibiotic resistance of NG (Unemo and Shafer 2014), a vaccine is urgently needed. There is currently no NG vaccine licensed. However, a candidate vaccine was recently shown to provide good protection against NG in pre-clinical testing, with specific immunity lasting more than six months, which included the gonococcal outer membrane vesicle and microencapsulated IL-12 (Liu *et al*. 2017).

1.4.3 *T. vaginalis*

T. vaginalis (TV) is an anaerobic protozoan parasite that mostly infects the squamous epithelium of the lower FGT (Kissinger *et al*. 2015) but can also be detected in endocervical secretions (Meites *et al*. 2015). In 2008, the WHO estimated that there were 276.4 million global cases, an 11.2% increase from 2005 (World Health Organization 2012). In 2013, the global prevalence of TV was 5% among women aged 15-49 years (Newman *et al*. 2015), similarly to a prevalence of 4.6% for South African women aged 19-24 years in 2016 (Francis *et al*. 2018).

The majority of cases of TV infection in both men and women are asymptomatic (Seña *et al*. 2007; Sutton *et al*. 2007). One third of asymptomatic women develop symptoms within six months (Rein 1990), while spontaneous resolution is common in men (Cudmore *et al*. 2004). Among men, the most common clinical indications of infection are urethral discharge and dysuria (Seña *et al*. 2007), while women have vaginal discharge, itching or an abnormal odour (Sutton *et al*. 2007).

TV is associated with substantial reproductive morbidity, including preterm deliveries, low birth weight and developmental delay in newborns (Cotch *et al*. 1997; Mann, McDermott, Gregg, *et al*. 2009; Mann, McDermott, Barnes, *et al*. 2009), PID (P. Moodley *et al*. 2002) and infertility (Grodstein *et al*. 1993), in addition to increasing the risk of HIV transmission and

[4] Online Source: https://www.cdc.gov/std/gonorrhea/treatment.htm, Accessed 28 Aug 2018

acquisition (Patricia Kissinger 2013). This increased susceptibility to HIV infection it thought to be due to disruption of the epithelial barrier by TV, facilitating viral entry (Guenthner et al. 2005), increased HIV viral shedding with TV co-infection (Fastring *et al*. 2014), or TV infection might initiate changes in the vaginal microbiota, which results in BV (Prashini Moodley *et al*. 2002), thus increasing HIV susceptibility (van de Wijgert *et al*. 2009). In a community with a TV prevalence of 25%, almost a fifth of all HIV infections could be attributed to TV (Sorvillo & Kerndt 1998). In the US, it was estimated that 6.2% of new HIV infections were attributed to TV infections (Chesson *et al*. 2004).

Adhesion of TV to vaginal epithelial cells is crucial for the pathogenesis of trichomonosis (Kucknoor *et al*. 2005; Arroyo *et al*. 1992). High levels of IL-8 and leukotreine B4 [pro-inflammatory mediator synthesised in myeloid cells from arachidonic acid] have been found in the vaginal secretions from women with symptomatic trichomonosis (Shaio *et al*. 1995; Shaio & Lin 1995). Furthermore, TV induced IL-8 and MIP-3α secretion by human neutrophils and monocytes *in vitro* (Ryu *et al*. 2004; Fichorova *et al*. 2006; Shaio *et al*. 1995, Fichorova *et al*. 2006). Ina cohort of South African women, TV was the most prevalent STI (21% prevalence), but the least inflammatory compared to the other STIs (Masson *et al*. 2014).

To treat TV, the CDC recommends metronidazole or trinidazole (2g, single oral dose) as standard of care, and 500 mg metronidazole orally twice a day for 7 days as alternative regimen.[5] Though more recent recommendations have suggested a one week course of metronidazole is almost twice as effective for TV infection as a single dose (Kissinger et al. 2018). In addition, it is also recommended that partners are treated as this can prevent recurrences, reduce transmission and new cases in the community (Meites *et al*. 2015). Unfortunately, metronidazole-resistance of TV has been detected since the 1960s, and is currently detected in 2.5–10% of isolates tested (Smith & Garber 2014), strongly emphasizing the need to develop a vaccine. However, there is currently no TV vaccine available, and the identification of appropriate targets has been difficult (Smith & Garber 2014).

[5]Online Source: https://www.cdc.gov/std/tg2015/trichomoniasis.htm, Accessed 28 Aug 2018

1.4.4 *M. genitalium*

M. genitalium (MG) is a bacteria without a cell wall, and the smallest known bacterium with a genome size of 580kb (Svenstrup *et al*. 2005). The tissue damage seen during genital tract infections with MG is caused, in part, by the toxins and metabolites such as H_2O_2 (Lind *et al*. 1984), and it is assumed that immunological response to MG accounts for some of the tissue damage, as has been seen with *M. pneumoniae*, a common respiratory pathogen (Taylor-Robinson & Jensen 2011). The prevalence of MG varies widely between countries and study populations, from 8.7% to 1.2% in adult South African women in the Limpopo province (Hay *et al*. 2015) and in KwaZulu Natal (Mlisana *et al*. 2012), respectively, 13.6% in adolescents from the Midwestern US (Tosh *et al*. 2007), and only 1% in Japanese men (Takahashi *et al*. 2006).

MG infection presents with urethritis in men (Workowski & Bolan 2015), while women with MG can either be asymptomatic, have cervicitis or PID (Anagrius *et al*. 2005; Falk *et al*. 2005). In women, recurrent MG infection has further been linked to tubal infertility (Clausen *et al*. 2001). MG infection increases HIV risk (Napierala Mavedzenge & Weiss 2009), possibly due to persistent inflammation associated with MG infection (McGowin et al. 2012). In Uganda and Zimbabwe, women with MG were more likely to acquire HIV (Odds Ratio (OR) 2.42; 95% Confidence interval (CI) 1.01-5.80; Mavedzenge *et al*. 2012). Further, HIV-positive Kenyan women with MG infections were three times more likely to shed HIV DNA in their genital secretions, even though the STI was not highly inflammatory (Manhart *et al*. 2008), which could explain increased HIV transmission.

The MG protein MG-309 was shown *in vitro* to induce immune responses against the pathogen via NF-kB activation, through TLR 2 and 6, resulting in induction of IL-6, IL-8, G-CSF and GM-CSF (McGowin, Ma, et al. 2009; McGowin, Popov, et al. 2009). Similarly, others reported that MG lipoproteins triggered the production of TNF-α, IL-1β and IL-6, and induced apoptosis via the activation of NF-kB in a monocytic cell line (Wu *et al*. 2008).

A 1g single oral dose of azithromycin is commonly used to treat MG, but as approximately 50% of all MG infections are caused by organisms that are already resistant to azithromycin, fourth-generation fluoroquinolones are more frequently being used as an alternative

(Bradshaw *et al*. 2017). The CDC further recommends partner treatment and testing in settings with validated MG tests[6].

1.4.5 Herpes Simplex Virus Type 2 (HSV-2)

HSV-2 is an α-herpesvirus that mainly infects cells of the vaginal mucosa by binding to the herpesvirus entry mediator and to the immunoglobulins nectin-1 and -2 from vaginal epithelial cells (Linehan *et al*. 2004). In 2012, it was estimated that there were 417 million HSV-2-infected people aged 15-49 years old (Looker *et al*. 2015). Of these, women were 1.8-times more likely to have a prevalent HSV-2 infection than men (Looker *et al*. 2015). Surveillance data suggests that HSV prevalence was much higher in Africa than in the next highest region, the Americas (32% versus 14%, respectively; Looker *et al*. 2015). The lowest prevalence was in Western Europe, where prevalence reached a maximum of approximately 18% among women and 13% among men. In SSA, the prevalence was up to 70% among women and approximately 55% among men (Looker *et al*. 2015; World Health Organization 2009). HSV-2 prevalence is higher in women because sexual transmission of HSV is more efficient from men to women than *vice versa* (WHO 2016). Among South African young women aged 19-24, the HSV-2 prevalence was 28.7% in 2016 (Francis *et al*. 2018).

Genital herpes infections often have no or mild symptoms that are not recognized by those affected, which facilitates its spread in the population. However, even when apparently asymptomatic, genital shedding of HSV-2 occurs frequently (Tronstein *et al*. 2011). Typically, about 10-20% of people with a current HSV-2 infection report a prior diagnosis of genital herpes (WHO 2016). When symptoms do occur, genital herpes is characterized by one or multiple genital/anal blisters or ulcers. Despite the typically asymptomatic nature of genital herpes, HSV-2 is associated with considerable morbidity and mortality. Genital lesions are often very painful and can therefore lead to substantial psychological morbidity (Stanberry *et al*. 2000). HSV-2 can also be passed from mother to child during birth. Without treatment, 80% of infants with disseminated disease die, while those who do survive often experience brain damage (Al Aswad and Suryadevara 2014).

[6]Online Source: https://www.cdc.gov/std/tg2015/emerging.htm, Accessed 28 Aug 2018

Genital herpes is associated with a two- to three-fold increased risk of HIV acquisition, and up to five-fold increase in HIV transmission per sexual act, and it has been hypothesized that HSV-2 accounts for 40–60% of new HIV infections in populations with a high HSV-2 prevalence (Wald & Link 2002; Celum *et al*. 2004; Serwadda *et al*. 2003). Masese *et al.* (2015) reported that the overall population risk for HIV infection was largely attributable to HSV-2, even in the absence of ulcerative lesions, with prevalent HSV-2 accounting for 48.3% and incident HSV-2 infections accounting for 4.5% of the HIV acquisition risk. Incident HSV-2 infections have also been associated with up to seven-fold increase in HIV acquisition in women (Freeman *et al*. 2006; Johnston & Cprey 2016). Although HSV-2 ulcers disrupt the mucosal barrier, higher numbers of DCs and CCR5+ CD4+ T-cells were observed in the FGT of women who have HSV-2, even in the absence of HSV-2 shedding or genital ulceration (Zhu *et al*. 2009; Rebbapragada *et al*. 2007). In addition, subclinical inflammatory responses in the mucosa were present for months after a HSV-2 reactivation event (Masese *et al*. 2015), which may indirectly increase the risk of HIV acquisition. HSV-2 may further indirectly increase HIV risk, as the acquisition of BV, another HIV risk factor, has been associated with HSV-2 infections (Cherpes *et al*. 2008; Nagot *et al*. 2007), and a causal link between incident HSV-2 infection and a higher risk of subsequent episode of BV has also been shown in Kenyan women (Masese *et al*. 2014)

HSV-2 may contribute to genital inflammation via its chemokine-binding protein glycoprotein G that binds to CXCR4 and induces a strong chemotactic response (Martinez-Martin *et al*. 2015). Johnston *et al*. (2011) also showed that HSV-2 interacts with plasmacytoid DCs and other cells via TLR-2, TLR-3, and TLR-9, and subsequently induces IFN-α production. It has been suggested that blood CD4+ T-cell responses to HSV-2 secreted membrane-bound glycoprotein G can be used to differentiate symptomatic from asymptomatic infections, as symptomatic HSV-2 infections were characterized by lower frequencies of blood CD4+ T-cells and lower plasma concentrations of IFN-γ, TNF-α and IL-2 than asymptomatic infections (Eriksson *et al*. 2004). However, Rebbapragada *et al.* (2007) found that symptomatic HSV-2 infections in women were associated with increased genital CD4+ T-cell activation (using surface expression of CD69+ and CCR5+) and higher concentrations of the chemokines IP-10, monocyte chemoattractant protein (MCP), monokine induced by gamma interferon (MIG) and RANTES. In contrast, even asymptomatic infections were characterized by increased CD4+ T-cell counts at the genital mucosa, increased CCR5 and CD38/HLADR expression, and

increased mannose receptor CD206 (HIV-binding lectin) expression in monocytes and myeloid-derived DCs (Shannon *et al*. 2014).

The CDC recommends that all patients with first episodes of genital herpes should receive antiviral therapy (Acyclovir 400 mg orally three times a day for 7–10 days, or acyclovir 200 mg orally five times a day for 7–10 days, or valacyclovir 1 g orally twice a day for 7–10 days, or famciclovir 250 mg orally three times a day for 7–10 days), as newly acquired genital herpes can cause a prolonged clinical illness with severe genital ulcerations and neurologic involvement[7]. The CDC further recommends that symptomatic sex partners should be treated in the same manner as patients who have genital herpes, while asymptomatic sex partners of patients who are symptomatic should be questioned concerning histories of genital lesions and offered type-specific serologic testing for HSV infection.[7]

Currently, no vaccines are available for the prevention of genital herpes caused by HSV-2, although two prophylactic vaccines have been tested for efficacy and safety in large phase III clinical trials. Both vaccines were subunit vaccines targeted against either HSV-2 glycoprotein B and D (manufactured by Chiron Corporation, US), or glycoprotein D alone (HerpeVac trial, manufactured by GlaxoSmithKline (GSK) Biologicals, UK). While both vaccines induced high titers of HSV neutralizing antibodies in serum, and also elicited Th1 responses in the HerpeVac trial (Corey *et al*. 1999; Stanberry *et al*. 2002), the Chiron vaccine showed no efficacy in preventing infection with HSV-2, duration of the first episode of clinical symptoms (in those who became infected) or frequency of recurrence (Corey *et al*. 1999). In the first HerpeVac trial, men were unprotected by the vaccine, while 74% of women from discordant couples were protected (Stanberry *et al*. 2002). However, the follow-up trial using the same vaccine in a generalized group of women found no protection against HSV-2 infection or symptoms, but a higher rate of efficacy against HSV-1 (Belshe *et al*. 2012).

1.4.6 Human Papilloma Virus (HPV)

More than 200 HPV genotypes have been identified, and almost a quarter of these affect the uro-genital tract (Denny 2009; Munoz *et al*. 2003). HPV infects the basal cells of stratified

[7] Online Source: https://www.cdc.gov/std/tg2015/herpes.htm, Accessed 28 Aug 2018

squamous epithelium in the FGT, while other cell types appear to be relatively resistant. The replication cycle begins with entry of the virus into the cells of the basal layer of the epithelium, where HPV DNA integrates into the host cell DNA and replicates as the basal cells differentiate and progress to the surface of the epithelium (reviewed by Burd 2003). Based on their association with cervical disease and cancer, HPV have been divided into low-risk, probably high-risk and high-risk HPV types (Munoz *et al*. 2003). Of the high-risk HPV genotypes, HPV 16, 18, 45, 33, 35, 52, 51 and 31 are most commonly associated with cervical cancer in Africa (Li *et al*. 2011). A meta-analysis found that women acquire natural immunity against subsequent infection with HPV-16 (pooled risk ratio (RR), 0.65; 95% CI 0.50-0.80) and HPV-18 (RR 0.70; 95% CI 0.43-0.98; Beachler *et al*. 2016), possibly through naturally acquired polyclonal antibodies, as strong antibodies responses to prophylactic HPV vaccination are believed to facilitate the protection in vaccinated women (Schiller & Lowy 2012).

There is conflicting information in the literature about whether HPV infections influence the inflammatory environment in the FGT, with some authors reporting no inflammatory changes with prevalent, incident or cleared HPV infections, irrespective of HPV type (Kriek *et al*. 2016), while others have suggested that HPV infections result in increased levels of IL-2, IL-12 and INF-γ in endocervical secretions (Campos *et al*. 2012). Among women infected with high risk HPV types, TNF-α, IL-6, IL-10, and IFN-γ were not associated with HPV-16 but increased IFN-γ was significantly associated with HPV-16 E6- and E7-positivity (OR 28.20, 95% CI 2.66–299.11; OR 19.62, 95% CI 2.14–180.26, respectively), and elevated IFN-γ was significantly associated with increased HPV viral load (Song *et al*. 2007). Further, an increase of IL-10, IL-12, MIP-1α, and TNF were associated with significantly longer times to clear HPV (Scott *et al*. 2013). Up-regulation of IFN has been shown to be a good hallmark for HPV16 infection clearance (Barros et al. 2018) while the clearance of HR-HPV was more frequent in the presence of low levels of MIP-1α and IL-8 (Fernandes et al. 2015).

HPV seems to contribute to HIV risk in men and women. A study conducted among men who have sex with men found that having an anal infection with at least two HPV types was associated with HIV seroconversion (hazard ratio (HR) 3.5, 95% CI 1.2–10.60; Chin-Hong *et al*. 2009), and a meta-analysis found a strong evidence of an increased risk of HIV acquisition in women infected with HPV (summary HR=2.06, 95% CI 1.44-2.94; Houlihan *et al*. 2012). Like

other STIs, cervical HPV infection might increase HIV acquisition among uninfected women by recruiting an increased number of HIV target cells to the genital mucosa, as although HPV does not produce ulcerative genital lesions, recruitment of T-cells to the genital epithelium is required to clear the infection (Averbach *et al*. 2010). Further, HPV-associated dysplastic cervical lesions are associated with an increased number of CD4+ T-cells compared to normal cervical epithelium (Kobayashi *et al*. 2004), although this may be the result of the dysplasia rather than the virus as disease progresses.

HPV treatment is directed to the macroscopic (such as genital warts) or pathologic precancerous lesions caused by HPV (detected during cervical cancer screening). Subclinical genital HPV infection typically clears spontaneously, thus specific antiviral therapy is not recommended by the CDC to eradicate HPV.[8]

In SA, the prevalence of HPV has been found to range from 44% to 71%, in differing cohorts, genders and age groups (Adler *et al*. 2014; Mbulawa *et al*. 2015; Giuliano *et al*. 2015). Thus, the development and international rollout of vaccines against certain types of HPV has been a major breakthrough. The bivalent Cervarix® vaccine (against HPV 16, 18; GSK Biologicals, Belgium), the quadrivalent Gardasil-4® (against HPV 6, 11, 16, 18; Merck and Co., Inc., USA) and nonavalent Gardasil-9® vaccines (against HPV 6, 11, 16, 18, 31, 33, 45, 52 and 58; Merck and Co., Inc., USA) have been tested in randomized controlled trials and shown to be safe, immunogenic and highly effective ≤6.5 years after vaccination (Denny 2009; Mbulawa *et al*. 2018). Gardasil-9® was approved by the FDA in 2014 for use in girls and women aged 9–26 years and boys aged 9–15 years (Kirby 2015), while in SA the national HPV vaccination program has implemented rollout of the quadrivalent Cervarix®as a school-based program to girls aged 9–10 years, and the Western Cape Government, servicing the Cape Metropole, started the first vaccination campaign during February and March 2018 (Mbulawa *et al*. 2018).[9] This program currently gives two doses of Cervarix®, six-months apart in an academic year (January to December; Mbulawa *et al*. 2018).

[8]Online Source, Available at: https://www.cdc.gov/std/tg2015/hpv.htm, Accessed 28 Aug 2018
[9]Online Source, Available at: https://www.westerncape.gov.za/general-publication/hpv-vaccinations, Accessed 3 April 2018

1.4.7 BV

BV is the most common vaginal condition of women aged 15 to 44[10], and is a poly-microbial syndrome characterized by a depletion of commensal *Lactobacillus* spp. in the lower FGT (Macklaim *et al*. 2013; Eschenbach *et al*. 1989). This is accompanied by an overgrowth of pathogenic vaginal anaerobic bacteria (100 to 1000-fold above normal), resulting in significant increase in bacterial diversity (Macklaim *et al*. 2013). Women with BV typically have a positive association with a spectrum of Gram-positive anaerobic bacteria (including *Gardnerella vaginalis*, *Atopobium vaginae*, *Eggerthella* spp., *Peptoniphilus* spp. and BV-associated bacteria [BVAB1-3, Clostridia-like bacteria]), anaerobic Gram-negative bacteria (including *Mobiluncus* spp., *Prevotella* spp., *Fusobacterium nucleatum*, and *Megasphaera* type 1), and other bacteria without cell walls (including *Mycoplasma hominis* and *Ureaplasma urealyticum*; Srinivasan *et al*. 2012; Africa *et al*. 2014; Lennard *et al*. 2017). These vaginal microbial changes may be accompanied by clinical changes in some women, including a thin, greyish-white, malodorous discharge, a vaginal pH ≥4.5, and the presence of clue cells (vaginal epithelial cells coated with gram-variable bacteria; Taha *et al*. 1998). However, approximately 85% of women with BV are asymptomatic (Anukam *et al*. 2006; Koumans *et al*. 2007), and therefore do not seek health care and, crucially, are not treated. However, a lack of symptoms does not imply a lower risk of pathogenic outcomes associated with BV, as asymptomatic BV produces levels of genital inflammation similar to symptomatic BV (Kyongo *et al*. 2015; Masson *et al*. 2014; Mlisana *et al*. 2012).

Epidemiological studies have shown that BV prevalence varies between and within countries (Kenyon *et al*. 2013) and is partly influenced by socio-economic factors (Holzman *et al*. 2001). The prevalence is lowest in Asia and Europe and highest in SSA (Kenyon *et al*. 2013). Further, young black women have a higher vaginal pH than their white peers (Ness *et al*. 2003), suggesting that racial differences may be associated with varying BV rates and/or pathology in SSA.

[10]Online Source, Available at: https://www.cdc.gov/std/bv/treatment.htm, Accessed 1 April 2018

1.4.7.1 Diagnosis of BV

There are currently two different approaches that are used to diagnose BV. Amsel criteria are more commonly used in clinical practice, and require the presence of ≥ 3 of the following criteria to diagnose BV, including: (i) thin, homogeneous vaginal discharge; (ii) vaginal pH ≥ 4.5; (iii) "fishy" odour of vaginal fluid before or after addition of 10% potassium hydroxide (whiff test); (iv) presence of clue cells on microscopic slides (Amsel *et al*. 1983). In contrast, Nugent scoring, which is based on Gram staining of vaginal smears and the microscopic quantitation (with scores ranging from 0 to 10) of bacterial morphotypes (*Lactobacillus*, *Gardnerella*/*Bacteroides* and curved Gram variable rods) is increasingly being considered the gold standard for diagnosis of BV (Nugent *et al*. 1991). A Nugent score of ≤3 is considered normal (predominantly *Lactobacillus* rods, absence of *Gardnerella*/*Bacteroides* and curved Gram variable rods), a Nugent score of 4–6 is considered to indicate presence of "intermediate" microbiota (mixture of all bacterial morphotypes), and a Nugent score of ≥7 is considered BV positive (predominantly *Gardnerella* or *Bacteroides* and curved Gram variable rods, in addition to absence of *Lactobacillus* rods).

1.4.7.2 Etiology of BV

Multiple factors have been reported to influence the composition of the vaginal microbiome, including: (1) ethnicity (Simhan *et al*. 2008; Cherpes *et al*. 2008); (2) age and hormonal changes associated with adolescence, menstruation, menopause (Cauci *et al.* 2002; Gajer *et al.* 2012); (3) hormonal contraception (Riggs *et al*. 2007); (4) concomitant STIs (Cherpes *et al*. 2008); (5) psycho-social stress (Nansel *et al*. 2006); (6) smoking (Hellberg *et al*. 2000; Brotman *et al*. 2014; Cherpes *et al*. 2008); (7) having multiple partners, a recent new partner, and/or frequent sexual intercourse (Fethers *et al*. 2008; Yen *et al*. 2003; Verstraelen *et al*. 2010); and (8) vaginal cleansing habits (Merchant *et al*. 1999; Brotman *et al*. 2008).

Possibly due to the multitude of factors that may cause a disruption in vaginal microbiota, the aetiology of BV is still unclear and likely caused by any or the combination of these risk factors. Machado and Cerca (2015) dynamically modelled factors associated with emergence of BV, which suggested that sexual transmission of *G. vaginalis* displace *Lactobacillus* spp. from their ecological niche in the FGT by laying down a biofilm that allows other anaerobic pathogenic bacteria to colonize the genital tract. *G. vaginalis* also secretes sialidase, a mucolytic protein

that degrades sialic acids in vaginal mucus, which allows *G. vaginalis* and other pathogenic bacteria to attach to the vaginal epithelium that would normally be protected by vaginal mucous, thereby enabling the formation of stable biofilms (Hardy *et al*. 2017). Although this model has significant merit, it has not yet been scientifically proven, and the initial event triggering the displacement of healthy *Lactobacillus* spp. to one with BV is unknown.

1.4.7.3 BV increases genital inflammation

Several studies have shown that BV, even when asymptomatic, is associated with increased concentrations of pro-inflammatory cytokines in the lower FGT, including IL-1α, IL-1β and TNF-β, and this may partly be due to increasing concentrations of bacterial TLR ligands in the FGT under dysbiotic conditions (Masson *et al*. 2015; Anahtar *et al*. 2016; Cauci *et al*. 2003). TLRs play a fundamental role in the activation of the innate immune system, as they recognize PAMPs derived from various microorganisms, and mediate the production of cytokines necessary for the induction of effective immunity (Kawasaki & Kawai 2014). However, down-regulation of some chemotactic cytokines (like IP-10, growth-regulated oncogene [GRO], macrophage-derived chemokine [MDC] and MIP-1α), responsible for recruitment of immune cells, and haematopoietic cytokines (IL-7 and granulocyte-macrophage colony-stimulating factor [GM-CSF]) has been observed in women with BV (Ryckman, Kelli K; Williams, Scott M.; Krohn, Marijane A.; Simhan 2008; Masson et al. 2014). Downregulation of genital chemokine responses in women with BV may reflect that these are common commensals of the GI microbiome (Danielsson *et al*. 2011). Using proteomics, Borgdorff *et al.* (2016) showed significantly increased levels of the pro-inflammatory cytokine IL-36α (a member of the IL-1 family) and its receptor agonist (IL-36RA), complement component C5, glucose-6-phosphate isomerase, and macrophage migration inhibitory factor (MIF) in women with BV compared to those who did not have BV. Importantly, BV treatment with metronidazole reduced levels of the pro-inflammatory cytokine IL-1β and the chemokines IL-8 and RANTES in cervicovaginal secretions, as well as the number of activated cervical CD4+ T-cells, including those expressing the HIV co-receptor CCR5 in women who were BV negative after treatment (Rebbapragada *et al*. 2008).

Untreated BV leads to some severe reproductive complications in women, including premature delivery, miscarriage, intrauterine foetal death, post-operative infections and PID

(Haggerty *et al*. 2016; Nelson *et al*. 2014; Clark *et al*. 1994). Further, BV exposure during pregnancy has been associated with adverse neonatal outcomes, in both full- and pre-term infants, as BV was associated with a significantly increased risk of assisted ventilation/respiratory distress at birth (adjusted risk ratio [aRR] 1.28; 95% CI 1.02-1.61), neonatal intensive care unit admission (aRR 1.42; 95% CI 1.11-1.82) and neonatal sepsis (aRR 1.60; 95% CI 1.13-2.27; Dingens *et al*. 2016).

BV increases the risk of acquisition and transmission of HIV (Sewankambo *et al*. 1997; Cohen *et al*. 2012; Martin *et al*. 1999; Taha *et al*. 1998; Myer *et al*. 2005; Van de Wijgert *et al*. 2008; Anahtar *et al*. 2016) and other STIs, including *T. vaginalis*, *N. gonorrhoea, C. trachomatis,* HPV and HSV-2 (Guo *et al*. 2012; Brotman *et al*. 2010; Nagot *et al*. 2007). As STIs and genital inflammation associated with STIs and/or BV have been associated with an increased risk of HIV infection (including HSV-2, *C. trachomatis, T. vaginalis* and *N. gonorrhoea*; Ward & Rönn 2010; McClelland *et al*. 2007), it is unclear whether BV directly or indirectly increases the risk of HIV acquisition, by increasing risk for these other STIs. As BV is associated with a variety of adverse outcomes that puts women, their partners and their infants at risk, it is crucial to have effective and durable treatment strategies for BV.

1.4.7.4 Treatment of BV

Since 1996, the South African Department of Health has used syndromic management of STIs and BV as standard of care, which intends to treat the signs or symptoms (syndrome) of a group of diseases rather than treating any specific disease. The most recent guidelines were published in 2015 (The Department of Health 2015). Treatment for BV falls under the syndromic management guidelines for vaginal discharge syndrome, which means if a women presents with discharge, no STI testing or Nugent Scoring to diagnose BV and/or STIs is performed but clinical staff follow signs and symptoms to identify the underlying disease. In SA, the abnormal vaginal discharge chart recommends treating woman with symptoms suggestive of BV and/or vaginal candidiasis with metronidazole (oral, 2g single dose), a clotrimazole vaginal pessary (500 mg as single dose) or clotrimazole vaginal cream (inserted 12 hourly for 7 days). If a woman does not respond to the treatment, a higher dose of metronidazole (oral, 400 mg, 12 hourly for 7 days) is recommended. If this still does not resolve the symptoms after seven days, women are referred to the hospital for further

management (The Department of Health 2015). Antibiotic treatment of BV, however, only results in a temporary resolution of symptoms, as recurrence rates of microbial dysbiosis are high, with ~30% of cases recurring in less than three months and almost 50% recurring within six months (Barrons and Tassone 2008).

1.4.8 Vulvo-vaginal candidiasis (VVC)

The main cause of VVC is *C. albicans* but it can also be caused by other *Candida* species, such as *C. glabrata*, *C. parapsilosis*, *C. dubliniensis*, *C. krusei*, *C. lusitaniae* and *C. tropicalis* (ElFeky *et al*. 2016). Approximately 75% of women will experience at least one episode of VVC in their lives, and ~45% will experience more episodes (Machado *et al*. 2014). Clinical symptoms include pruritus (severe itching), vaginal soreness, dyspareunia (painful sexual intercourse), dysuria (painful urination), and abnormal vaginal discharge (reviewed by Sobel 2016).

Candida spp. are also commonly found in the FGT of healthy women (in about 75%), and therefore considered to be commensals in females, but only ~5% of these women will develop VVC. Certain risk factors, like pregnancy, antibiotic use, contraceptives containing oestrogen, douching, oral and receptive anal sex, lead to the development of VVC (Bradshaw et al. 2005; Iliev & Leonardi 2017; Reis Machado et al. 2014). Having multiple partners is, however, not associated with an increased risk of VVC despite being a common risk factor for other STIs and BV (Bradshaw *et al*. 2005). It has been shown that the species composition and diversity of microbial communities in the FGT does not differ between women with and without frequent episodes of VVC (Zhou *et al*. 2009). However, BV and VVC were found to be negatively associated in three different cohorts from South Africa, Zimbabwe and Uganda, where women with BV tended to have a higher vaginal pH, while those with VVC had a lower vaginal pH (Van De Wijgert *et al*. 2008; Gautam *et al*. 2015; Moodley *et al*. 2002). However, treatment of BV with antibiotics, like vaginal metronidazole, commonly leads to the development of VVC, one of the recognized side effects of vaginal metronidazole gel (Sobel *et al*. 2006).

VVC has been associated with strong inflammatory responses (Sobel 2016), which might explain why VVC has been associated with a three-fold higher risk of HIV infection, and to account for ~3% of HIV acquisitions in Zimbabwean and Ugandan women (Van De Wijgert et

al. 2008). Th1 immunity is thought to be protective against *Candida* infections, while Th2 immunity has been associated with increased susceptibility to infection (Weissenbacher 2014). It is thought that PRRs on immune cells, TLRs and C-type lectin receptors recognize cell surface components of *Candida* spp., and lead to innate immune activation (Sobel 2016). IL-17 and IL-22 seem also to be involved in the immune response to VVC (Pietrella *et al*. 2011; De Luca *et al*. 2010). In a mouse model, IL-17 secretion by CD4+ T-cells in the FGT led to the recruitment of neutrophils, while inhibition of IL-17 caused more severe pathology (Pietrella *et al*. 2011). The critical role of Th17 cells in protection against mucosal candidiasis is demonstrated by the relationship chronic mucocutaneous candidiasis and autoimmunity to Th17-associated cytokines (Kisand et al. 2010), the association of reduced IL17 production and oral candidiasis (Conti et al. 2009) and mutations in the CC domain of STAT1 leading to defective Th17 responses increasing susceptibility to fungal infections (van de Veerdonk et al. 2011).

The CDC recommends a topical short-course with antimycotic formulations (like clotrimazole or miconazole) to effectively treat uncomplicated VVC, which have treatment success rates of 80%–90% in those who complete therapy.[11]

1.5 Factors influencing STI and HIV risk in adolescents

Worldwide, the highest reported rates of STIs are found among adolescents (aged 15 to 24 years old).[12] Half of all people living with HIV are in this age group, and ~60% of the new STI infections (including HIV) occur in young adolescents (Dehne and Rieder 2006), emphasizing the vulnerability of young people. The highest rates of infections are observed in the 20-24 year old age group, followed by 15-19 year olds (WHO 1993; Cates and McPheeters 1997).

The current generation of adolescents is the largest in history, with more than 1.8 billion young people (Das Gupta *et al*. 2014), including 600 million adolescent girls and young women (AGYW) who are disproportionally affected by STIs (CDC STI Surveillance 2000). In 2004, the prevalence of STIs was 24.1% among all female adolescents in the US and 37.7% among those

[11]Online Source, Available at: https://www.cdc.gov/std/tg2015/candidiasis.htm, Accessed 1 April 2018
[12]Online Source, Available at: https://www.cdc.gov/std/stats16/adolescents.htm, Accessed 29 Aug 2018

who were sexually experienced. HPV was the most common STI among all female adolescents with a prevalence of 18.3%, followed by chlamydia with a prevalence of 3.9% (Forhan 2009). A recent report of the prevalence of STIs in young South African women (median age 24.7 years) found that 21% were infected with at least one STI, with chlamydia being the most common (14%), followed by trichomoniasis (6%), gonorrhoea (3%) and syphilis (1%; Chirenje *et al*. 2017). In another South African study including younger female adolescents (median age 16.6 years), 31.1% had at least one STI (including chlamydia, gonorrhoea, and trichomoniasis), and again chlamydia was the most prevalent (23.2%), while trichomoniasis (5.8%) was less common than gonorrhoea (10.7%; O'Leary 2015).

Sexually active adolescents are at a higher risk of acquiring STIs than their older adult counterparts (Braxton et al. 2015a). However, not all young people engaging in sexual activity are at an equal risk of acquiring an STI. Several factors influence STI risk in adolescents, including epidemiological factors (like participation in sexual networks with a high STI prevalence, reduced access to sexual and reproductive health services), socio-behavioral factors (discussed in more detail in section 1.5.1; including gender inequality, sexual violence, age-disparate relationships, poor knowledge around STIs, insufficient access to education including sex education, frequency and type of intercourse, number of concurrent sexual partners, inconsistent use of barrier protection), and biological factors (discussed in more detail in section 1.5.2; like concurrent STI infections, low rates of male circumcision, a lack of acquired immunity to STIs; Bana *et al*. 2010, Lince-Deroche *et al*. 2015, Ellen *et al*. 2005, Bearinger *et al*. 2007, Shisana *et al*. 2014).

1.5.1 Socio-behavioral factors

Younger age of sexual debut has been linked to an increased risk of HIV acquisition (Wand & Ramjee 2012; Shrestha *et al*. 2016). Cohort studies from KwaZulu Natal, SA showed that the highest HIV seroconversion rate was among women reporting sexual debut with <15 years of age, which may also be due to an increased number of lifetime partners in those who started having sex earlier (Wand and Ramjee 2012). In Nepal, participants with an early sexual debut were significantly more likely to report a history of STIs (adjusted odds ratio [aOR] of 1.2, 95% CI 1.06-1.35) and had a significantly higher risk behavior profile, including having multiple sex partners (aOR 2.1, 95% CI 1.86-2.47), inconsistent condom use (aOR 0.7, 95% CI 0.61-0.86),

being paid for sex (aOR 1.6, 95% CI 1.14-2.27), a history of sexual violence (aOR 2.0, 95% CI 1.63-2.43), or teenage pregnancy (aOR 12.9, 95% CI 11.62-14.26; Shrestha *et al*. 2016). In SA, the likelihood of an early sexual debut seems to be dependent on the social environment in which adolescents grow up in, as early debut was significantly more likely among male and female adolescents with an older partner, and among girls who experienced forced sex (Pettifor *et al*. 2009).

Another important socio-behavioral factor to consider in adolescents is intergenerational relationships, defined as >10 years age difference. More than one third of South African AGYW aged 16-24 years old reported having had sexual intercourse with partners ≥ 5 years older (Maughan-Brown *et al*. 2014), therefore being exposed to older partners with a higher HIV prevalence. Although these young women were shown to be approximately three times more likely to have sex with an HIV-infected man than young women who had sexual partners with a similar age (Maughan-Brown *et al*. 2014), another South African study showed no increased HIV risk for female adolescents engaging in intergenerational relationships (Harling *et al*. 2014). A community-wide phylogenetic study to examine the underlying HIV transmission dynamics and the source and consequences of high rates of HIV infection in young women in SA found that sexual partnering between young women and older men, who might have acquired HIV from women of similar age, is a key feature of the sexual networks driving transmission. When these young women become older, they transmit HIV to their adult partners, who then again have sex with younger women, and the "cycle of infection" repeats (De Oliveira *et al*. 2017).

Gender based violence has also been shown to play a significant role in determining HIV risk. In a rural community in the Eastern Cape in SA, 31.8% of men reported physical or sexual violence against their female partners (Dunkle *et al*. 2006). In these communities, gender-based violence correlated with higher numbers of lifetime sexual partners, more recent intercourse, and men who reported physical and sexual violence against a partner within the past 12 months reported also significantly higher levels of HIV risk behaviour than men who reported less severe or less frequent episodes of violence (Dunkle *et al*. 2006). This indicates that women in violent relationships are more frequently exposed to STI- and/or HIV-positive partners. In concordance with that, HIV seropositivity in women was associated with sexual

violence (OR 1.48; 95% CI 1.15-1.89) and high levels of male control in a women's current relationship (OR 1.52; 95% CI 1.13-2.04) in SA (Dunkle *et al*. 2004). However, another South African study found that women experiencing limited sexual power (aOR 2.10; 95% CI 1.17-3.78) or forced sex (aOR 5.77; 95% CI 1.86-17.91) in a relationship was associated with inconsistent condom use, but not directly with HIV risk (Pettifor *et al*. 2004), which most likely indirectly increases the STI/HIV risk in the long-term. Indeed, inconsistent condom use was, in turn, significantly associated with HIV infection in the same study (aOR 1.58, 95% CI 1.10–2.27).

Thus, inconsistent use of barrier protection (like condoms) remains to be one of the major HIV risk factors in SSA (Giannou *et al*. 2015). Among both South African men and women, increasing partner numbers and inconsistent condom use are associated significantly with an increased risk of HIV infection (Pettifor *et al*. 2005), and a Cochrane review stated that consistent use of male condoms could potentially decrease the risk of HIV transmission by 80% in serodisconcordant couples (Weller & Davis-Beaty 2002), emphasizing the importance of barrier protection for the reduction of HIV risk.

1.5.2 Biological factors

1.5.2.1 Endogenous hormones and the menstrual cycle

The genital mucosa is the portal of entry for pathogens causing STIs, and the concentrations of genital tract immunoglobulins (Igs) vary through the menstrual cycle in adult women, being highest during menstruation and lowest around ovulation (Lu *et al*. 1999; Kutteh *et al*. 1998). Animal models have also confirmed that changes in Ig concentrations are related to ovarian hormone concentrations (Lu *et al*. 1999). In FGT secretions, the levels of IgG are higher than those of IgA (Mestecky & Russell 2000). Adolescents were found to have a greater drop in IgG during their follicular phase than adult women, but a similar rate of IgG increase in the luteal phase (Shrier *et al*. 2003). Changes in IgA, in contrast, did not differ between adolescent and adult women for either phase. This suggests that adolescents may be more sensitive to free oestrogen than adult women, and therefore hormone levels could have a greater effect on genital tract immunity during pubertal maturation.

In healthy women, the FGT microbiota is also influenced by the menstrual cycle. Menstruation is accompanied by a decrease in the abundance of *L. crispatus* and an increase in *G. vaginalis* concentrations (Srinivasan *et al*. 2010; Gajer *et al*. 2012). Srinivasan *et al*. (2010) suggested that low oestrogen levels during menses resulted in lower glycogen stores in epithelial cells of vaginal mucosa, which subsequently inhibits growth of *Lactobacillus* spp. that depend on glycogen as source of energy. In contrast, menstrual blood might provide an iron source for *G. vaginalis* that grows better in the presence of blood *in vitro* (Srinivasan *et al*. 2010).

Studies have also suggested that changes of oestrogen levels over the menstrual cycle may influence concentrations of inflammatory cytokines and recruitment of activated immune cells in the FGT (Wira *et al*. 2015). High concentrations of oestrogen are thought to be anti-inflammatory, leading to dampening cellular activation and cell-mediated immune responses, by downregulating ICAM-1, e-selectins and vascular cell adhesion molecule (VCAM)-1 [all of these are cell adhesion molecule expressed only on cells activated by cytokines], resulting in decreased T-cell and macrophage migration to the genital tract (Straub 2007). In women treated with exogenous oestradiol, expression of genital IL-8, IL-19, CXCL1 and CXCL6 were down-regulated, further suggesting that oestrogen dampens inflammation and immune cell recruitment to the FGT (Cotreau *et al*. 2007). On the other hand, low concentrations of oestrogen have been associated with induction of TNF-α, IL-6 and IL-1β (Straub 2007). While low oestrogen levels have also been associated with higher STI/HIV transmission rates (De Vincensi & HIV 1992; Martin *et al*. 1998), animal studies have shown that the topical application of oestrogen protected against STIs (Smith *et al*. 2000).

1.5.2.2 Hormonal contraceptives (HCs)

HCs give many women control over their own reproduction and more power in their sexual relationships where negotiation of condom use is often difficult, although some of the long-acting progestin-only injectable HCs have been associated with an increase in STI and HIV risk (Heffron et al. 2012; Polis & Curtis 2013; Byrne et al. 2016; Polis et al. 2016; Morrison et al. 2015). About 70% of young sexually active women aged 15–19 in SA use contraception, and the most common HC used by South African young women are the injectable progestin-only contraceptives Norethisterone enanthate (Net-En) and depot medroxyprogesterone acetate (DMPA) because they are seen to be discreet, long-acting, effective and convenient (Smit &

Beksinska 2013). There are now many options available for HCs, with combined oral contraceptive pills, vaginal rings, intrauterine devices, patches and progestin-only implants being some other popular choices.

Long-acting progestin-only HCs have been shown to increase the risk of HIV infection in women, while the effect of contraceptive pills, vaginal rings and implants have not been as thoroughly investigated yet (World Health Organization 2017; Deese et al. 2015). DMPA has been shown to increase the HIV transmission risk by almost two–fold (Heffron *et al*. 2012), and non-human primate and human studies have suggested that one of the main mechanisms for this may be the thinning of the vaginal and/or ectocervical epithelia, although evidence of epithelial thinning is more evident in primates than human studies (Achilles & Hillier 2013; Mitchell *et al*. 2014). A recent study in young women from Durban, SA, found that that increased progestin levels were associated with a higher availability of HIV target cells (Byrne *et al*. 2016), which might be another mechanism of increased infection and transmission.

Women using DMPA were also greater than two-fold more susceptible to HSV-2 compared to those not using HC (Grabowski *et al*. 2015). Similarly, women who were HSV-2 seropositive were significantly more likely to be shedding HSV-2 if they were using HCs (Cherpes *et al*. 2005). Calla *et al.* (2016) found that DMPA increased permeability of the vaginal mucosal epithelium in both mice and humans, and favored colonization of the mouse FGT by Gram-negative bacteria, thereby facilitating HSV-2 infection (Calla *et al*. 2016). DMPA use was also associated with a three-fold increased risk of infection with chlamydia or gonorrhoea, while oral contraceptives did not affect the risk of STI infection (Morrison *et al*. 2004). Interestingly, in contrast to the increased acquisition of STIs in DMPA users, use of all oral and injectable contraceptives as well as implants seems to be protective against BV (McClelland et al. 2008; Riggs et al. 2007; Pettifor, Delany, et al. 2009), and decrease the bacterial load of the BV-associated pathogen *G. vaginalis* but not of beneficial *Lactobacillus* spp. (Roxby *et al*. 2016).

1.5.2.3 The mucosal epithelial barrier and cervical ectopy

The multiple layers of stratified squamous epithelium that line the most exposed regions of the female genital mucosa constitute a significant physical barrier to an incoming pathogen, as it acts as the first line of defense. The squamous epithelium of the lower reproductive tract

mediates a range of innate defenses against microbes, and secretes various host-derived defensins, AMPs, enzymes and cytokines via TLRs and PAMPs (Wira *et al*. 2005). Futher, chemokines and cytokines recruit DCs that mediate innate defenses and inflammation (Cremel *et al*. 2005). These defenses provide a series of inhibitory activities against HIV entry and replication. For example, CXCL12 blocks HIV entry mediated by the CXCR4 co-receptor, while MIP-1α/β and RANTES block CCR5-based entry (Marozsan *et al*. 2001). In addition, MIP-3α recruits IFN-α/β-producing plasmacytoid dendritic cell to the endocervix and lead to an increase in IFN-γ expression in the vaginal tissues (Ghosh *et al*. 2009). Further, serpin and cystatin antiproteases have been identified as host factors that could be playing a role in providing protection against HIV in the female genital tract of exposed but uninfected females (Burgener *et al*. 2011). This would explain the low per-sex act probability of HIV transmission in humans, although the incidence of transmission is clearly unacceptably high.

Impairment in epithelial integrity is thought to be required for HIV-1 transmission *in vivo*. Epithelial micro-lesions have been detected in ~50% of women after consensual sexual intercourse, in addition to also frequently being observed on the inner foreskin and penile glans (Astrup *et al*. 2012). Protection provided by the stratified epithelium is further diminished by ulcerative STIs that decrease epithelial integrity, and thereby increase the risk of transmission. In general, anything that impairs epithelial integrity, such as, physical abrasion or trauma, ulceration, inflammation, hormonal status, or the use of intravaginal products can lead to an increased risk of HIV acquisition (Baleta 1998, Novell 1984, Baeten 2001, Hilber *et al*. 2010).

The endocervix is lined with a single-layered columnar epithelium, and desmosomes and tight junction proteins between columnar epithelial cells restrict intercellular virus permeability (Greenhead *et al*. 2000). Whereas the columnar epithelium is usually restricted to the endocervical canal, the eversion of these cells onto the ectocervix during cervical ectopy can increase the likelihood of microtrauma during intercourse (Coombs *et al*. 2003). Due to this thin, vascularized epithelium, ectopic tissue is fragile and is more likely to breakdown than the stratified epithelium. Observational epidemiological studies have suggested that cervical ectopy can increase the risk of acquiring some STIs, such as *C. trachomatis* (Critchlow *et al*. 1995), HPV (Toon *et al*. 1986), and cytomegalovirus (CMV; Collier *et al*. 1995).

The prevalence of cervical ectopy is more common in adolescents and pregnant women, as well as among women using certain HCs (Jacobson *et al*. 2000; Deinise L Jacobson *et al*. 1999; Gray *et al*. 2005). The transformation of columnar cervical epithelium into squamous epithelium does not occur until puberty, and the area of cervical ectopy decreases with aging and sexual activity, when squamous epithelium replaces columnar epithelium, (Jacobson *et al*. 2000; Moscicki *et al*. 2001). Hence, adolescents are more likely to have immature epithelium or larger areas of ectopy that could increase their risk of acquiring HIV and other STIs (Moscicki *et al*. 2001), as higher levels of cervicovaginal inflammatory and regulatory cytokines were described in healthy young women with cervical ectopy (Hwang *et al*. 2011).

Further, a cross-sectional HIV-serodiscordant study conducted in Nairobi, Kenya found that cervical ectopy significantly increased HIV risk in women with HIV-positive partners (Moss *et al*. 1991). Another study in discordant couples from Kenya reported that cervical ectopy was associated with a significantly increased risk for cervical HIV shedding (OR 5.0; 95% CI 1.5-16.9), suggesting its importance in the transmission of HIV (Clemetson *et al*. 1993). Similarly, a study in Cape Town, SA, found that ectopy extending over more than 20% of the cervix was associated with HIV seroconversion (aOR 2.18; 95% CI 1.01–4.69; Myer *et al*. 2006). However, other observational studies did not find an association between cervical ectopy and HIV infection (Nicolosi *et al*. 1994; Moscicki *et al*. 2001), and Venkatesh and Cu-Uvin (2013) reported that only about half of the studies they reviewed found cervical ectopy to be a significant risk factor for HIV acquisition. Therefore, the contribution of cervical ectopy to increased HIV risk remains unclear, and the majority of women in these trials were co-infected with other STIs, which could independently interrupt epithelial barriers and lead to an increased risk of HIV acquisition. However, given that cervical ectopy is more common in adolescents, it may contribute to the disproportionally high risk of STIs including HIV infection among sexually active female adolescents.

1.5.2.4 STIs, BV and yeast infections

Early in the HIV epidemic, bacterial and viral STIs were shown to increase both the acquisition and transmission of HIV between sexual partners (Piot and Laga 1989). More recently, Mlisana *et al.* (2012) confirmed that *C. trachomatis*, *N. gonorrhoeae*, and *M. genitalium*

infections were still the strongest predictors of HIV infection in high burdened communities (Mlisana *et al*. 2012). In addition, BV and vaginal candidiasis, although not sexually transmitted but sexually associated, contribute significantly to HIV transmission (Johnson *et al*. 2012). Despite knowledge that curable STIs were estimated to account for 39% of heterosexual HIV transmission in 1990 and substantial efforts to reduce and/or treat these more aggressively (Johnson *et al*. 2012), they were still shown to account for 14% of new HIV infections worldwide and over 50% of new HIV infections in women in SA in 2010 (Johnson *et al*. 2012). HSV-2 infection was the most influential in determining HIV risk in women (Johnson *et al*. 2012). Although successful management of STIs could secondarily reduce HIV incidence rates, this has proved very difficult to achieve (Celum *et al*. 2008; Gray *et al*. 2001; Wawer *et al*. 1999; Kaul *et al*. 2004; Watson-Jones *et al*. 2008; section 1.6.2). It is also likely that the contribution of curable STIs to HIV risk is greater in adolescent than in older adults, because the burden of these common infections is high in this naïve at-risk age group.

While the increased presence of HIV target cells and genital inflammation are important mechanisms that increase HIV risk in STI-positive populations, there is also evidence that STIs promote HIV transmission by increasing HIV susceptibility by other biological mechanisms (Galvin & Cohen 2004). A meta-analysis and systematic review have found that women with BV, HSV-2, HPV, *C. trachomatis*, *N. gonorrhoeae*, *Candida*, genital ulceration and vaginal discharge were more likely to have higher HIV viral loads in their genital secretions than those without these conditions (Johnson & Lewis 2008; Kalichman *et al*. 2011). In men, *N. gonorrhoeae*, *T. vaginalis*, CMV, urethritis and genital ulcer disease have also been associated with increased HIV viral loads in semen (Coombs *et al*. 2003). Ulcer-causing STIs generally increase concentrations of HIV in the genital tract, by direct shedding of HIV from the ulcerative lesion (Paz-Bailey *et al*. 2010; Coombs *et al*. 2003). The levels of HSV-2 shedding also correlated with HIV plasma viral loads (Schacker *et al*. 2002), and even asymptomatic HSV-2 shedding was found to be associated with increased HIV shedding (McClelland *et al*. 2002). Similarly, STIs that cause inflammation increase the levels of HIV that can be detected in urethral swabs, semen and FGT secretions (meta-analysis by Johnson and Lewis 2008). In men diagnosed with urethritis, gonorrhoea seems to have a greater effect on HIV viral load in than chlamydia (Rotchford *et al*. 2000), which might be due to the higher degree of inflammation usually caused by gonorrhoea. However, even less symptomatic STIs in men

have been linked to an increased semen HIV concentrations (Sadiq *et al*. 2005; Sadiq *et al*. 2002), and this correlated with the extent of urethral inflammation. Boily *et al.* (2009) found that current and healed genital ulcers in the HIV susceptible partner increased infectivity per-act five-fold compared with those not having experienced a genital ulcer before. These data show that both previous and current STIs greatly influence HIV acquisition and transmission risk in males and females.

1.5.2.5 FGT inflammation

BV and STIs as well as other biological factors have been shown to impact the risk of young women acquiring HIV. Genital inflammation may represent a common mechanism by which these diverse factors drive HIV risk (Masson *et al*. 2015; Anahtar *et al*. 2016; Mlisana *et al*. 2012). Several previous studies have demonstrated that STIs are a major cause of inflammatory cytokine upregulation and immune cell recruitment to the genital mucosa (Rebbapragada *et al*. 2007; Fichorova *et al*. 2001; Masson *et al*. 2014). In a study that included adult women from Kwa-Zulu Natal in SA, *C. trachomatis* was associated with the highest genital cytokine levels, followed by N*. gonorrhoeae*, HSV-2, *T. vaginalis* and BV (Masson *et al*. 2014). Besides STIs, HCs have been associated with cervical and vaginal inflammation (Wira *et al*. 2011; Morrison *et al*. 2014; Deese *et al*. 2015), increased FGT T-cell CCR5 expression (Huijbregts *et al*. 2013; Prakash *et al*. 2005), and T-cell and macrophage mucosal recruitment (Ildgruben *et al*. 2003; Wira *et al*. 2011). Other possible causes of genital inflammation include vaginal practices (Alcaide *et al*. 2017), exposure to seminal fluid (Sharkey *et al*. 2017; Adefuye *et al*. 2016), use of certain lubricants during sex (Fichorova *et al*. 2001), cyclical changes in hormone levels (Kyongo *et al*. 2012), as well as host genetics (Ryckman, Kelli K; Williams, Scott M.; Krohn, Marijane A.; Simhan 2008).

Non-human primate studies showed that an inflammatory response at the genital mucosa was required for successful infection following vaginal SIV exposure (Li *et al*. 2009). They showed in this non-human model that inflammation in the FGT not only leads to the recruitment of HIV target cells, but also creates an environment that favors HIV replication directly, as well as the establishment of a productive infection. Cytokines and chemokines have been shown to be useful biomarkers of inflammation in the FGT (Mlisana *et al*. 2012; Masson *et al*. 2015). Genital levels of α-defensins and antimicrobial peptides (AMPs) that

induce pro-inflammatory cytokines were associated with increased HIV acquisition rates in women (Levinson *et al*. 2009) and men (Hirbod *et al*. 2014). Women with elevated concentrations of proinflammatory cytokines, including MIP-1α, MIP-1β and IP-10, in their genital tracts were found to be at increased risk of HIV acquisition (Masson *et al*. 2015). IP-10, MIP-1α, and MIP-1β are chemotactic for HIV target cells, including T-cells, macrophages, and DCs (Li *et al.* 2009; Wira *et al.* 2005), and MIP-1α and MIP-1β are also ligands for the HIV co-receptor CCR5 and specifically recruit CCR5+ target cells into tissues (Farzan *et al*. 1997). Pro-inflammatory cytokines in the lower reproductive tract have been associated with increased frequencies of neutrophils, T- and B- cells, as well as higher levels of cellular activation (Arnold et al. 2016; Nkwanyana et al. 2009). Pro-inflammatory cytokines and chemokines that are involved in activation, differentiation, and recruitment of immune cells to the genital tract may therefore increase HIV transmission, as a productive infection depends on the presence of susceptible immune cell targets (CD4+), the level of immune cell activation (CCR5+) and monocyte differentiation to macrophages or DCs (Nkwanyana et al. 2009; C. R. Wira et al. 2005).

Arnold *et al.* (2015) found that elevated mucosal cytokine levels were associated with more abundant expression of several matrix metallopeptidases (including MMP9 and MMP8), and enriched for proteomic-pathways associated with cell motility, and actin cytoskeletal rearrangement, suggesting tissue degradation and/or remodeling (Arnold et al. 2016). Furthermore, they also showed that expression of protease inhibitors (including SLPI), epidermal cell differentiation, and cornified envelope pathways that are important for an effective physical barrier of the epidermis were decreased in the presence of high levels of mucosal cytokines. In addition, they further showed that genes associated with neutrophil, CD8 T-cells and pathogen-stimulated DC stimulation were enriched, suggesting that increased activity of several immune cell subsets was associated with elevated mucosal cytokines. Increased cytokines were also associated with increased numbers of cervical CD4 T-cells. Together, these data give strong evidence for the linkage between mucosal cytokines, barrier function, proteases, and immune cell recruitment, which are potential mechanisms that increase risk of HIV acquisition.

1.5.2.6 Vaginal pH

The pH of the lower reproductive women thought to be healthy is less than 4.5, and associated with a *Lactobacillus* dominated vaginal microbiome (Boskey *et al*. 1999; Aroutcheva *et al*. 2001). One of the beneficial effects of commensal vaginal *Lactobacillus* spp. is their ability to lower vaginal pH by their production of metabolites like hydrogen peroxide and lactic acid (Tomás *et al*. 2003; Boskey *et al*. 1999). Maintenance of this low vaginal pH is thought to play an important role in protecting women from reproductive complications and the acquisition of STIs including HIV.

Typically, the pH of lower FGT is typically around pH 4, depending on the combination and abundance of *Lactobacillus* spp. present (Aroutcheva *et al*. 2001; Boskey *et al*. 1999). When the microbiota is dominated by *L. crispatus*, the vaginal mucosa reaches a pH of 4.0 ± 0.3, while the dominance of *L. gasseri*, *L. jensenii* and *L. iners* results has been associated with vaginal pHs of 5.0, 4.7 and 4.4, respectively (Ravel *et al*. 2011). Generally, the low pH of the vaginal mucosa is believed to be the main strategy to prevent both bacterial and viral infections in the FGT. While O'Connor *et al*. (1995) observed that HIV is stable at pH levels as low as 4.5, Ongradi *et al*. (1990) reported that cell-free HIV-1 was inactivated at acidic pHs, in a pH and time dependent manner. Ongradi and colleagues (1990) further observed that incubation of cell-free HIV at pH 5.7 for two hours is enough to inhibit the infectivity of HIV, whereas only 20 min is required to completely inactivate the virus at a lower pH of 5.4. Cell-associated viral infectivity was not inhibited by low pH, however (Ongradi *et al*. 1990). More recently, Lai *et al*. (2009) reported that human cervico-vaginal mucus with low pH efficiently traps HIV, causing it to diffuse >1000-fold slower compared to water. Lactic acid, but not other acids like HCl, could abolish the negative surface charge on HIV without lysing the viral envelope at acidic pHs, which they argued may alter HIV surface protein structures and/or possibly inactivate the virus by disrupting the envelope membrane and exposing the capsid (Lai *et al*. 2009). Subsequently, this was confirmed in *in vitro* experiments, which showed that cell-free HIV was inactivated by L-lactic acid at an acidic but not neutral pH (Aldunate *et al*. 2013), and *ex vivo* using cervicovaginal explant tissue (ñahui Palomino et al. 2017). Further, culture supernatants of *L. crispatus, L. acidophilus, L. paracasei ssp. casei,* and *L. rhamnosuus* were shown to significantly inhibited HIV-1 replication, and co-culture with *L. gasseri* inhibited HIV-1 replication by more than 60% (Zabihollahi *et al*., 2012). Taken together, these results

support the observation that maintaining a low pH in the FGT by production of lactic acid is important to reduce HIV transmission.

An acidic environment additionally appears to be associated with lower activation of T-cells, which may lead to decreased susceptibility to HIV-1 infection (Hill & Anderson 1992; Olmsted *et al*. 2005). Monocytes, macrophages, and T-cells, which might act as target cells or the primary vectors for sexual transmission of HIV from the genital mucosa, were found to completely lose motility at pHs below 6.0 (Olmsted *et al*. 2005).

Apart from contributing to lowering vaginal pH, H_2O_2 has been suggested as an important anti-pathogenic strain-specific characteristic of vaginal lactobacilli. Various strains of *L. crispatus*, *L. jensenii*, and *L. gasseri* have been shown to produce H_2O_2 (Antonio *et al*. 1999; Balkus *et al*. 2012). The production of H_2O_2 by *Lactobacillus* spp. seems to be a non-specific host defense mechanism against different pathogens. Klebanoff and Coombs (1991) demonstrated a virucidal effect of a natural *L. acidophilus* isolate (at a concentration of 10^7 CFU/mL) against HIV by measuring the decrease of viral replication in T cells, which was shown to depend on their ability to produce H_2O_2, (Klebanoff & Coombs 1991). The same anti-HIV activity was not observed when this *L. acidophilus* strain was heat-inactivated or when it was replaced by a strain that was not capable of producing H_2O_2, suggesting the important role of H_2O_2 in inhibition of the HIV infections. However, the protective capacity of H_2O_2 concentrations produced by vaginal *Lactobacillus* spp. *in vivo* remains unclear. While *Lactobacillus* spp. produce high levels of H_2O_2 under aerobic conditions, they produce lower levels (0.5 mM – 1.0mM) under anaerobic conditions such as those found in the FGT *in vitro* and *in vivo* (O'Hanlon *et al*. 2011; O'Hanlon *et al*. 2010). With this recent knowledge, it is now thought that lactic acid may play a more important role in HIV inactivation *in vivo* than production of H_2O_2.

In contrast to the acidic environment in the lower FGT, semen is slightly alkaline (~pH 8.0) (Harraway *et al*. 2000), leading to pH neutrality of the vagina during and for a short period after intercourse (Nakra *et al*. 2016; Bouvet *et al*. 1997). This semen-induced neutral vaginal pH after sexual intercourse may favor male-to-female transmission of HIV during vaginal intercourse. Additionally, as this pH change persists after sex, the acidity-associated loss of

infectivity of the female-derived virions is prevented, and therefore female-to-male transmission is favored during the next sexual act. This is of special importance in SSA where transmission of HIV still mainly occurs via heterosexual intercourse. Comparing the effect of protected and unprotected sex on vaginal pH, concentrations of immune molecules, and antimicrobial activity of cervicovaginal lavages, Nakra *et al.* (2016) recently reported that vaginal pH remains elevated for two to six hours after unprotected sex, while no change was observed in pH following condom-protected sex. Further, the level of TGF-β increased, but HBD 2, -3, and IL-8 levels decreased after unprotected sex (Nakra *et al.* 2016).

1.5.2.7 HIV-1 viral load in the transmitting partner

Baten *et al.* (2011) showed that the concentration of HIV in the blood of HIV-infected men correlated directly with the amount of HIV contained in their semen, and therefore their potential to transmit HIV to their serodiscordant female partners during heterosexual sex. Furthermore, they noted that HIV transmission was not observed when the concentration of HIV was <1,500 copies/ml, and that the risk of transmission increased directly with increasing blood viral burden, with a 2.5-fold increased risk of seroconversion in the uninfected partner for every log increase in viral load of the infected partner (Quinn *et al*. 2000). HIV concentrations in those who are infected tended to be highest during acute HIV infection, in late untreated HIV disease, and in those who other co-infections, such as tuberculosis, malaria, HSV outbreaks and intestinal worms, putting those individuals at a higher risk of transmitting HIV to their partner (Pilcher *et al*. 2007; Pilcher, Jr, *et al*. 2004; Rutstein *et al*. 2017; Sulkowski *et al*. 1998; Toossi *et al*. 2001; Hoffman *et al*. 1999; Mole *et al*. 1997; Wolday *et al*. 2002). Wawer *et al.* (2005) calculated that HIV transmission during early HIV infection was 0.0081 per coital act, compared with 0.0010 and 0.0043 per coital act in individuals with more established or late HIV infection, respectively. Similarly, Pilcher and colleagues (2004) calculated that the probability of heterosexual transmission during acute HIV infection was 20-fold greater than established infection.

1.6 Syndromic management of STIs and BV

Effective STI management is critical for successful control, as it decreases the spread of infections in the community and prevents the development of STI-associated complications.

In many settings in SSA, a laboratory diagnosis of STIs is not feasible for health care providers due to time, resource, and financial limitations. Further, many health care facilities lack the trained personnel required for etiological diagnosis of STIs. In addition, the sensitivity and specificity of commercially available rapid tests varies significantly, thereby negatively affecting the reliability of an STI diagnosis (World Health Organization 2003). To circumvent the need for an etiological STI diagnosis, the WHO developed a syndrome-based approach for STI management in 1991 (Vuylsteke 2004) and promoted it in countries in the developing world. This approach, which relies on clinical algorithms, allows health workers to diagnose an infection on the basis of consistent, easily recognizable signs and symptoms (such as vaginal discharge, urethral discharge, genital ulcers, abdominal pain), and then provides treatment that targets the most likely organisms responsible for causing a syndrome (World Health Organization 2003).

Syndromic management for genital ulcers has been found to be inexpensive, simple and very cost-effective, in addition to being valid and feasible. Therefore, these conditions are in most cases appropriately treated (World Health Organization 2011). In contrast, the syndromic management approach has been less successful in the diagnosis of discharge causing STIs in women. While the WHO flowcharts for abdominal pain in women have been shown to be relatively successful for diagnosing pelvic inflammatory disease, those for vaginal discharge have limitations, especially in the management of cervical infections (such as *N. gonorrhoea* and *C. trachomatis*; World Health Organization 2003). To date, attempts to increase the sensitivity and specificity of the WHO vaginal discharge flowchart by including appropriate and situation-specific risk assessment have not been successful (World Health Organization 2011). Some of the risk assessment questions that have been based on demographics (such as age and marital status) have tended to classify too many adolescents as being at risk of cervical infection, thereby causing an overtreatment of STIs, while others who are indeed infected but asymptomatic are not identified using the syndromic approach (World Health Organization 2011). Therefore, there is a need to identify the main STI risk factors for adolescents in the local population and tailor the risk assessment accordingly.

It also needs to be taken into consideration that women need to recognize their “abnormal” discharge to gain access to these treatment algorithms, and every woman has her own sense

of what is considered normal and which amount of vaginal discharge is excessive. Vaginal discharge may be physiological or pathological, and symptomatic vaginal discharge is a poor predictors of STIs (Mlisana *et al*. 2012). Further, hormone changes (induced by HCs, cyclic hormonal changes, and pregnancy) and other factors, such as menstruation, semen, and personal habits and hygiene influence physiological discharge (Mitchell 2004), and thus it may be difficult for young women to determine whether their vaginal discharge is physiological or pathological.

With all of these limitations, syndromic management is still considered simple, facilitates rapid, same-day treatment, and avoids expensive etiological tests overall (WHO 2016), although it needs to be emphasized that this approach misses women experiencing asymptomatic infections, which are the majority of STIs globally. These asymptomatic infections in women are, however, associated with similar levels of genital inflammation (measured by cytokines), and therefore HIV risk, as symptomatic disease (Mlisana *et al*. 2012; Masson *et al*. 2015). As the prevalence of these STIs remains unacceptably high, particularly in resource-limited settings, there is an urgent need to refine the current STI management strategies for women in order to identify asymptomatic infections more effectively. Several commercial high quality laboratory and rapid nucleic acid amplification tests (NAATs), such as GeneXpert®, are available for common discharge-causing STIs, particularly in the developed world, and offer rapid accurate point-of-care (POC) diagnosis. However, the expense of these tests limits their use in resource-limited settings (Tabrizi *et al*. 2013; Pearce *et al*. 2013). Thus, the development of an inexpensive method for identifying women with STIs and/or BV, with potential to be used at the POC, would be favourable.

1.6.1 Syndromic management of STIs in South Africa

Syndromic management was implemented in South Africa in the mid-1990s and, together with an increased use of barrier protection, resulted in a notable decline in gonorrhoea, chancroid, and syphilis infections (Johnson *et al*. 2012). However, there is little evidence that the prevalence of other STIs including chlamydia, trichomoniasis, HSV-2 and BV decreased. Importantly, after the introduction of syndromic management, the proportion of new HIV infections attributable to curable STIs decreased from 39% in 1990 to 14% in 2010. There was little evidence that syndromic management changed HSV-2 and BV prevalence. In fact, the

proportion of HIV infections attributable to HSV-2 infections increased over this period and the contribution of BV to HIV acquisition remained unchanged (Johnson *et al*. 2012). However, these estimates could not be balanced by data on which antibiotics were prescribed once women were diagnosed, as this was not collected, or whether women (and their partners) completed their treatment course.

1.6.2 STI management as a strategy to reduce incident HIV-1 infections

Population-wide STI treatment interventions have been successful for the prevention of some STIs, although they have been largely unsuccessful for HIV prevention (Celum et al. 2008; Watson-Jones et al. 2008; Gray et al. 2001; Wawer et al. 1999; Kaul et al. 2004). While some studies in SA report on the successful decrease in HIV incidence with syndromic management (Johnson *et al*. 2012), the majority of syndromic STI management interventions in Africa caused no change in the rates of HIV acquisition, suggesting that symptomatic and asymptomatic infections may significantly contribute to HIV risk. STIs and BV are frequently asymptomatic in women although cause as much genital inflammation as symptomatic infections do (Mlisana *et al*. 2012).

HSV-2 was identified as the most significant STI increasing HIV risk (Johnson *et al*. 2012). Despite this, treatment HSV-2 seropositive women and men with acyclovir (400 mg, twice daily) was found to be ineffective at reducing HIV infection rates (Celum *et al*. 2008). Although HSV-2 suppressive therapy may reduce symptoms like genital ulceration, HSV-2 may still induce a persistent state of susceptibility to HIV infection because of the ongoing sub-clinical inflammation it causes (Rebbapragada *et al*. 2007).

BV might also significantly contribute to the failure of STI treatment interventions, as BV is difficult to diagnose and treat with antibiotics like metronidazole, and has recurrence rates of ~50% within six months of antibiotic treatment (Barrons & Tassone 2008). Monthly presumptive treatment with high-dose intravaginal metronidazole and miconazole reduced the risk of BV, did not meet the predetermined criteria as a means to reduce HIV acquisition (McClelland *et al*. 2015) .

Although the currently practiced symptom-based algorithms offer rapid and cheap benefits against some sexually acquired pathogens, there poor performance against other more fastidious STIs and BV emphasize the urgent need for etiological testing of STIs to ensure appropriate treatment of both symptomatic and asymptomatic STIs, which would secondarily affect HIV risk substantially.

1.7 Aims and objectives

The overall aim of this dissertation was to investigate the role of behavioural, physiological and biomedical characteristics that may influence HIV risk factors in AGYW, including endogenous hormone levels, hormone contraceptive choices, vaginal pH, prevalent and recurrent STIs, BV and yeast infections.

Specific objective 1: To determine the prevalence of sexual risk behavior, STIs, BV and yeast infections in South African AGYW, and the relationship of these infectious conditions to clinical symptoms.

Hypothesis: There will be a high prevalence of STIs, BV and yeast infections in AGYW with poor correlation to the presence of symptoms and high levels of risk sexual behaviour.

Specific objective 2: To investigate the impact of sexual risk data collection by paper-based and electronic methods in AGYW on reporting bias.

Hypothesis: The electronic method of data collection will increase the reporting of risk behaviour by decreasing reporting bias and will improve completeness of data entry.

Specific objective 3: To investigate the effect of BV, STIs and yeast infection on cytokine concentrations in the genital tract of AGYW

Hypothesis: BV/STI/yeast-negative women will have a low genital pH (pH <4.5), low levels of inflammatory cytokines and high levels of anti-inflammatory cytokines in genital secretions. Endogenous hormones, hormonal contraceptives and vaginal pH will influence levels of genital inflammation. BV or infection with STIs or yeast will be accompanied by an increase in genital inflammatory cytokines.

Specific objective 4: To investigate the use of cytokine biomarkers (IL-1α, IL-1β and IP-10) for identifying adolescents with STIs and vaginal dysbiosis.

Hypothesis: Cytokine biomarkers will identify women with BV, intermediate microbiota and STIs who are at risk of HIV acquisition, independently of the presence of symptoms. These genital conditions will be accompanied by changes in genital cytokines concentrations.

Chapter 2

Sexual risk behavior, prevalence and symptoms of STIs, yeast and BV in South African AGYW

2.1 Introduction

STIs are one of the top five disease categories for which healthcare is accessed globally, with more than one million people infected daily (World Health Organization 2013). Sexually active adolescents are at higher risk of acquiring STIs than their older adult counterparts (Braxton *et al*. 2015). This increased risk may be influenced by several factors, including higher risk behavior, poor knowledge around STIs (Bana *et al*. 2010), reduced access to sexual and reproductive health services (Lince-Deroche *et al*. 2015), participation in sexual networks with a high STI prevalence (Ellen *et al*. 2005), and a lack of or only partial acquired immunity to STIs (Bearinger *et al*. 2007). Untreated STIs may influence reproductive morbidity including pelvic inflammatory disease, infertility and cervical cancer in women (World Health Organization 2013).

In SA, AGYW (aged 15-24 years) bear the highest burden of HIV risk (Shisana *et al*. 2012), and STIs have an important influence on this risk (Laga *et al*. 1993; Masese *et al*. 2015; Napierala Mavedzenge & Weiss 2009; Galvin & Cohen 2004). Curable STIs account for ~14% of new HIV infections in women in South Africa (Lewis 2011), and high HIV prevalence rates in this region may be driven by the synergy between HIV and the other common STIs (Galvin & Cohen 2004; McClelland *et al*. 2005). The strength of this association between HIV and STIs appears to be strongest for STIs that result in genital ulcers (such as chancroid, syphilis and HSV), and weaker for STIs that cause discharge (such as *C. trachomatis N. gonorrhoea, M. genitalium* and *T. vaginalis*; Altini & Coetzee, 2005). *C. trachomatis* and *N. gonorrhea*, which infect the upper reproductive tract in women, can directly disrupt the columnar epithelial layer (Brunham & Rey-Ladino 2005; Prozialeck *et al*. 2002; Sun & Schoborg 2009), while HSV targets the squamous epithelial layer of the lower reproductive tract (Galvin & Cohen 2004; Johnston, Koelle & Wald 2011). STIs can also cause the recruitment of activated T-cells to the mucosa, increasing the chance of an HIV infection (Coombs, Reichelderfer & Landay 2003).

Although earlier studies suggested that HIV preferentially infects women at the cervical transformation zone at the squamo-columnar junction (Pudney, Quayle & Anderson 2005), more recent experiments in female macaques have shown that SIV is able to infect at any region of the upper and lower reproductive tracts, with replicating SIV being found within

cells in the ovaries, fallopian tubes and uterine lining of vaginally challenged animals (Stieh *et al*. 2014). In women, STIs are frequently asymptomatic and it is thought that the lack of observable symptoms may be due to the fact that common infections like *C. trachomatis* and *N. gonnorhoea* infect columnar epithelial cells of the upper reproductive tract (Edwards & Butler 2011). Even when asymptomatic, however, STIs are known to influence HIV risk by causing inflammatory changes in the reproductive tract of women (Mlisana *et al*. 2012). Additionally, the HIV-infected person is more infectious to their partner in the presence of these STIs, as viral shedding is increased when an HIV positive partner has a STI (Manhart *et al*. 2007).

The widespread use of syndromic management for STIs and poor national surveillance programs have resulted in poor reporting on the prevalence of individual STI pathogens in SA (Lewis 2011). South African STI surveillance data are mainly obtained from women attending antenatal or family planning clinics (Altini & Coetzee 2005). A recent study of adult women in SA with a median age of 28 years showed a STI prevalence (including either *N. gonorrhoeae, C. trachomatis*, and *T. vaginalis* or syphilis) of 13% at screening (Naidoo *et al*. 2014). In a second South African study of pregnant women with an average age of 25.5 years, 32.3% tested positive for a STI at baseline (including *N. gonorrhoeae, C. trachomatis,* and *T. vaginalis*; Moodley *et al*. 2015).

Although not considered a STI, BV is the most common vaginal infection among women of childbearing age (Turovskiy, Sutyak Noll & Chikindas 2011). BV describes an altered vaginal microbiota which is dominated by a diverse array of anaerobes, such as *Prevotella*, *Gardnerella*, and *Atopobium* species, and increases both the acquisition and transmission of STIs including HIV (Kenyon, Colebunders and Crucitti 2013; McClelland *et al*. 2018; Klatt *et al*. 2017), despite the majority of women being asymptomatic (Klebanoff *et al*. 2004).

STI prevalence varies in different regions around the world (Kenyon, Buyze and Colebunders 2014) and within various geographical areas within one country (Hughes & Field 2015) and within cities (Zenilman *et al*. 2002; Jennings *et al*. 2005). A similar pattern has been seen with BV, with variation both between and within countries (Kirakoya-Samadouglougou, Nagot and Defer 2008). Further, variation is seen regarding genital inflammation in different populations

of BV positive women that is thought to be due to both host factors and microbial diversity (Mitchell & Marrazzo 2014).

In this study it was hypothesized that the prevalence of STIs and BV in AGYW and the immune response to these conditions varied by region, even within the same country. The objective of this Chapter was to compare the prevalence of symptoms associated with laboratory-diagnosed STIs and BV in order to identify risk factors associated with HIV acquisition in adolescents, and to explore these as drivers of variability in risk across the country. Thus, young women from resource-poor communities in two major cities in different geographical locations (1800 km apart) in SA were enrolled: Masiphumelele in Cape Town and Soweto in Johannesburg. Although both are previously disadvantaged areas, socio-behavioural aspects differed between adolescents from these two cities (described in detail in Chapter 3). It was further hypothesized that differences in behavior and disease burden could result in differences in STI and/or BV prevalence between Masiphumelele and Soweto.

2.2 Materials and Methods

2.2.1 Cohort description

The (WISH) study enrolled 298 adolescent girls and young women (16-22 years old). Cape Town (Masiphumelele) participants were recruited from the family planning clinic of the Desmond Tutu HIV Youth Center (DTHF, University of Cape Town, PI Prof Linda-Gail Bekker), while Johannesburg (Soweto) participants were recruited from community outreach programs through collaboration with the Perinatal HIV Research Unit (PHRU, University of the Witwatersrand, PI Prof Glenda Grey). Both sites offer adolescent-friendly service with free syndromic management of STIs and BV, condoms, and HIV counseling, testing, and referral if appropriate. Women from Soweto attended a single study visit, while those from Masiphumelele were followed longitudinally for three visits for a total of four to six months [baseline and every two months for four months if they were using Net-EN (Nur Isterate, 200mg intramuscularly), combined oral contraception (Oralcon®, COP, 0.15mg levonorgestrel and 0.03mg ethinylestradiol), NuvaRing® (Etonogestrel/ethinyl estradiol vaginal ring; releases 0.12mg/day of etonogestrel and 0.015mg/day ethinyl oestradiol) or Implanon® (etonogestrel containing subdermal rod, which has 68 mg of progestin etonogestrel that is released at 60-70 µg/day). If they used DMPA (Depo Provera®, 150mg/ml intramuscularly), they were followed every three months for six months (enrollment and two further visits) for ease of follow-up. Of the 149 AGYW from Masiphumelele, 127 (85%) attended two visits and 88/149 (59%) attended all three visits. Since hormonal fluctuations are thought to influence important physiological and immunological properties of the female reproductive tract, AGYW using COPs, condoms or hormonal implants had visits scheduled to coincide with the luteal phase of their cycles on day 21, while those using injectable progesterone-only contraceptives, like Net-En or DMPA, had study visits scheduled two weeks after their last injection, after hormone peak levels had normalised.

2.2.2 Ethical approval processes

AGYW who were ≥18 years old provided informed written consent, while those <18 years old provided written assent, with written informed consent being obtained from their parent(s)/legal guardian(s). Ethics approval was obtained from the Human Research Ethics Committee of the University of Cape Town (UCT HREC, reference 267/2013) and University of

Witwatersrand Research Ethics Committees (Clearance certificate number: M1307) for this study. In addition, UCT HREC approved this study as part of this PhD (S. Barnabas HREC reference 503/2016).

2.2.3 Enrolment criteria

AGYW were enrolled if they were generally healthy, HIV-negative, sexually active, and not pregnant nor menstruating at the time of their study visit. All participants were asked to refrain from unprotected sex or douching ≤48 hours, and from antibiotics in the last two weeks prior to their visit. Prior to enrollment, all adolescents underwent HIV risk-reduction counseling and had rapid HIV and pregnancy tests, which were also performed at every study visit in the longitudinal arm of the study in Masiphumelele.

2.2.4 HIV and pregnancy tests

2.2.4.1 HIV tests

Finger prick blood was collected for a HIV rapid test (Alere Determine™ HIV-1/2 Ag/Ab Combo, Alere, Waltham, MA, United States), which is a lateral flow, POC qualitative immunoassay that detects HIV-1 p24 antigen, as well as antibodies to HIV-1 and HIV-2. Whole blood was transferred to the capillary tube, which was then placed on the sample pad. Once all the blood transferred to the sample pad, a drop of chase buffer was added to the pad. The test result was read between 20 and 30 minutes later, with a red line in either the antibody or antigen section along with a red control line indicating a positive test result. A positive test result would have then been confirmed with a second HIV rapid test of a different make – the Acon HIV 1/2/0 Tri-line Rapid Test (Acon Laboratories, Inc., San Diego, CA, USA).

2.2.4.2 Pregnancy tests

Pregnancy status was determined using a urine pregnancy test (U-test Pregnancy strip, Humor Diagnostica, Pretoria, South Africa). This is a rapid diagnostic screening test that detects human Chorionic Gonadotropin hormone (hCG) from midstream urine. Participants were asked to urinate into a receptacle. The pregnancy strip was then placed into the urine below the maximum mark, and the result was read two to three minutes later. A positive result consisted of a coloured line in the control region and a second one in the pregnancy region of the strip.

2.2.5 Socio-demographic questionnaire

AGYW enrolled on the study were asked to complete the questionnaire at enrolment visit (Appendix I). The first half of the cohort at each site was enrolled into the paper-based arm, while the second half of the cohort completed the same questionnaire on an iPad (described in more detail in Chapter 3). The questionnaire was divided into five sections: (i) demographics and socio-economic status, (ii) health care and support services utilization, (iii) emotional wellbeing and resiliency, (iv) reproductive and (v) sexual health.

2.2.6 Symptom questionnaire

Women from Masiphumelele (43%; 155/364) had an additional, voluntary paper-based questionnaire (Appendix II), administered by the study nurse at every visit, that recorded information about any symptoms suggestive of STIs or BV (abnormal vaginal discharge, dysuria and vaginal itching/ burning) that would trigger the use of the vaginal discharge algorithm used in syndromic management (The Department of Health 2015a).

2.2.7 Blood sample collection

Two vials of blood (9 ml each) were collected from each participant in a Serum Separating Tube (SST) and in an Acid Citrate Dextran Tube (ACD), respectively, by venipuncture. Serum from the SST tube was collected for endogenous hormone testing (methods in section 2.2.7.1, results in Chapter 4). Peripheral blood mononuclear cells (PBMCs) and plasma to measure cotinine (section 2.2.7.2) and HSV-2 IgG (section 2.2.7.3) was processed by Ficoll Density gradient centrifugation using Leucosep tubes as described previously (Jaumdally *et al*. 2017). PMBCs were stored for future assays.

2.2.7.1 Measurement of endogenous hormone concentrations in serum

Serum from the SST tube was tested for oestrogen (E2), progesterone and LH concentrations on the day of collection at the National Health Laboratory Service (NHLS) Chemical Pathology Laboratory at Groote Schuur Hospital, using a competitive electro-chemiluminescence immunoassay (Cobas®, Roche Diagnostics, Switzerland). The E2 assay range was 5.00-4300pg/mL with values below the limit of detection recorded as half the detection value (2.5pg/mL) in statistical analyses. The range for the progesterone assay was 0.03- 60.0ng/mL and values below the detection limit were taken as 0.015 ng/ml. For LH, the range was 0.100-

200mIU/mL and the lower undetected values were taken as 0.050 mIU/mL. Internationally, the 95% CI for these hormones are reported to be 43.8-211pg/mL for oestrogen, 1.7-27ng/mL for progesterone, and 1.0-11.4 mIU/mL for LH. Although no data was available from South African women as comparison, ranges for samples processed by the NHLS laboratory fell within the reported international ranges (Dr Philip Fortgens, personal communication, NHLS, Cape Town, SA).

2.2.7.2 Measurement of plasma cotinine concentrations

Cotinine, a nicotine metabolite used as marker for recent smoking, was measured as smoking has been associated with a higher risk of BV (Kenyon, Colebunders & Crucitti, 2013; Smart, Singal & Mindel, 2004). Cotinine ELISA kits (Sigma-Aldrich®, St. Louis, MO, US) were used to measure the level of cotinine in the blood plasma from Masiphumelele participants at visit one only. The assay was carried out according to the manufacturer's instructions. A solid phase competitive ELISA was used, where the cotinine from the samples compete with a horseradish peroxidase-cotinine conjugate. The intensity of the colour was inversely proportional to the cotinine concentration of the sample. Absorbance was read at 450nm and cotinine was reported as a binary variable (presence/absence).

2.2.7.3 Measurement of HSV-2 IgG

HSV-2 serology was performed using the HerpeSelect HSV-2 ELISA Kits (Focus Diagnostics, Cypress, CA, USA) on plasma samples from the Cape Town cohort only. Briefly, 100 µL of specimen or control were dispensed into the appropriate wells and incubated. The wells were then washed and 100 µL conjugate was added. The plate was then incubated, washed and 100 µL of substrate reagent was added. After an additional 10-minute incubation, stop reagent was added. Absorbance was measured with a spectrophotometer at a wavelength of 450 nm and the results recorded. An optical density value of > 1.10 was considered positive.

2.2.8 Genital sample collection

From each participant, at each study visit, genital mucosal samples were collected in the following order:

1. Dacron vulvovaginal swab to test for STIs (section 2.2.8.1).

2. FLOQSwab™ lateral vaginal wall swab for a wet mount slide to detect BV (Nugent Scoring), candidiasis (by gram stain) and vaginal pH (section 2.2.8.2).
3. Lateral vaginal wall swab to measure presence of prostate specific antigen (PSA) as a marker of semen contamination (section 2.2.8.3).
4. An endocervical swab to detect HPV (section 2.2.8.4).

Sample 1 was collected just prior to speculum insertion. Samples 2, 3 and 4 were collected during the speculum examination by a clinician.

2.2.8.1 Detection of STIs

At each visit, a vulvo-vaginal swab was collected to test for STIs. The participant undressed, was asked to lie supine and draw her legs up. The first swab collected was a vulvovaginal dacron swab (DryswabsTM, Medical Wire and Equipment, England) to test for *C. trachomatis, C. trachomatis* serovars L1-L3 (lymphogranuloma venereum serovars), *N. gonorrhoeae, T. vaginalis, M. genitalium, H. ducreyi, T. pallidum,* HSV-1*,* and HSV-2. All STI screening was performed at the STI Surveillance laboratory at the Centre for HIV and STIs (CHIVSTI), National Institute for Communicable Diseases (NICD), Sandringham, Johannesburg, SA. The swab sampled the vulva, the introitus and the lower vagina and was placed back into its container immediately after the procedure. The swabs were then transported at 4^{o}C to the lab and stored at -80 ^{o}C until they were transported weekly on dry ice to the CHIVSTI laboratory.

STI swabs were assayed for *N. gonorrhoeae, C. trachomatis, T. vaginalis* and *M. genitalium* using a discharge real-time multiplex polymerase chain reaction (M-PCR) assay using a Rotor-Gene 3000 platform (Corbett Robotics Pty Ltd., Sydney, Australia) as described previously (Mhlongo *et al*. 2010), using DNA extracted from vulvo-vaginal swabs using the Xtractor Gene (Corbett Robotics Pty Ltd., Brisbane, Australia). The primers and probes targeted the *N. gonorrhoeae* cytosine-specific DNA methyltransferase gene, the cryptic plasmid of *C. trachomatis*, the *T. vaginalis* repeated DNA fragment, and the *M. genitalium* pdhD gene, encoding for dihydrolipoamide dehydrogenase. Briefly, extracted DNA was incubated at 50°C (2 min) and Taq was activated at 95°C (10 min), following which 40 cycles of denaturation (95-°C, 20 sec) and annealing/extension (60°C, 60 sec) took place. Genomic DNA extracts prepared from the following ATCC® reference strains served as controls in each

experiment: *N. gonorrhoeae* (ATCC® 700825), *C. trachomatis* (ATCC® VR-885), *T. vaginalis* (ATCC® 30001), and *M. genitalium* (ATCC® 33530; Mhlongo *et al*. 2010). Specimens that tested positive for *C. trachomatis* with the ulcer M-PCR were further tested for *C. trachomatis* serovars L1-L3 (lymphogranuloma venereum serovars) by real-time, in-house simplex PCR, as described by Mhlongo *et al*. (2010). The primers and probe targeted the pmpH gene encoding the polymorphic membrane protein H of *C. trachomatis* L2b. After incubation for 2 min at 50°C, Taq was activated for 10 min at 95°C, followed by 45 cycles of denaturation for 15 seconds each, and finally annealing/extension at 95°C and one min at 60°C (Mhlongo *et al*. 2010).

Similarly, a real-time M-PCR assay on the same platform was used to detect HSV*, T. pallidum and H. ducreyi,* according to the method described by Mhlongo *et al*. (2010), using DNA extracted from the swabs using the Xtractor Gene (Corbett Robotics Pty Ltd., Brisbane, Australia). The primers and probes targeted the genes encoding the HSV glycoprotein D protein, the *T. pallidum* 47-kd lipoprotein, and the *H. ducreyi* HhdA haemolysin protein. After incubation at 50°C (2 min) and Taq activation at 95°C (10 min), 40 cycles of denaturation (95°C, 20 sec) and annealing/ extension (60°C, 60 sec) took place (Mhlongo *et al*. 2010). Specimens with a positive HSV result were typed into HSV-1/HSV-2 using the HSV-I/II typing Real-TM PCR assay (Sacace Biotechnologies, Como, Italy). Specimens with indeterminate results on the M-PCR assays were further tested and confirmed using commercially available real-time PCR assays specific for different STI organisms (Sacace Biotechnologies, Como, Italy).

Women determined to be positive for any of the treatable STIs (*N. gonorrhoeae, C. trachomatis, T. vaginalis*, *M. genitalium, T. pallidum,* Lymphogranuloma venereum *and H. ducreyi*) were recalled for treatment, either immediately or at their next follow-up visit. *C. trachomatis* infections were treated with a seven-day doxycycline course at the beginning of the study. Appoximately two months after the study began the treatment for *C. trachomatis* infections was changed to a single 1 g dose of azithromycin to improve adherence. *M. genitalium* was treated with a single 1 g dose of azithromycin, *N. gonorrhoeae* with a 250 mg intramuscular injection of ceftriaxone, and *T. vaginalis* with a 2 g single dose of oral

metronidazole. There were no cases of *T. pallidum,* Lymphogranuloma venereum *and H. ducreyi* detected. AGYW were given the choice of participant- or provider-initiated partner(s) notification or treatment. Women with vaginal discharge were treated immediately according to the national STI management guidelines.

2.2.8.2 BV Nugent scoring and inspection of fungal infections using wet mount slides

After the vulvovaginal swab was collected, a speculum was inserted, and additional samples obtained. A lateral wall/posterior fornix swab (Floqswabs, Copan Flock Technologies, Brescia, Italy) was used to collect genital fluids for BV Nugent scoring and fungal assessment (presence of fungal hyphae) by microscopy. The swab was also used to measure vaginal pH using an indicator strip (described below). The swab was first dipped into the posterior fornix and then rubbed against the lateral vaginal wall, midway down the vagina. It was firstly rolled across a glass slide, which was air-dried and transported to the lab at room temperature. The remaining fluid on the swab was used to obtain the vaginal pH by rubbing the swab on a colour-fixed indicator strip (pH range: 3.6-8.2; Macherey-Nagel, Düren, Germany), which was recorded at the time of sampling. On reaching the laboratory, the glass slide was heat-fixed and sent to the CHIVSTI, NICD for Nugent scoring and fungal screening.

At the NICD, the slides were Gram stained for Nugent scoring. The stained slide was read, and the number of morphotypes was evaluated based on a standardized scoring method, by two independent trained readers, who each evaluated 20-50 fields (Ms Venessa Maseko, personal communication). The diagnosis of BV is based on Nugent scoring, with the samples being assigned a score of 0 to 10. A BV Nugent score ≥7 was considered positive, 4-6 intermediate flora, and ≤3 was negative. The presence of candida hyphae, indicating the overgrowth of the yeast *Candida* was also evaluated on the Gram stain slides by microscopy.

Symptomatic fungal infections were treated with 1% Clotrimazole cream. Symptomatic BV (Nugent ≥7) was treated with 2 g single dose of oral metronidazole, while asymptomatic BV was not treated as per local protocols at the DTHF or PHRU.

2.2.8.3 Measuring concentrations of PSA

A swab (FLOQSwabTM, Copan Diagnostics, CA, USA) was rubbed on the opposing lateral wall of the genital tract, which was then placed in 1mL of phosphate-buffered saline (PBS) and transported to the laboratory at 4^{o}C. These samples were stored at -80^{o}C until used. The concentration of PSA was measured using the Quantikine Human Kallikrein 3/PSA Immunoassay kit (R&D Systems, Minneapolis, USA). The lateral wall swabs were allowed to slowly thaw in ice overnight. The swabs were vortexed for about 1 min in the transport medium and squeezed against the side of the cryovial wall to remove all the liquid from the swab. 50µL of each sample was then used for the assay. The assay was carried out according to the manufacturer's instructions. Having PSA levels ≥4ng/mL was used as a marker of unprotected sex within the last 48 hours (Evans *et al*. 2013).

2.2.8.4 HPV testing using the Roche Linear Array Genotyping assay

An endocervical swab (Longhorn Hyrda, Puritan Diagnostics, Maine, USA) for HPV typing was taken after the final lateral wall swab. The swab was placed into the cervical os and rotated 360^{o} twice, placed into 1.5mL of Primestore Molecular Transport Medium (Longhorn Vaccine and Diagnostics, San Antonio, USA) and transported to the laboratory at 4^{o}C. The samples were stored at -80^{o}C till testing. DNA for HPV typing was extracted from endo-cervical swabs (MagNa Pure Compact Nucleic Acid Isolation Kit Roche Diagnostics, Germany). The Roche Linear Array HPV Genotyping assay that detects 37 different HPV genotypes was used for HPV typing, and sample adequacy with β-globin. High-risk (hr) HPV types included HPV-16, -18, -26, -31, -33, -35*, -39, -45, -51, -52*, -53, -56, -58*, -59, -66, -68, -73 and -82, while low-risk (LR) HPV types included HPV-6, -11, -40, -42, -54, -55, -61, -62, -64, -67, -69, -70, -71, -72, -81, -83, -84, -89 (HPV-CP6108) and IS39 (Mbulawa *et al*. 2009). The α-9 HPV species included HPV-16, -31, -33, -35, -52, -58 and -67 (Mbulawa *et al*. 2014). *Co-infection with type 52 cannot be ruled out in cases of infection with types 35 or 58. This is due to the intellectual rights specific to detection of HPV52 prohibiting its direct identification using the Roche linear array test. HPV52 is thus measured indirectly using a mixed probe capable of detection of HPV33, 35, 52 and 58 (Marks *et al*. 2009). Dr Nai-Chung Hu and Dr Zizipho Mbulawa (NHLS HPV STI Surveillance Laboratory, UCT) performed these experiments.

2.2.9 Statistical analyses

STATA 12.0 was used for all statistical analyses and for multivariate model building (Stata Corp, College Station, USA). Graphs and figures were generated using GraphPad Prism 6 (GraphPad Software San Diego, USA). Non-parametric tests were used, unless otherwise indicated. The Mann-Whitney U test was used to compare two groups of continuous variables while a Fisher's exact test was used for categorical variables. The Kruskal-Wallis one-way analysis of variance was used for categorical variables with three or more groups. Confidence intervals of 95% and p values of ≤0.05 were used to assess statistical significance. Descriptive measures (such as median, standard deviation, interquartile range (IQR), frequencies, and percentages) were used to summarize data. Sensitivity and specificity analyses were carried out to compare the use of symptoms in the diagnosis of STIs to laboratory-based diagnosis. Sensitivity, specificity and the corresponding 95% CIs assessed the predictive accuracy of each clinical condition.

2.3 Results

2.3.1 Socio-behavioural characteristics and reported sexual risk behaviour

The majority of AGYW included in this study were high school students living in local communities in Masiphumelele or Soweto. Their median age was 18 (IQR 17-20) at enrollment; they reported a median age of sexual debut of 16 years (IQR 15-17) and a median of two sexual partners in their lifetimes (IQR 1-10; Table 2.1). They were predominantly black (99%) AGYW (100%) who self-identified as heterosexual (99%). Details of their socio-behavioural characteristics are described in detail in Table 2.1.

Sexual behaviour indicators (including HIV discordant relationships, intergenerational relationships and unprotected sex in the last three months) were generally more frequent in AGYW from Soweto than Masiphumelele. Almost three times as many adolescents from Soweto reported having a discordant, HIV-infected partner (33% versus [vs.] 9%, p<0·0001), and a higher frequency of unprotected sex in the last three months (52% vs. 31%, p=0·003), inter-generational relationships (defined as partner age difference ≥ 10 years; 14% vs. 4%, p=0·008) and using injected drugs or tattoos (16% vs. 6%, p=0·044) than those from Masiphumelele. Reported transactional sex rates were low in both communities (1% in Masiphumelele vs. 3% in Soweto, p=0·321), as was the frequency of vaginal practices (Table 2.1). Despite reporting a lower frequency of unprotected sex, young women from Masiphumelele reported lower rates of regular condom use than their Soweto counterparts (6% vs. 34% women reporting always using a condom, respectively; p<0·0001), or condom use at their last sex act (76% vs. 90%, p=0·005).

However, the objective markers of risk behavior (such as pregnancy rates and prevalence of PSA) were similar between the groups. Prior rates of pregnancies in both groups of young women were high: 23% in Masiphumelele and 27% in Soweto (p=0·648), and frequencies of women with detectable genital PSA (a marker of unprotected sex in the last 48 hours) was similar in both groups: 19% in Masiphumelele and 18% in Soweto (p>0·999). The majority of AGYW (74%; 166/224) that reported condom use at last sex act were PSA negative (suggesting that no semen was detected). Of those with discordant results (being PSA positive but

reported using condoms at last sex act), 13% (13/102) were from Soweto and 14% (17/122) were from Masiphumelele.

The use of vaginal products and vaginal insertion practices (VIPs) was generally low, although it tended to be higher in Masiphumelele than Soweto, with douching being significantly more frequent in Masiphumelele than in Soweto (13% vs. 3%, p= 0·001). The use of vaginal drying agents was also reported more frequently in Masiphumelele (8%) compared to Soweto (2%, p=0·070), while 4% of the AGYW from Masiphumelele had used traditional vaginal medicines compared to the 1% in Soweto (p=0·268). Unfortunately, no further information about the vaginal drying agents or traditional vaginal medicines was obtained in the questionnaire.

Since tobacco smoking has been identified as a co-factor for HPV-associated cervical disease progression and development of BV, cotinine levels were measured in plasma as a surrogate marker for cigarette smoking. Cotinine was only measured in Masiphumelele (as plasma was not collected in Soweto): only 19% of blood plasma samples tested (26/149) were positive, which could be evidence of recent smoking.

Overall, contraceptive use was higher in Masiphumelele (100%; 149/149) than Soweto (90%, 133/147; p=0·001), probably reflecting the recruitment strategy used in Masiphumelele that enrolled from a family planning clinic associated with the DTHF Youth Centre. The Soweto cohort, in contrast, was recruited from a more general population via several recruitment drives in the community. Male condoms were the most common contraception method used in Soweto (52%, 77/147), while the injectable progestin-only contraceptive Net-En was the most common contraceptive method in Masiphumelele (70%, 104/149). Injectable progestin-only contraceptive use was higher in Masiphumelele than Soweto for both DMPA (19% vs. 10%, respectively; p=0·001) and Net-En (70% vs. 16%, respectively; p<0·001).

Table 2.1. Reported sexual risk behaviour in adolescent and young South African women from two urban socio-economically deprived communities

Characteristic	Total n = 298[#]	Masiphumelele n = 149[#]	Soweto n = 149[#]	p-Value
Demographics				
Heterosexual [% (n/N)]	97 (227/225)	96 (122/127)	99 (97/98)	0.140
Age in years [median (IQR)]	18 (17-20)	18 (17-20)	18 (17-20)	0·060
Black African [% (n/N)]	99 (224/225)	100 (127/127)	99 (97/98)	0·452
Age of sexual debut (years) [median (IQR)]	16 (15-17)	16 (15-17)	16 (16-17)	0·153
Lifetime number of sex partners [median (IQR)]	2 (1-10)	2 (1-8)	2 (1-10)	0·072
Cotinine [% (n/N)]	n/a	19 (29/149)	n/a	n/a
Pregnancies [% (n/N)]				
Ever pregnant	26 (58/225)	24 (30/127)	29 (28/98)	0·648
Planned pregnancies	**7 (4/58)**	**0 (0/30)**	**14 (4/28)**	**0·048**
Miscarriages	5 (3/58)	0 (0/30)	11 (3/28)	0·106
Pregnancy termination	3 (2/58)	0 (0/30)	7 (2/28)	0·228
Multiple pregnancies	10 (6/58)	7 (2/30)	14 (4/28)	>0·999
Condom use [% (n/N)]				
Reported condom used always	**25 (56/225)**	**6 (19/127)**	**34 (37/98)**	**0·000**
Condom use during last sex act	**84 (188/225)**	**76 (96/127)**	**92 (92/98)**	**0·005**
Semen present (PSA+)	19 (55/291)	19 (28/148)	18 (27/143)	>0·999
Concordance between PSA and condom use	74 (166/224)	82 (100/122)	65 (66/102)	>0·999
Sexual risk factors [% (n/N)]				
Sex with HIV+ partner	**20 (44/225)**	**9 (12/127)**	**33 (32/98)**	**<0·001**
Multiple partners in the past 3 months	14 (32/225)	11 (15/127)	17 (17/98)	0·256
Intergenerational relationship (>10yr gap)	**8 (19/225)**	**4 (5/127)**	**14 (14/98)**	**0·008**
Transactional sex (ever)	2 (4/225)	1 (1/127)	3 (3/98)	0.321
Injected drugs/tattoos	**10 (23/225)**	**6 (8/127)**	**16 (15/98)**	**0·044**
Unprotected vaginal sex last 3 months	**40 (91/225)**	**31 (40/127)**	**52 (51/98)**	**0.003**
Unknown partner HIV status	58 (131/225)	64 (81/127)	51 (50/98)	0.056
Previous treated STI last 6 months	12 (26/225)	9 (12/127)	14 (14/98)	0·298
Anal sex (ever)	6 (13/225)	7 (9/127)	4 (4/98)	0·398
Vaginal practices [% (n/N)]				
Vaginal drying agent used (ever)	5 (12/225)	8 (10/127)	2 (2/98)	0·070
Ever douched	**8 (19/225)**	**13 (16/127)**	**3 (3/98)**	**0·001**
Traditional vaginal medicine	3 (7/225)	3 (5/127)	2 (2/98)	0·268
Contraception [% (n/N)]				
Any contraception	**95 (282/296)**	**100 (149/149)**	**90 (133/147)**	**0.001**
DMPA	**14 (42/296)**	**19 (28/149)**	**10 (14/147)**	**0.001**
Net-EN	**43 (128/296)**	**70 (104/149)**	**16 (24/147)**	**<0.001**
NuvaRing	**0 (1/296)**	**1 (1/149)**	**0 (0/147)**	**0·043**
COP*	5 16/296)	5 (7/149)	6 (9/147)	<0.618

Etonogestrel implant (Implanon)	3 (10/296)	6 (9/149)	1 (1/147)	>0·999
Injectable (not classified)	2 (7/296)	0 (0/149)	5 (7/147)	>0·999
Male condom only	**26 (77/296)**	**0 (0/149)**	**52 (77/147)**	**0·020**
Female condom only	**0 (2/296)**	**0 (0/149)**	**1 (2/147)**	**0.0145**
No contraception	**5 (14/296)**	**0 (0/149)**	**10 (14/147)**	**<0·0001**

[#]Complete questionnaire, STI and BV data was not available for all participants, thus participant numbers (n) may vary; *OralCon and Nordette. Text in bold indicates significant values. n/a = not available

2.3.2 Prevalence of STI, BV and candidiasis at enrolment

2.3.2.1 High burden of C. trachomatis in young women in Masiphumelele

Overall, *C. trachomatis* DNA was detected in genital samples from 30% (88/297) of the young women enrolled in this study. However, the prevalence of *Chlamydia* in Masiphumelele was almost three-times higher than in Soweto [42% (62/149) vs. 18% (26/148), respectively; p<0·0001; Table 2.2]. None of the chlamydia cases involved serovars L1-L3, which are the lymphogranuloma venereum serovars.

2.3.2.2 Prevalence of other vaginal discharge-associated STIs

The prevalence of *N. gonorrhoeae* was 8% (24/297) overall and tended to be higher in Soweto than Masiphumelele [5% (7/148) vs. 11% (17/149) p=0·0534, Table 2.2]. The prevalence of *T. vaginalis* [5% (16/297)] and *M. genitalium* [4% (11/297)] were similar in both cohorts (Table 2.2).

2.3.2.3 Prevalence of genital ulcer-associated pathogens

The prevalence of HSV-2 DNA was low in both cohorts at the enrollment visit (indicative of either an active or re-activated infection), being detected in seven of AGYW in Masiphumelele (5%) and one (1%) woman from Soweto (p=0·6660, Table 2.2). Furthermore, only one participant (from Masiphumelele) had HSV-2 genital ulcers that were visible during the clinical examination. In Masiphumelele, where blood was collected, 21% (31/149) of adolescents were HSV-2 seropositive at enrollment. No HSV-1 DNA positive participants were detected at either site, and no cases of *T. pallidum* or *H. ducreyi* were detected.

2.3.2.4 Prevalence of HPV infections

HPV was detected in 68% of women in Masiphumelele (100/147) and 65% of those in Soweto (63/97; p= 0.6774; Table 2.2), of which >50% were infected with high-risk HPV types (including HPV-16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 81, 82), which did not differ by site (p=0·900). Co-infection with HPV-52 could not be ruled out in cases of infection with types 35 or 58. The majority (74%) of AGYW infected with HPV had multiple infections. In this cohort, the most frequently detected HPV types detected overall were HPV-16 (19%, 31/163), with HPV-58 the most common in Masiphumelele (18%, 18/100) and HPV-16 in Soweto (24%, 15/93). Despite the high prevalence of HPV in both cohorts, only two adolescents in Masiphumelele were diagnosed with cervical squamous intraepithelial neoplasia 1 (CIN1) by Pap smear.

2.3.2.5 High burden of BV in South African AGYM

Overall, AGYW had a median vaginal pH of 5·0 [IQR 4·4-5·3], with 74% having a vaginal pH >4.5, considered to indicate BV according to Amsel criteria (Amsel *et al*. 1983). Interestingly, vaginal pH was higher in Soweto [5.0 (IQR: 4.7-5.6) than in Masiphumelele [4.7 (IQR: 4.4-5.3); p=0·002]. More than 40% (135/290) of the young women in the two communities had BV (defined as having a Nugent score ≥7), with similar prevalence in Soweto and Masiphumelele. In addition, 14 % had Nugent scores between 4-6, indicative of intermediate microbiota [17% (24/143) in Soweto vs. 12% (17/147) in Masiphumelele; p=0·396; Table 2.2].

2.3.2.6 Prevalence of fungal infections

Fungal infections, evident as hyphae or spores on the gram stain used for BV scoring, tended to be higher in Soweto than Masiphumelele [14% (20/143) vs. 7% (10/148); p=0·053].

Table 2.2. STI, BV and candidiasis prevalence in AGYW from Soweto and Masiphumelele at enrolment

Characteristic	Total n=290[#]	Soweto n=143[#]	Masiphumelele n=149[#]	p-value
Bacterial vaginosis				
Nugent score 7-10 [% (n/N)]	47 (135/290)	45 (64/143)	48 (71/147)	0·483
Intermediate Nugent 4-6[% (n/N)]	14 (41/290)	16 (24/143)	12 (17/147)	0·396
Vaginal pH [median (IQR)]	5·0 (4·4-5·3)	5·0 (4·7-5·6)	4·7 (4·4-5·3)	**0·002**
Fungal hyphae and spores [% (n/N)]	10 (30/291)	14 (20/143)	7 (10/148)	0·053
Viral infections [% (n/N)]				
HPV [% (n/N)]	67 (163/244)	65 (63/97)	68 (100/147)	0·677
HPV hr types*	54 (135/252)	53 (55/103)	54 (80/149)	0.898
HPV LR types**	42 (105/252)	36 (37/103)	46 (68/149)	0·341
Multiple HPV infections	74 (121/163)	71 (45/63)	76 (76/100)	0·582
HPV-16	19 (31/163)	24 (15/63)	16 (16/100)	0·226
HPV-18	9 (15/163)	8 (5/63)	10 (10/100)	0·784
HPV-6	10 (16/163)	10 (6/63)	10 (10/100)	>0·999
HPV-11	4 (7/163)	2 (1/63)	6 (6/100)	0·250
HSV-2 DNA	3 (8/297)	1 (1/148)	5 (7/149)	0·067
Bacterial and Protozoal infections [% (n/N)]				
C. trachomatis	30 (88/297)	18 (26/148)	42 (62/149)	**<0·001**
N. gonorrhoeae	8 (24/297)	5 (7/148)	11 (17/149)	0·053
T. vaginalis	5 (16/297)	3 (5/148)	7 (11/149)	0·198
M. genitalium	4 (11/297)	3 (5/148)	4 (6/149)	>0·999
T. pallidum	0 (0/297)	0 (0/148)	0 (0/149)	>0·999
C. trachomatis L1-L3 serovars	0 (0/297)	0 (0/148)	0 (0/149)	>0·999
H. ducreyi	0 (0/297)	0 (0/148)	0 (0/149)	>0·999

[#]Complete STI and BV data was not available for all participants, thus participant numbers (n) may vary
HPV hr types = 16, 18, 26, 31, 33, 35, 39, 45, 51, 52*, 53, 56, 58*, 59, 66, 68, 73, 81, 82
**HPV LR types =6,11,40,42,54,55,61,62,64,67,69,70,71,72,81,82,83,84, CP6108, IS39

2.3.2.7 Influence of age on STI and BV prevalence at enrolment

Age did not influence the prevalence of *N. gonorrhoea, T. vaginalis, M. genitalium,* HSV-2 or HPV, although younger AGYW (<20 years) tended to have higher rates of *C. trachomatis* than their older counterparts (≥20 years; 36% vs. 23%, p=0·079) and were more likely to have multiple STIs detected (41% vs. 27%, p=0·049; Table 2.3). The prevalence of co-infection of STIs with BV was high, and >25% in all age categories, with no difference seen between age groups.

Table 2.3. STI and BV prevalence over various age categories

Prevalence [% (n/N)]	Age groups		p-value
	<20 years	≥20 years	
C. trachomatis	35 (69/200)	23 (17/75)	0·079
N. gonorrhoea	7 (15/200)	12 (9/75)	0·239
T. vaginalis	8 (11/200)	4 (3/75)	0·765
M. genitalium	5 (8/200)	3 (2/75)	0·733
HSV-2	3 (5/200)	4 (3/75)	0·454
HPV	73 (135/200)	63 (46/73)	0·563
Multiple STIs	17 (27/199)	14 (10/74)	>0·999
Any STI with BV	41 (80/199)	27 (20/74)	0·049

2.3.2.8 Treatment of partners

The sexual partners of all AGYW who tested positive for STIs were offered treatment, either as a referral letter for the local clinic to give to their partners or by being given appropriate medication to deliver to their partners with detailed instructions. Despite partner notification being offered, only two partners came in for treatment, and ten participants took medication for their partners.

2.3.3 STI and BV recurrence or reinfection in AGYM

In AGYW from Masiphumelele who were followed longitudinally, the most prevalent bacterial STI at all three visits was *C. trachomatis*. Although the prevalence dropped from 42% at visit one to 18% at visit three, the majority of the 16 AGYW infected with *C. trachomatis* at visit three had been infected at all three visits (63%; 10/16). The prevalence of *N. gonorrhoeae* also declined from visit one (11%) to visit three (8%). In contrast, the prevalence of *T. vaginalis* remained constant from visits one (7%) to visit three (7%), although it was not present in the same participants over time. The prevalence of *M. genitalium* increased slightly between enrollment (4%) and the last visit (7%), with three participants having *M. genitalium* infection at all three visits (Table 2.4).

The rates of BV remained high throughout the study, with a prevalence of 48% at visit one, 46% at visit two, and 48% at visit three. Of the 71 AGYW with BV at enrollment, 69% (49/71) also had BV at their second visit and 37% (26/71) had BV at all three visits, suggesting either high recurrence rates or poor response to treatment. Similarly, the prevalence of HPV infection remained high throughout the study, with 68% of AGYW being positive at visit one, 65% at visit two, and 72% at visit three (Table 2.4).

Table 2.4. Longitudinal STI and BV prevalence in Masiphumelele, Cape Town

Characteristics [% (n/N)]	Visit 1 n=149	Visit 2 n=127	Visit 3 n=88	P-value
Bacterial vaginosis				
Nugent score 7-10	48 (71/147)	48 (61/126)	46 (39/84)	0·953
Intermediate Nugent score 4-6	12 (17/147)	9 (11/126)	7 (6/84)	0·428
Fungal hyphae and spores	7 (10/148)	11 (14/126)	13 (11/84)	0·207
Viral infections				
HPV	68 (100/147)	60 (75/126)	72 (64/88)	0·157
Multiple HPV	76 (76/100)	68 (51/75)	66 (42/64)	0·800
HPV-16	16 (16/100)	15 (11/75)	17 (11/64)	0·733
HPV-18	10 (10/100)	8 (6/75)	3 (2/64)	0·667
HSV-2 DNA	5 (7/149)	27 (34/127)	27 (24/88)	>0·999
HSV-2 IgG	21 (31/149)	27 (34/127)	27 (24/88)	0.485
Bacterial and Protozoal infections				
C. trachomatis	42 (62/149)	34 (43/127)	18 (16/88)	**0·001**
N. gonorrhoeae	11 (17/149)	6 (8/127)	8 (7/88)	0·340
T. vaginalis	7 (11/149)	8 (10/127)	7 (6/88)	0·601
M. genitalium	4 (6/149)	6 (7/127)	7 (6/88)	>0·999

2.3.4 Clinical symptoms are poor indicators for STIs or BV in adolescents

Symptoms that were captured in the study questionnaire (Appendix II) fall under the vaginal discharge algorithm of the National Syndromic Management guidelines (South African Department of Health 2015), including abnormal vaginal discharge, vaginal itching or burning, dysuria (painful/difficulty with urination), and any combination of these. Despite the high rates of laboratory-diagnosed STIs or BV detected in these adolescents from Masiphumulele (Table 2.4), only 15% were considered to be symptomatic. At each study visit, participants were asked about both abnormal and normal discharge. The most frequently reported symptom was what the AGYW considered normal vaginal discharge (in the choice between no discharge, normal discharge and a discharge that causes worry, Appendix II): 41% (16/39) at visit one, 53% (31/58) at visit two and 59% (34/58) at visit three (Table 2.5). In a typical reproductive health interview in the public health care sector available in SA, this symptom would not be considered abnormal by the clinician and would therefore not allow the participant to access care according to the National Syndromic Management guidelines.

Among the symptoms that would elicit care and access to the syndromic management guideline, vaginal discharge that the participants considered abnormal was the most common, but with only 8% (3/39) reporting discharge at visit one, 9% (5/58) at visit two and 2% (1/58) at visit three. Other symptoms linked to the syndromic management algorithm,

such as vaginal itching or burning, and dysuria, were less common (Table 2.5). When any combination of the symptoms that would initiate use of the syndromic management algorithm (including any combination of abnormal discharge, vaginal irritation, and/or dysuria) were considered, the frequency of symptoms that would elicit access to care improved but was still poor [maximum 22% (13/58)].

Table 2.5. Longitudinal symptom prevalence in Masiphumelele, Cape Town

Symptoms [% (n/N)]	Visit 1 n=39	Visit 2 n=58	Visit 3 n=58	P-value
Abnormal vaginal discharge	8 (3/39)	9 (5/58)	2 (1/58)	0·254
Dysuria	5 (2/39)	5 (3/58)	5 (3/58)	>0·999
Vaginal irritation/burning	5 (2/39)	9 (5/58)	2 (1/58)	0·278
Any symptom	13 (5/39)	22 (13/58)	9 (5/58)	0·640
Normal vaginal discharge	41 (16/39)	53 (31/58)	59 (34/58)	0·333

Sensitivity and specificity of each symptom was compared to the laboratory diagnosis of the discharge-causing STIs (including *C. trachomatis N. gonorrhoea, M. genitalium* and *T. vaginalis*), BV, or any combination of BV/STIs. In this study, the positive predictive value (PPV) was the probability that a AGYW with the symptoms truly have an STI or BV, while the negative predictive value (NPV) was the probability that AGYW with no symptoms truly didn't have the STI or BV (Umberger, Hatfield and Speck 2017). Overall, symptoms alone were insensitive in diagnosing any of these conditions.

Dysuria was the most sensitive for correctly diagnosing a laboratory-diagnosed STI (Figure 2.1): It had a 50.0% sensitivity for diagnosing any STI alone [no BV]; with 63.7% specificity, 7.0% PPV, and 95.9% NPV. Dysuria had a 37.5% sensitivity for diagnosing a STI together with BV; with 80.8% specificity, 9.7% PPV, and 95.3% NPV. It had 50.0% sensitivity for diagnosing BV only, with 49.3% specificity, 5.1% PPV, and 94.7% NPV.

Abnormal vaginal discharge was the least sensitive as a symptom to predict a laboratory-diagnosed STI only (Figure 2.1): Discharge the adolescents identified as abnormal had 22.2% sensitivity for diagnosing any STI alone, with a specificity of 62.3%, PPV 3.5%, and NPV 98.9%. It had 22.1% sensitivity for diagnosing a STI together with BV, with a specificity of 80.1%, a

PPV of 6.5%, and a NPV of 94.4%. Abnormal discharge ischarge had 55.6% sensitivity for diagnosing BV only, with a specificity of 50.0%, a PPV of 6.4%, and a NPV of 94.8%.

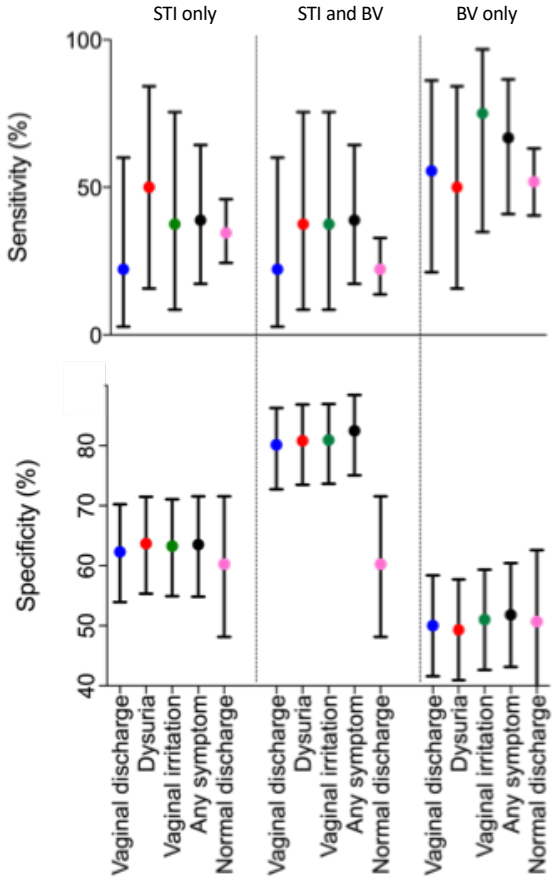


Figure 2.1. Sensitivity and specificity of clinical symptoms used in diagnosis of syndromic management (including "abnormal" vaginal discharge, genital itching or burning, and dysuria) for correctly diagnosing a laboratory-diagnosed STI and/or BV. Each colour represents a specific symptom/collection of symptoms and the bars represent the standard error bars for each sensitivity or specificity value. Sensitivity in this analysis shows the ability of symptoms to correctly identify those with an STI while specificity shows the ability of symptoms to correctly identify those without an STI. The error bars denote are graphical representations of the variability of data.

2.3.4.1 Poor receiver operating characteristic (ROC) curve of clinical symptoms for correctly predicting an STI or BV in AGYW

ROC curve accuracy was determined via a plot of sensitivity versus (1-specifity; Hajian-Tilaki 2013). It is possible to assess the overall diagnostic accuracy by calculating the area under the curve (AUC). A test with perfect discriminatory ability will have an AUC of 1.0, while a model unable to distinguish between individuals with or without the chosen outcome will have an AUC of 0.50 (Linden 2006). For this study, the area under the receiver operating characteristic

curve (AUROC) was poor for all the symptoms used for clinical diagnosis of an STI and/or BV, or any combination of symptoms (Figure 2.2): “Any symptom being present” (Appendix II) was the most accurate for predicting an actual STI and/or BV, with an AUROC of 51.2% in those who were both STI+/BV-, 60.7% in those who were STI+/BV+, and 59.3% in those who were BV+/STI-. In contrast, “abnormal vaginal discharge” performed the worst, with an AUROC 42.3% in those who where STI+/BV-, 51.2% in those who were STI+/BV+, and 52.8% in those who were BV+/STI- (Figure 2.2).

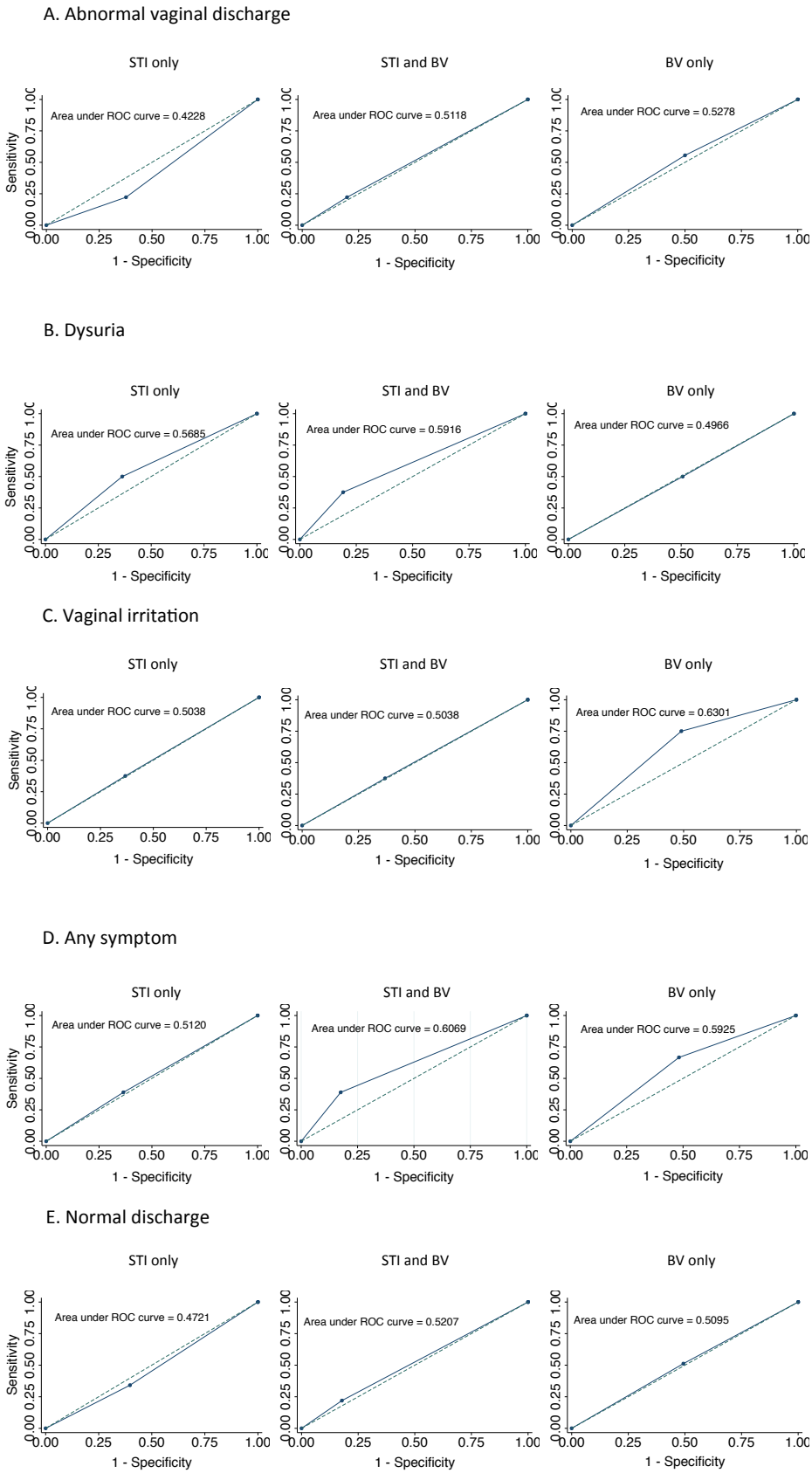


Figure 2.2 AUROC of clinical symptoms for diagnosing a STI or BV in AGYW. A. The symptoms (A)"abnormal vaginal discharge", (B) dysuria, (C) vaginal irritation, (D) any symptom and (E) normal discharge were evaluated for correctly identifying AGYW with a STI only, both an STI and BV or BV only. ROC curve accuracy was determined via a plot of sensitivity versus (1-specifity; Hajian-Tilaki 2013).

2.3.4.2 Concurrent genital conditions (multiple STIs or STIs with BV) did not increase evident symptom severity in AGYW

Many AGYW in the WISH cohort had multiple concurrent genital conditions, with 49% (50/103) of those with one STI also having another concurrent STI. Further, 39% (41/103) of the adolescents with a STI had concurrent BV. Of the adolescents infected with *C. trachomatis,* 36% (22/61) had another concurrent STI and 67% (41/61) had a concurrent STI or BV. Even in these individuals with multiple vaginal conditions, symptoms were still found to be a poor indication of whether a treatable condition was present. In the case of combinations of genital symptoms, a *T. vaginalis* infection with an intermediate Nugent score of 4-6 was the most symptomatic condition: 80% (4/5) of the women with concurrent *T. vaginalis* infection and intermediate Nugent score were symptomatic, half of these (2/4) had with vaginal irritation and half (2/4) had abnormal vaginal discharge. The next most symptomatic condition was yeast infection alone (evident on the Gram stain slide), although the numbers were very low (n=3). Of these three adolescents, two had symptoms present: one had dysuria and one had abnormal discharge. *C. trachomatis* infection either alone, or concurrent infection with HPV or another STI, had no symptoms. Of the eight AGYW with dysuria, 25% (2/8) had STI or BV and 25% (2/8) had concurrent infection with yeast and BV or intermediate Nugent. Of the nine AGYW with an abnormal discharge, 22% (2/9) had BV, 22% (2/9) had BV and concurrent *C. trachomatis* infection, and 22% (2/9) had *T. vaginalis* infection with an intermediate Nugent score. Half (4/8) of the cohort who complained of vaginal irritation had BV (Figure 2.3).

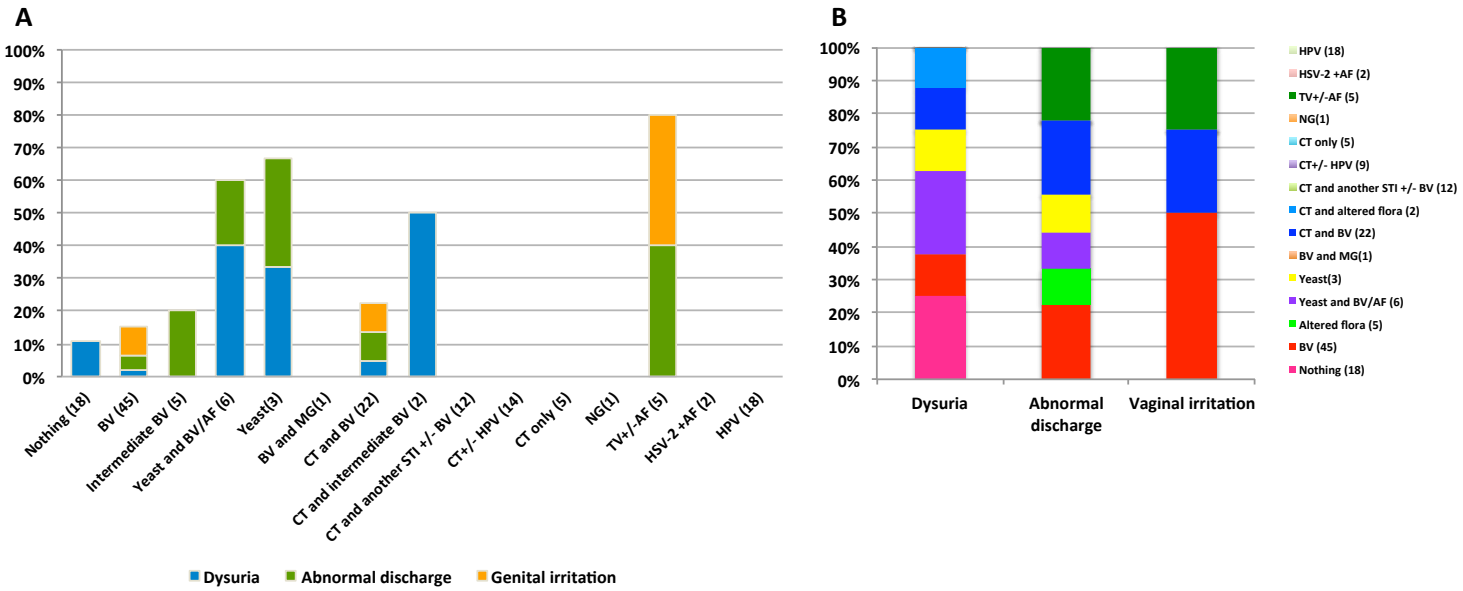


Figure 2.3. Clinical symptoms associated with each laboratory-diagnosed STI or BV. (A) Each stacked bar represents the presence of specific STIs (including *C. trachomatis, N. gonorrhoeae, T. vaginalis, M. genitalium*, and HSV-2), BV, yeast (by Gram stain) or a combination of the different conditions. Dysuria being reported as a symptom is shown by blue bars, abnormal discharge is shown by green bars and genital irritation by orange bars. (B) Each stacked bar represents a specific symptom – dysuria, abnormal discharge and genital irritation, with each of the stacked bars composed of the various conditions that were present when those symptoms were reported, including HPV only (light green), HSV-2 and altered flora (peach), *T. vaginalis* and altered flora (dark green), *N. gonorrhoae* (light orange), *C. trachomatis* (light blue), *C. trachomatis* and HPV (light purple), *C. trachomatis* and another STI (green), *C. trachomatis* and altered flora (light blue), *C. trachomatis* and BV (dark blue), *M. genitalium* and BV (orange), yeast (yellow), yeast and BV or altered flora (purple), altered flora (neon green), BV (red), or no STIs or BV (pink).

2.4 Discussion

Two low-income communities of AGYW at high risk for HIV infection, from major cities situated ~1800km apart in SA, were enrolled in this study to better understand the granularity of HIV risk in young and adolescent females from SSA. What this study revealed was that there was an extremely high prevalence of laboratory-confirmed STIs, in particular *C. trachomatis* and HPV, as well as BV in these AGYW, which would place them at higher risk for HIV infection and reproductive complications. Clinical symptoms, which are part of the vaginal discharge algorithm used to manage STIs and BV in South Africa, were shown to be a poor tool in the diagnosis of vaginal discharge causing STIs or BV, with a maximum sensitivity of 50% in this age group, showing that >80% of treatable infections in adolescent females would be missed using syndromic management. The majority of AGYW with a STI or BV were asymptomatic (85%), including those with *C. trachomatis* or *N. gonorrhoeae* infections that would typically cause discharge (though more so in males). This alone suggests that reliance on symptoms alone is not effective in managing STIs in young women in this setting. This results in a highly prevalent, silent epidemic of undiagnosed genital tract infections that increase risk of HIV and/or reproductive complications (WHO 2013; Johnson *et al*. 2012; Haggerty *et al*. 2010). In contrast, the presence of clinical symptoms did not necessarily mean that the young women were infected with a STI/BV, with PPVs as low as 3.5%. The majority of these young women, however, was asymptomatic and thus, would not access the vaginal discharge syndromic treatment algorithm practiced in SA.

It is recognized that there are significant barriers that prevent AGYW for accessing health care, especially for reproductive health, and these include strong traditional norms and beliefs found within communities (Regmi *et al*. 2010), poor knowledge among adolescents of the type of services available (Bernard *et al*. 2004), poor life science/biology literacy among youth in SA, concerns around privacy and confidentially of information shared with healthcare workers (Coker *et al*. 2010), limited accessibility of services (Booth *et al*. 2004), barriers to making appointments, and long waiting times at local clinics (Lim *et al*. 2012). Intervention models that have shown success among adolescents include those that target behaviours that are amenable to change in SA, such as condom use (Beksinska, Smit & Mantell 2012), and

interventions that have been specifically tailored towards adolescence populations (Jemmott *et al*. 2010), including peer education programs (Mash & Mash 2012).

It was interesting that sexual risk behaviour differed between sites, with the adolescents from Soweto reporting generally riskier sexual behavior than those in Masiphumelele, including higher frequencies of HIV-infected partners, intergenerational relationships (>10 years age difference), unprotected sex in the last three months and higher frequencies of injected drug use or tattoos. Possible reasons behind this are not clear. The Masiphumelele cohort was drawn from a family planning clinic setting offered by the DTHF Youth Centre, while the Soweto cohort was drawn from more generalised recruitment drives. Soweto is also a larger, more urban, heterogeneous population (including 39% Zulu, 32% Xhosa, 8% Sotho and 7% Tswana speaking South Africans), while the Masiphumelele population is smaller, more traditional and a lot more homogenous (98% Xhosa speaking). It is possible that common behavioural norms in the Masiphumelele community influenced the adolescents in that population, while Soweto is too big and heterogeneous for such influencing of the population to occur. It is also possible that recruitment through a youth centre (like the DTHF Youth Centre) would enrich for youth that would choose “safer” options.

Overall the most reported risky sexual behaviour in this cohort was not knowing the HIV status of their sexual partners (reported by 58% overall: 64% from Masiphumelele and 51% from Soweto). This was similar to the rates found in other South African adolescent populations (Davoren *et al*. 2014; Exavery *et al*. 2011). In contrast, the rates of transactional sex and intergenerational relationships (1% and 8%, respectively) were relatively low overall. This is in contrast to higher rates seen in other African populations (Wamoyi *et al*. 2016; Magni *et al*. 2015).

The use of vaginal products (douching, traditional medicines and vaginal drying agents) was also infrequently reported (<15% overall). This is similar to what has been reported in another Western Cape study (Reddy *et al*. 2009) but lower than what has been seen in Kwa-Zulu Natal (Hull *et al*. 2011). However, studies that focus on very high risk groups, such as South African sex-workers, have reported a much higher prevalence of vaginal product use (94-97%), likely associated with the sex trade (Morar, Ramjee and Al 2003). Some of the most commonly

reported substances that are used to douche include antiseptic solutions, detergents and medication such as ibuprofen (Gafos *et al*. 2010; Reddy *et al*. 2009), with the main reasons for use of these products being hygiene, health and sexual enhancers (Hilber *et al*. 2010). In the case of sex-workers, the main reasons given were to increase men's sexual pleasure and thus improve income (Morar, Ramjee and Al 2003).

Nineteen percent (29/149) of AGYW from Masiphumelele had positive plasma cotinine results, indicating that tobacco use in this community was not common. Although the detection of cotinine in plasma is used as a biomarker of cigarette smoking in the health care industry, some studies have also described the use of snuff (crushed tobacco) as a prevalent intravaginal insertion practice in some areas of SA (Gafos *et al*. 2010), with the most common reason being reported to achieve 'hot' sex, to increase sexual desirability and for the own sexual pleasure of the women (Gafos *et al*. 2010). Snuff has also been used in Tanzania to decrease lower abdominal cramps (Lees *et al*. 2014). Since the adolescents were not explicitly questioned about vaginal insertion of snuff, it was not clear whether the cotinine detected in plasma was a result of smoking or vaginal insertion. Only three of the 29 adolescents from Masiphumelele reported previous use of any vaginal products.

The very high prevalence of *C. trachomatis* infection in these adolescent populations was striking compared to reported *C. trachomatis* prevalence rates in other parts of Africa and worldwide: 2.6% in African adult women (Rowley, Toskin & Ndowa 2012), 4.7% in the US, (Torrone, Papp & Weinstock 2014) and 18% in sentinel sites looking at women with vaginal discharge (Kularatne *et al*. 2017). The reported cases of chlamydia in the US are highest among AGYW aged 15–24 years, and have increased by 4.1% from 2014–2016 (Centers for Disease Control and Prevention 2017). Recent South African data further confirmed that older adult women tended to have lower prevalence of chlamydia than their adolescent and young counterparts (Kularatne *et al*. 2017), which is thought to possibly reflect spontaneous resolution associated with acquired immunity, thereby decreasing risk for reinfection (Geisler *et al*. 2013; Batteiger *et al*. 2010). These high prevalence rates seen in the Masiphumelele cohort may be due to a combination of factors including: the young age of the cohort, a high proportion of asymptomatic infections, re-infection from untreated partners, inconsistent condom use, and participation in dense sexual networks harbouring *C. trachomatis*. This high

burden of disease could possibly support the case for community-based interventions in addition to more traditional, individually focused interventions (Bunnell *et al*. 1999).

The prevalence rates of chlamydia, gonorrhea, and syphilis in AGYW in the 15–19 year and 20–24 year age groups in the US during 2012–2016 has increased (Center for Disease Control 2016). There have been concerns that the use of PrEP may lead to sexual disinhibition and decreased use of barrier contraception (Rudy *et al*. 2010; Bekker, Gill and Wallace 2015). Despite this increasing trend in the US and elsewhere, there has been no data as yet to support this hypothesis.

The difference in *C. trachomatis* prevalence seen in Masiphumelele (42%) compared to Soweto (18%) was marked, despite both cohorts being enrolled from similar socio-economic communities. There were some important differences that could possibly account for this. The Masiphumelele cohort was drawn from a smaller and more isolated community, possibly allowing high incidence sexual networks to evolve, while Soweto is bigger and more integrated into the urban environment. In addition, the Masiphumelele cohort was recruited from a family planning clinic while the Soweto population was recruited from a more general population on recruitment drives.

While the longitudinal *C. trachomatis* prevalence in Masiphumelele did significantly decline over time, the other easily treatable STIs (*N. gonorrhoeae*, *T. vaginalis* and *M. genitalium*) did not drop significantly despite the availability of treatment. This suggests either unresolved infections or re-infection (despite efforts to treat partners). In this study, only two sexual partners opted to come to the clinic for treatment, ten adolescents took treatment for their partners, while the majority took STI referral notifications. It is difficult to determine the adequacy of partner treatment and to what extent this may have contributed to the persistence of these easily treatable infections. EPT has previously been well accepted (in 70%) among adolescents (Ricks *et al*. 2015) with risk behaviours (such as ≥ 4 lifetime sex partners or infrequent partner discussion about STI prevention) being associated with increased acceptance of expedited treatment (Ricks *et al*. 2015).

In Masiphumelele, where adolescents were followed for a total of three study visits, the longitudinal prevalence of BV remained high and did not significantly decline over the course of the study. A possible reason for this is that asymptomatic cases of BV were not treated in this study despite Nugent scores of 7-10, as per local management guidelines. Another possible reason for this is that a single 2g dose of metronidazole was used as treatment. While this is still recommended under the South African syndromic guidelines, this regimen results in higher failure rates than a one week's course of treatment (Bradshaw and Sobel 2016). Furthermore, recent studies have found that vaginal *G. vaginalis*, commonly associated with BV, frequently is resistant to metronidazole (Schuyler *et al*. 2016).

Rates of HPV were high in this young, sexually active population. This is in keeping with worldwide trends that shows a high prevalence in young populations who are close to the age of sexual debut, with HPV infection rates reaching 50–80% seen within two to three years of initiating sexual intercourse (Moscicki *et al*. 2007). The South African National HPV vaccination program was introduced as a school-based program for the first time in 2014 to girls aged 9–10 years (Mbulawa *et al*. 2018). This program uses the bivalent Cervarix® HPV vaccine that protects against two HR HPV types, HPV-16 and -18, given as two doses, six-months apart (Mbulawa *et al*. 2018). Thus, the AGYW in this study would not have received this vaccination, as they were beyond the age target for the vaccine campaign in 2014 and no catch-up vaccination has been offered to date. The other two HPV vaccines being rolled out in international programs are the Gardasil® vaccine which protects against two LR in addition to the two HR-HPV types found in Cervarix® HPV-6, and -11 and Gardasil-9® that protects against five additional types: HPV-31, -33, -45, -52 and -58 (Mbulawa *et al*. 2018). Overall in this cohort, 27% (44/163) of the AGWY were infected with either HPV-16 and/or HPV-18 and thus, would have been protected by the current national program that uses Cervarix®. If the Gardasil® vaccine had been used, an additional 14% (23/163) would have been protected, while the Gardasil-9® vaccine would have increased protection by an additional 25% (40/163) of participants. In Masiphumelele, the targets of the Cervarix® (HPV-16/18) vaccine were found in 25% (25/100) and thus, would have been protected by the current national program if they were in the correct age category. Women with HPV while the targets of the Gardasil® vaccine (HPV-6/11/16/18) were seen in 37% (37/100). A slightly lower prevalence was seen

in Soweto with the Cervarix® vaccine targets present in 20% (19/93) of women and the Gardasil® in 26% (24/93) of AGYW.

The longitudinal prevalence of HPV remained stable over the duration of the study, which is expected with a condition where the median duration of persistence was 168 days (Brown *et al*. 2008). The high prevalence and persistence of HPV, particularly HR HPV is, however, concerning as HPV is a further risk factor for HIV acquisition and for the development of cervical cancer (Houlihan *et al*. 2012). While the national cervical cancer screening program promotes three pap smears per lifetime, starting after the age of 30 at 10-year intervals (The Department of Health 2015b), only 19.3% of the adult South African population had ever had a Pap smear in a WHO evaluation of the program (WHO 2010). While the US Preventive Services Task Force does not recommend pap smears in women under 21 (U.S. Preventive Services Task Force 2017), this in is a setting of high pap smear uptake: 77.5% in women aged 30–39 had had a least one pap smear (Watson, Benard & Flagg 2018). The low uptake seen in SA could possibly be offset by the introduction of a vaccine that could protect the majority of women from HR HPV. There were only two cases of CIN1 seen in this cohort, the majority of these cases are known to regress spontaneously (Ho *et al*., 2011).

HSV-2 seroprevelence was only determined for participants from Masiphumelele, as blood was only collected from the Cape Town cohort. Only 7/149 (5%) AGYW from Masiphumelele were shedding HSV-2 actively in their genital secretions at visit one, with one woman shedding HSV-2 at two consecutive visits. Of these seven, six were also seropositive for HSV-2. However, a total of 31/149 (21%) of AGYW was seropositive for HSV-2 in Masiphumelele at visit one. In addition, seven adolescents that were seronegative at enrollment subsequently became HSV-2 seropositive at visits two or three, suggesting possible incident infections during the study. The median age of those with incident HSV-2 infections was 18.4 years, which was similar to what is has been reported in young reproductively active Indian women whose highest incidence was in those aged 15-20 years (Madhivanan *et al*. 2011).

This study highlighted some of the problems when using the vaginal discharge algorithm and syndromic management of STI and BV in AGYW, as their performance in predicting infections was unacceptably poor. While some studies, involving both men and women, have shown

relatively good performance of syndromic management (Mathews, van Rensburg & Coetzee 1998), the very low sensitivity seen here and in other studies involving women (Desai *et al*. 2003; Ghebremichael 2014) would result in a large number of STIs going untreated in young women. The low PPV would also suggest that a significant number of women would be over-treated (Vishwanath *et al*. 2000). Interestingly, in this cohort of adolescent females, the presence of multiple genital conditions did not increase the frequency or severity of symptoms.

Findings from this Chapter could have been influenced by some limitations. Self-reported data, in the form of socio-behavioral questionnaires, were used to collect information and reporting bias may have occurred. Only the Masiphumelele cohort was followed longitudinally, and similar follow-up in Soweto would have enabled evaluation of treatment in a different population. There were different strategies for enrollment, with the women from Masiphumelele being recruited primarily from the family planning clinic while those in Soweto were recruited from a more general population, which could result in selection bias. Data on health-seeking behavior outside of the clinics was not available. Partners were not enrolled in our study and no information on their treatment or STI prevalence was available. The role of re-infection in maintaining STIs can therefore not be established. There was no examination for the possibility of antimicrobial resistance for *C. trachomatis* or *N. gonorrhoeae*. Vulvovaginal swabs are not a sensitive nor specific method for the diagnosis in fungal infections which may have led to a misclassification if some infections.

In conclusion, this study showed an extremely high prevalence of treatable STIs and BV in adolescent females from two communities in southern Africa who are at high risk for HIV infection, with very poor performance of syndromic management in diagnosing STIs or BV. Given the finding that symptom-based treatment in young and adolescent women had an overall diagnostic accuracy of ~50%, equivalent to flipping a coin, this study calls for urgent review of the syndromic management policy, particularly for adolescent females.

Chapter 3

The influence of electronic data collection in collecting self-reported sexual risk behaviour in South African AGYW

3.1. Introduction

While comprising only 25% of the sexually active population, adolescents and young adults constitute almost 50% of all newly acquired STIs (Da Ros & da Silva Schmitt 2008). This disproportional rate is likely due to both biological and behavioural factors (Gewirtzman *et al*. 2011; Santelli *et al*. 2013; Dellar, Dlamini & Karim,2015). Obtaining accurate information on sensitive behavioural factors is crucial to help understand the transmission dynamics of HIV and other STIs, and to the development and evaluation of risk-reduction strategies tailored towards this population. However, acquiring this data is often challenging, as much of sexual health research relies on participants' self-report, and these may be difficult conversations to have with young people. In addition, there are frequently concerns about the confidentiality of shared sensitive information, which can often result in reporting bias, defined as participants reporting responses deliberately at odds to their actual behavior, which is particularly prevalent when collecting sexual behavior data (Jaspan *et al*. 2007; Lehrer *et al*. 2007; Kelly *et al*. 2013). Importantly, reporting bias occurs more frequently when the participants' actual behavior is considered socially unacceptable (Catania *et al*. 1990), and may be especially prominent in adolescents who may fear stigmatization or judgment by adult caregivers (Beguy *et al*. 2009).

Traditional methods of acquiring sexual behavior data include face-to-face interviews and paper-based questionnaires. Face-to-face interviews can, however, be influenced by the inaccurate rewording of questions, nonverbal cues, or directive questioning by the counselor collecting the data (Fowler Jr. & Mangione 1990). It has previously been estimated that only 35% of the abortions performed were accurately reported in face-to-face interviews (Jones & Forrest 1992). Paper-based surveys are thought to encourage more accurate reporting of sensitive behaviour by allowing respondents to complete a paper-and-pencil self-administered questionnaire (Vosylis, Žukauskienė & Malinauskienė 2012). However, this method has several limitations, including a reliance on sufficient literacy in participants to understand the questions being asked and complete all the questions accurately. The participants' answers and identification number are recorded together, which may result in concerns around privacy, and skip patterns may require complicated instructions that are difficult to follow (Turner, Danella & Rogers 1995). In addition, collating the research data

obtained by traditional paper-based methods can be costly and time-consuming (Vosylis, Žukauskienė & Malinauskienė, 2012).

In order to try and mitigate such challenges, computer-based questionnaires have been evaluated to improve participant confidence in the anonymity of the data collection (Jaspan *et al*. 2007). This has included the use of computer-assisted self-interviewing since the early 1990's (Couper *et al*. 1998). These technologies offer several advantages over traditional collection methods, including reduction in data entry errors, more complete data collection, and rapid result generation (Jaspan *et al*. 2007). Additionally, computerized systems can handle complex skip patterns, which may confuse participants in paper-based systems (Gwaltney, Shields & Shiffman 2008), have automatic consistency checks that review the participants' previous answers, and offer a greater level of confidentiality as written records do not exist (Wang *et al*. 2005). Some widely used computer-based methods include internet-based surveys (Dolezal *et al*. 2012; Gross *et al*. 2000; Turner 1998), personal digital assistants (Jaspan *et al*. 2007), and audio computer-assisted self-interviewing (Morrison-Beedy, Carey & Tu 2006).

The ongoing evolution of technology may improve the administration of computer-based questionnaires, using tablet or smart devices with touch screens that are user- and youth-friendly. I therefore hypothesized that sexual risk will be "underreported less" and would manifest as increased, reported risk taking in the electronically derived data. However, studies comparing computer-based technologies to traditional methods, either face-to-face interviews or paper-based surveys, have yielded conflicting results. Some previous studies have found no difference in prevalence of risky behaviour reporting in either arm (Dolezal *et al*. 2012; Johnson *et al*. 2001), while others reported that computer-based methods elicited more complete answers to sexual behaviour questions (Spark *et al*. 2015) and higher reporting of stigmatized behaviours, coerced sex or sex with a relative, stranger, or older men (Hewett, Mensch & Erulkar 2004; Le *et al*. 2006). In particular, adolescents have previously evaluated internet-based surveys as more pleasant compared with paper-based ones, which may infer increased comfort in answering sensitive issues electronically (Mangunkusumo *et al*. 2005). Few studies have evaluated the use of smart-devices among adolescents in developing countries. In this Chapter, I compared the use of a tablet-based questionnaire with

face-to-face, paper-based interviews in the collection of sexual risk and demographic data from AGYW who were at high risk for HIV and STI acquisition, residing in two major South African cities.

3.2 Materials and Methods

3.2.1 Cohort description

The 298 HIV negative black AGYW (16-22 years), previously described in Chapter 2, were enrolled in this study from two clinical trial sites in Masiphumelele, Cape Town and Soweto, Johannesburg in SA.

3.2.2 Development of the questionnaire and selection of online survey package

The questionnaire was developed in conjunction with the PHRU at the University of Witwatersrand (Dr Janan Dietrich) and the DTHF at UCT. The socio-demographic questionnaire had previously been developed and validated by the PHRU and was divided into the following sections: (i) demographics and socio-economic status, (ii) health care and support services utilization, (iii) emotional wellbeing and resiliency, (iv) reproductive and (v) sexual health.

The tablet-based questionnaire was adapted from the paper-based questionnaire and developed using SurveyGizmo version 4 (Boulder, US) software. SurveyGizmo was chosen as it enabled an electronic questionnaire with interactive questions matching the paper-based one to be created, was the most user-friendly, was more attractive in terms of the look and feel of the survey, and synchronized efficiently to a master computer. Other survey packages that were assessed included SurveyMonkey (San Mateo, CA, USA, https://www.surveymonkey.com) and Typeform (Typeform, Barcelona, Spain, https://www.typeform.com) but these were felt to be less appropriate to a detailed socio-demographic and sexual health questionnaire. Based on prevalent languages spoken at each site, questionnaires were translated from English into isiXhosa and Afrikaans in Masiphumelele and Zulu, isiXhosa and Sotho in Soweto.

3.2.3 Administration of the questionnaire

The first half of both cohorts was interviewed using the paper-based questionnaire and the latter half used the tablet. A delay in ethical approval of the questionnaire in Soweto resulted in the questionnaire being introduced later into this cohort and less paper-based questionnaires being completed at this site.

A trained counsellor at both sites instructed adolescents on the use of the tablet device. If participants struggled to complete the questionnaire on the device, the counsellor would provide further training and help to complete the questionnaire. The paper-based surveys were completed via face-to-face interviews. With the paper-based survey, the counsellors were trained to read the questions exactly as written, avoid comments related to answers, and not discuss risky behaviour until after the interview. The counsellors would further explain the questions if needed. All interviews (both paper- and tablet-based) were completed in private, with only the interviewer and the respondent being present, and respondents were offered the questionnaires in the language of their choice (Xhosa, Zulu, Sotho, Afrikaans and English) by multilingual interviewers. The interviewers were present for the completion of the tablet-based questionnaire to provide any additional support to participants in order to complete the questionnaire.

3.2.4 Statistical analyses

Statistical analyses were performed using STATA™ version 12 (StataCorp, Tx USA). Fisher's exact and Mann-Whitney tests were performed to evaluate differences in sociodemographic characteristics between survey type, and between sites. Logistic regressions were used to investigate the relationship between questionnaire method and reporting of demographic/behavioural factors, adjusting for possible risk factors for [such as participant age (Khasakhala & Mturi 2008), Xhosa-speaking (Flisher *et al*. 1996), in secondary school (Yi *et al*. 2010) and having parents as main source of income (Dinkelman & Lam 2008)]. Indicators of high risk behavior were considered to be: young age of sexual debut, unknown HIV status of sexual partner, HIV discordancy with sexual partner, unprotected sex or multiple sexual partners during previous three months, intergenerational relationships (>10 years age difference), anal or transactional sex, known to have had a STI in the previous six months, and use of injected drugs or having a tattoo in the previous six months.

3.3 Results

3.3.1 Completeness of questionnaires by method

Seventy five percent (225/298) of the AGYW completed in the questionnaire at enrollment: 127 from Masiphumelele (66 paper, 61 tablet) and 98 from Soweto (30 paper, 68 tablet), with a total of 96 paper-based and 129 tablet-based questionnaires being completed.

AGYW who used the paper-based questionnaire completed a higher proportion of the questions (99.7%; 2392/2400) than those who used the tablet (98.7%; 3184/3225; p=0·0003). Fewer participants using the tablet-based questionnaire answered the question about current marriage status than the paper-based one [89% (85/96) vs. 100% (129/129) p<0·0001]. However, there were no significant differences in answer completeness for any of the other questions answered.

3.3.2 Demographic differences by questionnaire method

Adolescents assigned to the tablet-based questionnaire arm tended to be slightly older (19 vs. 18 years; p=0·002), were less likely to have their parents as their main source of income (52% vs. 72%; p=0·002), or to currently be in secondary school (74% vs. 91%; p=0·002) compared to those in the paper-based survey arm. These differences were more marked in Soweto than Masiphumelele (Table 3.1). The Masiphumelele cohort was much more homogenous than the Soweto cohort, with 98% of the population isiXhosa-speaking compared to only 32% in Soweto (p<0·0001).

Table 3.1 Socio-demographic characteristics of the AGYW cohort assigned to the tablet or paper arm.

Characteristic	Total	Sites combined		Masiphumelele		Soweto	
		Tablet	Paper	Tablet	Paper	Tablet	Paper
	n=225	n=129	n=96	n=61	n=66	n=30	n=68
Age [median (IQR)]	18 (17-20)	19 (17-20)	18 (17-19)*	19 (18-20)	18 (17-19)	19 (17-20)	16 (16-17)*
isiXhosa [% (n/N)]	69 (156/225)	62 (80/129)	79 (76/96)*	98 (60/61)	98 (65/66)	29 (20/68)	37 (11/30)
Married [% (n/N)]	1 (3/225)	2 (3/129)	0 (0/96)	2 (1/61)	0 (0/66)	3 (2/68)	0 (0/30)
Secondary School [% (n/N)]	81 (183/225)	74 (96/129)	91 (87/96)*	84 (51/61)	89 (58/66)	66 (45/68)	97 (29/30)*
Low house hold income [% (n/N)]	72 (163/225)	72 (93/129)	73 (70/96)	74 (45/61)	70 (46/66)	71 (48/68)	80 (24/30)
Main Source income parents [% (n/N)]	60 (136/225)	52 (67/129)	72 (69/96)*	61 (37/61)	74 (49/66)	44 (30/68)	67 (20/30)#
≥1 financial dependents [% (n/N)]	20 (46/225)	26 (34/129)	13 (12/96)	20 (12/61)	15 (10/66)	32 (22/68)	7 (2/30)

*p<0·01; #p<0·05

3.3.3 Sexual risk behavior reporting

The most common risky behaviour reported overall by this cohort was the unknown HIV status of partners. The majority (57%) of AGYW in this study did not know their partners' HIV status. The second most common risk behavior was unprotected sex in the last three months, reported by 40% of participants. The third most common risk behavior identified was discordancy, where 20% of participants reported that their sexual partner was HIV infected while they were HIV negative (Table 3.2).

3.3.1.1 Sexual risk behavior reporting by questionnaire method

AGYW were more likely to report the use of injected drugs and/or having a tattoo in the tablet versus paper-based survey [14% vs. 5%; p=0.044]. The users of the tablet-based survey were, however, also more likely to report knowing their partner's HIV status (52% vs. 67%; p=0·040), although neither of these differences were significant after adjusting for multiple comparisons (Table 3.2).

Differences in condom use reporting were interesting comparing questionnaire methods. AGYW were significantly less likely to report "always using a condom" when completing the tablet-based rather than the paper-based questionnaire (aOR 0.13, 95%CI 0.03 - 0.66; p=0·014] but were significantly more likely to report "mostly using a condom" (aOR 10.17, 95%CI 2.10 - 49.26; p=0·004). Both remained significant after adjusting for the demographic factors described above (including participant age, isiXhosa speaking, currently being in secondary school and having their main source of income being their parents; Table 3.2). "Mostly using a condom" (40%, 52/129) was the most frequent response captured by tablet while "always" (30%, 29/96) or "occasional condom use" (33%, 32/96) were the most frequent responses captured on paper (Figure 3.1). This may reflect evidence of bias in the paper-based questionnaire towards reporting "always using a condom" compared to the tablet, where a more moderated response of "mostly using a condom" was the most common response captured.

Table 3.2 Sexual risk behavior by questionnaire method in AGYW

*p<0.01; #p<0.05

Characteristic	Total n=255	Tablet n=129	Paper n=96	OR	p-value	aOR (95% CI)	p-value
Age sexual debut [median (IQR)]	16 (15-17)	16 (15-17)	16 (15-17)		0.370		
Heterosexual [% (n/N)]	97 (219/225)	98 (127/129)	96 (92/96)	0.36	0.406	0.26 (0.04 - 1.82)	0.175
Unknown partner HIV status [% (n/N)]	58 (131/225)	52 (67/129)	**67 (64/96)#**	1.82	**0.040**	1.23 (0.66 - 2.29)	0.506
Discordant@ relationship [% (n/N)]	20 (44/225)	9 (27/129)	33 (17/96)	0.80	0.611	1.08 (0.48 - 2.42)	0.856
Unprotected vaginal sex last 3 months [% (n/N)]	40 (91/225)	31 (46/129)	52 (45/96)	1.57	0.102	1.61 (0.87 – 23.00)	0.132
Multiple partners last 3 months [% (n/N)]	14 (32/225)	12 (15/129)	18 (17/96)	1.62	0.247	1.64 (0.68 - 3.94)	0.266
Intergenerational relationships [% (n/N)]&	8 (19/225)	11 (14/129)	5 (5/96)	0.45	0.151	0.79 (0.22 - 2.85)	0.714
Anal sex [% (n/N)]	6 (13/225)	5 (6/129)	7 (7/96)	1.60	0.565	3.56 (0.79 - 15.96)	0.097
Transactional sex [% (n/N)]	2 (4/225)	3 (4/129)	0 (0/96)	0	0.137	1.00	-
Treatment for STI last 6 months [% (n/N)]	12 (26/225)	14 (18/129)	8 (8/96)	0.56	0.211	0.54 (0.20 - 1.43)	0.210
Injected drugs/tattoos [% (n/N)]	10 (23/225)	**14 (18/129)**	5 (5/96)#	0.33	**0.044**	0.39 (0.12 - 1.25)	0.113
Condom use [% (n/N)]							
Always	25 (56/225)	21 (27/129)	30 (29/96)	1.63	0.121	**0.13 (0.03 - 0.66)**	**0.014**
Mostly	34 (77/225)	40 (52/129)	**26 (25/96)#**	0.52	**0.032**	**10.17 (2.10 - 49.26)**	**0.004**
Occasionally	28 (64/225)	**25 (32/129)**	33 (32/96)*	3.08	**0.001**	3.21 (0.51 - 20.28)	0.214
Never	2 (4/225)	2 (2/129)	2 (2/96)	0.53	0.701	0.11 (0.01 - 1.37)	0.086

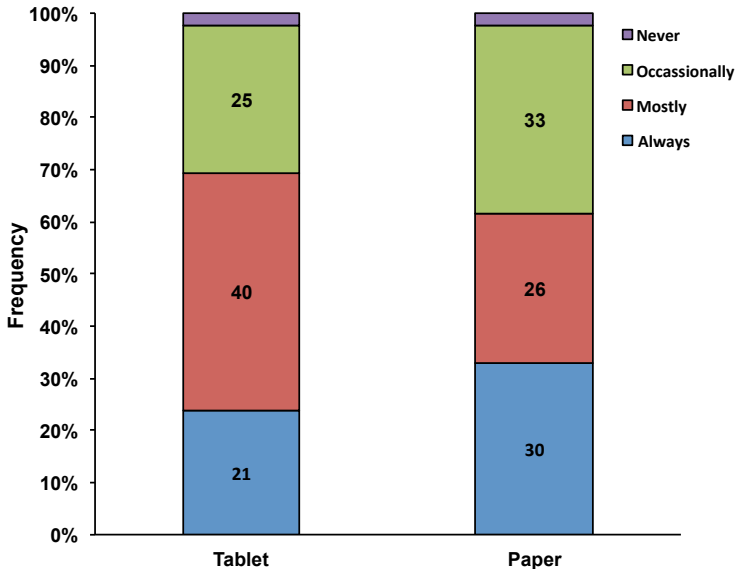


Figure 3.1 Comparison of reported condom use in AGYW in Cape Town and Soweto. The frequency of AGYW reposting "no condom use" (purple), "occasional condom use" (green), "mostly using condoms" (red) and "always using condoms" (blue) was compared between the tablet and paper-based questionnaire arm.

3.3.1.2 Sexual risk behavior reporting by questionnaire method within sites

In AGYW from Masiphumelele, those completing the paper-based survey reported a higher frequency of multiple partners in the last three months (18% vs. 5%; unadj p=0·028, adj. p=0·026) and previous anal sex (11% vs. 3%; unadj. p=0·168, adj. p=0·043) but were also more likely to report "always using condoms" (23% vs. 7%; unadj. p=0·013, adj. p=0·030) than those using the tablets. The tablet users were more likely to report the use of intravenous drugs or tattoos (12% vs. 2%; unadj. p= 0·027, adj. p=0·044, Table 3.3).

Within Soweto, reporting of risk behaviour was very similar in those completing the questionnaire on paper versus tablet, with the exception of a trend in AGYW who used the tablet arm to report more common treatment for STIs in the last six months than those using the paper-based questionnaire, which remained a trend after adjustment (3% vs. 19%; unadj. p=0·058, adj 0·051; Table 3.3).

3.3.1.3 Sexual risk behavior reporting by site

Irrespective of method used, AGYW from Soweto reported generally risker behaviour than those from Masiphumelele. There was a higher frequency of serodiscordant relationships (33% vs. 9%; unadj. p<0·0001), intergenerational relationships (14% vs. 4%; unadj. p=0·008), unprotected vaginal sex in the last three months (52% vs. 31%; unadj. p=0·003), and use of injected drugs/having tattoos (15% vs. 6%; unadj. p=0·044) in Soweto compared to Masiphumulele. Of these, only the proportion of women with serodiscordant relationships remained significantly different after adjustment for confounders (such as age, being Xhosa-speaking, currently attending secondary school and having parents as main source of income; adj. p=0·044). Interestingly, a greater proportion of the Soweto AGYW also reported "always using condoms" than the AGYW from Cape Town (34% vs. 6%; unadj. p<0.0001, adj. p=0·043; Table 3.4).

Table 3.3 Sexual risk behavior reporting by questionnaire method within AGYW from Masiphumelele and Soweto

	Masiphumelele						Soweto					
Characteristic	**Tablet n=60**	**Paper n=66**	**OR**	**p-value**	**aOR (95% CI)**	**p-value**	**Tablet n=68**	**Paper n=30**	**OR**	**P-value**	**aOR (95% CI)**	**p-value**
Age sexual debut [median (IQR)]	16 (15-17)	16 (15-17)		0.316			16 (16-17)	**16 (15-16)#**		**0.020**		
Heterosexual [% (n/N)]	98 (60/61)	94 (62/66)	0.26	0.367	0.19 (0.02 - 2.00)	0.168	99 (67/68)	100 (30/30)	-	1.000	-	-
Unknown partner HIV status [% (n/N)]	57 (34/60)	71 (47 /66)	1.89	0.098	1.65 (0.77 - 3.55)	0.199	46 (33/68)	57 (17 /30)	1.39	0.515	0.47 (0.14 - 1.57)	0.219
Discordant@ relationship [% (n/N)]	10 (6/61)	9 (6/66)	0.90	1.000	0.88 (0.24 - 3.14)	0.843	31 (21/68)	37 (11/30)	1.30	0.643	1.34 (0.41 - 4.49)	0.626
Unprotected vaginal sex last 3 months [% (n/N)]	25 (15/61)	38 (25/66)	1.83	0.131	1.69 (0.77 - 3.70)	0.191	46 (31/68)	67 (20/30)	2.39	0.079	1.57 (0.49 - 5.08)	0.451
Multiple partners last 3 months [% (n/N)]	5 (3/60)	**18 (12/66)#**	4.22	**0.028**	**4.88 (1.21 - 19.76)**	**0.026**	18 (12/68)	17 (5/30)	0.93	1.000	0.50 (0.11 - 2.31)	0.375
Intergenerational relationships [% (n/N)]&	3 (2/60)	5 (3/66)	1.38	1.000	9.97 (0.58 - 170.95)	0.113	18 (12/68)	7 (2/30)	0.33	0.215	0.23 (0.04 - 1.36)	0.105
Anal sex [% (n/N)]	3 (2/60)	**11 (7/66)**	3.44	0.168	**10.98 (1.08 - 111.31)**	**0.043**	6 (4/68)	0 (0/30)	0.00	0.310	-	-
Transactional sex [% (n/N)]	2 (1//60)	0 (0/66)	0.00	0.476	-	-	8 (3/68)	0 (0/30)	0.00	0.551	-	-
Treatment for STI last 6 months [% (n/N)]	8 (5/60)	11 (7/66)	1.31	0.767	1.15 (0.33 - 3.98)	0.828	**19 (13/68)**	3 (1/30)	0.15	**0.058**	**0.11 (0.01 - 1.01)**	**0.051**
Injected drugs/tattoos [% (n/N)]	**12 (7/60)**	2 (1/66)#	0.34	**0.027**	**0.11 (0.01 - 0.94)**	**0.044**	16 (11/68)	13 (4/30)	0.80	1.000	1.29 (0.24 - 7.02)	0.772
Condom use [% (n/N)]												
Always	**7 (4/60)**	23 (15/66)#	2.64	**0.013**	**3.76 (1.14 - 12.44)**	**0.030**	34 (23/68)	47 (14/30)	1.71	0.262	0.96 (0.29 - 3.19)	0.944
Mostly	60 (36/60)	**30 (20/66)***	0.41	**0.001**	**0.30 (0.14 - 0.64)**	**0.002**	24 (16/68)	17 (5/30)	0.65	0.595	0.82 (0.19 - 3.63)	0.795
Occasionally	23 (14/60)	38 (25/66)	1.35	0.084	2.02 (0.90 - 4.52)	0.087	6 (4/68)	23 (7/30)#	4.87	**0.031**	**12.83 (1.33 - 123.48)**	**0.027**
Never	1(1/60)	3 (2/66)	0.00	1.000	1.75 (0.15 - 20.63)	0.655	6 (4/68)	0 (0/30)	0.00	0.310	-	-

@Relationship with HIV+ partner

&>10 years age difference

*p<0.01; #p<0.05

Table 3.4 Comparison of sexual risk behavior reporting in AGYW from Soweto and Masiphumelele by site

Characteristic	Soweto n=98	Cape Town n=127	OR	p-value	aOR (95% CI)	P-value
Age sexual debut [median (IQR)]	16 (15-17)	16 (15-17)				
Heterosexual [% (n/N)]	99 (97/98)	96 (122/127)	0.25	0.236	0.14 (0.00 - 9.57)	0.359
Unknown partner HIV status [% (n/N)]	51 (50/98)	64 (81/127)	1.73	0.056	1.11 (0.47 - 2.61)	0.816
Discordant[@] relationship [% (n/N)]	**33 (32/98)**	9 (12/127)*	0.22	**0.000**	**0.34 (0.12 - 0.97)**	**0.044**
Unprotected vaginal sex last 3 months [% (n/N)]	**52 (51/98)**	31 (40/127)*	0.43	**0.003**	0.44 (0.19 - 1.04)	0.060
Multiple partners last 3 months [% (n/N)]	17 (17/98)	11 (15/127)	0.64	0.256	0.94 (0.27 - 3.22)	0.918
Intergenerational relationships [% (n/N)][&]	**14 (14/98)**	4 (5/127)*	0.25	**0.008**	0.88 (0.12 - 6.32)	0.897
Anal sex [% (n/N)]	4 (4/98)	7 (9/127)	1.81	0.398	0.57 (0.10 - 3.33)	0.531
Transactional sex [% (n/N)]	3 (3/98)	1 (1/127)	0.25	0.321	0.82 (0.24 – 6.58)	0.782
Treatment for STI last 6 months [% (n/N)]	14 (14/98)	9 (12/127)	0.63	0.298	0.97 (0.27 - 3.49)	0.964
Injected drugs/tattoos [% (n/N)]	**16 (15/98)**	6 (8/127)[#]	0.38	**0.044**	0.99 (0.21 - 4.70)	0.995
Condom use [% (n/N)]						
Always	34 (37/98)	**6 (19/127)***	0.29	**0.000**	**0.37 (0.14 - 0.97)**	**0.043**
Mostly	**24 (21/98)**	59 (56/127)	2.89	**0.000**	**4.02 (1.39 - 11.64)**	**0.010**
Occasionally	**6 (11/98)**	23 (39/127)	3.51	**0.001**	2.72 (0.90 - 8.30)	0.077
Never	6 (4/98)	2 (3/127)	0.57	0.473	0.28 (0.04 - 1.90)	0.191

[@]Relationship with HIV+ partner
[&]>10 years age difference
*p<0.01; #p<0.05

3.3.1.4 Sexual risk behavior reporting by site within questionnaire method

When analyzing risky behavior reporting by site within the tablet- and paper-based arm, a similar trend was seen as previously, when sexual risk behavior reporting in AGYW from Soweto and Masiphumelele was compared (section 3.3.1.3).

In the AGYW who completed the tablet survey, those from Soweto reported much higher risk sexual behaviour than those from Masiphumelele (Table 3.5), and the proportion of women reporting "always using condoms" was also higher in the Soweto group that completed the tablet-based questionnaire (34% vs. 6%; unadj. p<0·0001, adj. p= 0·014, Table 3.5) than those from Cape Town. A similar pattern of more risky behavior reporting in AGYW from Soweto was also observed in AGYW who completed the paper-based survey (Table 3.5), along with a higher frequency of condom use always (47% vs 23%; unadj. p<0·030). This indicates that irrespective of the questionnaire method used, AGYW from Soweto tended to report more risky behaviour than women from Masiphumelele aside from condom use, suggesting that the social environment in which adolescents live may influence reporting of behaviours more than the questionnaire method.

Table 3.5 Risk behavior reported in Masiphumelele, Cape Town vs. Johannesburg, Soweto within methods

	Tablet						Paper					
Characteristic	**Soweto n=68**	**Cape Tow n=61**	**OR**	**p-value**	**aOR (95% CI)**	**p-value**	**Soweto n=30**	**Cape Town n=66**	**OR**	**p-value**	**aOR (95% CI)**	**p-value**
Age sexual debut [median (IQR)]	**16 (16-17**)	16 (15-17)*		**0.008**		0.247	16 (15-16)	16 (15-17)		0.400		0.207
Heterosexual [% (n/N)]	99 (67/68)	98 (60/61)	0.90	1.000		0.997	100 (30/30)	94 (62/66)		0.306	0.00	
Unknown partner HIV status [% (n/N)]	46 (33/68)	56 (34 /61)	1.39	0.380	1.24 (0.40 – 3.90)	0.705	57 (17 /30)	71 (47/66)	1.89	0.171	0.28 (0.05 - 1.59)	0.150
Discordant[@] relationship [% (n/N)]	**31 (21/68)**	10 (6/61)*	0.25	**0.005**	**0.18 (0.05 – 0.72)**	**0.015**	**37 (11/30)**	9 (6/66)	0.003	**0.003**	0.73 (0.09 - 5.99)	0.772
Unprotected vaginal sex last 3 months [% (n/N)]	**46 (31/68)**	25 (15/61)[#]	0.40	**0.017**	**0.28 (0.09 – 0.90)**	**0.032**	**67 (20/30)**	38 (25/66)	0.015	**0.015**	0.62 (0.16 - 2.44)	0.493
Multiple partners last 3 months [% (n/N)]	**18 (12/68)**	5 (3/61)[#]	0.25	**0.030**	0.18 (0.03 - 1.01)	0.052	17 (5/30)	18 (12/66)	1.11	1.000	6.72 (0.62 - 72.60)	0.117
Intergenerational relationships [% (n/N)][&]	**18 (12/68)**	3 (2/61)[#]	0.16	**0.011**	0.14 (0.01 - 1.76)	0.129	7 (2/30)	5 (3/66)	0.67	0.646	0.00	0.997
Anal sex [% (n/N)]	6 (4/68)	3 (2/61)	0.55	0.684	0.15 (0.01 - 2.20)	0.164	0 (0/30)	11 (7/66)	-	0.094	-	-
Transactional sex [% (n/N)]	8 (3/68)	2 (1/61)	0.37	0.622			0 (0/30)	0 (0/66)	-	-	-	-
Treatment for STI last 6 months [% (n/N)]	19 (13/68)	8 (5/61)	0.38	0.125	0.55 (0.11 - 2.71)	0.462	3 (1/30)	11 (7/66)	3.441	0.428	4.95 (0.17 - 144.79)	0.353
Injected drugs/tattoos [% (n/N)]	16 (11/68)	11 (7/61)	0.68	0.612	1.04 (0.18 - 6.02)	0.967	**13 (4/30)**	2 (1/66)	0.032	**0.032**	0.65 (0.01 – 52.70)	0.850
Condom use [% (n/N)]												
Always	34 (23/68)	**6 (4/61)***	0.137	**0.000**	**0.13 (0.03 - 0.66)**	**0.014**	47 (14/30)	**23 (15/66)**	0.37	**0.030**	0.47 (0.101 - 2.03)[#]	0.311
Mostly	**24 (16/68)**	59 (36/61)*	4.68	**0.000**	**1.17 (2.10 - 49.26)**	**0.004**	17 (5/30)	30 (20/66)	2.17	0.212	2.12 (0.41 - 11.06)	0.372
Occasionally	**6 (4/68)**	23 (14/61)*	4.77	**0.009**	3.21 (0.51 - 20.28)	0.214	23 (7/30)	38 (25/66)	2.00	0.243	1.77 (0.38 - 8.35)	0.469
Never	6 (4/68)	2 (1/61)	0.267	0.369	0.11 (0.01 - 1.37)	0.086	0 (0/30)	3 (2/66)	0.00	1.000	1 (0)	-

[@]Relationship with HIV+ partner

[&]>10 years age difference

*p<0.01; #p<0.05

3.4 Discussion

Respondents' perceptions of social norms may influence their willingness to answer personal questions candidly and this may be exaggerated during adolescence (Hewett, Mensch & Erulkar 2004). I hypothesized that a tablet-based questionnaire would increase the reporting of risky behaviours compared to face-to-face administered paper-based surveys in adolescents, as it offers the perception of increased anonymity, in addition to being more a youth-orientated method of data collection. However, in this study, no major differences in adolescent reporting of sexual risk behavior between the two data capture methods was noted, leading to the conclusion that use of technology to assist with disinhibition of reporting of sensitive data collection in this age group of South African females did not offer significant risk reporting gains.

Further, administering the sociobehavioural risk questionnaires using an electronic device did not improve completeness of the data collection in this study. In fact, there was a marginally better completion rate with the more traditional method. It is possible that this particular population was not familiar or comfortable with the use of tablet devices, and therefore did not use the device as effectively as their more technologically savvy counterparts from better-resourced countries. Thus, it would be important to include a measure of smart-device competence in future studies to normalize questionnaire completion data. While previous studies have shown that adolescents from socially-disadvantaged communities may experience difficulty with the use of technology (Potdar & Koenig 2005), a survey conducted previously in SA has suggested that access to smart phone technologies is relatively high, even amongst socioeconomically-deprived families (James & Versteeg 2007). It is likely that, with the increased penetration of smart phones into the South African market since this survey was completed, familiarity with the use of these devices in this population has increased.

What emerges in review of the reported risk behavior data is a very high prevalence of risky behavior in this adolescent population. Not knowing their partner's HIV status was the most common risk factor and identified in the majority of AGYW (57%) asked. The rates seen here were similar to those seen in an adolescent study from the Eastern Cape Province of SA (58.5%; Toska *et al*. 2015). The next most common behavioral risk factor identified was

unprotected vaginal sex (40%), which, while high, was lower than the rates that had been reported in Kenya (in adolescents 14 to 21 year olds) and Uganda (in youth between 15 and 24 years; 87.5% and 57%. Respectively; Lightfoot *et al*. 2007; Ankunda, Atuyambe & Kiwanuka 2016). Finally, despite this survey being done in a cohort of adolescents with relatively few lifetime partners (median 2), almost 20% of respondents were in discordant (HIV+ partners) relationships. This rate was, again, lower that the prevalence of 36% seen in adolescents in Uganda (Ankunda, Atuyambe & Kiwanuka 2016).

Location more than method seemed to influence the reporting of risk behaviour. Geographical differences in the reported risk behaviour data were seen between adolescents in Masiphumelele in Cape Town and Soweto in Johannesburg, two major cities in SA. Adolescents from Soweto reported higher frequency of HIV discordant relationships, intergenerational relationships, and the use of injected drugs or getting a tattoo than those in Masiphumelele. When comparing the two methods within this high-risk group of AGYW from Soweto only, neither method showed superiority in reporting of more frequent risk behavior. This finding suggests that the social environment in which adolescents live may influence reporting of behaviours more so than the method of questioning. Interestingly, AGYW from Soweto were found to have lower rates of laboratory-diagnosed STIs and BV than those in Masiphumelele (Chapter 2, Barnabas *et al*. 2017), suggesting that reporting of risky behaviour rather than the risk behaviour itself was higher in Soweto than in Masiphumelele. Importantly, the Soweto AGYW also reported more frequent condom use than those from Masiphumelele, which may have also influenced the lower STI prevalence in this population, despite otherwise riskier sexual activity.

Within Masiphumelele, where the demographics between those who were assigned to either of the questionnaire methods were the most similar, the reported risk behaviour was, again, not dramatically different. Those who completed the paper-based questionnaire reported more multiple partners in the last three months and higher frequencies of anal sex, while the tablet-based arm reported a higher frequency of injectable drug use or tattoos. This again seems to emphasize the precedence of the socio-geographic environment over the method by which the data was obtained though there may be a suggestion that the reporting of more

taboo practices, such as the use of injectable drugs, was higher with the electronic method of data collection.

Socioeconomic status and family structure have been previously shown to influence adolescent sexual behaviours, with adolescents from poorly resourced settings possibly displaying increased risk behaviours (Santelli *et al*. 2000). The AGYW who took part in this study were generally all from poorer South African circumstances, and therefore possibly had access to fewer resources. In those who completed the paper-based survey, no socioeconomic differences were present by site (Masiphumelele vs. Soweto). Despite this there was more frequent reporting of sexual risk behavior noted in respondents from Soweto than Masiphumelele (more discordant relationships, unprotected sex in the last three months and the use of injected drugs or tattoos), suggesting, again, that location was important in sexual risk behavior reporting. Further, in Masiphumelele, where the socioeconomic status between the two groups was similar, there was no difference in risk reporting by method.

Limitations of this questionnaire evaluation include the fact that the adolescents were not contemporaneously but sequentially assigned to data collection methods. There were some demographic differences being noted between those that completed the questionnaire on tablet versus paper. Although this may have introduced bias, multivariate modeling was used to attempt to limit this. Both questionnaire methods were not administered to the same participant, which could have shown more accurately how the responses varied by method. A different counselor was used to administer the questionnaire in Masiphumelele and Soweto, which may also have influenced reporting. The participants who completed the survey on the tablet tended to be slightly older and less likely to have their parents as the main income source (particularly in Soweto), although age did not influence survey type assigned, as the AGYW did not have a choice in which survey type to use. Finally, it is also possible that the presence of the interviewer in the room while the participant completed the electronic questionnaire could have influenced how some of the data was reported by decreasing the perception of anonymity and, thus, increasing reporting bias.

Future technological innovations may allow questionnaires to be administered via smartphone apps allowing participants to complete questionnaires at home, with multiple

participants accessing the questionnaires simultaneously, simplifying the collection of sexual risk behaviour data (Robinson *et al*. 2013). Software to increase ease in administration, along with the significant cost savings and quicker data collation, remain strong drivers for the increasing use of technology to administer such questionnaires. The finding of equivalence between the methods of data collection may, in fact, further encourage the use of technology, as this could emphasize that the data is not biased by the collection method, but rather depends on the social environment participants live in. Further improvements of both the hardware and software could see an increase in the significant technical advantages of electronic data collection.

Further, what is striking in this cohort is the very high prevalence of risk behavior present despite years of behavior change messaging in both the media and in schools. This would suggest that these methods have been ineffective and that more targeted research is required into how to effect real and persistent changes in these at risk populations. The accurate reporting of sexual risk behaviour data in this group could help improving the message delivered.

In conclusion, this study found that reporting of sexual risk behaviour in South African AGYW was not influenced by the method of questioning, and that use of tablet-based questionnaires did not improve risk behavior reporting. Instead, risk appeared to be quite granular with geographic restrictions in different cities within SA. The relatively small difference between the two reporting methods suggests that a lack of technical insight in this population is not the reason for the result found. The technical and methodological advantages of electronic data collection discussed earlier would however suggest that the use of electronic collection of data become the preferred method for collection of risk behaviour data in adolescent populations at risk of HIV.

Chapter 4

Evaluating the effect of vaginal pH, hormones, BV, yeast and viral STIs on cytokine markers for genital inflammation in South African AGYW

4.1 Introduction

Genital inflammation, measured by the elevation of cytokine biomarkers, has been associated with increased risk for HIV acquisition in adult South African women (Masson *et al*. 2015a). Production of these inflammatory biomarkers in the lower FGT is thought to be influenced by various factors, including HC use, phase of the menstrual cycle, trauma, VIPs and infection with pathogens and pathobionts (Francis *et al*. 2016; Van de Wijgert 2017).

Long-acting injectable progesterone-only hormonal contraceptives, such as DMPA and Net-En in particular, are thought to influence the inflammatory milieu in the lower FGT (Hapgood, Kaushic & Hel 2018). Deese *et al.* (2015) showed that DMPA use was associated with higher concentrations of the chemokines MIP-1α, MIP-1β, IL-8, IP-10 and RANTES in the lower genital tract (using ectocervical swabs for measurement), as well as the pro-inflammatory cytokine IL-6, while Net-En users had higher genital concentrations of IL-8, RANTES, and IL-6 than women not using hormonal contraception. In contrast, Ngcapu *et al.* (2015) reported that genital tract cytokines were generally dampened in cervicovaginal lavages from adult South African women using DMPA compared to those not using hormonal contraception, including eotaxin, MCP-1, MDC, IL-15, platelet-derived growth factor (PDGF)-AA and a metalloproteinase TIMP-2.

Other factors that have been shown to influence genital tract inflammation include immune-modulatory factors in seminal fluid from male partners, which has been shown, by microarray analysis, to upregulate the pro-inflammatory cytokines IL-1α, IL-1β and IL-6, the growth factor GM-CSF, and the chemokine IL-8 (Sharkey *et al*. 2012b). Rametse *et al*. (2018) recently showed that semen from men with higher levels of genital inflammation further exacerbate inflammatory responses (mRNA and protein) by cervical epithelial cells *in vitro* compared to semen with lower concentrations of cytokines (Rametse *et al*. 2018). Adjusting for condom use during sex, Nakra *et al.* (2016) reported that unprotected (condomless) sex was associated with an increase in TGF-β1 but a decrease in human beta-defensin (HBD)-2, HBD-3, and IL-8 levels in genital samples from HIV negative women.

BV is a genital condition characterized by a relatively low proportion of lactobacilli and a diverse population of anaerobic bacteria, including *Gardnerella*, *Prevotella*, *Atopobium*, *Sneathia*, BVAB1-3, or *Mobiluncus* species (Ravel *et al*. 2011; van de Wijgert & Jespers 2017). Globally, BV is the most frequent cause of vaginal discharge in reproductive aged women (Mitchell & Marrazzo 2014). A high prevalence of BV has been reported in South African adult women, with the highest rates seen in women presenting to STI clinics and in sex workers (Puran *et al*. 2014; Johnson, Coetzee & Johnson 2005; Kenyon, Colebunders & Crucitti 2013a). AGYW are thought to be more susceptible to BV compared to older women, as they are closer to their age of sexual debut and tend to have more irregular, new or multiple sexual partners (Shisana *et al*. 2014; Fethers *et al*. 2008).

BV is frequently asymptomatic (Klebanoff *et al*. 2004; Kenyon, Colebunders & Crucitti 2013b), although several studies have shown that even asymptomatic BV is associated with increased concentrations of pro-inflammatory cytokines, including IL-1α, IL-1β and TNF-β (Masson *et al*. 2015b; Anahtar *et al*. 2016; Cauci *et al*. 2003). Conversely, *in vivo* studies have also described that certain chemotactic cytokines, like IP-10, GRO, MDC and MIP-1α, which are responsible for recruitment of immune cells, and haematopoietic cytokines (IL-7, GM-CSF) are down-regulated in women with BV compared to those with healthy vaginal microbiomes (Masson *et al.* 2015; Ryckman *et al.* 2008). Suppression of chemotactic cytokine responses may result in fewer immune cells being recruited to the genital tract, which may contribute to the lack of symptoms seen in some women with BV (Mlisana *et al.* 2012; Masson *et al.* 2015). Further, the inflammatory cytokines IL-1α and IL-1β were lowered while the chemokines IP-10, MIP-3α and MIG were increased in women treated with oral metronidazole who resolved BV after treatment, possibly implying that resolution of BV resolved these biomarker signatures (Joag *et al.* 2018).

Like BV, vulvovaginal yeast infections are common in the FGT, predominantly being caused by *Candida albicans* (Pirotta & Garland 2006; Bitew & Abebaw 2018). VVC has been associated with sexual activity in adolescents (Barousse *et al.* 2004), although it has been shown to occur more frequently in women with a *Lactobacillus*-dominated vaginal microbiota compared to women with BV-associated microbiota (Van De Wijgert et al. 2008). Genital inflammatory responses to yeast infections include an increase in IL-1α and TNF-α (Steele and Fidel 2002),

although a decrease in the mucosal chemokine IL-17 has also been reported (Masson *et al.* 2015).

Both BV and yeast infections are thought to increase risk for HIV acquisition in women (1.7- and 1.5-fold, respectively), with these two common vaginal conditions thought to further enhance HIV risk synergistically (Van De Wijgert et al. 2008). Both BV and yeast colonization in the lower reproductive tract alter mucosal epithelial barrier integrity, in addition to causing inflammation and HIV target cell activation (Zevin et al. 2016; Van De Wijgert et al. 2008).

HPV infection is the most common viral infection of the reproductive tract (World Health Organization 2018) and will infect most sexually active men and women at some point in their lives. While HPV has previously been shown not to increase genital inflammation (Kriek *et al.* 2016), HPV infection has been associated with an increased risk of HIV acquisition (Averbach *et al.* 2010; Smith-McCune *et al.* 2010).

The prevalence of HSV-2 in SSA is high, and in a large cohort of adult, HIV negative women from Durban, SA the prevalence was found to be 65% (Daniels *et al.* 2016). HSV-2 infection is lifelong and is characterized by frequent genital tract shedding in both symptomatic and asymptomatic persons (Wald *et al.* 2000). Genital dysbiosis, characterized by a decline of lactobacilli, has been associated with increased viral HSV-2 shedding (Cherpes *et al.* 2005). HSV-2 generates a strong immune response in cells resident at the site of infection (Posavad *et al.* 2015), and HSV-2 infection has been shown to interact with plasmacytoid DCs and other cells, and to induce IFN-α production (Johnston *et al.* 2011). Infection with HSV-2 has also been shown to trigger a significant increase in TNF-α and IL-6 *in vitro* (Stefanidou *et al.* 2013).

Although genital inflammation has been shown to be a major, independent risk factor for the acquisition of HIV (Masson *et al*. 2015), the drivers of inflammation in this vulnerable population are not clear. Understanding how inflammation is influenced by non-infective and infective factors, including vaginal pH, hormonal contraception, semen, BV, yeast and STIs, is crucial to inform on new intervention strategies targeting genital inflammation. Thus, the one of the objectives of this dissertation was to define how in AGYW infective and non-infective factors influence genital inflammation and potentially increase the risk of HIV acquisition in

these young women. The aims of this Chapter were therefore to assess the major drivers of genital inflammatory cytokine biomarkers in healthy adolescents (with no STIs, no yeast infections, and a Nugent of ≤ 3); and then to evaluate how these cytokine biomarkers change in the presence of vaginal dysbiosis associated with BV (Nugent score 7-10), viral STIs (HSV or HPV) or VVC. The effect of bacterial and parasitic STIs on cytokine biomarkers will be evaluated in detail in Chapter 5.

4.2 Methods

4.2.1 Cohort description

To determine the cytokine profile in healthy women and the impact of viral STIs, BV and yeast on AGYW in this cohort, all women enrolled in visit one of the study (n=298; Chapter 2) were evaluated. Thirty-five women (12 Masiphumelele and 23 Soweto) were STI- (including *C. trachomatis, N. gonorhhoea, T. vaginalis, M. genitalium*, HSV-2, and HPV), BV- (Nugent 0-3) and yeast-negative and served as controls. There were no cases of HSV-1 diagnosed. In addition, women from Masiphumelele who had persistent BV, or those who cleared their BV longitudinally over their three visits were chosen for further analyses.

4.2.2 Ethics statement

AGYW ≥18 years provided written informed consent. Those <18 years old provided written assent, with written informed consent obtained from their parent(s)/legal guardian(s). Ethical approval was obtained from the Universities of Cape Town (HREC Ref 267/2013) and Witwatersrand Research Ethics Committees (Clearance certificate number: M1307; Chapter 2, section 2.2.2).

4.2.3 Collection and processing of genital specimens

Cervicovaginal mucus was collected from each participant using a disposable menstrual cup (MC; Softcup®, Evofem Inc., San Diego, CA, USA). The clinician inserted the cup into the vagina behind the pubic bone, over the cervix, as instructed by the manufacturer. According to the protocol, MCs were held in place for 1 hour, and the time of insertion and removal were recorded in the participant folder by the study clinician. At the end of an hour, the MC was removed by the clinician, placed in a sterile 50mL propylene container (Greiner Bio One, Germany), and transported to the lab at 4°C, where it was processed within 4 hours of collection. The weight of cervicovaginal secretions collected by the MC was derived by subtracting the average weight of an empty 50ml tube with an unused Softcup® from that of the weight of the tube containing the genital sample (Jaumdally *et al*. 2018). The average weight was obtained from 10 empty 50mL tubes with unused menstrual cups of the same brand. In the laboratory, the 50mL Greiner tube was opened in a biosafety cabinet and, using the end of a sterile 1000µL pipette tip, and the menstrual cup was inverted to allow the

cervicovaginal mucus to flow to the bottom of the tube while being centrifuged. The tube was closed and centrifuged at 453g at 4°C for 10min, with the brakes off. The MC was then removed and discarded. The secretions pooled in the 50mL tube were resuspended with phosphate buffer saline (PBS) at a ratio of 1mL of undiluted cervicovaginal mucus: 4ml of PBS (where 1g of mucus was assumed to equal to 1mL; Jaumdally *et al*. 2018). The suspension was gently mixed using a Pasteur pipette and aliquotted into three 2mL cryovials, which were stored at -80°C until needed.

A range of other genital mucosal samples were collected from each woman at each study visit, including in the following order: (1) a Dacron vulvovaginal swab to test for viral STIs (described in Chapter 2 section 2.2.8.1); (2) a FLOQSwab™ lateral vaginal wall swab for a wet mount slide to detect BV (Nugent Scoring), VVC (gram stain) and vaginal pH (Chapter 2 section 2.2.8.2). One unit pH change 0.3 as the strips were in 0.3 increments. The STI and HPV swabs were transported at 4°C to the lab and stored at -80 °C. The STI swabs were then shipped on dry ice to the CHIVSTI, NICD, Johannesburg, South Africa for testing. At the clinic, the BV swab was rolled across a glass slide, air-dried and transported to the lab at room temperature. The slide was then heat-fixed and also transported to the CHIVSTI, NICD for Nugent scoring and fungal screening. The HPV swab was stored at until -80 °C until testing at UCT (Chapter 2, section 2.2.8.4).

4.2.4 Measurement of cytokines in genital secretions

Multiplex luminex flow cytometry was used to measure the concentrations of 48 cytokines in cervicovaginal mucus from MCs. Prior to measuring cytokines, the genital samples were filtered by centrifuging at 1950g for 10 min at 4°C in SPIN-X® 0.2µm cellulose acetate filters to exclude debris. The Human Cytokine Group I 27-plex (measuring the concentrations of regulatory cytokines: IL-10 and IL- 1RA; adaptive cytokines: IL-17, IL-13, IL-4 and IFN-γ; pro-inflammatory cytokines: IL-12p70, IL-6, IL-1β, RANTES and TNF-α; growth factors: vascular endothelial growth factor [VEGF] basic fibroblast growth factor [FGF-basic], G-CSF, GM-CSF, IL-7, IL-9 and PDGF-BB; and chemokines: eotaxin, IP-10, MCP-1 [MCAF], MIP-1α, MIP-1β, IL-2, IL-5, IL-8, IL-15), and the Human Cytokine 21-plex kit (measuring adaptive cytokine: IL-2RA; pro-inflammatory cytokines: TNF-β, TNF-related apoptosis-inducing ligand [TRAIL] MIF, IL-18, IL-12(p40) and IL-1α; growth factors: stem cell factor [SCF], hepatocyte growth factor [HGF],

SDF-1α, IL-3, β-nerve growth factor [NGF], stem cell growth factor [SCGF]-β, LIF, and macrophage colony-stimulating factor [M-CSF]; and chemokines: Cutaneous T-cell attracting chemokine [CTACK], IL-16, GRO-α, IFN-α2, MCP-3, and MIG Bio-Rad Laboratories Inc®) were used, according to the manufacturer's instructions. The lower limits of detection for the 48 cytokines ranged from 1-307 pg/mL. A 5PL regression line was used to determine the cytokine concentrations. Values below the lower limit were recorded as half the lowest concentration measured for (Masson *et al*. 2014). Sample placements on the assay plate were randomly selected based on site and date of collection. For quality control, ten samples that were used as inter-plate controls (included in all plates) while ten other samples were used as intra-plate controls (run in duplicates). These 20 samples were randomly chosen from those that had a large enough volume to be included on all the plates. Inter-plate correlations were carried out using the ten samples included on all plates and intra-plate correlations were carried out using the ten samples run in duplicates within each plate (using Spearman correlations) to confirm that the data was comparable. IL-15 was excluded, as the values did not pass quality control. The cytokines IL-2, IL-5, and RANTES were reported as binary variables (presence/absence) as 76.0%, 64.2% and 52.6% of the values, respectively, were below detectable range, thus only 44 cytokines were used in further analysis.

4.2.5 Screening for viral STIs, yeast and BV

Multiplex PCR was carried out on fluids collected using a vulvovaginal swab taken from all participants (n=298) to screen for HSV-1 and -2 (Chapter 2, section 2.2.8.1). HPV typing was performed using the Roche Linear Array HPV Genotyping assay (Chapter 2, section 2.2.8.4). BV was assessed by Nugent scoring, and fungal screening was assessed using the same slides (Chapter 2, section 2.2.8.2).

4.2.6 Measurement of HSV-2 IgG

HSV-2 serology was performed using the HerpeSelect HSV-2 ELISA Kits (Focus Diagnostics, Cypress, CA, USA) on plasma samples from the Masiphumelele cohort only, and is described in detail in Chapter 2, section 2.2.7.3.

4.2.7 Measurement of cotinine as a biomarker for smoking

Cotinine levels were measured from blood plasma using ELISA, as described in Chapter 2, section 2.2.7.2.

4.2.8 Measurement of PSA as a biomarker for unprotected sex

The presence of PSA was measured from genital secretions using ELISA, as described in Chapter 2, section 2.2.8.3.

4.2.9 Measurement of endogenous sex hormones

The concentrations of E2, progesterone and LH were measured from blood plasma, as described in Chapter 2, section 2.2.7.1. These levels would represent hormone concentrations during the luteal phase of the menstrual cycle in women not on injectable HCs, or two weeks after their injection for women using Net-En or DMPA.

4.2.10 Statistical analyses

STATA 12.0 was used for all statistical analyses and for multivariate model building. Graphs and figures were generated using Prism 6. Non-parametric tests were used. The Mann Whitney U test was used to compare two groups of continuous variables, while a Fisher's exact test was used for categorical variables. The Kruskal Wallis one-way analysis of variance was used for categorical variables with ≥ 3 groups. A false discovery rate step down procedure was used to adjust p values for multiple comparisons. 95% confidence intervals and p values of ≤0.05 were used to assess statistical significance. All values were reported to three significant figures. Multivariate logistic regressions were carried out to assess the impact of BV/STIs/yeast infection on genital inflammation using a step-up strategy where the different variables that could potentially confound the relationship between BV/STIs/yeast infection and inflammation were included one at a time, and R^2 values were compared as a measure of model fit. The R^2 value gives the proportion of the variance that can be accounted for by the regression model, and R^2 values are a good measure of how well the dependent variable (the individual cytokines or cytokine classes in this case) predicts the outcome (Nagelkerke 1991). In healthy women, the model with the best fit adjusted for injectable contraceptive use and pH.

4.3 Results

A total of 298 adolescents (16-22 years) from Masiphumelele and Soweto were included in this analysis (described in detail in Chapter 2, Table 2.1). At enrolment, 47% (135/290) had BV (Nugent 7-10), and a further 14% (41/290) had an intermediate Nugent score of 4-6, suggesting altered microbiota. In addition, 10% (30/291) had fungal infections that were evident on the Gram stain (Chapter 2, Table 2.2). HPV was not considered in the linear regressions analysis, as it was so ubiquitous [prevalence of 67% (163/244)], and a previous paper from our laboratory has shown that HPV did not influence inflammatory cytokine levels in the FGT (Kriek *et al*. 2016).

The prevalence of BV (Nugent 7-10) was high in this cohort, as 48% (71/147) of AGYW in Masiphumelele and 45% (64/143) of those in Soweto had BV (Chapter 2, Table 2.2). Compared to adolescents who were BV and STI negative, the BV-positive group had a significantly higher vaginal pH (5.0 vs. 4.4, p=0·0002), were more likely to use a HC implant (Implanon; 14% vs. 0%, p=0·022), and more likely to report a lower frequency of male condom use as the only form of contraception (19% vs. 51%, p=0·016). As mentioned in Chapter 2, the prevalence of HPV was also very high, with 67% (163/244) overall, and prevalences of 65% (63/97) seen in Masiphumelele and 68% (100/147) in Soweto, with 54% (135/252) overall infected with high risk HPV. There were no differences seen in contraceptive use, frequency of condom use, or pH between HPV-positive and –negative groups (Table 4.1).

Table 4.1. Characteristics of adolescents from Masiphumelele and Soweto at enrolment

Characteristics	Total n=298	Masiphumelele n=149	Soweto n=149	p-value
Age at Visit [median (IQR)]	18 (17-20)	18 (17-20)	18 (17-20)	0·1273
Lateral wall pH [median (IQR)]	5.00 (4.40-5.30)	4.70 (4.40-5.30)	5.00 (4.70-5.60)	**0·0006**
Endogenous hormones [median (IQR)]				
Oestradiol	92.70 (61.7-211.30)	71.70 (52.55-102.00)	210.00 (89.00-406.00)	**<0·0001**
Progesterone	1.10 (0.60-1.70)	0.70 (0.05-1.20)	1.25 (0.90-4.30)	**<0·0001**
Luteinising Hormone	4.70 (2.80-7.90)	4.35 (2.75-6.30)	6.10 (3.00-10.10)	**0·0006**
Endogenous hormones those not on contraception [median (IQR)]				
Oestradiol			212.73 (92.7-420.5)	
Progesterone			1.84 (0.83-4.15)	
Luteinising Hormone			7.02 (4.14-11.84)	
Contraceptives [% (n/N)]				
Injectable contraceptive	57.43 (170/296)	88.59 (132/149)	25.05 (38/147)	**<0·0001**
DMPA	13.85 (41/296)	18.12 (27/149)	9.53 (14/147)	0·032
Net-En	43.58 (129/296)	70.46 (105/149)	17.00 (25/147)	**<0·0001**
COC	5.41 (16/296)	4.70 (7/149)	6.12 (9/147)	0·588
Nuvaring	0.34 (1/296)	0.06 (1/149)	0 (0/147)	0·320
Implanon	3.38 (10/296)	6.04 (9/149)	0.68 (1/147)	**0·011**
Male Condom only	25.68 (76/296)	0 (0/149)	51.70 (76/147)	**<0·0001**
None	4.73 (14/296)	0 (0/149)	9.52 (14/147)	**<0·0001**
Recent smoking [% (n/N)]	n/a	19.46 (29/149)	n/a	n/a
Viral STIs [% (n/N)]				
HSV-2 (HerpeSelect HSV-2)	n/a	22.07 (32/145)	n/a	n/a
HSV-2 (DNA)	2.68 (8/298)	4.70 (7/149)	0.67 (1/144)	**0·067**
HPV (Any)	66.80 (163/244)	64.95 (63/97)	68.02 (100/147)	0·677
HPV High risk *	53.57 (135/252)	53.36 (55/103)	53.69 (80/149)	0·898
HPV Low risk **	41.66 (105/252)	35.92 (37/103)	45.63 (68/149)	0·341
Multiple HPV infections	74.23 (121/163)	71.42 (45/63)	76.00 (76/100)	0·582
HPV-16	19.01 (31/163)	23.80 (15/63)	16.00 (16/100)	0·226
HPV-18	9.20 (15/163)	7.93 (5/63)	10.00 (10/100)	0·784
HPV-6	9.81 (16/163)	9.52 (6/63)	10.00 (10/100)	>0·999
HPV-11	4.29 (7/163)	1.58 (1/63)	6.00 (6/100)	0·250
No STI/BV/Yeast [% (n/N)]	11.74 (35/298)	15.44 (23/149)	8.05 (12/149)	0·071
PSA [% (n/N)]	19.97 (55/290)	18.91 (28/148)	19.01 (27/142)	0·984

HPV high risk types = 16, 18, 26, 31, 33, 35, 39, 45, 51, 52*, 53, 56, 58*, 59, 66, 68, 73, 81, 82
**HPV low risk types =6,11,40,42,54,55,61,62,64,67,69,70,71,72,81,82,83,84, CP6108, IS39
n/a = not available

4.3.1. Genital cytokine profiles in control adolescents

Genital cytokine concentrations were measured in cervicovaginal fluid collected from STI/ yeast-negative AGYW who had a Nugent score ≤3, to evaluate the influence of non-infective processes on genital inflammation (Figure 4.1). In these "healthy" adolescents of reproductive age, IL-1RA, an anti-inflammatory cytokine that functions as a receptor agonist of IL-1, was detected at the highest concentrations (42073.2 pg/mL; IQR 17220.5-64626.6), while the adaptive cytokine IL-4 was detected at the lowest median concentration (1.4 pg/ml;

IQR 0.9-2.6). Concentrations of IL-8, a chemoattractant cytokine produced by blood cells and various tissues (Bickel 1993) varied the most between individuals, with concentrations ranging from 10.0 to 749921.7 pg/mL in vaginal fluid. In the absence of vaginal dysbiosis, yeast infections or STIs, it was interesting to note that there was no obvious difference between healthy AGYW living in Masiphumelele and Soweto in any of the 44 cytokines measured, despite significant differences in the hormonal contraceptive use patterns by site (Figure 4.1 and Table 4.1).

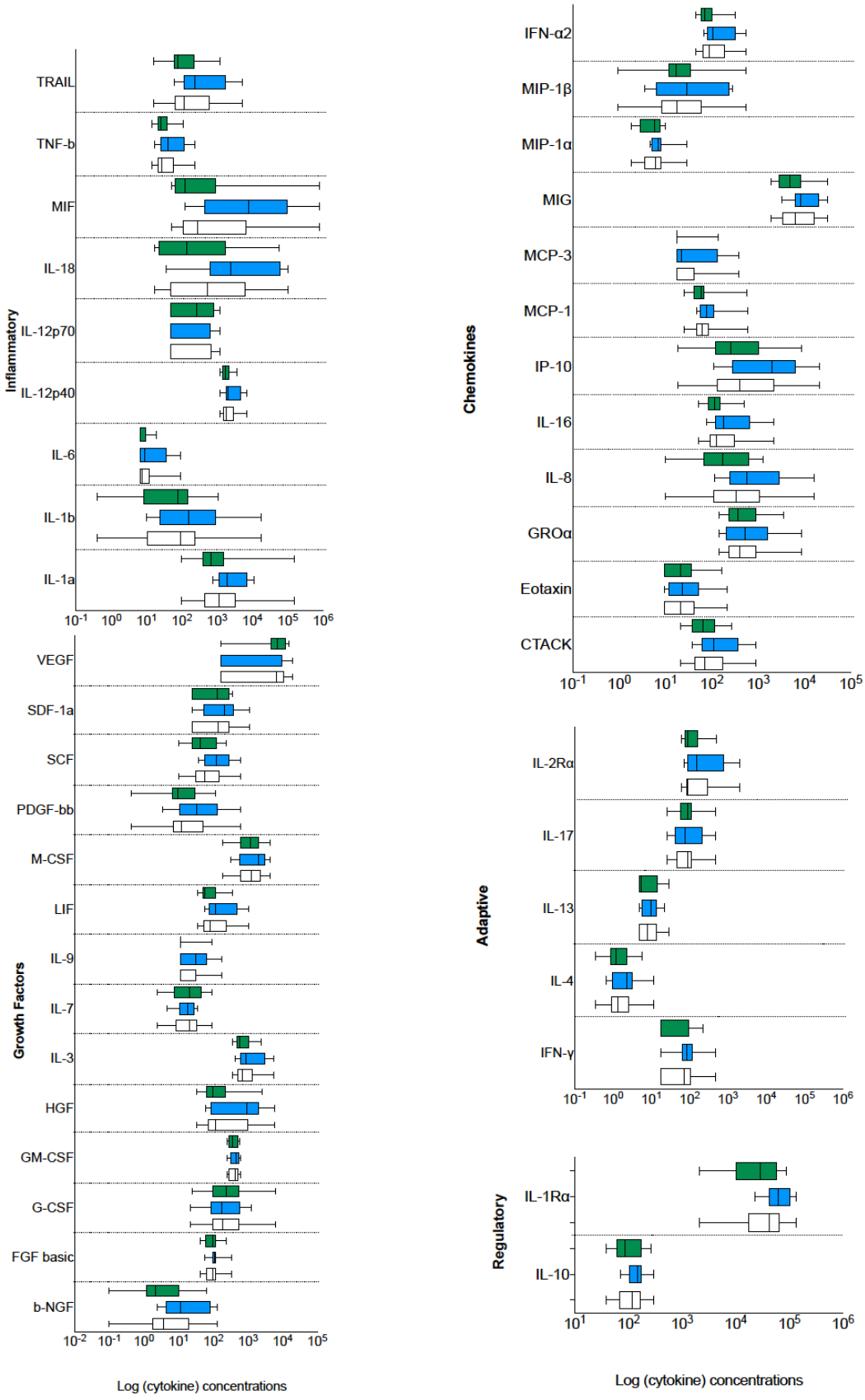


Figure 4.1. Comparison of cytokine concentrations in reproductively healthy adolescents (with no STI, yeast infections or vaginal dysbiosis) for the whole cohort (white bars), women from Masiphumelele only (blue bars) and women from Soweto only (green bars). Classes of cytokines are shown on Y-axis. The box-and-whisker plots show the median, IQR and 5-95% range.

4.3.1.1 Vaginal pH and genital cytokine profiles in healthy AGYW

The median vaginal pH for healthy AGYW (Nugent≤3/STI-/yeast-) was 4.4 (IQR 4.1-5.0), which did not differ significantly by site (Figure 4.2). Since Francis *et al*. (2016) reported that vaginal pH correlates inversely with the genital tract concentrations of IL-18, IL-2, GM-CSF, TGF-β and HBD3, the relationship between vaginal pH and genital cytokine concentrations in healthy AGYW in this study was investigated. As reported by Francis *et al*., (2016), vaginal pH in adolescents was positively associated with elevated concentrations of half of the cytokines measured, including: MIF (β=1.260; p=0·001), IL-18 (β=1.043; p=0·001), HGF (β=0.619 p=0·001), SCGF-β (β=0.595; p=0·005), SDF-1α (β=0.594; p<0·0001), IP-10 (β=0.584; p=0·022), IL-1α (β=0.549; p=0·005), G-CSF (β=0.526; p=0·009), GRO-α (β=0.458; p=0·001), IL-8 (β=0.442;p=0·031), TRAIL (β=0.438; p=0·017), IL-7 (β=0.408; p=0·004), CTACK (β=0.300; p=0·01), IL-2Rα (β=0.299; p=0·012), LIF (β=0.289; p=0·009), IL-16 (β=0.275; p=0·013), MCP-3 (β=0.242; p=0·043), IL-3 (β=0.232; p=0·018), IFN-α2 (β=0.227; p=0·007), TNF-β (β=0.227; p=0·025), IL-4 (β=0.225; p=0·043), and IL-12(p40) (β=0.128; p=0·037). The β-coefficient indicates the degree of change in the individual cytokine concentrations for a one unit change in pH: for example, an increase in one unit pH would be associated with a 1.260 unit increase in log MIF cytokine concentration and a 1.043 unit increase in log IL-18 concentrations. SDF-1α, IL-18, MIF, GRO-α, HGF, IL-7, IL-1α, SCGF-β, IFN-α2, G-CSF, LIF, CTACK, IL-2Rα, IL-16, TRAIL and IL-3 remained significant after correction for multiple comparisons (Figure 4.3).

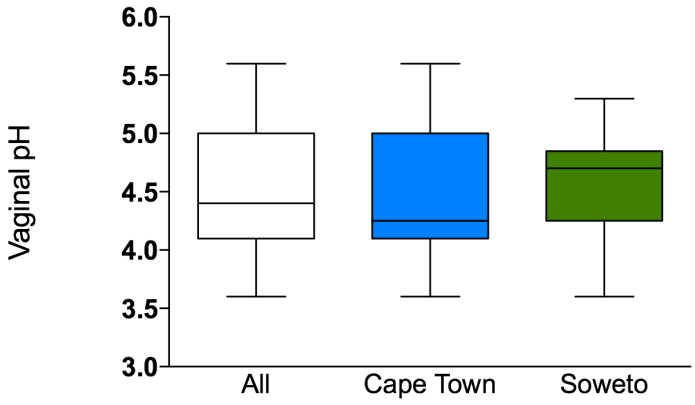


Figure 4.2. Comparison of vaginal pH in healthy adolescents (STI-/yeast-/Nugent≤3) from Masiphumelele (Cape Town; blue) versus Soweto (green). Each box-and-whisker plot shows the median, IQR and 5-95% range.

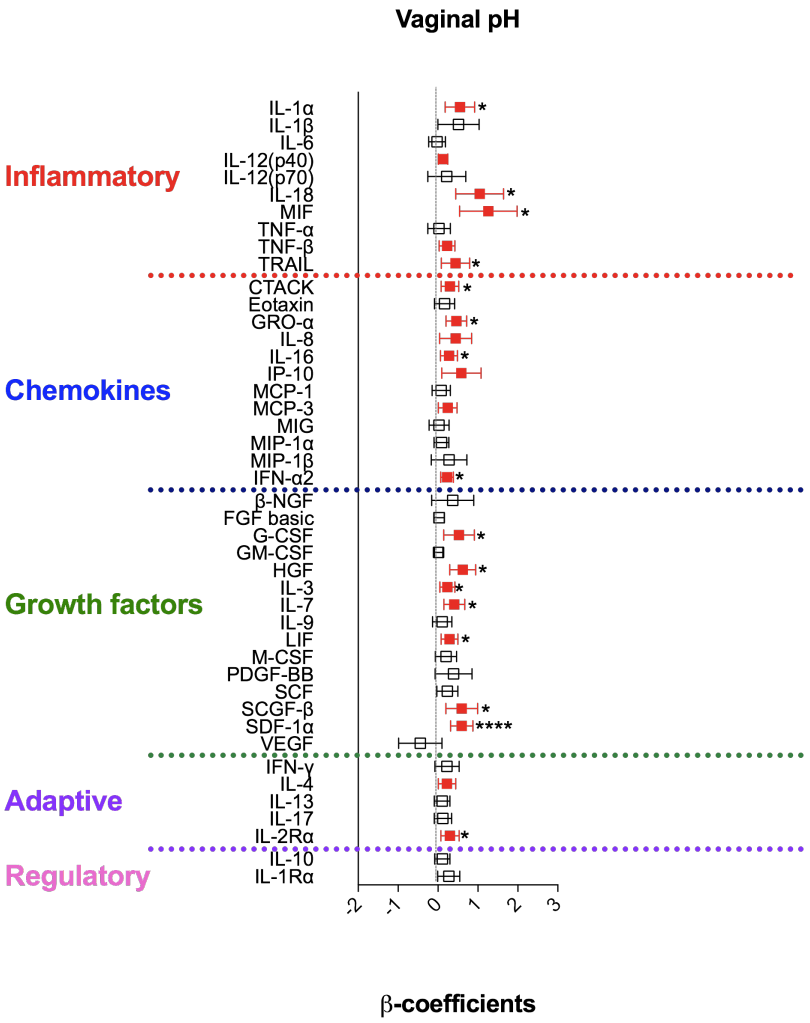


Figure 4.3 Relationship between vaginal pH and cytokine concentrations in healthy AGYW (STI-/yeast/Nugent≤3), assessed by multivariate linear regression analysis, adjusted for injectable hormone contraceptive use. Using IL-18 as an example, the β-coefficients can be interpreted as a 1.260 pg/mL change in vaginal MIF concentrations for every 1 unit change in vaginal pH. Significant associations are shown in red boxes. The associations that were significant after adjustment for multiple comparisons (false discovery rate step down procedure) are marked with an asterisk (*). P-values ≤0.05 are considered significant.

4.3.1.2 Endogenous hormone concentrations and genital cytokine profiles in healthy AGYW

Endogenous hormones may affect inflammatory responses (Straub 2014; Tait *et al*. 2008) and influence HIV/STI risk (Smith *et al*. 2000). Therefore, it was hypothesized that endogenous hormone levels could affect genital cytokine concentrations in healthy (STI-/ yeast-/Nugent≤3) AGYW. Endogenous hormonal fluctuations during the menstrual cycle are also thought to influence genital inflammation in the female reproductive tract (Kayisli *et al*. 2002). Thus, for this study, AGYW using combined COCs, condoms or hormonal implants (Implanon) were sampled during the luteal phase of their cycles. Those using the long-acting injectable Net-En or DMPA were sampled two weeks after their last injection, after hormone peak levels had normalized.

Overall, the median plasma concentration of E2 in adolescent females was 210 pmol/L (IQR 70.95-581.5), progesterone 1.25 mnol/L (IQR 0.9-2.5), and LH 5.3 IU/L (IQR 2.7-9.8). According to international published reference ranges, these levels are lower than those seen in adult women [E2 388 pmol/L, progesterone 25.76 mnol/L], while LH concentrations were similar [5.7 IU/L (Roth *et al*. 2014; Reed & Carr 2000)]. Comparisons of the endogenous hormonal concentrations between sites were not reliable as all (12/12) of the AGYW from Masiphumelele were using injectable contraceptives, while only 3/23 of adolescents from Soweto used injectable contraceptives: two were using DMPA and one Net-En (Figure 4.4).

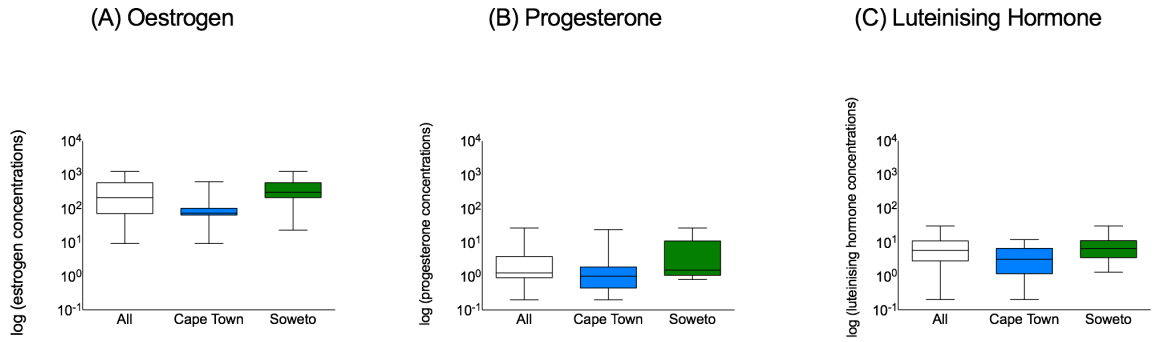


Figure 4.4. Oestrogen (A), Progesterone (B) and Luteinising hormone (C) levels in adolescents with no STI, or yeast infections and a Nugent score≤3. Women from the whole cohort are shown by the white bars, women from Masiphumelele (Cape Town) only by the blue bars and women from Soweto only by the green bars. The box-and-whisker plots show the median, IQR and 5-95% range.

After correcting for injectable HC use and vaginal pH, endogenous E2 concentrations were weakly associated with IL-1RA (β= 0.0008; p=0·022), IL-18 (β =0.002; p=0·044), IL-7 (β =0.0008; p=0·031), and MIG (β = 0.0006; p=0·04) concentrations, although these were not significant after adjusting for multiple comparisons, while progesterone was not associated with altered genital cytokine levels (Figure 4.5).

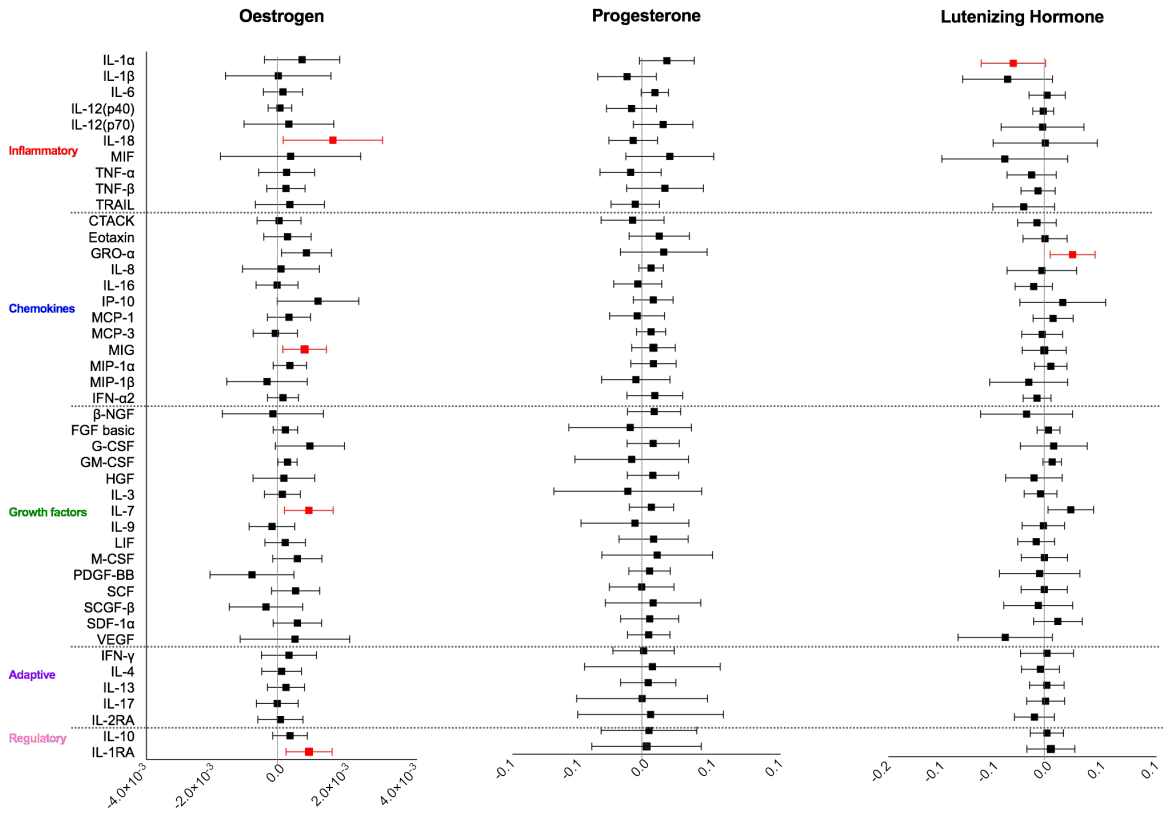


Figure 4.5. Comparison between genital cytokine concentrations and endogenous hormone concentrations in adolescents. Multivariate linear regression showing the associations (before correcting for multiple comparisons) between oestrogen, progesterone and luteinising hormone and cytokines for STI, BV, altered flora and yeast negative participants, while correcting for injectable contraceptive use and vaginal pH. Each association is shown as a β-coefficient and the error bars are the 95% CI. Statistically significant associations are shown in red boxes. P values of ≤0.05 are considered statistically significant.

4.3.1.3 Effect of exogenous hormones on genital cytokines in healthy AGYW

Injectable progestin-only contraception (DMPA or Net-EN) has been associated with alterations of the genital inflammatory profile, although there has been some disagreement about whether they increase or decrease genital inflammatory markers (Deese *et al*. 2015; Ngcapu *et al*. 2015). Genital tract inflammatory cytokines concentrations were thus compared by HC use group in these healthy (Nugent≤3/STI-/yeast-) adolescents. Similar to the findings of Deese *et al.* (2015), injectable HC use was associated with significantly elevated cytokine concentrations, across several functional classes (Figure 4.6). More than half of the cytokines measured in the three "healthy" adolescents using DMPA (27/44) were significantly elevated compared to those not using hormonal contraception, including: IL-1α, IL-1β, IL-6, IL-12p40, IL-18, MIF, TNF-β, Eotaxin, IL-8, IL-16, IP-10, MCP-3, MIG, MIP-1α, IFN-α2, FGF basic, HGF, IL-3, IL-9, LIF, MCSF, SCF, SCF-β, SDF-Iα, IL-4, IL-17 and IL-2RA. Similarly, adolescents using Net-En (n=11) had significantly elevated concentrations of almost three quarters of the cytokines

measured (31/44) compared to women not using HCs, including: IL-1α, IL-1β, IL-18, MIF, CTACK, IL-8, IL-16, IP-10, MCP-3, IFNα2, βNGF, HGF, IL-9, LIF, PDGF-bb, SCF, SCF-β and IL-1RA. Only four cytokines were commonly increased in AGYW using DMPA and Net-EN (MCP-3, MIP-1α, FGF-basic, and M-CSF), of which DMPA users had significantly higher concentrations than Net-En users.

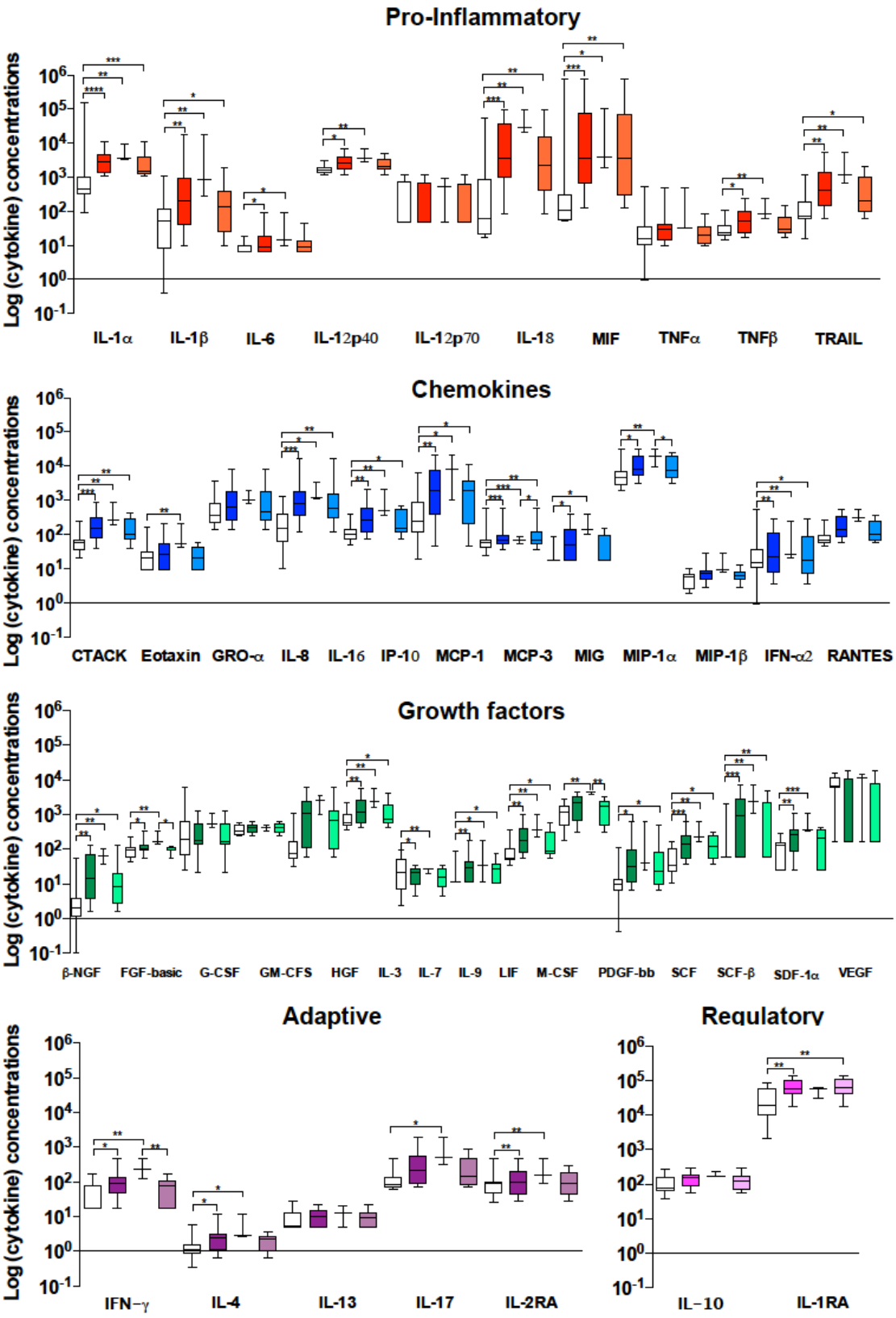


Figure 4.6. Comparison of genital cytokine concentrations according to hormonal contraceptive group in healthy (Nugent≤3/STI-/yeast-) AGYW. The box and whiskers show women using no contraception (clear boxes, n=20), those using any injectable (dark boxes; n=14), DMPA (line only; n=3) and Net-En (lighter shade boxes, n=11). Mann-Whitney U tests were used to compare median cytokine concentrations. P-values≤0.05 were considered significant.

4.3.1.4 Effect of unprotected sex (PSA+) on genital cytokine profiles in healthy AGYW

Semen has been reported to increase the concentrations of several pro-inflammatory and immune-regulating cytokines, including CSF2, IL-6, IL-8, IL-1α and TGF-β1, in genital secretions collected from women (Nakra *et al*. 2016; Sharkey, Tremellen, Jasper, Gemzell-Danielsson & S. a Robertson 2012), which was also associated with an influx of neutrophils into the lower reproductive tract in pigs (Schuberth *et al*. 2008). To investigate if the presence of semen influenced cytokines in genital secretions in adolescents, those who were PSA+ (17%, 6/35; indication of sex within last 48-72 hours; MacAluso *et al*. 1999) were compared to those who were PSA- (83%, 29/35). Amongst those that were PSA+, no difference was seen in the frequency of HC use: 20% (3/15) used HCs, while 15% (3/20; p>0·999) did not. Using linear regression to correct for injectable HC use and vaginal pH (the factors were determined by the step-up model building), a marginal increase was only in IL-1β and GRO-α noted (β=0.03; p=0·03 and β=0.02; p=0·041 respectively; data not shown), although these were not significant after correcting for multiple comparisons (p=0·32 and 0·14, respectively)

To evaluate the most influential factors affecting genital cytokine classes in healthy adolescents (STI-/yeast-/Nugent≤3; controls), various models were considered in which cytokine functional classes were considered into a single factor (Table 4.2): (1) Unadjusted model, without correcting for the effect of genital pH or injectable contraceptive use (2) a model to test for the confounding effect of genital pH alone; and (3) a model to test the effect of genital pH and injectable contraceptive use. Overall, a linear regression model including genital pH and progestin-only injectable contraceptive use gave the best fit, explaining up to 50% of the variance in the genital cytokines in healthy women. The strongest association was found with chemokines. Concentrations of the endogenous hormone levels (LH, progesterone or E2) and the presence of PSA from semen exposure did not seem to influence the association.

Table 4.2. Modeling influential factors the best explain variance in cytokine functional classes in healthy (Nugent≤3/STI-/yeast-) AGYW

	Model		
Cytokine class	**Unadjusted[#]**	**Correcting for genital pH[%]**	**Correcting for genital pH and injectable contraceptive[$]**
Regulatory	0.0016 (p<0·001)	0.0292 (p=0·0001)	0.3323 (p<0·0001)
Adaptive	0.0361 (p<0·0001)	0.1070 (p<0·0001)	0.2822 (p=0·0023)
Chemokine	0.1087 (p<0·0001)	0.1575 (p<0·0001)	0.5564 (p<0·0001)
Growth Factors	0.0312 (p<0·0001)	0.1904 (p<0·0001)	0.5071 (p<0·0001)
Inflammatory	0.0045 (p<0·0001)	0.0863 (p<0·0001)	0.5060 (p<0·0001)

[#]β-coefficients from simple linear regression models, with no covariates
[%]β-coefficients after adjusting for the effect of genital pH
[$]β -coefficients after adjusting for both genital pH and injectable HC use

4.3.2 Viral STIs and genital inflammation in AGWY

4.3.2.1 HPV infection and genital inflammation

The vast majority of sexually active people (>80%) will acquire HPV in their life (Einstein *et al*. 2009), most of which (>90%) will resolve without any sequelae (Wentzensen *et al*. 2010). As stated earlier, previous work from our lab has shown no influence on genital cytokine levels with HPV infection (Kriek *et al*. 2016). In agreement with this data, no association was seen between genital inflammation and HPV infection in AGYW from in this cohort when compared to healthy women without HPV infection in a multivariate model adjusting for adjusting for injectable HC use, genital pH and co-infections (Figure 4.7). This was also true when the Soweto and Masiphumelele cohorts were looked at individually (data not shown).

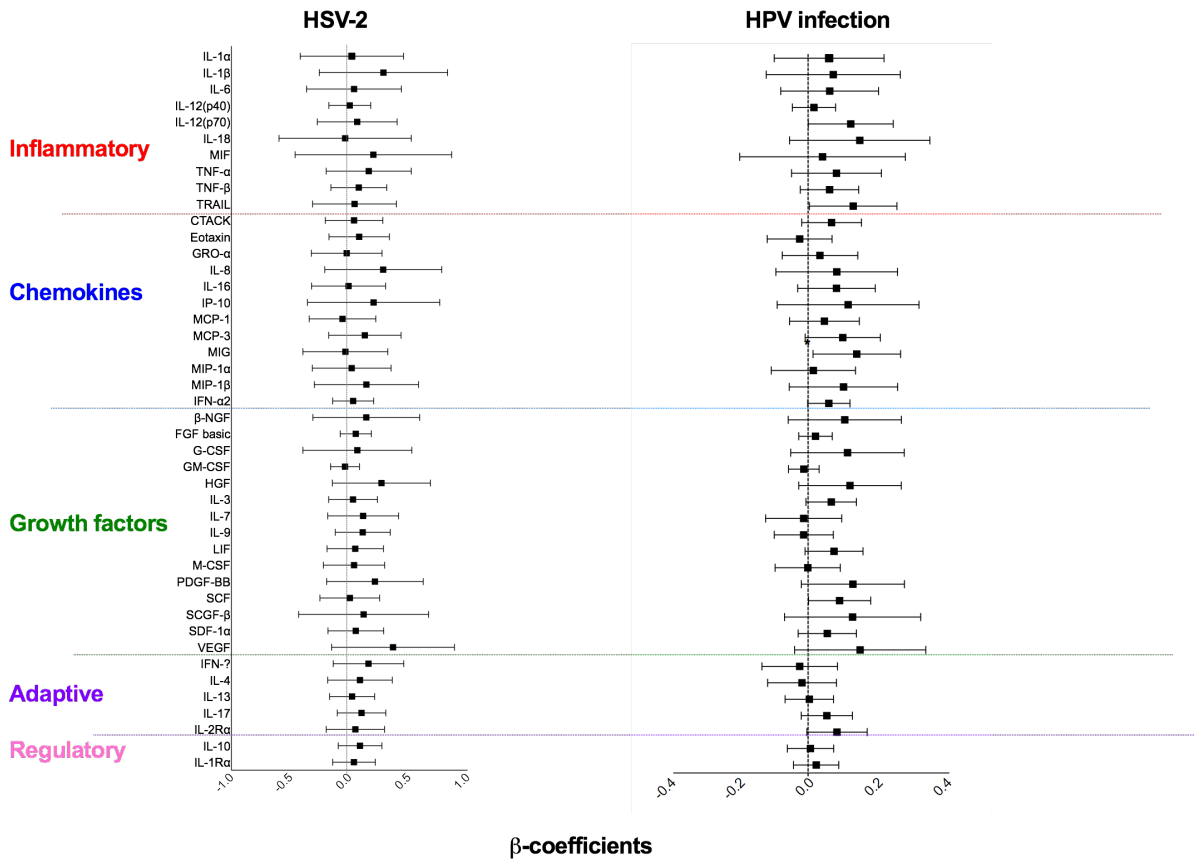


Figure 4.7. Multivariate linear regressions showing the associations between HSV-2 or HPV infection and the 44 cytokines in AGYW from the WISH cohort, adjusting for injectable HC use, genital pH and co-infections. Each association is shown as a β-coefficient and the error bars are the 95% CI. Statistically significant positive associations are shown in red. P values of ≤0.05 are considered significant.

4.3.2.2 HSV-2 infection and genital inflammation

Previous studies have identified the lifetime number of sex partners as the strongest predictor for genital HSV-2 infection (Beauman 2005). Infection occurs via direct contact, and is characterized by periodic symptomatic or asymptomatic viral shedding (Looker *et al*. 2015). Only few adolescents [3% (8/298)] enrolled in this study were actively shedding HSV-2 (DNA+), 5% (7/149) in Masiphumelele and 1% (1/144) in Soweto (Chapter 2), one of whom had ulcers being visible. The prevalence of HSV-2 DNA (viral shedding) was not associated with significant alterations in any of the cytokines measured, overall (Figure 4.7) or in Masiphumelele and Soweto separately (data not shown).

Since active shedding of HSV-2 is episodic and may not fully reflect the burden of HSV-2 in the adolescents, HSV-2 serology was measured in AGYW from Masiphumelele, where plasma was collected. Despite low rates of active HSV-2 shedding, 22% (32/145) of Masiphumelele AGYW

were HSV-2 seropositive, representing previous infections. Being HSV-2 seropositive was associated with significant changes in the genital cytokine profiles of adolescents, with 24/44 cytokines significantly being increased, and 18/44 remained significant after adjusting for multiple comparisons (Figure 4.8). These included SCGF-β (β=0.48; p=0·009), IL-18 (β= 0.47; p=0·011), β-NGF (β=0.42; p=0·007), IL-6 (β=0.39; p<0·0001), G-SCF (β=0.37; p=0·025), HGF (β=0.28; p=0·051), TNF-α (β=0.27; p=0·039), MCP-3 (β=0.26; p=0·020), TRAIL (β=0.26; p=0·025), LIF (β=0.26; p<0·0001), SCF (β=0.24; p=0·006), IL-3 (β=0·24; p<0.0001), IL-2Rα (β=0.24; p=0·008), CTACK (β=0.22; p=0·008), TNF-α (β=0.20; p=0·11), MIP-1α (β=0.17; p=0·009), IFN-α2 (β=0.17; p=0·010), SDF-1a (β=0.16; p=0·049) and IL-12 (p40) (β=0.15; p=0·026). Of the 32 adolescents who were HSV-2 seropositive, 16% (5/32) were actively shedding HSV-2. In this small group, no significant change in genital cytokine profiles was observed compared to the STII-/yeast-/Nugent≤3 AGYW (Figure 4.8).

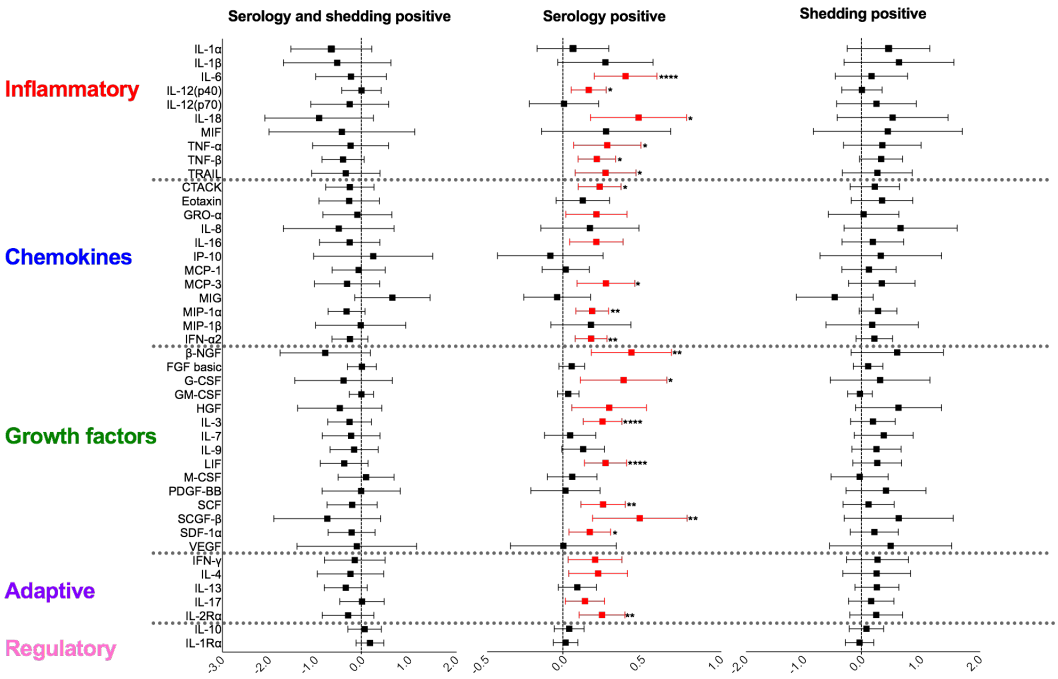


Figure 4.8. Multivariate linear regressions showing the associations between HSV-2 shedding/HSV-2 seropositive, seropositive only, only shedding and the 44 cytokines in Masiphumelele AGYW adjusting for injectable HC use, genital pH and co-infections. Each association is shown as a β-coefficient and the error bars are the 95% CI. Statistically significant positive associations are shown in red* indicate values that remained significant after correction for multiple comparisons, ** indicate values that remained significant after correction for multiple comparisons to more than three decimal places, and **** indicate values that remained significant after correction for multiple comparisons to more than five decimal places. P-values ≤0.05 are considered significant.

4.3.3 BV, yeast and genital inflammation in AGWY

4.3.3.1 BV and genital inflammation

BV was highly prevalent in this cohort (47% overall, Chapter 2, Table 2.2). After correcting for injectable HC use, genital pH and co-infections, BV was associated with more than three quarters of the cytokines that were measured (34/44) being upregulated compared to the control group, after correcting for multiple comparisons (Figure 4.9). These included: IL-1α (β=0.46; p<0·0001), IL-1β (β=0.44; p<0·0001), MIF (β=0.78; p<0·0001), IL-18 (β=0.75; p<0·0001), β-NGF (β=0.47; p<0·0001), TRAIL (β=0.41; p<0·0001), SCGF-β (β=0.35; p=0·001), G-CSF (β=0.35; p<0·0001), LIF (β=0.30; p<0·0001), IL-12p70 (β=0.29; p<0·0001), MCP-3 (β=0.29; p<0·0001), TNF-β (β=0.28; p<0·0001), IL-7 (β=0.28; p<0·0001), IL-2Rα (β=0.27; p<0·0001), PDGF-BB (β=0.26; p<0·0001), HGF (β=0.26; p=0·002), CTACK (β=0.26; p<0·0001), VEGF (β=0.25; p=0·012), IFN-ɣ (β=0.24; p<0·0001), SDF-1α (β=0.23; p<0·0001), M-CSF (β=0.22; p<0·0001), IL-9 (β=0.20; p<0·0001), IL-4 (β=0.19; p<0·0001), IL-3 (β=0.19; p<0·0001), IFN-α2 (β=0.19; p<0·0001), IL-16 (β=0.17; p=0·003), TNF-α (β=0.17; p=0·014), Eotaxin (β=0.16; p=0·002), IL-13 (β=0.13; p<0·0001), IL-10 (β=0.08; p=0·017), IL-12(p40) (β=0.08; p=0·014), IL-1Rα (β=0.07; p=0·034), GM-CSF (β=0.06; p=0·006) and FGF-basic (β=0.06; p=0·020). On the other hand, five chemokines were significantly down regulated, after correcting for multiple comparisons, including: IP-10 (β=-0.61; p<0·0001), MCP-1 (β=-0.17; p=0·002), IL-8 (β=-0.19; p=0·038), GRO-α (β=-0.22; p<0·0001), and MIG (β=-0.48; p<0·0001; Figure 4.9).

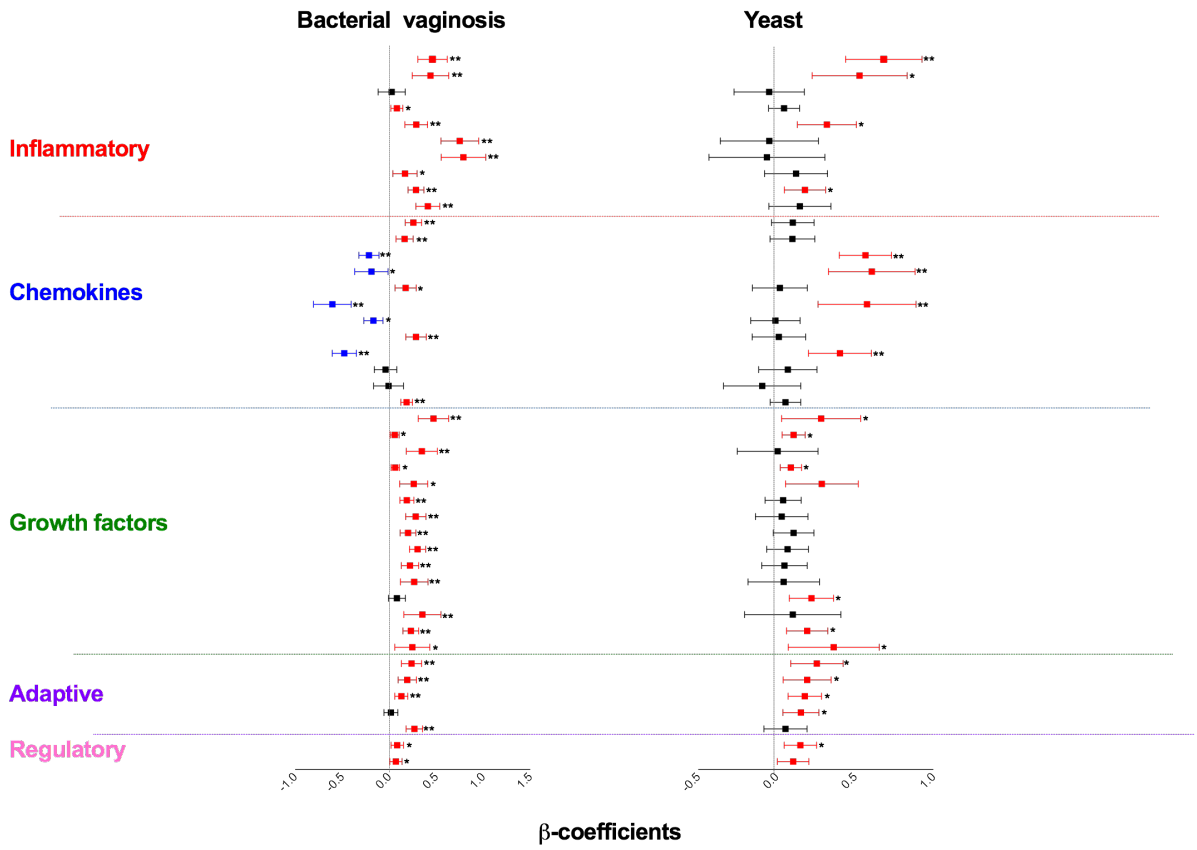


Figure 4.9. Multivariate linear regressions showing the associations between BV or yeast and the 44 cytokines correcting for injectable HC use, genital pH, LH and co-infections. Each association is shown as a β-coefficient and the error bars are the 95% CI. Statistically significant positive associations are shown in red and negative associations in blue. * indicate values that remained significant after correction for multiple comparisons, ** indicate values that remained significant after correction for multiple comparisons to more than three decimal places. P values of ≤0.05 were considered significant.

BV was associated with a similar genital cytokine profiles in adolescents from Masiphumelele and Soweto, with 27/44 and 29/44 cytokines being upregulated, respectively. The chemokines GRO-α, IP-10 and MIG were significantly down regulated at both sites (Figure 4.10).

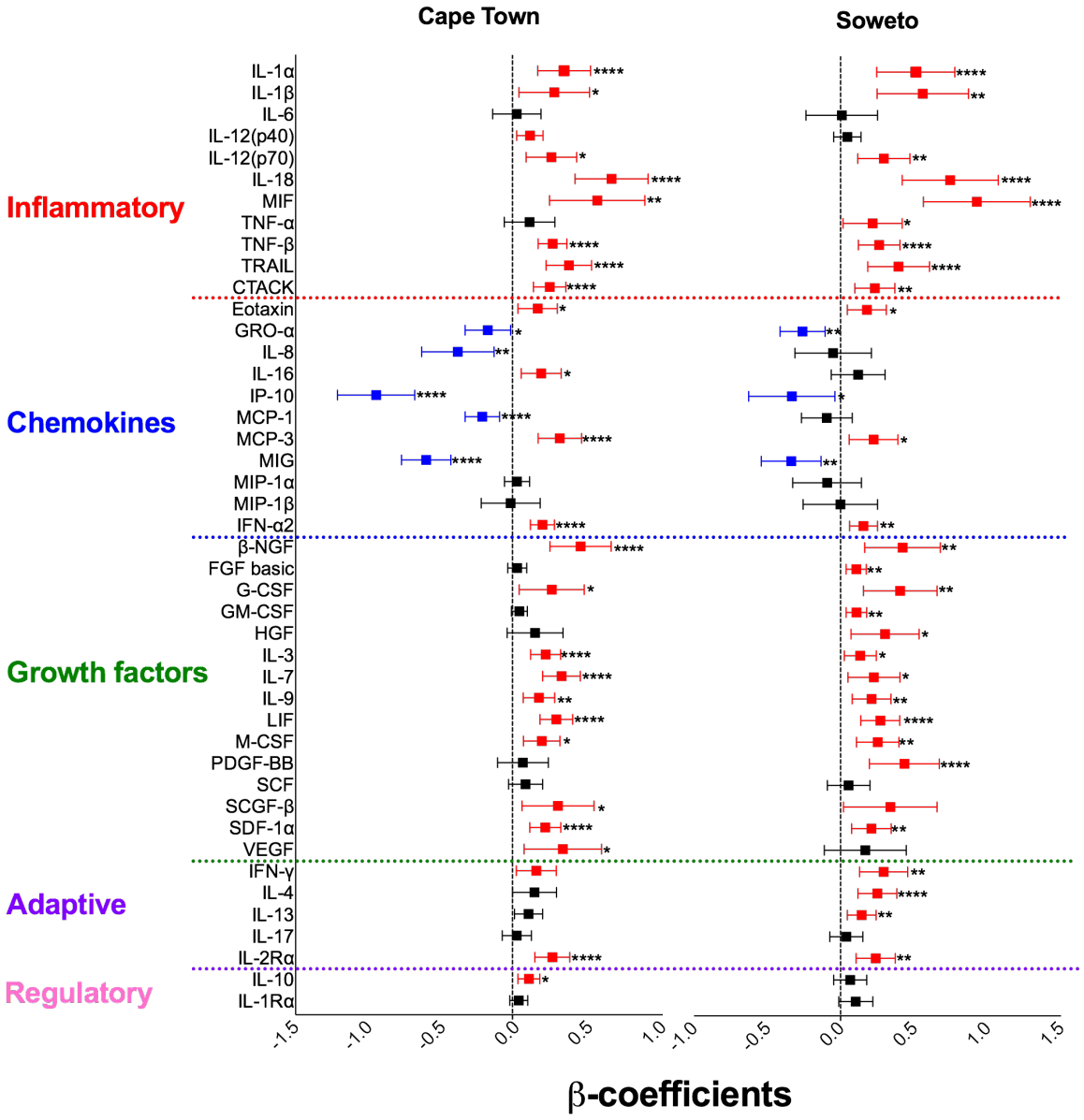


Figure 4.10. Relationship between BV and genital tract cytokines in Masiphumelele (Cape Town) and Soweto, assessed by multivariate linear regression analysis adjusting for injectable HC use, genital pH and co-infections. Each association is shown as a β-coefficient and the error bars are the 95% CI. Significant positive associations are shown in red and negative in blue. * indicate values that remained significant after correction for multiple comparisons, ** indicate values that remained significant after correction for multiple comparisons to more than three decimal places, and **** indicate values that remained significant after correction for multiple comparisons to more than five decimal places. P-values ≤0.05 are considered significant.

BV is thought to be a risk factor for the acquisition of certain other common STIs, including HPV, HSV-2, *N. gonorrhoea* and *C. trachomatis* (Bautista *et al*. 2017). In this study, adolescents with BV were significantly more likely to also be infected with a STI (20.8%; 62/298,) with only 7.2% (21/298) having BV alone (Chi-square p<0·0001). In order to better understand the interaction between STIs and BV, the inflammatory profile of AGYW with (1) BV and *C.*

trachomatis (the most common bacterial STI, Chapter 2), (2) BV and the other STIs and (3) BV, *C. trachomatis* and other STIs was compared. BV alone was more inflammatory than any of these other conditions, either in combination or alone (Figure 4.11).

Further, a comparison was made between the median cytokine levels for BV positive (Nugent 7-10), intermediate microbiota (Nugent 4-6) and BV negative (Nugent$\leq$ 3). Both the BV positive and intermediate microbiota had levels of inflammation that were consistently greater than what was seen in the BV negative group (Figure 4.11).

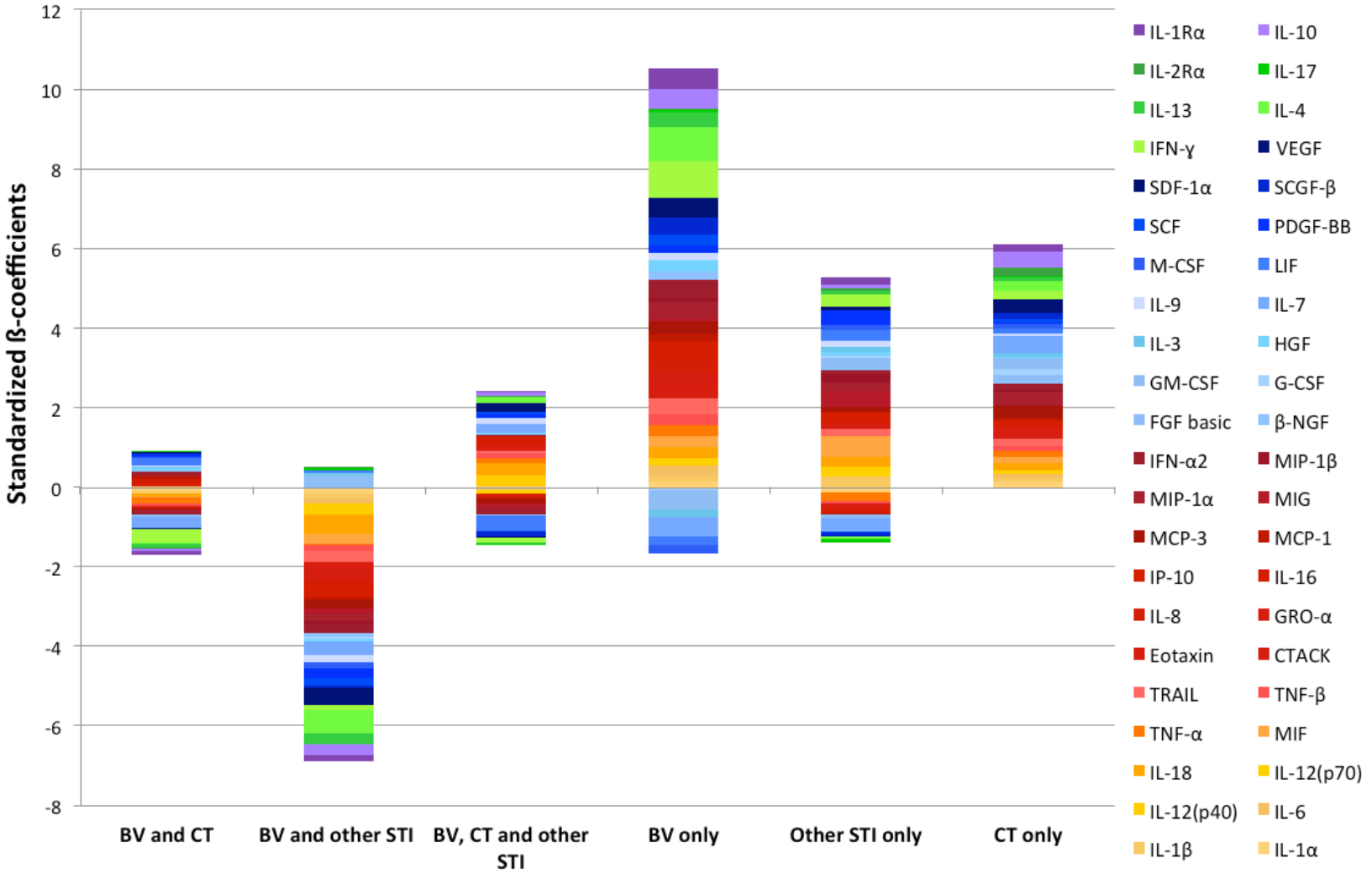


Figure 4.10 Comparison of cumulative genital cytokine concentrations in response to infection with BV, *C. trachomatis* (CT), other STIs (*N. gonorrhea, M. genitalium or T. vaginalis,* and HSV-2) or in combination (BV+CT, BV+STI and BC+CT+STI) in South African AGYW. Inflammatory cytokines are shown in shades of orange, chemokines in shades of red, growth factors in shades of blue, adaptive cytokines in shades of green and regulatory cytokines in shades of purple. Stacked bars of standardized β-coefficients for the regressions between each cytokine, adjusting for injectable HC use, genital pH and co-infections, in AGYW stratified by BV, CT or other STIs are shown.

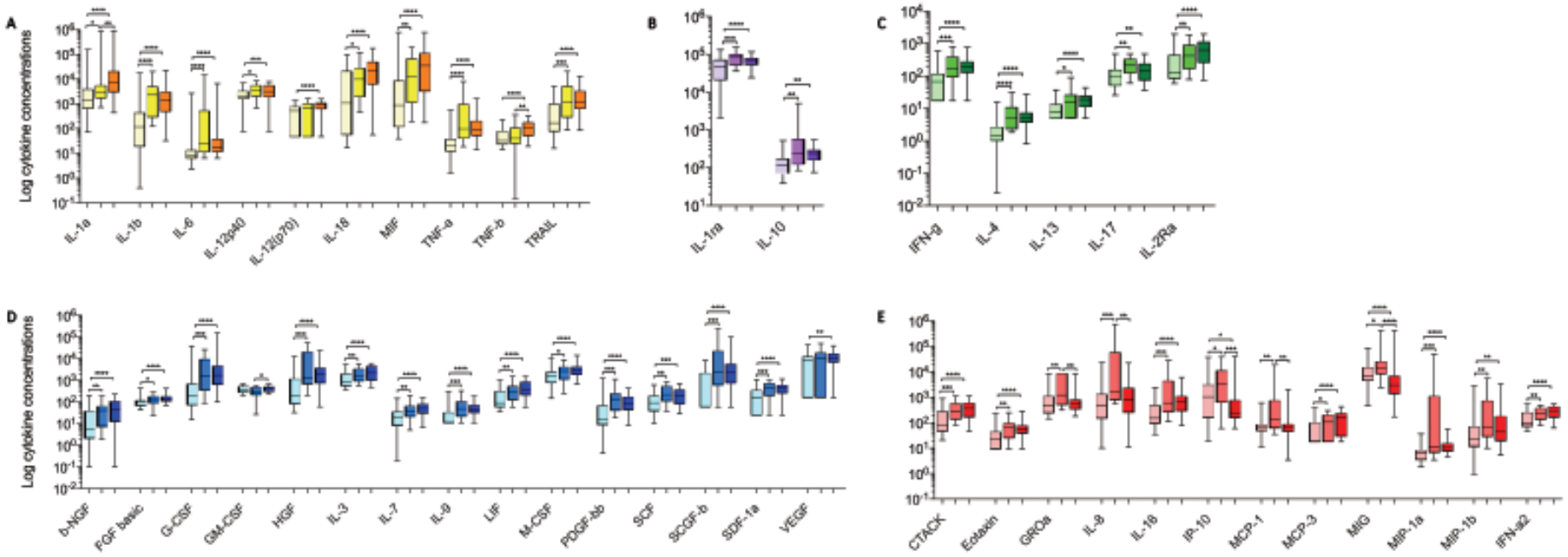


Figure 4.11. Genital cytokine concentrations by BV status in AGYW. The box and whiskers show women using no BV (clear boxes), those with intermediate microbiota (medium boxes), and BV positive (Nugent 7-10; dark boxes). Cytokines were classified as (A) pro-Inflammatory, (B) regulatory, (C) adaptive, (D) growth factors, and (E) chemokines. Mann-Whitney U tests were used to compare median cytokine concentrations. * indicate significant values, ** indicate values significant to more than three decimal places, and **** indicate significant values to more than five decimal places. P≤0.05 was considered significant.

Only seven adolescents were determined to be consistently healthy (STI-/BV-/yeast-) over the course of the study in Masiphumelele, where longitudinal follow-up was possible. Genital tract inflammatory cytokine levels in these healthy adolescents were compared with the six participants who appeared to be BV+ (Nugent 7-10) but with no other co-infections at all three visits (Figure 4.14). AGYW who were persistently BV+ tended to have consistently elevated levels of several cytokines, including the pro-inflammatory cytokine IL-6, the growth factor IL-7 and the adaptive cytokine IL-4, compared to persistently healthy individuals. In contrast, the chemokines IP-10 and MIG were consistently down regulated in those with BV compared to those who remained healthy (Figure 4.12).

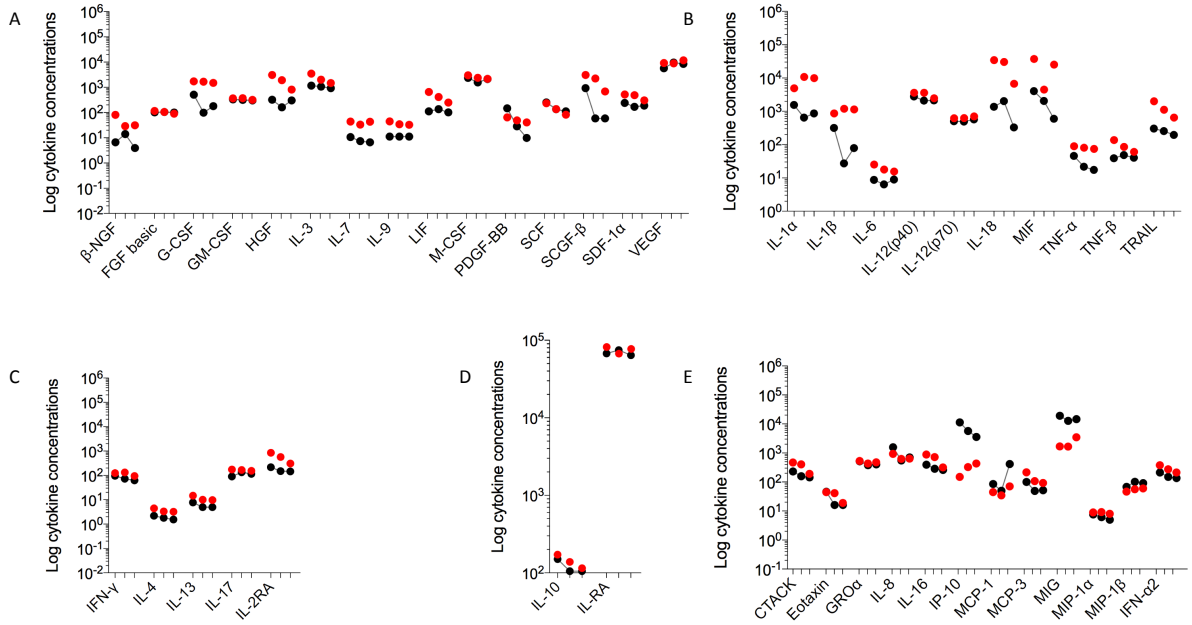


Figure 4.12. Longitudinal profiling of genital cytokine concentrations in adolescents who were BV+ (Nugent 7-10) at all three visits (red) compared to healthy (Nugent≤3/BV-/STI-) at all three visits (black). Cytokines were classified as growth factors (A), inflammatory (B), adaptive (C), regulatory (D,) and chemotactic (E).

Syndromic management of STIs and BV is the standard of care in SA, which is based on women presenting to health care facilities with complaints that trigger a treatment algorithm (The Department of Health 2015). However, it has previously been shown that genital inflammation, and thus HIV risk, is evident in both symptomatic and asymptomatic women (Mlisana *et al*. 2012). To investigate if this was true in this cohort, levels of genital inflammation were compared in symptomatic and asymptomatic women. No differences in the overall genital cytokine profiles were observed in BV+ AGYW with and without symptoms of BV (data not shown).

4.3.3.2 Yeast infections and genital inflammation

Vaginal yeast infection will affect approximately three-quarters of all women during their reproductive years (Dovnik *et al*. 2015), with a high proportion of these being *C. albicans* (Sobel 1997). While the diagnosis is often made clinically, microscopy can be used to detect the mycelia, which is the network of fungus hyphae (Dovnik *et al*. 2015). Approximately 10% (30/291) of AGYW in this cohort had a yeast infection at enrolment, diagnosed from the Gram stain. Microscopic presence of yeast was found to be highly inflammatory, with almost half (21/44) of the cytokines measured being up-regulated, 19 of which remained significant after correcting for multiple comparisons (Figure 4.8). These included IL-1α (β=0.68; p<0·0001), IL-1β (β=0.53; p=0·001), IP-10 (β=0.58, p <0·0001), GRO-α (β=0.57; p <0·0001), IL-8 (β=0.61; p<0·0001), MIG (β=0.40; p<0·0001), IL-12p70 (β=0.32; p=0·001), IL-13 (β=0.18; p=0·001), SCF (β=0.22; p=0·002), IFN-ɣ (β=0.26; p=0·003), FGF basic (β=0.11; p=0·004), SDF-1α (β=0.20; p=0·004), IL-10 (β=0.15; p=0·004), TNF-β (β=0.18; p=0·007), IL-17 (β=0.16; p=0·008), GM-CSF (β=0.09; p=0·009); IL-4 (β=0.20; p=0·012), VEGF (β=0.36; p=0·014) and HGF (β=0.29; p=0·015).

Examining yeast-associated genital inflammation by site, the chemokines GRO-α, IL-8 and IP-10 were elevated in genital secretions collected from both Masiphumelele and Soweto. However, cytokine up-regulation tended to be less pronounced in adolescents from Masiphumelele than Soweto. In addition, IL-17 was up regulated in Masiphumelele only, although this did not remain significant after correcting for multiple comparisons. In Soweto, the cytokine profile was similar to the overall pattern, with 17 cytokines upregulated, 14 of which remained significant after adjustment for multiple comparisons, including: IL-1α (β=0,86; p<0·0001), IL-1β (β=0,67; p=0·002), IP-10 (β=0.51; p=0·012), GRO-α (β=0.57; p<0·0001), IL-8 (β=0.67; p<0·0001), MIG (β=0.47; p=0·001), FGF basic (β=0.15; p=0·002), IL-12p70 (β=0.37; p=0·003), IL-13 (β=0.20; p=0·004), SCF (β=0.28; p=0.005), SDF-1α (β=0.26; p=0·006), IFN-ɣ (β=0.31; p=0·007), IL-10 (β=0.21; p=0·007) and GM-CSF (β=0.12; p=0·015; Figure 4.13).

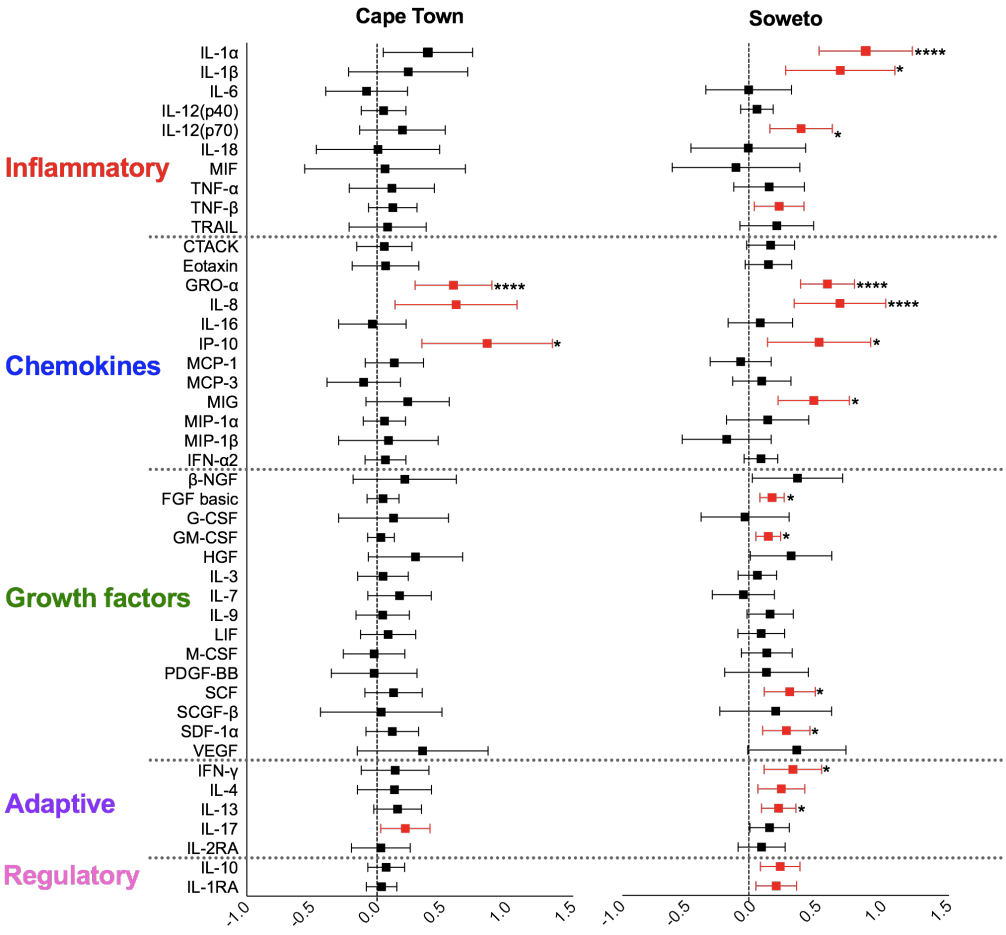


Figure 4.13. Relationship between genital yeast infection and cytokines in AGYW from Cape Town and Soweto, assessed by multivariate linear regression analysis adjusting for injectable HC use, genital pH and co-infections. Each association is shown as a β-coefficient and the error bars are the 95% confidence interval. Statistically significant positive associations are shown in red. * indicate values that remained significant after correction for multiple comparisons, and **** indicate values that remained significant after correction for multiple comparisons to more than five decimal places. P-values of ≤0.05 are considered significant.

4.4 Discussion

This Chapter evaluated some of the non-infective factors that influence inflammation in healthy adolescents from SA, as well as the impact of viral STIs, BV and vaginal yeast infections on the genital inflammatory milieu. Endogenous E2 and progesterone levels in AGYW were lower than those seen in adult women, while LH levels were similar. While it has been previously reported that E2 influences inflammation in a dose-dependent manner, with higher levels causing immune dampening, and lower concentrations inducing inflammation (Straub 2007), no significant association was seen in this cohort in the regression analysis (that controlled for HC use) after adjustment for multiple comparisons.

In the Masson paper (2015) HIV seroconversion was associated with raised genital inflammatory cytokines (including chemokines MIP-1α, MIP-1β, and IP-10). The risk of HIV acquisition was significantly higher in women with evidence of genital inflammation, defined by at least 5 of 9 inflammatory cytokines being raised (Odds Ratio 3.2). Further, genital cytokine concentrations were persistently raised (for about 1 year before infection) with no readily identifiable cause found.

The use of progestin-only HCs like DMPA and Net-En has been shown to almost double the risk of vaginal HIV transmission in observational trials (Heffron *et al*. 2012). Primate studies conducted with SIV suggest that this may be due to the thinning of the vaginal or ectocervical epithelium (Veazey *et al*. 2012). This, however, has yet to be shown in women (Achilles & Hillier 2013; Bahamondes *et al*. 2014; Mitchell *et al*. 2014). While a recent South African study showed that both DMPA and Net-En users had a higher risk of HIV acquisition (Byrne *et al*. 2016), most studies have focused on the risk attributable to DMPA (which tends to be more commonly used) rather Net-En. Systematic reviews by Polis *et al.* (2013 and 2016) reported no significant association between Net-En and increased HIV acquisition, although the trend appeared to be positive (Polis & Curtis 2013; Polis *et al*. 2016). There have, however, been concerns about inferring associations from observational trials, as socio-behavioural biases that influence contraceptive choice may also influence HIV risk. The Evidence for Contraceptive Options and HIV Outcomes (ECHO) study, which is currently enrolling in four counties (SA, Kenya, Zambia and Swaziland), is a randomized control study that will address

some of these socio-behavioural factors (http://echo-consortium.com/). These results are expected in 2019.

An early study of HIV-infected sex workers showed significantly higher genital mucosal levels of TNF-α and IFN-γ compared to HIV-uninfected sex workers and non-sex workers. However, the serum levels of all the cytokines tested were lower in HIV-infected sex workers compared to with the other groups. The increased production of genital mucosal pro-inflammatory cytokines in HIV infected sex worker possibly reflects susceptibility to HIV-1 infection and disease progression/perpetuation at the initial site of exposure (Lajoie et al. 2008)

In the WISH cohort, there appeared to be an association between exogenous hormone contraceptives and inflammation. The most commonly used HC was Net-En, which was associated with an increase in three quarters of the cytokines measured compared to the less commonly used DMPA, which resulted in increased concentrations of half of the cytokines measured. This finding was similar to what was reported by Deese *et al*. (2015) and Morrison *et al*. (2014), who found higher genital concentrations of IL-6, IL-8 and RANTES in DMPA and Net-En users compared to women not on contraceptives, while increases in genital ICAM-1, MIP-1α, MIP-1β, and IFN-γ, and IP-10 were only seen in DMPA users. In addition, Morrison *et al*. (2014) reported decreased concentrations of IL-1RA, a regulatory cytokine, which helps regulate imbalances in tissue inflammation, associated with IL-1 signaling. The association between DMPA use and increase in RANTES concentrations in the genital secretions has also previously been reported (Wira et al. 2011a). In contrast, Ngcapu *et al*. (2015) reported lower levels of IL-12p40, eotaxin (CCL11), MCP-1, MDC, fractalkine, IL- 15 and PDGF-AA in women on injectable contraceptives (both Net-En and DMPA; Ngcapu *et al*. 2015). Interestingly, genital levels of the anti-HIV antimicrobial peptides HBD-2 and -3 were also reduced with DMPA use (Wira et al. 2011b). DMPA is thought to regulate inflammatory genes in the FGT via the glucocorticoid receptor (Govender *et al*. 2014). *In vitro* work has shown that DMPA has a higher binding affinity for the glucocorticoid receptor than Net-En (Koubovec *et al*. 2005), which may cause immune suppression.

The use of DMPA does appear to be negatively associated with BV, since women using DMPA tend to have lower rates of BV than women not using DMPA (Baeten *et al*. 2001; Pettifor *et*

al. 2009; Jespers *et al*. 2014; McClelland *et al*. 2008; Riggs *et al*. 2007; Rifkin *et al*. 2009). Although it is still not clear why women using DMPA would be less likely to develop BV, a possible mechanism could be that the amenorrhea experienced by DMPA users (in whom regular menstruation is not common) is protective, as menstruation has been linked to decreases in *Lactobacillus* numbers and increases in BV-associated organisms (Srinivasan *et al*. 2010). Additionally, a Kenyan study showed a significant decrease in the BV-associated pathogen *G. vaginalis* and an overall decrease in bacterial load after DMPA initiation, but no change in *Lactobacillus* spp. numbers (Roxby *et al*. 2016).

In this study, recent unprotected sex, measured by the presence of PSA in genital secretions, was not associated with any observable change in genital inflammation, unlike what was has been reported in earlier studies (Sharkey *et al*. 2012; Nakra *et al*. 2016). However, vaginal pH in women without vaginal dysbiosis (Nugent 0-3) was associated with an increase in a quarter of the cytokines measured, after adjustment for multiple comparisons. Vaginal pH is a relatively sensitive although non-specific method to detect BV (Hemalatha *et al*. 2013). However, vaginal pH may be associated with ethnicity, with Ravel *et al*. (2011) describing a higher pH in African American and Hispanic women, than their Asian American or white counterparts. A higher vaginal pH has been associated with an increased in IL-1β (Hemalatha *et al*. 2012).

Active HSV-2 shedding was relatively rare in this cohort of adolescents (5%; 7/149 in Masiphumelele), although 22% (32/145) were seropositive. In those who were determined to be actively shedding HSV-2, no significant change in genital cytokine levels were noted, despite previous reports of an increase in TNF-α, IL-6, IL-8, IL-10, IFN-ɣ and CCL2 with HSV-2 re-activation (Ferreira *et al*. 2013; Martha Stefanidou *et al*. 2013; Reske *et al*. 2008). A study in South African adult women showed that active HSV-2 shedding downregulated markers of inflammation (Masson *et al*. 2014). In contrast, adolescents who were HSV-2 seropositive had quite a substantial increase in genital cytokines, with 40% (18/44) of those measured being higher than healthy adolescents, after adjustment for multiple comparison.

Vaginal yeast infections were found to be inflammatory in these adolescents, and associated with an increase in almost half of cytokines measured. Cytokine profiles did appear to differ

by geographic location, being more inflammatory in Soweto than Masiphumelele adolescents. Most symptomatic fungal infections of the FGT are due to *C. albicans* (Pirotta & Garland 2006) though high rates (41.6%) of non-*albicans Candida* species has been reported in Ethiopian adult women (Bitew & Abebaw 2018), thus it is possible that differences in inflammation were due to a variety of fungal species causing infection. Yeast infections can induce a strong immune response via triggering the hosts pattern recognition receptors on innate cells (Sobel 2016), and yeast infections have been associated with a two-fold increase in HIV risk (Johnson and Lewis, 2008). This could partly be due to the increased levels of inflammatory cytokines and chemokines secreted in response to the infection, in addition to causing disruption of the epithelial barrier function.

The condition that was associated with the most pronounced effect on genital inflammation was BV. BV is consistently found to be a risk factor for HIV acquisition (Atashili *et al*. 2008; Mirmonsef *et al*. 2012), STI acquisition (Abbai *et al*. 2016; Bautista *et al*. 2017) in addition to adverse pregnancy outcomes (Nelson *et al*. 2014; Haggerty *et al*. 2004; Govender *et al*. 1996; Clark *et al*. 1994). While BV is highly prevalent in Africa (Cohen *et al*. 2012; Abbai *et al*. 2016), it is often asymptomatic (Koumans *et al*. 2007; Klebanoff *et al*. 2004) and has no effective durable treatment (Bradshaw & Sobel 2016; Bradshaw *et al*. 2006; Bradshaw & Brotman 2015; Wilson 2004). In this cohort, BV was associated with a significant increase in more than three quarters of the cytokines from all functional classes tested (pro-inflammatory, chemokines, growth factors, adaptive and regulatory), but a significant decrease in several of the chemokines (IP-10, MCP-1, IL-8, GRO-α, and MIG). This chemokine downregulation may reflect tolerance to microbes that may be derived from gut flora (Danielsson, Teigen and Moi, 2011). A decrease in IP-10 and MCP-1 has previously been reported in South African women with BV (Masson *et al*. 2015; Masson *et al*. 2014), while *Prevotella bivia*, a common BV species, has been shown to suppress IP-10 *in vitro* (Fichorova *et al*. 2013). An increase in levels of pro-inflammatory cytokines, such as TNF-α, TNF-β, IL-1α and IL-1β, has also previously been associated with BV in other studies (Thurman *et al*., 2015; Cauci *et al*., 2003; Masson *et al*., 2015; Arnold *et al*., 2016). A study of adult Kenyan women also found that BV was associated with an increase in the growth factor GM-CSF, which was seen here, but also in the adaptive cytokine IL-17 and the chemokine IL-8 (Arnold et al. 2016), both of which were decreased in

this study. It is possible that lipopolysaccharides from bacteria may downregulate chemokines levels (Bennett *et al*. 2011).

Co-infection with STIs and BV were common in both cohorts of adolescents. Surprisingly, co-infection did not increase levels of genital inflammation, with the highest levels seen with BV alone, in comparison to either BV with *C. trachomatis,* or BV with another STI, or BV, *C. trachomatis* and another STI. This has been previously reported in pregnant women (Cauci & Culhane 2007). It is possible that co-infection resulted in more immune tolerance due to bacterial adaptation and/or immune evasion mechanisms found in *C. trachomatis* (Brunham *et al*. 2005), such as the suppression of the NF-κB activation signalling pathway, down regulation of HLA Class I expression, and degradation of other host proteins to evade host defenses (Ibana *et al*. 2011; Le Negrate *et al*. 2008; Zhong 2009).

Limitations of this Chapter include that while cervical ectopy has previously been associated with an increase in the proinflammatory cytokines IL-1 β, IL-6, IL-12(p70), MIP-1β, IL-8 and G-CSF it was not assessed in this study (Kyongo *et al*. 2015). Adjustment for co-infections was necessary in linear regressions when determining the effect of viral STIs, BV and yeast on the genital mileu, as the numbers of AGYW who had single infections (but no other STIs, or yeast, or BV) were too small. Further, the data was not longitudinal on both sites, thus the effect of persistent BV on genital inflammation could only be evaluated in the Masiphumelele cohort. Despite being asked not to be engaged in sexual intercourse 48 hours before genital sampling, approximately 20% were PSA positive, which may have influenced cytokine levels (although no clear differences were seen between PSA+ and PSA1 AGYW in this Chapter).

In summary, Net-En, fungal infections and BV were most strongly associated with genital inflammation while, endogenous hormones, HSV infection and semen did not seem to influence genital inflammation as markedly. The effect of Net-EN was more pronounced than was seen with DMPA. There was a high prevalence of HPV and BV seen in this cohort of young women. While HPV and HSV shedding did not influence genital inflammation markedly, yeast infections and BV did. BV was the most inflammatory condition examined with similar levels of inflammation seen in Masiphumelele and Soweto, and amongst symptomatic and asymptomatic women. The generation of a strong inflammatory response and the possible

recruitment of highly activated cells to the genital mucosa would put these BV positive adolescent women at an even higher risk of subsequent HIV acquisition.

Chapter 5

Evaluating the effect of bacterial and parasitic STIs on cytokine markers for genital inflammation in South African AGYW

5.1 Introduction

Pathogen clearance begins with the recognition of PAMPS, recognized by PRR which results in the production of inflammatory proteins (Svanborg, Godaly & Hedlund 1999), although this can also result in upregulation of immune receptors, recruitment and activation of inflammatory cells, and tissue destruction (Darville & Hiltke 2010). STIs are especially prevalent during adolescence when sexual contact is often not planned, condom use is inconsistent, immunity to sexually-acquired infections is low, and stable partnerships are not common (Pettifor *et al*. 2004; Marston & King 2006; Shisana *et al*. 2014). The most common bacterial STI reportedly acquired by adolescents is *C. trachomatis* (Walker et al. 2012; Lee et al. 2006). The VOICE study, carried out in adult women (mean age 25.3 years) in three African countries including SA, Uganda and Zimbabwe, showed the highest *Chlamydia* incidence in SA (14% compared to 6% and 3%, respectively), with most infections being asymptomatic (Chirenje et al. 2017).

Bacterial STIs are known to increase the risk of HIV transmission - up to 2-fold with *C. trachomatis* infections (Johnson & Lewis 2008) - and are thought to increase HIV risk in women. Their influences on HIV risk are likely to differ based on their individual pathogenic mechanisms. *C. trachomatis* and *N. gonorrhoea* can directly disrupt the columnar epithelial layer of the endocervix or upper reproductive tract (Brunham & Rey-Ladino 2005; Prozialeck et al. 2002; Sun & Schoborg 2008). An early study by Coombs *et al*. (2003) showed that STIs may also result in the recruitment of activated T-cells to the mucosa, including CD4+ T-cells, thereby increasing the chance of HIV encountering its host target cell and establishing infection.

STIs cause genital inflammation and are associated with increased susceptibility to HIV infection (Mlisana *et al*. 2012). Masson *et al*. (2015) found that chemokines IP-10, MIP-1α, MIP-1β, and IL-8 in genital secretions were associated with >3-fold higher HIV risk in South African women. Furthermore, increased concentrations of the pro-inflammatory cytokine IL-1β in genital secretions have also been associated with increased production of the vaginal enzymes sialidase and prolidase, frequently associated with BV (Cauci & Culhane 2007). These may, in turn, be able to degrade important mucosal protective factors, such as cyclic

adenosine monophosphate (cAMP; (Yudin et al. 2003), a secondary messenger that mediates numerous biological responses via cAMP-dependent protein kinase A (Mathiesen et al. 2013; Keshwani et al. 2015) and may help suppress HIV replication (Moreno-fernandez *et al*. 2011). Female genital mucosal inflammation has also been associated with altered expression of mucosal barrier proteins, such as Coronin-1A, Filamin-A, and Fibrinogen beta chain, and the influx of HIV-susceptible target cells (Arnold *et al*. 2015).

The establishment of a productive SIV infection in the genital tracts of vaginally-challenged rhesus macaques was dependent on the production of inflammatory cytokines, such as MIP-1α, MIP-1β, and IL-8 (Li *et al*. 2009). These inflammatory cytokines were shown to recruit CD4+ T-cell targets to the site of infection, facilitating local SIV infection and systemic spread. Blocking these inflammatory cytokines using an immune dampening gel like glycerol monolaurate (GML), which reduced IL-8 concentrations, protected macaques from becoming infected (Li *et al*. 2009).

In humans, genital inflammation may directly stimulate HIV replication through activation of the nuclear factor NF-κB (Osborn, Kunkelt & Nabel 1989). Alternatively, genital inflammation may be an intermediate step in increasing susceptibility to HIV infection by altering mucosal barrier function (Arnold *et al*. 2016), or by facilitating chemotaxis of susceptible HIV target cells into the genital tract (Levine *et al*. 1998).

For this study, it was hypothesized that STIs are a major cause of inflammatory cytokine up-regulation in the human FGT, thereby increasing the risk of HIV acquisition. Thus, the aim of this Chapter was to examine the role of bacterial and parasitic STIs (with *C. trachomatis, N. gonorrhoeae, T. vaginalis* and *M. genitalium*) in perturbation of cytokine biomarkers of genital inflammation. This Chapter evaluates the effects of prevalent bacterial and parasitic STIs on the inflammatory cytokine profile of the lower genital tract of South African AGYW. Defining the way in which STIs impact the mucosal immune system and potentially increase HIV risk in young South African women is crucial in ameliorating the high prevalence rates of STIs in SA.

5.2 Methods

5.2.1 Cohort description

There were 298 HIV negative, young women between the ages of 16 and 22 years recruited from two sites – Masiphumelele and Soweto (as described in detail in Chapter 2, section 2.2.1). Thirty-five women (12 Masiphumelele and 23 Soweto) were STI-, BV- (Nugent 0-3) and yeast-negative and served as controls.

5.2.2 Ethics statement

Ethics approval was obtained from the Human Research Ethics Committee of the University of Cape Town (UCT HREC, reference 267/2013) and University of Witwatersrand Research Ethics Committees (Clearance certificate number: M1307) for this study (Chapter 2, section 2.2.2).

5.2.3 Cytokine evaluation

Cervicovaginal mucus was collected from each participant for evaluation of genital cytokines using a disposable menstrual cup (Softcup®, Evofem Inc). The cytokines measured included FGF-basic, Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1β, IL- 1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, IP-10, MCP-1 [MCAF], MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, VEGF, IL-1α, IL-2RA, IL-3, IL-12(p40), IL-16, IL-18, CTACK, GRO-α, HGF, IFN-α2, LIF, MCP-3, M-CSF, MIF, MIG, β-NGF, SCF, SCGF-β, SDF-1α, TNF-β, and TRAIL (described previously in detail in Chapter 4, section 4.2.4).

5.2.4 Screening for STIs, BV and yeast

Multiplex PCR was carried out on fluids collected using a vulvovaginal swab taken from all participants (n=298) to screen for the STIs *C. trachomatis, N. gonorrhoeae, T. vaginalis, M. genitalium, H. ducreyi, T. pallidum and C. trachomatis serovars* L1-L3 (lymphogranuloma venereum serovars), as described in detail in Chapter 2, section 2.2.8.1. BV Nugent scoring and inspection of fungal infections was performed using wet mount slides after Gram staining (Chapter 2, section 2.2.8.2).

5.2.5 MLST of *Chlamydia* isolates

To evaluate sequence types of *C. trachomatis* circulating in women enrolled in the study, MLST was performed at the Geneeskundige en Gezondheids Dienst (GGD), Amsterdam by Drs Bart Versteeg and Sylvia Bruistein on samples positive for *C. trachomatis*. As described previously by Klint *et al*. (2007a), MLST is a high-resolution genotyping system based on five target (non-housekeeping genes: hctB, CT058, CT144, CT172 and pbpB) regions and the ompA gene. PCR amplification, sequencing and genotyping of these five variable regions and the ompA sequence were carried out according to the protocol published on the high resolution-MSLT database website (http://mlstdb.bmc.uu.se/). The primer-to-primer sequences were checked against the *C. trachomatis* high resolution-MLST database (http://pubmlst.org/chlamydiales/) for allele comparison and sequence type (ST) allocation. Only samples for which all six loci were correctly amplified and sequenced were used to obtain a full high resolution-MLST sequence type (Herrmann *et al*. 2015; Versteeg *et al*. 2017). The BioNumerics software version 7 (Applied Maths, Sint-Martens-Latem, Belgium) was used to construct a minimum spanning tree of all the sequence types in the dataset.

5.2.6 Measurement of cotinine as a biomarker for smoking

Cotinine levels were measured from blood plasma using ELISA, as described in Chapter 2, section 2.2.7.2.

5.2.7 Measurement of PSA as a biomarker for unprotected sex

The presence of PSA was measured from genital secretions using ELISA, as described in Chapter 2, section 2.2.8.3.

5.2.8 Measurement of endogenous sex hormones

The concentrations of E2, progesterone and LH were measured from blood plasma, as described in Chapter 2, section 2.2.7.1. These levels would represent hormone concentrations during the luteal phase of the menstrual cycle in women not using HCs and those using COCs, or two weeks after their injection for women using Net-En or DMPA.

5.2.9 Statistical analyses

All statistical analyses and multivariate models were carried out using STATA 12.0 (Stata Corp, College Station, TX, USA). Graphs and figures were generated using Graphpad Prism 6 (GraphPad Software, San Diego, CA, USA). Mann-Whitney U tests were carried out to compare two groups of non-parametric continuous variables, while the two-sided Fisher's exact test was used for categorical variables. The Kruskal-Wallis one-way analysis of variance was used for variables with three of more categories. Multivariate logistic regressions were carried out to assess the impact of bacterial and parasitic STIs on genital inflammation using a step-up strategy where the different variables that could potentially confound the relationship between BV/STIs/yeast infection and inflammation were included one at a time, and R^2 values were compared as a measure of model fit. To assess statistical significance, 95% confidence intervals and p values of ≤0.05 were used.

5.3 Results

Of the 298 AGYW enrolled in the WISH cohort (described in Chapter 2), 39% (115/298) had at least one STI (including *C. trachomatis, N. gonorrhoeae, T. vaginalis, M. genitalium*, and HSV-2), with 10% having multiple infections (30/298). *C. trachomatis* was the most common infection in adolescents in both Masiphumelele and Soweto, with 42% (62/149) and 17% (26/149) being infected at their enrolment, respectively (p<0·0001). Of those with any STI, 21% also had concurrent BV (Nugent 7-10; 62/298), VVC (11/298) or both (5/298). The prevalence of *T. vaginalis* (16/298) and *M. genitalium* (11/298) were similar between the two sites. Women from Masiphumelele were more likely to have multiple STIs than those from Soweto (16% vs. 4%; p=0·001), or have BV at the same time as a STI (28% vs. 14%; p=0·006; Table 5.1). There were no cases of *H. ducreyi*, *T. pallidum* or *C. trachomatis* serovars L1-L3 in the cohorts. There were 35 women (12 Masiphumelele and 23 Soweto) who were STI negative, had no yeast infections and a Nugent score ≤3 who were used as controls.

Adolescents with bacterial or parasitic STIs (either *C. trachomatis, N. gonorrhoeae, T. vaginalis* or *M. genitalium*) were slightly older than those without any infection (STI-/BV/yeast-controls; median 19 years [IQR 18-20] vs. 18 years [IQR 17-20], p=0·042). These adolescents with any bacterial STI had similar vaginal pH (median 4.7 vs. 4.6) and similar rates of detectable PSA (17.6% vs. 16.4%) as those without a STI. They were also more likely to report using any injectable contraceptive (DMPA and Net-EN) than those in the control group (69% vs. 40%, p=0.010), although this was not significant when the analysis focused on individual contraceptives (DMPA p=0·241 or Net-En p=0·052). There was lower reported condom use in the STI+ than STI- adolescents (27% vs. 54%, p=0·006).

Table 5.1. Prevalence of STIs, BV, yeast and co-infections in AGYW from Masiphumelele and Soweto at enrolment

Characteristics	All participants n=298	Masiphumelele n=149	Soweto n=149	p-value
Frequencies of STIs [%(n/N)]				
Any STI	39 (115/298)	52 (77/149)	26 (37/149)	**<0·0001**
C. trachomatis	30 (88/298)	42 (62/149)	17 (26/149)	**<0·0001**
N. gonorrhoea	8 (24/298)	11 (17/149)	5 (7/149)	**0·053**
T. vaginalis	5 (16/298)	7 (11/149)	3 (5/149)	0·198
M. genitalium	4 (11/298)	4 (6/149)	3 (5/149)	>0·999
HSV-2	3 (8/298)	5 (7/149)	1 (1/149)	**0·067**
Multiple STIs	10 (30/298)	16 (24/149)	4 (6/149)	**0·001**
Median number STIs (IQR)	0 (0-3)	1 (0-3)	0 (0-2)	-
Co-infections [%(n/N)]				
STIs+BV	21 (62/298)	28 (41/149)	14 (21/149)	**0·006**
STIs+Yeast	4 (11/298)	3 (4/149)	5 (7/149)	0·541
STIs+BV+Yeast	2 (5/298)	2 (3/149)	1 (2/149)	>0·999
No STI/BV/Yeast [%(n/N)]	12 (35/298)	15 (23/149)	8 (12/149)	0·071

Values in bold indicate those that were considered significant.

5.3.1 Genital inflammatory cytokine profiles associated with bacterial and parasitic STIs in South African AGYW

Adolescents enrolled into this cohort were relatively close to sexual debut with few sexual partners, and it was therefore interesting to investigate their host immune responses to common STIs. To investigate the effect of various bacterial and parasitic STIs on their genital inflammatory milieu, cytokine profiles for each individual STI (including *C. trachomatis, N. gonorrhoeae, T. vaginalis,* and *M. genitalium*) were evaluated compared to the control (STI-/yeast- and Nugent score ≤3) group. After correcting for potential confounders (including injectable HC use, genital pH and co-infections), *C. trachomatis* and *T. vaginalis* were the two most inflammatory genital infections of those evaluated, with 23/44 of the cytokines that were measured being elevated with either STI (Figure 5.1). *C. trachomatis* infections in this young cohort were associated with significantly increased concentrations of IL-1β (β=0.37; p=0·001), IP-10 (β=0.31; p=0·006), HGF (β=0.28; p= 0.001), G-CSF (β=0.28; p=0·002), MIG (β-estimate=0.27; p<0·0001), IL-18 (β=0.25; p=0·022), TNF-α (β=0.24; p=0·001), β-NGF (β=0.22; p=0·014), IL-6 (β=0.22; p= 0·007), IL-8 (β=0.19; p=0·046), IL-4 (β=0.17; p=0·001), IFN-ɣ (β=0.17; p=0·004), TRAIL (β=0.17; p=0·015), GRO-α (β=0.15; p=0·014), SCF (β=0.14; p=0·005), MIP-1α (β=0.13; p=0·045), LIF (β=0.12; p=0·013), IL-9 (β=0.11; p=0·019), IL-2Rα (β=0.11; p=0·027), TNF-β (β=0.10; p=0·024), IL-3 (β=0.10; p=0·019), IL-1RA (β=0.09; p=0·017) and IFN-α2 (β=0.08; p= 0·016). Similarly, *T. vaginalis* was associated with significantly elevated genital

concentrations of IL-1β (β=0.71; p=0·001), MIF (β=0.70; p=0·007), IL-1α (β=0.69; p<0·0001), HGF (β=0.66; p<0·0001), G-CSF (β=0.48; p=0·007), GRO-α (β=0.48; p<0.0001), VEGF (β=0.47; p=0·02), SCGF-β (β=0.46; p=0·032), β-NGF (β=0.45; p=0·01) ,IL-16 (β=0.37; p=0·003), TRAIL (β=0.31; p=0·026), IFN-ɣ (β=0.29; p=0·014), MIG (β=0.28; p=0·043), TNF-α (β=0.28; p=0·046), TNF-β (β=0.27; p=0·004), SCF (β=0.23; p=0·022), IL-2Rα (β=0.22; p=0·025), SDF-1α (β=0.21; p=0·026), LIF (β=0.20; p=0·033), IL-3 (β=0.16; p=0.05) ,IFN-α2 (β=0.14; p=0·043) and FGF basic (β=0.11; p=0·032). In contrast, infections with *N. gonorrhoeae* (n=24) and *M. genitalium* (n=11) were not associated with evident changes in genital cytokine concentrations, compared to "healthy" AGYW (Figure 5.1).

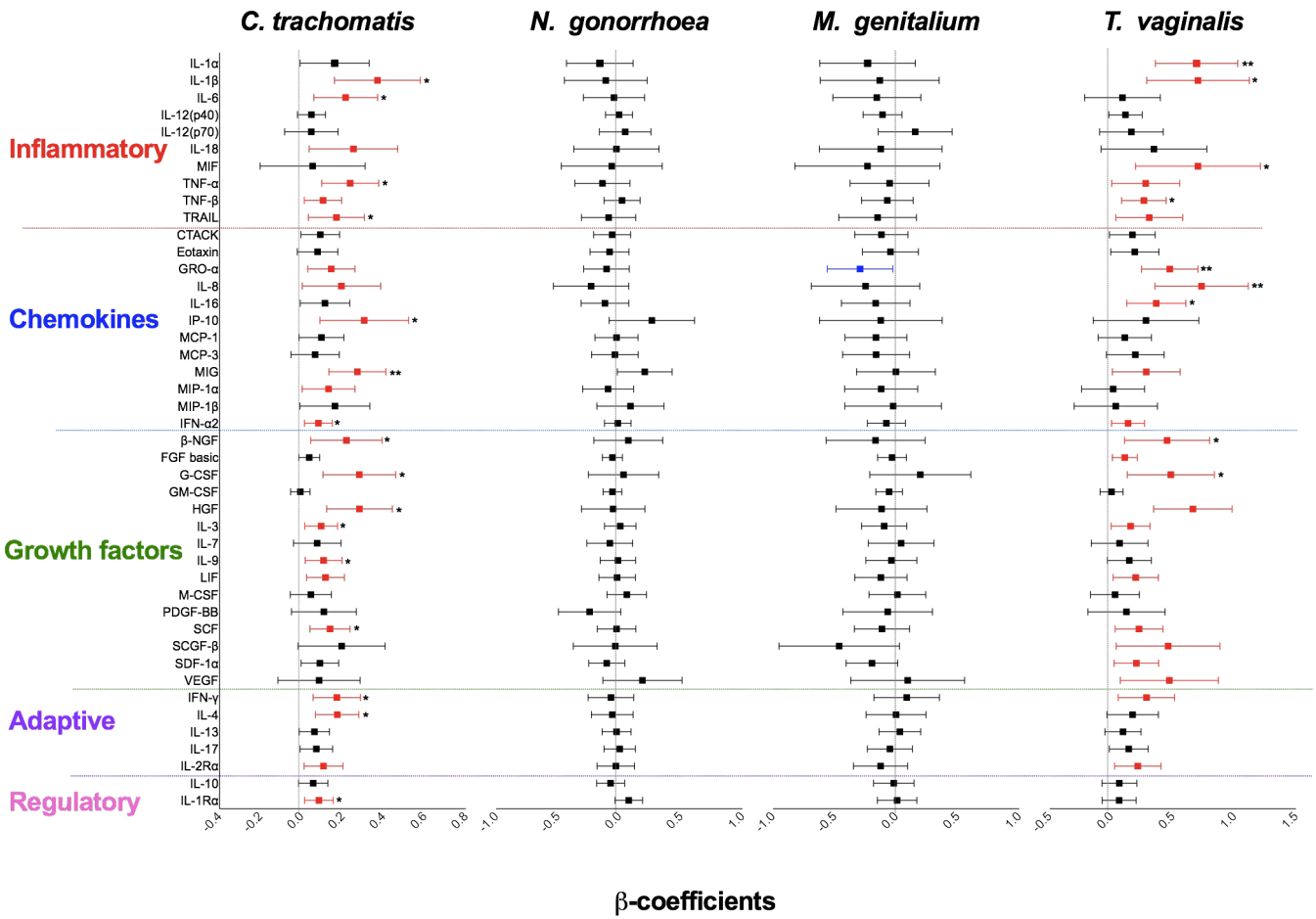


Figure 5.1. Impact of *C. trachomatis* (n=88), *N. gonorrhoea* (n=24), *T. vaginalis* (n=16), and *M. genitalium* infections (n=11) on concentrations of inflammatory, regulatory, adaptive cytokines, chemokines and growth factors in genital tract secretions from AGYW, evaluated by multivariate linear regressions (adjusting for vaginal pH, injectable contraceptives, co-infection with *C. trachomatis, N. gonorrhoea, T. vaginalis* , and *M. genitalium,* HSV-2, yeast *or* BV). Each association is shown as a β-coefficient and the error bars are the 95% CI. The β-coefficient indicates the degree of change in the individual cytokine concentrations for a positive STI result compared to AGYW who were STI-/yeast- and had a Nugent score ≤3. Statistically significant positive associations are shown in red and negative associations in blue. * indicate values that remained significant after correction for multiple comparisons; ** indicate values that remained significant after correction for multiple comparisons to more than three decimal places. P-values ≤0.05 are considered significant.

5.3.2. Genital cytokine profiles associated with *M. genitalium* infections in AGYW by site

M. genitalium is a common cause of cervicitis and upper genital tract infections in women (Couldwell & Lewis 2015), and adhesion is the primary mechanism of pathogenesis of *M. genitalium* (Sethi *et al*. 2012), although inflammatory host immune responses may exacerbate pathogenesis (Taylor-Robinson & Jensen 2011). Despite commonly causing cervicitis, *M. genitalium* infections in this cohort of adolescents did not substantially alter inflammatory cytokine signatures in cervicovaginal secretions collected from the lower reproductive tract, with the chemokine GRO-α being significantly lower in those who were infected (p=0·028; Figure 5.1), although not after correction for multiple comparisons. In Masiphumelele, GM-CSF (β=-0.23; p=0·001) and GRO-α (β=-0.40; p=0·044) were downregulated compared to women without STIs, yeast and a Nugent score ≤3. In Soweto, SDF-1α (β=-0.37; p=0·026) alone was down regulated. Only down-regulation of GM-CSF concentrations in Masiphumelele adolescents remained significant after correction for multiple comparisons (p=0·044; Figure 5.2).

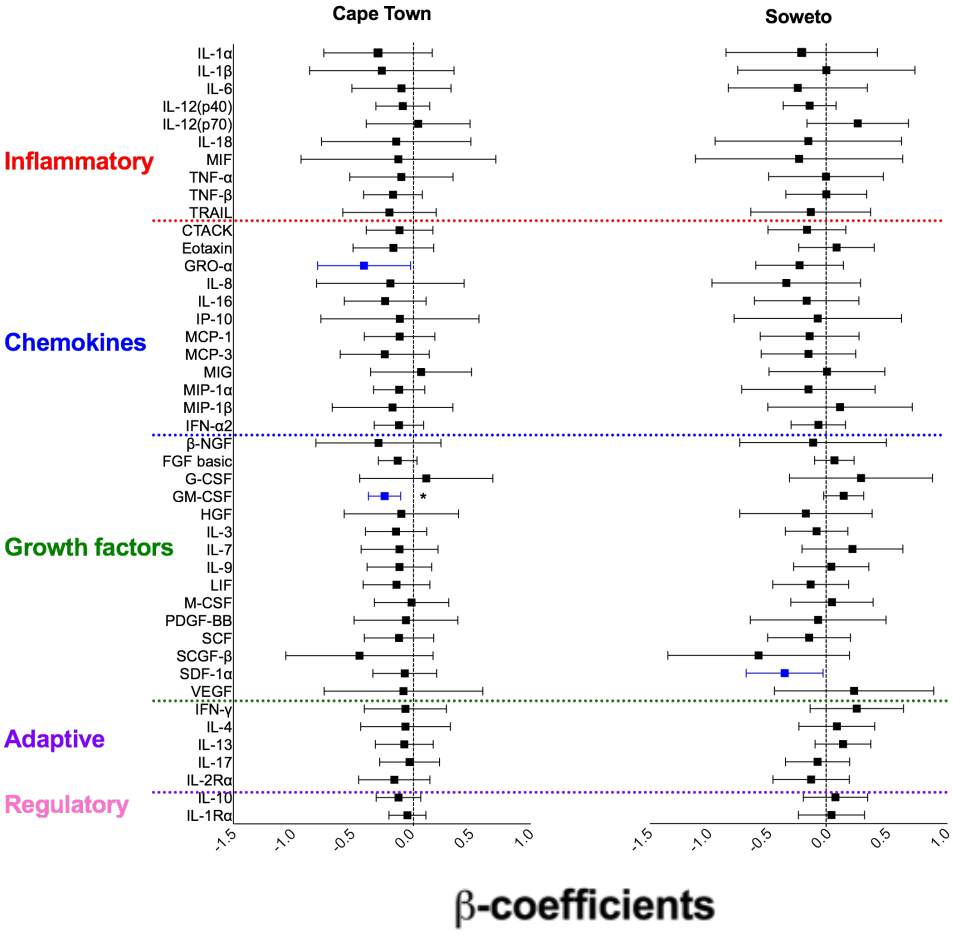


Figure 5.2. Impact of *M. genitalium* infection on lower genital tract cytokines in AGYW from Masiphumelele (Cape Town) and Soweto. The impact was assessed using multivariate linear regressions (adjusting for vaginal

pH, injectable contraceptives, co-infection with *C. trachomatis, N. gonorrhoea, T. vaginalis,* HSV-2, yeast and BV). Each association is shown as a β-coefficient and the error bars are the 95% CI. The β-coefficient indicates the degree of change in the individual cytokine concentrations for a positive *M. genitalium* result compared to AGYW who were STI-/yeast- and had a Nugent score ≤3. Significant positive associations are shown in red and negative associations in blue. P values ≤0.05 are considered significant.

5.3.3 Genital inflammatory profiles associated with *N. gonorrhoea* in AGYW by site

N. gonorrhoea infections are asymptomatic approximately half of the time in adult women, but typical symptoms in those who are symptomatic include a mucopurulent discharge, with evidence of a friable cervix on speculum examination (Chan *et al*. 2012). *N. gonorrhoea* infections tend to elicit a stronger host inflammatory response than *C. trachomatis* infections in symptomatic women (Hook & Handsfielsd 2007). However, while *N. gonorrhoea* was the second most prevalent STI in this cohort of AGYW (8.1%, 24/298), infections were typically asymptomatic (Chapter 2, section 2.3.4), and did not significantly impact the genital inflammatory milieu in the cohort as a whole (Figure 5.1) as well as in Masiphumelele and Soweto individually (Figure 5.3).

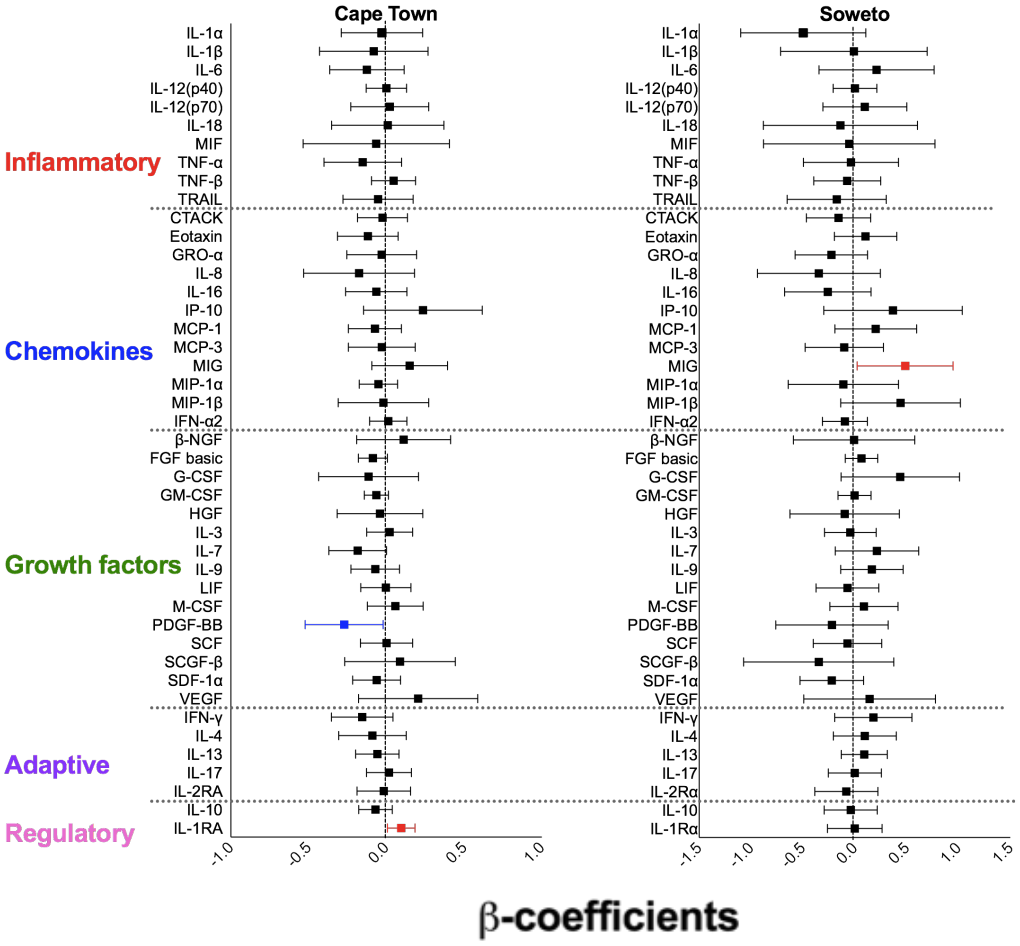


Figure 5.3. Impact of *N. gonorrhea* infections on lower genital tract cytokines in adolescents from Masiphumelele (Cape Town) and Soweto. The impact was assessed by multivariate linear regression analysis (adjusting for vaginal pH, injectable contraceptives, co-infection with *C. trachomatis, M. genitalium, T. vaginalis,*

HSV-2, yeast and BV). Each association is shown as a β-coefficient and the error bars are the 95% CI. The β-coefficient indicates the degree of change in the individual cytokine concentrations for a positive *N. gonorrhoea* result compared to AGYW who were STI-/yeast- and had a Nugent score ≤3. Significant positive associations are shown in red and negative associations in blue. P-values of ≤0.05 are considered statistically significant.

However, of the 24 *N. gonorrhoea* infections in this cohort, more than half (62.50%; 15/24) were also co-infected with *C. trachomatis*. To examine the effect of *C. trachomatis* co-infection, cytokine profiles were compared to those of women with single infections with either *C. trachomatis* or *N. gonorrhoea* (Figure 5.4). Adolescents who were co-infected with *N. gonorrhoea* and *C. trachomatis* tended to have less cytokine inflammation than those infected with either *C. trachomatis* or *N. gonorrhoea* alone. In Masiphumelele, due to the more moderate levels of genital inflammation associated with *C. trachomatis* infection (n=50), co-infection with *C. trachomatis* and *N. gonorrhea* (n=12) resulted in a similar level of inflammation as was seen with *N. gonorrhea* alone (n=5). AGYW from Soweto co-infected with *N. gonorrhea* and *C. trachomatis* appeared to have a more upregulated inflammatory profiles than was seen in Masiphumelele (Figure 5.4).

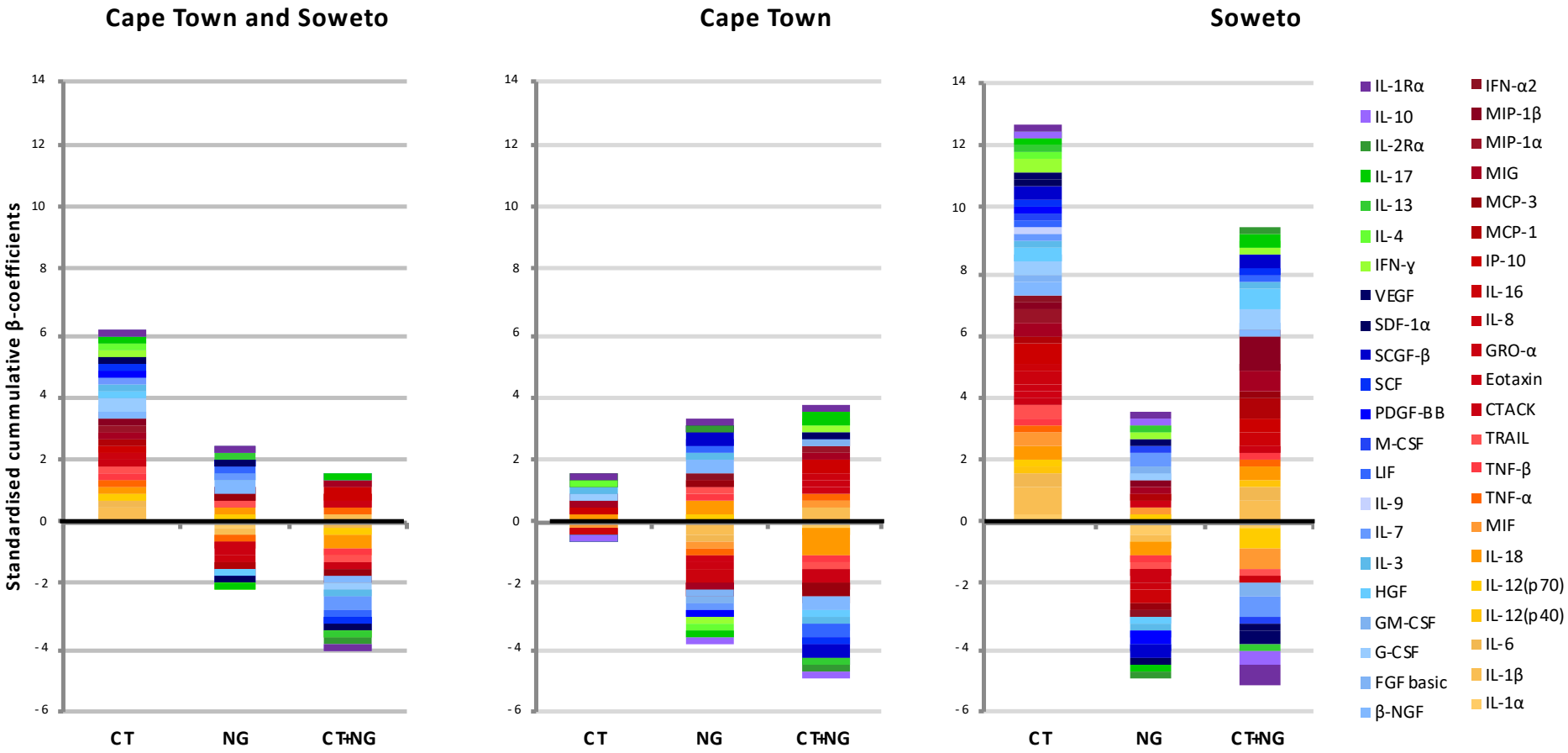


Figure 5.4. Comparison of cumulative genital cytokine concentrations in response to infection with *C. trachomatis* (CT), *N. gonorrhea* (NG) or both in combination (CT+NG) in South African AGYW, stratified according to site. Inflammatory cytokines are shown in shades of orange, chemokines in shades of red, growth factors in shades of blue, adaptive cytokines in shades of green and regulatory cytokines in shades of purple. Stacked bars of standardized β-coefficients for the regressions between each cytokine, adjusting for injectable hormonal contraceptive use and vaginal pH are displayed.

5.3.4 Inflammatory cytokine profiles associated with *T. vaginalis* in AGYW by site

T. vaginalis is a flagellated parasitic protozoan that can infect the vagina and vulva, and is associated with PID and preterm delivery (Seña *et al*. 2007). While many women infected with *T. vaginalis* are asymptomatic, the infection can result in a frothy green vaginal discharge and the appearance of a "strawberry" cervix (when small, bright red lesions caused by tiny hemorrhages are seen on the surface of the cervix; Swygard *et al*. 2004). Overall, *T. vaginalis* was associated with up-regulation of a quarter of the cytokines measured (Figure 5.1), including IL-1α (β=0.70; p<0·0001), IL-1β (β=0.71; p=0·0088), IL-8 (β=0.74; p<0·0001), MIF (β=0.71; p=0·033), HGF (β=0.67; p<0·0001), G-CSF (β=0.48; p=0·034), GRO-α (β=0.48; p<0·0001), β-NGF (β=0.46; p=0·044), IL-16 (β=0.37; p=0·022) and TNF-β (β=0.27; p=0·025), all of which were significant after adjusting for multiple comparisons. Genital inflammation in the Masiphumelele cohort was more pronounced than Soweto, with IL-1α (β=0.68; p=<0·0001), IL-1β (β=0.66; p=0·003), IL-8 (β=0.70; p=0·002), HGF (β=0.68; p<0·0001), VEGF (β=0.67; p=0·005), and GRO-α (β=0.51; p<0·0001) being significantly up-regulated compared to AGYW who were STI-/yeast- with Nugent score ≤3, after adjusting for multiple comparisons. In Soweto, in contrast, no significant increase in any of the cytokines were seen in adolescents infected with *T. vaginalis*, although only five adolescents were infected with *T. vaginalis* in Soweto (Figure 5.5).

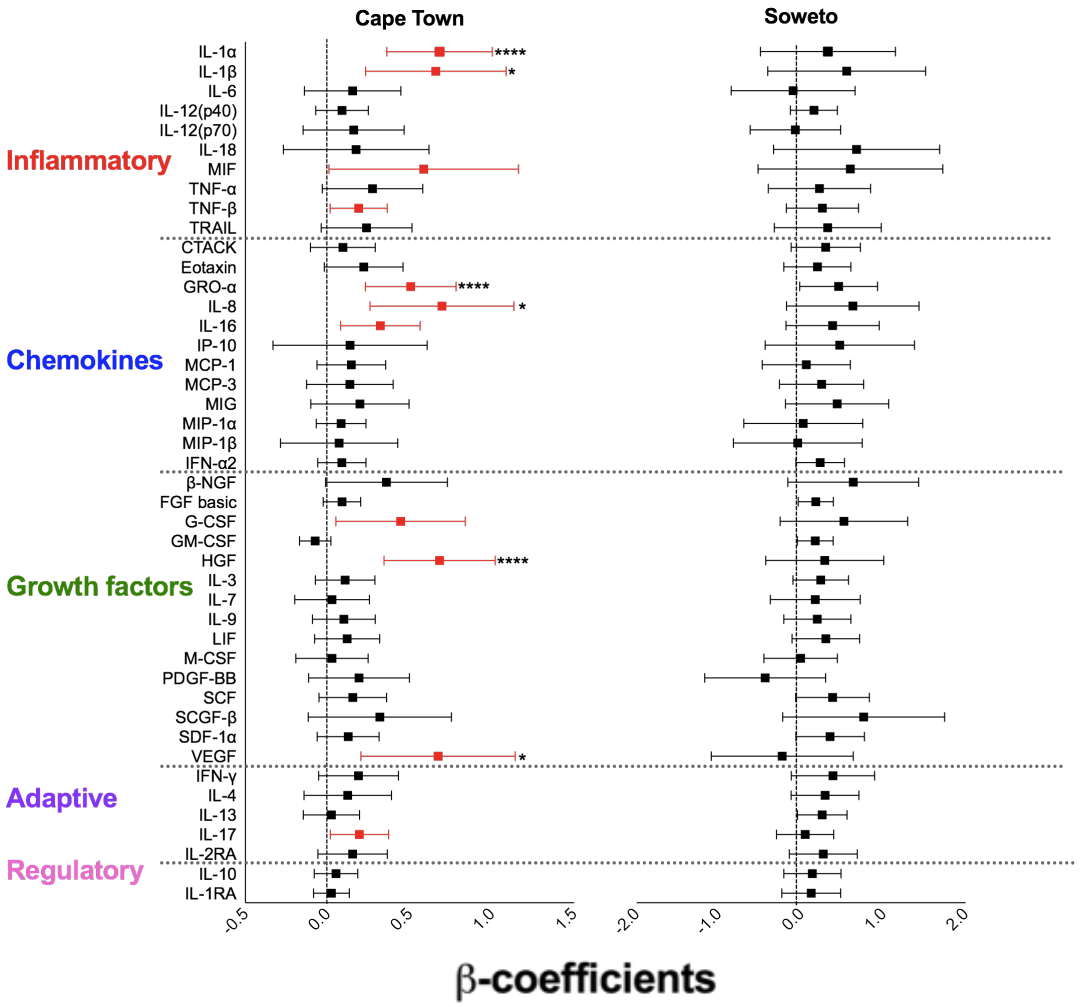


Figure 5.5. Impact of *T. vaginalis* infections on lower genital tract cytokines in AGYW from Masiphumelele (Cape Town) and Soweto. The impact was assessed using multivariate linear regression, adjusting for vaginal pH, injectable contraceptives, co-infection with *C. trachomatis, M. genitalium, N. gonorrhoea,* HSV-2, yeast and BV. Each association is shown as a β-coefficient and the error bars are the 95% CI. The β-coefficient indicates the degree of change in the individual cytokine concentrations for a positive *T. vaginalis* result compared to AGYW who were STI-/yeast- and had a Nugent score ≤3. Significant positive associations are shown in red and negative in blue. * indicate values that remained significant after correction for multiple comparisons and **** indicate values that remained significant after correction for multiple comparisons to more than five decimal places. P values of ≤0.05 are considered significant.

5.3.5 Genital cytokine responses associated with *C. trachomatis* in AGYW

More than a third of the cytokines measured (43.2%; 19/44) were significantly elevated in AGYW infected with *C. trachomatis* compared to their STI-/BV-/yeast- counterparts, after adjusting for multiple comparisons (Figure 5.1). Cytokines that were significantly elevated spanned all functional classes, with the effect size being larger for inflammatory cytokines IL-1α (β=0.68; p<0·0001), IL-1β (β=0.52; p=0·001), IP-10 (β=0.58; p<0·0001), IL-8 (β=0.61; p<0·0001) and GRO-α (β=0.57; p<0·0001) to

those with more moderate [including MIG (β=0.40; p<0·0001), VEGF (β=0.36; p=0·014), IL12(p70) (β=0.32; p=0·001)] to weak relationships [including HGF (β=0.29; p=0·015), IFN-γ (β=0.25; p=0·0003), SCF (β=0.22; p=0·002), SDF-1α (β=0.20; p=0.004), IL-4 (β=0.20; p=0·012), TNF-β (β=0.18; p=0·007), IL-17 (β=0.16; p=0·008), IL-10 (β=0.15 p=0·004), IL-13 (β=0.12; p=0·001), FGF basic (β=0.11; p=0·004), and GM-CSF (β=0.10; p=0·009)]. Of note, IL-1α, IL-1β and IP-10, markers that will be discussed in Chapter 6, were all elevated in AGYW infected with *C. trachomatis.*

5.3.5.1 Differences in cytokine profiles for C. trachomatis by site

Like the overall prevalence, the inflammatory profile of *C. trachomatis* was markedly different in Masiphumelele (n=62) compared to Soweto (n=26; Figure 5.6). Compared to infections in Masiphumelele, where only MIG was elevated in adolescents infected with *C. trachomatis* (but did not remain so after correction for multiple comparisons, p>0·9999), those in Soweto were significantly more inflammatory, upregulating 31/44 cytokines across all functional classes, of which 23 remained significant after adjustment for multiple comparisons, including IL-1β (β=0.79; p=<0·00001), IP-10 (β=0.51; p=0·006), IL-6 (β=0.43; p=0·001), TNF-α (β=0.40; p=0·001), MIG (β=0.40; p=0·001), MIP-1α (β=0.35; p=0·001), IFN-ɣ (β=0.31; p=0·001), HGF (β=0.41; p=0·003), IL-4 (β=0.27; p=0·003), G-CSF (β=0.43; p=0·005), SCF (β=0.23; p=0·007) ,MCP-1 (β=0.24; p=0·008), β-NGF (β=0.38; p=0·01) ,TRAIL (β=0.30; p=0·011) ,LIF (β=0.20; p=0·011) ,IL-9 (β=0.19; p=0·012), IL-10 (β=0.15; p=0·012), IL-7 (β=0.24; p=0·013), SDF-1α (β=0.19; p=0·014), MIP-1β (β=0.33; p=0·022), CTACK (β=0.18; p=0·023), GRO-α (β=0.22; p=0·026) and IFN-α (β=0.13; p=0·027) but not the pro-inflammatory cytokine IL-1α.

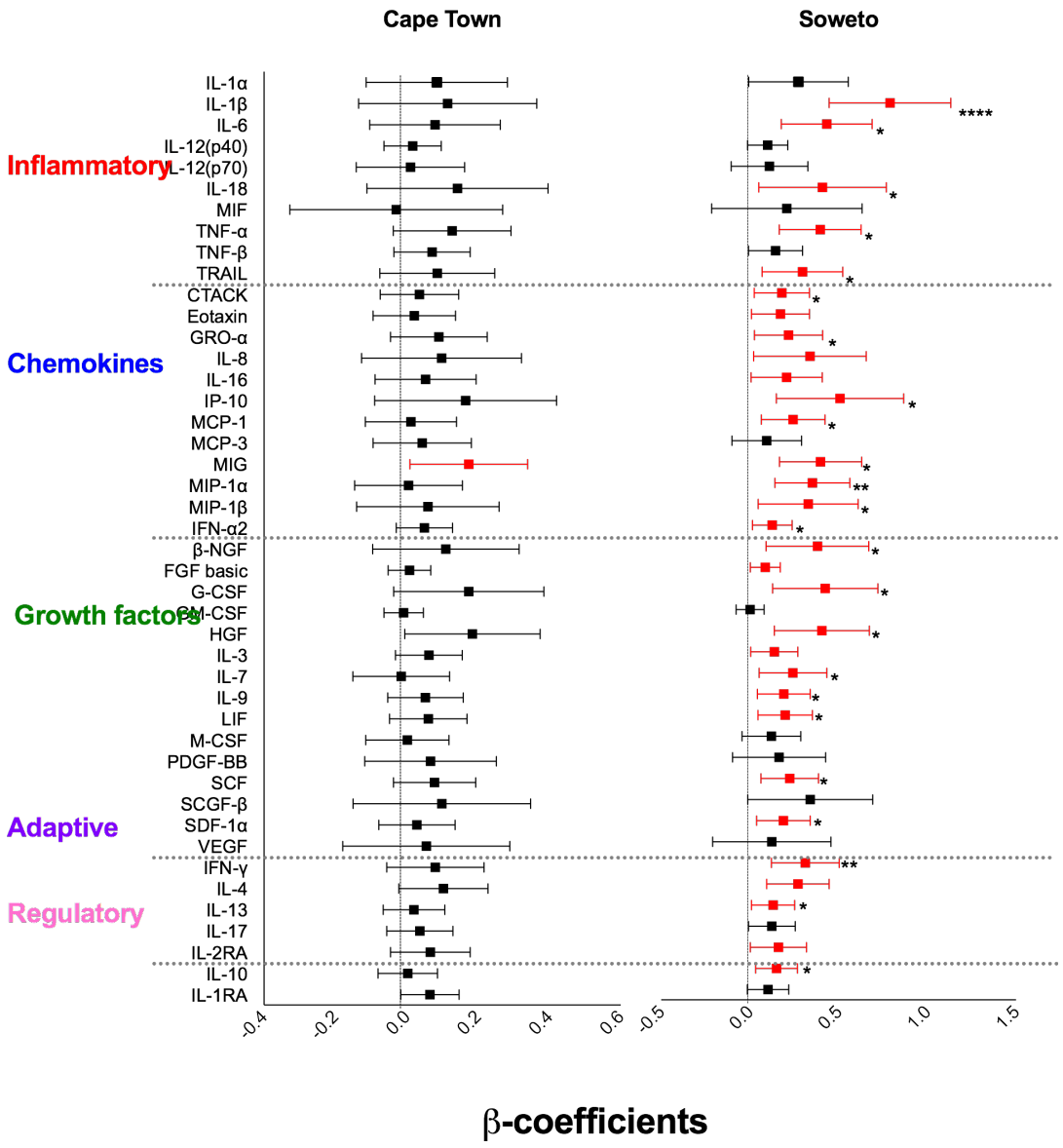


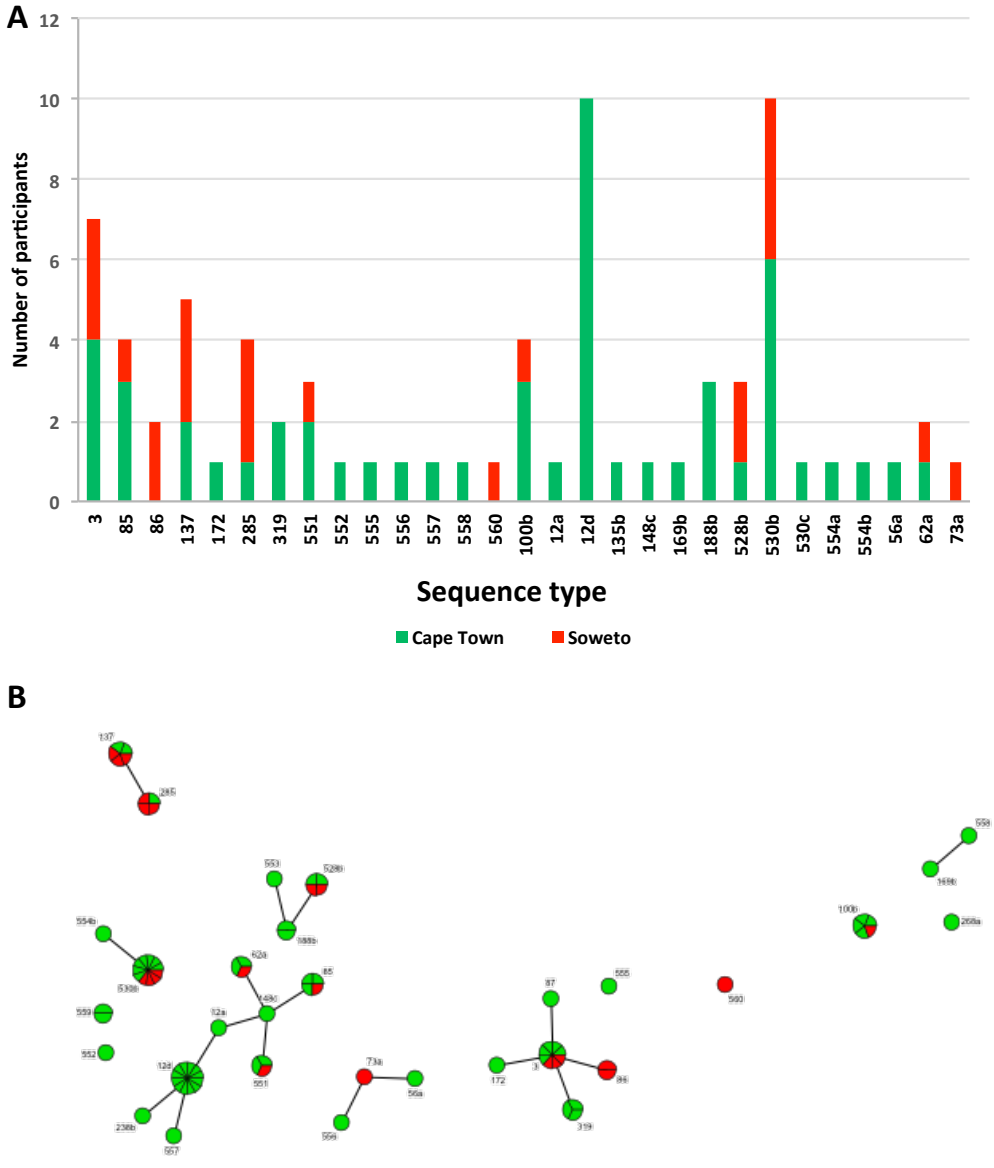
Figure 5.6. Multivariate linear regressions showing the associations between *C. trachomatis* and the 44 cytokines in Masiphumelele (Cape Town) and Soweto, adjusting for vaginal pH, injectable contraceptives, co-infection with *M. genitalium, N. gonorrhoea, T. vaginalis,* HSV-2, yeast and BV. Each association is shown as a β-coefficient and the error bars are the 95% CI. The β-coefficient indicates the degree of change in the individual cytokine concentrations for a positive *C. trachomatis* result compared to AGYW who were STI-/yeast- and had a Nugent score ≤3. Statistically significant positive associations are shown in red. *indicate values that remained significant after correction for multiple comparisons; ** indicate values that remained significant after correction for multiple comparisons to more than three decimal places; **** indicate values that remained significant after correction for multiple comparisons to more than five decimal places. P values of ≤0.05 are considered significant.

5.3.5.2 Differences in C. trachomatis cytokine profiles by Chlamydia ST

Menon *et al*. (2015) suggested that specific serovars and genotypes of *C. trachomatis* might influence host inflammatory responses and thus pathology. In this study, it was hypothesized that difference in genital inflammatory profiles between Soweto and

Masiphumelele were due to *C. trachomatis* STs circulating in each cohort. Using MLST (Klint *et al*. 2007), 34 different *C. trachomatis* STs (from 7 genovars) were detected in 52 samples from Masiphumelele and 23 samples from Soweto. Of these 34 STs, 15 were novel types that had not been detected previously. The three most common *C. trachomatis* STs were ST12d (13.3%; 10/75), ST530b (12%; 9/75) and ST3 (9.3%; 7/75; Figure 5.7A). There were no significant differences (p=0·187) in distribution by geographical site, as shown in the minimum spanning tree in Figure 5.7B.

Figure 5.7. Distribution (A) and minimum spanning trees (B) of *C. trachomatis* genotypes in AGYW



living in Soweto (red) and Masiphumelele/Cape Town (green). The minimum spanning tree shows the high resolution-MLST pattern of 85 *C. trachomatis* samples (one sample per unique participant). Each circle represents one high resolution-MLST ST. The size of each circle is proportional to the number of identical STs in the cohort. Figure 5.7B provided by Dr Bart Versteeg (GGD, Amsterdam).

Although this study was not powered to compare host genital cytokines responses by *C. trachomatis* STs, distinct cytokine profiles were observed by ST. *C. trachomatis* ST3 appeared to be the most inflammatory despite not being the most prevalent, associated with increased concentrations of 14/44 (32%) of the cytokines measured compared to AGYW that were STI-/BV-/ yeast-. These included genital IL-6 (β=1.07; p<0·0001), IL-8 (β=0.95; p=0·006), MIP-1β (β=0.88; p=0·006), SCGF-β (β=0.85; p=0·032), G-CSF (β=0.72; p=0·029), GRO-α (β=0.58; p=0·005), IL-16 (β=0.58; p=0·012), MCP-1 (β=0.55; p=0·007), TNF-α (β=0.53; p=0·044), CTACK (β=0.45; p=0·01), IL-2Rα (β=0.40; p=0·025), SDF-1α (β=0.39; p=0·021), LIF (β=0.35; p=0·042) and IL-17 (β=0.35; p=0·018). Interestingly, changes in the pro-inflammatory cytokines IL-1α, IL-1β and the chemokine IP-10 were not associated with infection by this *C. trachomatis* ST, although this profile was not generally seen with global *C. trachomatis* infections. In contrast, *C. trachomatis* ST137 did not appear to influence genital inflammatory responses. *C. trachomatis* ST100b was found more commonly in Masiphumelele (n=3 cases) than Soweto (n=1 case), and was the least inflammatory ST found in this cohort, with an eclectic group of four cytokines being downregulated: MCP-3 (β=-0.43; p=0·037); SCGF-β (β= -0.73; p=0·048); FGF basic (β=-0.24; p=0·006); and IL-13 (β=-0.26; p=0·038). MCP-3 is a chemokine that activates various inflammatory cells (Ben-Baruch *et al*. 1995), SCGF-β is a growth factors that stimulates granulocyte/macrophage colonies (Stefanantoni *et al*. 2014), FGF-basic induces host cell proliferation and migration (Holland & Varmus 1998), while IL-13 increases human leukocyte antigen (HLA) class II expression on B-cells and monocytes (Wynn 2003). Increased concentrations of the inflammatory cytokine IL-6 remained significantly associated with *C. trachomatis* ST3, after adjusting for multiple comparisons (Figure 5.8).

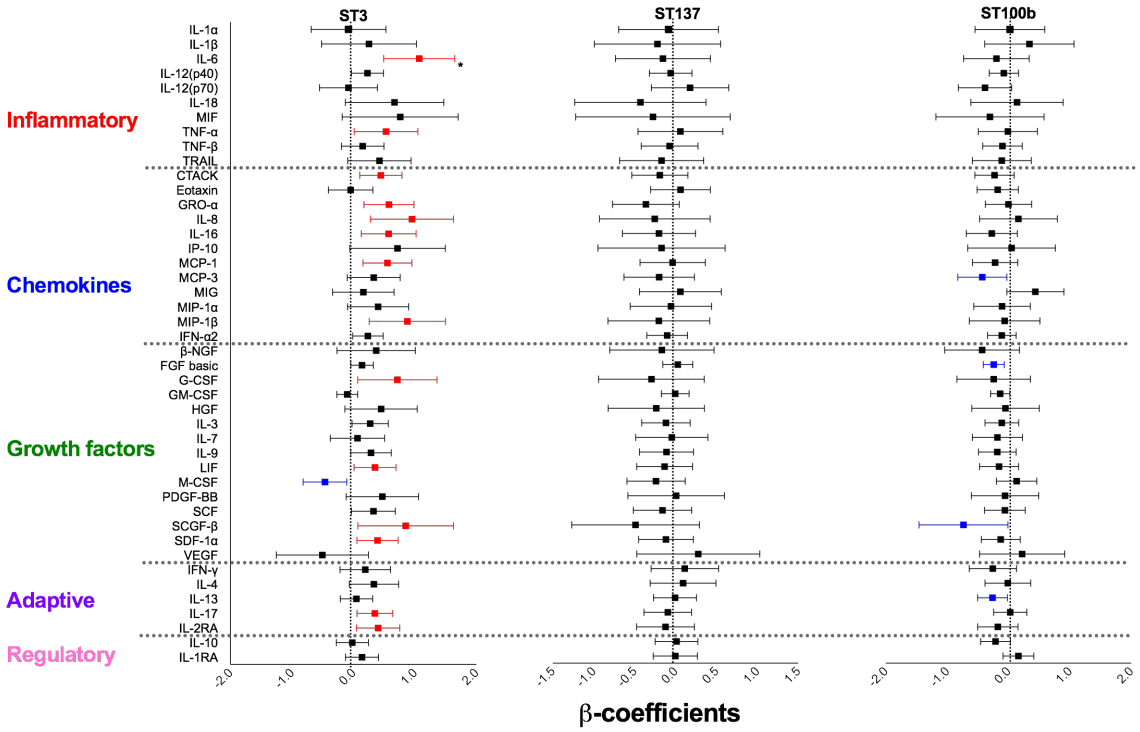


Figure 5.8. *C. trachomatis* STs (determined by MLST) appeared to influence genital cytokine changes in AGYW. This was determined by multivariate linear regression analysis adjusting for other ST types, vaginal pH, injectable contraceptives, co-infection with *M. genitalium, N. gonorrhoea, T. vaginalis,* HSV-2, yeast and BV. Each association is shown as a β-coefficient and the error bars are the 95% CI. Significant positive associations are shown in red and negative associations in blue. Dr Bart Versteeg (GGD, Amsterdam) conducted MLST typing. *indicate values that remained significant after correction for multiple comparisons. P-values ≤0.05 are considered significant.

5.3.5.3 Genital cytokine profiles to specific C. trachomatis STs vary by geographical location

Differences in genital cytokine profiles were seen in *C. trachomatis* STs that were detected in both Masiphumelele and Soweto, including ST3 (n=4 Masiphumelele and n=3 in Soweto) and ST530b (n=6 in Masiphumelele and n=4 in Soweto). Overall, adolescents infected with *C. trachomatis* ST530b had significantly increased genital concentrations of MIG, G-CSF and IL-9 compared to STI-/BV-/yeast- AGYW. However, the same ST appeared to be associated with more significant changes in the cytokine profile in Soweto than in Masiphumelele: 23/44 cytokines were significantly upregulated in Soweto before adjusting for multiple comparisons, and 10/44 remained significant after adjusting. These included IL-1β (β=2.32; p<0·0001), G-CSF (β=1.79; p<0·0001), HGF (β=1.45; p=0.001), MIP-1β (β=1.31; p=0·006), TRAIL (β=1.18; p=0·004), TNF-α (β=1.17; p=0·003), IFN-ɣ (β=0.93; p=0·004), IL-9 (β=0.77; p=0·002), IL-4 (β=0.76; p=0·003) and LIF (β=0.65; p=0·011). In contrast, only one cytokine was

significantly increased in adolescents from Masiphumelele while four were downregulated. Of these, none remained significant after adjustment for multiple comparisons.

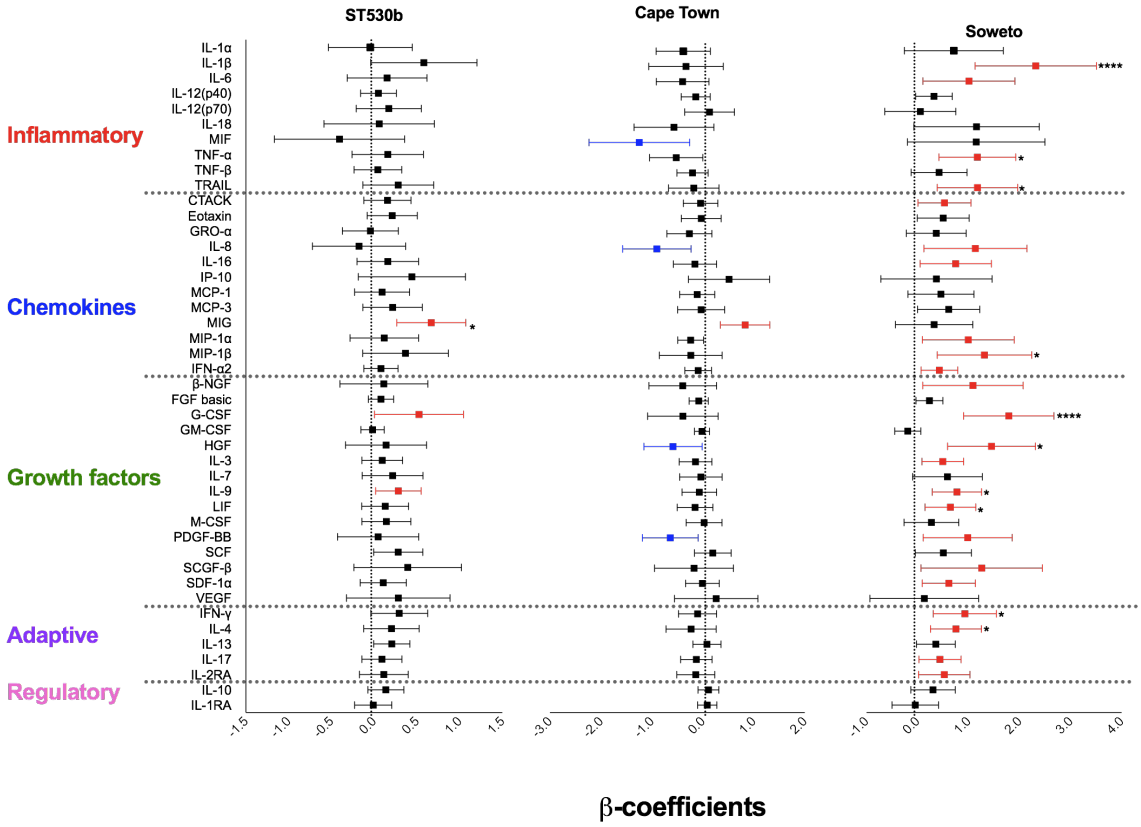


Figure 5.9. Multivariate linear regressions showing the associations between *C. trachomatis* ST530b infection and the 44 cytokines overall (ST530b), in Masiphumelele (Cape Town) and Soweto, after adjusting for other ST types, vaginal pH, injectable contraceptives, co-infection with *M. genitalium, N. gonorrhoea, T. vaginalis,* HSV-2, yeast and BV. Each association is shown as a β-coefficient and the error bars are the 95% CI. Statistically significant positive associations are shown in red and negative in blue. Dr Bart Versteeg (GGD, Amsterdam) conducted MLST typing. *indicate values that remained significant after correction for multiple comparisons; **** indicate values that remained significant after correction for multiple comparisons to more than five decimal places. P values ≤0.05 are considered significant.

C. trachomatis ST3 (n=7) had the most inflammatory profile of all the *C. trachomatis* STs, and infection with ST3 in Masiphumelele was more inflammatory than ST530b, with 7/44 cytokines being increased [including MIP-1β (β=0.69; p=0·042),IL-6 (β=0.64; p=0·025), IL-16 (β=0.54; p=0·022), MCP-1 (β=0.51; p=0·008), CTACK (β=0.45; p=0·016), SDF-1α (β=0.42; p=0·021) and IL-17 (β=0.34; p=0·044)]. In contrast, the concentration of growth factors GM-CSF (β=-0.24; p=0·003) and M-CSF (β=-0.59; p=0·003) were significantly lower compared to the control group. However, none remained significant after adjustment for multiple comparisons. In Soweto, *C. trachomatis*

infection with ST3 was associated with increased concentrations of IL-6 (β=0.67; p=0·003) and decreased concentrations of VEGF (β=-1.63; p=0·042). None remained significant after adjusting for multiple comparisons (Figure 5.10). Interestingly, IL-6 was increased in both sites, while the biomarkers IL-1α, IL-1β and IP-10 were not increased for this ST.

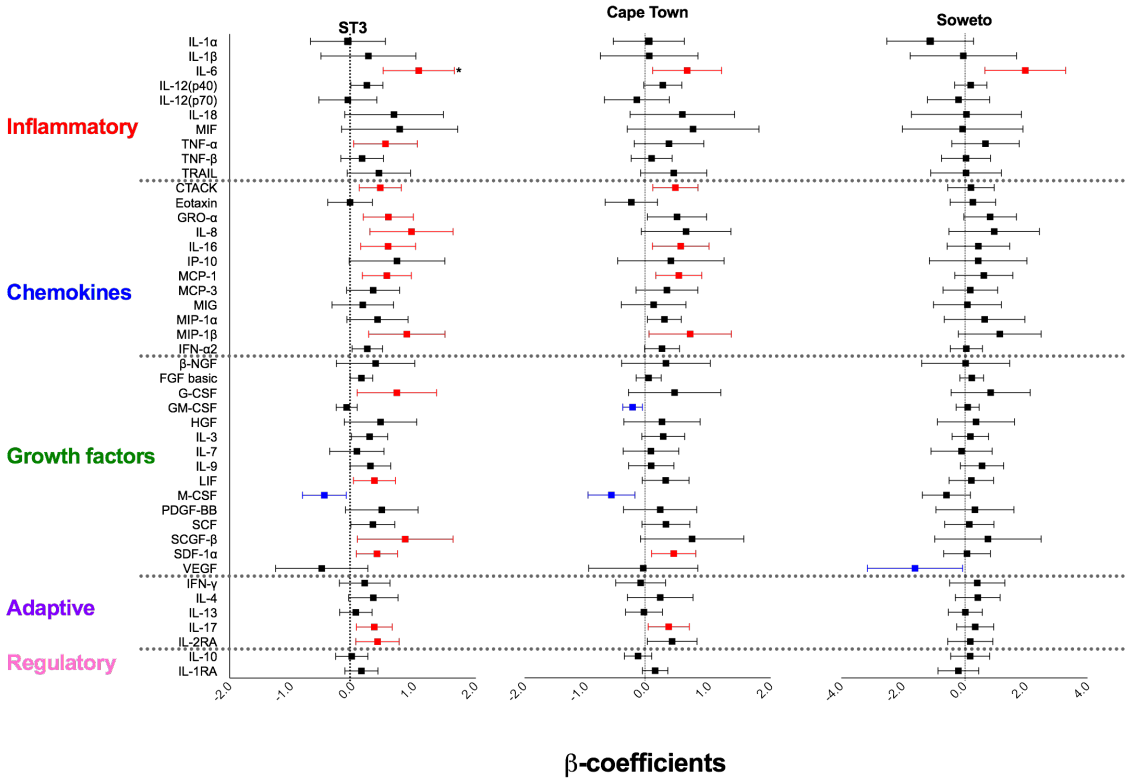


Figure 5.10. *C. trachomatis* ST3 infection influences genital tract cytokines overall (ST3), and differently in adolescents from Masiphumelele (Cape Town) and Soweto, assessed by multivariate linear regression analysis adjusting for other ST types, vaginal pH, injectable contraceptives, co-infection with *M. genitalium, N. gonorrhoea, T. vaginalis,* HSV-2, yeast and BV. Each association is shown as a β-coefficient and the error bars are the 95% CI. Significant positive associations are shown in red and negative in blue. Dr Bart Versteeg (GGD, Amsterdam) conducted MLST typing. *indicate values that remained significant after correction for multiple comparisons. P values of ≤0.05 are considered significant.

5.3.5.4 Cytokine profiles associated with persistent/repeated C. trachomatis infection

There is some evidence for development of acquired immunity against *C. trachomatis* infection. Acquired host immunity may explain decreasing prevalence with increasing age (Arno et al. 1994). Furthermore, bacterial burden appears to be inversely correlated with age, with younger individuals having higher bacterial counts than their older counterparts (Barnes *et al*. 1990). In this study, 69% (49/71) AGYW were *C. trachomatis* infected at two consecutive visits (over 4-6 months) and 37% (26/71)

were infected at all three visits (over 6-9 months). Since partner treatment was not widely accepted (Chapter 2, section 2.3.2.8), despite being offered to those who returned for treatment, it is possible that this may indicate either re-infection from their sexual partners or persistence. Thus, it was hypothesized in this study that the level of genital inflammation may be lower at the second visit because of better pathogen control in the presence of adaptive host immunity. A limitation of this analysis was that only three AGYW fit these criteria (*C. trachomatis* infected at two consecutive infections, with a Nugent score ≤3 and no other co-infections), so this analysis was restricted to these three participants. Their average genital tract cytokines levels of these participants were very similar at both study visits, with no significant decline in levels on genital inflammation seen for any cytokine in any of the functional classes (Figure 5.11).

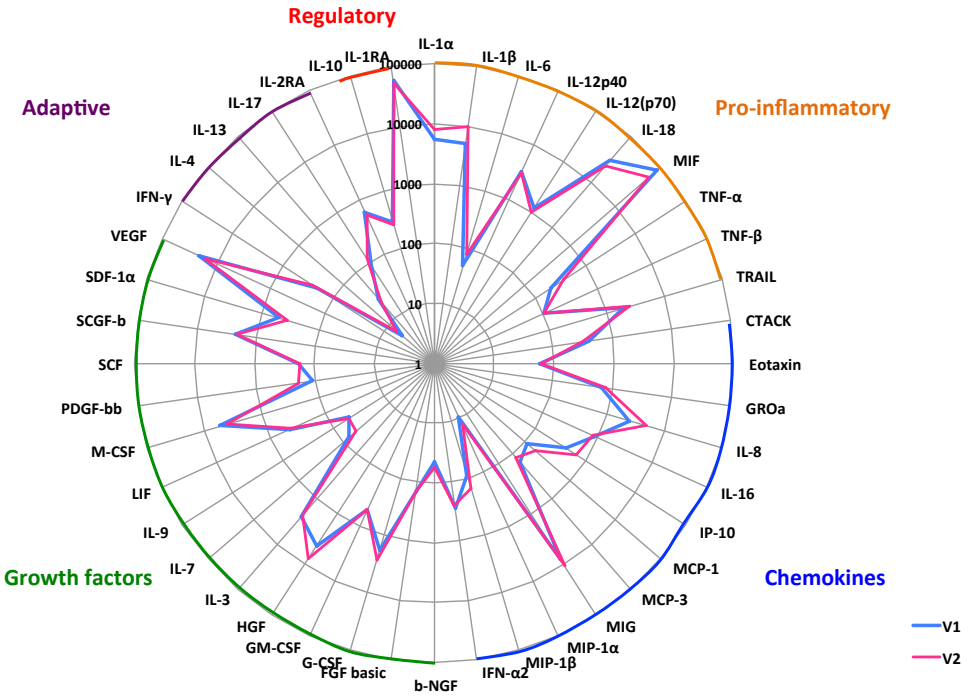


Figure 5.11. Comparison of mean genital cytokine concentrations (pg/mL) in AGYW (n=3) with persistent/repeated *C. trachomatis* infection. Blue line graph indicates cytokine levels of first visit (V1) with *C. trachomatis* infection, and pink line graph shows levels measured at the second visit (V2) with persistent/repeated *C. trachomatis* infection. Cytokines are colour-coded according to the class they belong to, including pro-inflammatory (orange), chemokines (blue), growth factors (green), adaptive (purple) and regulatory (red) cytokines.

5.3.5.5 Impact of age of sexual debut on cytokine responses to C. trachomatis infection

Adolescents in this cohort reported a median of two years of sexual experience and a median of two lifetime sexual partners (IQR 1-10), which was similar in Masiphumelele and Soweto (Chapter 2, Table 2.1). To understand if the duration of sexual activity influenced genital tract inflammatory responses to *C. trachomatis* infection, genital cytokine profiles in AGYW were analysed according to their years since sexual debut, grouping AGYW with either ≤1 year sexual experience (n=24; median age 17, IQR 16-17), 2-3 years (n=26; median age 19, IQR 18-19) or 4-8 years (n=16; median age 20, IQR 19-20) of sexual experience (Figure 5.12). In *C. trachomatis* infected adolescents who had only recently started to have sex (≤1 year of sexual experience), MIG was the only cytokine upregulated (β=0.29; p=0·0120). In contrast, being sexually active for 2-3 years was associated with an increase in more than half of the cytokines measured, including IP-10 (β=0.58; p=0·001), IL-18 (β=0.47; p=0·005), MIG (β=0.42; p<0·0001), IL-1β (β=0.42; p=0·008), SCGF-β (β =0.37; p=0·031), HGF (β=0.37; p=0·004), β-NGF (β=0.31; p=0·026), TRAIL (β=0.31; p=0·026), PDGF-BB (β=0.28; p=0·021), TNF-α (β=0.27; p=0·009), SCF (β=0.26; p=0·001), MCP-3 (β=0.24; p=0·01), IL-4 (β=0.23; p=0·003), IFN-γ (β=0.22; p-0·018), IL-2Rα (β=0.22; p=0·003), IL-16 (β=0.21; p=0·028), CTACK (β=0.20; p=0·007), LIF (β=0.18; p=0·006), TNF-β (β=0.19; p=0·013), IL-3 (β=0.18; p=0·004), IL-9 (β=0.16; p=0·028), SDF-1α (β=0.15; p=0·039), IFN-α2 (β=0.14; p=0·004), IL-12p40 (β=-0.14; p=0·018) and FGF-basic (β=0.09; p=0·031). Of these, MIG, IP-10, SCF, TRAIL, IL-4, IL-2Rα, IFN-α2, HGF, IL-3, IL-18, LIF, CTACK, IL-1β, TNF-α, MCP-3, TNF-β, IL-12p40, IFN-γ and PDGF-BB remained significant after adjusting for multiple comparisons. The young women who were the most sexually experienced (having between 4-8 years since debut) did not seem to have an inflammatory response to *C. trachomatis* (Figure 5.12).

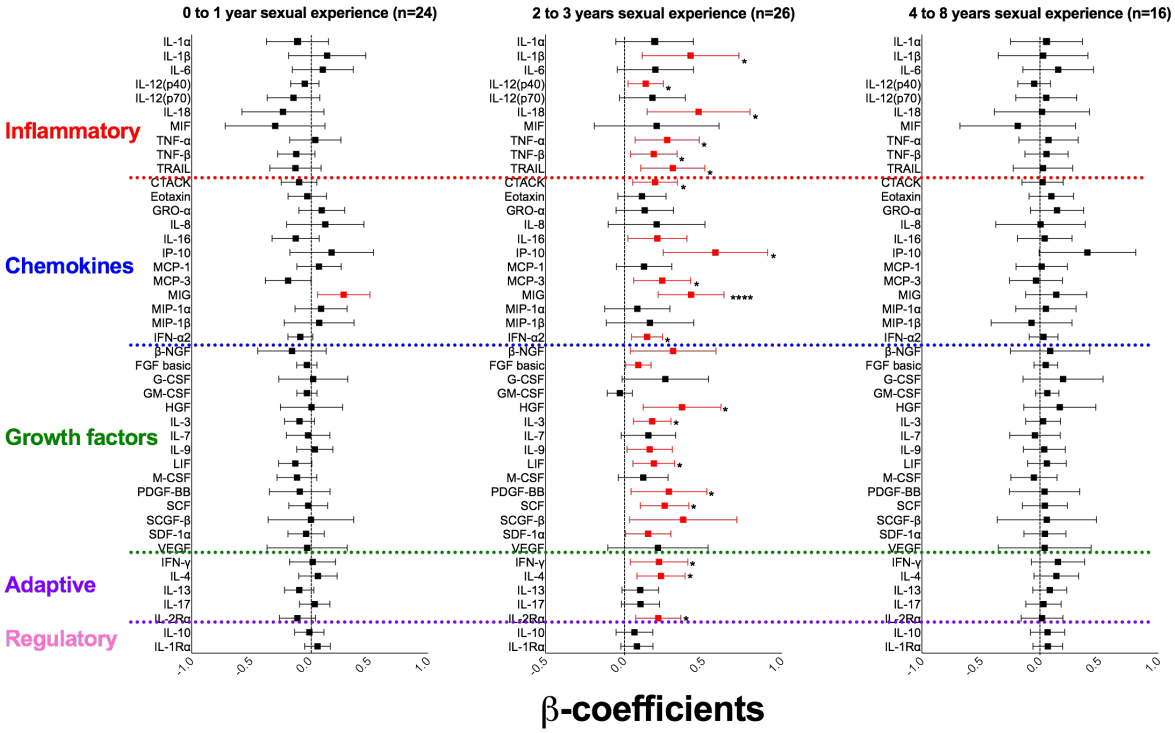


Figure 5.12. Impact of years of sexual experience on host inflammatory responses to *C. trachomatis* infection, assessed by multivariate linear regression analysis, adjusting for vaginal pH, injectable contraceptives, co-infection with *M. genitalium, N. gonorrhoea, T. vaginalis,* HSV-2, yeast and BV. AGYW were grouped according to the duration that they had been sexually active: ≤1 years; 2-4 years; and 4-8 years. Each association is shown as a β-coefficient and the error bars are the 95% CI. Statistically significant positive associations are shown in red and negative associations in blue. * indicates comparisons that remained significant after correction for multiple comparisons. P values of ≤0.05 are considered statistically significant.

Because inflammatory responses to *C. trachomatis* appeared to differ in adolescents from Masiphumelele and Soweto, the impact of time since sexual debut on cytokine responses to *C. trachomatis* was evaluated for each of the sites (Figure 5.13 Masiphumelele and Figure 5.14 for Soweto). In Masiphumelele, it was interesting that the less sexually experienced *C. trachomatis*-infected adolescents (≤1 year sexual experience; n=16) had significantly decreased genital tract concentrations of IL-1α (β=-0.33; p=0·024), LIF (β=-0.19; p=0·039), TNF-β (β=0.19; p=0·016), IL-13 (β=-0.20; p=0·021), CTACK (β=-0.20; p=0·04), IL-16 (β=-0.26; p=0·029), and IL-12(p70) (β=-0.33; p=0·023) compared to AGYW who were STI-/yeast- and had a Nugent score ≤3, after adjusting for pH, injectable contraceptives, co-infection with *M. genitalium*, *N. gonorrhoea*, *T. vaginalis*, yeast and BV. In contrast, Masiphumelele AGYW with 2-3 years sexual experience (n=21) had increased concentrations of MIG (β=0.37; p=0·004) and no significantly decreased cytokines, while adolescents with 4-8 years of

sexual experience (n=15) had no significant changes in their genital cytokine profiles compared to women who were STI-/yeast- and had a Nugent score ≤3 (Figure 5.13).

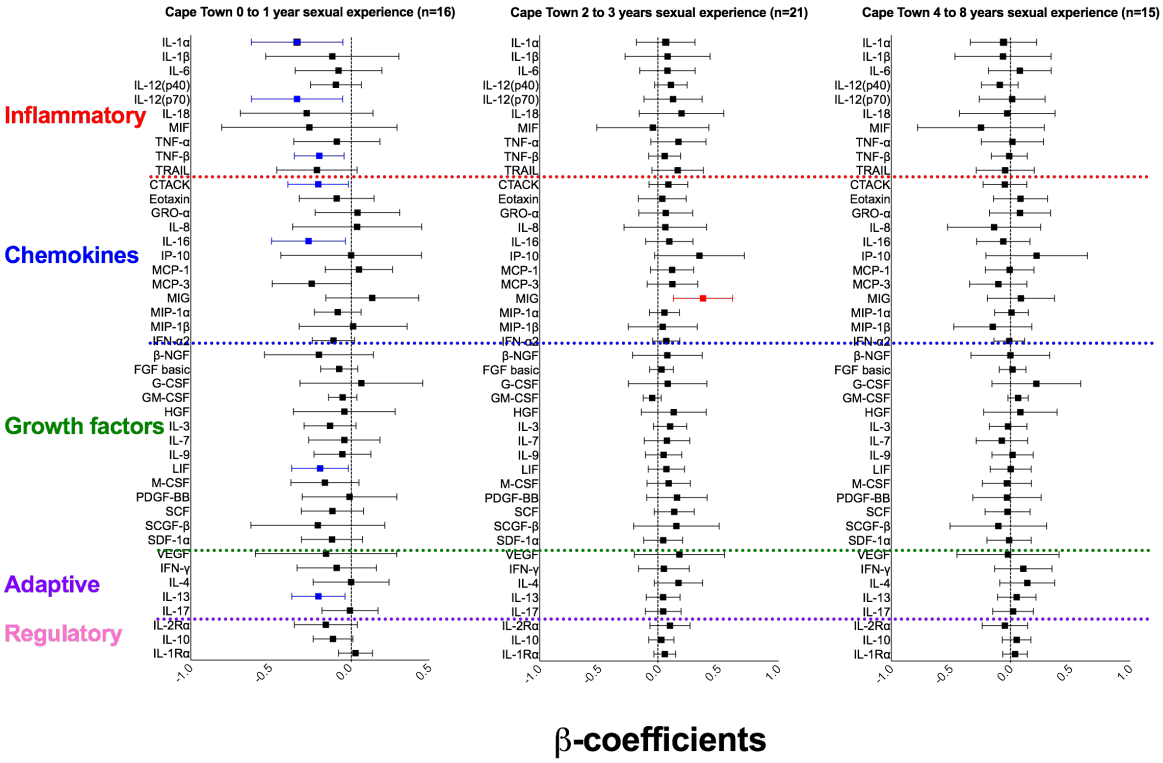


Figure 5.13. Impact of years of sexual experience on genital cytokine responses to *C. trachomatis* infection in AGYW from Masiphumelele (Cape Town). The impact was evaluted using multivariate linear regression, adjusting for vaginal pH, injectable contraceptives, co-infection with *M. genitalium, N. gonorrhoea, T. vaginalis,* HSV-2, yeast and BV. Cape Town AGYW were grouped according to the duration that they had been sexually active: ≤1years; 2-4 years; and 4-8 years. Each association is shown as a β-coefficient and the error bars are the 95% CI. Statistically significant positive associations are shown in red and negative in blue. *Indicates comparisons that remained significant after correction for multiple comparisons. P values of ≤0.05 are considered statistically significant.

In contrast, adolescents from Soweto had a different cytokine pattern emerging with age since sexual debut (Figure 5.14). AGYW who had recently started having sex (≤1-year; n=8) had significantly upregulated levels of IL-1β (β =0.66; p=0·016) and MIG (β =0.47; p=0·0187) in the presence of *C. trachomatis* compared to uninfected counterparts. There was a substantial upregulation of 27/44 cytokines in adolescents with *C. trachomatis* who had been sexually active for 2-3 years (n=5), including IL-18 (β=1.26; p=0·001), IL-1β (β=1.20; p<0·0001), IP-10 (β=1.04; p=0·005), MIF (β=0.94; p=0·032), β-NGF (β=0.92; p=0·005), SCGF-β (β=0.89; p=0·032), HGF (β=0.86; p=0·002), TRAIL (β=0.67; p=0·008), G-CSF (β=0.66; p=0·021), PDGF-BB (β=0.58; p=0·039), SCF (β=0.52; p=0·006), TNF-α (β=0.51; p=0·038), LIF (β=0.50; p<0·0001), IL-2Rα (β=0.49; p=0·001), MCP-3 (β=0.49; p=0·009), IL-16 (β=0.48; p=0·032), IL-7 (β=0.47; p=0·022),

SDF-1α (β=0.46; p=0·004), IFN-ɣ (β=0.45; p=0·013), TNF-β (β=0.45; p=0·024), CTACK (β=0.44; p=0·003), IL-4 (β=0.39; p=0·005), IL-3 (β=0.39; p=0·001), IFN-α2 (β=0.36; p<0·0001), IL-17 (β=0.30; p=0·014), Eotaxin (β=0.26; p=0·045) and FGF basic (β=0.16; p=0·032). Of these, 17/44 remained significant after correction for multiple comparisons: IL-1β, IFN-α2, LIF, IL-18, IL-3, IL-2Rα, HGF, CTACK, SDF-1α, IP-10, β-NGF, IL-4, SCF, TRAIL, MCP-3, IFN-ɣ and IL-17 (Figure 5.14). There were not enough participants in the 4 to 8-year group (n=1) from Soweto to complete the analysis for this group.

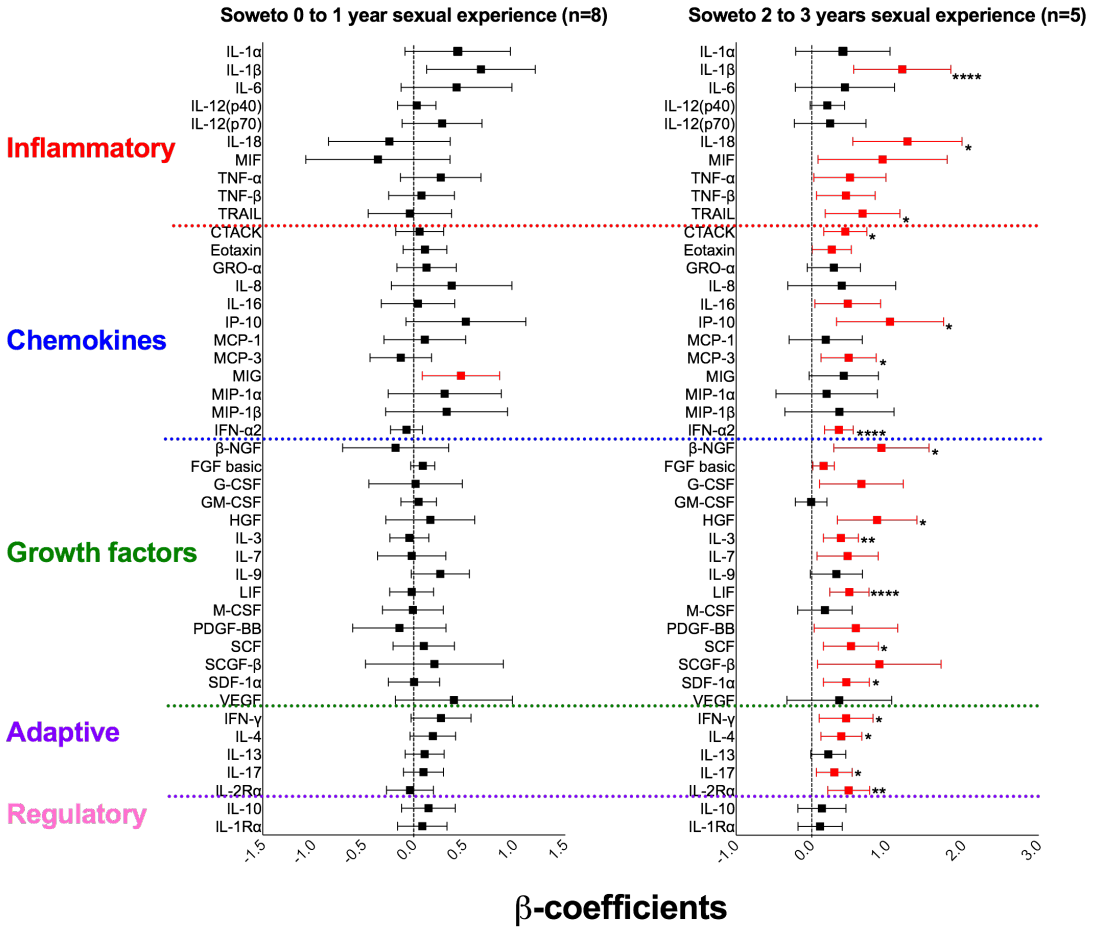


Figure 5.14. Impact of years of sexual experience on genital cytokine responses to *C. trachomatis* infection in AGYW from Soweto. The impact was evaluated using multivariate linear regressions and adjusting for vaginal pH, injectable contraceptives, co-infection with *M. genitalium, N. gonorrhoea, T. vaginalis,* HSV-2, yeast and BV. AGYW were grouped according to the duration that they had been sexually active: ≤1 year or 2-4 years. Each association is shown as a β-coefficient and the error bars are the 95% CI. Statistically significant positive associations are shown in red and negative in blue. * Indicates comparisons that remained significant after correction for multiple comparisons. P-values of ≤0.05 are considered statistically significant.

5.3.6 Relationship between cytokine changes induced by bacterial and parasitic STIs and clinical symptoms

Despite syndromic management underpinning STI management in SA (The Department of Health 2015), several studies from SA have demonstrated that the majority of STIs do not present with symptoms that would trigger the use of the treatment algorithm (Mlisana *et al*. 2012; Barnabas *et al*. 2017). In order to evaluate the relationship between genital inflammatory cytokine changes and clinical symptoms of a STI, genital cytokine concentrations in the asymptomatic AGYW with any of the bacterial and parasitic STIs in the Masiphumelele cohort (88%; 49/56) were compared with those who had symptoms 12% (7/56). As was previously described, no difference was seen in genital cytokine levels between AGYW with asymptomatic and symptomatic STIs (Figure 5.15). While medians in IL-8 and MCP-1 are different between the symptomatic and asymptomatic groups [8217 pg/µL (IQR 799.7 – 81670) vs. 1992 (IQR 1348 – 6912) and 267.3 (IQR 54.75 – 610.7) vs. 72.75 (IQR 48.48 - 138.3), respectively], these are not statistically significant (p-values 0.4656 and 0.2916, respectively).

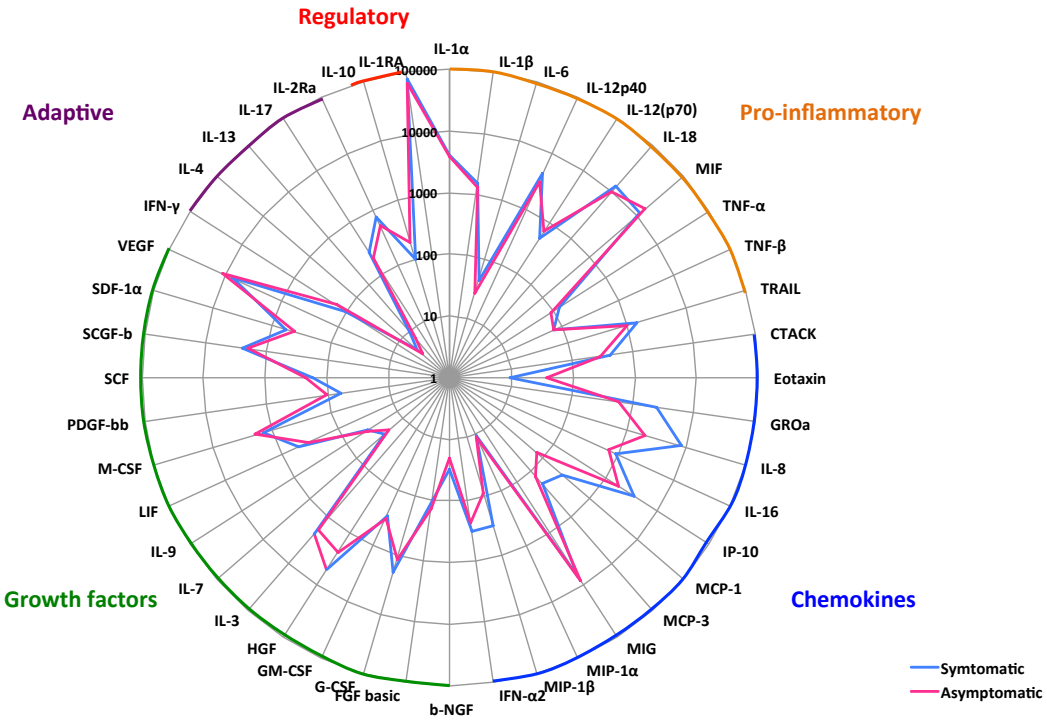


Figure 5.15. Comparison of median genital cytokine concentrations (pg/mL) in symptomatic (blue line graph) and asymptomatic (pink line graph) AGYW with laboratory-diagnosed STIs. Cytokines are colour-coded according to the class they belong to, including pro-inflammatory (orange), chemokines (blue), growth factors (green), adaptive (purple) and regulatory (red) cytokines.

5.4 Discussion

This Chapter describes the prevalence of bacterial and parasitic STIs in a cohort of South African AGYW and their effect on genital inflammatory cytokine levels. Adolescents had a relatively high prevalence of STIs, with *C. trachomatis* being the most common prevalent and inflammatory infection. In addition, one in ten adolescents in this cohort had multiple concurrent STIs, in which *C. trachomatis* was mostly detected concurrently with *T. vaginalis* or *M. genitalium*. Furthermore, about one in five women had at least one STI at the same time as having BV (Nugent 7-10).

Despite frequently been reported to cause inflammation and discharge in the reproductive tract (Makepeace *et al*. 2001; Naumann *et al*. 1997; Velasquez *et al*. 2012; Château & Seifert 2016), *N. gonorrhea* and *M. genitalium* infections in this cohort of AGYW tended to be associated with no or slightly dampened inflammatory signatures. Co-infection with *N. gonorrhea* and *C. trachomatis* together did not exacerbate inflammation compared to each STI alone, as has been seen previously (Hedges *et al*. 1998). Persistent *M. genitalium* infection has previously been linked with elevated genital IL-8, MCP-1, and MIP-1α responses (McGowin *et al*. 2012). Similarly, *in vitro* experiments have reported an increase in IL-6, IL-8, GM-CSF and MCP-1 protein levels in epithelial cell lines cultured with *M. genitalium* (McGowin, Popov & Pyles 2009). As was observed in response to *N. gonorrhea* infection, there was no change in genital inflammatory cytokine profiles in this cohort of AGYW. This lack of inflammation with *N. gonorrhea* has been seen previously with levels of IL-1, IL-6, and IL-8 in genital secretions not elevated in women with gonococcal cervicitis (Hedges et al. 1998). This lack of association between inflammation with *N. gonorrhea* has been seen more recently in a South African cohort (Anahtar et al. 2015). Additionally, the presence of other co-infections has been shown previously to not increase the local inflammatory responses (Hedges et al. 1998). It is thus possible that the more vigorous immune response to these co-infections or BV (which were present in most cases) could result in the body responding less strongly to *N. gonorrhea*. Recurrent, uncomplicated gonococcal infections have also been shown to not alter the antibody levels in patients with a current gonococcal infection, suggesting that

immunological memory is not induced by repeat infections (Hedges et al. 1999). It has also been shown that the binding of *N. gonorrhea* to CEACAM1 arrests the activation and proliferation of CD4+ T lymphocytes and thus could have a suppressive effect on the host immune response (Boulton & Gray-Owen 2002).

Of the four bacterial and parasitic STIs (*N. gonorrhoea*, *C. trachomatis, M. genitalium* and *T. vaginalis*) evaluated in this Chapter, *T. vaginalis* appeared to be the most inflammatory (together with *C. trachomatis*), being associated with increased expression of almost a quarter of the cytokines measured, after adjusting for multiple comparisons. Previously, *T. vaginalis* was reported to increase expression of IL-8 and IL-10 *in vivo* (Fichorova *et al*. 2006; Ahn, Song & Ryu 2008). However, *T. vaginalis* was also reported to suppress TNF-α and IL-6 levels in infected women (Ahn et al. 2008). While this young cohort appeared to have quite a bit of pathology associated with their *T. vaginalis* infections, it was shown to be the least inflammatory infection in another study from our group of adult South African women (>18 years), with no cytokines being increased after adjustment for multiple comparisons (Masson *et al*. 2014). As with *C. trachomatis,* geographical sites appeared to have varying levels of inflammation, with genital cytokine levels being higher in Soweto than Masiphumelele. Furthermore, there was no apparent change in the levels on genital inflammation in AGYW with persistent *T. vaginalis* infections. While the pathogenic mechanisms of *T. vaginalis* infections and host-parasite interactions remain mainly undefined (Lin *et al*. 2015), virulence properties associated with *T. vaginalis* include adhesion to ectocervical epithelium which is thought to initiate degradation of cells in the FGT resulting in cytolysis (Lustig *et al*. 2013). It would be interesting in future experiments to isolate *T. vaginalis* strains from AGYW with persistent infection from this cohort and from more symptomatic cohorts to compare the absence or presence of various presumptive virulence factors. It is possible that the lack of apparent change in the levels on genital inflammation with persistent *T. vaginalis* infections may be due to immune evasion techniques, such as molecular mimicry (Alderete *et al*. 2001) or the secretion of immunogenic proteins that may neutralize antibodies and T-cells (Lehker & Alderete 2000).

In this cohort of adolescents, *C. trachomatis* was not only the most prevalent bacterial STI but it was associated with the most apparent changes in genital inflammation, with more than a third of the cytokines measured being significantly higher compared to adolescents who were STI-/yeast- and had a Nugent score ≤3, including cytokines across all functional classes after adjustment for multiple comparisons. This is similar to what has been reported previously with an upregulation of IFN-ɣ, TNF-α, IL-10, IL-12, IL-11, IL-8, IL-6, GM-CSF, IL-1α, IL-6, IL-4 (Reddy *et al*. 2004; Hafner 2015; Ziklo *et al*. 2016; Zhong 2009). Furthermore, Agrawal *et al*. (2007, 2009) found higher levels of IL-1β, IL-6, IL-10, IFN-ɣ and TNF-α in genital secretions from women with persistent *C. trachomatis* infection, while women seeking health care for fertility disorders had higher levels of genital IL-1β, IL-6, IL-8 and IL-10 but lower levels of IL-12 and IFN-γ compared to fertile women. This general inflammation was similar to what has been previously seen in adult South African women infected with *C. trachomatis,* where >40% of the cytokines measured were increased (Masson *et al*. 2014).

What was striking was the difference in inflammation seen between *C. trachomatis* infection in Masiphumelele and Soweto, with much higher levels of inflammation seen in Soweto than Masiphumelele. A possible reason for this could be the difference in HC use between the sites. HC has been shown to influence genital inflammation, with DMPA being associated with higher MIP-1α, MIP-1β, IL-6, IL-8, and IP-10 concentrations, while Net-EN users had higher IL-6 and IL-8 (Deese *et al*. 2015). Higher endogenous hormones levels were seen in Soweto, which have previously been associated with immune dampening (Straub 2007). Hormones have been shown to influence: the innate (Kaushic *et al*. 2010) and adaptive immune (Fainboim *et al*. 2018), as well as cytokine responses (Willis *et al*. 2003). Ethnicity may have played a role, as there was a more heterogeneous population with a lower prevalence of Xhosa speakers in Soweto. The higher burden of disease in Masiphumelele could also pay a role as certain human leukocyte antigen (HLA) variants have been associated with protection from *C. trachomatis* infection with individuals with two class II alleles, HLA-DR1*1503 and DRB5*0101 being less susceptible to tubal damage and thus infertility from *C. trachomatis* infection (Cohen *et al*. 2003). HLA typing was not conducted in this study and there is a paucity of data as to whether HLA types vary between Xhosa

and other black South Africans (Tshabalala, Mellet & Pepper 2015) though some differentiation has been seen with the frequency of HLA types Bw35 and A2 possibly being increased in in Xhosa people with the juvenile-onset diabetes (Briggs *et al*. 1980). Taking evidence from another intracellular bacterium, *Mycobacterium tuberculosis*: certain widely circulating *M. tuberculosis* strains induced lower inflammation in the lung mucosa/tissue culture/PBMCs, accompanied with less pronounced induction of IL-1β, IFN-ɣ, and IL-22 by *M. tuberculosis* lysates in *ex vivo* induction of cytokines in peripheral PBMCs from healthy volunteers than less common strains (van Laarhoven *et al*. 2013).

To determine whether genetic variability could account for differences in inflammatory responses to *C. trachomatis* (as described previously; Dean *et al*. 1995; Srivastava *et al*. 2008; Workowski *et al*. 2014) in Soweto and Masiphumelele, MLST was performed. No difference in circulating STs by site was observed, although the ST diversity was greater in Masiphumelele than Soweto. However, there was a difference in the levels of genital inflammation associated with the same *C. trachomatis* STs circulating in Masiphumelele and Soweto, which may indicate that genetic variability in *C. trachomatis* alone may not determine the level of genital inflammation caused, but other factors that are site-specific, including HC as discussed above, may contribute.

Reinfection with *C. trachomatis* has been associated with a strong secondary immune response (Hillis *et al*. 1997) and repeated *C. trachomatis* infection has been associated with PID and other reproductive sequelae (Haggerty *et al*. 2010). However, there is also some evidence from *in vitro* studies that primary *C. trachomatis* infections are highly inflammatory compared to persistent infections (Schrader *et al*. 2007), and it has been suggested that women can develop protective immunity *C. trachomatis*, as it has been observed that the rate of incident infections decreases over time, and reinfections are associated with lower bacterial loads (Batteiger *et al*. 2010). Thus, it was of interest to investigate if the duration of sexual activity influenced genital tract inflammatory responses to *C. trachomatis* infection. The highest level of genital inflammation associated with *C. trachomatis* infection was observed in AGYW who

where more sexually experienced (2-3 years of sexual experience compared to <2 years), with almost half of the cytokines measured being elevated in more sexually experienced youth compared to only a single cytokine (MIG) being elevated in *C. trachomatis*-infected adolescents with less sexual experience. A similar pattern was seen in adolescents from both sites, with one major difference. Less sexually experienced AGYW infected with *C. trachomatis* in Masiphumelele also had decreased concentrations of seven cytokines [IL-1α, LIF, TNF-β, IL-13, CTACK, IL-16, and IL-12(p70)] compared to no changes in this group in Soweto. While these results are contrary to what was expected, it needs to be acknowledged that pathogen or host factors influencing differential *C. trachomatis* pathogenesis are not well understood. Various factors influencing virulence and inflammatory responses have been described [including a membrane attack complex/perforin protein, type III secretion effectors, stress response proteins, and antigens (e.g. HSP60, MOMP and LPS) that are able to elicit host immunity; Elwell *et al*. 2016; Bonner *et al*. 2015; Koehler *et al*. 1997; Gerard *et al*. 2004], but it is not clear which factors of combinations of factors determine the extent of inflammation associated with *C. trachomatis* infection. Dampened inflammatory responses (as observed in women with <2 years sexual experience) have been associated with poor bacterial clearance (Yang *et al*. 1996; Igietseme *et al*. 2000), while IFN-γ responses are thought to be key components in resolving *C. trachomatis* infections (Farris *et al*. 2010; Menon *et al*. 2015). However, at the same time strong inflammatory responses to *C. trachomatis* may contribute to immune-mediated pathology, including PID and infertility (Beatty *et al*. 1994). Overall, this highlights the urgent need to clearly understand correlates of pathogenesis (and/or protection) for *C. trachomatis* infection, in order to identify treatable factors that can be targeted in the design STI prevention strategies.

One of the limitations of this analysis was the distinct age category, as those with less sexual experience were younger than those who had more sexual experience, a potential confounder. Another potential confounder would be the number of partners in each category. Further, while *C. trachomatis* infection is initially cervical, it commonly migrates to the upper genital tract (Peipert 2003). Thus, collecting

secretions from the lower FGT to evaluate inflammatory responses induced *C. trachomatis* by may be sub-optimal.

In this Chapter, inflammatory mechanisms by which STIs could influence higher risk for HIV infection in AGYW were evaluated by focusing on cytokine biomarkers detected in the lower reproductive tract. Certain bacterial and parasitic STIs, especially *T. vaginalis* and *C. trachomatis*, were found to generate a strong genital inflammatory response, although there appeared to be some heterogeneity in the inflammatory response at the two different geographical sites, indicating that other physiological and socio-behavioural factors also influence the immune response. Together, this data provides more insight into how STIs play a role in young women's risk of HIV acquisition, although risk associated with genital inflammation is likely to be distinct according to the types of STIs that are prevalent in communities, as well as difference in pathogenicity of the circulating strains. The discovery of biological signatures of genital inflammation (Masson et al. 2016) in specific populations could help with the development of point-of-care based on cytokines that could provide a cheaper, more accessible alternative to PRC testing.

Chapter 6

Evaluating the use of IL-1α, IL-1β and IP-10 as biomarkers to identify AGYW with STIs and vaginal dysbiosis

6.1 Introduction

Identifying women with asymptomatic infections remains a critical gap in current BV and STI management. Although absence of symptoms caused by BV or a STI may suggest that women do not need treatment, research from SA has suggested that asymptomatic infections carry the same level of inflammatory cytokine upregulation as symptomatic infections (Mlisana *et al*. 2012) that could influence HIV risk (Masson *et al*. 2015).

As mentioned in previous Chapters, the current management of STIs and BV in SA is based on symptomatic women entering a treatment algorithm, which was implemented nationally since the mid-1990's (The Department of Health 2015). Syndromic management has been of benefit in reducing new HIV infections, as syndromic management was estimated to have reduced the proportion of new adult HIV infections attributable to curable STIs from 39% in 1990 to 14% in 2010 (Johnson *et al*. 2012). Further, this strategy seems to have been effective for ulcerative conditions (such as chancroid and syphilis) but it has not served women well for discharge-causing STIs (such as *C. trachomatis, N. gonorrhoeae, T. vaginalis* and *M. genitalium*) or BV (Johnson *et al*. 2011).

Previous analyses from our laboratory in a cohort of ~200 HIV-uninfected high-risk women from Durban, SA (CAPRISA 002), showed that only 12% had clinical signs of a STI or BV. Since genital cytokines were similarly elevated in asymptomatic and symptomatic women, it was hypothesized that cytokines could be used as biomarkers for the presence STIs or BV. Using cervicovaginal lavage (CVL) concentrations of the inflammatory cytokines IL-1α, IL-1β and IP-10, Masson *et al.* (2016) showed that a combination of these three cytokines (upregulation of either IL-1α and IL-1β together with downregulation of IP-10) correctly classified 76% of women according to STI and/or BV status, with a sensitivity of 72%, a specificity of 81%, a PPV of 86% and NPV of 64% (Masson *et al*. 2016). These cytokine biomarkers, demonstrating pathology associated with STIs or BV rather than the specific etiologic agents themselves, could

possibly be used to identify women with STIs and BV, independently of the presence or absence of symptoms.

While SA and many less developed countries in Africa and worldwide still practice syndromic management of STIs and BV, a whole range of more accurate and reliable methods are available for the diagnosis of STIs based on specific nucleic acid testing for the presence of the etiologic agents (Pearce *et al*. 2013; Gaydos 2014). However, their use nationally and in other regions of Africa is limited by their costs and relatively sophisticated technical requirements. The gold standard for BV diagnosis is Nugent Scoring (Nugent *et al*. 1991), but again, the widespread application of this test is limited by technical requirements. POC tests that are based on antigen detection for the diagnosis of STIs have been shown to have a predictive value that is inconsistent, performing better at high antigen loads than at lower loads (Hislop *et al*. 2010; Watchirs Smith *et al*. 2012). Thus, alternative methods that accurately diagnose BV and STIs and easy to use at low costs are urgently needed, especially in setting like SA, where STI, BV and HIV rates are high.

The hypothesis for this Chapter was that the previously identified biomarkers IL-1α, IL-1β and IP-10 would be elevated in women with STIs and vaginal dysbiosis, independent of the genital sample type. Thus, the aim of this Chapter was to validate the use of these biomarkers for the identification of women with STIs and vaginal dysbiosis, independently of the presence of symptoms, in the two distinct cohorts of AGYW that formed part of the WISH cohort (described in detail in Chapter 2). This will help developing an inexpensive POC test that could be offered to all women attending family planning clinics, primary healthcare clinics, community health centers or pharmacy clinics, or for self-testing. These women have initiated healthcare provider contact but are not necessarily seeking healthcare for genital conditions. This would potentially enable screening and detection of a greater number of women with asymptomatic STIs and vaginal dysbiosis who would not seek healthcare due to the absence of symptoms and are thus not treated, thereby improving syndromic management.

Furthermore, as cytokines were measured in CVLs (which required speculum examination) in the CAPRISA 002 discovery study, biomarker performance by genital sample type was compared; including genital samples collected using MC (Softcup®) samples, lateral vaginal wall and vulvovaginal swabs (which are samples that could be collected without a speculum or self-collected). Lastly, as the standard of care for STI/BV management in resource limited settings is to treat women according to clinical signs or symptoms, the performance of the cytokine biomarkers were compared to clinical signs and/or symptoms, in order to evaluate whether the biomarkers model would identify a greater number of women.

6.2 Methods

6.2.1 Cohort description

The 298 (149 adolescents from Soweto and 149 from Masiphumelele) sexually experienced HIV-negative AGYW (described in Chapter 2, section 2.2.1) were included in the analysis in this Chapter, regardless of their symptoms of a STI or vaginal dysbiosis.

6.2.2 Ethics statement

The Universities of Witwatersrand (Clearance certificate number: M1307) and Cape Town (HREC Ref 267/2013) Human Research Ethics Committees approved this study. (described in Chapter 2, section 2.2.2)

6.2.3 Collection and processing of genital specimens

As described previously in Chapter 4, genital secretions for cytokine measurements were collected using disposable MCs (n=285), vulvovaginal swabs (n=281) and lateral vaginal wall swabs (n=274). The use of MC to collect genital secretions and the processing of the sample are detailed in Chapter 4, section 4.2.3. Vulvovaginal and lateral vaginal wall swabs were collected from participants by the study clinician by rotating each swab 360° on the vulva and/or lateral vaginal wall, placed in 1ml PBS, transported to the laboratory at 4°C and then stored at -80°C until cytokine testing. In order to evaluate the cytokine biomarkers in minimally processed samples, vaginal swab samples were not filtered or centrifuged prior to cytokine measurement. Each vaginal swab sample was vortexed for 30 sec; the mucus was scraped off the swab on the side of the tube and vortexed for an additional 10 sec. Other genital mucosal samples collected included a Dacron vulvovaginal swab to test for STIs (described in Chapter 2, section 2.2.8.1); and a FLOQSwab™ lateral vaginal wall swab for a wet mount slide to detect BV (Nugent Scoring), candidiasis (gram stain) and vaginal pH (Chapter 2, section 2.2.8.2).

6.2.4 Measurement of biomarkers in genital secretions

The cytokine biomarkers IP-10, IL-1α and IL-1β were measured in vulvo-vaginal and lateral vaginal wall swab samples by ELISA (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Briefly, assay diluent was added to each well. Next, the standard, control, or samples were added. The plate was incubated for 2 hours at room temperature and then washed. Human conjugate was added; the incubation and wash steps were repeated. Next, substrate solution was added, the plate was incubated for 20-30 min at room temperature and finally 50 µL of stop solution added. The optical density was determined within 30 min, using a microplate reader set to 450 nm. IP-10, IL-1α and IL-1β levels were measured in MC samples by Luminex® as part of the 21- and 27-plex kits (Bio-Rad Laboratories Inc®, USA) on a Bio-Plex™ Suspension Array Reader (Bio-Rad Laboratories Inc®, Hercules, CA, USA) as previously described in Chapter 4, section 4.2.4.

6.2.5 16S sequencing and analysis

16S sequencing of the microbiome from vaginal swabs collected during the WISH study was performed by Drs Katie Lennard and Heather Jaspan [Computational Biology Unit, UCT, Cape Town, SA; and University of Washington, Seattle, USA; (Lennard *et al*. 2018)], following amplification of the V4 region of the 16S rRNA gene, by using modified universal primers [and quality checked with a Bioanalyzer (Agilent)]. While there are benefits to choosing the V1-V3 region over the V4 region for 16S gene amplification, the V4 region was chosen as it allowed better resolution of BV-associated bacteria (Lennard *et al*. 2018). Pooled triplicate samples were purified with AMPure XP beads (Beckman Coulter), and quantified using the Picogreen dsDNA assay (Invitrogen). Amplicons were pooled and quantified using the Kapa Library Quantification kit. Purified libraries consisting of ~100 pooled samples were paired-end sequenced on an Illumina MiSeq platform (300 bp paired-end with V3 chemistry). Following de-multiplexing, raw reads were pre-processed as follows: forward and reverse reads were merged using usearch7 (Edgar 2010), allowing a maximum of three mismatches; merged reads were quality filtered using usearch7 (reads with E scores larger than 0.1 were discarded); primer sequences were removed using a custom python script; following which merged, filtered reads were truncated at 250bp. Next,

sequences were de-replicated whilst recording the level of replication for each sequence using usearch7. De-replicated sequences were sorted by abundance (highest to lowest) and clustered *de novo* into operational taxonomic units (OTUs) at 97% similarity using usearch7 Chimeric sequences were detected (against the Gold database) using UCHIME (Edgar *et al*. 2011) and removed. Individual sequences were assigned to the specific identifiers using a 97% similarity threshold. Taxonomic assignment was performed in QIIME 1.8.0 (Caporaso *et al*. 2010) using the RDP classifier (using the default confidence level of 0.5) against the GreenGenes 13.8 reference taxonomy for 97% identity. Samples with ≥ 5000 reads were selected for downstream analyses. The OTU table was standardized (i.e. transformed to relative abundance * median sample read depth), and filtered so that each OTU had to have at least 10 counts in at least 2% of samples or have a relative abundance of at least 0.001%. Dr. Katie Lennard (UCT, Cape Town) provided this data and did the analysis.

6.2.6 Screening for viral STIs, yeast and BV and symptoms

Vulvovaginal swabs were collected for STI testing as described in section 2.2.8.1 using M-PCR assays for discharge-causing STIs (*C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, *M. genitalium)* and ulcerative STIs (HSV-1 and -2, *T. pallidum* and *H. ducreyi*; described in detail in Chapter 2, section 2.2.8.1). Lateral wall/posterior fornix swabs were collected, rolled onto glass slides and fixed for BV assessment (using Nugent scoring) and fungal hyphae assessment. The pH of lateral vaginal wall swab samples was measured using colour-fixed indicator strips (Macherey-Nagel, Düren, Germany; described in detail in Chapter 2, section 2.2.8.1). An endocervical swab was obtained for HPV typing (described in detail in Chapter 2, section 2.2.8.4). HSV-2 serology was performed using the HerpeSelect HSV-2 ELISA Kits (Focus Diagnostics, Cypress, CA, USA) (described in detail in Chapter 4 section 4.2.5)

A subset of women residing in Cape Town (n=36) was questioned on the presence of symptoms of STIs or vaginal dysbiosis (abnormal vaginal discharge, dysuria and vaginal itching/burning; described in Chapter, section 2.2.6).

6.2.7 Measurement of cotinine as a biomarker for smoking

Cotinine levels were measured from blood plasma using ELISA, as described in Chapter 2, section 2.2.7.2

6.2.8 Measurement of PSA as a biomarker for unprotected sex

PSA was measured in lateral vaginal wall swabs as a biomarker of semen exposure in the previous 48 hours using Human Kallikrein 3/PSA Quantikine ELISA kits (R&D Systems, Minneapolis, USA), as described in Chapter 2, section 2.2.8.3.

6.2.9 Measurement of endogenous sex hormones

The concentrations of E2, progesterone and LH were measured from blood plasma, as described in Chapter 2, section 2.2.7.1.

6.2.10 Statistical analysis

Statistical analyses were performed using STATA^{TM} version 12 (StataCorp, College Station, TX, USA) and Qlucore Omics Explorer (Qlucore, Sweden). Cytokine data were log_{10}-transformed. Logistic regression was used to evaluate the predictive value of IP-10, IL-1α and IL-1β and other variables included in this study. Genital samples from AGYW with intermediate Nugent scores (4-6) were included as an endpoint in this model, as work from our group in the CAPRISA 002 biomarkers discovery study found that including intermediate Nugent scoring samples significantly reduced the number of women who were incorrectly classified as STI/BV positive using these cytokine biomarkers (p=0.0048), even though inclusion or exclusion of women with intermediate microbiota did not significantly influence the accuracy of the cytokine model (Masson *et al*. 2015). Additionally, AGYW in the WISH cohort with intermediate microbiota had higher levels of inflammatory cytokines in their genital tracts compared to STI-/BV- women and had inflammatory levels similar to those with BV (Chapter 4, section 4.3.3.1). Youden's Index was used to determine appropriate probability cutoffs for each model, assuming that sensitivity and specificity are of equal importance. The likelihood ratio test was used to compare nested models. For evaluation of factors that may have influenced the predictive value of the cytokine biomarkers model, women were grouped as follows: True positive (STI positive by PCR

or Nugent score ≥4, and grouped as positive using the biomarkers model), true negative (STI negative by PCR and Nugent score ≤3, and grouped as negative using the biomarkers), false positive (STI negative by PCR and Nugent score ≤3, but grouped as positive using the biomarkers) and false negative (STI positive by PCR or Nugent score ≥4, but grouped as negative using the biomarkers). Proportions were compared between these groups using Fisher's Exact test, while continuous variables were compared using Mann Whitney U test. P-values <0.05 were considered statistically significant.

6.3 Results

6.3.1 Performance of IL-1α, IL-1β and IP-10 as biomarkers of STIs and vaginal dysbiosis

In the adolescents included in the WISH study, 205/285 (72%) had a bacterial/protozoan STI, BV (Nugent 7-10) or intermediate (Nugent 4-6) microbiota determined by laboratory testing (Chapter 2, section 2.3.2). Despite collecting fluid from distinct anatomical locations in the lower reproductive tract, IL-1α, IL-1β and IP-10 together were similarly predictive of a STI, BV or intermediate microbiota in each sample type, correctly classifying 76% of women using their MC samples, 76% of women using their lateral wall swabs and 73% of the women using their vulvovaginal swabs (Table 6.1). Although both, the lateral wall swabs and MCs performed similarly, the lateral wall swabs were easier to collect, required less processing and were thus a more feasible sample collection method for a POC test.

6.3.2 Cytokine biomarker performance compared to clinical signs or symptoms

The performance of the cytokine biomarkers was compared to the current standard of care, syndromic management. In a subset of women from the WISH cohort for whom clinical symptoms were recorded (n=36), symptoms detected 41% of those with laboratory-diagnosed STIs, BV or intermediate microbiota, with a specificity of only 57%, suggesting that only 44% of women would have been correctly classified if all women with a symptom had sought healthcare in a syndromic management setting (Chapter 2, section 2.3.4). IL-1α, IL-1β and IP-10 identified a greater number of women with these genital conditions than clinical symptoms (Fischer's exact p=0.0003; Table 6.1) Including clinical symptoms to the biomarkers model did not improve the accuracy or fit [Likelihood ratio (LR) χ^2: 0.14, p=0.711].

Table 6.1 Performance of IL-1α, IL-1β and IP-10 as biomarkers of STIs and vaginal dysbiosis

Biomarker	Sample type	Model Classification	True STI/BV diagnosis* (n)		Sensitivity	Specificity	PPV	NPV	Correctly classified
			Pos.	Neg.	% (95% CI)	% (95% CI)	% (95% CI)	(95% CI)	% (IQR)
IL-1α + IL-1β+ IP-10	Menstrual cup	Pos.	152	18	72 (65-78)	85 (75-92)	93 (87-96)	54 (45-63)	76 (70-80)
		Neg.	53	62					
IL-1α + IL-1β+ IP-10	Vulvo-vaginal swab	Pos.	148	21	73 (67-79)	73 (62-83)	88 (82-92)	52 (42-61)	73 (68-78)
		Neg.	54	58					
IL-1α + IL-1β+ IP-10	Lateral vaginal wall swab	Pos.	154	22	77 (71-83)	71 (59-81)	88 (82-92)	54 (44-64)	76 (70-81)
		Neg.	45	53					
IL-1α + IL-1β+ IP-10 + PSA	Lateral vaginal wall swab	Pos.	145	19	73 (66-79)	74 (63-84)	88 (83-93)	51 (41-61)	74 (68-79)
		Neg.	53	55					
Clinical symptoms	Lateral vaginal wall swab	Pos.	12	3	41 (24-61)	57 (18-90)	80 (52-96)	19 (5-42)	44 (28-62)
		Neg.	17	4					
IL-1α + IL-1β + IP-10 + clinical symptoms	Lateral vaginal wall swab	Pos.	21	1	72 (53-87)	86 (42-100)	95 (77-100)	43 (18-71)	75 (58-88)
		Neg.	8	6					
pH	Lateral vaginal wall swab	Pos.	83	11	77 (68-84)	61 (41-78)	88 (80-94)	40 (26-57)	74 (65-81)
		Neg.	25	17					
IL-1α + IL-1β+ IP-10 + pH	Lateral vaginal wall swab	Pos.	94	10	87 (79-93)	64 (44-81)	90 (83-95)	56 (38-74)	82 (75-88)
		Neg.	14	18					
IL-1α + pH	Lateral vaginal wall swab	Pos.	93	9	86 (78-92)	68 (48-84)	91 (84-96)	56 (38-73)	82 (75-88)
		Neg.	15	19					
		Neg.	50	80					

The table was adapted from Masson, Barnabas, *et al*. Sex Transm Infect 2018;0:1–8. doi:10.1136/sextrans-2017-053506; STIs were assessed by PCR and BV/intermediate microbiota by Nugent Scoring. Pos.= Positive; Neg.= Negative

6.3.3 Identifying drivers of incorrect classification

As multiple factors may influence cytokine concentrations in the lower reproductive tract (other than bacterial/protozoan discharge-causing genital infections, BV and intermediate microbiota), the influence of potential confounders including: ulcerative and/or viral infections [HSV, HPV, syphilis (*T. pallidum*), chancroid (*H. ducreyi*)], candidiasis, hormone contraceptive use, age of study participants, semen contamination, blood contamination of mucosal samples, vaginal product insertion, hormone levels in plasma and the vaginal microbiota on the performance of the cytokine biomarkers were investigated (Table 6.2).

HSV-2 PCR positivity, HPV and fungal infections were not associated with false positive results and none of the women in this cohort were shedding HSV-1 at the time of sample collection, nor had a positive syphilis or chancroid PCR tests. Slightly more women who were classified as false positive using the cytokine biomarkers model were using the long-acting hormone contraceptive Nur-Isterate, compared to true negative women, although this was not statistically significant and may have been a spurious false positive result due to the number of statistical tests performed (Table 6.2).

Although false negative women had lower blood E2 concentrations than true positive women ($p=0.02$, Table 6.2), E2 concentrations were not associated with cytokine concentrations and E2 inclusion in the biomarkers model did not improve the fit (LR $\chi 2$: 1.61, $p=0.204$) or accuracy.

Table 6.2 Drivers of incorrect classification

	TRUE NEGATIVE N/total (%)	FALSE POSITIVE N/total (%)	P-value (TN vs FP)	TRUE POSITIVE N/total (%)	FALSE NEGATIVE N/total (%)	P-value (TP vs FN)
STI, BV or fungal infection						
C. trachomatis PCR+	0/53 (0)	0/22 (0)	1.00	61/154 (39.6)	23/45 (51.1)	0.18
N. gonorrhoeae PCR+	0/53 (0)	0/22 (0)	1.00	15/154 (9.7)	8/45 (17.8)	0.18
T. vaginalis PCR+	0/53 (0)	0/22 (0)	1.00	13/154 (8.4)	3/45 (6.7)	1.00
M. genitalium PCR+	0/53 (0)	0/22 (0)	1.00	7/154 (4.6)	4/45 (8.9)	0.54
BV (Nugent 7-10)	0/53 (0)	0/22 (0)	1.00	**113/154 (73.4)**	**15/45 (33.3)**	**<0.0001**
Intermediate microbiota (Nugent 4-6)	0/53 (0)	0/22 (0)	1.00	34/154 (22.1)	6/45 (13.3)	0.29
HSV-2 2 PCR+	0/53 (0)	0/22 (0)	1.00	8/154 (5.2)	0/45 (0)	0.20
HSV-1 PCR+	0/53 (0)	0/22 (0)	1.00	0/154 (0)	0/45 (0)	1.00
H.s ducreyi PCR+	0/53 (0)	0/22 (0)	1.00	0/154 (0)	0/45 (0)	1.00
T. pallidum PCR+	0/53 (0)	0/22 (0)	1.00	0/154 (0)	0/45 (0)	1.00
Fungal infection	7/53 (13.2)	3/22 (13.6)	1.00	11/154 (7.1)	6/45 (13.3)	0.22
HPV PCR+	21/45 (46.7)	9/19 (47.4)	1.00	97/129 (75.2)	24/36 (66.7)	0.39
High Risk HPV*	17/45 (37.8)	7/19 (36.8)	1.00	85/129 (65.9)	21/36 (58.3)	1.00
Low Risk HPV**	14/45 (31.1)	7/19 (36.8)	0.77	62/129 (48.1)	17/36 (47.2)	1.00
Non-infectious characteristics						
Using DMPA	5/37 (13.5)	2/19 (10.5)	1.00	22/149 (14.8)	5/43 (11.6)	0.80
Using Nur-Isterate	16/37 (43.2)	13/19 (68.4)	0.09	67/149 (45.0)	21/43 (48.8)	0.73
PSA positive	11/52 (21.2)	4/22 (18.2)	1.00	45/153 (29.4)	10/45 (22.2)	0.45
Residence [no. Capetonians/total (%)]	18/53 (34.0)	13/22 (59.1)	0.07	86/154 (55.2)	27/45 (60.0)	0.73
Vaginal product insertion	4/41 (9.8)	2/16 (12.5)	1.00	13/119 (10.9)	4/37 (10.8)	1.00
Blood contamination	2/53 (3.8)	2/22 (9.1)	0.58	19/154 (12.3)	6/45 (13.3)	0.80
	Median (IQR)	**Median (IQR)**		**Median (IQR)**	**Median (IQR)**	
Age	18 (17-20)	17.5 (17-19.75)	0.68	18 (17-20)	19 (18-20)	0.57
Vaginal pH	**4.1 (4.1-4.4)**	**4.7 (4.1-5.1)**	**0.02**	**5.0 (4.7-5.3)**	**4.7 (4.1-4.9)**	**0.0005**
Nugent score	0 (0-0.5)	0 (0-1)	0.16	**8 (6-9)**	**2 (0-7.5)**	**<0.0001**
E2 (pmol/l)	81.0 (61.7-98.9)	71.4 (56.9-82.4)	0.35	**79.6 (52.9-125.1)**	**63.9 (46.8-71.0)**	**0.02**
Progesterone (nmol/l)	1.0 (0.5-1.3)	1.0 (0.6-1.6)	0.70	0.7 (0.4-1.2)	0.6 (0.4-1.1)	0.47
LH (IU/l)	4.2 (1.8-9.4)	4.3 (1.8-6.4)	0.73	4.5 (3.2-6.4)	3.7 (2.4-5.1)	0.26
Testosterone (nmol/l)	1.2 (0.3-1.5)	1.7 (1.1-1.8)	0.33	0.9 (0.3-1.2)	1.0 (0.7-1.4)	0.13

TN: True negative; FP: False positive; DMPA: Depot medroxyprogesterone acetate; PSA: Prostate specific antigen; IQR: Interquartile range; nucleic acid amplification test (NAAT), E2: Oestrogen, LH: Luteinising hormone

STI-/BV- women who were incorrectly grouped as false positive using the biomarker panel had significantly higher vaginal pH than women who were true negative (p=0.0005; Table 6.2), suggesting altered vaginal microbiota that was not detected by Nugent scoring may influence genital cytokine concentrations. Thus, 16S rRNA microbiome data from the WISH cohort (Lennard *et al*. 2017) was used to evaluate

the bacteria associated with each grouping. Women classified as false positive by the biomarkers model had increased relative abundance of several organisms associated with dysbiosis and BV, including *P. bivia* and *Gardnerella* (Figure 6.1).

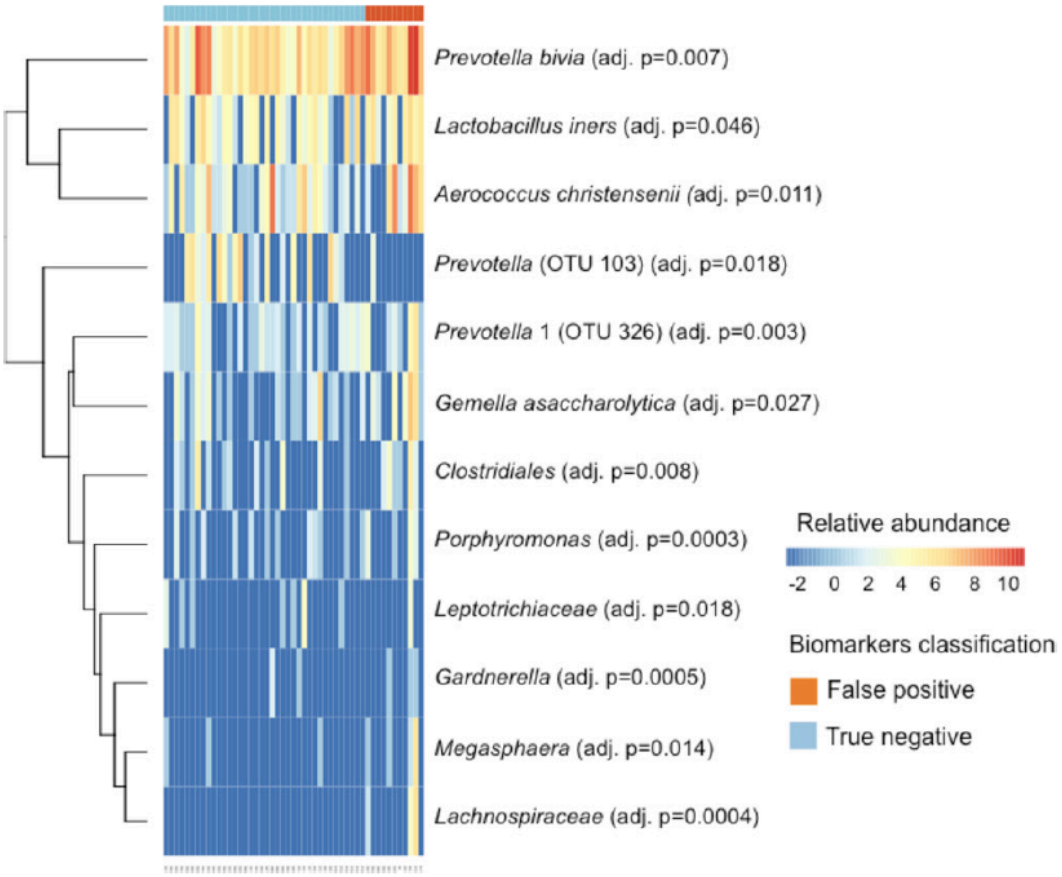


Figure 6.1 Vaginal bacteria that were significantly differentially abundant between a subset of true negative (n=38) and false positive (n=11) women. Differential abundance testing showed that false positive women had increased relative abundance of BV-associated organisms, including *Megasphaera, Gardnerella, Lachnospiraceaebacteria, Clostridiales, Prevotella bivia, Aerococcus christensenii, Gemella asaccharolytica, Porphyromonas and Leptotrichiaceae* and a lower abundance of *Prevotella* OTU 103 compared with true negatives (Masson, Barnabas, *et al*. Sex Transm Infect 2018;0:1–8. doi:10.1136/sextrans-2017-053506).

6.3.4 Vaginal pH alone as a predictor of asymptomatic STIs or BV

As vaginal pH differed between false positive and true negative women and between true positive and false negative women, pH was investigated as a biomarker of AGYW having STIs, BV and intermediate microbiota in the WISH cohort. Vaginal pH correctly classified 74% of women (sensitivity 77%, specificity 61%, PPV 88.3% and NPV 41%), however, the specificity of vaginal pH alone was substantially lower than the

biomarkers model (61% vs. 71%). Inclusion of vaginal pH in the biomarkers model significantly improved the accuracy, detecting a higher proportion of women with STIs, BV or intermediate microbiota (with a sensitivity 87%), albeit with reduced specificity (64%), and correctly classifying a higher proportion of women (82%) compared to the model including only cytokines (76%). Including vaginal pH in the model also reduced the number of cytokines that were needed for the model to achieve a similar predictive value as the model including all three cytokines, since IL-1α and vaginal pH correctly classified 82% of women (with a sensitivity of 86%, a specificity of 68%, a PPV of 91% and a NPV of 56%; Table 6.1).

6.4 Discussion

This Chapter validated the use of IL-1α, IL-1β and IP-10 as biomarkers for STIs and vaginal dysbiosis in AGYW from two separate geographical regions in SA and in three genital sample types. Of the sample types evaluated, the lateral wall swabs was determined as the best for biomarker measurement, based both on the accuracy (76% correctly classified) and ease of collection. Further, the inclusion of vaginal pH may help refine this tool as a model, since including pH and one cytokine, IL-1α, correctly classified 82% of women. Compared to the current standard of care, which relies on women presenting with clinical symptoms of STIs/BV, the cytokine biomarkers identified 77% of women with higher specificity (71% cytokine biomarkers vs. 57%), whereas only 41% of women with STIs or dysbiotic microbiota reported having symptoms.

Discharge-causing STIs and BV are important healthcare issues that are not being adequately addressed in resource-limited settings, as the majority of these conditions are asymptomatic (Mlisana *et al*. 2012). However, these genital conditions cause inflammation in the FGT that is in turn associated with increased risk of HIV acquisition and adverse reproductive outcomes (Masson *et al*. 2015).

An inexpensive POC biomarkers triage test for identifying women with STIs and BV, independently of the presence of absence of symptoms, could be used in family planning clinics, primary health centers, midwife obstetric units, private practices and pharmacies to screen women who, while accessing the healthcare system, may not necessarily be concerned about genital infections. A large proportion (approximately 34%) of reproductive-aged, sexually active women in SA use hormonal contraception (Chersich *et al*. 2017). Most would receive their contraception from one of the above facilities and thus, offering the biomarkers POC test at these facilities could potentially identify a large pool of at risk women with asymptomatic genital infections. Women identified using such a test could be triaged to either receive presumptive antibiotic therapy or further testing to determine the underlying etiology of their inflammation.

For the purposes of this study, sensitivity and specificity were considered to be of equal importance. However, depending on the implementation strategy for the device, the cytokine concentration cut-offs could be altered to optimize either sensitivity, or specificity, or both. It may be useful to maximise sensitivity should the device be used as a triage test to identify women who likely have a genital condition, and to then refer the women for etiological diagnosis. However, should the women identified using the test be offered immediate antibiotic treatment, both sensitivity and specificity would be important and both would need to be maximised. It was found that, in addition to higher sensitivity and specificity compared to clinical symptoms, the cytokine biomarkers also had a higher PPV (88% vs. 80%), suggesting that presumptive antibiotic treatment according to the biomarkers would result in the overtreatment of a smaller proportion of women compared to women treated according to clinical symptoms.

Another important factor of a POC test strategy is the complexity of sample collection and processing. In this study, cytokine biomarkers appeared to perform well in several different genital sample types (lateral vaginal wall swabs, vulvovaginal swabs and MCs), demonstrating the robust nature of these biomarkers across different regions of the lower reproductive tract. Although CVLs (requiring speculum examination and clinician input) were used for cytokine measurement in the initial CAPRISA 002 biomarker discovery study (Masson *et al*. 2015), lateral vaginal wall swab samples performed similarly to CVLs and marginally better than the other samples that were assessed, and would be more amenable to POC testing than MCs (which need to be kept in place for at least 30 minutes and may require some processing). As lateral vaginal wall swab samples are easy to collect, it is possible that a test device including these biomarkers could make use of self-collected samples, or the test could be self-administered, which would dramatically reduce the overall cost.

FGT inflammatory responses, and hence the concentrations of inflammatory cytokines including IL-1α, IL-1β and IP-10, may be influenced by many factors other than discharge-causing STIs, BV and intermediate microbiota. Studies have found (or suggested) that viral and/or ulcerative STIs (HSV, syphilis, chancroid), parasitic

infections, candidiasis, anti-inflammatories, antibiotics and antiretroviral initiation, semen exposure, the microbiome, blood reproductive hormone levels, menstrual cycle phase, pregnancy, cervical ectopy, hormonal contraceptive use, vaginal product use and blood contamination may influence the FGT inflammatory environment (Sharkey *et al*. 2012; Masson *et al*. 2014; Anahtar *et al*. 2015; Deese *et al*. 2015; Kaushic *et al*. 2011). In the South African setting, the aim of such a test would be to improve detection of both symptomatic and asymptomatic discharge-causing STIs and vaginal dysbiosis. These conditions are frequently asymptomatic and symptoms, when present are non-specific, whereas STIs such as HSV-1/2, syphilis and chancroid cause genital ulcers and condylomata lata (syphilis) and can be managed syndromically (Johnson *et al*. 2011). Furthermore, women who have syphilis can be identified using an existing, accurate *T. pallidum* rapid test and studies have shown that HPV is not associated with substantial changes in inflammatory cytokine concentrations (Kriek *et al*. 2016), which would thus be poor biomarkers of HPV. These infections (when present), as well as other potentially confounding factors, including candidiasis, hormonal contraceptive use, vaginal product use and blood contamination did not appear to impact participant classification using the biomarkers model. Semen exposure did not differ significantly between false positives and true negatives and between false negatives and true positives in this cohort. Blood oestradiol concentrations were lower in women who had a STI/BV/intermediate microbiota but were incorrectly classified negative (false negatives), compared to women correctly classified as positive. Oestradiol increases cytokine expression *in vitro* and during the follicular phase of the menstrual cycle (associated with elevated blood oestradiol concentrations), cytokine concentrations are elevated in the FGT (Wira *et al*. 2015). In this study, oestradiol was not significantly associated with changes in IL-1α, IL-1β or IP-10 concentrations, however it is possible that in some cases, women with lower oestradiol concentrations were incorrectly classified as STI negative/Nugent≤3 because they had lower cytokine levels as a result of lower oestradiol concentrations. It is also possible that this observed difference in oestradiol concentrations is a spurious false positive result due to the number of comparisons made in this study.

Women who had a bacterial/protozoan discharge-causing STI, BV or intermediate microbiota, but were classified as negative using IL-1α, IL-1β and IP-10 biomarkers, may not have been identified because they had low levels of genital inflammation as a result of having resolving or less severe infections, or these women may have been recently treated and may have PCR positive results due to the presence of dead organisms. Measurement of inflammatory cytokine biomarkers likely identifies women with the most severe inflammatory genital infections. Inclusion or exclusion of women with intermediate microbiota (Nugent scores 4-6) as endpoints in the model did not materially influence the predictive value. Women diagnosed with intermediate microbiota would not normally receive antibiotic treatment, however, the finding that these women had high levels of inflammation in this cohort (Chapter 4, section 4.3.3.1) suggests that treatment strategies for intermediate microbiota may need to be revisited, particularly as these women appear to have comparable levels of inflammation as women with BV.

Women who were falsely identified by the cytokine biomarker test as being positive (false positives who were STI, BV and intermediate microbiota negative by laboratory PCR tests and Nugent scoring, but classified as positive using the biomarkers model), had higher vaginal pH levels and increased abundance of dysbiotic and BV-associated bacteria compared to true negatives. Although Nugent scoring, the gold standard for BV diagnosis, is considered subjective, it is dependent on the individual reading the Gram-stained slide and influenced by the relative abundance of only a small subset of bacterial species that typically comprise BV (in particular *Gardnerella, Mobiluncus* and *Lactobacillus* species; Nugent *et al*. 1991). It is thus likely that, in some cases, women were incorrectly diagnosed as BV or intermediate microbiota negative using Nugent scoring. All of the taxa that were differentially abundant, aside from *L. iners*, *Leptotrichiaceae* and *Porphyromonas,* were also significantly more abundant in women with genital inflammation (Lennard *et al*. 2017; Anahtar *et al*. 2015). This suggests that these women have genital inflammation caused by these pathogenic bacteria and could also benefit from antibiotic treatment.

Although pH alone was marginally less accurate than the combined biomarkers model (74% vs. 76%) and had lower specificity (61% vs. 71%), the addition of pH to one of the inflammatory cytokines (IL-1α) had a better predictive value than all three cytokines in combination. Vaginal pH is one of the markers included in Amsel criteria for BV diagnosis, which has been found to perform poorly relative to Nugent scoring (Sha *et al*. 2005). Vaginal pH likely detects women with altered vaginal microbiota, which was very prevalent in this particular cohort, but does not detect common STIs that do not cause vaginal pH alterations. In contrast, the cytokine biomarkers also detect women with inflammatory STIs and those with the highest levels of genital inflammation who may be at increased risk of HIV (Masson *et al*. 2015; McKinnon *et al*. 2018). Therefore, the combination of an inflammatory marker and pH is likely to be particularly effective at identifying genital infections/conditions in general. In future, it will be important to evaluate the performance of pH together with an inflammatory marker in larger cohorts of women and cohorts of women in which BV is less prevalent. The use of a single cytokine together with vaginal pH could substantially reduce the cost a POC test compared to a test including three cytokines.

A limitation of this study is that, although a thorough evaluation of multiple potential confounding factors was conducted, the impact of HIV infection status, parasitic infections, the use of anti-inflammatories, antibiotics and antiretrovirals, menstrual cycle phase, pregnancy and cervical ectopy was not evaluated, which could influence genital inflammatory status and hence the accuracy of the biomarkers model. Another limitation is that the absolute cytokine concentration cut-offs for classification were not validated, as different sample types were evaluated in the different cohorts in order to identify the best sample type for this biomarkers test.

This test and proposed algorithm does, however, suggest an alteration of current practice, which is not to treat asymptomatic BV or women with a Nugent score of 4-6. It creates a new clinical pathway, however, management pathways and therapeutic options after identifying asymptomatic women with STIs and vaginal dysbiosis using such a biomarker test need to be further delineated. Thus, further investigations are needed to establish a clear risk/benefit profile prior to implementing such a change.

In conclusion, the robustness of cytokine biomarkers was validated across two different populations of women and three different genital sample types, showing that IL-1α, IL-1β and IP-10 concentrations consistently predicted the presence of discharge-causing STIs, BV and intermediate microbiota. Measurement of vaginal pH together with an inflammatory marker may further refine this diagnostic test. Although the accuracy of cytokine biomarkers model was approximately 76% (or 82% for the model including IL-1α and vaginal pH), this represents a substantial improvement on the current standard of care for identifying STIs and BV in resource-limited settings. Additionally, as the cytokines included in this model are markers of inflammatory processes, by measuring these cytokines the women with subclinical genital inflammation would likely be identified. Until more accurate, inexpensive, rapid, POC tests are developed and implemented in resource-limited settings, an inexpensive, true POC test including these biomarkers would likely prove extremely useful to decrease the prevalence of these genital conditions, which may in turn reduce the incidence of HIV infection and reproductive complications.

Chapter 7

Discussion

Although several probable risk factors have been suggested, none perfectly explain why young women, particularly in SSA, are at such high risk of HIV infection. It is estimated that more than 70% of all new HIV infections occur in SSA (Kharsany & Karim 2016) and, within this group, young women make up the majority. More basic and clinical research into how and why these young women acquire HIV is warranted. Physiological factors, including genital inflammation and hormonal contraceptives, are thought to impact genital health and increase HIV risk in women (Masson et al. 2015; Heffron et al. 2012). Some of the best understood drivers of genital inflammation include STIs and BV (Masson et al. 2014), which are inadequately diagnosed and managed in SA and in many other regions where syndromic management is the standard of care (Mlisana et al. 2012). Although there is convincing evidence that young women bear the burden of HIV risk in South Africa, few studies have focused on adolescence, in cohorts that are likely to be close to sexual debut. Although there are obvious differences between humans and primates, an intriguing study of natural SIV infection in a troop of African Green monkeys showed rapid acquisition of SIV in juvenile Green monkeys shortly after they become sexually mature, that was largely associated with increased frequencies of activated CD4+ T cells in their genital tracts (Ma et al. 2014). Data from this study in natural primate infection suggest that adolescence is a time of particular susceptibility to females.

This dissertation determined the prevalence of STIs and BV and their association with symptoms in the two cohorts of AGYW from SA; to examine the role of risky behavior on the prevalence of STIs in this age group and the proportion that were symptomatic; to investigate how factors such as endogenous hormone concentrations, hormonal contraceptive use, recent unprotected sex, or presence of STIs, BV and yeast infections influence cytokine biomarkers of adolescent genital inflammation previously associated with HIV risk and, finally, to validate the use of cytokine biomarkers to identify those with asymptomatic STIs or BV that may be at risk for HIV infection.

7.1 Relationship between STIs, BV or yeast infections and their symptoms in AGYW

Chapter 2 focused on characterizing the prevalence of risky behaviours, STIs and BV in AGYW that enrolled in the study. Almost 40% of AGYW in this study had at least one STI, with a higher overall prevalence in Masiphumelele than Soweto (52% vs. 26% with any STI, respectively). Adolescents with bacterial or parasitic STIs (including *C. trachomatis, N. gonorrhoeae, T. vaginalis* and *M. genitalium*) were older than the uninfected controls (median age 19 years vs. 18 years; p=0.042). However, both the infected and control groups had similar vaginal pH, detectable PSA and cotinine, and endogenous hormone concentrations. Adolescents with an infection were more likely to use injectable contraceptives (DMPA and Net-EN) than the control group (69% vs. 40%, p= 0.010) and to report lower condom use (27% vs. 54%, p=0.006).

C. trachomatis was the most common STI detected in the cohort and was more prevalent in Masiphumelele than Soweto (42% vs. 18%, respectively). *C. trachomatis,* previously shown to be one of the most common bacterial STIs both worldwide and in SSA (WHO 2016), is known to be recurrent and persistent in young women, with the major risk factor being resumption of sex soon after completion of antibiotic treatment with either azithromycin or doxycycline and failure to treat sexual partners (Whittington et al. 2001; Batteiger et al. 2010). As *C. trachomatis* was so prevalent, it was interesting to determine whether the circulating *C. trachomatis* isolates differ by geographic regions, which could be used to detect any underlying sexual networks. Thus, MLST was used to determine STs and to further explore this. Thirty-four STs were seen, 15 of which were novel. The three most common STs were ST12d (13.3%; 10/75), ST530b (12%; 9/75) ST3 (9.3%; 7/75), with no significant differences in ST distribution by geographical site.

N. gonorrhoeae was more prevalent in Cape Town than in Soweto (11% vs. 5%, respectively), while *T. vaginalis* and *M. genitalium* were similar in both cohorts. Several risk factors contribute to *T. vaginalis* incidence and prevalence, including a concurrent *C. trachomatis* infection, multiple sexual partners and black race (Helms et al. 2008). While susceptibility to and/or prevalence of most other STIs decrease with age, *T. vaginalis* infections have been

shown to be more common in older than younger women (Helms et al. 2008). A review by Gray-Swain and Peipert (2006) also suggested that young women were more likely to have PID caused by *N. gonorrhoea* than older women. In a longitudinal African study, women with *M. genitalium* at the previous visit were more likely to acquire HIV (OR of 2.42; 95% CI 1.01-5.80) (Mavedzenge et al. 2012).

The prevalence of HSV-2 shedding was similarly low between sites with low HSV-2 DNA positivity rates in both Masiphumelele and Soweto (5% vs. 1%, respectively) although prevalence of HSV-2 seropositivity was 21 % in Masiphumelele. High rates of HPV were seen at both sites (68% vs. 65%, respectively). Incident HSV-2 infections has previously been associated with a 30% increase in the odds of having a BV episode (Masese et al. 2014), irrespective of HIV status (Nagot et al. 2007).

Similarly, there appeared to be no significant difference seen between Masiphumelele (14%) and Soweto (7%) in prevalent fungal infections and BV (48% and 45%, respectively). VVC was diagnosed by the presence of hyphae on Gram stained microscopic slides, which is a traditional method to diagnose vaginal candidiasis (besides culture of *Candida* spp.). Some molecular methods and POC tests capture of *Candida* mannan from vaginal secretions have higher sensitivities and specificities than traditional methods (reviewed by Ilkit & Guzel 2011). However, it needs to be considered that while *Candida* spp. are also commonly found in the FGT of healthy women (in about 75%), only ~5% of these women will develop VVC (Bradshaw *et al*. 2005; Iliev & Leonardi 2017). Thus, it needs to be ensured that sensitive molecular methods do not over-diagnose VVC.

While indicators of risky behavior (such as having HIV serodiscordant partners, being in an intergenerational relationship, and reporting unprotected sex in the last three months) were more prevalent in adolescents from Soweto than Masiphumelele, so too was condom use (both at their last sex act and stating that they always used condoms) indicating that adolescents in Soweto maybe had better perception of their risk and may also explain the lower STI rates seen in Soweto compared to Masiphumelele. This is contrary to what has been previously been reported in Tanzania in which reporting of condom use correlated negatively

with risky behaviours, such as multiple sexual partnerships, in adolescents (14 to 18 years) (Njau et al. 2013).

As previously reported (Mlisana et al. 2012), symptoms were a poor tool to diagnose the presence of STIs or BV, with only 24% of those with a discharge-causing STIs or BV being symptomatic. Of the clinical symptoms that were evaluated, dysuria was the most sensitive for correctly diagnosing an STI while having a vaginal discharge that the adolescents considered abnormal was the least sensitive. Interestingly, the presence of concurrent conditions (like having multiple STIs or an STI together with BV) did not increase the symptom severity.

The continued reliance on syndromic management of STIs in this vulnerable population is of great concern. The high rates of STIs and BV in adolescents, and the poor predictive value of syndromic management is likely to contribute to the disproportionate HIV incidence seen in this population (Dellar et al. 2015). Routine speculum exams (Woo et al. 2011) and the development of care packages that would include point-of-care tests, immediate treatment and EPT have been suggested as alternatives to syndromic management. This package-of-care model was successfully used in Kwa-Zulu Natal, South Africa in a population of 267 young women (median age 23) that were attending a large public healthcare clinic for STI care, of whom 23.6% (63/267) were diagnosed with STIs (*C. trachomatis* 18.4%, *N. gonorrhoea* 5.2% and *T. vaginalis* 3.0%) using two POC tests: the GeneXpert® (*C. trachomatis* and *N. gonorrhea*) and the OSOM-1 Rapid Trichomonas Test. If diagnosed with any STI, infections were treated immediately. Using this approach, all but two *C. trachomatis* and one *N. gonorrhoea* infections were cleared by 12 weeks of follow up. Additionally, the majority of women that were offered EPT accepted it (54/62) and most partners were reported to having taken their treatment one week later (48/54) (Garrett et al. 2018). The study by Garrett *et al.* (2018) did not conduct a cost-effectiveness evaluation, which would be crucial if such a program was rolled out on a larger scale.

7.2 Collection of sexual risk behavior data in adolescents using an electronic questionnaire

Obtaining accurate sexual risk behavior data is critical to understanding and engaging AGYW with preventative programs that are tailored for this age group. However, these data are historically difficult to obtain and it has been suggested that the use of electronic questionnaires could aid with data collection, as it increases confidence in the anonymity of the data and speaks to the younger generation. Familiarity and access to web-based technologies may however better serve more developed countries, than the two socio-economically deprived communities from which the WISH study enrolled. The use of a paper-based method to collect demographic and risk behaviour data from adolescents is compared to an electronic method in Chapter 3.

In this study, the use of electronic methods did not alter the reporting of risky behavior in either cohort, neither did it improve the completeness of the questionnaires. There was a high prevalence of risky behaviour reported, with the majority of AGYW (57%) not knowing their partner's HIV status and 40% reporting unprotected vaginal sex in the last three months. The socio-geographic environment appeared to play a more important role in reporting of risk, with adolescents from Soweto reporting higher frequency of HIV discordant relationships, intergenerational relationships, use of injected drugs or getting a tattoo than those from Masiphumelele. There were no major differences in reported risk behaviour between the two questionnaire methods used within Masiphumelele and Soweto.

Despite not offering benefit in terms of data collection, the electronic questionnaire may be preferable to paper-based systems as they allow easier data extraction and minimize transcription errors that would decrease costs and improve accuracy. With greater acceptance of technical innovation and increased familiarity with this method of data collection, it is also possible that completion of questionnaires improve.

7.3 Characterising genital cytokine profiles in AGYW

Chapter 4 describes the genital cytokine profiles in "healthy" adolescents and examines some of the physiological factors that may influence this profile. Cytokines are key modulators of

inflammation, participating in acute and chronic inflammation *via* a complex and sometimes seemingly contradictory network of interactions (Turner et al. 2014). While it is possible to classify cytokines based on the nature of the immune response individual cytokines also performing specific roles dependent upon cell type and location (Turner et al. 2014). Further, a better understanding of molecular crosstalk in inflammatory cascades and the emergence of anti-cytokine therapies against chronic diseases associated with immune dysregulation has facilitated the discovery of non-canonical inflammatory markers and immune response modifiers (Fichorova 2004).

There was no obvious difference seen in genital cytokine profiles of healthy women in Masiphumelele or Soweto. Vaginal pH tended to be slightly higher in Soweto than Masiphumelele (4.7 vs, 4.3, respectively), and changes in the vaginal pH in this healthy cohort appeared to be associated with quite substantial changes in the cytokines measured.

Although endogenous hormones (E2, progesterone or LH) appeared not to have a significant impact on genital tract cytokines, exogenous hormonal contraceptives (Net-En and DMPA) had a marked inflammatory effect. The role of progesterone-only contraceptives in altering the genital inflammation is unclear with studies showing DPMA associated with both increases (IL-6, IL-8 and RANTES, ICAM-1, MIP-1α, MIP-1β, and IFN-γ, IP-10) (Deese et al. 2015; Morrison et al. 2014) and decreases (IL12p40, CCL11, MCP-1,MDC, IL-15, PDGF-AA and fractalkine) in cytokines (Ngcapu et al. 2015). Furthermore, observational studies have shown progestin-only HCs are associated with a two-fold increase in risk of vaginal HIV transmission (Heffron et al. 2012).

Recent sex, as indicated by the presence of PSA, did not appear to substantially influence genital inflammation with only IL-1β and GRO-α concentrations being slightly elevated. This is in contrast to previous studies which have shown that exposure to semen associated with increases in inflammatory cytokine and growth factor concentrations in the female genital tract, and with recruitment of leukocytes to the genital mucosa (Introini et al. 2016; Doncel et al. 2014).

7.4 Genital inflammatory cytokine changes associated with BV, STIs and fungal infections in adolescents

Chapter 4 further describes the changes in the inflammatory cytokine profile associated with BV, viral and yeast infections in the lower FGT of adolescents. BV was the most prevalent conditions in the cohort, with an overall prevalence of 47%. In addition, BV was the most inflammatory condition seen with more than three quarters of the cytokines being upregulated. Despite some differences in the bacterial communities associated with BV in Masiphumelele and Soweto (Lennard *et al.* 2017), genital cytokine profiles associated with BV appeared to be similar between the two sites. Cytokines commonly elevated in adolescents with BV from both sites were IL-1α, IL-1β, MIF, IL-18, β-NGF, TRAIL, SCGF-β, G-CSF, LIF, IL-12(p70), MCP-3, TNF-β, IL-7, IL-2Rα, PDGF-BB, HGF, CTACK, VEGF, IFN-ɣ, SDF-1α, M-CSF, IL-9, IL-4, IL-3, IFN-α2, IL-16, TNF-α, Eotaxin, IL-13, IL-10, IL-12(p40), IL-1Rα, GM-CSF and FGF-basic. Interestingly, BV was also associated with downregulation of five chemokines, including IP-10, MCP-1, IL-8, GRO-α and MIG three of which, GRO-α, IP-10 and MIG, which were downregulated in adolescents from Soweto and Masiphumelele. In adult African women, Masson *et al.* (2014) previously described that BV was linked with an increase in pro-inflammatory markers (IL-1α, IL-1β and TNF-β) but downregulation of several chemokines (IP-10, GRO, MDC and MIP-1α, IL-7 and GM-CSF). The up-regulation of inflammatory cytokines associated with BV seen in this younger adolescent cohort was more marked than has been described in other populations. Adolescents with BV were also more likely to be infected with a concurrent STI (20.8%). However, BV alone was more inflammatory than coinfection of BV and *C. trachomatis*, or BV and another STIs. This finding emphasizes the need to urgently define more adequate screening and treatment algorithms for BV.

Vaginal yeast infections were also highly inflammatory, with almost half of the cytokines measured being up-regulated cytokines. Yeast infections in Soweto appeared to cause more pronounced cytokine up-regulation than in Masiphumelele. Vulvovaginal fungal infections have previously been associated with strong inflammatory responses (Sobel 2016), although Th1 responses are thought to be protective against fungal infections, while Th2 responses are associated with increased susceptibility (Weissenbacher 2014).

Chapter 5 examines the role of prevalent bacterial and parasitic STIs and changes in genital tract inflammation. Almost three quarters of the adolescents enrolled in the WISH study were infected with at least one STI (including *C. trachomatis, N. gonorrhoeae, T. vaginalis, M. genitalium*, and HSV-2), with one in ten having multiple infections. Although *C. trachomatis* tended to be more prevalent than *T. vaginalis*, they appeared to be equally inflammatory, with almost half of the cytokines measured being elevated.

While it has been reported that *T. vaginalis* infection increases IL-8 and MIP-3a production (Fichorova et al. 2006), no such a pronounced change in genital inflammation as observed in this study has previously been reported. Geographical variation in inflammation with *T. vaginalis* infection was seen, with infections in Masiphumelele adolescents appearing to be slightly more inflammatory than those in Soweto.

In contrast, *C. trachomatis* infection was associated with marked changes in genital inflammatory cytokine levels (Rasmussen et al. 1997; Masson et al. 2014). Geographical variability was also seen with *C. trachomatis* infection, with more pronounced inflammatory response seen in Soweto than Masiphumelele (were no cytokines changed). Pattern of inflammation were associated with the various STs with ST3 exhibiting the most inflammatory pattern, with a quarter of the cytokines measured being elevated. In contrast, *C. trachomatis* ST137, the fourth most prevalent ST, was not inflammatory, with no significant changes in cytokine markers compared to uninfected women; and ST100b, the least inflammatory, caused down regulation of four cytokines. Geographic variation was also seen STs: ST530b in Soweto adolescents caused significant increases in the concentrations of ten of the cytokines measured compared to no changes seen in Masiphumelele. *Chlamydia* infection has been associated with increases results in an increase in pro-inflammatory cytokine IL-α, IL-β, IL-6, IL-12p70, TNF-α and chemokine production such as IL-8, MIP-1β, and RANTES (Masson et al. 2014). *C. trachomatis* is recognized by pattern recognition receptors including nucleotide-binding oligomerization domain-like receptors and Toll-like receptors (TLR) (Hafner et al. 2014). TLR2 and TLR4 are activated by live *Chlamydia* and highly expressed in female genital tissues (Horne et al. 2008).

In the adolescents with ≤2 years of sexual experience who were infected with *C. trachomatis* had very moderate changes in the genital cytokine profiles, with only MIG being upregulated. In contrast, those who had been sexually active for 2+ years had almost half of their genital cytokines elevated.

N. gonorrhoea infection did not impact on the genital inflammatory milieu in this cohort. As more than half of the adolescents infected with *N. gonorrhoea* were also co-infected with *C. trachomatis,* the effect of co-infection on the genital milieu was examined. AGYW co-infected with *N. gonorrhoea* and *C. trachomatis* tended to have less cytokine inflammation than those infected with either *C. trachomatis* or *N. gonorrhoea* alone, though higher levels of inflammation were seen in AGYW from Soweto that were co-infected than those from Cape Town. Similarly, *M. genitalium* infections appeared to cause only a moderate change in cytokines, with no significant changes after correction for multiple comparisons.

7.5 Using cytokine biomarkers to detect women with asymptomatic infections

While syndromic management, practiced in SA since the 1990's (The Department of Health 2015) has proven to be effective for ulcerative conditions, such as chancroid and syphilis, it has not been of benefit for women with discharge-causing STIs, such as *C. trachomatis, N. gonorrhoeae, T. vaginalis and M. genitalium*, or BV (Johnson *et al.* 2011). Thus, many women with asymptomatic but equally inflammatory conditions are not treated in SA.

Thus, Chapter 6 validated the use of IL-1α, IL-1β and IP-10 as biomarkers of asymptomatic STIs, BV and intermediate microbiota. The majority of AGYM in the cohort had a discharge-causing bacterial/protozoan STI, BV or intermediate microbiota. From this group, IL-1α, IL-1β and IP-10 together correctly classified 76% of women using their MC samples, 76% of women from LWS and 73% from their VVS. Additionally, these biomarkers identified significantly more women with these conditions than clinical symptoms, and the inclusion of clinical symptoms to the model did not improve the accuracy or fit. Importantly, the combination of these three cytokines identifies generally women with STIs and/or vaginal dysbiosis, independently of the etiological agent that causes the STI and/or vaginal dysbiosis. Bacterial

lipopolysaccharides have been shown to induce the production of several cytokines including TNFα, the IL-1 family, IL-6, IL-8, the IL-10 family, the IL-12 family, IL-15 and TGFβ via TLR-4 (Amoroso et al. 2011).

Identifying possible drivers of incorrect false-positive classification using this model, it was noted that false positive results were seen in women with a higher vaginal pH than true negative results. This suggested that a possible altered vaginal microbiota that was not detected by Nugent scoring that may change the genital milieu confirming the relationship between pH influence genital inflammation was seen in Chapter 4. Women who were considered to give false positive results using these biomarkers appeared to have increased relative abundance of organisms associated with BV, including *P. bivia* and *Gardnerella*. (Amoroso et al. 2011) In contrast, conditions such as HSV-2 DNA positivity and fungal infections were not associated with false positive results.

While vaginal pH alone correctly classified 74% of women, the specificity of vaginal pH alone was lower than the biomarkers model (61% versus 71%). The inclusion of pH in the biomarkers model improved the classification (82%) and sensitivity (87%) of results, but reduced specificity (64%). Additionally the inclusion of vaginal pH reduced the number of cytokines needed to achieve a similar predictive value: IL-1α and vaginal pH correctly classified 82% (sensitivity 86%, specificity 68%, PPV 91% and NPV 56%) while IL-1β and vaginal pH correctly classified 81% (sensitivity 84%, specificity 68%, PPV 91% and NPV 53%). Furthermore, there was very poor correlation between symptoms and the etiological diagnosis which would result in very few of this population accessing appropriate care. Despite this low levels of symptomology levels of genital inflammation and thus HIV risk are high.

7.6 Limitations

This study had several limitations. Due to logistical constraints, only the Masiphumelele cohort was followed longitudinally, while for the Soweto cohort data from only one time point was available. Future studies following AGYW from different sites on SA over time would provide more insight into time-dependent variations in cytokine levels and within- and between-participants variation. Further, self-reported behavioral data was included, which

might have been influenced by reporting bias. Symptom data was only collected form a sub-set of the participants, thus resulting in a limited sample size. Further, no detailed treatment data for participants themselves and their partners was collected, therefore the effect of treatment on cytokine levels could not be evaluated. Multivariate linear regression models were used to determine associations between non-infectious (such as vaginal pH and hormone concentrations) and infectious (such as STIs, BV and yeast) factors and FGT cytokine profiles. These statistical models allow for substantial collinearity, and alternatively decision trees where only the most influential cytokines and chemokines are selected for further analyses (Liebenberg *et al.* 2017; Arnold *et al.* 2015) could be used to address collinearity but infer cytokine influence. Another limitation is that while this thesis focused on evaluating factors associated with HIV risk, HIV seroconversion was not included as endpoint in the study design. Another limitation of this study was that stratification of women into groups with or without specific inflammatory conditions was difficult due to a combination of limited sample size and recruitment strategy.

7.7 Future Directions

Syndromic management is non-specific and results in both over- and under-treatment of these conditions. The development of better cytokine signatures of genital inflammation would allow the development of a cheap point-of-care device. This could allow more efficient treatment of STIs and BV, lower inflammation and thus lower HIV transmission (Masson et al. 2015).

7.8 Final conclusion

In summary, this dissertation provides a detailed description of behavioural and biological risk factors for HIV in young and adolescent South African women. The major findings were the high levels of behavioural risk seen in this population, the high prevalence of STIs and BV and the strong inflammatory responses, with up regulation of several cytokines, associated with the highly prevalent BV, *C. trachomatis*, *T. vaginalis*, and yeast infections. Previous epidemiological studies have suggested a strong relationship between BV/STIs and increased odds of HIV acquisition. The very poor correlation between symptoms and the etiological STI diagnosis would result in very few of this population accessing appropriate care and it is a clear indication that syndromic management, currently in place in South Africa, is not enough

to prevent HIV acquisition. Here, the findings suggest that a mechanism through which HIV risk increases is through a strong cytokine response to STIs, BV and fungal infections. Finding ways to reduce the prevalence of these conditions in AGYW, or devising new methods of dampening the immune response to these conditions could potentially decrease the incidence of new infections in this vulnerable population of young women. This thesis validates one potential method – the use of the biomarkers IL-1α, IL-1β and IP-10, which could potentially be used to develop a POC test that would allow the rapid identification and triage of at risk AGYW.

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Appendices

Appendix I. Socio-demographic questionnaire

Appendix II. Symptom question

Appendix I. Socio-demographic questionnaire

WISH: Women's initiative in sexual health

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Introduction

Hello my name is _____. I am part of a research team from the University of Cape Town (UCT). In this study we would like to know more about how young people in Soweto live. We want to know about the challenges that young people face in their daily lives. So, we are going to ask you in particular about sexual and substance use behavior, psychological, emotional and physical well being as well as questions about social and family support.

You have been invited as a possible participant because you are between 16-22 years of age, and because you live in Soweto. If you agree to participate in this study, please fill in the questionnaire attached to this information sheet. You will be asked to sign a consent form before you fill in the questionnaire and you will get a copy to keep. Please read the information provided on the consent form, and ask questions about anything you do not understand, before deciding whether you would like to participate or not.

Interview details

Participant Identification ☐☐☐☐ ☐☐ ☐☐☐

Date of Interview (DD/MMM/YYYY) _____

Location of interview: _____

Interviewer Name: _____

Interview start time: (HH:MM AM/PM): _____

Interview stop time: (HH:MM AM/PM): _____

Section 1: Demographics and Socio-Economic Status

1.1: Demographics

What is your date of birth?
_____ DAY/MONTH/YEAR

With respect to your sexual orientation, how do you currently identify?
Select one.

- ❑ Heterosexual/Straight
- ❑ Lesbian
- ❑ Gay
- ❑ Queer
- ❑ Bisexual
- ❑ Questioning
- ❑ Other, please specify: _____
- ❑ Don't know
- ❑ Prefer not to answer

Have you ever been married?

- ❑ Yes
- ❑ No
- ❑ Prefer not to answer

What is your current marital status?
Select one.

- ❑ Married (legal or traditional) or living as married
- ❑ Single
- ❑ Separated / Divorced
- ❑ Widowed
- ❑ Other, please specify: _____
- ❑ Prefer not to answer

If ever married, how old were you when you were first married?
_____ years.

- ❑ Don't know
- ❑ Prefer not to answer

What do you consider to be your racial and/or ethnic background?
Select all that apply.

- ❑ Zulu
- ❑ Pedi
- ❑ Tsonga
- ❑ Sotho
- ❑ Swati
- ❑ Tswana
- ❑ Venda
- ❑ Xhosa
- ❑ Ndebele

- ❑ Coloured
- ❑ White
- ❑ Indian
- ❑ Other, please specify: _____
- ❑ Don't know
- ❑ Prefer not to answer

What is the primary (main) language you speak at home?
Select one.

- ❑ Afrikaans
- ❑ English
- ❑ IsiNdebele
- ❑ IsXhosa
- ❑ IsiZulu
- ❑ Sepedi
- ❑ Sesotho
- ❑ Setswana
- ❑ SiSwati
- ❑ Tshivenda
- ❑ Xitsonga
- ❑ Other, please specify: _____
- ❑ Don't know
- ❑ Prefer not to answer

1.2 Socio-economic Status

Are you currently a student?

- ❑ Yes
- ❑ No
- ❑ Don't Know
- ❑ Prefer not to answer

If YES, what type of school are you currently enrolled?

- ❑ Primary / Grade school [Grades 1-7]
- ❑ Secondary / Highschool [Grades 8-12]
- ❑ Post-secondary school (Trade or technical training, college, or university)
- ❑ Other (specify) _____
- ❑ Don't Know
- ❑ Prefer not to answer

If NOT IN SCHOOL, what is the highest level of formal education you have completed?
Select one.

- ❑ No formal education
- ❑ Incomplete primary school (up to grade 7)
- ❑ Complete primary school (completed grade 7)
- ❑ Incomplete secondary school (up to grade 12)
- ❑ Complete secondary school (completed grade 12)
- ❑ Incomplete post-secondary training (Trade or technical training, college, or university)
- ❑ Complete post-secondary training (Trade or technical training, college, or university)
- ❑ Other (specify): _____
- ❑ Don't Know
- ❑ Prefer not to answer

Young people get money in a variety of ways; for instance, a full-time job, a part-time job, odd jobs or piece work, from social grants, and from parents, friends, or relatives. Over the last 6 months, what were the different ways YOU have made money? *Read list and select all that apply.*		**[of selected] Which was your main source of income?** *Select ONLY one.*
Employment (full-time or part-time job)	❑	❑
Self-employed (income from my own business)	❑	❑
Odd jobs or piece work (e.g.,	❑	❑
Receive social grants (e.g. Disability, Child support, Foster care)	❑	❑
From your parent(s)	❑	❑
From your boyfriend(s)/girlfriend(s)/partner(s)	❑	❑
From other friend(s)/ relative(s)	❑	❑
Personal savings	❑	❑
Loan(s) / Student Loan(s)	❑	❑
Honoraria (for participating in research, workshops, or trainings)	❑	❑
Scholarships		
Sex work *(ensure confidentiality)*	❑	❑
Selling drugs *(ensure confidentiality)*	❑	❑
Pan-handling/begging	❑	❑
Other, please specify: _____	❑	❑
None. I haven't made any money over the last 6 months	❑	❑
Don't know	❑	❑
Prefer not to answer	❑	❑

In an average month, how much money do you make in total (before any taxes)?
Select one.

- ❑ I do not make any money
- ❑ 0 to 499 Rand
- ❑ 2,000 to 2,999 Rand
- ❑ 1,000 to 1,999 Rand
- ❑ 3,000 to 3,999 Rand
- ❑ 4,000 to 4,999 Rand
- ❑ 500 to 999 Rand
- ❑ 5,000 or more Rand
- ❑ Don't know
- ❑ Prefer not to answer

How many people are financially dependent on you, not including yourself?

Indicate the number of people:_____

- ❑ Don't know
- ❑ Prefer not to answer

Read each item and decide whether it is true (T) or false (F) for you.	True	False
1. It is sometimes hard for me to go on with my work if I am not encouraged.	❑	❑
2. I sometimes feel resentful when I don't get my way.	❑	❑
3. On a few occasions, I have given up doing something because I thought too little of my ability.	❑	❑
4. There have been times when I felt like rebelling against people in authority even though I knew they were right.	❑	❑

	True	False
5. No matter who I'm talking to, I'm always a good listener.	❑	❑
6. There have been occasions when I took advantage of someone.	❑	❑
7. I'm always willing to admit it when I make a mistake.	❑	❑
8. I sometimes try to get even rather than forgive and forget.	❑	❑
9. I am always courteous, even to people who are disagreeable.	❑	❑
10. I have never been irked when people expressed ideas very different from my own.	❑	❑
11. There have times when I was quite jealous of the good fortune of others.	❑	❑
12. I am sometimes irritated by people who ask favors of me.	❑	❑
13. I have never deliberately said something that hurt someone's feelings.	❑	❑

Section 2: Health Care and Support Service Utilization

In this section we will ask you questions about health services you may have needed and/or received in the last six months. These include medical services, counselling, or social services offered through government clinics, private clinics, non-government organizations, community centres, or hospitals.

2.1 Health Care and Support Service Utilization

Type of health service	In the past 6 months, have you NEEDED any of the following services?		In the past 6 months, have you RECEIVED any of the following services?	
	Yes	No	Yes	No
Primary medical care (e.g., for a cold, minor injury)	❑	❑	❑	❑
Medical services for testing or treatment of sexually transmitted infections (STIs)	❑	❑	❑	❑
Family planning services, including access to birth control methods to prevent pregnancy	❑	❑	❑	❑
Medical services for abortion	❑	❑	❑	❑
Medical care for circumcision referral or procedure	❑	❑	❑	❑
Medical services for TB screening and treatment	❑	❑	❑	❑
HIV testing, prevention, treatment, and/or care services	❑	❑	❑	❑
Medical care for chronic conditions (e.g., asthma, diabetes)	❑	❑	❑	❑
Medical care during or after pregnancy	❑	❑	❑	❑
Dentist	❑	❑	❑	❑
Services from a social worker (e.g., access to social grants, housing, school access)	❑	❑	❑	❑

Mental health services or counseling (e.g, from a psychiatrist, psychologist, or counselor)	❑	❑	❑	❑
Counselling and other services for support related to physical violence	❑	❑	❑	❑
Counselling and other services for support related to sexual violence	❑	❑	❑	❑
Counselling for addictions (e.g., alcohol, drugs)	❑	❑	❑	❑
Emergency hospital services (e.g., car accidents, pregnancy complications, mental health)	❑	❑	❑	❑
Emergency hospital services for infectious diseases (e.g., STIs, HIV, TB, Hepatitis, malaria)	❑	❑	❑	❑
Emergency hospital services for physical violence / trauma (e.g, rape, gunshot wounds).	❑	❑	❑	❑
Admitted to the hospital	❑	❑	❑	❑
Other (please specify): _____				

Section 3: Emotional Wellbeing and Resiliency

3.1 Perceived Stress

Below is a list of questions that ask you about your feelings and thoughts. Please read each one carefully and indicate how often you felt or thought a certain way during the last month.
Select one response per row.

In the last month, how often have you:							
	Never	**Almost never**	**Sometimes**	**Fairly often**	**Very often**	**Don't know**	**Prefer not to answer**
Been upset because of something that happened unexpectedly?	❑	❑	❑	❑	❑	❑	❑
Felt that you were unable to control the important things in your life?	❑	❑	❑	❑	❑	❑	❑
Felt nervous and "stressed"?	❑	❑	❑	❑	❑	❑	❑
Felt confident about your ability to handle your personal problems?	❑	❑	❑	❑	❑	❑	❑
Felt that things were going your way?	❑	❑	❑	❑	❑	❑	❑
Found that you could not cope with all the things that you had to do?	❑	❑	❑	❑	❑	❑	❑
Been able to control irritations in your life?	❑	❑	❑	❑	❑	❑	❑
Felt that you were on top of things?	❑	❑	❑	❑	❑	❑	❑
Been angered because of things that were outside of your control?	❑	❑	❑	❑	❑	❑	❑
Felt difficulties were piling up so high that you could not overcome them?	❑	❑	❑	❑	❑	❑	❑

3.2 Depression

Below is a list of the ways you might have felt or behaved during the past week. Please tell me how often you have felt this way during the past week.
Select one response per row.

During the past week:	**Less than 1 day)**	**1-2 days**	**3-4 days**	**5-7 days**	**Don't know**	**Prefer not to answer**
I was bothered by things that usually don't bother me.	❑	❑	❑	❑	❑	❑
I had trouble keeping my mind on what I was doing.	❑	❑	❑	❑	❑	❑
I felt depressed.	❑	❑	❑	❑	❑	❑
I felt that everything I did was an effort.	❑	❑	❑	❑	❑	❑
I felt hopeful about the future.	❑	❑	❑	❑	❑	❑
I felt fearful.	❑	❑	❑	❑	❑	❑
My sleep was restless.	❑	❑	❑	❑	❑	❑
I was happy.	❑	❑	❑	❑	❑	❑
I felt lonely.	❑	❑	❑	❑	❑	❑
I could not get "going".	❑	❑	❑	❑	❑	❑

3.3 Anxiety

Below is a list of questions about things that might have bothered you. Please read each one carefully and indicate how often you have been bothered by each problem during the past two weeks.

During the past two weeks, how often have you….	**Not at all**	**Rarely, less than a day or two**	**Several days**	**More than half the days**	**Nearly every day**	**Don't Know**	**Prefer not to answer**
Felt nervous, anxious, or scared?	❑	❑	❑	❑	❑	❑	❑
Not been able to stop worrying?	❑	❑	❑	❑	❑	❑	❑
Not been able to do things you wanted to or should have done, because they made you feel nervous?	❑	❑	❑	❑	❑	❑	❑

Section 4: Girls' and Women's Reproductive Health

4.1 Menstrual History

Have you ever had a menstrual period?
Select one.

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

How old were you when you had your first menstrual period?
Probe best estimate.
Age in years: _____
- ❑ Don't know
- ❑ Prefer not to answer

On what date did you start your last menstrual period?
Probe best estimate.
DD / MM/ YYYY: _____
- ❑ Don't know
- ❑ Prefer not to answer

In the last six months, what was the usual length of your menstrual cycles?
Select one.
- ❑ Less than 24 days
- ❑ Between 24-35 days
- ❑ Greater than 35 days
- ❑ Too variable or irregular to say
- ❑ Don't know
- ❑ Prefer not to answer

Which of the following describes the duration of your menstrual period in the last six months?
Select one.
- ❑ Less than 4 days
- ❑ Between 4-7 days
- ❑ Greater than 7 days
- ❑ Too irregular to say
- ❑ Don't know
- ❑ Prefer not to answer

How would you describe your menstrual flow in the last six months? My menstrual bleeding has been or was:
Select one.
- ❑ Light
- ❑ Medium
- ❑ Heavy
- ❑ Very heavy
- ❑ Too irregular to say
- ❑ Don't know
- ❑ Prefer not to answer

In the last six months, have you at any time gone more than three months (90 days) without your menstrual period?
Select one.
- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

In the last six months, have your periods been regular or irregular?
Select one.
- ❑ Regular
- ❑ Irregular

- ❑ Don't know
- ❑ Prefer not to answer

Do you bleed in-between periods?
Select one.
- ❑ Yes
- ❑ No
- ❑ Don't know
  Prefer not to answer

4.2 Vaginal Hygiene Practices

Now I'd like to ask you some questions about vaginal hygiene practices.

	Have you EVER…			(IF 'EVER'…) In the last 30 days, how often have done this?						
	Yes	No	Prefer not to answer	Never	Once or twice	Weekly	A few times a week	Almost daily	Daily	Prefer not to answer
Washed with water inside your vagina?	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Washed with soap inside your vagina?	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Washed with something else besides water or soap inside your vagina, including detergents or antiseptics?	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Used your fingers to wash inside your vagina?	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Used anything else to wash inside your vagina, including cloths or sponges?	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Put or kept traditional medicines or herbs inside your vagina?	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Put or kept medicine from a doctor/nurse/pharmacy inside your vagina?	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Put or kept paper, cloth, or cotton wool inside your vagina?	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Used a tampon?	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Put or kept anything else inside your vagina?	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Used anything to dry or tighten your vagina for sex?	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Practiced douching to clean your vagina?	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Had sex during your period?	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑

4.3 Pregnancy History

Have you ever been pregnant? This includes all pregnancies, whether the outcome was a live birth, miscarriage, stillbirth, termination of pregnancy (abortion), or an ectopic/tubal pregnancy.
Select one.

- ❑ Yes
- ❑ No
- ❑ Prefer not to answer

How many times have you ever been pregnant?
Ensure response includes current pregnancy, if applicable.
Select one.

- ❑ 1
- ❑ 2
- ❑ 3
- ❑ 4
- ❑ 5
- ❑ 6
- ❑ 7
- ❑ 8 or more
- ❑ Don't know
- ❑ Prefer not to answer

[if 1 or more pregnancies] For your most recent pregnancy what was the outcome of the pregnancy?
Select one.

- ❑ Not Applicable, Currently Pregnant
- ❑ Single live birth
- ❑ Multiple live birth
- ❑ Miscarriage
- ❑ Stillbirth
- ❑ Pregnancy termination
- ❑ Ectopic pregnancy
- ❑ Don't know
- ❑ Prefer not to answer

[if multiple live births] How many live births occurred?

- ❑ Two
- ❑ Three
- ❑ Other, please specify: _____

[All pregnancies] Was this a planned pregnancy?
Select one.

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

[if not current pregnancy] Did you undergo antenatal HIV screening during this pregnancy?
Select one.

- ❑ Yes
- ❑ No

- ❑ Don't know
- ❑ Prefer not to answer

[if live birth] When did you have the baby?

Indicate month and year of delivery:

- ❑ Don't know
- ❑ Prefer not to answer

[if not live birth] When did the pregnancy end?

Indicate month and year:

- ❑ Don't know
- ❑ Prefer not to answer

[if not live birth] How many weeks had you been pregnant when the pregnancy ended?

Indicate number of weeks: _____

- ❑ Don't know
- ❑ Prefer not to answer

4.4 Contraceptive use

In the past have you used any form of contraception, safer sex method, or any other means to prevent pregnancy, to avoid getting or giving sexually transmitted infections, or to regulate your periods?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

In the past, what forms of contraception, safer sex method, or any other means to prevent pregnancy, to avoid getting or giving sexually transmitted infections, or to regulate your period have you used?
Interviewer to read through list and select all that apply.

- ❑ Oral contraceptive, also known as 'the pill'
- ❑ An injection/injectable, also known as 'Depo-Provera'
- ❑ NuvaRing, a vaginal ring that you insert once a month
- ❑ A contraceptive patch, also known as Ortho Evra and used once a week
- ❑ Implanon, also known as a "progestin implantable contraceptive"
- ❑ An Intrauterine Device, also known as an "IUD" or "Copper IUD"
- ❑ An Intrauterine System, also known as an "IUS" or "Mirena"
- ❑ A diaphragm, also known as a "cervical cap"
- ❑ The sponge
- ❑ Any vaginal creams, jellies, or foams
- ❑ Any emergency contraception, commonly known as "Escapelle", "the morning after pill", or "Norlevo"?
- ❑ Male condoms
- ❑ Female condoms
- ❑ The rhythm method, also known as "periodic abstinence"
- ❑ The withdrawal method
- ❑ Chosen to practice abstinence with male partners
- ❑ Any other method? (please specify) _____

How old were you when you first used contraception, safer sex method, or any other means to prevent

pregnancy, to avoid getting or giving sexually transmitted infections, or to regulate your periods?

- ❑ Age in years: ____ _____ years of age.
- ❑ Don't know
- ❑ Prefer not to answer

(For individuals whose partners use male condoms) In the past, how consistently have you used male condoms?

- ❑ Always, every time I've had sex
- ❑ Most of the time
- ❑ Occasionally
- ❑ Never
- ❑ Unsure
- ❑ Prefer not to answer

(For individuals who use female condoms) In the past, how consistently have you used female condoms?

- ❑ Always, every time I've had sex
- ❑ Most of the time
- ❑ Occasionally
- ❑ Never
- ❑ Unsure
- ❑ Prefer not to answer

(For individuals who use oral or injectable contraception) In the past 6 months, have you ever forgotten to take your contraception tablets or were you ever more than 5 days late for your injection?

- ❑ Yes
- ❑ No
- ❑ Don't Know
- ❑ Prefer not to answer

(For individuals who report using emergency contraception) How many times have you taken emergency contraception?

Indicate number of times: _____

- ❑ Don't know
- ❑ Prefer not to answer

What are the main reasons that you have not used contraception in the past 6 months?

Select all that apply, even if the reasons have changed.

- ❑ I am currently pregnant
- ❑ I am trying to become pregnant
- ❑ I don't mind becoming pregnant
- ❑ I don't believe in using birth control
- ❑ I don't think I would become pregnant
- ❑ I cannot become pregnant
- ❑ I cannot become pregnant because my sexual partner is infertile
- ❑ I don't like using contraception
- ❑ My sexual partner doesn't like using contraception
- ❑ My sexual partner refuses to use/will not let me use contraception
- ❑ I am not having sex with a biological man (e.g., my sexual partner is a woman, transman, etc.)
- ❑ **I am not having any sex (Do Not Proceed with the study)**
- ❑ I am in a mutually faithful sexual relationship
- ❑ I knew my partner and I had the same HIV status (e.g., "we are both HIV-positive" or "we are both HIV-negative")
- ❑ I thought my partner(s) was/were at low risk of getting HIV or AIDS

- ❑ I do not know where to get contraception or do not have access to contraception
- ❑ I feel uncomfortable/embarrassed to go to a family planning clinic
- ❑ I went to a family planning clinic but I was not treated well
- ❑ Other, please specify: _____
- ❑ Don't know
- ❑ Prefer not to answer

What is/ would be your preferred method of contraception?

Select one.

- ❑ Oral contraceptive, also known as 'the pill'
- ❑ An injection/injectable, also known as 'Depo-Provera'
- ❑ NuvaRing, a vaginal ring that you insert once a month
- ❑ A contraceptive patch, also known as Ortho Evra and used once a week
- ❑ Implanon, also known as a "progestin implantable contraceptive"
- ❑ An Intrauterine Device, also known as an "IUD" or "Copper IUD"
- ❑ An Intrauterine System, also known as an "IUS" or "Mirena"
- ❑ A diaphragm, also known as a "cervical cap"
- ❑ The sponge
- ❑ Any vaginal creams, jellies, or foams
- ❑ Any emergency contraception, commonly known as "Escapelle", "the morning after pill", or "Norlevo"?
- ❑ Male condoms
- ❑ Female condoms
- ❑ The rhythm method, also known as "periodic abstinence"
- ❑ The withdrawal method
- ❑ Chosen to practice abstinence with male partners
- ❑ Another method (please specify): _____

4.5 Fertility Intentions

Do you intend to have (more) children in the future?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

[if intend to have children] When in the future do you intend to have children?

Select one.

- ❑ I'd like to have children now
- ❑ Not now, but within 1 year
- ❑ In 1 to 2 years from now
- ❑ In 3 to 4 years from now
- ❑ More than 4 years from now
- ❑ Don't know
- ❑ Prefer not to answer

4.5 Risk factors		**Low risk**	**At risk**	**Total**
1. Have all your current sexual partners tested for HIV? (Unknown partner status)	n/a	yes	No / don't know (1 point)	
2. Are any of your sexual partners HIV positive? (Discordancy)	n/a	no	Yes/ Don't know (1 point)	
3. Did you use condoms every time you had sex in the past 3 months? (Unprotected sex)	n/a	Yes	No (1 point)	
4. Have you had more than one sexual partner in the past 3 months or is your partner having other partners? (Multiple partners)	n/a	No	Yes (1 point)	
5. Is your partner more than 10 years older/ younger than you (Intergenerational sex)	n/a	no	Yes (1 point)	
6. Did you have anal sex with someone in the past 6 months?	n/a	no	Yes (1 point)	
7. Did you have sex with someone for food, airtime, clothes, money, etc. in the past 6 months? (Transactional sex)	n/a	no	Yes (1 point)	
8. Have you or your partner had a STI in the past 6 months?	n/a	no	Yes (1 point)	
9. Have you injected drugs/ had a tattoo/ needed blood products in the past 6 months?	n/a	no	Yes (2 points)	
			TOTAL	

0 = low risk = test yearly unless in window period

1 = At risk = test in 3 months – 12 months (depending on risk factor and if in window period)

2 or more = high risk= test in 3 months time

Is there a risk factor which can be addressed? No❑ Yes❑

Section 5: Women's Sexual Health

The next section includes some personal questions about your sexual activities. Some of these questions are very personal and may make you feel uncomfortable. Since the survey is confidential, no one will know your answers. We would appreciate your participation in answering these questions as truthfully as possible. If you would like to complete this section yourself, you are welcome to do so. If you would like me to guide you through this whole section, that's okay too.

How would you like to proceed?
Select one.
- ❑ I'd prefer to complete this section myself
- ❑ I'd prefer to complete this section together

5.1 Sexual History

Have you ever had consensual sex? Consensual sex means that both you and your partner wanted to have sex.
Select one.
- ❑ Yes
- ❑ No
- ❑ Prefer not to answer

How old were you the first time you had consensual vaginal or anal sex?
Indicate age in years: _____
- ❑ Don't know
- ❑ Prefer not to answer

The first time you had consensual sex, what was/were the main reason(s)? Please select all that apply.
- ❑ In love
- ❑ Wanted to feel loved/protected
- ❑ For pleasure/I like having sex/It's fun
- ❑ Friends were/everyone was/did not want to feel left out
- ❑ Peer pressure from friends
- ❑ Forced/pressured by partner
- ❑ For food, shelter, clothes or other goods
- ❑ Other (please specify): _____
- ❑ Prefer not to answer

The first time you had sex with a partner, what was the nature of this relationship?
- ❑ Friends
- ❑ Boyfriend/girlfriend
- ❑ Husband/wife
- ❑ Stranger
- ❑ Other (please specify): _____
- ❑ Prefer not to answer

How many people have you had sex with in your lifetime?

Number of different people: _____

- ❑ Don't know
- ❑ Prefer not to answer

During the past 12 months, have you had vaginal or anal sex?

- ❑ Yes
- ❑ No
- ❑ Don't Know
- ❑ Prefer not to answer

If Yes with how many partners? ____ ____

How many of these relationships were "exclusive" or "mutually monogamous" (meaning while you were in the relationship, you and your partner only had sex with each other, and had no other sexual partners outside the relationship)? _____ _____

If you are using condoms with your partners, how often do you use them?

- ❑ Always, every time I've had sex
- ❑ Most of the time
- ❑ Occasionally
- ❑ Never
- ❑ Unsure
- ❑ Prefer not to answer

During the past 12 months, have you had vaginal or anal sex?

- ❑ Yes
- ❑ No
- ❑ Don't Know
- ❑ Prefer not to answer

During the past 12 months, have you used condoms regularly?

- ❑ Yes
- ❑ No
- ❑ Don't Know
- ❑ Prefer not to answer

Did you use barrier protection (male or female condoms) with your last sexual act?

- ❑ Yes
- ❑ No
- ❑ Don't Know
- ❑ Prefer not to answer

During the past 12 months, have you had vaginal or anal sex with someone you know or suspected to be HIV-infected?

- ❑ Yes
- ❑ No
- ❑ Don't Know
- ❑ Prefer not to answer

During the past 12 months, have you had vaginal or anal sex with someone you know or suspected injected

drugs?

- ❑ Yes
- ❑ No
- ❑ Don't Know
- ❑ Prefer not to answer

During the past 12 months, have you had vaginal or anal sex with someone in exchange for giving or receiving food, clothes, money, drugs, or favours?

- ❑ Yes
- ❑ No
- ❑ Don't Know
- ❑ Prefer not to answer

Have you ever been diagnosed with or been treated for a sexually transmitted infection?
Examples:
Syphilis, gonorrhoea, non-gonococcal urethritis, herpes simplex virus type 2 (HSV-2), chlamydia, pelvic inflammatory disease (PID), trichomonas, mucopurulent cervicitis, epididymitis, procitis, lymphogranuloma vernereum, chancroid, hepatitis B.

- ❑ Yes
- ❑ No
- ❑ Don't Know
- ❑ Prefer not to answer

During the past 12 months, have you been diagnosed with a sexually transmitted infection?

- ❑ Yes
- ❑ No
- ❑ Don't Know
- ❑ Prefer not to answer

How many sexually transmitted infections have you had in your lifetime?

- ❑ Yes
- ❑ No
- ❑ Don't Know
- ❑ Prefer not to answer

Appendix II. Symptom questionnaire

WISH Study Symptom Check Sheet Version 1.0

Date: __ __ / __ __ __ / __ __ __ __

PID Number: WISH - 01 - __ __ __

Participant symptom checklist (please mark which symptoms are present):

	Yes	No
Does the participant have an abnormal burning sensation when urinati	☐	☐
Does the participant have a vaginal discharge?	☐	☐
If yes, is the discharge abnormal or cause worry?	☐	☐
Does the participant have vaginal irritation, burning or itching?	☐	☐

Other notes: