The Impact of Point-of-care Testing and Treatment of Sexually Transmitted Infections and Bacterial Vaginosis on the Genital Epithelial Barrier Integrity

By

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Submitted in fulfillment of the academic requirements for the degree of Master of Medical Science

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School of Laboratory Medicine and Medical Science

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Durban

2022

As the candidate's supervisor I have approved this thesis for submission

Signed: [redacted] Name: Lenine Liebenberg Date: 22 Dec 2022

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3) To the CAPRISA organization for the opportunity and for providing the facilities to conduct my research. I would also like to thank the CAPRISA 083 participants. I am thankful to the CAPRISA staff for their kindness and willingness to assist whenever needed.
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5) To my family and my friends, thank you for making this journey so much easier with all the words of encouragement and cheering.

POSTER PRESENTATIONS

1. Impact of point-of-care testing and treatment of sexually transmitted infections and bacterial vaginosis on the genital epithelial barrier integrity.

Ndlela N, Garrett N, Mtshali A, Osman F, Singh R, Mitchev N, Ngobese N, Abdool Karim S1, Mlisana K, Passmore J, Rompalo A, Mindel A, and Liebenberg LJP

The 24th International AIDS Conference, Virtually, 29 July – 2 August 2022

Impact of Point-of-care Testing and Treatment of Sexually Transmitted Infections and Bacterial Vaginosis on the Genital Epithelial Barrier Integrity

Ndlela N[1], Garrett N[1,2], Mtshali A[1], Osman F[1], Singh R[3,4], Mitchev N[3], Ngobese N[5], Abdool Karim S[1,6], Mlisana K[3,4], Passmore J[1,7,8], Rompalo A[9], Mindel A[1], and Liebenberg LJP[1,3]

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INTRODUCTION

- Genital inflammation and epithelial barrier damage are factors responsible for women's unique vulnerability to HIV.
- Because sexually transmitted infections (STIs) and bacterial vaginosis (BV) directly contribute to these, effective treatments are necessary to improve genital health and limit HIV risk in women.[1,2]
- A programme of point-of-care (POC) STI/BV testing, immediate treatment, and expedited partner therapy (EPT) is shown to clear STIs and reduce inflammation in a cohort of young women in South Africa[3]
- Here we investigate the impact of this approach on the integrity of the genital epithelial barrier against HIV/STIs.

RESULTS

MMP concentrations are higher in women with STIs/BV

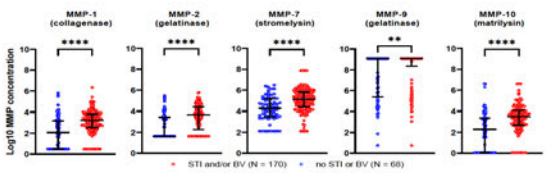

Figure 1: MMP concentrations in women with and without STI/BV. MMP concentrations were measured in MC fluid of women with STI/BV (170) and women with neither STI nor BV (n=68) at baseline. MMP data are logged, and Mann-Whitney tests were used to compare the non-parametric data. P-values <0.01 and <00001 are indicated by asterisks * * and ****, respectively. Significant differences were denoted by p-values <0.05.

STI treatment is associated with reduced MMP-1

- Sixty of the 170 women with detectable STI (*C. trachomatis, N. gonorrhoeae, T. vaginalis,* and *M. genitalium*) or BV at baseline (35.29%) were followed up at both 6- and 12-weeks post-treatment.
- Of these, STIs and BV were resolved in 88.57% (31/35) and 24.56% (14/57) women by 12 weeks post-treatment, respectively.
- MMP concentrations were positively associated with several pro-inflammatory cytokines
- STI treatment, regardless of BV status (n=35), was associated with a marked reduction in MMP-1 (p=0.0039; **Fig.2)**, although not reduced to the level observed in the healthy baseline population.
- No changes in MMP concentrations were observed in women treated for BV (n=57), regardless of their STI status **(Fig.3).**

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Presented at AIDS 2022 – The 24th International AIDS Conference

METHODS

- HIV-negative women (N= 238) with BV or POC diagnosis of Chlamydia trachomatis, Neisseria gonorrhoeae, or Trichomonas vaginalis (STI/BV) received immediate treatment and EPT for STIs. Participants were retested 6- and 12-weeks post enrolment.
- Concentrations of 48 cytokines and 5 matrix metalloproteinase (MMP) biomarkers of epithelial barrier integrity were measured in menstrual cup (MC) fluid using multiplex ELISA technology.
- Mann-Whitney tests were used to assess the relationship between MMP concentrations and STI/BV at baseline, with ANOVA and multivariable linear mixed models used to assess the impact of treatment on MMP concentrations.

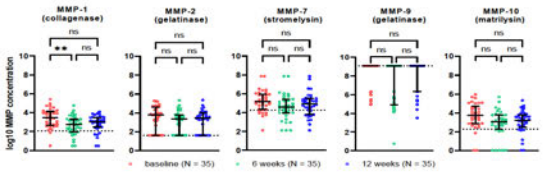

Figure 2: MMP concentrations in women treated for STI. Median MMP concentrations were assessed in matched MC fluid across all three visits to evaluate the impact of STI treatment only (N=35). The dotted lines indicate average MMP concentrations in uninfected individuals. Linear mixed models controlling for age, pelvic examination, and BV confirmed these observations. P-values >0.05 are indicated by ns and p-values <0.01 are indicated by asterisks **.

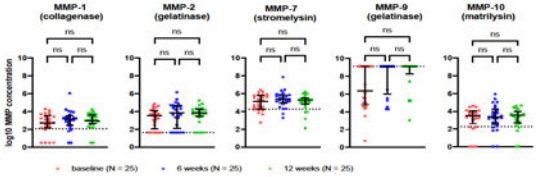

Figure 3: MMP concentrations in women treated for BV. Median MMP concentrations were assessed in matched MC fluid across all three visits to evaluate the impact of BV treatment only (N= 25). The dotted lines indicate average MMP concentrations in uninfected individuals. All p-values >0.05 are denoted by ns. Linear mixed models controlling for age, pelvic examination, and STIs confirmed these observations.

CONCLUSION

- Our findings indicate that while POC STI/BV testing, immediate treatment, and EPT can reduce STIs and genital inflammation, only MMP-1 levels were reduced, but not restored completely.
- BV treatment did not alter genital MMP levels.
- Additional strategies to improve epithelial barrier integrity may be required to reduce HIV infection risk

CAPRISA · NRF National Research Foundation · AIDS 2022 29 July – 2 August

2. Investigating the impact of POC STI/BV treatment on genital epithelial barrier integrity

Ndlela N, Garrett N, Mtshali A, Osman F, Singh R, Mitchev N, Ngobese N, Abdool Karim S1, Mlisana K, Passmore J, Rompalo A, Mindel A, and Liebenberg LJP

11th Infectious Diseases in Africa Symposium, Cape Town, Stellenbosch, 5-10 September 2022

Investigating the impact of POC STI/BV treatment on genital epithelial barrier integrity

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Introduction

- The genital epithelial barrier is an essential first line of defense in women, limiting access of pathogens to susceptible HIV target cells in the mucosa.
- Sexually transmitted infections (STIs) and bacterial vaginosis (BV) are common causes of inflammation in the genital epithelial barrier.
- Although this inflammation may be important in pathogen elimination, it may influence HIV susceptibility through epithelial barrier disruption and HIV target cell recruitment.
- The contribution of STIs and BV to this inflammation implies that their treatment could potentially reduce HIV susceptibility in women.
- Point-of-care (POC) testing and treatment of STIs/BV and expedited partner therapy (EPT) has recently been shown to clear STIs and reduce genital inflammation in young women. Here, we evaluated its impact on genital epithelial barrier integrity.

Methods

- 238 women received a POC diagnosis of *Chlamydia trachomatis* and *Neisseria gonorrhea* by Xpert and *Trichomonas vaginalis* using OSOM rapid tests.
- BV was diagnosed using microscopy by Nugent scoring, and *Mycoplasma genitalium* was detected using PCR.
- Women with STIs/BV received immediate treatment and EPT and were retested 6- and 12 weeks post-treatment.
- Concentrations of 48 cytokines and 5 matrix metalloproteinase biomarkers of barrier integrity (MMP) were measured in Menstrual Cup (MC) fluid at each visit by Multiplex Elisa technology.
- Mann-Whitney tests and multivariable linear mixed model analyses were used to determine the relationship between MMP and STI/BV at baseline and the treatment effect on MMP concentrations, respectively.

Results

Women with STIs/BV have higher levels of MMPs

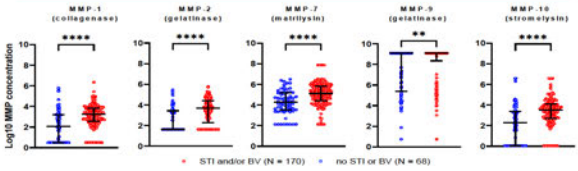

Figure 1: Comparisons of MMP concentrations in MC fluid of women with STIs/BV (n=170) and women without STIs/BV (68) at baseline. The Mann-Whitney test was used to compare median MMP concentrations between the two groups. The MMP data is logged, and significant p-values are denoted by asterisks (*).

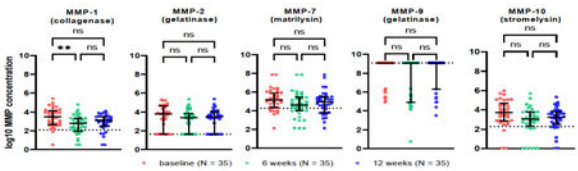

Figure 2: MMP concentrations in matched MC fluid of women treated for STIs (n=35), regardless of BV status over three visits. Friedman test was used to compare the median MMP concentrations, and these observations were confirmed by linear mixed models controlling for age, pelvic examination, and BV. MMP concentrations in healthy individuals are denoted by the dotted lines. Significant and insignificant p-values are denoted by asterisks (*) and ns, respectively.

Temporary MMP-1 reduction on STI treatment

- Of the 238 women enrolled, 170 had any STI/BV at baseline and the prevalence of these were 14.29%(CT), 4.62% (NG), 4.2% (TV), 4.62% MG and BV was detected in 68.49%.
- Sixty of the 170 (35.29%) women with STI/BV at baseline were followed up and had samples for 6- and 12 weeks post-treatment.
- Associations were observed between MMPs and several inflammatory cytokines
- STI treatment, regardless of BV status (n=35), was associated with a marked reduction in MMP-1 (p=0.0039; **Fig.2**), although the reduction was modest and did not reach the level in the healthy baseline population.
- No changes in MMP concentrations were observed in women treated for BV (n=57), regardless of their STI status (**Fig.3**).

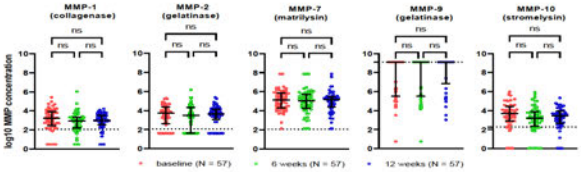

Figure 3: MMP concentrations in matched MC fluid of women treated for BV (n=57), regardless of STI status. Friedman test was used to compare the median MMP concentrations, and these observations were confirmed by linear mixed models controlling for age, pelvic examination, and STIs. MMP concentrations in healthy individuals are denoted by the dotted lines. All the insignificant p-values were denoted by ns.

Conclusion

- While BV treatment does not alter genital MMP levels, STI treatment significantly reduces but does not restore genital MMP-1 concentrations.
- These findings suggest that although the POC testing and treatment intervention effectively clears STIs and reduces genital inflammation, the integrity of the genital epithelium remains compromised, and infection risk is still high in women.
- There is a need for more effective treatment strategies for both STI and BV.

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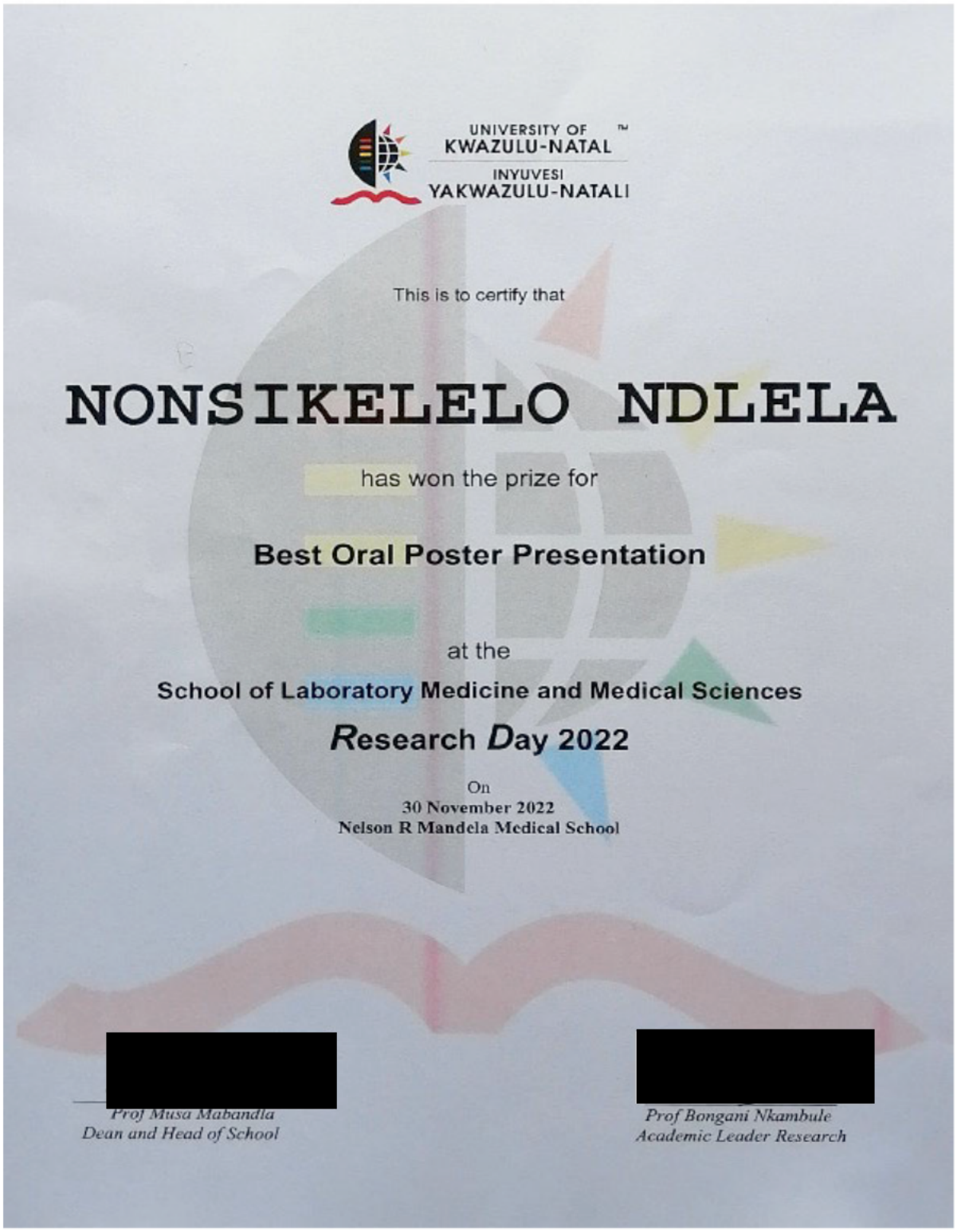
ORAL POSTER PRESENTATIONS

1. Investigating the impact of POC STI/BV treatment on the genital epithelial barrier integrity of CAPRISA 083 participants

Ndlela N, Garrett N, Mtshali A, Osman F, Singh R, Mitchev N, Ngobese N, Abdool Karim S1, Mlisana K, Passmore J, Rompalo A, Mindel A, and Liebenberg LJP

School of Laboratory Medicine and Medical Science Researcg Day, 30th November 2022



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Prof Bongani Nkambule
Academic Leader Research

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ABBREVIATIONS

A. vaginae	*Atopobium vaginae*
BV	Bacterial vaginosis
C. trachomatis	*Chlamydia trachomatis*
CCR5	C-C chemokine receptor 5
CTACK	Cutaneous T-cell attraction chemokine
CVL	Cervicovaginal lavage
ELISA	Enzyme-linked immunosorbent assay
EPT	Expedited partner therapy
FGT	Female genital tract
FGF	Basic fibroblast growth factor
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp	Glycoprotein
GRO-α	Growth regulated oncogene
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSV	Herpes simplex virus
IL	Interleukin
IN	Integrase

INF	Interferon
IP-10	Interferon gamma induced protein
L. crispatus	*Lactobacillus crispatus*
L. gasseri	*Lactobacillus gasseri*
L. iners	*Lactobacillus iners*
LIF	Leukaemia inhibitory factor
M. genitalium	Mycoplasma genitalium
MC	Menstrual cup
MCP	Monocyte chemotactic protein
M-CSF	Macrophage colony-stimulating factor
MIF	Macrophage migration inhibitory factor
MIG	Monokine induced by gamma-Interferon
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteiases
N. gonorrhoeae	Neisseria gonorrhoea
NGF-β	Nerve growth factor
NHLS	National Health Laboratory Services
NAATs	Nucleic acid amplification tests
P. bivia	*Prevotella bivia*
PCR	Polymerase chain reaction
PDGF-ββ	Platelet-derived growth factor

POC	Point-of-care
PIC	Pre-integration complex
RANTES	Regulated on activation normal T-cell expressed and presumably secreted
RT	Reverse transcriptase
RTC	Reverse transcriptase complex
SCF	Stem cell factor
SCGF-β	Stem cell growth factor
SDF-1α	Stromal-derived factor
SIV	Simian immunodeficiency virus
STI	Sexually transmitted infection
T. pallidum	*Treponema pallidum*
T. vaginalis	*Trichomonas vaginalis*
TIMPs	Tissue inhibitors of metalloproteinases
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
VEGF	Vascular endothelial growth factor

ABSTRACT

Background

In sub-Saharan Africa, women and young girls suffer the highest burden of HIV infections. Inflammation at the genital tract is a factor responsible for the increased susceptibility to HIV risk in women, presumably through related epithelial barrier damage and target cell recruitment. Considering the direct contribution of sexually transmitted infections (STIs) and bacterial vaginosis (BV) to this inflammation, their effective treatment could potentially reduce HIV risk in this vulnerable population. It has recently been shown in South African women that a point-of-care (POC) STI/BV detection model, immediate treatment, and expedited partner therapy (EPT) resolved STIs and reduced concentrations of genital proinflammatory cytokines. This study investigated an additional impact of the model on the genital epithelial barrier.

Methods

POC STI/BV screening was conducted on HIV-negative women (n=238) enrolled in the CAPRISA 083 trial between May 2016 and June 2017. *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections were detected by Xpert CT/NG test, while the OSOM Rapid test detected *Trichomonas vaginalis*. Women were further tested for *Mycoplasma genitalium* and BV using PCR and microscopy, respectively. Multiplex ELISA was used to quantify 48 cytokines and five matrix metalloproteinase (MMP) biomarkers of epithelial barrier integrity from menstrual cup (MC) specimens (MMP-1, MMP-2, MMP-7, MMP-9, MMP-10). Mann-Whitney U tests were used to assess the relationship between MMPs and STI/BV at baseline, with ANOVA and multivariable linear mixed models used to determine the impact of treatment on MMP concentrations.

Results

At baseline, women diagnosed with STI/BV (170/238) had higher concentrations of all MMPs compared to women with neither STI/BV (68/238; $p>0.05$). Several proinflammatory and chemotactic cytokine concentrations correlated significantly with that of MMPs at baseline. By 12 weeks post-treatment, 31/35 (88.57%) women resolved their baseline STIs, while only 14/57 (24.56%) resolved their baseline BV status. Most participants received concurrent treatment for STIs and BV (n=60), with few receiving treatments for STI (n=3) or BV alone

(n=25). Significant reductions in MMP-1 concentrations were observed after 6 weeks in women treated for STI and BV (2.763 pg/ml; p= 0.0066) or STIs regardless of BV status (2.760 pg/ml; p= 0.0048). No changes in MMP concentrations were observed in women treated for BV.

Conclusion

POC STI/BV treatment was associated with a reduction in MMP-1 concentrations. This implies that although POC STI/BV treatment may treat STIs and reduce inflammation, the integrity of the genital epithelial barrier may not be fully restored, and women remain susceptible to genital infections, including HIV, as a result. Additional strategies may be needed to repair the genital epithelium after treatment.

CHAPTER 1

INTRODUCTION

Despite the tremendous progress in attempts to curb the HIV epidemic, the global estimates of people living with HIV remain high, as 38.4 million people were living with HIV in 2021 (UNAIDS, 2022). The Eastern and Southern Africa region bears the highest burden of HIV infections, with an estimated 20.6 million people infected (UNAIDS, 2022). It is worth noting and particularly concerning that most of these infections in sub-Saharan Africa are recorded in women and young girls (UNAIDS, 2022). Several studies seeking to understand the unique vulnerability to HIV infections seen in this population have explored a variety of factors, including socio-economic, behavioral, religious, cultural as well as biological factors that may be implicated (Fasciana et al., 2021, Ramjee et al., 2016, Mabaso et al., 2018, Mtshali et al., 2021a, Murewanhema et al., 2022).

In as much as these factors may contribute to the increased susceptibility to infection observed in women, a deeper understanding of the interactions between the virus and the genital epithelium may probably be more critical, considering that penile-vaginal sex is the commonest transmission mode of HIV in women. The genital epithelial barrier, in its optimum state, can effectively hinder pathogen entry into the female genital tract (Shattock and Moore, 2003). Its efficiency is maintained by a combination of different components that make up this barrier, i.e., mucus layer covering the barrier, tight junction molecules, and epithelial cell arrangement (Shukair et al., 2013, Blaskewicz et al., 2011, Carias and Hope, 2019). It is clear, though, that despite its robustness, pathogens still bypass this cell layer, and successful infection is established (Hladik and McElrath, 2008). In an intact barrier, such events for HIV are less likely; however, damage to this epithelium reduces its competence and increases its permeability to invading pathogens (Carias and Hope, 2019). This was demonstrated in a study of factors promoting barrier penetration by HIV, where penetration was higher in epithelial areas lacking cellular junctions, with only 3 out of 124 virions penetrating beyond an intact tight junction (Carias et al., 2013a). Furthermore, Cherne et al, 2020 observed decreased epithelial resistance on endocervical epithelial cells in the context of bacterial vaginosis (BV), and subsequent increased HIV transmigration (Cherne et al., 2020).

For instance, studies have established that the risk of acquiring HIV is higher in the context of genital inflammation (Masson et al., 2015, McKinnon et al., 2018), most probably due to cytokine-mediated HIV target cell recruitment associated with genital inflammation (Arnold et al., 2016a). In support of this, higher levels of inflammatory cytokines MIP-1α, MIP-1β, IL-8, and IP-10 predicted HIV seroconversion in a cohort of young South African women (Masson

et al., 2015). The functions of these soluble proteins (β-chemokines) as ligands for HIV co-receptor CCR5 and their role in the recruitment of HIV target cells to the site of infection may explain this increased susceptibility to HIV in an inflammatory state (Alkhatib et al., 1996, Cocchi et al., 1995). MIP-1α and MIP-1β expressing plasmacytoid dendritic cells were involved in the recruitment of CD4+ T cells in a Simian immunodeficiency virus (SIV) macaque model (Li et al., 2009a). The role of inflammatory cytokines in HIV risk is further elucidated by the active function of IP-10 in recruiting activated T cells and the established association between IL-8 and increased susceptibility to HIV cells (Farber, 1997, Dufour et al., 2002, Narimatsu et al., 2005).

In addition to HIV target cell recruitment, genital inflammation is associated with epithelial barrier damage. It has been suggested that inflammation weakens the epithelium by disrupting the tight junction molecules in between the epithelial cells (Stockmann et al., 2000, Nazli et al., 2010a). The observations of an increased local expression of proteases, notably matrix metalloproteinases (MMPs), in the context of genital inflammation are of particular interest (Arnold et al., 2016a). MMPs are zinc endopeptidases responsible mainly for extracellular matrix degradation (Nagase and Woessner, 1999). Because MMPs have defined functions in wound healing and tissue remodeling (Parks, 1999, Caley et al., 2015, Nguyen et al., 2016), their upregulation in an inflammatory state could imply a level of barrier defect under these conditions. For these reasons, several studies have used MMP as epithelial barrier function biomarkers (Ngcapu et al., 2015, Arnold et al., 2016a, Ghosh et al., 2018, Aldous et al., 2021).

Sexually transmitted infections (STIs) and the gynecological condition bacterial vaginosis (BV) have long been established as inducers of genital inflammation in women (Masson et al., 2014b, Masson et al., 2019). Both curable (e.g., *Chlamydia trachomatis, Neisseria gonorrhea*) and incurable (Herpes Simplex Virus, Human papillomavirus) STIs have been associated with inflammation in the female genital tract (Masson et al., 2014b, Parr and Parr, 2000, Liebenberg et al., 2019). In addition, STI induced inflammation can be observed even in the absence of STI symptoms, i.e., asymptomatic infection (Masson et al., 2014b). In the same way that STIs are associated with compromised barrier integrity (Guenthner et al., 2005), observations of altered barrier proteins are reported in the context of BV (Borgdorff et al., 2016). Evidence from numerous studies has identified similar patterns of MMP upregulation upon infection with STIs (i.e., *Chlamydia trachomatis, Neisseria gonorrhea*), as well as in response to BV in women (Ault et al., 2002, Juica et al., 2017, Rodas et al., 2017, Cherne et al., 2020). MMP

upregulation during these inflammatory conditions supports their role in tissue degradation and remodeling in women with genital inflammation.

Taken together, the relationship between genital inflammation and both STI and BV suggests that treating these could potentially decelerate the high HIV infection rates in women. Laboratory-based techniques such as nucleic acid amplification tests (NAATs), immunological assays, microscopy, and culture are the gold standard for the detection of STIs and BV (Caruso et al., 2021). However, these are uncommon in resource-constrained settings as issues regarding affordability, longer turnaround time, and technical expertise frequently challenge their applicability (Caruso et al., 2021, Tucker et al., 2013). As such, diagnosis and treatment in these settings depend on syndromic management, which tends to be less effective given the asymptomatic nature of most BV cases and STIs (Mlisana et al., 2012, Karim et al., 2021, Francis et al., 2018). Point-of-care (POC) testing for STI/BV has recently been explored as an alternative to the symptom-based approach. In addition to enabling accurate detection and immediate treatment of infections, they have also been shown to be acceptable by young women (Garrett et al., 2018). Their potential to reduce HIV risk has been demonstrated through their ability to resolve genital inflammation (Garrett et al., 2021b). The model's impact on the genital epithelial barrier integrity, another significant contributor to HIV risk, remains unassessed. We hypothesize that after the specific diagnosis and timely treatment using the POC model, the inflammatory response would be reduced, and the integrity of the epithelium would be restored.

CHAPTER 2
LITERATURE REVIEW

2.1. HIV epidemiology

In 2021 alone, 650 000 people lost their lives from AIDS-related illnesses. Despite this loss, significant progress has been made to counteract the devasting effects of the HIV epidemic. A decline in the global number of new infections from 2.2 million in 2010 to 1.5 million in 2021 has been reported (UNAIDS, 2022). The number of people living with HIV is, however, considerably high, as 38.4 million people were reported to be living with HIV in 2021. Most of these people living with HIV (28.7 million) are aware of their status and accessing antiretroviral therapy (UNAIDS, 2022).

The Eastern and Southern African regions are the most affected regions, with approximately 20.6 million people living with HIV (UNAIDS, 2022). Sub-Saharan Africa accounts for an estimated 71% of global infections (Kharsany and Karim, 2016). Of particular concern is the disproportionate burden of infection in women and girls (aged 15-24), who accounted for 63% of new infections in sub-Saharan Africa (UNAIDS, 2022). In Southern Africa, women are six times more likely to be infected by HIV than men (Karim and Baxter, 2019). A community-based survey conducted in KwaZulu-Natal, South Africa, demonstrated that HIV prevalence in women was three times higher than that observed in men of the same age (de Oliveira et al., 2017). Other studies have also reported on the unique vulnerability to HIV infections in women compared to men in this setting (Kharsany et al., 2018), and this has been attributed to a combination of factors, including specific behaviours by women, biological and host-related factors (Mtshali et al., 2021a)

HIV is predominantly transmitted via anogenital mucosal exposure through heterosexual transmission (Shaw and Hunter, 2012). Alternative transmission routes include mother-to-child transmission and the exchange of blood through sharps (Shaw and Hunter, 2012, Patel et al., 2014). Two types of HIV strains exist, HIV-1 and HIV-2 and these originated from zoonotic transfer from simian immunodeficiency virus in chimpanzees and sooty mangabey monkey, respectively (Sharp and Hahn, 2011). Although these share similarities, there are significant differences in geographical distribution, clinical manifestation, and pathogenesis. HIV-1 also differs in that it is associated with faster progression to AIDS (Nyamweya et al., 2013). Three stages of infection (primary stage, asymptomatic stage, and late stage) have been described for HIV, where infectiousness varies depending on the stage of infection (Hollingsworth et al., 2008). Individuals with chronic HIV infections are known to harbour viruses of the highest

genetic diversity, referred to as quasispecies (Cohen et al., 2011). These genetically diverse viral populations are present in the blood, semen, and cervicovaginal secretions. Still, restricting bottlenecks exist during acute infection resulting in only a few viruses from the diverse viral population successfully establishing infection. These include donor-related factors (i.e., compartmentalization of genital tract-specific viral lineages, selection in the transmission fluid, donor viral load, general fitness of the virus to establish infection, etc.) and recipient related factors such as such as the number of target cells present at the site of infection (Joseph et al., 2015). The level of barrier integrity, mucous composition and thickness of the genital epithelium are also implicated in the success of HIV infection in the genital tract (Carias et al., 2013b, Nunn et al., 2015, Hoang et al., 2020).

2.2. The basic structure and replication of HIV

The HIV viral particle is enveloped by a lipid bilayer that the virus derives from the host cell membrane during the process of budding (Freed, 2015). Below the viral envelope is the capsid core which houses the viral genome comprising two copies of single-stranded RNA (Gill et al., 2019). The virus has glycoprotein structures, gp120, projecting on its surfaces and connected to the virus through the transmembrane protein pg41, which the virus uses to fuse into the host (Chen, 2019). The gp120 on the virus's surface binds to the CD4 receptor on immune cells such as T cells, macrophages, dendritic cells, Langerhans cells. Its binding to available coreceptors CCR5 or CXCR4 on the host cell's surface induces conformational changes on the gp41 and facilitates fusion with the host cell membrane (Keller et al., 2018, Chen, 2019). Viral fusion enables the release of the capsid with its contents (viral RNA, integrase, and reverse transcriptase) to the cytoplasm (Rossi et al., 2021).

Within the viral capsid are two enzymes, integrase (IN) and reverse transcriptase (RT), that the virus uses, respectively, to convert ssRNA to double-stranded DNA and subsequent integration of that vial DNA into the host genome (Gill et al., 2019). Gene transcription follows integration, and the viral RNA is transported to the cytoplasm for viral protein synthesis (Freed, 2015). The Gag and the GagPol polyproteins are translated and translocated to the plasma membrane, the assembly site. This is then followed by encapsidation of the RNA into the viral particles. Gag-gag interactions in the plasma membrane allow the progeny virions to be assembled. Env glycoproteins synthesis proceeds in the endoplasmic reticulum, where it is cleaved into surface glycoprotein (gp120) and transmembrane glycoprotein (gp41) and transported to the cell

surface through the Golgi apparatus and internalized or incorporated into the virus particles. Budding is stimulated through the viral coding of late domains, which interact with host factors to facilitate virus release. After the release, the Gag and GagPol polyproteins are cleaved by a protease to form mature Gag and Pol proteins (Freed, 2015, Kleinpeter and Freed, 2020).

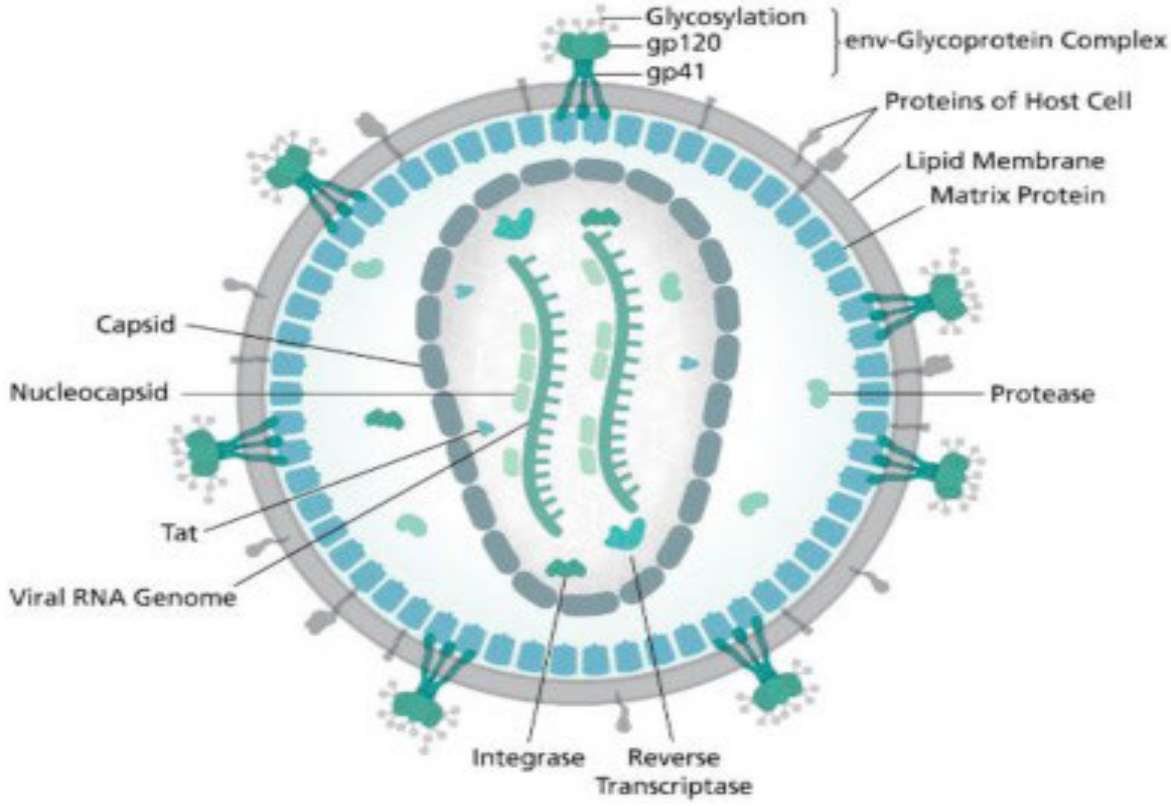

Figure 1. The basic structure of HIV-1 particle, adapted from (Gill et al., 2019).

2.3. Factors associated with increased risk of HIV in women

The increased vulnerability to HIV infections in women has been attributed to specific behaviors by men and women, socioeconomic factors, as well as to biological factors (Ramjee et al., 2016, Mabaso et al., 2018, Mtshali et al., 2021a). Sexual relations with multiple sexual partners and low condom use are behaviors commonly linked with an increased risk of HIV infections in both men and women (Naicker et al., 2015, Pettifor et al., 2005). Young women in relationships with older partners are also more likely to acquire HIV (Evans et al., 2016, de Oliveira et al., 2017). Vaginal douching, a common practice by women for various reasons, increases HIV risk in women through epithelial cell death and damage (Hesham et al., 2021).

It has also been demonstrated that women who have experienced sexual abuse in their childhood or adolescent years are more likely to engage in HIV risk behaviors (Parillo et al., 2001). Furthermore, early first intercourse (Becker et al., 2018), transactional sex (Kilburn et al., 2018), few years of schooling, and food insecurity have been associated with increased HIV risk in young women (Dellar et al., 2015).

Increased HIV acquisition risk is also influenced by several biological factors, such as genital epithelial barrier damage (Abbai et al., 2016, Arnold et al., 2016a), genital inflammation (Masson et al., 2015, Kaul et al., 2015), STIs (Laga et al., 1993, Mlisana et al., 2012), use of certain hormonal contraceptives women (Heffron et al., 2012, Edfeldt et al., 2020), BV (Gosmann et al., 2017), and the infection stage of a women's sexual partner (Hollingsworth et al., 2008)

2.4. Sexual transmission of HIV to women

Because heterosexual transmission to the genital mucosa through penetrative sex accounts for most HIV infections, careful discernment of how infection is established in this area is crucial in developing effective prevention strategies. Approximately 80% of HIV infections occur in the mucosa surfaces (Cohen et al., 2011). In women, both cell-associated and cell-free viruses are carried through semen to the female genital tract (Cavarelli and Le Grand, 2020). Despite the robustness of the epithelial barrier and the associated factors in preventing pathogen entry in the genital tract, successful infection with HIV is still achieved (Rodriguez-Garcia et al., 2021). There are different ways in which infection occurs. In some instances, the vaginal epithelial cells can capture the virus for cell-to-cell transmission or reach the target cells by transcytosis (Wu et al., 2003, Kohli et al., 2014). The primary target cells for HIV, however, include CD4 +T cells, particularly the ones expressing the HIV coreceptor CCR5 as well as Th17 surface markers (Rodriguez-Garcia et al., 2014). Other implicated immune cells include dendritic cells, which, in addition to producing antiviral responses, capture the virus for subsequent infection of CD4 T cells (Rodriguez-Garcia et al., 2017). Macrophages (Shen et al., 2009), neutrophils, and natural killer cells (Rodriguez-Garcia et al., 2021, Moreno de Lara et al., 2021) are also targets for infection. Although HIV can be transmitted through an intact barrier (Carias et al., 2013a), it can be anticipated that factors such as tissue trauma from sexual intercourse or genital ulceration resulting from an infection with STIs would provide the virus with easier access to the target cells. The infection then spreads to the bloodstream and

disseminate first to the draining lymph nodes and to other lymphoid tissue compartments (Haase, 2005).

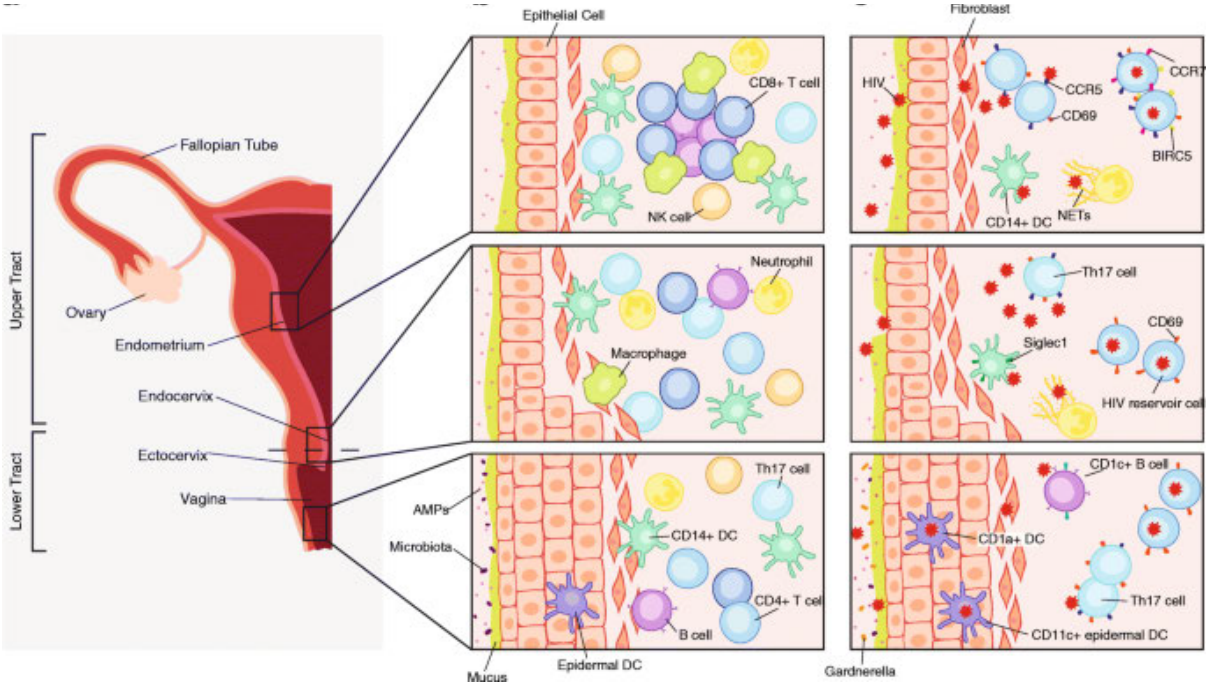

Figure 2. A graphical representation of HIV pathogenesis in the female genital tract, adapted from (Rodriguez-Garcia et al., 2021).

2.5. Defense mechanisms against HIV infection in the female genital tract

The genital epithelial barrier is an effective defense mechanism against pathogens in the female genital tract (Shattock and Moore, 2003). Although heterogenicity exists, the genital epithelium hinders most infections in the genital tract with relatively low infection rates per sexual act (Boily et al., 2009). The female genital tract is divided into two compartments: the endocervix and the ectocervix (**Figure 2**). The endocervix comprises simple columnar epithelial cells held together by tight junction molecules and desmosomes. This differs from the ectocervix, where stratified squamous epithelial cells that lack tight junction molecules between the cells are found (Blaskewicz et al., 2011, Carias and Hope, 2019). The ectocervical epithelial cells are further divided into different layers: the basal, parabasal, clear, condensation, and cornified layers (Shattock and Moore, 2003). The uppermost layer is made up of dead keratinized and flattened cells, which are exfoliated every 4 hours, a potential mechanism to eliminate pathogen entry in this area (Anderson et al., 2014).

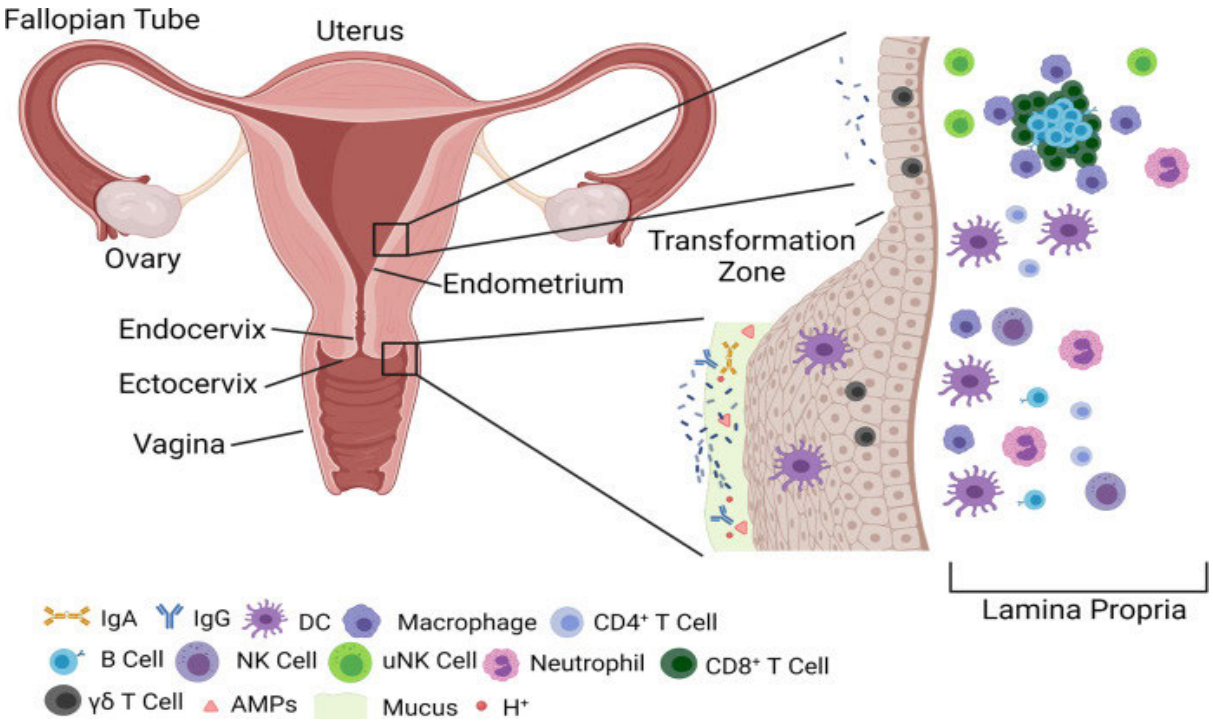

Figure 3. The endocervical and the ectocervical compartments of the female genital tract, adapted from (Plesniarski et al., 2021).

In addition to the inhibitory effect of epithelial cells against pathogens, the cervicovaginal mucous, vaginal microbiota, and pH contribute significantly to maintaining a healthy genital environment. Ectocervical and endocervical epithelial cells express gel-forming mucins, the major constituents of the cervicovaginal mucous, which, together with water and other molecules released by epithelial cells, protects against trauma from sexual intercourse and invading pathogens (Gipson et al., 1997, Lacroix et al., 2020). Cervicovaginal mucous also contain antimicrobial molecules, e.g., secretory leukocyte peptidase inhibitor (SLPI) and high-mobility group nucleosomal-binding domain 2, contributors to its inhibitory effect against invading pathogens in the genital tract (Ming et al., 2007).

Similarly, the role of a healthy vaginal microbiome as a defense mechanism against colonization by pathogens is critical in maintaining a healthy genital tract. A healthy vaginal microbiome is generally associated with a dominance of *Lactobacillus* species (Chen et al., 2021). *Lactobacillus* contributes to genital health through glycogen degradation from local epithelial cells, (Mirmonsef et al., 2014), which leads to lactic acid production and subsequent maintenance of a low vaginal pH (3.5 +/- 0.2) (O'Hanlon et al., 2013). Animal models have also demonstrated the inhibitory effect of lactic acid and hydrogen peroxide production by

Lactobacillus species to *G. vaginalis* (Choi et al., 2022). This was further shown in HIV, where direct inhibition of HIV replication and infectivity was inhibited by acid production from *Lactobacillus* (Ñahui Palomino et al., 2017, Aldunate et al., 2013). Some *Lactobacillus* strains can reduce bacterial viability (Foschi et al., 2017), and some have demonstrated antifungal activity against an agent of vaginal candidiasis, *Candida albicans* (MacAlpine et al., 2021).

2.6. BV and genital inflammation in women

Despite the contribution of *Lactobacillus* species and associated factors in maintaining a healthy vaginal environment in women, alterations in the vaginal microflora from a *Lactobacillus*-dominated microbiota to more diverse microbial populations dominated by anaerobic bacteria are common. This usually results in a condition referred to as bacterial vaginosis (BV) (Chen et al., 2021). *Prevotella bivia, Atopobium vaginae, Gardnerella vaginalis, Megasphaera type 1, Sneathea, Mobiluncus,* and *Clostridiales* are microorganisms commonly found in women with BV (Muzny et al., 2018, Onderdonk et al., 2016). The contribution of these microorganisms in BV pathogenesis varies: from cytokine upregulation, epithelial barrier disruption, and production of specific virulence factors (Onderdonk et al., 2016). *G. vaginalis* and other BV-associated microorganisms can form biofilms (Machado and Cerca, 2015), and these be responsible for the high recurrance rates after tretatmnt and may significantly contribute to treatment resistance (Swidsinski et al., 2008, Verstraelen and Swidsinski, 2013).

Although the cause of BV remains unclear, microbial dysbiosis can be explained by certain behaviors by women, such as smoking (Brotman et al., 2014), douching (Ranjit et al., 2018), concurrent partners (Mulinganya et al., 2021), condomless sex or exposure to semen (Jespers et al., 2014, Jewanraj et al., 2021), new sex partner (Vodstrcil et al., 2015), etc. Microbiome composition also differs by ethnicity, and black women have been shown to have a more diverse, low *Lactobacillus* abundance vaginal microbiomes (Anahtar et al., 2015, Borgdorff et al., 2017, Gupta et al., 2017).

While some women are asymptomatic and do not show any observable signs and symptoms (Mulinganya et al., 2021, Mlisana et al., 2012), BV is often characterized by vaginal discharge (Majigo et al., 2021), malodor (Abou Chacra et al., 2022), wetness (Klebanoff et al., 2004), and vaginal itching. Amsel's criteria are one-way through which BV is detected (Amsel et al., 1983). Detection of BV based on Amsel's criteria relies on the presence of three of the four

characteristics (a positive whiff test, vaginal pH >4.5, detection of clue cells, and vaginal discharge) that have been associated with BV. However, the gold standard for BV diagnosis is by Nugent scores through gram staining (Nugent et al., 1991, Anukam et al., 2014). Scores from 1-3, 4-6, and 7-10 are used to assign vaginal microbiota as either normal, intermediate, or BV positive, respectively. Hay-Ison criteria are also based on the identification of bacterial morphotypes as either normal, intermediate, or BV positive with the addition of non-BV associated criteria (Sherrard et al., 2018).

Accurate diagnosis is critical because of the adverse health challenges associated with BV in women. For instance, BV is associated with pelvic inflammatory disease (Turpin et al., 2021), endometritis (Ravel et al., 2021), and infertility (Sherrard et al., 2018). These can be prevented with accurate early diagnosis. Pregnant women infected with BV are more likely to experience premature labor (Nelson et al., 2009, Arena and Daccò, 2021). Adverse pregnancy outcomes such as babies with low birth weight (Aduloju et al., 2019) and neonatal sepsis (Dingens et al., 2016) are also linked to BV. In addition, BV is associated with an increased risk of STIs (Bautista et al., 2017, Ahmed et al., 2022), and women with BV are at an even greater risk of acquiring HIV (Gosmann et al., 2017, Apalata et al., 2021, Cohen et al., 2012).

High-diversity microbiomes lacking *Lactobacillus* have been associated with genital inflammation in South African women, even in women without BV (Anahtar et al., 2015, Gosmann et al., 2017, Lennard et al., 2018b). In these studies, microorganisms that were shown to trigger cytokine upregulation (IL-8, IL-1α, IL-1β) include *Prevotella amnii, Mobiluncus mulieris, Sneathia amnii*, and *Sneathia sanguinegens*, and *Gardnerella, Prevotella.* Women with BV had higher levels of pro-inflammatory cytokines TNF-β, IL-1α, and IL-1β in their cervicovaginal lavages than women negative for BV (Masson et al., 2014b). Upregulation of genital inflammatory cytokines has also been demonstrated by epithelial cells *in vitro*, where treatment with *G. vaginalis* induced IL-6, IL-8, IL-1β, and TNF-α, which was modulated by treatment with *Lactobacillus* (Santos et al., 2018). Further, an anti-inflammatory effect of *Lactobacillus* is also demonstrated in BV-infected mice upon its administration (Choi et al., 2022).

2.7. STIs and genital inflammation in women

Infections with chlamydia, syphilis, gonorrhea, and trichomonas are relatively common, with 374 million infections occurring each year globally (WHO, 2022). Although these can be cured when detected, some viral STIs (i.e., hepatitis B, human papillomavirus, and herpes simplex virus) are incurable and pose a major global health threat. Even with curable STIs, management is challenging, as most people infected are asymptomatic (Mlisana et al., 2012, Francis et al., 2018). Those with symptoms typically present with genital ulcers (herpes simplex virus-1 and -2, HSV-1 and -2; *Treponema pallidum*), vaginal discharge (*Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and *Mycoplasma genitalium*) and warts (human papillomavirus, HPV) (Wagenlehner et al., 2016). Beyond these physical manifestations, several sexual and reproductive health challenges have been linked with STIs. These include infertility (Tsevat et al., 2017), cervical cancer (Burd, 2003), ectopic pregnancies (Xia et al., 2020), pelvic inflammatory disease, as well as tubal factor infertility (Hoenderboom et al., 2019). In some cases, the impact of STIs can go to the extent of causing neonatal infections with detrimental effects, and adverse pregnancy outcomes may result (Moodley and Sturm, 2000). These necessitate effective methods of diagnosis and treatment of STIs.

Infections with either a bacterial (*C. trachromatis*, *N. gonorrhea*, *M. genitalium*, *T. vaginalis*, *T.pallidum*) or a viral STI (e.g., HSV-2, HPV) are often associated with genital inflammatory cytokine elevation in young South African women (Mlisana et al., 2012, Masson et al., 2014a, Garrett et al., 2021a, Liebenberg et al., 2019). One study showed that women infected with *C. trachomatis* had IL-10, IL-12, IFN-α, and IFN-γ upregulation compared to uninfected controls (Reddy et al., 2004). Similar trends of cytokine upregulation have also been demonstrated in *in vitro* studies on both ectocervical and endocervical epithelial cells following infection with either *C. trachomatis* (Tang et al., 2021), *N. gonorrhea* (Fichorova et al., 2001), or *T. vaginalis* (Fichorova et al., 2006).

In South Africa, STIs are highly prevalent in women and young girls (Jongen et al., 2021, Kharsany et al., 2020). Given the association between STIs and HIV acquisition risk (Barker et al., 2022, Cohen et al., 2019), the reports of high HIV infection rates among this population are not surprising. For the most part, the consistent associations between genital inflammation and STIs may probably be the main reason driving the increased susceptibility to HIV in this group.

2.8. Inflammation increases HIV risk in women

Inflammation is generally a defense mechanism in the human body used to fight infection or resolve tissue injury and forms part of the innate immune system. Inducers of inflammation, which can either be endogenous (e.g. stressed/ damaged cells, malfunctioning tissue) or exogenous (e.g. microbial/non-microbial) depending on their origin, result in the production of inflammatory mediators (including cytokines and chemokines) by immune cells (e.g. macrophages, mast cells), which influence vasculature and leukocyte recruitment to the site of injury/infection (Medzhitov, 2008). Several pro-inflammatory, e.g., TNF-α, IL-1, IL-6, IL-8, and IL-12, and anti-inflammatory cytokines, e.g., IL-10 and TGF-β, are involved in the inflammatory response. Additionally, chemotactic cytokines (chemokines) facilitate immune cell migration toward the site of infection through chemotaxis (Arango Duque and Descoteaux, 2014). Although inflammation is an essential biological process responsible for restoring tissue functionality, it can be detrimental if not controlled.

Genital epithelial cells from the endocervix and the ectocervix secrete different types of pro-inflammatory cytokines and chemokines as a physiological process to prevent infections (Fahey et al., 2005). Evidence from primates, where inflammation was necessary to initiate infection with simian immunodeficiency virus (SIV) in endocervical cells, emphasized the role of genital inflammation in HIV susceptibility (Li et al., 2009b). In this study, MIP-3α was associated with plasmacytoid dendritic cell recruitment at the site of infection. Through the production of chemokines MIP-1α and MIP-1β, a mechanism through which the plasmacytoid dendritic cells recruited CD4+ T cells to the endocervix, which led to the establishment of successful infection, was explained. Further validating the role of inflammation infection is the inhibition of productive infection upon SIV challenge in macaques without inflammatory responses (Li et al., 2009b).

The association between inflammation and HIV susceptibility was further substantiated in a study of young South African women, where inflammation, defined as having elevated levels of five in a panel of nine pro-inflammatory cytokines and chemokines, was strongly associated with HIV risk (Masson et al., 2015). Additionally, higher concentrations of genital pro-inflammatory cytokines (IP-10, MIP1-α, MIP-1β, and IL-8) predicted HIV seroconversion in these women, and HIV subsequently infected women with inflammation with relatively low infectivity (Selhorst et al., 2016). Various studies support the role of inflammation on HIV

acquisition risk in the context of both STIs and BV (Anahtar et al., 2015, Gosmann et al., 2017, Masson et al., 2015, Masson et al., 2014b).

2.9. Mechanisms for the relationship between genital inflammation and increased HIV acquisition risk

2.9.1. Target cell influx

As mentioned above, the influence of cytokine-mediated inflammatory responses on HIV acquisition in women is well-documented (Masson et al., 2014b, Masson et al., 2015). It has become apparent that inflammation fosters an environment that favors HIV replication.

HIV preferentially replicates in CD4+ T cells in the female genital tract to establish successful infection (Zhang et al., 1999, Haase, 2010). This is supported by several studies reporting on the depletion of CD4+ T cells during HIV infection (Nkwanyana et al., 2009, Li et al., 2005, Jaspan et al., 2011). Several studies have also demonstrated a relationship between genital pro-inflammatory cytokines and CD4+ T cell influx to the female genital tract (Arnold et al., 2016a, Dabee et al., 2019, Nkwanyana et al., 2009). This is further supported by the role that proinflammatory cytokines and chemotactic cytokine concentrations have as predictors of increased HIV acquisition risk (Masson et al., 2015, Gosmann et al., 2017). The chemotactic cytokines MIP-1α and MIP-1β are chemotactic for CCR5 expressing cells, a coreceptor for HIV (Li et al., 2009b).

Genital pro-inflammatory cytokine-related CD4+ T cell recruitment has also been described in the context of STIs and BV (Gosmann et al., 2017), which is not surprising considering the previously established relationship between HIV and other STIs, and between HIV and BV (Masson et al., 2014b, Masson et al., 2015). Women with high-diversity vaginal microbial populations were reported to have high numbers of activated CCR5+ CD4+ T cells compared to women with low abundance *Lactobacillus*-dominated vaginal microbial populations (Anahtar et al., 2015). In the same cohort, further analysis showed that women with diverse microbial communities with *Gardnerella* dominance had a higher number of Th17 cells, one of the susceptible target cells for HIV in the female genital tract (Rodriguez-Garcia et al., 2014). This was particularly beneficial to HIV infection as a 4-fold increase in infection rates were observed (Gosmann et al., 2017). Another study found higher proinflammatory cytokine concentrations (IL-1α) in women with higher levels of anaerobic bacteria (Shannon et al., 2017).

2.9.2. Epithelial barrier damage

In addition to HIV target cell recruitment, epithelial barrier impairment is thought to be another way inflammation in the genital tract influences HIV risk in women. The associations between inflammation and epithelial barrier damage have long been established (Arnold et al., 2016a). By modulating tight junction barriers towards increased permeability, specific pro-inflammatory cytokines, including IFN-γ, IFN-α, IL-1β, and IL-4, can reduce intestinal epithelial barrier competency (Al-Sadi et al., 2009).

As it is well established that both STIs and BV are inducers of inflammation, it is expected that the integrity of the genital epithelial barrier would be compromised in the context of these. In support of this, the exposure of ectocervical epithelial cells to *L. iners or G. vaginalis*, microorganisms whose inflammatory effects have been established (De Seta et al., 2019, Lennard et al., 2018a), has been associated with increased cell permeability and more than five-fold increase in several cytokines and chemokines, including IL-6 and IL-8 (Anton et al., 2018). These results were similar to the observations of increased IL-1 and IL-8 and subsequent epithelial cell breakdown, evident through increased cell permeability in cervical cells exposed to bacterial lipopolysaccharide (Nold et al., 2012). *T. vaginalis* alone or in combination with *G. vaginalis, P. bivia,* and *A. vaginae* are capable of increasing ectocervical cell monolayer permeability, possibly through phosphate activity and exposure of ectocervical cell monolayer to *T. vaginalis* or the BV-associated microorganism was associated with a decrease in occludin and upregulation of TNF-α and IL-6 (Hinderfeld et al., 2019). The direct contribution of TNF-α to weakening epithelial cell integrity has been reported (Nazli et al., 2010b). Additionally, supernatants from vaginal microbial dysbiosis-associated microorganisms, including *A. vaginae*, reduced cell viability in cervical epithelial cells (Nicolò et al., 2021).

The effect of inflammation on the effectiveness of the epithelial barrier is observed *in vitro* and *in vivo* studies of animal models, where increased cytokine levels (TNF-α, IL-6, IL-8, and IL-10) accompanied by lower levels of soluble E-cadherin, a biomarker of epithelial barrier integrity, have been reported in mice inoculated with *G. vaginalis* (Sierra et al., 2018). In humans, the evidence of inflammation-related barrier damage is mainly obtained from mucosal proteome analysis. Increased inflammatory responses coupled with proteolytic activity were observed in women with severe microbial dysbiosis (Borgdorff et al., 2016). Further supporting these observations is the decrease in protease inhibitors, e.g., serine leukocyte inhibitor (SLP), in women with STIs compared to uninfected women (Draper et al., 2000).

It is worth noting that protease expression associated with epithelial barrier damages was increased in women during the luteal phase of menstruation in other studies and may have implications for HIV risk (Birse et al., 2015). Of particular interest are the observed associations between specific proteases MMP-8 and MMP-9 with higher levels of genital pro-inflammatory cytokines in a cohort of Kenyan women (Arnold et al., 2016a). Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that function primarily in the well-timed degradation of the extracellular matrix (Visse and Nagase, 2003). A total of 28 MMPs have been identified, 23 of which are expressed in the human genome (Cui et al., 2017). Under normal conditions, the expression of MMPs is relatively low; however, the expression is increased when tissue remodeling or wound healing is required (Page-McCaw et al., 2007a). Some of the other physiological processes that MMPs mediate include embryogenesis, morphogenesis, and angiogenesis.

MMPs are assigned into six different groups. Collagenases (MMP-1, MMP-8, MMP-13, and MMP-18), Gelatinases (MMP-2 and MMP-9), Stromelysin (MMP-3, MMP-10, and MMP-11), Matrilysin (MMP-7 and MMP-26), Membrane-type MMPs (MMP-14, MMP-15, MMP-16, MMP-24, MMP-17, and MMP-25) and other MMPs (MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27 and MMP-28) (Jabłońska-Trypuć et al., 2016). The secretion of MMPs as pre-proMMPs requires them to be activated during translation through the removal of the signal peptide to form a pro-MMP that is further activated by the cleavage of the cysteine switch (the association between the cysteine in the prodomain and the Zinc)(Cui et al., 2017).

The specific inhibitors of MMPs are tissue inhibitors of MMPs (TIMPS); however, MMP activity may also be regulated by α1-protease and α2-macroglobulin (Baker et al., 2002, Nagase et al., 2006). A total of four different TIMPs (TIMP-1, TIMP-2, TIMP-3, TIMP-4) are encoded by the human genome and are involved in the inhibition of MMP activity in humans (Brew and Nagase, 2010). The affinity at which TIMPs bind to different MMPs differs depending on the MMP-TIMP pair (Brew and Nagase, 2010). TIMPs bind MMPs at a 1:1 ratio, where dysregulation may result in diseases such as arthritis, cancer, ulcers, brain-degenerative diseases, multiple sclerosis, etc. (Laronha and Caldeira, 2020).

2.10. The basic structure of MMPs

The basic structures of all MMPs besides MMP-7, MMP-23, and MMP-26 include a signal N-terminal peptide, a pro-domain, a catalytic domain, a linker peptide, a hemopexin domain, and

in some MMPs, an additional transmembrane domain with the small cytoplasmatic C-terminal domain (Laronha and Caldeira, 2020). A set number of amino acids makes up these components and is consistent across different MMPs, except the signal N-terminal peptide and the linker peptide, which differs in length within various MMPs. The pro-domain consists of 80 amino acids, the catalytic domain consists of about 160 amino acids, and the hemopexin domain generally comprises about 210 amino acids.

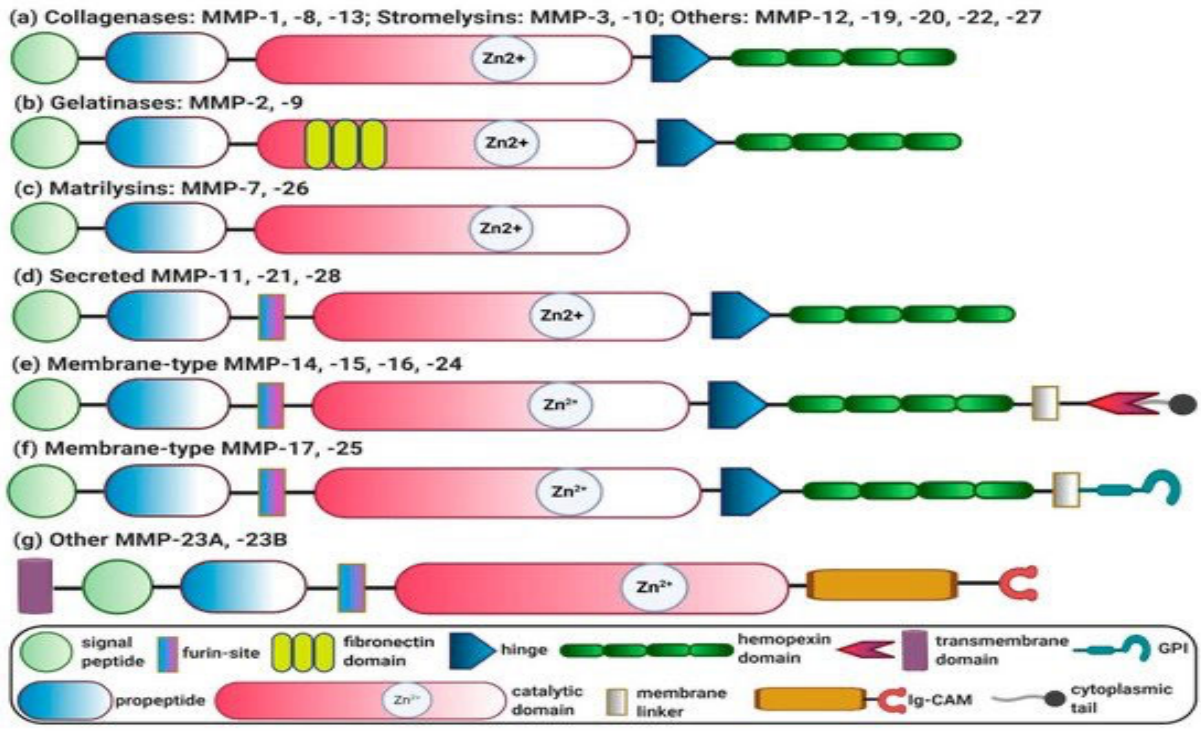

Figure 4: The different classes of MMPs grouped according to their domain organization, adapted from (Geervliet and Bansal, 2020).

A relationship between MMPs and inflammation has been established (Mauviel, 1993, Fingleton, 2017, Manicone and McGuire, 2008). In this regard, tissue degradation and remodeling may be upregulated in women with genital inflammation. In fact, increased MMP expression has been observed due to STIs, including *N. gonorrhea* (Juica et al., 2017) and *C. trachomatis* (Ault et al., 2002) in tissue explants. BV-associated microorganisms (e.g., *A. vaginae, G, vaginalis*) have also been shown to induce MMP expression in cervical epithelial cells (Cherne et al., 2020, Gerson et al., 2022). With numerous studies in animal models highlighting the role of MMPs in wound healing (Hayden et al., 2011, Keskin et al., 2021), their upregulation suggests

a role in the restoration of barrier integrity. For this reason, MMP detection is being widely used as a biomarker of epithelial barrier integrity (Ngcapu et al., 2015, Arnold et al., 2016b, Ghosh et al., 2018, Aldous et al., 2021). Taken together, the role of MMPs in wound healing and tissue remodelling and their upregulation in the context of STIs and BV suggest that effectively treating these could aid in the restoration and maintenance of intact genital epithelial barrier, and potentially reduced HIV susceptibility.

2.11. BV treatment

While treatment for BV is available (oral/gel metronidazole and clindamycin cream) (Workowski et al., 2021), its effectivity against BV is challenged by several factors. The reduction of BV anaerobes following treatment for BV has been reported (Verwijs et al., 2020). However, a relatively rapid re-emergence of these microorganisms shortly after treatment with metronidazole is a significant challenge (Mayer et al., 2015, Mtshali et al., 2021b). One possible reason for this may include treatment-resistant biofilms (Swidsinski et al., 2008). Although the possibility of sexual transmission is also not being overlooked (Vodstrcil et al., 2021). sexual partner treatment does not reduce BV recurrence, which may suggest otherwise (Schwebke et al., 2020). Bacterial abundance before treatment may also contribute to treatment failure (Gustin et al., 2022).

The use of probiotics after treatment with BV may be justified, as evidence suggests that Metronidazole does not necessarily increase the *lactobacillus* levels in the genital microbiome but rather affect the BV-associated bacteria (Armstrong et al., 2022a). Another study reported that incorporating probiotics in addition to standard treatment with antibiotics resulted in a long time of relapsing (Heczko et al., 2015). Additionally, *lactobacillus* containing Verum antibiotic was associated with reduced BV recurrence (Reznichenko et al., 2020).

2.12. STI treatment

Currently, the recommended treatment options for *C. trachomatis* are Doxycycline, azithromycin, or Levofloxacin, *N. gonorrhea* is treated with ceftriaxone, and metronidazole is recommended for *T. vaginalis* (Workowski et al., 2021). *M. genitalium* is treated with Doxycycline followed by either azithromycin or moxifloxacin, depending on the sensitivity to macrolide (Workowski et al., 2021). Effective STI treatment would mostly depend on effective diagnosis. Laboratory diagnosis of STIs is the gold standard for treating STIs, offering

relatively high sensitivity and specificity (Caruso et al., 2021). Although effective, factors such as costs, lack of equipment, and shortage of experience laboratory technicians, delays in treatment challenge its feasibility, more so in resource-constrained settings (Cristillo et al., 2017, Gupta and Sharma, 2019). In these settings, the detection and treatment of STIs rely on the syndromic approach (treating STIs based on observable clinical signs and symptoms) (Gupta and Sharma, 2019).

Factors such as overtreatment and asymptomatic infections remaining undetected have challenged the applicability of the syndromic management of STIs (Wi et al., 2019, Kaida et al., 2018). In addition, antibiotic resistance from overtreatment associated with the method can be anticipated and could have implications for treatment in the future. For instance, more than half of *N. gonorrhea* and *T. palladium* infections that were diagnosed based on clinical signs could not be confirmed by laboratory diagnosis (Ayalew et al., 2022)—as such, using the POC test to overcome these challenges is being explored. POC tests are easy-to-use tests that enable quick result turnaround, allowing patients to receive a diagnosis and immediate treatment upon one visit (Muralidhar, 2015, Cristillo et al., 2017). POC tests are developed to meet specific criteria (affordability, specificity, sensitivity, user-friendly, rapid, equipment-free, and delivered to end user) (Adamson et al., 2020). Several POC tests are available for common STIs such as syphilis, trichomoniasis (OSOM TV detection assay), gonorrhea, and chlamydia (Xpert CT/NG test) (Peng et al., 2020, Garrett et al., 2019). Xpert CT/NG and OSOM Rapid tests enable adequate and appropriate testing within 90 minutes of specimen collection and facilitate partner treatment, which is important in efforts to reduce reinfection from sexual partner (Adamson et al., 2020, Garrett et al., 2019). They offer several benefits, including enabling much higher accuracy in detecting STIs, affordability, short time to results, and not requiring extensive (Caruso et al., 2021, Cristillo et al., 2017, Adamson et al., 2020). These tests are easily acceptable by women, and their ability to effectively treat STIs and reduce levels of genital inflammation is appealing (Garrett et al., 2018, Garrett et al., 2021a).

Evidence from several studies has suggested that women, especially in sub-Saharan Africa, suffer the highest burden of HIV infections. It has become apparent that genital inflammation, immune cell recruitment as well as the associated epithelial barrier damage play an integral role in increasing susceptibility to HIV that is observed in this group. Taken together, the contribution of STIs and BV to both genital inflammation and epithelial barrier damage suggests, in this context, that prioritizing their effective treatment could have implications for HIV risk. To overcome the challenges associated with the current treatment options available

for both STIs and BV, POC testing and treatment options are being explored. In as much as these are effective in clearing STIs and reducing inflammation, their impact on the genital epithelium, the first line of defense against infections, including HIV in the genital tract, remains unconfirmed. Discerning the effect of this POC testing and treatment on the genital epithelial barrier is critical to designing effective strategies to reduce HIV risk in women.

2.14. AIM

This study aims to assess the impact of POC treatment for STI/ BV on the integrity of the genital epithelial barrier.

2.15. HYPOTHESIS

Specific and timely diagnosis of STIs and BV using the POC testing and treatment model improves the integrity of the female genital epithelial barrier, creating an environment less susceptible to HIV and other STIs.

1.16. OBJECTIVES

1) To quantify concentrations of MMP biomarkers of the epithelial barrier integrity
2) To compare the concentrations of these biomarkers (MMPs) before and after POC treatment for STI/BV
3) To determine the relationship between MMPs and genital cytokine biomarkers of inflammation

CHAPTER 3

METHODOLOGY

3.1. Study design

This is an ancillary study of the CAPRISA 083 (Garrett et al., 2021a, Garrett et al., 2018) and evaluated the impact of POC testing and treatment of STIs and BV on the integrity of the genital epithelial barriers of CAPRISA 083 participants. The CAPRISA 083 study, conducted between May 2016 and January 2017, evaluated the role of POC testing, treatment, and EPT in reducing STIs/BV and cytokine biomarkers of HIV risk (Garrett et al., 2018). Briefly, participants were tested immediately onsite (POC) for *C. trachomatis, N. gonorrhea, T. vaginalis* and BV, they received the appropriate targeted treatment for the detectable infection/condition, and infected participants were also provided with the appropriate STI treatment for their sexual partners (EPT). This ancillary study of the CAPRISA 083 trial generated complementary assessments of the impact of the POC model on the epithelial barrier against STIs. Here, the genital concentrations of MMPs, commonly used as biomarkers of epithelial barrier integrity (Ngcapu et al., 2015, Arnold et al., 2016b), were measured and compared with corresponding genital cytokine and STI/BV data in the genital fluid specimens of CAPRISA 083 participants at baseline, and at two follow-up visits post-treatment for STI/BV. Ethical approval for this ancillary study was granted by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BREC/00003122/2021, Supplementary document 1).

3.2. Study population

The CAPRISA 083 study included 267 women at high risk of HIV infection presenting at Prince Cyril Zulu Communicable Disease Center in Durban KwaZulu-Natal for STI care. Women between the ages of 18-40 years were eligible and enrolled if their baseline HIV antibody test was negative and if they had not had antibiotic treatment seven days prior to enrollment. Pregnant women and sex workers were excluded from the study. All participants provided informed consent before enrolling and screening for STIs.

3.3. Specimen collection

At baseline, the participants provided two vaginal swabs collected by a professional nurse to enable GeneXpert STI testing and microbial examination to diagnose BV and candidiasis. While the GeneXpert STI testing was underway, professional nurses assisted the participants

in inserting and removing menstrual cups (Soft Cup, Instead, San Diego, California, USA) for genital secretion collection to enable soluble protein or cytokine analysis, as previously described (Archary et al., 2015). After 2 hours, the menstrual cups were removed and placed in labeled sterile 50 ml conical tubes for transport on ice to the CAPRISA laboratory. In addition, participants were interviewed to record their sexual behavior and reproductive health information. Essential information regarding the reason for presenting to the clinic, the number of sexual partners, the type of sexual activity they engage in, frequency of condom use, and contraceptive use was obtained through questionnaires administered by professional nurses. The nurses conducted pelvic assessments under speculum examination for any observable signs of STIs and any vulval and pelvic abnormalities.

3.4. Processing of Menstrual Cup samples

The menstrual cup (MC) sample was processed within 24 hours of collection at the CAPRISA laboratory. Briefly, the MC was centrifuged at 2000 rpm for 10 minutes at 20-25^{o}C. The MC was then removed and discarded, and the remaining pellet and supernatant were centrifuged again at 200 rpm for 5 minutes to enhance the separation of genital fluid from the mucous pellet. After centrifugation, 50 µl of the supernatant was transferred into a 2.0 mL sterile cryovial which was mixed with 200 µl of cold PBS and aliquoted into labeled cryovials. Samples that had 200 µl or more of the sample were aliquoted and labeled as neat supernatants. The mucus pellets were transferred into labeled cryovials, and both the supernatant and the pellet aliquots were stored at -80^{o}C at the CAPRISA Laboratory Repository.

3.5. Point-of-care testing, immediate treatment, and expedited partner therapy

Participants had their genital swabs analyzed for STIs by laboratory technologists with experience in GeneXpert technology, and the results were made available within 2 hours of testing. The combined Xpert CT/NG assay (Cepheid, Sunnydale, California, US) was used to test for chlamydia and gonorrhea, and the OSOM Trichomonas Rapid test (Sekisui Diagnostics, Lexington, MA, US) was conducted to test for trichomonas (Garrett et al., 2018). Gram stain microscopy was undertaken to diagnose BV and candida by Nugent scoring. Further testing at the National Health Laboratory Services (NHLS) was performed using quantitative PCR to analyze the DNA from genital swabs for *Atopobium vaginae*, BV-associated bacterium-2, *G. vaginalis*, Megasphaera-1, *Prevotella bivia*, *Lactobacillus crispactus*, *Lactobacillus jensenii*,

Lactobacillus inners and *Mycoplasma genitalium* (Singh et al., 2020). Participants diagnosed with gonorrhea were treated with 250 mg intramuscular ceftriaxone and 1 gram of azithromycin orally. Chlamydia was treated with 1 gram of azithromycin, and trichomonas and BV were treated with 2 grams of metronidazole orally. Participants diagnosed with any of the STIs or BV were given treatment for their sexual partners.

3.6. Measurement of soluble protein concentrations

Pre-and post-treatment cytokines and MMPs were quantified in the MC specimens using the Bio-Plex Pro Human Cytokine Group I and II panels (Bio-Rad Laboratories, USA, (Garrett et al., 2021a)) and the MILLIPLEX MAP Human MMP Magnetic Bead Panel 2, respectively (MILLIPORE, Billerica, MA).

3.6.1. Multiplex ELISA principle

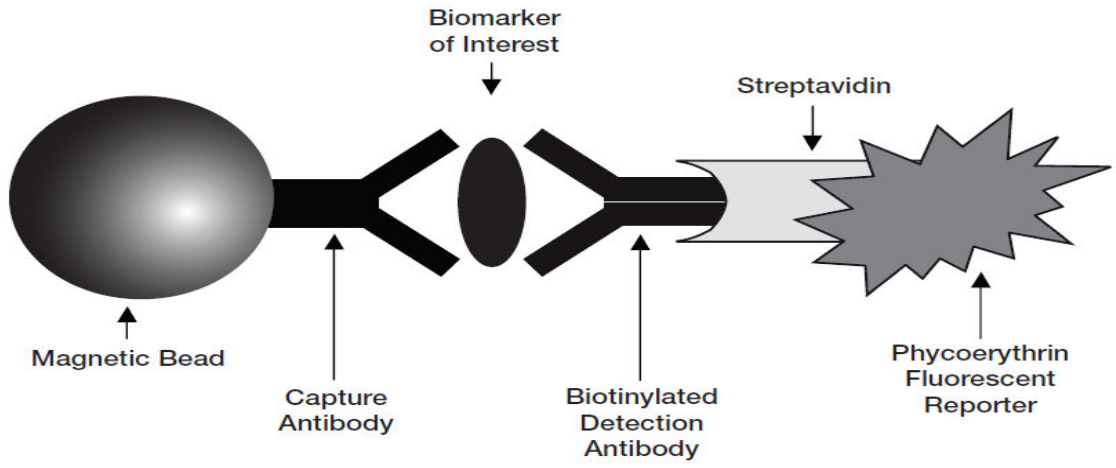

Figure 5. A graphical representation of the Bio-Plex immunoassay (Adapted from Bio-Plex Pro Human MMP Assays Instruction Manual).

Multiplex ELISA enables the simultaneous detection of multiple analytes in a sample using distinctly colored bead sets infused with multiple fluorescent dyes (e.g., red and infrared) at different ratios. Antibodies specific to the analyte of interest are precoated onto the bead. The antibody-coupled bead sets are incubated with the sample and will bind to the analyte of interest. A biotinylated detection antibody specific to the analyte of interest is then added to

amplify the signal of the reporter molecule, Streptavidin-Phycoerythrin, which binds to the biotinylated antibody. The beads are then passed through an array reader, where the red classification and the green reporter lasers excite the beads to enable the identification and quantification of the analyte, respectively.

3.6.2. Measurement of matrix metalloproteinase concentrations in genital fluid

The concentrations of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-12p40, IL-12p70, IL-18, MIF, TNF-α, TNF-β, TRAIL), chemotactic cytokines (CTACK, Eotaxin, Gro-α, IL-8, IL-16, IP-10, MCP-1, MCP-3, MIG, MIP-1α, MIP-1β, RANTES, IFN-α2), growth factors (B-NGF, FGF, G-CSF, GM-CSF, HGF, IL-3, IL-7, IL-9, LIF, M-CSF, PDGF-BB, SCF, SCGF-B, SDF-1α, VEGF), adaptive response cytokines (IFN-γ, IL-2, IL-4, IL-5, IL-13, IL-15, IL-17, IL-2Rα) and regulatory cytokines (IL-10, IL-1Rα) were assessed in CAPRISA 083 participants and the Multiplex ELISA procedure was described elsewhere (Garrett et al., 2021b). This section will focus on the methodology for MMP quantifications conducted as part of this degree. The MILLIPLEX MAP Human MMP Magnetic Bead Panel 2 (MILLIPORE, Billerica, MA) was used to quantify five relevant MMPs spanning the majority of the functional groups: the collagenase MMP-1, the gelatinases MMP-2 and MMP-9, the matrilysin MMP-7, and the stromelysin MMP-10.

3.6.3. Plate layout

Prior to beginning the assay, a plate layout was designed to diagram the placement of the blank, standards (S1 to S7), quality controls (ctrl 1 and ctrl 2), and the samples (X; Figure 8). Standards, controls, and two samples were duplicated within the same plate to assess the variability of intraplate measurements, and three samples were additionally duplicated over consecutive plates to assess plate-to-plate (interplate) variability. No significant differences were observed between the magnitude of the duplicated variables within each plate (**supplementary Table 5**) and across two plates (**Supplementary Table 6**; $p<0.05$, $r>1$). To further ensure consistency in the results, % CVs and standard recovery were assessed. % CVs above 20% were flagged, and standard recovery between 70 and 130% was acceptable. Most of the % CVs were below 20% (**Supplementary Table 7**), and standards recovery was within the 30-70% range (**Supplementary Table 8**). A total of 6 experimental plates were required to quantify the MMP concentrations in 377 genital specimens included in this study.

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	blank	ctrl 1	ctrl 1	X 11	X 19	X 27	X 35	X42	X50	X 58	X 66
B	S1	S1	ctrl 2	ctrl 2	X12	X20	X 28	X36	X43	X51	X59	X67
C	S2	S2	X1	X1	X13	X21	X29	X37	X 44	X 52	X60	X68
D	S3	S3	X2	X2	X14	X 22	X30	X 38	X 45	X53	X61	X69
E	S4	S4	X3	X7	X15	X23	X31	X 38	X46	X 54	X62	X70
F	S5	S5	X4	X8	X16	X24	X32	X 39	X47	X55	X63	X71
G	S6	S6	X5	X9	X 17	X 25	X 33	X40	X 48	X56	X 64	X72
H	S7	S7	X6	X10	X18	X26	X 34	X41	X49	X57	X65	X73

Figure 6. **A plate layout demonstrating the assigned positions for standards, controls, blanks, and samples on a 96 well plate.**

3.6.4. Sample preparation

The MC supernatant (50ul) was removed from the CAPRISA Laboratory Repository and thawed on ice for approximately 30 minutes before conducting the assay. After thawing, a threefold dilution of the sample was performed by adding 100 µl of cold PBS to each sample. After vortexing for approximately one minute to mix, a sample master plate was prepared by transferring a 40 µl volume of diluted sample into the corresponding wells of a 96-well conical bottom plate. The placement of samples in a master plate in preparation for direct transfer to the experimental plate promotes high throughput, accuracy, and efficiency in the assay.

3.6.5. Bio-Plex Multiplex Immunoassay System Start-Up

The Bio-Plex200 Array Reader system was turned on an hour before use and was calibrated before each run by following the software prompts to allow for standardization of the

fluorescent signal. Careful attention was paid to ensuring that the sheath fluid bottle was full and the waste bottle was empty before starting. The protocol in the manager software was prepared each day before running the assay to avoid any delays after the experiment was complete. The protocol was described, analytes were selected (MMP1, MMP2, MMP7, MMP9, and MMP10), and the plate was formatted according to the plate layout.

3.6.6. Reagent preparation

3.6.6.1. Antibody-Immobilized Beads

Five MMP antibody immobilized bead vials were vortexed for about 5 minutes. A volume of 750 µl (150 µl from each antibody bead vial) was added to a mixing bottle. A volume of 2.25 ml bead diluent was added to the mixing bottle to bring to a final volume of 3.0 ml to dilute the beads. After mixing well by vortexing, the beads were stored at room temperature for later use.

3.6.6.2. Quality Controls

Quality controls 1 and quality control 2 were reconstituted by adding 250 µl of deionized water before they were used. After adding water, the controls were mixed well by inverting the vial a few times. Before transferring the controls to their designated, labeled polypropylene microfuge tubes, the tubes were allowed to sit for about 5-10 minutes.

3.6.6.3. Wash buffer

The 60 ml 10X wash buffer which comes with the kit, was first brought to room temperature before use. The contents of the buffer were mixed thoroughly to bring all the salts into solution. The wash buffer was then diluted to 1X by adding 540 ml of deionized water to bring it to a final volume of 600 ml.

3.6.6.4. Human MMP panel 2 standards

Before use, the human MMP panel 2 standard was reconstituted by adding 250 ul of deionized water. The vial was inverted a few times to mix the content and vortexed for about 10 seconds. Before transferring the standards to the assigned labeled polypropylene microfuge tube, the

vial was allowed to sit for about 5-10 minutes. The reconstituted standard was labeled as standard seven and was used to prepare the working standards.

To prepare the six working standards, each of the six polypropylene microfuge tubes was labeled from standard 1 to standard 6. Each of the six tubes was filled with 200 µl of Assay Buffer. 1:3 serial dilutions were performed by adding 100 µl of the reconstituted standard 7 to the tube labeled standard 6. The contents of standard six were mixed by vortexing for a few minutes and 100 µl was transferred to a tube labeled standard 5. This was done for all the tubes up to standard 1. The assay buffer only was used as a blank.

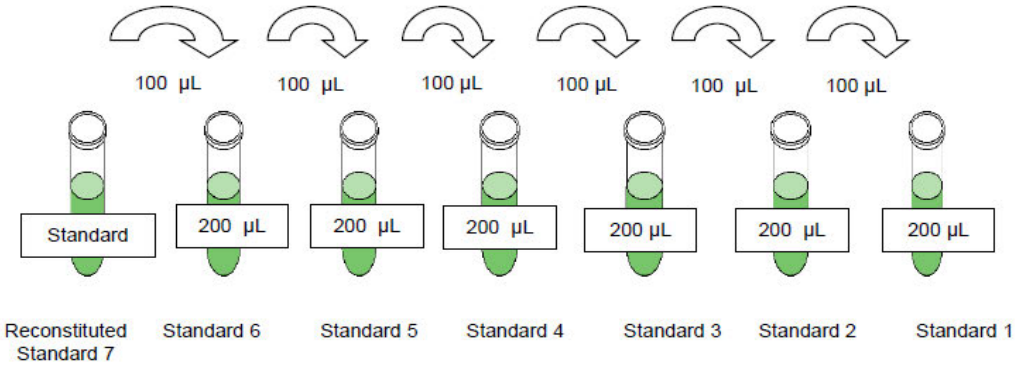

Figure 7. A graphical representation of the three-fold serial dilutions conducted for standard preparation. (Adapted from MILLIPLEX MAP Human MMP Magnetic Bead Panel 2 Assay protocol).

3.6.6.5. Immunoassay procedure

Before conducting the assay, all the reagents were allowed to warm to room temperature (20-25°). The assay was initiated by washing all the 96-plate wells with 200 ul wash buffer using a plate washer at room temperature (20-25°C). This was followed by the addition of 25 ul of standards and controls in their designated wells individually. A volume of 25 ul assay buffer was then added to the blank and all the sample wells using a multichannel pipette. After adding the assay buffer, 25 ul of PBS, which was used as a matrix solution, was added to the blank, standards, and control wells. This was then followed by adding a 25 ul sample to the appropriate wells from the master plate.

Before adding the beads, the mixing bottle was vortexed for a few minutes in case of beads settled at the bottom of the bottle. The mixed beads were discarded into reservoirs, and 25 ul of the mixed beads were added to each well of the plate using a multichannel pipette. The plate was sealed, placed on a plate shaker, covered with foil, and incubated with agitation for 2 hours at room temperature (20-25^{o}C). After incubation, the plate was placed on a plate washer and washed two times with 200 ul wash buffer.

After washing, 25 ul of Detection Antibodies were added to each well on the plate. The plate was sealed, placed on a plate shaker, covered with foil, and incubated with agitation for 1 hour at room temperature (20^{o}C). After incubation, 25 ul of Streptavidin phycoerythrin was added to all the sample wells containing the Detection Antibodies. The plate was sealed, placed on a plate shaker, covered with foil, and incubated with agitation for 30 minutes at room temperature (20^{o}C). Following incubation, the plate was washed two times with 200 ul wash buffer to remove all the well contents. Finally, a multichannel pipette was used to add 100 ul sheath fluid into each of the plate wells. The plate was then incubated on a plate shaker for about 5 minutes, and data were acquired on the Bio-Plex 200 Array reader system.

6.7. Statistical considerations

The CAPRISA 083 participants' demographic characteristics were summarized based on their baseline STI/BV profile. The proportions between the two groups were measured using the Fishers test. Nugent scoring ws used to diagnose BV as BV negative (0-3), intermediate (4-6) and BV positive (7-10). A BV postive result in this cohort was defined by a score of (4-10) (Garrett et al., 2021a). Women were calssified into different subgroups depending on the various permutations of STIs and BV. For example mutually exclussive set of owmen with STIs only, mutually exclussive set of women with BV only, women with both STIs and BV detectable, women with detectable STI (irrespective of their BV status), women with detectable STI irrespecyive of their BV status, and the overlapping population of women with STI and/or BV. Before treatment intervention, Kruskal-Wallis tests with Dunn's post tetsting were used to compare median MMP concentrations of the infected and uninfected groups. Friedman test for non-parametric data was used to compare MMP concentrations at baseline, 3- and 12 weeks post-treatment. Multivariable regression analyses were conducted at baseline to confirm the relationship between untreated STI/BV and MMPs. This FDR adjustment for multiple comparisons was done by using the Benjamin-Hochberg test. Linear mixed models, adjusting

for participant age, pelvic examination, and inflammation, were fitted to assess the effect of POC STI/BV treatment on MMPs. Statistical analyses were performed using GraphPad Version 8.43.3 (GraphPad Software, San Diego, CA), SAS version 9.4 (SAS Institute, Cary, North Carolina) and STATA version 15.0 (StataCorp., College Station, TX, USA).

CHAPTER 4
RESULTS

4.1. Baseline characteristics of the CAPRISA 083 participants

From a total of 267 HIV-uninfected women enrolled in the CAPRISA 083 prospective cohort study, only 238 had data available for MMP analysis and were included in this study. The median age of these women was 23 years (Interquartile range 21-27). More than half of these women, 71.43% (170/238), were positive for BV and/or at least one of the tested STIs (C. *trachomatis (n=34)*, *N. gonorrhea (n=11)*, *T. vaginalis (n=10),* or *M. genitalium (n=11)*) at baseline (i.e., "STI/BV," **Table. 1**). More women with STI/BV had BV 95. 88% (163/170), and STIs were detectable in 32.94 % (56/170). At enrolment, 24.12% of women with STI/BV had genital inflammation defined by elevated cytokine biomarkers (Masson et al., 2015, Garrett et al., 2021a), compared to 16.18% of women negative for STI and BV. Vaginal inflammation defined by elevated cytokine biomarkers (Masson et al., 2015, Garrett et al., 2021a) was confirmed in 24.12% of women with STI/BV compared to 16.18% of women negative for STI and BV. Age was treated as a categorical variable with two groups (<25 years vs ≥ 25 years), in keeping with prior publications from the cohort (Garrett et al., 2021a) and documented evidence for differences in HIV susceptibility between these two age categories (Dellar et al., 2015).

4.2. MMP concentrations were higher in women with STI/BV compared to women without STI/BV

Women with STI/BV (n=170) had significantly higher median concentrations of all the MMPs evaluated in this study (MMP-1, -2, 7, -9, and -10) compared to women without any STI/BV (n=68; **Figure 9**). Because most participants had both STIs and BV, we compared the baseline genital MMP concentrations of women with neither STI/BV (n=68) with subgroups of women with various permutations of detectable STIs and BV i.e., (1) the mutually-exclusive sets of women with only STI detectable (n=7), only BV detectable (n=114), both STI and BV detectable (n=49; Supplementary figure 12); and (2) the overlapping populations of women with STI and/or BV (n=170), women with detectable STI (irrespective of BV detection; n=56), and women with detectable BV (irrespective of STI detection, (n=163); Supplementary figure 13). **Figure 8** is included to illustrate the subsets of enrolled women included in this study.

Table 1. Demographic characteristics of the CAPRISA 083 participants.

Variable	Category	Overall N=238 n/N (%)	STI/ BV detected N=170; n/N (%)	STI and BV negative N=68; n/N (%)	p-value
Age	Less than 25	154 (64.71)	110 (64.71)	44 (64.71)	>0,999
	25 and over	84 (35.29)	60 (35.29)	24(35.29)	
Highest level of education	Primary	1/234 (0.43)	1/168 (0.60)	0	0.219
	Secondary	169/234 (72.22)	126/168 (75)	43/66 (65.15)	
	Tertiary	64/234 (27.35)	41/168 (24.40)	23/66 (34.85)	
Reason for presentation	STI symptoms	214 (89.92)	151 (88.82)	63 (92.65)	0.306
	No symptoms (check-up)	17 (7.14)	12 (7.06)	5 (7.35)	
	Other	7 (2.94)	7 (4.12)	0	
Number of sexual partners in the past 12 months	None	3 (1.26)	2 (1.18)	1 (1.47)	0.189
	One	162 (68.07)	110 (64.71)	52 (76.47)	
	More than one	73 (30.67)	58 (34.12)	15 (22.06)	
Number of sexual partners in the past 2 months	None	12 (5.04)	8 (4.71)	4 (5.88)	0.831
	One	205 (86.13)	146 (85.88)	59 (86.76)	
	More than one	21 (8.82)	16 (9.41)	5 (7.35)	
Gender(s)of sexual partners	Men	234 (98.32)	167 (98.24)	67 (98.53)	>0,999
	Men and women	4 (1.68)	3 (1.76)	1 (1.47)	
Type of sexual activity	Anal	12 (5.04)	7 (4.12)	5 (7.35)	0.331
	No Anal	226 (94.96)	163 (95.88)	63 (92.65)	
	Oral	68 (28.57)	52 (30.59)	16 (23.53)	0.341
	No Oral	170 (71.43)	118 (69.21)	52 (76.47)	
Frequency of condom use	Always	10 (4.20)	7 (4.12)	3 (4.41)	0.928
	Sometimes	155 (65.13)	112 (65.88)	43 (63.24)	
	Never	73 (30.67)	51 (30)	22 (32.35)	
Symptoms of vaginal discharge or genital ulcers	Yes	129 (54.20)	84 (49.41)	45 (66.18)	**0.022**
	No	109 (45.80)	86 (50.59)	23 (33.82)	
Contraceptive use	Yes	84 (35.29)	63 (37.06)	21 (30.88)	0.453
	No	154 (64.71)	107 (62.94)	47 (69.12)	
Contraceptive type	Condom only	7/84 (8.33)	3/63 (4.76)	4/21(19.05)	0.281
	IUD	2/84 (2.38)	2/63 (3.17)	0	
	Oral contraceptive pill	9/84 (10.71)	7/63 (11.11)	2/21 (9.52)	
	Progesterone injections	49/84 (58.33)	37/63 (58.73)	12/21 (57.14)	
	Subdermal implant	17/84 (20.24)	14/63 (22.22)	3/21 (14.29)	
Inflammation	Yes	52 (21.85)	41 (24.12)	11(16.18)	0.225
	No	186 (78.15)	129 (75.88)	57 (83.82)	
Candidiasis	Positive	46 (19.33)	31 (18.24)	15 (22.06)	0.586
	Negative	192 (80.67)	139 (81.76)	53 (77.94)	
Pelvic exam	Normal	123 (51.68)	83 (48.82)	40 (58.82)	0.197
	Abnormal	115 (48.32)	87 (51.18)	28 (41.18)	

BV, bacterial vaginosis; STI, sexually transmitted infection; IUD, intrauterine device; STIs (N. gonorrhoea, C. trachomatis, T. vaginalis, M. genitalium)

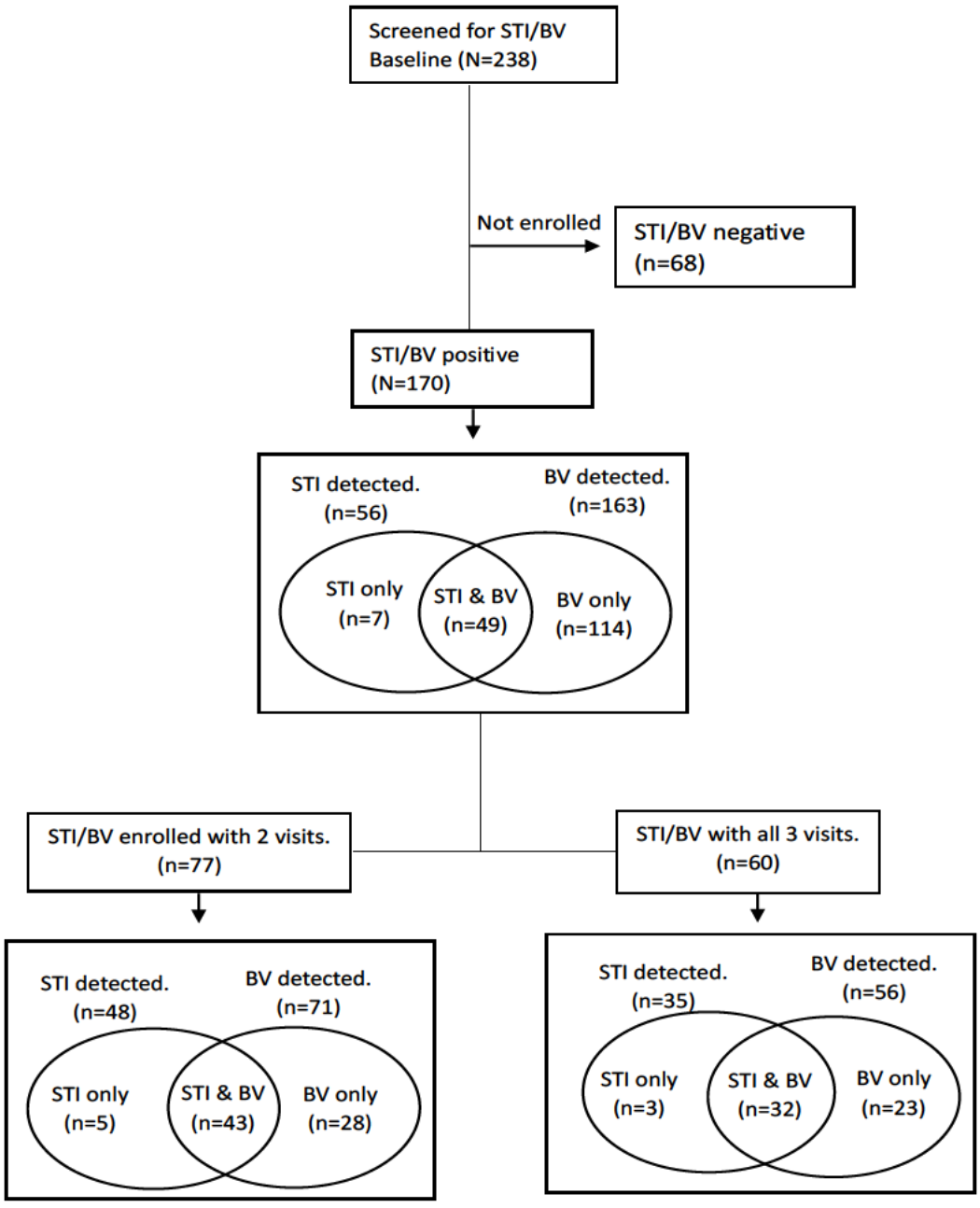

Figure 8: A diagram illustrating the subclassifications of the CAPRISA 083 cohort according to STI/BV status.

Consistently lower concentrations of MMP-1, -2, -7, and -10 were observed in women with neither STI/BV than women with detectable STIs (i.e., regardless of their BV status; n=56),

women with detectable BV (i.e., regardless of their STI status; n=163), women with BV only (n=114), and women infected with both STIs and BV (n=49; p<0.05, **Supplementary Figure 12**). MMP-9 concentrations only differed among women with detectable STI (n=56; median 9.093 pg/ml; IQR (9,093- 9,093)) and women with neither STI nor BV (n=68; median 9.093 pg/ml; IQR (5,397- 9,093)). Multivariable regression analysis controlling for age, pelvic examination, and inflammation confirmed these observations, and statistical significance was maintained after adjusting for multiple comparisons by FDR (**Table 2**).

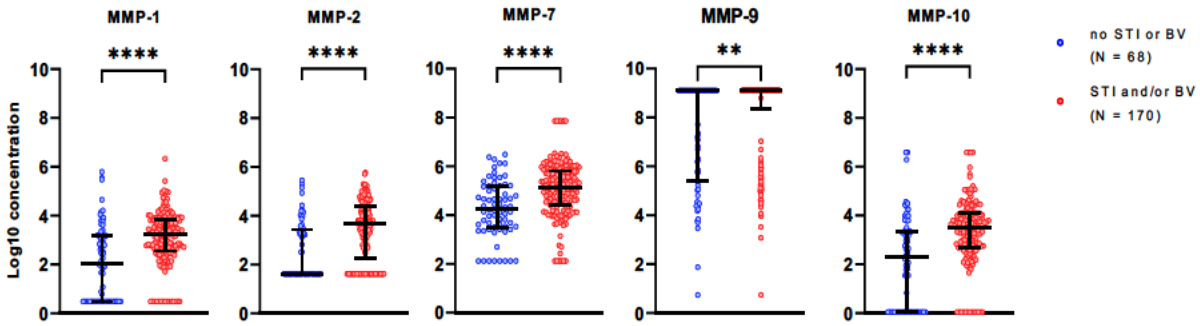

Figure 9: Comparisons of MMP concentrations between women with and without STI/BV. MMP concentrations were measured in the MC fluid of 238 women at baseline. Women with STI/BV (n=170) had significantly higher median MMP concentrations (MMP-1, -2, -7, -9, and -10) compared to women without any STI/BV (n=68). Red dots present women with STI/ BV, while women with neither STI/BV are presented by blue dots. Mann-Whitney U tests were used to compare the non-parametric data between the two groups. Significant differences were denoted by p-values <0.05.

Table 2. Association between MMPs and STI/BV (N=238)

MMP	β-coeficient[1]	95% CI	p-value	FDR
MMP-1	0.582	0.330 – 0.834	<0.001	<0.001
MMP-2	0.640	0.376 – 0.905	<0.001	<0.001
MMP-7	0.613	0.353 – 0.873	<0.001	<0.001
MMP-9	0.441	-0.007 – 0.889	0.054	0.054
MMP-10	0.843	0.526 – 1.160	<.0001	<0.001

[1] *Multivariable linear regression model adjusting for participant age, pelvic examination, and inflammation status. P<0.05 were considered statistically significant. MMP, Matrix metalloproteinase; FDR, False Discovery Rate*

4.3. MMPs were associated with several pro-inflammatory cytokines and chemotactic cytokines

Because of the previously established relationship between MMPs and inflammation cytokines (Mauviel, 1993, Fingleton, 2017, Manicone and McGuire, 2008), we investigated associations between MMP concentrations and the 48 cytokines that were previously assessed in this cohort (Garrett et al., 2021a). Multivariable linear mixed models controlling for age, pelvic examination, STIs, and BV were used to determine the relationship between MMPs and cytokines (**Table 3**). Associations maintained statistical significance after adjusting for multiple comparisons between MMPs and either pro-inflammatory or chemotactic cytokines. These include positive associations between concentrations of MMP-1and IL-1β, IL-6, MIP-1α, RANTES; MMP-2 and IL-6, MIP-1α; MMP-7 and IL-6, MIP-1α, MIP-1β; MMP-9 and IL-1α, MIP-1α and MMP-10 and IL-1β, IL-6, MIP-1α, RANTES, IL-15 (**Figure 10**). Additionally, MMP-10 concentrations were also associated with increases in the adaptive response cytokine IL-15.

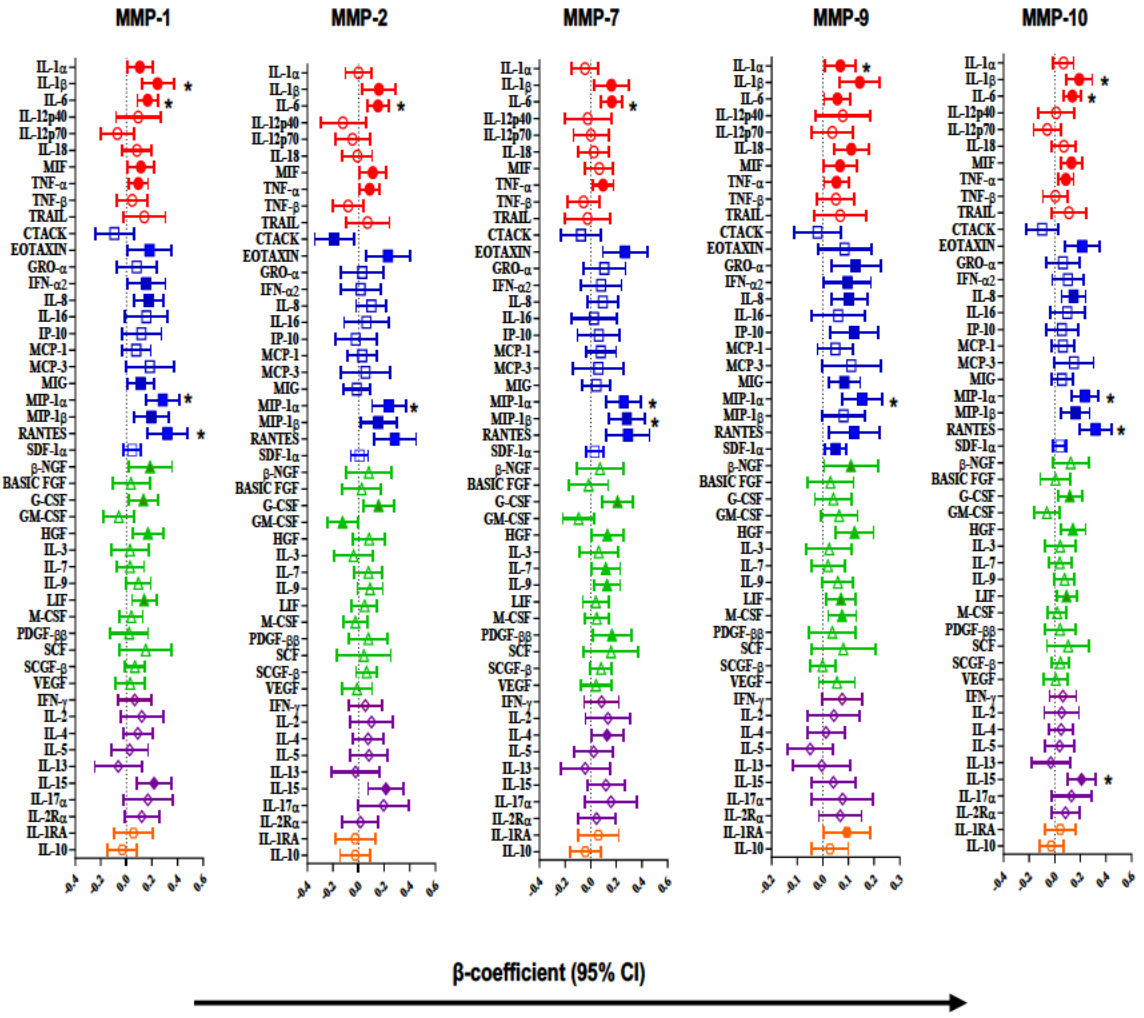

Figure 10. Associations between MMPs and cervicovaginal cytokine concentrations. Multivariable linear mixed models controlling for age, pelvic examination, STIs (*N. gonorrhea, C. trachomatis, T. vaginalis, and M. genitallium*), and BV were used to assess the relationship between MMPs and genital cytokines concentrations at baseline. A total of 238 participants with both cytokine and MMP data were included in this analysis. Cytokines involved in inflammation were represented by red circles, chemotaxis by blue squares, hematopoiesis by green triangles, the adaptive response by purple diamonds, and regulation by orange hexagons. Filled shapes indicate significant p-values ($p < 0.05$), and $p < 0.05$ after adjusting for multiple comparisons by FDR is indicated by asterisks (*).

Table 3. Associations between MMPs and genital inflammation (N=238)

MMP	β-coeficient[1]	95% CI	p-value
MMP-1	0.831	0.456 – 1.205	0.001
MMP-2	0.622	0.251 – 0.993	0.001
MMP-7	0.369	0.002 – 0.737	0.049
MMP-9	0.117	-0.518 – 0.752	0.718
MMP-10	0.976	0.513– 1.438	0.001

Multivariable linear regression model adjusting for participant age, pelvic examination, STIs, and BV. P<0.05 were considered statistically significant. MMP, Matrix metalloproteinase; FDR, False Discovery Rate, STIs (C. trachomatis, N. gonorrhoea, T. vaginalis, and M. genitalium)

4.4. STI/BV treatment does not comprehensively alter MMP concentrations

Of the 170 women diagnosed with STI/BV at baseline, only 45% (77/170) were immediately treated with antibiotics at baseline. Only 35.29% (60/170) of those treated retested at 6- and 12 weeks following treatment. Matched MC fluid from women with all three visits (n=60) was used to assess the impact of POC treatment on the genital epithelial barrier integrity. From those with all three visits (n=60), STIs and BV were diagnosed in 35 and 57 women at baseline, respectively. STIs cleared in 88.57% (31/35) women, and BV was resolved in 24.56% (14/57) women by 12 weeks post-treatment. As per previous reports by (Garrett et al., 2021a), concentrations of most cytokine biomarkers of inflammation were reduced by weeks -6 and 12 post-treatment (**Supplementary Table 9).** The 60 women with STI/BV were grouped according to their STI/BV status as STI/BV (n=60), STI and BV (n=32), detectable STIs (n=35), detectable BV (n=56), STIs only (n=3) and BV only (n=23), and MMP concentrations assessed in matched samples across three visits in the different groups to evaluate the independent effect of STI or BV treatment on MMP concentrations. Although a trend in the reduction of MMP concentrations of the women enrolled in the study with at least two visits (n=77) was generally observed after six weeks of treatment in all the MMPs, these alterations did not reach statistical significance (**Table 4**). Only MMP-1 concentrations demonstrated an alteration, with lower 6-week median concentrations of MMP-1 observed in women who were treated for detectable STI (n=32; p=0.0048; **Figure 11a**, and the subset of women presenting with both STIs and BV (n=35; p=0.0066; **Figure 11a**). BV treatment was not associated with any changes in MMP concentrations. Linear mixed models assessing alterations in MMP

concentrations post-treatment, controlling for participant age, pelvic examination, and inflammation demonstrated that a relationship between MMP-1 (β coefficient 0.484 (95% CI 0.127-0.842), p=0.008), MMP-7 (β coefficient 0.431 (95% CI 0.056-0.807), p=0.024), MMP-10 (β coefficient 0.001(95% CI 0.414-1.704), p=0.001) was maintained after STI treatment. No associations were found between MMPs and BV (**Table 4**).

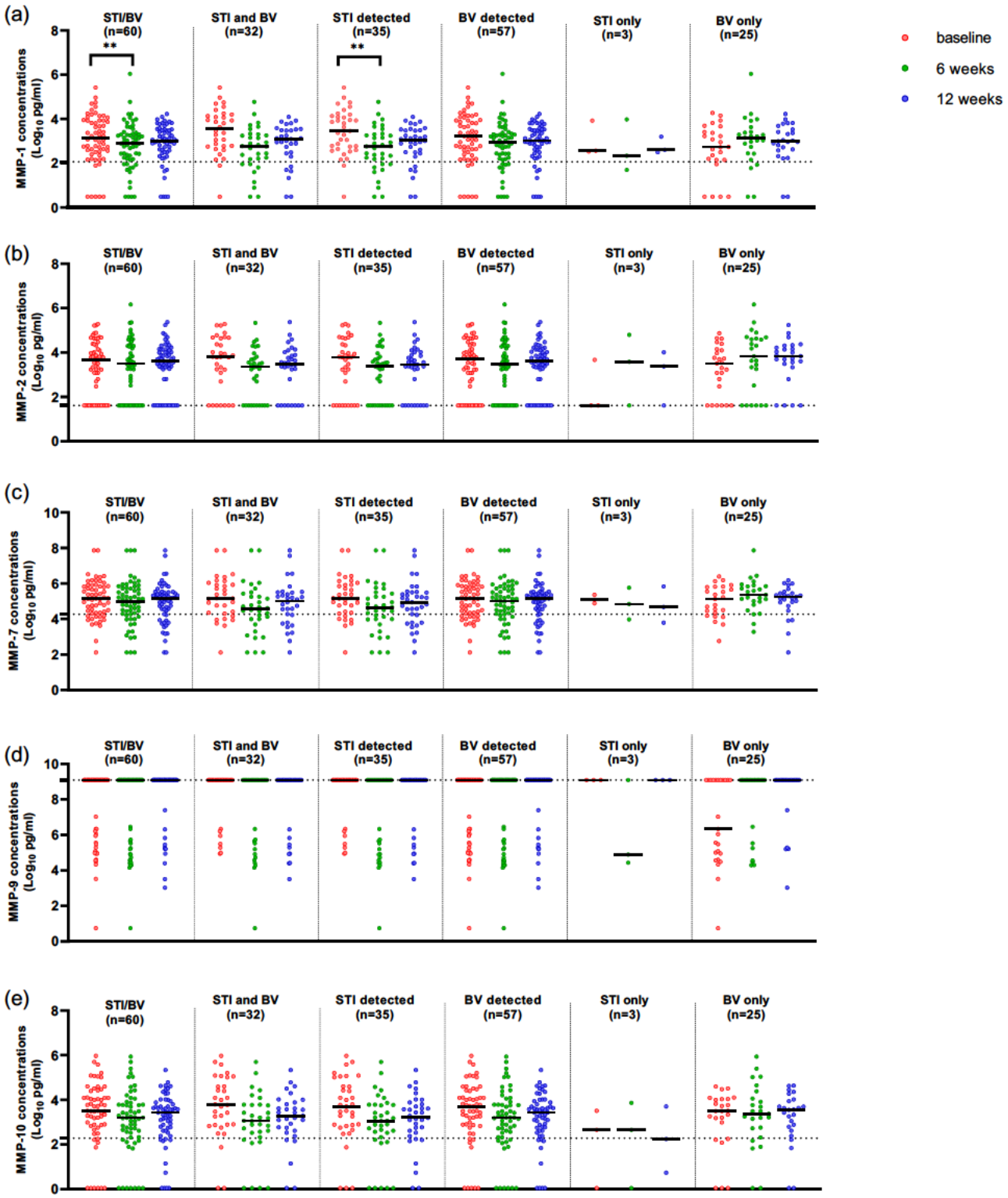

Figure 11. Comparisons of MMP concentrations in the MC fluid of women with STI/BV at baseline who were treated and retested at 6- and 12- weeks post-treatment. To assess the independent effect of STI and BV treatment, women with STI/BV (n=60) at baseline with two other visits were further grouped as having STI/BV, STI and BV, detectable STI, detectable BV, STI only and BV only. Baseline, six-week, and 12-week visits are presented by

red, green, and blue dots, respectively. Friedman test for non-parametric data compared the concentrations of MMP-1, -2, -7, -9, and -10 from matched MC fluid across all visits. The dotted lines indicate the baseline median MMP concentrations in women with neither STI/BV.

Table 4: Association between MMP and STI or BV after treatment

	Median MMP concentration Log10 pg/ml (IQR)					Any STI		
	Baseline (n=77)	Week 6 (n=71)	Week 12 (n=67)	p-value	Effect	β-coefficient	95% CI	p-value
MMP-1	3.226 (2.547-3.925)	3.029 (2.210-3.628)	2.998 (2.488-3.520)	0.2140	Any STI	0.484	0.127 - 0.842	0.008
					Nugent Score	0.014	-0.054 - 0.082	0.684
MMP-2	3.670 (1.619-4.430)	3.547 (1.619-4.318)	3.608 (2.806-4.136)	0.9891	Any STI	0.335	-0.063 - 0.733	0.099
					Nugent Score	0.067	-0.011 - 0.145	0.094
MMP-7	5.141 (4.437-5.842)	5.011 (4.204-5.711)	5.180 (4.230-5.532)	0.5427	Any STI	0.431	0.056 - 0.807	0.024
					Nugent Score	0.015	-0.061 - 0.090	0.700
MMP-9	9.093 (6.273-9.093)	9.093 (5.533-9.093)	9.093 (6.313-9.093)	0.7866	Any STI	1.059	0.414 - 1.704	0.001
					Nugent Score	-0.108	-0.234 - 0.019	0.095
MMP-10	3.508 (2.784-4.449)	3.214 (2.527-4.057)	3.308 (2.493-3.897)	0.1613	Any STI	0.450	0.000 - 0.899	0.050
					Nugent Score	0.054	-0.036 - 0.143	0.241

Linear mixed models, controlling for participant age, pelvic examination, and inflammation. P<0.05 were considered statistically significant. Any STI; C. trachomatis, N. gonorrhoeae, T. vaginalis, and M. genitalium.

CHAPETER 5

DISCUSSION

While successful clearance of STIs and BV is important, a deeper understanding of the treatment effect beyond infection clearance may be equally important. This is especially true in efforts to reduce HIV infection rates in women, where several other biological factors, such as the genital epithelium, are implicated. This study aimed to assess the impact of POC testing and treatment of STIs and BV on the integrity of the female genital epithelial barrier. An intact epithelial barrier in the female genital tract is critical for preventing pathogen entry in this area (Carias and Hope, 2019). Because STIs and BV directly contribute to barrier impairment, treatment of infection only, without ensuring restoration of barrier competency, is insufficient.

Here we assessed established biomarkers of epithelial barrier integrity, MMPs, to evaluate POC testing and treatment effect on the genital epithelial barrier integrity. The data presented shows that women with STI/BV have significantly higher levels of genial MMPs than those without STI/BV. We anticipated the observed associations between MMP biomarkers of epithelial barrier integrity and that several genital pro-inflammatory cytokines and chemokines. Surprisingly, POC STI/BV testing and treatment did not significantly impact the genital MMP concentrations over time. This is despite the previous observations of STI clearance and reduction of inflammation upon POC treatment in this cohort.

Our results are not the first to show an association between increased MMP expression and STI/BV. *C. trachomatis* and *N. gonorrhea* have previously been associated with higher levels of MMP-2 and -9 (Ault et al., 2002) and MMP-8 and -9 (Juica et al., 2017, Rodas et al., 2017), respectively. MMPs are known mainly for their function in extracellular matrix degradation (Nagase et al., 2006). These proteases have been described as significant regulators of wound healing and tissue remodeling (Page-McCaw et al., 2007b, Caley et al., 2015). MMP-1 is a collagenase with specific functions in re-epithelization during wound healing and has been used in some studies as an indicator of good healing (Stevens and Page-McCaw, 2012, Muller et al., 2008). The role of MMP-1 in accelerated wound healing was further shown in full-thickness skin wounds created in rats (Keskin et al., 2021). Impaired re-epithelization was reported in the absence of MMP-9 in mice, suggesting a significant role in epithelization (Kyriakides et al., 2009). The upregulation of MMP-9 soon after coitus (Mohammadi et al., 2022), known to induce genital lesions in the genital epithelium (Astrup et al., 2012), further substantiate a role in tissue repair. MMP-10 expression is observed in migrating keratinocytes in wounds (Rechardt et al., 2000). Lastly, MMP-7 activity was increased during normal wound healing with delays in wound closure upon its inactivation (Hayden et al., 2011).

The role of STIs (e.g., HSV-2) as inducers of genital epithelial barrier impairment has long been established and can more directly be attributed to their role as inducers of genital ulcers (Celum et al., 2004). The contribution of STIs in weakening the barrier integrity and increasing its permeability to pathogens is in sync with the increased susceptibility to HIV observed in their context (Horbul et al., 2011). Taking this into consideration, the data presented here support our hypothesis that MMP upregulation in the context of STIs and BV may signify a role in tissue repair to restore barrier integrity.

Like STIs, high levels of MMP are consistently elevated in the context of BV. Women with BV expressed higher levels of MMP-1, -3, -10, and -13 in their cervicovaginal fluid (Cherne et al., 2020). *G. vaginalis*, a key pathogen in women with BV (Morrill et al., 2020), has been associated with MMP-1 and MMP-9 (Gerson et al., 2022). In addition, expression of MMP-10 and -13 was increased in response to *A. vaginae* (Cherne et al., 2020), a microorganism consistently found in BV biofilms with *G. vaginalis* (Hardy et al., 2016, Castro et al., 2020) Taking into consideration the role of MMPs in tissue repair, there are two likely causes for increased expression of MMPs in BV condition. Firstly, BV has been shown in several studies to be associated with epithelial cell shedding (O'Hanlon et al., 2020, Amegashie et al., 2017). Secondly, BV induces epithelial cell apoptosis (Roselletti et al., 2020). Both these are mechanisms through which barrier integrity is compromised.

Results from this study further show associations between genital pro-inflammatory cytokines and chemokines with MMPs, which is comparable to previous studies showing associations between MMPs and genital inflammatory cytokines (Arnold et al., 2016a, Short et al., 2021). Increased levels of genital pro-inflammatory cytokines and chemokines, an indication of genital inflammation, have been associated with STIs and BV in young South African women. While it is possible that inflammatory responses are beneficial for pathogen clearance (Medzhitov, 2008), the influence that this has on HIV susceptibility through epithelial barrier damage and immune cell recruitment is concerning (Arnold et al., 2016a, Gosmann et al., 2017, Masson et al., 2015). Additionally, the adverse reproductive challenges associated with both STIs (cancer, infertility, ectopic pregnancies) (Burd, 2003, Tsevat et al., 2017, Mpiima et al., 2018) and BV (preterm birth, pelvic inflammatory disease, infertility) cannot be ruled out (Shimaoka et al., 2019, Ravel et al., 2021), collectively suggesting that effective STI and BV treatment should be prioritized.

Although proinflammatory cytokine concentrations were reduced on STI/BV treatment in this study (Garrett et al., 2021a), MMP concentrations were not significantly altered 12 weeks post-BV treatment. Reductions in MMP-1 concentrations, however, were observed only six weeks post-treatment in the subset of women treated for STIs (**Figure 11**). Although MMP-1 levels were reduced, these results may need to be interpreted cautiously since these reductions were very modest. Based on this, it can be concluded that STI treatment only and not BV was associated with MMP reduction. Women treated for BV maintained their higher MMP concentrations despite having received metronidazole, the standard gold therapy for BV (Workowski et al., 2021). This was unsurprising as a high rate of BV recurrence was reported after treatment reported in these women (Mtshali et al., 2021b), which has also been observed in other studies (Bradshaw et al., 2006, Francis et al., 2016). The temporal genital microbial shifts imply that the benefit of the healthy vaginal microbiome on the genital epithelium may have been short-lived, and women had regressed to their compromised state by the time repeat testing was conducted. One of the significant challenges in women treated for BV is the failure of long-term *Lactobacillus* spp. recolonization after treatment (Goje et al., 2021). Resistance to Metronidazole (Schuyler et al., 2016, Ruiz-Perez et al., 2021) and even sexual transmission of microbes (reseeding) have been suggested as possible reasons for high BV for treatment failure and high recurrent rates (Vodstrcil et al., 2021, Ratten et al., 2021).

The present results have implications for the evaluation of treatment options for women as the POC model, although effective in reducing genital inflammation and clearing STIs, may be insufficient to restore barrier integrity after treatment. More effective treatment strategies are needed. Research towards improving vaginal health in women is increasingly focusing on ensuring recolonization of the female genital microbiome by the *Lactobacillus* spp. considered representatives of a healthy female genital microbiome. The benefits of *Lactobacillus* spp. in maintaining barrier integrity range from re-epithelization of genital epithelial cells (Takada et al., 2018) to increasing transepithelial resistance and reducing the permeability of the genital epithelial cells (Dizzell et al., 2019, Anton et al., 2018).

Evidence of decreased BV recurrence rates (Cohen et al., 2020) and improved barrier integrity was observed recently when a live biotherapeutic product, LACTIN-V containing *L. crispatus* was applied after treatment (Armstrong et al., 2022b). Studies have also investigated the potential vaginal microbiome transfer in efforts to revert to *Lactobacillus* abundance in women with BV (Lev-Sagie et al., 2019). More recently, bacteriophage therapy using endolysin has

also been explored as an alternative to BV treatment (Arroyo-Moreno et al., 2022). In non-human primates, the potential of prebiotic maltose gel to foster the proliferation of *Lactobacillus* and decrease BV-associated bacteria has also been demonstrated (Zhang et al., 2020). With the results of this study clearly demonstrating the inability of metronidazole treatment alone to restore barrier integrity, integrating any of these strategies with treatment could possibly address this challenge. This could either be through promoting *Lactobacillus* proliferation or repairment of the genital epithelium.

This study acknowledges a few limitations. First, the functionality of MMPs is not clear since the assay could not distinguish between active MMPs and proMMPs (inactive MMPs). However, the predominance of active MMPs in the context of BV has been observed (Cherne et al., 2020), and the contribution of proMMPs is likely minimal. Second, the assessment of the independent effects of STI or BV treatment on the genital epithelial barrier integrity was challenged by the relatively low numbers of participants with either STI only or BV only. Although MMPs may be valuable biomarkers of wound healing and barrier integrity, future confirmatory assessments of other such biomarkers, e.g., tissue inhibitors of metalloproteinases (TIMPs), soluble E-cadherin, tight junction proteins, fibrinogen, and claudins. may address more comprehensively the relationship between STI/BV treatment and the restoration of barrier function. Lastly, we recommend that further research is conducted to assess the impact of the treatment intervention on immune cell activation, another critical determinant of HIV susceptibility in the female genital tract.

Nonetheless, this work has clearly demonstrated the impact that STI/BV have in disrupting the female genital epithelium, potentially compromising its effectiveness against invading pathogens. Of more clinical relevance was probably the observation that treating STIs and BV with antibiotics may not be enough to restore barrier function. This therefore raises concerns on the current treatment options and signify a need for better, more effective treatment in women with STIs/BV.

CHAPTER 6

CONCLUSION

In conclusion, this study has shown that although POC testing and treatment of STI/BV may be effective against STIs and reduce genital inflammation in the genital tract, a transient reduction in MMP-1 only was observed after treatment. . Even though the concentrations of MMPs, biomarkers of epithelial barreir compromise are higher in women with STIs/BV compared to healthy women with neither STI/BV, treatment did not significantly alter these profiles in women with STI/BV after 12 weeks. These findings suggest that POC STI/BV treatment does not restore epithelial barrier integrity, and women remain susceptible to infection after treatment. Additionally, more effective treatment options are necessary to restore barrier integrity and improve vaginal health in women with STI/BV.

CHAPTER 7
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CHAPTER 8

SUPPLEMENTARY DATA

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

26 August 2021

Miss Nonsikelelo Ndlela (217071075)
School of Lab Med & Medical Sc
Medical School

Dear Miss Ndlela,

Protocol reference number: BREC/00003122/2021
Project title: Impact of Point-of-Care Treatment on Sexually Transmitted Infections and Bacterial Vaginosis on Genital Epithelial Barrier Integrity
Degree: MMedSci

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 26 August 2021. Please ensure that outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is subject to national and UKZN lockdown regulations, see (http://research.ukzn.ac.za/Libraries/BREC/BREC_Amended_Lockdown_Level_3_Guidelines.sflb.ashx). Based on feedback from some sites, we urge PIs to show sensitivity and exercise appropriate consideration at sites where personnel and service users appear stressed or overloaded.

This approval is valid for one year from 26 August 2021. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2020) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 14 September 2021.

Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Supplementary table 5: Intraplate variability within each of the six 96-well plates measured between duplicated standards, controls, and unknown samples.

	MMP-1		MMP-2		MMP-7		MMP-9		MMP-10	
	p-value	r	p-value	r	p-value	r	p-value	r	p-value	r
Plate 1	0.131	0.998	**0.021**	0.998	0.475	0.985	0.160	0.998	0.147	0.993
Plate 2	0.971	0.989	0.612	0.994	0.453	0.996	0.444	0.959	0.401	0.980
Plate 3	0.254	0.990	0.941	0.997	0.255	0.998	0.496	1.000	0.777	0.996
Plate 4	0.099	0.996	0.227	0.994	0.089	0.998	>0.999	1.000	0.053	0.997
Plate 5	0.570	0.998	0.311	0.913	0.407	0.975	0.317	0.984	0.325	0.969
Plate 6	0.223	0.962	0.802	0.991	0.512	0.995	0.205	0.981	0.265	0.958

Paired t test for nonparametric was used to determine the difference between duplicated standards, controls, and unknown samples. Significant differences were denoted by p-values >0.05. Pearson correlation coefficients (r) were used to measure the magnitude of correlation between the 7 duplicated standards, 2 duplicated controls and 2 duplicated unknown samples.

Supplementary table 6: Interplate variability between two of the six 96-well plates measured between standards, controls, and unknown samples.

	MMP-1		MMP-2		MMP-7		MMP-9		MMP-10	
	p-value	r	p-value	r	p-value	r	p-value	r	p-value	r
Plate 1 and 2	0.6669	0.995	0.8875	0.9911	0.831	0.9872	0.2248	0.884	0.1931	0.8346
Plate 2 and 3	0.5701	0.9962	0.2969	0.9815	0.9623	0.9878	0.2579	0.9838	0.2572	0.9544
Plate 3 and 4	0.1793	0.958	0.172	0.7348	0.1746	0.9737	0.3465	0.9925	0.1428	0.9054
Plate 4 and 5	0.2796	0.869	0.1635	0.8429	0.5215	0.7154	0.5798	0.9816	0.2806	0.8112
Plate 5 and 6	0.6909	0.9357	0.1308	0.9426	0.2295	0.9024	0.6919	0.8864	0.383	0.9031

Paired t test for nonparametric data was used to measure the differences between the 14 standards, 4 controls and 3 unknown samples that were duplicated in two consecutive plates. Significant p-values were denoted by p-values >0.05. Pearson correlation coefficients (r) were used to measure the magnitude of correlation between the standards, controls, and unknown samples.

Supplementary table 7: Summary of the % CVs obtained from the six 96-well plates.

		MMP-1	MMP-2	MMP-7	MMP-9	MMP-10
	Type			%CV		
Plate 1	Blank	0	0	2.48	3.45	7.44
	Standard 1	7.54		15.18	7.64	2.48
	Standard 2	8.41	2.67	1.85	4.26	4.49
	Standard 3	0.22	0	2.41	7.89	2.89
	Standard 4	23.91	22.06	13.36	11.21	14.24
	Standard 5	10.78	7.64	9.96	9.06	7.86
	Standard 6	10.16	14.4	5.99	4.1	9.91
	Standard 7	2.98	0.78	4.66	0.53	0.99
	Control 1	23.91	17.25	12.3	19.64	20.54
	Control 2	11.21	20.8	15.13	15.79	15.87
	unknown 1	7.07	0	7.87	7.65	4.29
	Unknown 2	6.98	5.18	4.38	3.2	14.1
Plate 2	Blank	0	0	0	8.32	3.63
	Standard 1	2.93		1.59	0	5.89
	Standard 2	5.49	5.66	6.05	6.15	2.57
	Standard 3	22.8	1.63	22.47	5.84	9.65
	Standard 4	7.42	6.67	3.15	6.68	1.61
	Standard 5	4.46	10.31	3.74	16.68	0.79
	Standard 6	17.9	0	15.35	0	0
	Standard 7	8.44	4.3	1.92	14.98	4.25
	Control 1	7	20.2	16.86	17.06	16.7
	Control2	6.95	2.23	4.47	1.41	2.35
	unknown 1	0	17.68	4.96	2.71	4.88
	Unknown 2	21.76	7.44	3.21	3.81	5.66
Plate 3	Blank	0	0	0	7.44	7.44
	Standard 1	18.25	0	16.16	14.43	5.05
	Standard 2	27.04	9.03	19.36	22	6.33
	Standard 3	10.71	6.43	9.66	9.37	18.72
	Standard 4	6.41	2.14	4.43	9.89	1.86
	Standard 5	1.55	1.2	4.81	2.75	0.87
	Standard 6	4.19	10.82	3.97	7.77	1.03
	Standard 7	1.05	2.5	0.02	0.54	1.37
	Control 1	6.45	3.93	0.94	2.4	4.51
	Control2	0.06	12.46	1.43	3.24	2.11
	unknown 1	0	10.1	5.05	1	3.29
	Unknown 2	55.58	0	12.8	0.83	2.63
Plate 4	Blank	2.77	4.88	14.14	3.82	28.28
	Standard 1	1.32		5.83	1.43	15.71
	Standard 2	5.43	0	3.12	10.67	5.48
	Standard 3	0.35	8.84	14.09	0	3.43
	Standard 4	13.39	12.12	5.97	7.61	12.74
	Standard 5	18.79	8.47	2.24	11.62	1.9

	Standard 6	5.45	11.16	4.69	0.77	0.42
	Standard 7	6.14	4.3		0.75	1.59
	Control 1	15.32	7.86	3.69	17.04	10.01
	Control2	16.81	19.78	12.32	14.71	12.41
	unknown 1	4.88	9.43	14.43	0.54	16.97
	Unknown 2	57.82	20.2	29.56	7.24	27.95
Plate 5	Blank	6.53	0	0	12.86	3.63
	Standard 1	18.4		18.17	10.88	2.77
	Standard 2	16.15	6.15	10.83	5.77	14.73
	Standard 3	1.12	3.63	0.94	6.15	7.68
	Standard 4	12.52	0.76	8.66	3.36	7.14
	Standard 5	0.54	7.22	2.99	2.34	0.88
	Standard 6	6.3	4.5	1.93	3.4	3.76
	Standard 7	9.03	28.5	5.15	9.84	9.15
	Control 1	10.7	3.63	4.62	16.39	13.25
	Control2	1.67	4.9	1.75	8.2	8.19
	unknown 1	24.15	14.14	95.2	52.85	55.71
	Unknown 2	2.57	0	3.01	0	10.88
Plate 6	Blank	10.88	23.57	14.14	0	0
	Standard 1	11.47		3.01	4.04	6.73
	Standard 2	0.34	8.32	2.67	4.18	7.64
	Standard 3	22.12	11.79	25.95	31.38	11.17
	Standard 4	14.52	14.43	15.72	9.12	5.03
	Standard 5	21.82	2.14	6.17	15.55	5.16
	Standard 6	8.02	7.28	4.68	1.82	3.98
	Standard 7	10.62	14.14	8.48	17.22	6.41
	Control 1	16.85	6.96	5.37	0.87	3.9
	Control2	16.66	11.94	6.64	5.23	10.21
	Unknown 1	3.82	20.2	0	4.61	3.29
	Unknown 2	7.34	21.11	5.65	3.82	3.72

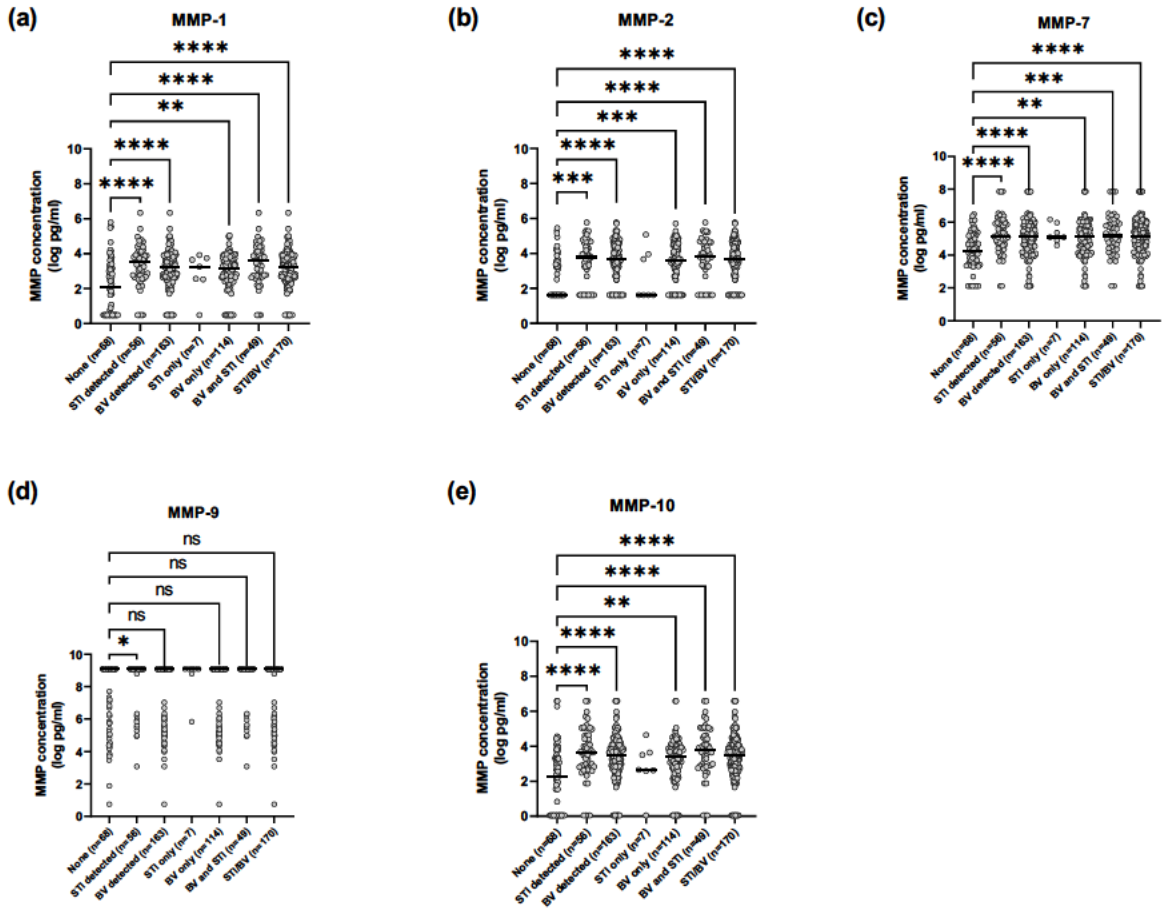
A % CV below 20 was acceptable.

Supplementary table 8: Summary of the recovery of the standards from the six 96-well plates.

		MMP-1	MMP-2	MMP-7	MMP-9	MMP-10
	Type	(Observed /Expected) 100				
Plate 1	Standard 1	99		101	98	98
	Standard 2	101	91	95	106	103
	Standard 3	103	108	115	100	101
	Standard 4	93	94	90	92	93
	Standard 5	111	108	113	116	112
	Standard 6	94	96	89	93	95
	Standard 7	103	101	131	102	101
Plate 2	Standard 1	100		101	100	94

	Standard 2	98	89	97	93	106
	Standard 3	105	106	102	117	104
	Standard 4	94	100	102	100	102
	Standard 5	104	109	94	89	90
	Standard 6	98	90	112	108	107
	Standard 7	101	106	93	73	86
Plate 3	Standard 1	96	121	99	99	101
	Standard 2	108	91	104	105	98
	Standard 3	96	100	95	95	102
	Standard 4	101	103	104	103	99
	Standard 5	99	98	100	100	100
	Standard 6	101	101	96	100	100
	Standard 7	100	100	104	100	100
Plate 4	Standard 1	92		97	84	95
	Standard 2	119	107	109	124	111
	Standard 3	89	97	92	89	93
	Standard 4	106	102	106	105	103
	Standard 5	102	98	98	104	101
	Standard 6	95	101	103	95	99
	Standard 7	104	100		103	101
Plate 5	Standard 1	103		101	105	106
	Standard 2	95	94	98	91	90
	Standard 3	107	107	103	113	111
	Standard 4	97	96	98	96	100
	Standard 5	99	95	100	100	95
	Standard 6	102	112	102	101	104
	Standard 7	99	97	97	100	99
Plate 6	Standard 1	97		98	92	94
	Standard 2	108	77	114	116	109
	Standard 3	98	114	89	95	100
	Standard 4	97	96	107	101	96
	Standard 5	101	97	101	96	99
	Standard 6	101	107	97	103	102
	Standard 7	100	98	104	100	100

A standard recovery between 70-130% was acceptable.



Supplementary figure 12: **Comparisons of genital MMP concentrations in the MC fluid of women with and without detectable STI or BV at baseline (N=238).** Median MMP concentrations were compared neither STI or BV (n=68; "None") and women with STIs detected (n=56), BV detected (n=163), STIs only (n=7), BV only (n=114), BV and STI (n=49) and STI/BV (n=170) at baseline. Kruskal-Wallis tests with Dunn's post-testing were used to compare the non-parametric data. Significant differences in median MMP concentrations were denoted by p-values < 0.05.

Supplementary table 9: Changes in the genital cytokine biomarkers of inflammation -6 and 12 post-treatment (n=60).

Cytokine	Median (IQR)			p-value
	Baseline	6 weeks	12 weeks	
b-NGF	1.89 (1.13-2.29)	1.66 (0.76-2.19)	1.42 (-1.57-2.07)	0.1149
CTACK	3.38 (0.85-3.72)	3.52 (3.20-3.69)	3.43 (2.92-3.65)	0.9446
Eotaxin	2.61 (-0.35-2.97)	2.18 (-0.35-2.85)	2.23 (-0.35-2.72)	0.3981
FGF basic	2.85 (-0.17-3.07)	2.78 (-0.17-3.02)	2.74 (-0.17-3.02)	0.9857
G-CSF	4.74 (4.18-5.12)	4.27 (3.88-4.66)	4.16 (3.41-4.58)	**0.0010**
GM-CSF	3.02 (2.51-3.26)	3.13 (2.36-3.30)	3.19 (2.99-3.29)	0.0590
GROa	4.65 (3.88-5.22)	4.61 (3.78-5.24)	4.43 (3.50-5.03)	0.9468
HGF	4.14 (3.61-4.76)	4.04 (3.47-4.49)	3.79 (3.27-4.39)	0.0732
IFN-a2	2.76 (1.72-2.99)	2.67 (2.09-3.02)	2.56 (-0.71-2.89)	0.2779
IFN-g	3.15 (2.86-3.45)	3.01 (2.79-3.21)	2.98 (2.66-3.29)	**0.0126**
IL-10	2.90 (2.50-3.11)	2.80 (2.56-3.11)	2.72 (2.35-3.00)	**0.0312**
IL-12p70	3.19 (2.72-3.49)	3.06 (2.64-3.39)	2.91 (2.37-3.32)	0.0609
IL-12p40	0.71 (0.71-4.05)	2.80 (0.71-4.08)	0.71 (0.71-3.73)	0.6276
IL-13	1.743 (1.32-1.89)	1.63 (1.26-1.96)	1.55 (0.87-1.90)	0.0605
IL-15	-0.56 (-0.56- -0.56)	-0.56 (-0.56-1.97)	-0.56 (-0.56- -0.56)	0.6096
IL-16	3.36 (0.16-4.09)	3.17 (0.16-3.95)	3.03 (0.16-3.64)	0.1763
IL-17	2.46 (1.77-2.77)	2.38 (1.88-2.84)	2.34 (2.08-2.72)	0.6207
IL-18	4.07 (3.51-4.55)	3.94 (3.39-4.48)	3.76 (3.25-4.55)	0.4493
IL-1a	4.15 (3.62-4.71)	3.85 (3.29-4.21)	3.65 (3.14-4.17)	**0.0163**
IL-1b	3.61 (3.22-4.14)	3.23 (2.90-3.87)	3.33 (2.76-3.73)	**0.0096**
IL-1ra	8.43 (6.14-8.43)	8.43 (6.06-8.43)	8.425 (6.06-8.43)	0.3095
IL-2	-2.30(-2.30- -2.30)	-2.30 (-2.30- -2.30)	-2.30 (-2.30- -2.30)	0.6412
IL-2Ra	2.85 (2.26-3.27)	2.69 (2.16-3.22)	2.59 (1.65-2.98)	**0.0386**

IL-3	3.69 (3.27-4.50)	3.62 (2.89-4.48)	3.55 (2.72-4.47)	0.3843
IL-4	1.59 (1.35-1.89)	1.46 (1.22-1.66)	1.46 (1.25-1.64)	0.1070
IL-5	1.52 (-0.74-2.06)	1.04 (-0.74-1.97)	1.48 (-0.74-1.83)	0.6115
IL-6	2.81 (2.28-3.23)	2.57 (2.16-2.98)	2.52 (1.99-3.01)	0.1165
IL-7	2.17 (1.87-2.37)	2.15 (1.68-2.39)	2.04 (1.67-2.28)	0.3156
IL-8	4.68 (4.06-5.11)	4.33 (4.03-4.89)	4.31 (3.81-4.76)	0.2194
IL-9	2.37 (2.07-2.54)	2.29 (2.11-2.48)	2.28 (1.88-2.49)	0.3060
IP-10	4.42 (3.39-5.03)	4.08 (3.35-4.89)	3.69 (2.77-4.57)	0.3539
LIF	3.09 (2.54-3.42)	2.89 (2.42-3.26)	2.65 (2.22-3.19)	**0.0328**
M-CSF	4.03 (3.66-4.29)	3.73 (3.45-4.03)	3.51 (3.13-4.02)	**0.0008**
MCP-1	3.13 (2.87-3.38)	3.05 (2.58-3.29)	2.96 (2.66-3.24)	0.1499
MCP-3	1.65 (-0.63-3.04)	2.18 (-0.63-2.97)	-0.63 (-0.63-2.76)	0.2237
MIF	4.86 (3.89-5.31)	4.85 (4.30-5.29)	4.81 (4.02-5.25)	0.4872
MIG	4.59 (3.99-5.21)	4.28 (3.75-5.03)	4.11 (3.42-4.72)	0.0541
MIP-1a	1.73 (1.31- 2.03)	1.61 (1.35-1.99)	1.63 (0.98-1.91)	0.5743
MIP-1b	2.35 (1.80-2.64)	2.39 (1.81-2.93)	2.17 (1.80-2.81)	0.7047
PDGF-bb	2.85 (2.47-3.13)	2.61 (2.27-2.99)	2.57 (1.86-3.05)	0.0713
RANTES	1.93 (-0.52-2.49)	1.33 (-0.52-2.37)	0.41 (-0.52-2.32)	0.1936
SCF	2.72 (-0.76-3.44)	2.36 (-0.76-3.32)	1.38 (-0.76-2.87)	0.1011
SCGF-b	2.07 (2.07-2.07)	2.07 (2.07-2.07)	2.07 (2.07-2.07)	0.5719
SDF-1a	3.96 (3.54-4.15)	3.92 (3.49-4.17)	3.88 (3.51-4.15)	0.9504
TNF-a	3.24 (2.89-3.47)	2.94 (2.75-3.29)	2.91 (2.68-3.16)	**0.0004**
TNF-b	2.33 (1.97-2.68)	2.17 (1.73-2.58)	2.12 (1.51-2.39)	**0.0228**
TRAIL	2.88 (0.85-3.29)	2.64 (-0.37-3.35)	2.38 (-0.37-3.18)	0.7671
VEGF	4.27 (3.94-4.65)	4.16 (3.59-4.53)	3.92 (3.39-4.43)	**0.0133**

IQR, median interquartile range; NGF-β, nerve growth factor; CTACK, cutaneous T-cell attraction chemokine; FGF, basic fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GRO-α, growth regulated oncogene; HGF, hepatocyte growth factor; IL, Interleukin; INF, interferon; interferon gamma induced protein, IP-10;

LIF, leukaemia inhibitory factor; MCP, monocyte chemotactic protein; M-CSF, macrophage colony-stimulating factor; MIF, macrophage migration inhibitory factor; MIG, monokine induced by gamma-Interferon; MIP, macrophage inflammatory protein; PDGF-ββ, platelet-derived growth factor; RANTES, regulated on activation normal T-cell expressed and presumably secreted; SCF, stem cell factor; SCGF-β, stem cell growth factor; SDF-1α, stromal-derived factor; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis inducing ligand; VEGF, vascular endothelial growth factor

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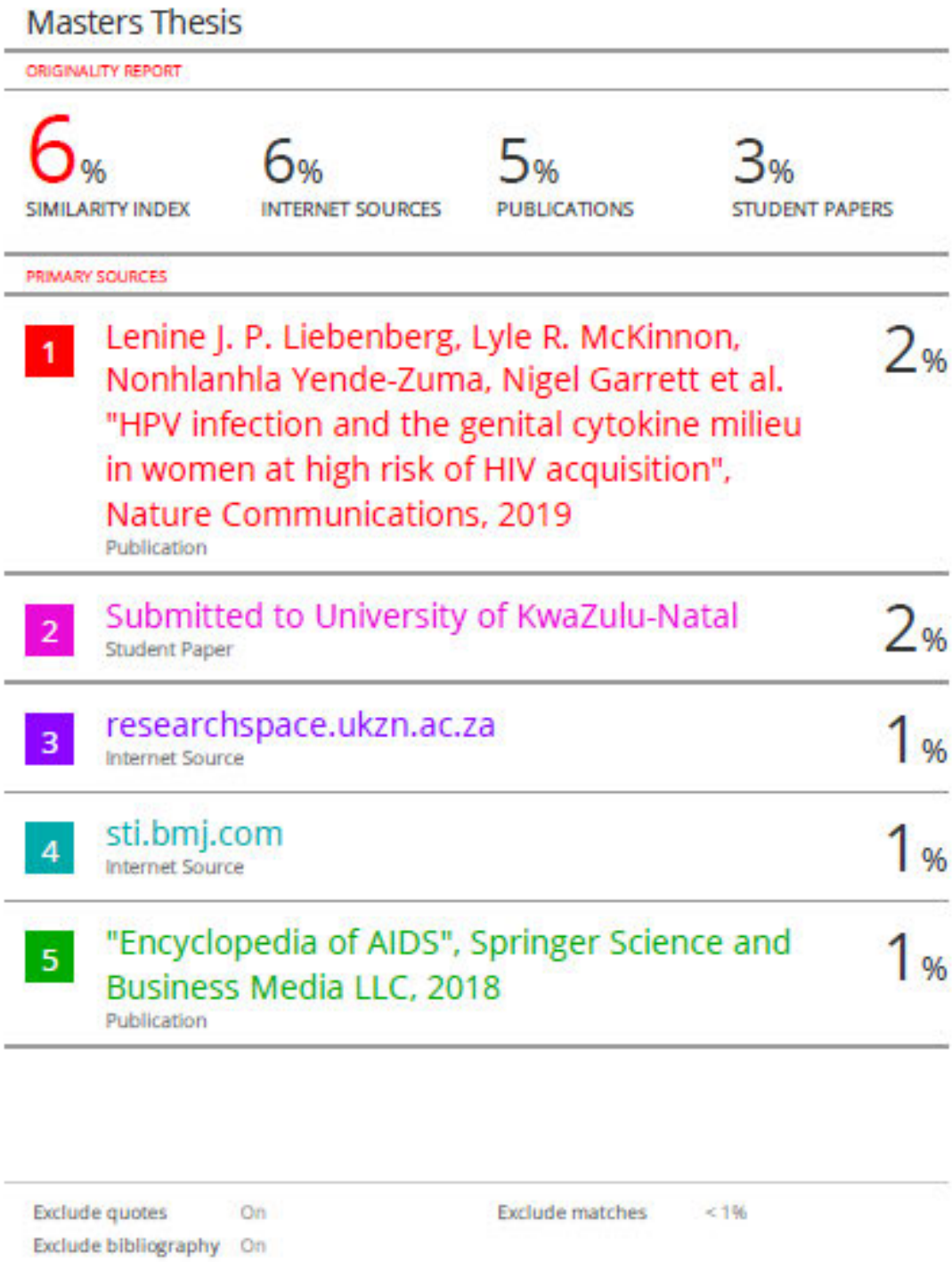

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