

The Relationship Between Vaginal Microbiota Composition, HPV Infection Status, and Associated Cytokine Profiles and Immune Cell Subsets

Presented by

Wenkosi Perez Qulu (209511420)

Submitted in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**

Principal Supervisor: Dr Sinaye Ngcapu

Co-supervisors: Dr Andile Mtshali and Dr Gugulethu Mzobe

School of Laboratory Medicine and Medical Sciences; Department of Medical Microbiology; Nelson R. Mandela School of Medicine, College of Health Sciences; University of KwaZulu-Natal; Durban, South Africa

2024

PREFACE

I, Wenkosi Qulu, declare that the experimental work in this dissertation is conducted solely by myself unless otherwise detailed by reference or acknowledged. The experimental work detailed in this thesis was conducted at the Center for the AIDS Program of Research in South Africa (CAPRISA) Laboratory, Doris Duke Medical Research Institute Building, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, South Africa, under the supervision of Dr Sinaye Ngcapu and Andile Mtshali.

This study embodies the novel work done by the author and has not been presented in any form to any other academic institution. In the event whereby others used the work, it is appropriately acknowledged in the text.

Signed: [redacted] Date: 11 August 2025

Wenkosi Perez Qulu (Candidate)

Signed: [redacted] Date: 11 August 2025

Sinaye Ngcapu (Supervisor)

Signed: [redacted] Date: 11 August 2025

Andile Mtshali (Co-Supervisor)

Signed: [redacted] Date: 11 August 2025

Gugulethu Mzobe (Co-Supervisor)

DECLARATION 1-Plagiarism

I, Wenkosi Qulu declare as follows:

The research described in this dissertation, except otherwise specified, is my original work.

This dissertation has not been presented for degree purposes or examination by any other academic institution.

This dissertation does not comprise other data, pictures, graphs, or information acquired from other researchers unless purposely acknowledged as being obtained from other researchers.

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Where I have repeated a publication in whereby I am an author, co-author, or editor, I specified in detail which portion of the journal was written by me only and had completely referenced the aforementioned publication.

This thesis does not contain texts, tables, or pictures copied and pasted from the internet unless exclusively acknowledged, and the information is detailed in the thesis and the reference section.

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DECLARATION 2 - Publications

The journals presented in this dissertation have originated from the CAPRISA 083 study, and the research was carried out as part of the DSI-NRF Centre of Excellence in HIV Prevention, which is sponsored by the Department of Science and Innovation and the National Research Foundation. The thesis comprises work accomplished by myself with research effort that covers the suggested requirement of three submitted articles in satisfaction of the degree of Doctor of Philosophy by research manuscript: One original, published first-authored article (Wenkosi et al., PLOS ONE), one first-authored article (Wenkosi et al., Multidisciplinary Publishing Institute), and an added coauthored manuscript in line with the theme of this thesis, in which I put in a significant amount of laboratory work, data analysis, and manuscript editing.

The following contributions were made to the publications of the following manuscripts

1. Wenkosi Qulu[1,2†], Andile Mtshali[1,2†], Farzana Osman[1], Nonsikelelo Ndlela[1,2], Lungelo Ntuli[1,2], Gugulethu Mzobe[1,2], Nivashnee Naicker[1], Nigel Garrett[1,3], Anne Rompalo[4], Adrian Mindel[1], Sinaye Ngcapu[1,2], *Lenine Liebenberg[1,2] **High-risk human papillomavirus prevalence among South African women diagnosed with STIs and BV.** PLOS ONE**,** *Published***. Contribution**: Together with Doctor Andile Mtshali, we carried out the laboratory experiment and wrote the manuscript, which has been carefully reviewed by all the co-authors. The article is featured in chapter 2 of the thesis.

2. Wenkosi Perez Qulu ,[2,†], Gugulethu Mzobe [1,2,†], Andile Mtshali [1,2], Marothi Peter Letsoalo 1, Farzana Osman [1], James Emmanuel San [3] , Asavela Olona Kama [1], Nigel Garrett [1,4], Adrian Mindel [1], Anne Rompalo [5], Lenine J. P. Liebenberg [1,2,6], Derseree Archary [1,2], Aida Sivro [1,2,7,8] and Sinaye Ngcapu [1,2,*] **Metronidazole Treatment Failure and Persistent BV Lead to Increased Frequencies of Activated T-and Dendritic-Cell Subsets,** Multidisciplinary Publishing Institute, *Published*. **Contribution**: This study was conceptualized by Dr Sinaye Ngcapu. Together with Mtshali, Drs Mzobe and Ngcapu, we conducted all laboratory experiments and analysed and wrote the manuscript, which has been published in the **Multidisciplinary Digital Publishing Institute** (**MDPI**) publishing journal. The article is featured in chapter 3 of the thesis.

PRESENTATIONS EMANATING FROM THIS THESIS

Oral Presentation

Annual Research Symposium of the School of Laboratory Medicine and Medical Sciences, K1-K2 rooms AHRI Building, 04 October 2023, University of KwaZulu-Natal, South Africa.

Wenkosi Qulu[1,2†], Andile Mtshali[1,2†], Farzana Osman[1], Nonsikelelo Ndlela[1,2], Lungelo Ntuli[1,2], Gugulethu Mzobe[1,2], Nivashnee Naicker[1], Nigel Garrett[1,3], Anne Rompalo[4], Adrian Mindel[1], Sinaye Ngcapu[1,2], *Lenine Liebenberg[1,2] **High-risk human papillomavirus prevalence among South African women diagnosed with STIs and BV.** School of Laboratory Medicine and Medical Sciences, 04 October 2023, University of KwaZulu-Natal, South Africa.

Poster Presentation

18th International Congress of Immunology (IUIS), 27 November–2 December 2023, Cape Town International Convention Centre, Cape Town, South Africa

Wenkosi Qulu[1,2†], Andile Mtshali[1,2†], Farzana Osman[1], Nonsikelelo Ndlela[1,2], Lungelo Ntuli[1,2], Gugulethu Mzobe[1,2], Nivashnee Naicker[1], Nigel Garrett[1,3], Anne Rompalo[4], Adrian Mindel[1], Sinaye Ngcapu[1,2], *Lenine Liebenberg[1,2] **High-risk human papillomavirus prevalence among South African women diagnosed with STIs and BV.** International Congress of Immunology Conference (IUIS), 27 November 2023, University of KwaZulu-Natal, South Africa.

DEDICATIONS

I would like to express my heartfelt gratitude to my dear family, whose constant support and encouragement have guided me throughout my academic journey. Your unwavering belief in me has been the driving force behind my perseverance and determination. I would like to extend my sincere thanks to my mentors for their invaluable guidance and wisdom. This thesis is a true testament to the collective efforts of all those who have believed in me and have contributed to my growth as both a scholar and an individual.

ACKNOWLEDGMENTS

I would like to thank my supervisor **Dr Sinaye Ngcapu** for his invaluable contribution, support, and inspiration in my pursuit of knowledge throughout the course of my PhD program.

Dr. Andile Mtshali and **Dr. Gugulethu Mzobe** for their continual support and availability to assist with research questions related to the CAP083 study cohort, during the course of my Ph.D. program

CAPRISA Mucosal laboratory colleagues, Laboratory staff, and fellows for their valuable support

CAPRISA study participants, and employees who without, this study would not have been possible

DST-NRF Center of Excellence in HIV Prevention, and KwaZulu-Natal College of Health Sciences for financial support

Poliomyelitis Research Foundation (PRF) (grant number: 19/108) for financial support

My family for their unwavering support, encouragement, and belief in me through my studying

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LIST OF ABBREVIATIONS

BV	bacterial vaginosis
CST	community state type
CTACK	cutaneous T-cell attraction chemokine
CT	*Chlamydia trachomatis*
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMMPRIN	extracellular matrix metalloproteinase inducer
bFGF	basic fibroblast growth factor
FGT	female genital tract
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GRO-α	growth-related oncogene-α
HGF	hepatocyte growth factor
HIV	human immunodeficiency virus
HPV	human papillomavirus
HSV	herpes simplex virus
IFN-α2	interferon alpha-2
IL	interleukin
IL-1RA	interleukin-1 receptor antagonist
IMI	intramuscular injection
IFN-γ	interferon gamma
IP-10	induced protein-10
LIF	leukemia 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
MgCl2	magnesium chloride
NFkβ	nuclear factor kappa beta
NG	Neisseria gonorrhoeae
NGF-β	nerve growth factor-beta
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
RANTES	regulated upon activation normal T cell expressed and presumably secreted
RPM	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
SCF	stem cell factor
SCGF-β	stem cell growth factor-beta
SDF-1α	stromal-derived factor-1alpha
SIV	simian immunodeficiency virus
STI	sexually transmitted infection
TBE	Tris borate EDTA
TLR-4	toll-like receptor-4
TNF-α	tumor necrosis factor-alpha
TRAIL	TNF-related apoptosis-inducing ligand

TV	*Trichomonas vaginalis*
UNAIDS	Joint United Nations Programme on HIV and AIDS
VEGF	vascular endothelial growth factor
WHO	World Health Organization

THESIS CONTEXT

Chapter 1 outlines the study's design, objectives, and methodologies in detail. This chapter also includes a literature review manuscript currently under preparation, which examines the immunoregulatory effects of HPV on mucosal immunity, inflammatory cytokine profiles, and the vaginal microbiome.

In **Chapter 2,** we explored the epidemiology of Human Papillomavirus (HPV) infection, including its interplay with sexually transmitted infections (STIs) and bacterial vaginosis (BV), and their impact on matrix metalloproteinases (MMPs) in South African women. In this chapter, we found that women with both STIs and BV had a higher prevalence of HPV compared to those without these conditions. BV was notably associated with an increased likelihood of detectable high-risk HPV infection. Additionally, women with STIs and/or BV were found to have a higher prevalence of HPV strains covered by the Gardasil9 vaccine.

In Chapter 3, we examined the interplay between high-risk HPV infection, mucosal immune responses, and vaginal microbiota in a group of 54 HIV-negative South African women who were also coinfected with STIs and/or BV and took part in the CAPRISA 083 study. It explores how HPV infection correlates with mucosal T cell expression and examines the link between specific high-risk HPV types and BV-related bacteria. The findings reveal that HPV infection is associated with lower frequencies of certain immune cells and altered levels of cytokines and chemokines. Women with HPV and *Lactobacillus*-dominant vaginal microbiota exhibited higher levels of some inflammatory markers, whereas those with non-*Lactobacillus* dominance had reduced levels of these markers and immune cell frequencies. These results highlight the impact of HPV on immune responses and the role of vaginal microbiota in influencing these effects.

In **Chapter 4,** we investigated the immune parameters associated with the response to Metronidazole treatment, specifically looking at changes in microbiota and immune cell frequencies. Post-treatment, a modest reduction in BV-associated bacteria was observed, although *lactobacillus* colonization remained limited. Significant increases in CCR5+ CD4+ T

cells and plasmacytoid dendritic cells (DCs) were noted at six and twelve weeks following treatment.

ABSTRACT

Background

Studying the interplay between vaginal microbiota, HPV, cytokines, and immune cell subsets is crucial for understanding female reproductive health and the risk of cervical cancer. A healthy vaginal microbiota, primarily composed of *Lactobacillus species*, helps maintain an acidic environment that inhibits pathogens and supports local immune function. Disruptions in this microbiota can influence HPV susceptibility and the immune response, as specific cytokines play key roles in inflammation and immune cell recruitment. Variations in cytokine profiles can influence the clearance or persistence of HPV infections, highlighting the importance of immune cell subsets, such as dendritic cells and T cells, in responding to the virus. The aims of this PhD dissertation were to investigate the relationship between vaginal microbiota composition, HPV infection status, and the associated cytokine profiles and immune cell subsets, by evaluating (i) the epidemiology of Human Papillomavirus (HPV) infection, including its interplay with STIs and BV, and their impact on matrix metalloproteinases (MMPs) in South African women (Chapter 2); (ii) the interplay between high-risk HPV infection, mucosal immune responses, and vaginal microbiota in HIV-negative South African women who were also coinfected with STIs and/or BV and took part in the CAPRISA 083 study (Chapter 3); and the immune parameters related to the response to Metronidazole treatment, specifically analyzing changes in microbiota composition and immune cell frequencies.

Methods

This study involved an assessment of 267 HIV-uninfected women with laboratory-confirmed STIs, candidiasis, and/or BV within the CAPRISA 083 cohort. The participants, aged 18-40, were recruited at the Prince Cyril Zulu Communicable Diseases Clinic in Durban, South Africa, and enrolled in the CAPRISA 083 study. HIV rapid testing and point-of-care STI screening using the GeneXpert (Cepheid) and standard PCR multiplex assays were conducted at each visit. Vaginal swabs were obtained for Gram staining to diagnose candidiasis and BV using Nugent's criteria, with scores of 0-3 considered BV negative, 4-6 intermediate, and 7-10 positive. HPV genotyping was performed using the Roche Linear Array on menstrual cup pellets. Screening for STIs and BV utilized the Xpert® CT/NG assay, while genomic DNA was extracted from vaginal swabs with the PowerSoil DNA Isolation Kit (MoBio, CA, USA).

Concentrations of five matrix metalloproteinases (MMPs) and 48 cytokines were measured in menstrual cup supernatants through multiplexed ELISA. Cervical T- and dendritic-cell subsets and monocytes were measured in endocervical cytobrushes samples using multiparameter flow cytometry, and the composition of vaginal microbial communities was evaluated using 16S rRNA gene sequencing. All statistical analyses were performed using R software version 4.3.1 (www.r-project.org). To compare characteristics between HPV-negative and HPV-positive groups, Fisher's exact test and Pearson's chi-squared tests were applied for categorical variables. For continuous variables, such as age and number of sexual partners, the Wilcoxon rank-sum test was utilized. Descriptive data for continuous variables were summarized using medians and interquartile ranges, while categorical data were presented as frequencies and percentages.

Results

As reported in chapter 2, HPV was detected in 34% of women (83 out of 243), with a median age of 23 years (interquartile range [IQR] 21-27 years). Among those infected, low-risk (lr) HPV (71%, 59/83) and high-risk (hr) HPV (54.2%, 45/83) were common. Hr-HPV was significantly more prevalent in women with STIs and/or BV compared to those without these conditions ($p = 0.029$). In multivariable analysis, BV was linked to higher odds of hr-HPV detection (odds ratio [OR]: 2.64, 95% confidence interval [CI]: 1.02-6.87, $p = 0.046$). Additionally, strains associated with the Gardasil®9 vaccine were more commonly found in women diagnosed with STIs and/or BV (55.2%, 32/58) compared to those without (24%, 6/25; $p = 0.009$). Among women with STIs and/or BV, HPV detection was significantly correlated with increased concentrations of MMP-10 ($b = 0.55$, 95% CI 0.79-1.01; $p = 0.022$). In the work reported in chapter 3, the presence of any HPV strains was inversely related to TRAIL, CTACK, MIG, and IFN-α2 concentrations, but this significance diminished for TRAIL and IFN-α2 after adjusting for age, *Lactobacillus* dominance, and STI presence. In addition, the multivariable analysis revealed that women with any HPV infection had significantly reduced frequencies of CCR5+ CD4+ T cells and HLADR+ CD14-CD123+ plasmacytoid dendritic cells. Oncogenic HPV specifically correlated with increased frequencies of HLADR+ CD14-CD123+ cells. In multivariate analyses adjusting for age and STIs, women with *Lactobacillus* dominance and any HPV infection showed higher levels of MIP-1β, RANTES, and IL-7, but lower CTACK levels compared to those without HPV. The *in vivo* experiments reported in Chapter 4 demonstrated that MDZ treatment modestly reduced BV-associated bacteria while

rare colonization by *Lactobacillus* species (excluding *L. iners*) was observed; additionally, by weeks 6 and 12, there was a significant increase in CCR5+ CD4+ T cells and plasmacytoid dendritic cells compared to pre-treatment levels. Moreover, MDZ non-responders exhibited significantly higher frequencies of activated CD4 T cells and monocytes than responders, underscoring the impact of MDZ treatment on immune cell profiles and microbial community dynamics.

Conclusion

In conclusion, this study highlights the complex interplay between HPV infection, BV, and immune responses in women. In Chapter 2, we observed a notable prevalence of HPV, particularly high-risk strains, in younger women, with significant associations found between hr-HPV and the presence of STIs or BV. The multivariable analysis further established BV as a significant risk factor for hr-HPV detection. Additionally, the presence of HPV was linked to increased MMP-10 concentrations among women with STIs and/or BV, indicating a potential inflammatory response. Findings from Chapter 3 reveal that HPV infections were associated with altered immune cell frequencies and cytokine profiles, suggesting that HPV may modulate local immune responses. Finally, Chapter 4's findings on MDZ treatment indicate its potential to influence microbial communities and immune cell dynamics, particularly in relation to BV. Collectively, these results underscore the need for continued research into the interactions between HPV, bacterial communities, and immune responses to inform better clinical management strategies. Collectively, the work reported in this thesis underscores the significant associations between HPV infection, bacterial vaginosis, and immune responses, highlighting the impact of these factors on cervical health and the potential implications for clinical management strategies.

CHAPTER 1

1.1 Introduction

Genital human papillomavirus (HPV), the most widespread sexually transmitted infection (STI) globally, is distinguished by its small, double-stranded DNA structure. HPV comprises over 230 fully characterized genotypes to date, with at least 40 of these infecting the mucosa of the genital tract [1-5]. Ongoing discoveries of new HPV variants continue. HPV genotypes are categorized based on their oncogenic potential, with low-risk types typically causing benign lesions, while high-risk types are associated with premalignant and malignant lesions. Anogenital warts are primarily caused by low-risk HPV (lrHPV) types 6 or 11, accounting for more than 90% of cases. In contrast, high-risk HPV (hrHPV) types (e.g., types 16 and 18) are detected in 99% of cervical precancers, highlighting their significant association with the development of cervical abnormalities [6, 7]. These hrHPV genotypes can infect cells on the cervix, leading to changes in the host DNA that can progress to cancer over time if left untreated.

Cervical cancer ranks as the fourth most common cancer in women worldwide, with approximately 660,000 new cases and approximately 350,000 deaths reported in 2022 [8]. The burden of HPV infection falls disproportionately on women of reproductive age (15–44 years) [9-11]. Several biological and behavioral factors have been associated with HPV natural history in women. These include early sexual debut, sexual practices, genital hygiene practices, host biological/immunological factors, host genetic factors, viral factors, concurrent STIs like HIV, and an elevated number of sexual partners over one's lifetime [12-19]. A considerable proportion (80%) of sexually active individuals contract genital HPV during their lifetime [20] with those experiencing persistent infection facing a notably heightened risk of developing cervical cancer [21-24]. Sexually transmitted HPV infections are typically transient, often asymptomatic, with more than 90% of detected HPVs naturally cleared within 1-2 years [25-28]. Multiple studies have suggested that host immune response, vaginal microbiome, and other factors within the female reproductive system may influence the clearance or persistence of HPV infection, thereby impacting the risk of developing cervical cancer [29].

HPV deploys various mechanisms to alter the host immune response, potentially creating a favorable environment for viral persistence and disease progression. Initially, the virus may evade

detection by the immune system, allowing it to establish infection in epithelial cells. As the infection progresses, HPV can interfere with the normal function of immune cells, such as Langerhans cells (LCs) and dendritic cells (DCs), which are crucial for initiating an immune response against viral infections [30]. Studies have reported increased levels of immunosuppressive cells such as transforming growth factor-beta (TGF-β)-producing Tregs and anti-inflammatory cytokine interleukin (IL)-10 in women with persistent HPV infection compared to those who cleared the infection. Cytokines such as IL-6, IL-10, and IL-17 have been linked to the persistence of HPV infection and create conditions favorable for the development of neoplastic lesions [31-34]. Furthermore, studies have demonstrated that E6 proteins of HPV-16 and HPV-18 can downregulate the transcription of type-1 interferons (IFNs) and the secretion of IFN Kappa [35, 36]. These HPV-driven interferences can impair innate and adaptive immune responses often linked to the cytotoxic activity of natural killer cells and CD8+ T cells, as well as CD4+ T cells, promoting conditions that suppress immune surveillance and facilitate viral persistence [37-39]. While studies have demonstrated that immune cells possess anti-viral activities and how HPV evades these mechanisms, there is less understanding of the cell-mediated mucosal immune response regarding HPV incidence, persistence, and clearance

Recent studies demonstrated that the genital microbiome may have a potential role in the infection and persistence of HPV, as well as susceptibility to HIV [40, 41]. Changes in the vaginal microbiome, such as decreased abundance of beneficial *Lactobacillus* species and increased diversity of anaerobic bacteria usually found in BV, have been associated with an increased risk of HPV infection and persistence [42, 43]. This dysbiosis may create an environment conducive to HPV acquisition and replication. Additionally, BV-linked bacterial species within the vaginal microbiome may directly interact with HPV, potentially influencing viral persistence and the development of cervical lesions [44-47]. Research evidence indicates that women harboring high-risk and multiple HPV types tend to exhibit significantly greater alpha diversity, characterized by elevated proportions of BV-associated bacteria such as *Sneathia, Prevotella,* and *Megasphaera*, compared to uninfected counterparts [48, 49]. Further research is needed to elucidate the complex interactions between HPV and the genital microbiome and to explore potential interventions that could mitigate the impact of dysbiosis on HPV infection and cervical cancer development.

While evidence suggests a relationship between the host immune responses, soluble markers, and HPV infection, the specific connection between the host responses and the progression to HPV-associated cervical cancer remains unclear. In addition, little is known about the relationship between vaginal microbiome and HPV infection, including the specific association in African populations regarding the persistence or clearance of HPV infections remains incompletely understood. To address these research gaps, this study explored the epidemiological factors of HPV, along with the prevalence of STIs and/or BV, and their respective influence on mucosal immunology and microbiome in South African women. The following literature review centers on prior studies that explore the connections between the genital microbiome and HPV infection.

1.1.1 The study aims and objectives

Aim 1: To investigate the epidemiology of HPV combined with STI/BV prevalence and the relative effects on matrix metalloproteinases among South African women

Rationale

Globally, the human papillomavirus (HPV) is the most prevalent sexually transmitted infection and a major contributor to cervical cancer [50]. Young women in South Africa under the age of 25 bear a disproportionate burden of HIV and HPV infections [51]. Concurrently, infections with STIs such as HIV, *Chlamydia trachomatis, Neisseria gonorrhoeae, and Trichomonas vaginalis* increase the risk of HPV infection, which can lead to chronic infections and precancerous lesions[52]. In addition, mucosal integrity is impacted by HPV's biological infiltration and modification of epithelial cells, resulting in loss of epithelial barrier integrity [53]. Matrix metalloproteinases (MMP) are biomarkers of epithelial barrier integrity essential for the breakdown of the extracellular matrix, including cell invasion, development, and modification of the tumour microenvironment [54]. Although the relationship between cervical cancer and MMP expression has been demonstrated, it remains unclear if HPV infection causes MMP expression in the female genital tract (FGT).

Hypothesis: We hypothesize that HPV, along with concurrent STIs and/or BV, weakens the epithelial barrier integrity in the genital mucosa through increased expression of matrix metalloproteinases.

Aim 2: To determine the immunomodulatory effect of HPV on the genital microbiome composition, including frequency of cervical T and DC subsets, and genital tract soluble immune mediators [including inflammatory cytokines, chemokines, growth factors, adaptive, and regulatory].

Rationale

HPV, genital immune response, and the vaginal microbiome are intricately linked and influence each other in significant ways. HPV, which infects the genital mucosa, can lead to a range of conditions from benign warts to more severe diseases such as cancer. The body's immune system is critical in controlling HPV infections; an effective immune response can lead to the clearance of the virus, while a compromised or dysfunctional immune response may result in persistent infection and progression to disease. Concurrently, the vaginal microbiome, a diverse community of microorganisms, plays a vital role in maintaining epithelial barrier integrity and supporting immune function [29, 44, 55]. A balanced vaginal microbiome helps protect against infections and supports the immune system, whereas dysbiosis, or microbial imbalance, can increase susceptibility to HPV infection and affect disease outcomes [56, 57]. Understanding how HPV interacts with genital immunity and the microbiome is crucial for developing more effective prevention and treatment strategies for HPV-related conditions.

Hypothesis: We hypothesize that host immune response and vaginal microbiome influence the clearance and persistence of HPV, as well as the risk of developing cervical cancer.

Aim 3: To determine the effects of MDZ treatment on the frequency of mucosal immune cell subsets in endocervical cytobrushes from women with a laboratory diagnosed STI and/or BV

Rationale

The diverse microbiota is reminiscent of bacterial communities found associated with the condition known as BV, which is also characterized by a lack of *Lactobacillus* species, can present clinically with vaginal discharge, a fish-like odor, vaginal discomfort, and urinary symptoms, and colonization with a diverse spectrum of primarily anaerobic bacteria. Antibiotic treatment of BV, the current standard of care, is often ineffective and recurrence rates following treatment are high [58]. Recurrence and identifying women with BV are key challenges, as a large proportion of cases are asymptomatic. BV has been associated with increased genital inflammation [59], and increased risk for HIV infection [60]. A recent study by Anahtar *et al.* (2015) demonstrated that high BV-associated bacterial-diversity enhanced stimulation of TLR4 and NFkB, leading to increased pro-inflammatory cytokines and a subsequent increase in activated CCR5+CD4+ T-cells in the female genital tract [59]. BV is associated with an increased risk of acquiring STIs, including *Trichomonas vaginalis*, herpes simplex virus type 2 (HSV-2), and *Neisseria gonorrhoeae* [61]. This is of particular concern as HPV related cancer and STIs (including HIV) are responsible for a large proportion of morbidity and mortality in South Africa [62].

Hypothesis: We hypothesized that BV treatment, along with an increase in the dominance of *Lactobacillus species*, would reduce the frequency and activation of the mucosal immune response. Additionally, we proposed that treatment failure or recurrent BV is associated with a more diverse vaginal microbiota, which in turn results in a higher number of activated HIV-targeted cells in the female genital tract.

1.2 LITERATURE REVIEW

1.2.1 Human Papillomavirus.

HPV is a prevalent sexually transmitted infection (STI) that affects both males and females. Approximately 70% to 90% of HPV infections are cleared by the immune system within two years [63]. However, persistent infections can lead to changes in cervical cells, transforming them into pre-cancerous cells that often present no symptoms. While early-stage pre-cancerous cells may return to normal on their own or with treatment, persistent pre-cancer cells can progress to cervical cancer [64]. Cervical cancer is the fourth most common cancer among women globally, with 570,000 new cases diagnosed in 2018 [65, 66]. In South Africa, there are approximately 5 743 new cases of cervical cancer each year, resulting in 3 027 deaths annually [67].

There are over 200 HPV genotypes [68], which are categorized into high-risk and low-risk types. High-risk types play a critical role in the development of various cancers [69]. Specifically, HPV types 16 and 18 are the most prevalent high-risk strains, responsible for approximately 70% of cervical cancer cases worldwide [70]. Additionally, HPV infections are linked to cancers of the vulva, penis, anus, and vagina. In contrast, low-risk HPV types typically cause warts on the skin and genitals, which can develop anywhere from a few weeks to several years after exposure [71].

1.2.2 HPV structure and genome organization

HPV has a circular, double-stranded DNA genome, which is non-enveloped and consists of about 7900 nucleotides. The HPV genome comprises roughly eight to ten open reading frames and is organized into three primary regions: the upstream regulatory region (URR), the early (E) gene region, and the late (L) gene region [72, 73]. The URR is a non-coding sequence located upstream of the early genes, including E6 and E7. This region houses various regulatory elements crucial for controlling the transcription of these early genes, which are essential for viral replication and disease progression [74].

The early gene region encodes six regulatory proteins: E1, E2, E4, E5, E6, and E7. Each of these proteins plays a distinct role at different stages of the HPV life cycle [75, 76]. The E1 protein functions as a multifunctional helicase that is critical for viral DNA replication. It binds to the viral

origin of replication (ORI), unwinding the DNA double helix to initiate replication. In contrast, E2 encodes a transcriptional regulator that interacts with specific DNA sequences within the viral genome, influencing the expression of viral genes. Its role as a transcriptional activator or repressor depends on interactions with various cellular factors [77, 78]. The E4 protein is involved in the later stages of the viral life cycle, aiding in viral assembly and release by disrupting the host cell's cytoskeleton and promoting genome amplification [79]. E5 functions as an early oncoprotein that contributes to tumor progression. E6 and E7 cooperate to create an environment conducive to viral replication; E6 binds to the tumor suppressor protein p53, directing it for degradation, while E7 interferes with retinoblastoma protein (pRb) function, further promoting cell cycle progression and viral replication [80, 81].

The late gene region encodes the capsid proteins L1 and L2. L1 is the major capsid protein responsible for forming the viral capsid, which encases the viral genome. L1 can self-assemble into viral-like particles (VLPs) that closely mimic infectious virions but lack viral DNA. L2, a minor capsid protein located beneath L1, is crucial for several stages of the virus's life cycle, including entry into host cells, intracellular trafficking, and uncoating of the viral genome (**Figure 1.1**) [82].

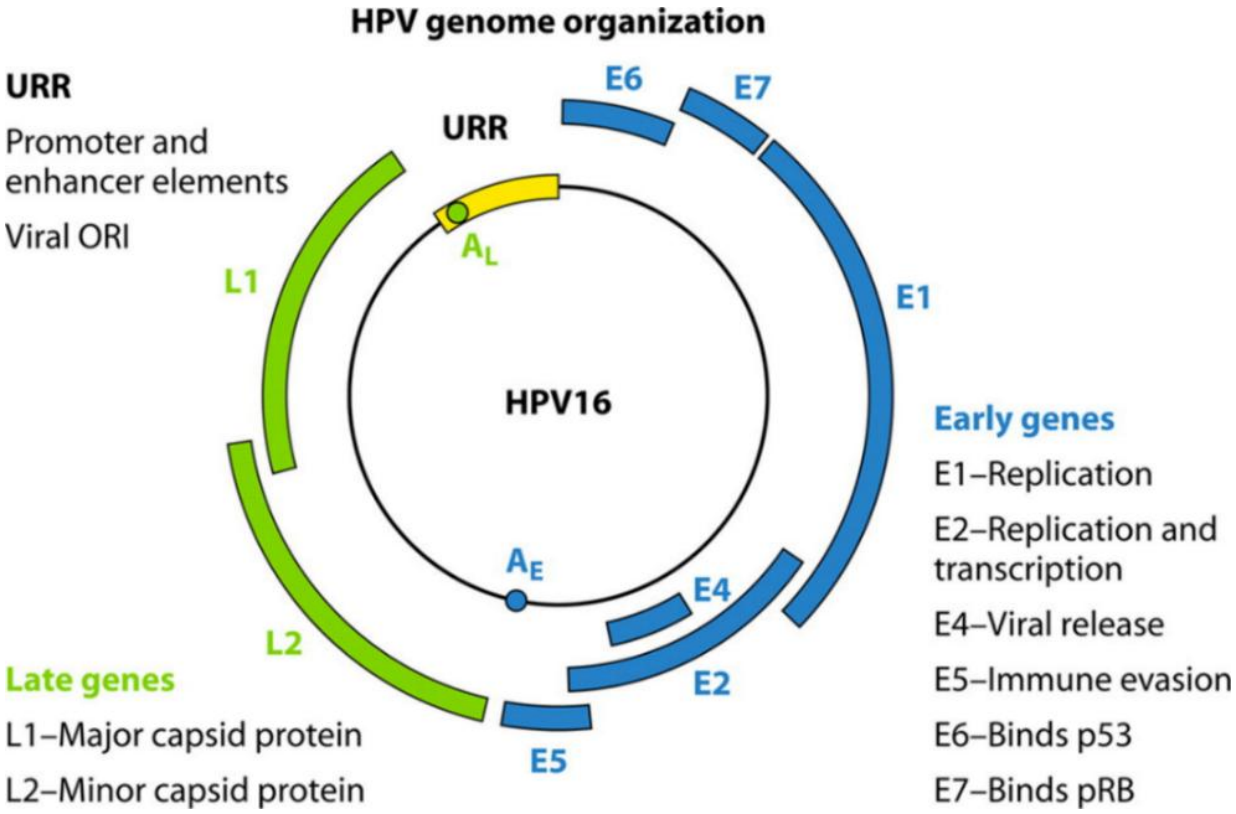


Figure 1.1: The genomic organization of mucosal high-risk HPV is illustrated in the adapted figure from Stanley *et al*., 2012. Key components include **E1 and E2 genes** that are crucial for viral replication and transcription. E1 encodes a helicase that facilitates DNA replication, while E2 encodes proteins that regulate transcription, initiate DNA replication, and partition the viral genome. **E6 and E7 genes** that influence cellular proteins that control the cell cycle. E6 binds to the p53 tumor suppressor protein, leading to its accelerated degradation via ubiquitination. E7 interacts with retinoblastoma protein (pRb), promoting cell cycle progression. **E4 Gene** regulates viral genome amplification, virus synthesis, and release, and is involved in the virus's transmission while the URR is critical for controlling early gene transcription and replication. It contains promoter and enhancer elements as well as the viral origin of replication [83].

1.2.3 Human Papillomavirus classification

HPV is a non-enveloped, double-stranded DNA virus belonging to the Papillomaviridae family [72, 84]. The particle structure of the HPV virus an icosahedral particle structure, measures approximately 50-60 nm in diameter and contains around 8000 base pairs [72, 84]. HPV

classification primarily relies on genetic sequences, especially the L1 gene that encodes the major capsid protein. Based on nucleotide sequence variations, HPV is categorized into five genera: **α-**papillomavirus**,** β-papillomavirus**,** γ-papillomavirus**,** μ-papillomavirus, and ν-papillomavirus. Mucosal and genital HPV types fall under the α-papillomavirus genus [85]. To date, over 200 genital HPV genotypes have been characterized, with classifications as either low-risk or high-risk. Low-risk HPV genotypes, such as types 6, 11, 40, 42, 43, 44, 54, 61, 70, and 81, are primarily associated with benign conditions like genital warts and mild cervical lesions [86-88]. In contrast, high-risk HPV (HrHPV) genotypes are linked to more severe conditions such as cervical intraepithelial neoplasia (CIN) and cervical cancer. Fifteen HrHPV types are recognized for their strong correlation with invasive cervical cancer and high-grade lesions. Notably, the International Agency for Research on Cancer (IARC), part of the World Health Organization (WHO), has classified several of these HrHPV types as Group 1 carcinogens. These include HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 [87].

1.2.4 Global Prevalence of HPV

HPV infection is firmly established as a leading cause of cervical cancer. Increasing evidence also highlights HPV as a significant factor in various other anogenital cancers, including those of the anus, vulva, vagina, and penis, as well as in head and neck cancers [89-91]. The prevalence of HPV varies greatly among regions of the world, higher rates have been reported in Sub-Saharan Africa which has the highest rates of HPV infection (24%), followed by Eastern Europe (21%), Latin America (16%), and the lowest in Western Asia (1.7%) [92]. Globally, the incidence rate of cervical cancer attributed to HPV is 13.1 per 100,000, with a mortality rate of 6.9 per 100,000. In Africa, the incidence rate is significantly higher, at 27.6 per 100,000, with a mortality rate of 20 per 100,000, making it the highest among all continents. Asia follows, with an incidence rate of 11.9 per 100,000 and a mortality rate of 6.2 per 100,000. [93].

Globally, HPV prevalence is notably higher in women than in men, with peak incidence observed primarily in women aged 25 years or younger. This age group has a global prevalence rate of 19.2%, which can rise to 43.9% in Africa [94, 95]. In KwaZulu-Natal, South Africa, the prevalence of HPV was found to be 76.3%. Among these women, 54.5% had high-risk HPV genotypes. This

indicates a significant proportion of women in the region are affected by high-risk strains of HPV [71]. In Gauteng province, South Africa, high HPV prevalence was reported to be 85% among women under 25 years of age. In comparison, the Western Cape exhibited an estimated HPV prevalence ranging from 44% to 71% in the same age group [10]. In 2018, the incidence rate of cervical cancer cases attributable to HPV in South Africa was estimated to be 43.5 per 100,000 population. The rate of anogenital cancers other than cervical cancer, including vulvar, vaginal, anal, and penile cancers, was estimated to be 1.7% in South Africa [94].

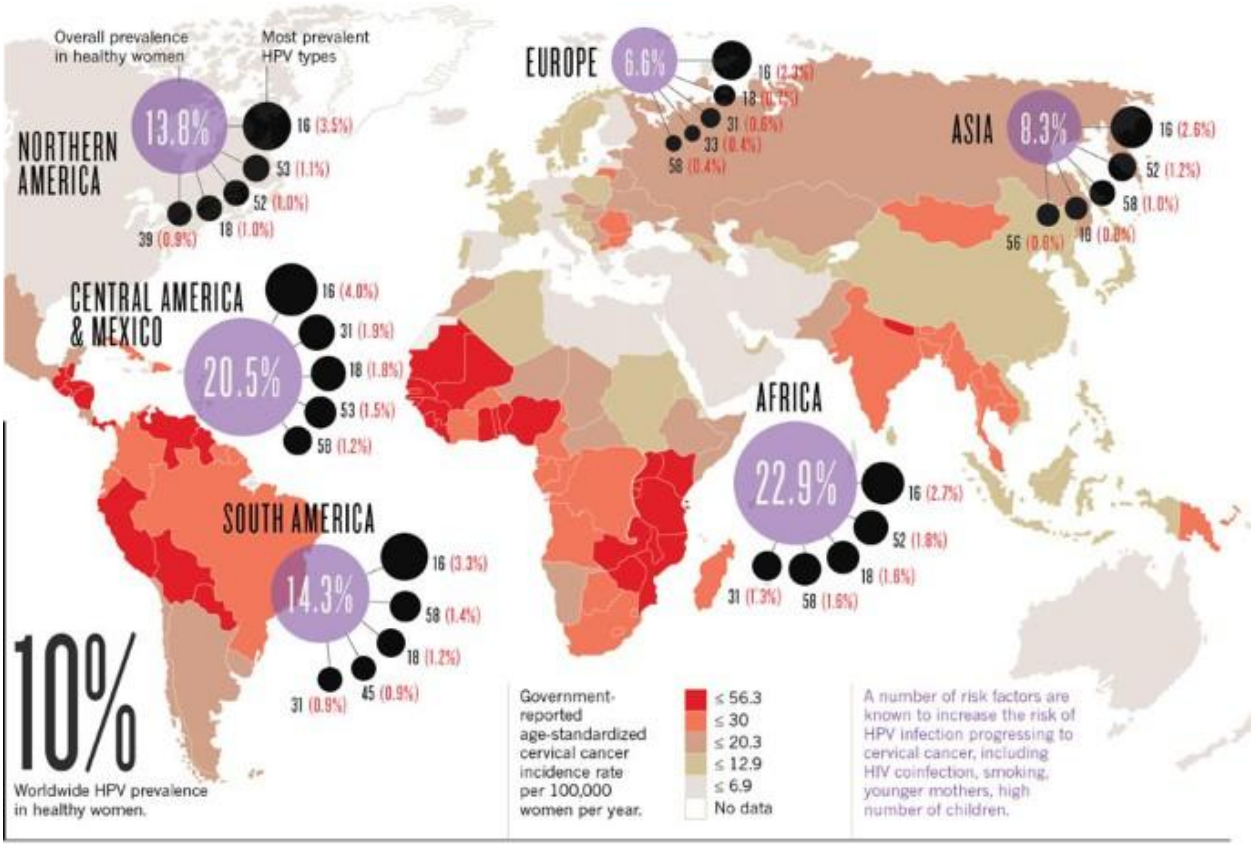


Figure 1.2: Global HPV prevalence. Sub-Saharan Africa had the highest global HPV prevalence in 2007. Figure adapted from De Sanjosé, et al. (2007) [96].

1.2.5 HPV genotype distribution patterns in Africa

Research across Southern Africa has revealed significant variation in the HPV genotypes responsible for cervical cancer. In South Africa and Tanzania, the predominant genotypes are HPV

16 and HPV 52 [97]. In Zimbabwe, HPV 16 and HPV 35 are most common [98], while in Madagascar, HPV 53 and HPV 68 are prevalent[99]. East African studies also show differing distributions of HPV genotypes. In Uganda, the leading high-risk HPV types are HPV 52 and HPV 58 [100], whereas in Tanzania, HPV 52 and HPV 16 are most common [101]. Kenya shows a prevalence of HPV 58 and HPV 16 [102], and in Ethiopia, HPV 16 and HPV 52 are predominant [103]. Research in West Africa highlights a different distribution of HPV genotypes compared to Southern and Eastern Africa [103]. In Senegal, Burkina Faso, and Nigeria, the prevalent genotypes associated with cervical cancer include HPV 52 and HPV 31, HPV 52 and HPV 59, and HPV 35 and HPV 16 [104, 105]. A study in Ethiopia identified the most common genotypes as HPV 16, 52, 58, and 45. Another study in Kenya found HPV 52, 51, 16, 35, and 18 to be the most prevalent during the first month of pregnancy [106]. Conversely, in Southern Africa, the distribution of high-risk HPV genotypes, including HPV 16, 52, 18, 56, and 58, differs from East Africa, with HPV 58 being the most prevalent, followed by HPV 52, 45, 16, and 18 [107]. In Mozambique, HPV 52 is the most common, with HPV 35 and HPV 16 following, contrasting with the genotype distribution in East Africa [108].

Given the diverse HPV genotype distribution across Africa, targeted public health strategies are crucial. HPV vaccination programs should be tailored to the most prevalent high-risk HPV genotypes in each country. Additionally, improving screening and diagnostic methods, especially in regions with limited healthcare infrastructure, is essential for the effective control and prevention of HPV-related diseases. Enhanced collaboration among African nations can facilitate the development and implementation of integrated strategies to address the HPV burden more effectively.

1.2.6 HPV diagnosis, management, and prevention strategies

1.2.6.1 HPV Diagnosis

Traditional viral diagnostic methods, like electron microscopy, cell culture, and some immunological techniques, aren't effective for detecting HPV. This is because HPV cannot be cultured in cell lines. HPV screening utilizes a range of methods including biopsy, colposcopy,

acetic acid tests, Pap smears, and nucleic acid-based tests [109]. Among these, the Pap smear is the primary tool for detecting precancerous cells in the cervix. Any abnormalities found in a Pap smear are further evaluated through colposcopy, biopsy, and molecular tests [109]. Colposcopy involves examining the cervix, vagina, and occasionally the vulva after applying an acetic acid solution. During this procedure, biopsies are taken from any lesions suspected of being neoplastic based on the colposcopic examination. The findings from a colposcopy are assessed based on the degree of acetowhitening, surface contour, mosaic pattern, and punctuation of the lesions. The severity of these abnormalities typically correlates with the severity of the lesions [110, 111]. The FDA has approved the Hybrid Capture HPV DNA Test 2 for use alongside the Pap test to detect as little as 1 picogram of HPV DNA per milliliter. This test offers sensitivity and specificity comparable to PCR-based methods. One of its main advantages is its relatively straightforward handling and high reproducibility, making it one of the most standardized HPV detection methods available. Although the test does not identify specific HPV types, it can detect groups of HPV genotypes, including “low-risk” types (6, 11, 42, 43, 44) and “high-risk” types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) [112].

1.2.6.2 HPV management and prevention

To prevent HPV-related diseases, prophylactic vaccines are employed to avoid initial HPV infection and subsequent lesions [113]. These vaccines are designed to prevent infections from the most common cancer-causing HPV types by stimulating the immune system to produce protective antibodies. Currently, there are six licensed HPV vaccines: three bivalent, two quadrivalent, and one nonavalent. Notable vaccines include Cervarix, which targets high-risk HPV types 16 and 18 [86]; Gardasil 4, which protects against HPV types 6, 11, 16, and 18 [114]; and Gardasil 9, which offers broader protection against additional HPV types 31, 33, 35, 45, 52, and 58 [115, 116]. Despite the availability and promotion of these vaccines, cervical cancer rates remain high in many parts of the world (**Table 1.1**) [117].

Table 1.1: FDA-approved vaccines

Vaccine type	Cervarix	Gardasil4	Gardasil9
Gender and age range	Females, 9-25 years	Males and Females, 9-26 years	Males and Females 9-26 years
Coverage (HPV types)	HPV 16 and 18	HPV 6, 11 (genital warts), 16 and 18	HPV 6, 11 (genital warts), 16, 18, 31, 33, 45, 52, and 58
Dose	2 doses: 9 – 14 years 3 doses: >15 years	2 doses: 9 – 13 years 3 doses: >14 years	2 doses: 9 – 14 years 3 doses: >15 years
Vaccination scheme	2 doses: 0, 5 months 3 doses: 0, 1, 6 months	2 doses: 0, 6 months 3 doses: 0, 2, 6 months	2 doses: 0, 6-12 months 3 doses: 0, 2, 6 months

While most HPV infections are cleared by the immune system on their own, persistent infections may require treatment. HPV-related diseases are traditionally managed through ablative or excisional methods to remove infected cells [3]. For genital warts, chemical treatments include trichloroacetic acid, podophyllin compounds, and imiquimod [118, 119]. Trichloroacetic acid is a caustic agent applied to lesions, podophyllin inhibits cell division, and imiquimod stimulates the production of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) [120]. Cryotherapy, which uses liquid nitrogen or a cryoprobe to freeze and destroy lesions, is effective but the cryoprobe is not suitable for vaginal use due to the risk of fistula formation [118, 121]. For more extensive HPV lesions, electrocautery and laser therapy can be employed, and surgical removal with a scalpel may be necessary to excise large lesions [118].

1.2.7 High burden of HPV infection and risk factors among women

In general, women are biologically more susceptible to HPV infection compared to men, particularly during sexual intercourse. HPV prevalence has been reported to be five times higher

among young women compared to their male peers. HPV prevalence typically peaks in younger women around the time of sexual debut and then declines in their late 20s and 30s [94, 122]. Ongoing research indicates that HPV prevalence rates differ based on geographical region, age group, sexual behavior, and biological factors [123].

1.2.7.1 Behavioral factors linked to HPV risk

Although the risk of HPV infection can vary among individuals, several well-documented behavioral factors contribute to increased susceptibility. These include the number of sexual partners, long-term use of combined oral contraceptives, smoking and exposure to environmental tobacco smoke, early sexual debut, unprotected sexual intercourse, and the number of pregnancies. [123-126]. Additionally, disparities in HPV infection rates and related diseases exist among different populations, influenced by factors such as socio-economic status, access to healthcare, education, and cultural beliefs [127]. Individuals from lower socio-economic backgrounds often experience higher rates of HPV infections and related illnesses [128]. Limited access to healthcare facilities, insufficient knowledge about HPV and prevention measures, and financial barriers to vaccination and screening further contribute to these disparities in HPV-related outcomes [129, 130]. Population density, increased mobility, and variations in sexual behavior may all contribute to higher HPV prevalence in urban areas compared to rural areas [126].

1.2.7.2 Biological risk factors for HPV infection

Women are biologically more susceptible to HPV infection due to several anatomical and physiological factors. The vaginal mucosa, with its large area, is more exposed to sexual secretions compared to the primarily skin-covered penis. Additionally, the volume of potentially infectious male ejaculate deposited in a woman's vaginal cavity during intercourse exceeds the amount of potentially infected cervical and vaginal fluids that males encounter [131]. STIs are other biological risk factors that may predispose women to become infected with HPV. Numerous studies have identified a connection between STIs and HPV infection, noting that various STIs often co-occur with HPV in women. For instance, *Trichomonas vaginalis* has been positively correlated with HPV presence, suggesting that this infection increases the risk of HPV in women

[132]. It is proposed that nitrosamines produced by *Trichomonas vaginalis* may induce cervical inflammation, creating conditions that facilitate HPV penetration into the basal layer of the cervical epithelium [133]. Additionally, HPV incidence has been significantly higher among women with co-infection by *Chlamydia trachomatis* compared to those without, though it remains unclear whether this association varies among different HPV genotypes [134]. Moreover, individuals who are immunocompromised, such as those living with HIV/AIDS or undergoing immunosuppressive therapy, may be at a higher risk of contracting HPV and developing related disorders [135].

1.2.8 The Role of HPV in Cancer Carcinogenesis

The HPV viral life cycle is relatively prolonged. Under optimal conditions, it takes about three weeks from initial infection to the release of new viral particles [83]. However, the time from host infection to the appearance of visible viral lesions can range from weeks to months. This variability indicates that HPV can sometimes evade the host's immune responses effectively [83]. HPV enters epithelial tissues through microabrasions and its lifecycle is closely tied to the differentiation of keratinocytes. As keratinocytes mature into the spinous layer, viral gene expression and replication are amplified. Although HPV gene amplification has only been observed in keratinocytes [136], the specific receptor through which HPV interacts with basal keratinocytes remains unidentified [137]. Nonetheless, heparin sulfate proteoglycans, α6-integrin, and laminin-5 are known to play crucial roles in this interaction. HPV enters cells via endocytosis, penetrates the nucleus, and begins replication [84, 138]. Following the infection of basal cells, including stem cells that divide rapidly, viral gene expression is activated, leading to the production of up to 100 extrachromosomal copies of HPV DNA per cell. During this phase, the E1 and E2 proteins form a complex that binds to the replication origin, recruiting cellular polymerases and other proteins essential for DNA replication. For high-risk HPV (Hr-HPV), the expression of the E6 and E7 oncogenes is tightly regulated, with E6 and E7 transcripts being minimally detectable in the epithelium's proliferative compartment [83]. As infected basal keratinocytes move into the differentiating stratum spinosum, viral gene expression increases and the number of viral copies per cell rises into the thousands. During this phase, there is heightened expression of E6 and E7 early and late genes driven by the late promoter. Viral assembly occurs in the upper layers of the

squamous epithelium, where virions are released and can infect adjacent tissues (**Figure 1.3**) [137-140].

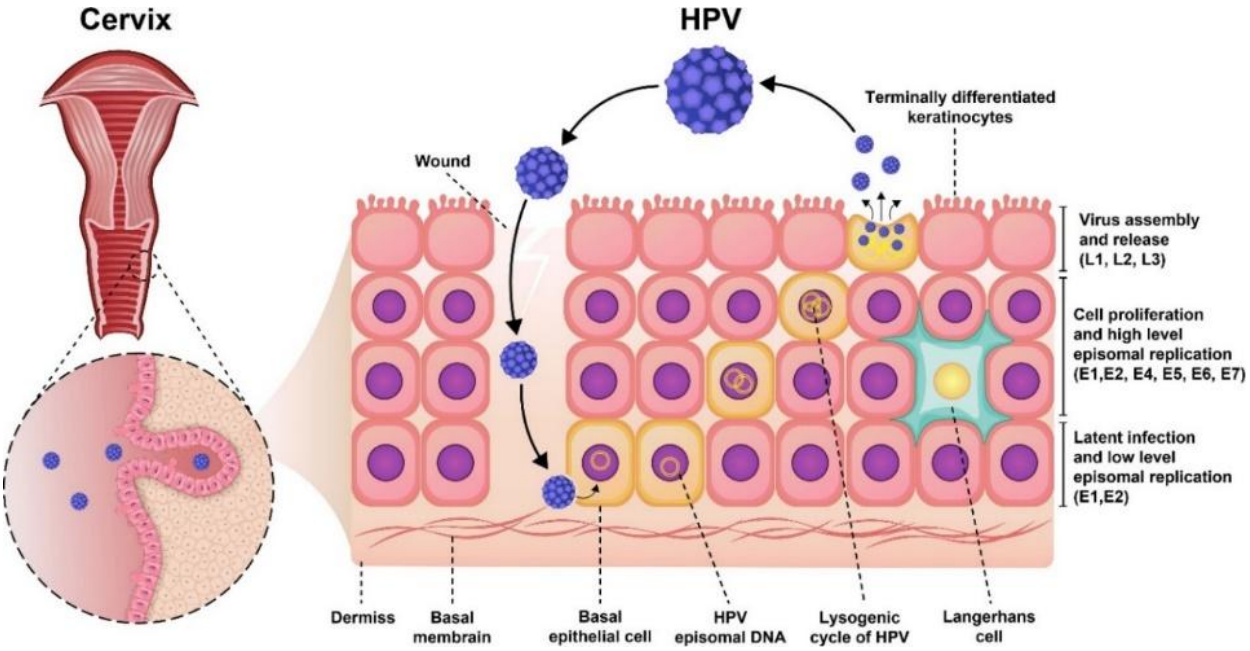


Figure 1.3: The HPV life cycle starts with the virus in a dormant state within epithelial cells, showing minimal proliferation. As it transitions to the lysogenic cycle, the proliferation rate increases. Eventually, virions are assembled and released from keratinocytes, initiating a new infection cycle (Adapted from Yousefi, 2022) [141].

1.2.9 HPV immune evasion strategies

The HPV life cycle is intricately linked with the differentiation of keratinocytes. Unlike many viruses, HPV does not produce secreted proteins, and its viral proteins are expressed in minimal amounts during infection. Additionally, HPV does not induce inflammation, viraemia, or host cell death [142]. By leveraging the keratinocyte differentiation process, HPV can effectively evade the host's immune responses [143]. Consequently, HPV infection does not lead to cell lysis or cytopathic death. Key stages of the virus's life cycle occur within terminally differentiated keratinocytes, which are destined for death and detachment in areas away from immune activation [143, 144]. As a result, HPV infections generally do not cause cell death or inflammatory

responses, leading to minimal production of proinflammatory cytokines, which are crucial for recruiting antigen-presenting cells (APCs) [143, 144]. Being an exclusively intraepithelial virus, HPV does not enter the bloodstream, resulting in only a small number of viral particles being exposed to the host's immune system. Consequently, individuals who are naturally exposed to HPV often show weak humoral immunity against the virus. This is evident in patients with cervical intraepithelial neoplasia, who display impaired T cell responses against HPV proteins E2, E6, and E7 compared to individuals with regressed lesions [142].

1.2.10 Evasion of host innate immunity in HPV infection

HPV employs several strategies to evade both innate and adaptive immune responses [83, 144, 145]. The innate immune system, which acts as the first line of defense, includes key players such as neutrophils, monocytes, macrophages, eosinophils, mast cells, and other cells that recognize and respond to pathogens through receptors like toll-like receptors, NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) [146]. High-risk HPV (Hr-HPV) has been shown to impair the expression of CCL20, a chemokine produced by keratinocytes, which diminishes the ability of Langerhans cells (LCs) to mount a cytotoxic immune response and reduces LC infiltration [30]. HPV infection also affects the differentiation of monocytes into mature dendritic cells (DCs), leading to altered DC function [147]. Natural killer (NK) cells, which target virus-infected and tumor cells that lack major histocompatibility complex (MHC) class I molecules, also play a crucial role in immune surveillance [144]. Studies have found low levels of NK cell receptors and reduced cytotoxic activity in cervical cancer and precursor lesions, indicating a diminished capacity to eliminate infected cells [148]. Although cells infected with HPV release type 1 interferons (IFNs), including IFN-α and IFN-β, which have antiviral, antiproliferative, and antiangiogenic effects and activate NK cells [144, 149], the E6 and E7 proteins of HPV downregulate the transcription of type-1 IFNs and inhibit their nuclear translocation. Furthermore, HPV-infected cells exhibit low levels of IFN-inducible genes compared to normal keratinocytes, highlighting a mechanism of immune tolerance [144]. Macrophages, activated by cytokines, are critical for the destruction of HPV-infected cells through mechanisms such as TNF-α secretion and antibody-dependent cytotoxicity [144, 150]. Chemokines like monocyte chemotactic protein (MCP-1) and macrophage inflammatory protein 3-α (MIP-3α) help recruit macrophages to the

infection site. However, HPV 16 E6 and E7 proteins downregulate these cytokines, inhibiting the recruitment of macrophages to the site of infection [151, 152].

1.2.11 Evasion of host adaptive immunity in HPV infection

Adaptive immunity involves antigen-specific T cell-mediated responses designed to eliminate viral, bacterial, or parasitic infections and malignant cells [153]. In this process, major histocompatibility complex (MHC) class I molecules present viral peptides on the cell surface to CD8+ T cells. However, HPV 16 E5 and E7 proteins have been shown to reduce MHC class I expression, enabling the virus to evade cytotoxic T lymphocyte (CTL) attacks [144]. Additionally, research has linked changes in leukocyte numbers and functions, including impaired CD8+ cytotoxic lymphocytes, to the progression of neoplastic lesions [154]. In women with persistent HPV infections, increased levels of the anti-inflammatory cytokine IL-10 have been observed compared to those who have cleared the infection. Similarly, high levels of immunosuppressive cells, such as regulatory T cells (Tregs) producing transforming growth factor-beta (TGF-β), are found in cervical tissues and vulvar intraepithelial neoplasia (VIN) lesions. The elevated presence of IL-10 and TGF-β may hinder T cell function by limiting the ability of antigen-presenting cells (APCs) to support the differentiation and proliferation of CD4+ T cells, thereby modulating the adaptive immune response [144]. Additionally, the reduction of MHC class I expression and impairment in APC trafficking and maturation further inhibit CD4+ and CD8+ T cell functions [155-157].

1.2.12 Cytokine and cellular immune response against HPV

Genital inflammation is the body's natural immune response to injury or infection [158]. This inflammatory process involves an increase in immune cells and proinflammatory cytokines within the genital tract [159]. Research indicates that cancerous cells can release various cytokines and chemokines, which attract a range of leukocytes to the site of infection, including lymphocytes, neutrophils, macrophages, dendritic cells, and mast cells. Tumor-associated macrophages (TAMs) are particularly crucial in this process, as they help in the destruction of virus-infected cells when activated by IL-2, IL-12, and interferons [160, 161]. In the context of HPV, TAMs produce

cytokines such as IL-10 and transforming growth factor beta (TGF-β), as well as inflammatory mediators like prostaglandin E2 (PGE2) and matrix metalloproteinase-7 (MMP-7). These substances can disrupt antigen presentation, reducing the effectiveness of T cells in recognizing and responding to antigens [162]. Laniewski *et al* 2018 [46], reported elevated levels of proinflammatory and chemotactic cytokines in invasive cervical carcinoma (ICC), including IL-36, TNF-α, RANTES (regulated upon activation, normal T cell expressed and presumably secreted), MIP-1α, MIP-1β, and IP-10 (interferon gamma-induced protein-10). Additionally, hematopoietic cytokines such as Fms-like tyrosine kinase-3 ligand (Flt-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF), along with adaptive immunity-related cytokines like IL-2, IL-4, and CD40 ligand (CD40L), were also significantly expressed in ICC [46]. Liebenberg *et al*. (2018) found that high levels of mucosal cytokines in the reproductive tract of HPV-infected women were linked to both increased HPV prevalence and a higher risk of acquiring HIV [55]. Another study revealed that women with high concentrations of mucosal cytokines, including IL-10, IL-12, MIP-1α, and TNF-α, were less likely to clear HPV infections. Conversely, lower levels of certain chemokines, such as IL-8, were associated with successful HPV clearance [163].

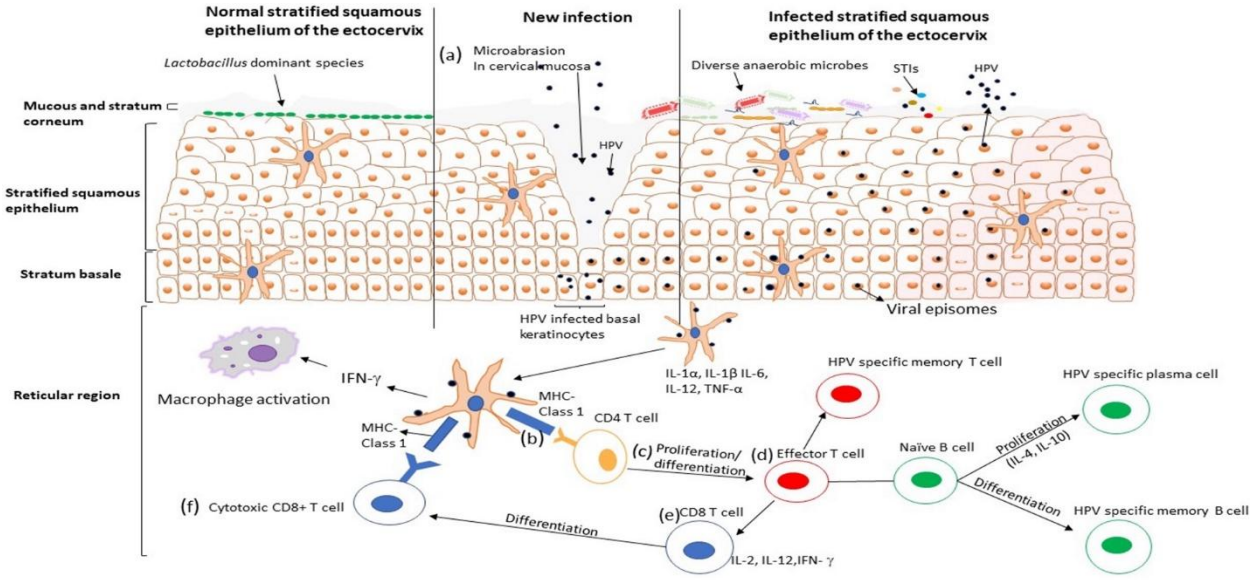


Figure 1.4: HPV Infection Cycle in the Ectocervix of the Female Genital Tract. **(A)** HPV virus particles penetrate the stratified squamous epithelium through microabrasions. They then infect the basal keratinocytes at the basement membrane, establishing an infection. Viral replication occurs, and the infected cells are transported upwards through the epithelium. **(B)** Viral particles are captured by dendritic cells, which release pro-inflammatory cytokines and chemokines (such as IL-1α, IL-1β, IL-6, TNF-α, IL-12) that attract and activate CD4+ T cells. **(C)** CD4+ T cells recognize HPV antigens presented by dendritic cells and undergo proliferation and differentiation into effector T cells. **(D)** Effector T cells proliferate into HPV-specific memory T cells and **(E, F)** cytotoxic CD8+ T cells. These effector cells bind with naïve B cells, which then differentiate into HPV-specific plasma cells and HPV-specific memory B cells. The activation of both cellular and humoral immunity contributes to the clearance of HPV infection. Adapted from Ntuli et al. (2022) [29].

1.2.13 HPV oncoproteins regulate MMP expression in cervical cancer

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases essential for the degradation of specific extracellular matrix components and tissue remodeling [164-166]. In the female genital tract, they play a crucial role during normal reproductive processes, such as the remodeling of the endometrial compartment during menstruation [167]. MMPs include several types of enzymes with specific functions. Collagenases, such as MMP-1, break down collagen by cleaving peptide bonds. Gelatinases, including MMP-2 and MMP-9, hydrolyze gelatin. Stromelysins, like MMP-3 and MMP-10, are involved in degrading the extracellular matrix. Matrilysins, such as MMP-7, are uterine metalloproteinases also engaged in extracellular matrix breakdown. The activity of MMPs is regulated through various mechanisms, including activation by other proteases, modulation at the gene transcription level, and hormonal control [168].

Altered MMP expression and distribution are pivotal in the progression of intraepithelial lesions to invasive malignancies [169]. The HPV oncoproteins E6 and E7 are particularly significant in disrupting MMP production and activation. HPV 16 has been associated with specific MMP expressions in cervical cancer progression compared to normal tissue [170-173]. Zhou *et al*. (2020) [174] found that Hr-HPV 16 E6 and E7 oncoproteins enhance the expression of MMP-2 and MMP-9, facilitating cervical cell migration. Similarly, studies by Sheu *et al*. (2003) [175] and Nasri *et al*. (2005) [176] have shown significantly higher levels of MMP-2 in high-grade cervical lesions compared to normal or low-grade lesions, highlighting its potential as a biomarker for cervical cancer and premalignant lesions. Research has also demonstrated that HPV oncoproteins E5, E6, and E7 can downregulate tissue inhibitors of metalloproteinases (TIMPs) and/or upregulate the expression of MMP-2, MMP-9, and membrane type-1 (MT1)-MMP in both normal and transformed epithelial cells. This alteration in TIMP and MMP levels enhances epithelial cell invasion and contributes to the tumorigenic properties of Hr-HPV [170-172, 174, 177-179]. Additionally, MMPs such as MMP-1, MMP-9, and MMP-13 have been linked to patient prognosis in HPV-induced cancers [180]. These MMPs are crucial in ECM degradation, tumor invasion, and metastasis. Oncoproteins E6 and E7 have been shown to upregulate MMP-1, MMP-9, and MMP-13 through various signaling pathways, including protein kinase B (AKT), the mitogen-activated protein kinase cascade (MEK/ERK), and activator protein-1 (AP-1) [180]. The increased

expression of these MMPs is associated with cancer progression, offering valuable insights into disease advancement and patient outcomes in HPV-related malignancies [180].

1.2.14 Vaginal Microbiome in Healthy Women

A healthy vagina maintains an acidic environment and hosts beneficial bacteria that help prevent infections and keep the pH balanced. Studies indicate that what constitutes a "normal and healthy" microbiome can vary among individuals and ethnic groups [181]. Typically, the pH of vaginal fluid in reproductive-aged women ranges from 3.8 to 4.5, but it can be as high as 4.9 in African women. The vaginal environment in a healthy state is predominantly characterized by *Lactobacillus species*. These bacteria produce several inhibitory agents, including lactic acid to acidify the environment and inhibit pathogen growth, hydrogen peroxide, and bacteriocins, which can directly inhibit HIV. Additionally, *Lactobacillus* helps maintain the integrity of the vaginal epithelium [182].

1.2.15 Vaginal dysbiosis and HIV acquisition

a) Bacterial vaginosis

BV is a polymicrobial condition marked by the replacement of normal *Lactobacillus species* with high concentrations of anaerobic bacteria such as *Gardnerella vaginalis*, *Fennyhessea*, *Mycoplasma hominis*, and members of the *Prevotella*, *Mobiluncus*, *Megasphaera*, *Sneathia*, and *Eggerthella* genera [183, 184]. Risk factors for BV include multiple sexual partners, a new partner, douching, intravaginal hygiene practices, recent antibiotic use, hormonal changes, and reduced levels of vaginal *lactobacilli*. These factors compromise the vaginal epithelium, leading to immune activation and inflammation, which can increase the risk of HIV acquisition [185].

In Sub-Saharan Africa, widespread vaginal practices such as the insertion of herbal or non-herbal preparations, antiseptic cleansing, and wiping vaginal secretions can disrupt the vaginal microbiome, leading to BV and an increased risk of HIV [186, 187]. Typical symptoms of BV include vaginal odor, increased vaginal discharge, and vulvar irritation. The overgrowth of

pathogenic anaerobic bacteria, often 100 to 1000 times above normal levels, leads to a significant increase in bacterial diversity [184]. BV prevalence varies by region and social factors, being lowest in Asia and Europe (4.5% to 24%) and highest in Sub-Saharan Africa (6% to 58%). In South Africa, nearly 70% of female sex workers have BV [188, 189]. Ryckman *et al*. (2008) demonstrated that while BV-positive women from different races have similar cytokine responses, baseline cytokine profiles differ between black and white women in the absence of BV, which may influence BV-related organisms' growth in certain populations [190]. Stress-related gene polymorphisms associated with race and BV susceptibility suggest that increased psychological stress experienced by black women could contribute to higher BV prevalence in this population [190]. Additionally, higher baseline vaginal pH in black adolescents compared to their white peers is another proposed factor [191].

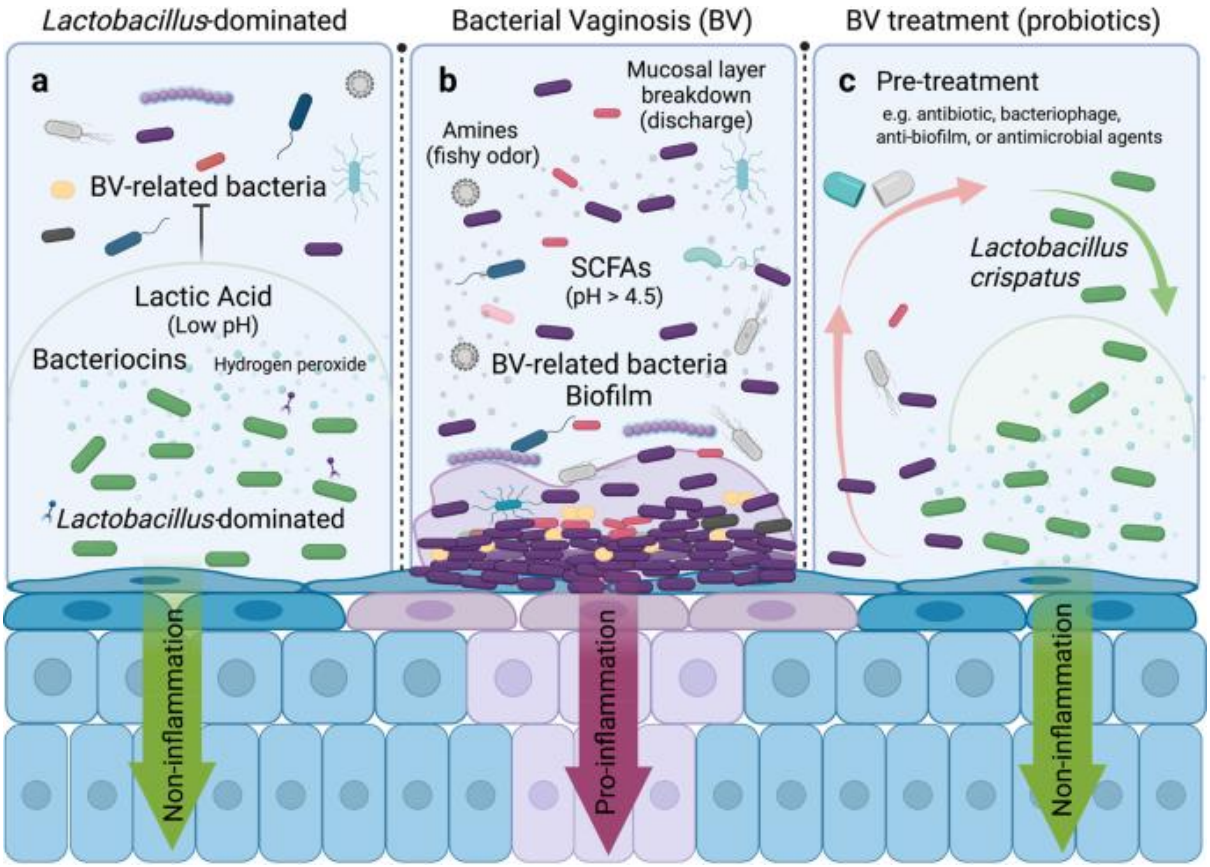


Figure1.5. Schematic diagram of an optimal and non-optimal vaginal microbiome, including BV treatment. (**a)** *Lactobacillus*-Dominated Vaginal Microbiome: In a vaginal microbiome dominated by *Lactobacillus* species, such as *Lactobacillus crispatus*, these bacteria produce lactic acid, bacteriocins, and hydrogen peroxide (H_2O_2). These substances help protect against BV-related bacteria and other infections. (**b)** BV Microbiome Environment: In a BV-associated microbiome, bacteria like *Gardnerella* induce inflammation in the vaginal tract and form a biofilm on vaginal epithelial cells. This biofilm can increase antibiotic resistance and make probiotic treatments less effective. BV-related bacteria also produce short-chain fatty acids (SCFAs) that raise vaginal pH. Additionally, the breakdown of amino acids and mucosal proteins by these bacteria results in the production of amines and a thinner mucosal layer. (**c)** Treatment of BV with Probiotics: Effective BV treatment often involves a combination of pretreatment methods, such as antibiotics, bacteriophages, anti-biofilm agents, or antimicrobials, along with the administration of vaginal probiotic species. However, WHO and CDC recommend treating BV with oral or vaginal antibiotics, such as metronidazole or clindamycin. Adapted from Wu et al. (2022) [192].

b) BV Diagnosis

BV is diagnosed based on clinical and microscopic findings using the Amsel criteria, which include: thin grayish discharge, a fishy odor upon adding potassium hydroxide (whiff test) in up to 70% of cases, a vaginal pH greater than 4.5 in up to 90% of cases, and the presence of clue cells (epithelial cells coated with bacteria) on wet-mount microscopy [193]. Approximately 85% of women with BV are asymptomatic [188], which can lead to under-treatment. However, asymptomatic BV is associated with genital inflammation similar to that in symptomatic cases [194]. Nugent's criteria, which use Gram staining of vaginal samples, can also quantify and grade bacterial populations. BV is characterized by a decrease in *Lactobacillus* and a significant increase in Gram-negative rod bacteria, genital mycoplasmas, and *Gardnerella vaginalis* [181].

This criteria evaluates the 3 types of bacteria and assigns scores to each as shown:

- *Lactobacillus* (score, 0-4)
- *Bacteroides Gardnerella* (score, 0-4)
- *Mobiluncus* (score, 0-2)

The total scores of all 3 are calculated and interpreted as follows:

- 0-3: Normal
- 4-6: Intermediate bacterial count
- 7-10: Bacterial Vaginosis is confirmed present

c) The adverse effects of BV

Although BV is not classified as an STI, it is sexually associated and is the most common vaginal health issue among women of reproductive age [188]. Risk factors for BV include smoking, vaginal douching, multiple sexual partners, having a partner with BV, and early sexual debut [195, 196]. BV increases the risk of reproductive complications, HIV acquisition, and transmission [197-199] and the acquisition or transmission of other STIs, including *trichomoniasis*, herpes simplex virus type 2 (HSV-2), HPV, and gonorrhea [200, 201]. Untreated BV can lead to premature delivery, miscarriage, intrauterine fetal death, postoperative infections, and pelvic inflammatory disease [197-199]. BV is also associated with elevated cervicovaginal inflammation [194, 202], which contributes to increased HIV risk. Women with BV have a higher prevalence of HIV

compared to those with normal vaginal microbiota [201, 203, 204]. Studies show a two- to four-fold increase in HIV acquisition risk among women with BV [193, 204-207]. Serodiscordant couples studies indicate that HIV-positive women with BV are more likely to transmit the virus to their male partners [200]. BV also heightens the risk of mother-to-child HIV transmission and the persistence of STIs, which is particularly concerning in regions with high HIV and STI prevalence, such as South Africa [208, 209]. This susceptibility may be secondary to the genital inflammation associated with BV [194, 210], which is of particular concern in South Africa with large and intertwined HIV and other STI populations [211]. BV is linked to elevated levels of pro-inflammatory cytokines like IL-1α, IL-1β, and TNF-β, and toll-like receptor (TLR) ligands in the female genital tract [194, 212]. However, some studies have observed downregulation of chemotactic cytokines, which might explain the asymptomatic nature of many BV cases and could be due to immune tolerance to commensal microbes [213].

d) Management of BV

Both the WHO and CDC recommend treating BV with oral or vaginal antibiotics, such as metronidazole or clindamycin. However, these treatments often provide only temporary relief, with high recurrence rates, approximately 30% of cases recur within three months and 50% within six months [214]. Research into combining probiotics with antibiotics for BV treatment has generally reported improved cure rates and no adverse effects [215, 216]. Despite these positive findings, there is significant variability among studies in terms of probiotic species and strains used, dosages, treatment duration, and study populations. The first clinical trial combining probiotics with antibiotics, conducted in 2005, involved administering 100 mg of vaginal clindamycin for three days, followed by tampons containing 10^8 CFU of *Lactobacillus gasseri*, *Lactobacillus casei rhamnosus*, and *Lactobacillus fermentum*, with a placebo group for comparison [217]. This study did not find a significant difference in BV cure rates between the probiotic group (56%) and the control group (62%). Another study used 400 mg of oral metronidazole for seven days, followed by a vaginal pessary containing 10^7 CFU of *Lactobacillus acidophilus* for twelve days, which similarly showed no significant difference in cure rates (72% for the probiotic group vs. 73% for controls) [218]. A subsequent trial used a seven-day course of 2% vaginal clindamycin cream, followed by vaginal capsules containing 10^8-10^9 CFU of

Lactobacillus gasseri and *Lactobacillus rhamnosus* for ten days, with the probiotic treatment repeated after each menstruation for ten days across three menstrual cycles. This study reported slightly higher BV cure rates in the probiotic group (65%) compared to the controls (46%) [219]. Similarly, other studies have shown that combining 500 mg of oral metronidazole for seven days with oral *Lactobacillus rhamnosus* and *Lactobacillus reuteri* (each 10^9 CFU) for 30 days resulted in an 88% cure rate compared to 40% in the control group [220]. Another study using 300 mg of oral clindamycin for seven days followed by vaginal capsules with 10^9 CFU of *Lactobacillus casei rhamnosus* for seven days reported an 83% cure rate versus 35% in the control group [221]. The most recent trial tested a combination of one probiotic capsule containing *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Lactobacillus gasseri* (10^8 CFU in total) with metronidazole twice daily for ten days, followed by one probiotic capsule daily for ten days. This regimen was associated with a 51% reduction in BV symptom relapse compared to placebo [222].

1.2.16 BV, vaginal microbiome, and HPV infection

Several studies, primarily focused on Western and Eurasian populations, have identified a link between BV and cervicovaginal bacterial communities with HPV infection and its progression. A meta-analysis of 12 studies involving 6,372 women aged 13-69 found a link between prevalent BV and uterine cervical HPV infection [223]. A follow-up study over 6 years on 329 Finnish women found that asymptomatic BV was associated with persistent cervical HPV infections [224]. Additionally, a U.S. study examined the relationship between common vaginal infections, including BV, and the natural history of HPV in women aged 15-56, including 756 HIV-positive individuals and 380 women at high risk for HIV [225]. This study found that current or prior BV was linked to prevalent HPV, new HPV infections, and delayed HPV clearance. However, no connection was found between prior HPV infection and BV. The impact of BV on new and existing HPV infections was not influenced by HIV status, but when accounting for combined HIV status and CD4 count, BV significantly increased the risk of both new and existing HPV infections [225].

A cross-sectional study examining the microbial diversity in 32 HPV-negative and 38 HPV-positive Chinese women with normal cervical cytology found that *L. gasseri* and *G. vaginalis* were more frequently detected in HPV-positive women compared to HPV-negative women [215].

Additionally, HPV-positive women exhibited significantly higher microbial diversity than HPV-negative women. The frequencies of *L. iners* and *L. gallinarum* did not differ between the two groups [215]. In women with prevalent HPV infection, certain bacterial taxa, such as *Prevotella*, *Megasphaera*, *Sneathia*, *Fusobacterium*, *Dialister*, *Pseudomonas*, *Brevibacterium*, *Anaerococcus*, and *Arcanobacterium*, were found in higher relative abundances compared to HPV-negative women [42, 47, 226]. A Korean twin cohort study exploring the link between cervical vaginal microbiomes and host genetics revealed that HPV-infected women had distinct microbial profiles based on their menopausal status [42]. In premenopausal HPV-infected women, *Lactobacillus* and *Sneathia* were predominant (55.4% and 11.6%, respectively), while in postmenopausal HPV-infected women, *Prevotella* (22.7%) was the most abundant non-heritable bacterium [227]. Conversely, *Alkanindiges*, *Anaerococcus tetradius*, *Corynebacterium accolens*, *Finegoldia magna*, *F. nucleatum*, *Raoultella planticola*, *Halorubrum*, and *Vampirovibrio* were found to be highly enriched in women without prevalent HPV infection [42, 47, 226, 228].

Despite some conflicting reports [43, 228, 229], the majority of studies on cervical vaginal microbiomes [43, 228-232] have found that higher abundances of *Lactobacillus* or *Lactobacillus*-dominated community state types (CSTs) are generally associated with lower rates of prevalent HPV infection. A cross-sectional study in Nigeria identified that HPV-negative women had higher relative abundances of *L. iners* compared to HPV-positive women [47]. Additionally, a North American cohort study that prospectively examined the relationship between vaginal microbiomes and HPV, while accounting for immune status [229] found that among various *Lactobacillus* species (*L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii*), only *L. crispatus* was significantly associated with reduced HPV or oncogenic HPV detection. There was no overall association between the relative abundance of the *Lactobacillus* genus and prevalent HPV [229]. Furthermore, a Korean twin cohort study [42] previously observed significantly higher relative abundances of *Lactobacillus* in HPV-negative women compared to HPV-positive. A prospective study by Shannon and colleagues (2017) [228], which examined the impact of HPV's natural history on cervical vaginal microbiomes and genital immune responses in 59 African and Caribbean women in Canada, found that both low bacterial load and high abundances of *L. gasseri* were associated with prevalent HPV.

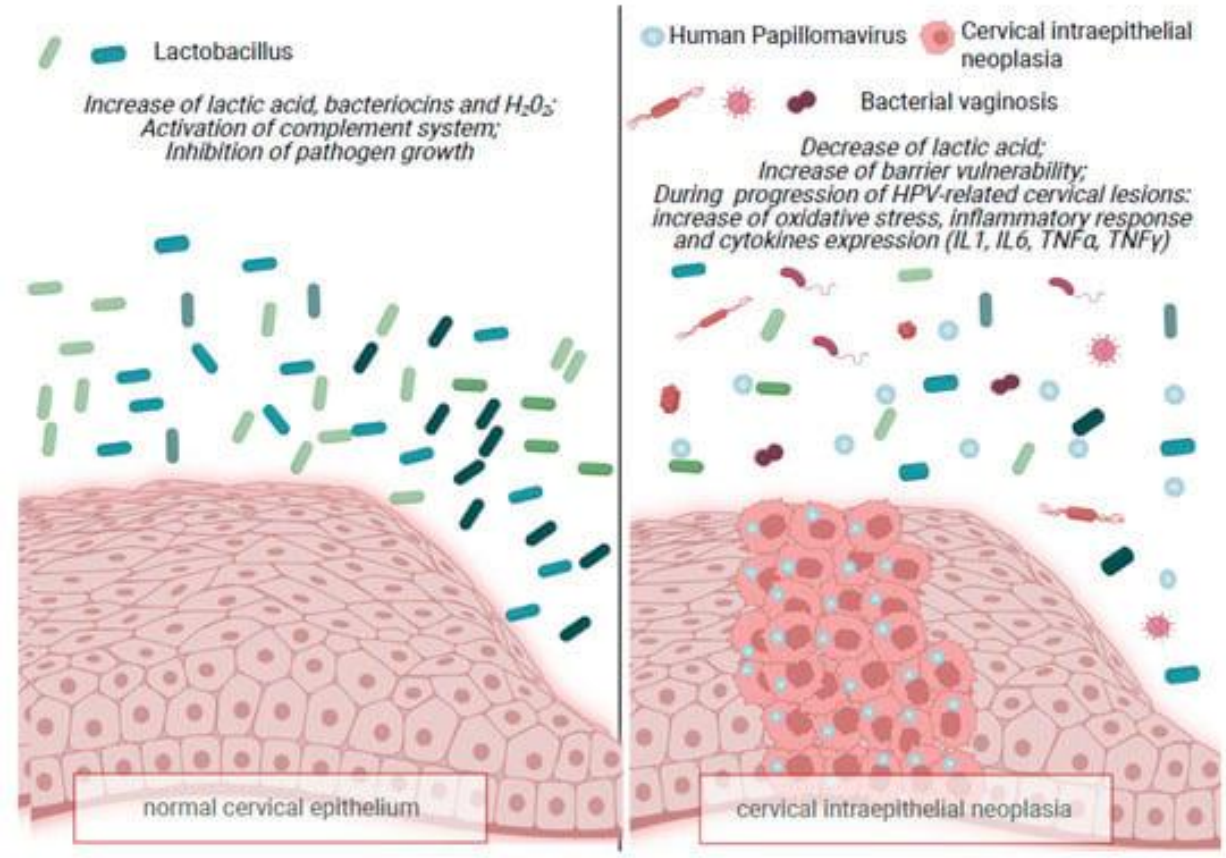


Figure 1.6. Complex association between HPV infection, the vaginal microbiome, and cancer development in the cervicovaginal environment. Figure adapted from Gardella et al. (2022) [233].

1.2.17 Conclusion

Human Papillomavirus (HPV) presents a complex challenge for the immune system due to its ability to evade detection and destruction. Upon infection, HPV targets epithelial cells where it replicates within the nucleus, avoiding exposure to immune cells that primarily surveil the cytoplasm. The virus employs various strategies to subvert the immune response, including the downregulation of major histocompatibility complex molecules, which are crucial for presenting viral antigens to T cells. Additionally, HPV can interfere with the activation and function of dendritic cells, key players in initiating a robust immune response. These evasion tactics allow HPV to persist in the host, leading to chronic infection and, in some cases, progression to

malignancies such as cervical cancer. Concurrently, the vaginal microbiome plays a significant role in modulating HPV infection and persistence. A balanced microbiome, characterized by a predominance of *Lactobacillus* species, helps maintain a low pH and produces antimicrobial peptides that inhibit HPV infection. Conversely, dysbiosis, or microbial imbalance, can compromise local immune defenses and create a more favorable environment for HPV persistence. This interplay between HPV, the immune system, and the vaginal microbiome underscores the importance of a holistic approach to understanding and managing HPV infections, emphasizing the need for continued research in these areas could enhance our understanding of HPV-host interactions and potentially lead to targeted interventions and more effective healthcare strategies.

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CHAPTER 2

High-risk human papillomavirus prevalence among South African women diagnosed with other STIs and BV

Globally, HPV, a highly transmissible sexually transmitted virus, is responsible for approximately 5% of all cancers and accounts for just over 30% of infections associated with cancers [8, 93]. Despite the high prevalence of HPV and its link to elevated risks of cervical cancer and HIV [234-237], there is limited information on the relationship between HPV and other STIs or BV. In addition, it is unclear whether coinfections with HPV and STIs or Bv have a compounding effect on biomarkers related to epithelial barrier integrity.

In Chapter 2, we explore the epidemiology of HPV infection in conjunction with STIs and BV, and their impact on MMPs among 243 South African women. We found that HPV was more frequently detected in women diagnosed with STIs and BV compared to those without these conditions. Specifically, BV was linked to a higher likelihood of detecting high-risk HPV (Hr-HPV) types, and Gardasil9 vaccine-type strains were more common in women with STI and/or BV. Furthermore, Hr-HPV detection in women with STI and/or BV was significantly associated with increased levels of MMP-10, suggesting a potential connection between HPV infection and changes in epithelial barrier integrity. These findings highlight the urgent need for integrated screening programs for STIs and BV, alongside enhanced cancer screening and HPV vaccination initiatives. The frequent coexistence of Hr-HPV with STI and BV underscores the importance of addressing multiple infections in prevention strategies. Additionally, this study emphasizes the need for further research to deepen our understanding of the complex interactions between MMPs and HPV infection.

Dr. Ngcapu, Dr Mtshali, Dr. Liebenberg, and I conceptualized and designed the study. Dr Mtshali and I were responsible for measuring cytokine levels, HPV genotyping, and flow cytometry in vaginal specimens. I analyzed and interpreted the data, which was validated by Mrs. Osman, the study statistician. I also wrote the manuscript, which was reviewed by my supervisors and approved by all coauthors for publication.

PLOS ONE

RESEARCH ARTICLE

High-risk human papillomavirus prevalence among South African women diagnosed with other STIs and BV

Wenkosi Qulu[1,2☯], Andile Mtshali[1,2☯], Farzana Osman[1], Nonsikelelo Ndlela[1,2], Lungelo Ntuli[1,2], Gugulethu Mzobe[1,2], Nivashnee Naicker[1], Nigel Garrett[1,3], Anne Rompalo[4], Adrian Mindel[1], Sinaye Ngcapu[1,2], Lenine Liebenberg[1,2,5]*

1 Centre for the AIDS Programme of Research in South Africa (CAPRISA), Durban, South Africa, **2** School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa, **3** School of Nursing and Public Health, Discipline of Public Health Medicine, University of KwaZulu-Natal, Durban, South Africa, **4** Johns Hopkins School of Medicine, Baltimore, Maryland, United States of America, **5** Centre for Epidemic Response and Innovation, Stellenbosch University, Cape Town, South Africa

☯ These authors contributed equally to this work.
* Liebenbergl@sun.ac.za

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Citation: Qulu W, Mtshali A, Osman F, Ndlela N, Ntuli L, Mzobe G, et al. (2023) High-risk human papillomavirus prevalence among South African women diagnosed with other STIs and BV. PLoS ONE 18(11): e0294698. https://doi.org/10.1371/journal.pone.0294698

Editor: Nontuthuzelo Iris Muriel Somdyala, South African Medical Research Council, SOUTH AFRICA

Received: August 8, 2023

Accepted: November 7, 2023

Published: November 30, 2023

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: https://doi.org/10.1371/journal.pone.0294698

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files. Data are available by request on a dedicated

Abstract

Introduction

Human papillomavirus (HPV) infection is a leading cause of cervical cancer. Although this relies on infection and persistence of HPV in epithelial cells, often occurring in the context of other sexually transmitted infections (STIs) and bacterial vaginosis (BV), data on the relationships between these and their relative effects on epithelial barrier integrity in women remain sparse. This study describes the epidemiology of HPV combined with STI and/or BV prevalence and the relative impact on matrix metalloproteinases (MMPs) among South African women.

Methods

Roche Linear Array was used for HPV genotyping in menstrual cup pellets of 243 HIV-negative women participating in the CAPRISA 083 cohort study. Vulvovaginal swabs were tested for *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis* using Xpert® CT/NG assay and lateral flow assay, and Gram staining was performed to diagnose BV using Nugent scoring criteria. Concentrations of 5 MMPs were measured in menstrual cup supernatants by multiplexed ELISA. Fisher's exact tests, Mann-Whitney U tests, and multivariable regression models determined associations between HPV infection, STI and/or BV, and MMP concentrations.

Results

HPV was prevalent in 34% of women (83/243; median 23 years, interquartile range (IQR) 21–27 years). Low-risk (lr) (71%, 59/83) and high-risk (hr)-HPV infections (54.2%, 45/83) were common. Hr-HPV was frequently detected in STI and/or BV-positive women compared to women without STIs or BV ($p = 0.029$). In multivariable analysis, BV was associated with

portal on the CAPRISA website (https://www.caprisa.org/Pages/CAPRISAStudies).

Funding: The study was funded by the Poliomyelitis Research Foundation (PRF grant number: 19/108) and the DST-NRF Center of Excellence in HIV Prevention, which is supported by the Department of Science and Innovation, and National Research Foundation (NRF). The CAPRISA 083 cohort study was funded by a United States – South African Program for Collaborative Biomedical Research grant through the South African Medical Research Council and the National Institute of Health (AI116759). WPQ received support from the CAPRISA Research Administration and Management Training Program (Grant # G11 TW010555-01). WPQ was funded by DST-NRF CoE in HIV Prevention (grant 96354). AM is funded by DST/NRF Innovation Postdoctoral Fellowship grant number: PDG210309589501. GM is funded by DST/NRF Innovation Postdoctoral Fellowship grant number: SFP180507326699. Poliomyelitis Research Foundation (PRF grant number: 18/16). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

increased odds of hr-HPV detection (OR: 2.64, 95%CI: 1.02–6.87, p = 0.046). Furthermore, Gardasil®9 vaccine-type strains were more frequently detected in women diagnosed with STI and/or BV (55.2%, 32/58 vs 24%, 6/25; p = 0.009). Among STI and/or BV-positive women, HPV detection was significantly associated with increased MMP-10 concentrations (b = 0.55, 95% CI 0.79–1.01; p = 0.022).

Conclusion

Most women with hr-HPV had another STI and/or BV, emphasizing an urgent need for STI and BV screening and intensive scale-up of cervical cancer screening and HPV vaccination programmes. Furthermore, the study highlights the need for more extensive research to confirm and understand the relationship between HPV infection and barrier integrity.

Introduction

Human papillomavirus (HPV), the most prevalent sexually transmitted virus worldwide, is the main etiologic agent for the development of cervical cancer [1, 2]. Although there are geographical disparities in HPV prevalence, sub-Saharan Africa has the highest global prevalence of HPV (ranging from 8.5 to 74.6%) among women with normal cervical cytology [3–5]. The risk for HPV infection is skewed toward young women who engage in sexual intercourse early in their reproductive years [6, 7]. In South Africa, young women <25 years of age are disproportionately affected by HPV and high-risk (hr)-HPV, with prevalence reaching up to 70% and 50%, respectively [8, 9]. The prevalence of multiple HPV infections and incidence of cervical cancer in young women varies widely depending on the different ethnic groups and geographic areas [10–12].

Young women are biologically susceptible to sexually transmitted infections (STIs), including HIV infection [13]. Studies have demonstrated that women living with HIV are 6 times more likely to develop cervical cancer compared to women without HIV [14]. STIs such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis* have been associated with HPV infection and persistence, leading to delayed infection clearance and possible induction of precancerous lesions or cervical intraepithelial neoplasia (CIN) type 1, 2, or 3 [15, 16]. Furthermore, bacterial vaginosis (BV), the most common cause of abnormal vaginal discharge among women of childbearing age, is associated with incident HPV infection and HPV persistence [17, 18]. The high prevalence of BV and multiple infections with sexually transmitted pathogens (such as HPV and other STIs) has been associated with increased HIV acquisition risk in sub-Saharan African women [19–21].

Although cervical cancer is preventable through HPV vaccination if it is administered before exposure, HPV vaccination coverage has been low while incidence rates remain high in resource-limited settings [19]. Data from South Africa have shown that approximately 3.2% of women in the general population harbour cervical HPV-16 or HPV-18 infection and 64.2% of invasive cervical cancers have been attributed to these genotypes [22]. In addition, HPV-35 and HPV-45 associated cervical precancers are more common in women of African descent and account for nearly 10% of cervical cancer cases [1, 23–25]. While the existing highly effective HPV vaccines have decreased HPV-16 prevalence, these do not target HPV-35 and may explain the increased prevalence observed in sub-Saharan African women [12, 25–27].

An intact squamous epithelium effectively protects against pathogen entry [28]. As HPV infects and replicates in the epithelium [29] cells might lose their epithelial characteristics and

develop a mesenchymal phenotype [30]. However the contribution of HPV infection to this transformation has not yet been fully elucidated. Matrix metalloproteinases (MMPs) are biomarkers of epithelial barrier integrity that play a critical role in the degradation of extracellular matrix, including remodelling of the tumour microenvironment, cell invasion, and development of metastatic disease [31–35]. In addition, MMPs are involved as essential molecules in multiple and diverse physiological processes of cancer, such as cell migration, differentiation, proliferation, apoptosis, inflammatory reactions, angiogenesis, and platelet aggregation [36]. While there is a relationship between MMPs and cervical cancer, it remains unknown whether HPV infection induces MMP expression in the female genital tract.

In this study, we determined the prevalence of HPV in a population of 243 young South African women attending primary healthcare in Durban, South Africa. We identified the most common circulating oncogenic genotypes and determined associations of BV and other STIs with HPV prevalence to test the hypothesis that HPV and concurrent STI and/or BV weaken the epithelial barrier integrity in the genital mucosa.

Materials and methods

Study design and population

This was a cross-sectional analysis of 243 young South African women who participated in the CAPRISA 083 cohort study between May 2016 and January 2017. All women provided written informed consent for participation in the study, and participants were only included in this study if consenting to specimen storage for assessment of the study's secondary objectives and any future research. CAPRISA 083 evaluated a combination of point-of-care (POC) STI testing, immediate treatment, and expedited partner therapy in non-pregnant, HIV-negative women, 18 years and older, who attended for sexual and reproductive care at a public healthcare clinic in eThekwini, South Africa [37, 38]. Gram stain (Nugent score with 0–3 considered BV negative, 4–6 intermediate BV, and 7–10 BV positive) was used for BV screening [39]. STI testing included screening for *C. trachomatis*, *N. gonorrhoeae* by Xpert® CT/NG (Cepheid, Sunnydale, California, US) and for *Trichomonas vaginalis* (TV) by the OSOM® Rapid Trichomonas Test (Sekisui Diagnostics, Lexington, MA, US). Women with confirmed STIs and/or BV were treated appropriately as per international guidelines. Any data that could potentially identify participants' information were anonymised throughout the research process. The authors adhered to the relevant data protection regulations and ethical guidelines to minimize the risk of unintentional disclosure of participants' identities. The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BE303/17).

Measurement of matrix metalloproteinases in SoftCup supernatants

Concentrations of 5 MMPs (MMP-1, -2, -7, -9, and -10; MMP Panel-2 kit, Merck-Millipore, Missouri, U.S.A.) were measured in SoftCup supernatants collected at enrollment were measured on a Bio-Plex 200 Array Reader system (Bio-Rad Laboratories Inc®, Hercules, California). Assays were conducted according to the manufacturer's instructions. The sensitivity of the kits ranged between 0.2 and 45.2 pg/ml for each cytokine measured and between 2 and 200 pg/ml for each MMP. Bio-Plex manager software (version 5.0; Bio-Rad Laboratories Inc®) was used to analyze the data and all analyte concentrations were extrapolated from the standard curves using a 5-parameter logistic (PL) regression equation. Analyte concentrations that were below the lower limit of quantification were reported as the mid-point between zero and the lowest concentration measured for each analyte. For analyte readings above the upper limit of detection, concentrations were reported as halfway between the highest concentration and the upper limit of the standard curve.

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This was a cross-sectional analysis of 243 young South African women who participated in the CAPRISA 083 cohort study between May 2016 and January 2017. All women provided written informed consent for participation in the study, and participants were only included in this study if consenting to specimen storage for assessment of the study's secondary objectives and any future research. CAPRISA 083 evaluated a combination of point-of-care (POC) STI testing, immediate treatment, and expedited partner therapy in non-pregnant, HIV-negative women, 18 years and older, who attended for sexual and reproductive care at a public healthcare clinic in eThekwini, South Africa [37, 38]. Gram stain (Nugent score with 0–3 considered BV negative, 4–6 intermediate BV, and 7–10 BV positive) was used for BV screening [39]. STI testing included screening for *C. trachomatis*, *N. gonorrhoeae* by Xpert® CT/NG (Cepheid, Sunnydale, California, US) and for *Trichomonas vaginalis* (TV) by the OSOM® Rapid Trichomonas Test (Sekisui Diagnostics, Lexington, MA, US). Women with confirmed STIs and/or BV were treated appropriately as per international guidelines. Any data that could potentially identify participants' information were anonymised throughout the research process. The authors adhered to the relevant data protection regulations and ethical guidelines to minimize the risk of unintentional disclosure of participants' identities. The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BE303/17).

Measurement of matrix metalloproteinases in SoftCup supernatants

Concentrations of 5 MMPs (MMP-1, -2, -7, -9, and -10; MMP Panel-2 kit, Merck-Millipore, Missouri, U.S.A.) were measured in SoftCup supernatants collected at enrollment were measured on a Bio-Plex 200 Array Reader system (Bio-Rad Laboratories Inc®, Hercules, California). Assays were conducted according to the manufacturer's instructions. The sensitivity of the kits ranged between 0.2 and 45.2 pg/ml for each cytokine measured and between 2 and 200 pg/ml for each MMP. Bio-Plex manager software (version 5.0; Bio-Rad Laboratories Inc®) was used to analyze the data and all analyte concentrations were extrapolated from the standard curves using a 5-parameter logistic (PL) regression equation. Analyte concentrations that were below the lower limit of quantification were reported as the mid-point between zero and the lowest concentration measured for each analyte. For analyte readings above the upper limit of detection, concentrations were reported as halfway between the highest concentration and the upper limit of the standard curve.

HPV DNA detection and genotyping in cervicovaginal specimens

HPV DNA was extracted in matching menstrual cup pellet specimen using an automated MagNA pure instrument (Roche Diagnostics, Indianapolis, IN, USA) and amplified using the Roche Linear Array® HPV Genotyping Test kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer instructions. The assay identifies 37 HPV genotypes [HPV-6, -11, -16, -18, -26, -31, -33, -35, -39, -40, -42, -45, -51, -52, -53, -54, -55, -56, -58, -59, -61, -62, -64, -66, -67, -68, -69, -70, -71, -72, -73, -81, -82, -83, -84, -89 (HPV-CP6108) and –IS39] [8]. Hr-HPV genotypes include HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, or -68, while low-risk HPV include HPV-6, -11, -26, -40, -42, -53, -54, -55, -61, -62, -64, -67, -69, -70, -71, -72, -73, -81, -82 subtype (IS39), -83, -84, and -89 (CP6108).

Statistical analysis

Baseline characteristics were summarized using median and interquartile ranges for continuous variables, and proportions for categorical variables. Differences between proportions were tested by Fisher's exact test, whilst the difference between medians was computed using the Mann-Whitney U test. Log binomial logistic regression was used to determine the association between HPV infection and STI or BV infection. HPV infection was defined as follows: infection with any HPV type, infection with any low-risk type, and infection with any high-risk type. Additionally, HPV infection was defined as the detection of any types targeted by the Cervarix® vaccine (HPV-16 and -18), the detection of any HPV types targeted by the Gardasil®4 vaccine (HPV-6, -11, -16, -18), and the detection of HPV types targeted by Gardasil®9 vaccine (HPV-6, -11, -16, -18, -31, -33, -45, -52, -58) to determine the association of vaccine-preventable HPV types with STI or BV acquisition. Single HPV infection is defined as the detection of only one HPV type in the same sample. Multiple HPV infections were defined as the detection of two or more HPV types in the same sample. In cases where multiple infections were identified, individuals were counted as infected for the specific category if they have one or more infections in that category. However, these women were counted more than once when determining the prevalence of lr-HPV, and hr-HPV, if they have HPV types that belong to more than one category. A p-value less than 0.05 was considered significant. Statistical analysis was carried out using STATA v17 (STATA Corp, College Station, TX, USA) and SAS ®9.4 software (SAS Institute Inc., Cary, NC, USA).

Results

Baseline characteristics by HPV infection status

A total of 243 sexually active women with a median age of 23 years (interquartile range IQR 21–27 years) were included in the analysis. HPV genotypes were detected in 34% (83/243) of women (Table 1). The majority of women reported condom use (69.5%, 169/243) and this was similar among women with detectable HPV genotypes and those without (71.1% and 68.8%, respectively; p = 0.770). Furthermore, there were no differences in the proportion of women using hormonal contraceptives (42.5%, and 34.0%, p = 0.204), and the median number of sex partners in the past 12 months was 1 partner (IQR 1–2; p = 0.834) in both groups. More than half of the women in this cohort had intermediate BV or BV, and this did not differ significantly between women with detectable HPV genotypes and those without HPV infection (36.1% and 31.3%, respectively; p = 0.359). Nearly 15% (36/243) of women were infected with *C. trachomatis* and this was similar between groups (16.9% versus 13.8%; p = 0.569). The prevalence of *N. gonorrhoeae* (4.5%) and *T. vaginalis* (3.3%) were relatively low in this cohort and were also similar between groups (Table 1).

Table 1. Sociodemographic and clinical characteristics of study participants.

Variable	Level	Overall (N = 243)	HPV+ (N = 83)	HPV- (N = 160)	p-value
		% (n/N)			
Age (years)	Median (IQR)	23 (21–27)	22 (21–25)	23 (21–27)	0.234
Level of education	Primary education	0.43 (1/233)	0 (0)	0.64 (1/154)	0.471
	Secondary education	72.1 (168/233)	68.4 (54/79)	74.0 (114/154)	
	Tertiary education	27.5 (64/233)	31.6 (25/79)	25.3 (39/154)	
Condom use	Yes	69.5 (169)	71.1 (59)	68.8 (110)	0.770
Frequency of condom use	Always	4.1 (10)	4.8 (4)	3.8 (6)	0.829
	Sometimes	65.4 (159)	66.3 (55)	65.0 (104)	
	Never	30.9 (75)	28.9 (24)	31.3 (50)	
Contraceptive use	Yes	36.9 (87/236)	42.5 (34/80)	34.0 (53/156)	0.204
Type of contraception	Injection	60.2 (53/88)	65.7 (23/35)	56.6 (30/53)	0.486
	IUD	1.2 (1/88)	2.9 (1/35)	0	
	Oral	11.4 (10/88)	5.7 (2/35)	15.1 (8/53)	
	Subdermal implant	19.3 (17/88)	17.1 (6/35)	20.8 (11/53)	
	Condom	8.0 (7/88)	8.6 (3/35)	7.6 (4/53)	
Number of sex partners in past 12 months	Median (IQR)	1(1–2)	1(1–2)	1(1–2)	0.834
Genital examination	Abnormal	88.2 (105/119)	84.1 (37/44)	90.7 (68/75)	0.378
Pelvic examination	Abnormal	49.0 (119)	53.0 (44)	46.9 (75)	0.417
Candida infection	Positive	19.3 (47)	14.5 (12)	21.9 (35)	0.176
BV status (Nugent score)	No BV (0–3)	30.9 (75)	33.7 (28)	29.4 (47)	0.359
	Intermediate BV (4–6)	36.2 (88)	30.1 (25)	39.4 (63)	
	BV (7–10)	32.9 (80)	36.1 (30)	31.3 (50)	
Chlamydia trachomatis	Yes	14.8 (36)	16.9 (14)	13.8 (22)	0.569
Neisseria gonorrhoeae	Yes	4.5 (11)	6.0 (5)	3.8 (6)	0.517
Trichomonas vaginalis	Yes	3.3 (8)	2.4 (2)	3.8 (6)	0.719
Any STI	Yes	21.0 (51)	20.5 (17)	21.1 (34)	1.000
STI and/or BV	Yes	71.6 (174)	69.9 (58)	72.5 (116)	0.668
	No	28.4 (69)	30.1 (25)	27.5 (44)	
Co-conditions	BV only	50.62 (123)	49.4 (41)	51.3 (82)	0.759
	STI only	2.5 (6)	3.6 (3)	1.9 (3)	
	BV and STI	18.5(45)	16.9 (14)	19.4 (31)	
	No BV or STI-	28.4 (69)	30.1 (25)	27.5 (44)	

Abbreviations: BV, bacterial vaginosis; IQR, interquartile range; IUD, Intrauterine device; STI, sexually transmitted infection. Any STI includes *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis*.

https://doi.org/10.1371/journal.pone.0294698.t001

Type-specific HPV frequency and genotype distribution

Of the 83/243 (34%) women with detectable HPV genotypes, the proportions of hr-HPV and lr-HPV infections were 54.2% (45/83) and 71% (59/83), respectively (Fig 1A). The most prevalent hr-HPV genotypes were HPV-51, HPV-52, HPV-45, and HPV-59 with corresponding proportions of 12% (10/83), 9.6% (8/83), 9.6% (8/83), and 9.6% (8/83), respectively. HPV-16 (8.4%, 7/83) was more prevalent than HPV-18 (1.2%, 1/83). The most frequently detected lr-HPV genotypes were HPV-81 (14.5%, 12/83), HPV-66 (9.6%, 8/83), HPV-62 (9.6%, 8/83), and HPV-11 (9.6%, 8/83). Infection with a single HPV type (54.2%, 45/83) was slightly more common than multiple HPV infections (45.8%, 38/83) in this cohort (Fig 1B). The multiple HPV infections were observed as follows: 28.9% of women were infected with 2 HPV types (24/83),

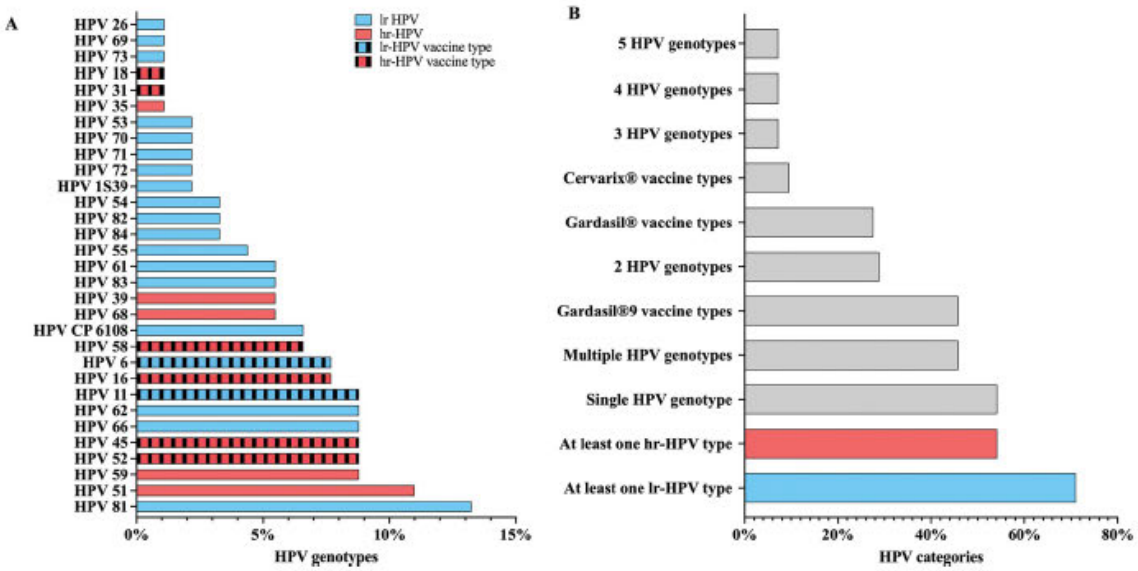


Fig 1. A: Genotype distribution of hr- and lr-HPV among women with HPV infection (N = 83). Frequency of HPV genotypes for all women at baseline. The salmon bars indicate hr- HPV genotypes, the blue bars indicate lr-HPV genotypes and the dotted bars indicate strains included in vaccines. **B: Type-specific HPV genotype frequency at baseline (N = 83).** The blue bars indicate lr-HPV genotypes, salmon bars indicate hr-HPV genotypes, and solid gray bars indicate, any HPV genotype, number of HPV genotypes, single and multiple HPV genotypes, and vaccine type strains. Hr-HPV is defined as infection with any of the following HPV genotypes: HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, or -68. Lr-HPV is defined as infection with any of the following HPV genotypes: HPV-6, -11, -26, -40, -42, -53, -54, -55, -61, -62, -64, -67, -69, -70, -71, -72, -73, -81, -82 subtype (IS39), -83, -84, and -89 (CP6108). Cervarix® HPV vaccine is defined as infection with HPV types-16 and -18. Gardasil®4 HPV vaccine is defined as infection with any of the following HPV types: HPV-6, -11, -16, or -18. Gardasil®9 HPV vaccine is defined as infection with HPV-6, -11, -16, -18, -31, -33, -45, -52, or -58.

https://doi.org/10.1371/journal.pone.0294698.g001

7.2% with 3 HPV types (6/83), 7.2% with 4 HPV genotypes (6/83), and 7.2% with 5 HPV genotypes (6/83), respectively. Hr-HPV types targeted by the Cervarix® HPV vaccine (HPV-16 and/or 18) were detected in 9.6% (8/83) of women with HPV infection. HPV types targeted by the Gardasil®4 vaccine (HPV-6, -11, -16 and -18) were detected in 27.7% (23/83) and those targeted by Gardasil®9 vaccine types (HPV-6, -11, -16, -18, -31, -33, -45, -52 and -58) were detected in 45.8% (38/83) of women (Fig 1B).

Factors associated with HPV prevalence in women

Fisher's exact tests were used to assess the relationship between HPV type and STIs and/or BV. Among women with HPV infection, infection with hr-HPV genotypes was more prevalent in women diagnosed with STIs and/or BV compared to those with no STI and/or BV (62%, 36/58 vs 36%, 9/25; p = 0.029). Gardasil®9 vaccine-type strains were more frequently detected in women diagnosed with STI and/or BV compared to women with no STI and/or BV (55.2%, 32/58 vs 24%, 6/25; p = 0.009) (Table 2).

Next, multivariable analysis was used to determine whether the presence of STIs and/or BV in this cohort was associated with hr-HPV or multiple HPV genotypes, as previously demonstrated [40, 41]. None of the STIs/BV were associated with any HPV or multiple HPV type infection (Table 3). In multivariate analysis adjusting for age, pelvic exam assessment, and number of sexual partners in the last 12 months, women diagnosed with intermediate BV or BV (aOR 2.64, 95% confidence interval (CI) 1.02–6.87, p = 0.046 and those co-infected with

Table 2. Co-detection of HPV and other STIs and/or BV (N = 83).

HPV types	HPV Positive (N = 83)		p-value
	No STI and/or BV %(n)	STI and/or BV %(n)	
	N = 25	N = 58	
lr-HPV	84.0 (21)	65.5 (38)	0.116
hr-HPV	36.0 (9)	62.1 (36)	0.029
Single HPV	56.0 (14)	53.4 (31)	0.831
Multiple HPV	44.0 (11)	46.6 (27)	
Vaccine type HPV			
Cervarix®	4.0 (1)	12.1 (7)	0.425
Gardasil®4	16.0 (4)	32.8 (19)	0.181
Gardasil®9	24.0 (6)	55.2 (32)	0.009

Abbreviations: HPV, human papillomavirus; hr-HPV, high-risk human papillomavirus; lr-HPV, low-risk-human papillomavirus. P-values less than 0.05 were considered significant. One p-value was used for single and multiple HPV types.

https://doi.org/10.1371/journal.pone.0294698.t002

BV and any STI (OR 3.16, 95% CI 1.15–8.64, p = 0.025) were more likely to have detectable hr-HPV genotype than women without BV (Nugent ≤3) or STI (Table 3).

The association between HPV infection and the exact mechanism such as markers of epithelial barrier integrity that may contribute to cervical cancer disease progression remains an active area of research. Next, we compared MMPs in women with and without HPV, and no

Table 3. Associations between HPV, STIs, and/or BV.

Variable	Univariable	P-value	Multivariable	P-value
	OR (95% CI)		aOR (95% CI)	
Any HPV (N = 243)				
BV	0.82 (0.46–1.44)	0.486	0.84 (0.47–1.49)	0.546
Chlamydia	1.27 (0.61–2.64)	0.517	1.23 (0.59–2.58)	0.584
Candida	0.60 (0.29–1.24)	0.168	0.53 (0.25–1.10)	0.089
STI and/or BV	0.88 (0.49–1.58)	0.668	0.88 (0.49–1.60)	0.685
Any STI	0.95 (0.50–1.84)	0.889	0.92 (0.47–1.80)	0.810
Multiple-type HPV infection (N = 83)				
BV	1.20 (0.48–2.99)	0.703	1.15 (0.44–2.98)	0.777
Chlamydia	0.87 (0.27–2.76)	0.810	0.86 (0.26–2.87)	0.809
Candida	0.82 (0.24–2.84)	0.757	0.85 (0.23–3.09)	0.806
STI and/or BV	1.11 (0.43–2.85)	0.831	1.04 (0.39–2.80)	0.936
Any STI	0.97 (0.27–2.33)	0.669	0.74 (0.24–2.26)	0.595
Hr-HPV types (N = 83)				
BV	2.50 (0.98–6.36)	0.054	2.64 (1.02–6.87)	**0.046**
Chlamydia	1.15 (0.36–3.68)	0.810	1.17 (0.36–3.78)	0.792
Candida	0.55 (0.16–1.91)	0.350	0.57 (0.16–2.00)	0.377
STI and/or BV	2.91 (1.10–7.70)	**0.032**	3.16 (1.15–8.64)	**0.025**
Any STI	0.94 (0.32–2.73)	0.906	0.95 (0.32–2.80)	0.930

CI; Confidence interval; Any STI includes *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis*. The odds ratio and corresponding p-values were determined using logistic regression analysis. aOR: adjusted Odds ratio in a multivariate model controlling for age, pelvic exam assessment, and number of sexual partners in the last 12 months. Estimates for *Trichomonas* and *Gonorrhoea* infection were excluded from the multiple and HR-HPV analysis due to the small sample size. P-values less than 0.05 were considered significant.

https://doi.org/10.1371/journal.pone.0294698.t003

Table 4. Association between HPV, STI, and/BV and biomarkers of epithelial barrier integrity.

Variable	Univariable	p-value	Multivariable	p-value
	β (95% CI)		β (95% CI)	
MMP-1	0.21 (-0.17–0.59)	0.276	0.14 (-0.24–0.52)	0.460
MMP-2	0.35 (-0.04–0.74)	0.082	0.30 (-0.09–0.70)	0.133
MMP-7	0.13 (-0.26–0.52)	0.503	0.12 (-0.27–0.527)	0.528
MMP-9	-0.12 (-0.69–0.45)	0.672	-0.13 (-0.69–0.44)	0.660
MMP-10	0.57 (0.11–1.03)	**0.016**	0.55 (0.79–1.01)	**0.022**

CI; Confidence interval; β-coefficients and corresponding p-values were determined using linear mixed regression analysis. A multivariable model controlled for age, pelvic exam assessment, and number of sexual partners in the last 12 months. P-values less than 0.05 were considered significant.

https://doi.org/10.1371/journal.pone.0294698.t004

differences in MMP concentrations were observed (S1 Fig). A linear mixed regression analysis was used to estimate the association of HPV and MMPs concentrations among STI and/or BV-positive women, adjusting for age, number of sexual partners in the last 12 months, and pelvic examination assessment. Of the five MMPs tested, the concentration of MMP-10 (β = 0.55, 95% CI 0.79–1.01; p = 0.022)] was significantly increased among HPV-positive women with STI and/or BV relative to women without HPV even after adjusting for multiple comparisons. No differences were observed in other MMP concentrations of women with and without HPV (Table 4).

Discussion

This study characterized the genital HPV prevalence and type-specific distribution in menstrual cup specimens of young women enrolled in a cohort study that combined an STI care model of point-of-care STI testing, immediate treatment, and expedited partner therapy. In addition, the relationship between HPV and STI and/or BV was assessed, including the relative effects on MMP concentrations. Our study demonstrated a high prevalence of hr and lr HPV infection among these women. Hr-HPV was frequently detected in STI and/or BV-positive women compared to women without. Women with STI and/or BV and with concurrent HPV exhibited significantly higher levels of MMP-10.

The overall HPV prevalence was 34% (83/243) and 54.2% of these were high-risk genotypes. This is consistent with previous studies that reported hr-HPV prevalence of 54% among women in South Africa [8, 42–44]. In contrast, other studies within the Southern Africa region have reported either lower (28–46%) [7, 45, 46] or higher (70–80%) [25, 47] hr-HPV prevalence than observed here. The inconsistency in hr-HPV prevalence within this region may be attributed to the population, sample type, collection method, and transient nature of HPV infection. Furthermore, our sample type of choice was menstrual cup pellets while others either used cervicovaginal lavage pellets [8], cervical biopsies [25], swabs or brushes [9, 48] collected from the cervix.

Studies have identified that STIs and/or BV are associated with HPV infection [17, 43], and may facilitate HPV persistence, leading to complications like cervical cancer [8]. In addition, there is evidence for multiple interactions between HPV, BV, and other STIs, and increased risk of HIV acquisition and transmission [49]. BV detection, but not STIs, showed a strong correlation with hr-HPV infection. These findings can be explained by a high burden of both BV and HPV among women of African descent. Additionally, many young, healthy, black African women have vaginal communities with low *Lactobacillus* abundance and high diversity [50, 51]. BV has been previously associated with prevalent or new HPV infections and low-grade squamous intraepithelial cervical lesions [52]. Studies have demonstrated that an

optimal female genital tract environment dominated by *Lactobacillus* species may be protective against HPV infection whereas those with diverse microbiota, reminiscent of BV, are not [53, 54]. Women with high relative abundances of *Atopobium vaginae* and *Gardnerella vaginalis* have been shown to increase the risk of acquiring new HPV infections and facilitate HPV persistence [55, 56]. It has been hypothesized that the mechanism behind increased HPV risk in women with BV may involve the disruption of epithelial barrier integrity by *G. vaginalis* [57]. These findings highlight the need for more effective treatments for BV, including *Lactobacillus*-based probiotics, which may support HPV clearance and other multi-component strategies for preventing sexually transmitted diseases (including HPV) among women.

HPV infection of the dividing basal layer requires disruption of epithelial barriers. Disruption of epithelial cells might involve the degradation of the extracellular matrix (ECM). Hr-HPV infection, including cancer progression, has been associated with increased expression of specific ECM-degrading MMPs in the cervical cancer [32, 58, 59]. In healthy tissue, MMP expression and activity is well coordinated, however, upon HPV infection this regulation is disrupted [60]. We showed that although HPV was not associated with MMP concentrations in the general population, the additive effect of concurrent HPV infection was associated with increased concentrations of MMP-10 in women with STI and/or BV. Elevated levels of MMP-10 may likely induce the expression of crucial molecules involved in angiogenesis, metastasis, and apoptosis, fostering a favourable environment that supports the survival and growth of malignant tumours [61]. Evidently, increased MMP-10 expression has been associated with resistance to apoptosis and stimulation of pro-angiogenic factors such as hypoxia-inducible factor-1 alpha (HIF)-1α and MMP-2, and pro-metastatic factors including plasminogen activator inhibitor-1 (PAI-1) and chemokine (CXC motif) receptor 2 (CXCR2) [61]. Accordingly, in vivo siRNA therapeutics targeting MMP-10 have demonstrated a reduction in tumour growth and angiogenesis [61]. In summary, the increased expression of MMP-10 in women with STIs and/or BV suggests a potential correlation with the development of cervical intraepithelial lesions. These findings collectively underscore the significance of MMPs as a viable target for therapeutic intervention in HPV-associated cervical cancer. The findings indicate that HPV alone may not be sufficient to induce the expression of MMP-10 but in combination with STIs and/or BV. The interplay between these factors could have implications for HPV persistence and the development of cervical cancer. Further research is needed to fully understand the complex interactions between cervical HPV, STIs, BV, and MMP-10 expression in women.

This study had some limitations. First, the sample size was relatively small, which impacted the statistical power and the generalizability, particularly to older women. Second, the study did not elicit whether participants were vaccinated for HPV. Although HPV vaccinations are available in the private healthcare sector, the high cost of the vaccines would have excluded this option for the majority of participants. Furthermore, the roll-out of the Cervarix® vaccine started in 2014 for 9–12-year-old girls in South Africa, while this study started in 2016 and it is therefore unlikely that participants received the vaccination in school. Nevertheless, HPV prevalence was as high or similar to reports from our region. Finally, this study only conducted a cross-sectional analysis of HPV prevalence among the cohort; hence, we were unable to assess HPV infection dynamics associated with STI and/or BV status.

In conclusion, the prevalence of hr-HPV infection among women in this population was high; underscoring an urgent need for improved cervical cancer screening and prevention programmes, including roll-out, and scale-up of HPV vaccines to curb the reported increase in HPV acquisition and cervical cancer incidence. Furthermore, the observed association between HPV infection and BV emphasizes the need to develop better BV treatments, such as biofilm dissolving agents and live biotherapeutics that could shift the vaginal microbiota

towards *Lactobacillus*-dominant communities, and multicomponent STI strategies with the potential to prevent sexually transmitted diseases and improve the reproductive health of women.

Supporting information

S1 Fig. Comparisons of MMP concentrations against HPV status in women at baseline (N = 243). The t-test was used to compare median log-transformed MMP concentrations between HPV-infected and uninfected groups.
(TIF)

Acknowledgments

We would like to thank the CAPRISA 083 study participants for their contribution to the research and the clinical and laboratory teams for the collection of clinical data and specimens.

Author Contributions

Conceptualization: Wenkosi Qulu, Andile Mtshali, Nigel Garrett, Adrian Mindel, Sinaye Ngcapu, Lenine Liebenberg.

Data curation: Andile Mtshali, Lungelo Ntuli, Gugulethu Mzobe, Nivashnee Naicker, Lenine Liebenberg.

Formal analysis: Andile Mtshali, Farzana Osman, Nonsikelelo Ndlela, Gugulethu Mzobe, Lenine Liebenberg.

Funding acquisition: Nigel Garrett, Anne Rompalo, Adrian Mindel, Sinaye Ngcapu, Lenine Liebenberg.

Investigation: Wenkosi Qulu, Andile Mtshali, Lungelo Ntuli, Nigel Garrett, Anne Rompalo.

Methodology: Wenkosi Qulu, Andile Mtshali, Nonsikelelo Ndlela, Lungelo Ntuli, Anne Rompalo, Lenine Liebenberg.

Project administration: Nivashnee Naicker.

Resources: Nigel Garrett, Adrian Mindel.

Supervision: Sinaye Ngcapu.

Validation: Wenkosi Qulu, Andile Mtshali, Nigel Garrett.

Visualization: Andile Mtshali.

Writing – original draft: Wenkosi Qulu, Andile Mtshali, Sinaye Ngcapu, Lenine Liebenberg.

Writing – review & editing: Wenkosi Qulu, Andile Mtshali, Farzana Osman, Nonsikelelo Ndlela, Lungelo Ntuli, Gugulethu Mzobe, Nivashnee Naicker, Nigel Garrett, Sinaye Ngcapu, Lenine Liebenberg.

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CHAPTER 3

Vaginal Microbiota Modulates HPV-Induced Immune Response: Differential Effects on Inflammatory Markers and Immune Cell Frequencies

HPV infection can significantly impact immune responses and alter the vaginal microbiome, which in turn affects immune function. The presence of HPV can disrupt the local immune environment by suppressing the activity of key immune cells and cytokines, leading to a diminished ability to effectively target and eliminate the virus. Concurrently, HPV-induced changes in the vaginal microbiota can exacerbate these effects. For instance, an imbalance in the vaginal microbiome, characterized by a reduction in beneficial *Lactobacilli* and an overgrowth of pathogenic bacteria, can further impair immune responses and create a more permissive environment for HPV persistence and progression. This dysbiosis can influence local immune defenses, potentially leading to increased susceptibility to HPV infection and a reduced ability to clear the virus, thereby affecting overall disease outcomes. For this reason, in **Chapter 3**, we hypothesized that the composition and diversity of the vaginal microbiome may influence the immune system's response to HPV, and that this interaction could be reciprocal. Specifically, we proposed that HPV-induced microbial dysbiosis alters the local environment of epithelial tissues and disrupts the local immune response, creating conditions that favor persistent viral infection and diminish immune surveillance. We aimed to examine the effects of type-specific HPV infection on the cervical cytokine profile and immune cell subsets. We also assessed how type-specific HPV infection impacts vaginal microbiome composition, aiming to test whether HPV infection increases genital microbiome diversity, inflammatory cytokines, and mucosal T-cell activation. Understanding these factors could provide insights into the biological mechanisms driving changes in the vaginal microbiota and genital immune response during HPV infection, potentially revealing targets for interventions to reduce HPV burden.

Vaginal Microbiota Modulates HPV-Induced Immune Response: Differential Effects on Inflammatory Markers and Immune Cell Frequencies

Wenkosi Perez Qulu[1,2], Andile Mtshali[1,2], Gugulethu Mzobe[1,2], Marothi Letsoalo[1], Asavela Olona Kama[1,] Aida Sivro[1,6,7], Lenine JP Liebenberg[1,2]and Sinaye Ngcapu[1,2*]

[1]Centre for the AIDS Programme of Research in South Africa (CAPRISA), Durban, South Africa; [2]Department of Medical Microbiology, University of KwaZulu-Natal, Durban, South Africa; [3]KwaZulu-Natal Research Innovation and Sequencing Platform, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa; [4]Discipline of Public Health Medicine, University of KwaZulu-Natal, Durban, South Africa; [5]Hopkins University, Baltimore, United States; [6]JC Wilt Infectious Disease Research Centre, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada. [7]Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, MB, Canada.

Keywords: Vaginal microbiota; genital cytokines; human papillomavirus; systemic immune activation

These authors contributed equally to this work

***Correspondence**: Dr Sinaye Ngcapu; sinaye.ngcapu@caprisa.org

Running Title: Vaginal Microbiota and HPV Immune Response

Abstract

Background

HPV infection can alter the composition of vaginal microbiota, potentially weakening the immune response and leading to persistent infections. This disruption may increase the risk of cervical dysplasia or cancer, but the exact effects of HPV on immune responses and how changes in vaginal microbiota influence these responses remain unclear. This study investigated how HPV infection affects the vaginal microbiome, endocervical cell subsets, and genital cytokine profiles in 54 women with a Nugent score >4.

Methods

HPV genotyping was performed on menstrual cup pellets using Roche Linear Array, while vulvovaginal swabs were analyzed for *C. trachomatis, N. gonorrhoeae,* and *T. vaginalis* with specific assays, and bacterial vaginosis was diagnosed through Gram staining and Nugent scoring. Cytokine levels in menstrual cup supernatants were measured with multiplexed ELISA, and T cell and dendritic cell subsets were analyzed by flow cytometry. Associations between HPV infection, vaginal microbiota, and mucosal immune response were evaluated using univariable and multivariable regression models.

Results

Among the 22/54 (40.7%) women with detectable HPV genotypes, 40.9% (9/22) had only oncogenic HPV types, 31.8% (7/22) had only non-oncogenic HPV types, and 27.3% (6/22) had both types. HPV types were detected more frequently in single infections (12/22, 54.5%) than in multiple infections (10/22, 45.5%). In univariate analysis, the presence of any HPV strains was inversely associated with the concentrations of TRAIL, CTACK, MIG, and IFN-α2; however, this significance was lost in TRAIL and, IFN-α2 after adjusting for age, *Lactobacillus* dominance, and the presence of at least one STI. In multivariable analysis, women with any HPV infection showed significantly reduced frequencies of CCR5+ CD4+ T cells and HLADR+ CD14-CD123+ plasmacytoid dendritic cells. Oncogenic HPV infection was specifically associated with increased frequencies of HLADR+ CD14- CD123+ plasmacytoid dendritic cells compared to HPV-negative

women. In multivariate analyses adjusting for age and at least one STI, women with *Lactobacillus* dominance and any HPV infection had significantly higher concentrations of MIP-1β, RANTES, and IL-7, but significantly lower concentrations of CTACK, compared to those with *Lactobacillus* dominance and no HPV infection. We observed decreased concentrations of TRAIL, IL-16, MIG, SDF-1α, and IP-10, in women with non-*Lactobacillus* dominance and any HPV infection compared to those with non-*Lactobacillus* dominance but no HPV infection, while TNF-β, eotaxin, IL-16, and MIG were significantly lower in women with non-*Lactobacillus* dominance and high-risk HPV infection compared to those with non-*Lactobacillus* dominance but without high-risk HPV infection. Women with dominant non-*Lactobacillus* communities and any HPV infection had reduced frequencies of cervical T cell subsets (CD38+ CD4+ and CCR5+ CD4+) compared to those with *Lactobacillus* dominance and no HPV infection.

Conclusion

In this study, we observed that HPV infection is associated with reduced frequencies of certain immune cells and altered cytokine/chemokine levels. Women with HPV and *Lactobacillus*-dominant vaginal microbiota had higher concentrations of some inflammatory markers, while those with non-*Lactobacillus* dominance showed decreased levels of these markers and immune cell frequencies. These results underscore the influence of HPV on immune responses and the role of vaginal microbiota in modulating these effects.

Keywords: oncogenic HPV, *Lactobacillus* dominance, cytokines, immune cells, T cells, dendritic cells.

3.1 Introduction

Cervical cancer is the fourth most common cancer among women worldwide, with approximately 660,000 new cases and around 350,000 deaths reported in 2022, underscoring its significant impact on global health. High-risk subtypes of human papillomavirus (HPV) are the primary cause of most cervical cancer cases [8]. A range of risk factors is associated with recurring HPV infections, with early sexual intercourse, a high number of sexual partners, and infrequent condom use being the most prevalent [238]. In addition, differences in prevalence related to health behaviors and sociodemographic factors such as age, marital status, income level, race, lack of information about HPV, and inadequate access to testing, vaccination, and treatment have also been documented [239]. Furthermore, it has been hypothesized that biological factors, including host defense mechanisms and the vaginal microbiome in the female genital tract, play a significant role in influencing the persistence or regression of HPV infections [29, 55, 240].

A *Lactobacillus-dominated* vaginal microbial community has long been characteristic of health in the female genital tract (FGT), by reducing the inflammatory response and inhibiting the colonization of genital pathogens through the production of lactic acid, bacteriocins, and other products, improving reproductive outcomes [241, 242]. *Lactobacillus crispatus* and *Lactobacillus gasseri* are the most frequently detected species in women who test negative for HPV [62]. Additionally, *Lactobacillus gasseri* may be linked to quicker clearance of acute HPV infections in women who test positive for HPV [43]. In contrast, meta-analysis and systematic review by Norenhag and his team found that women with *Lactobacillus iners* had a higher risk of HPV infection (OR 2.11; 95% CI 1.28–3.46) and squamous intraepithelial lesions (SIL) (OR 1.95; 95% CI 1.07–3.56) compared to those with *Lactobacillus crispatus* [44]. Furthermore, BV-linked diverse microbes (especially *Fannyhessea, Prevotella, and Gardnerella*) are often associated with HPV infection and transmission, rapid remission of HPV, and the progression to premalignant and invasive cervical cancer [243-245].

Immunosurveillance is essential for activating antigen-specific responses that target and remove HPV infections and virally transformed cells. In individuals with a competent immune system, 90% of anogenital HPV infections are resolved [246, 247]. Innate immune cells, including natural

killer (NK) cells, antigen-presenting cells [APCs such as dendritic cells (DCs), and Langerhans cells (LCs)], are crucial during the initial stages of infection. Meanwhile, HPV-specific CD8+ T cells focus on targeting early viral proteins within infected cells [248-250]. However, HPV employs sophisticated mechanisms to evade host immune responses, significantly contributing to its persistence and pathogenicity. One such strategy involves modifying APC functions, including the alteration of pathogen recognition receptors [29, 30, 147]. Another mechanism by which HPV evades the immune response is through the inhibition of macrophage and other immune cell recruitment to the infection site, a process mediated specifically by the E6 and E7 proteins of HPV16 [114]. Furthermore, other key strategies involve the manipulation of regulatory T cells (Tregs), particularly those producing immunosuppressive cytokines such as IL-10 and TGF-β, creating a microenvironment that inhibits effective antiviral activity and immune surveillance [144]. CD4+ and CD8+ T cell function can be inhibited by downregulating surface MHC I expression and disrupting the trafficking and maturation of APCs [251]. Collectively, these findings highlight the need for further research into how HPV evades the immune system and how this understanding can be used to improve the prevention and treatment of HPV-related diseases.

The role of the host microbiome and immune response in HPV infection is not fully understood. Here we hypothesize that the composition of vaginal microbiome and diversity could affect how well the immune system responds to HPV, and that the interaction might be reciprocal. Additionally, we proposed that HPV-induced microbial dysbiosis modifies the local environment of epithelial tissues and disrupts the local immune response, creating conditions that are more favorable for persistent viral infection and less effective at immune surveillance. In this study, we assessed the impact of type-specific HPV infection on cervical cytokine profile and immune cell subsets. We evaluated the impact of type-specific HPV infection on vaginal microbiome composition, to test the hypothesis that HPV infection increases genital microbiome diversity, inflammatory cytokines, and mucosal T-cell activation. Understanding these factors could reveal the biological mechanisms behind changes in the vaginal microbiota and genital immune response during HPV infection. This insight may help identify potential targets for interventions designed to reduce HPV burden.

3.2 Methodology

3.2.1 Study design, participants, and sample collection

This study included 54 HIV-uninfected women with a laboratory-diagnosed sexually transmitted infection (STI), and/or candidiasis and/or BV. The women were aged 18-40 years and recruited at Prince Cyril Zulu Communicable Diseases Clinic, Durban, South Africa, and enrolled in the CAPRISA 083 study [252]. HIV rapid testing was conducted at each visit, as was the point of care STI screening by GeneXpert (Cepheid) and standard PCR multiplex assay [253]. Furthermore, a vaginal swab was used for Gram staining for the diagnosis of Candidiasis [254] and BV using Nugent's criteria (a score of 0 - 3 was considered BV negative, 4 - 6 intermediate BV, and 7 - 10 BV positive) [255]. Participants diagnosed with genital symptoms in keeping with possible Candidiasis, *Chlamydia trachomatis* (CT)*, Neisseria gonorrhoeae* (NG)*, Trichomonas vaginalis* (TV), and/or BV (including intermediate group) were treated in line with South African STI treatment guidelines [252]. Enrolled and treated women were asked to return after 6 weeks for a follow-up examination and a test-of-cure 3 months later. Laboratory samples, including the genital specimens (SoftCup pellets and supernatants, genital swabs, and cytobrushes), were collected at baseline, week 6, and week 13 after treatment. Women who were pregnant, living with HIV, or who had received antibiotic treatment within the last 7 days were excluded from the study. The study received approval from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BE303/17).

3.2.2 Characterization of vaginal microbiota

Genomic DNA was extracted from vaginal swabs using the PowerSoil DNA Isolation Kit (MoBio, CA, USA). The V3-V4 region of the 16S rRNA gene was amplified by PCR with primers 319F (forward) and 806R (reverse) [256]. Library preparation involved sequencing 120 pooled samples on the Illumina MiSeq platform, generating paired-end reads with V3 chemistry. The data was processed with the 'DADA2' R package, which handled quality-based filtering, trimming, amplicon sequence variant (ASV) inference, chimera removal, and taxonomy assignment. ASVs were phylogenetically placed using QIIME 2's SEPP-based fragment insertion plugin and a reference tree. Taxonomic classification was performed using the SILVA ribosomal RNA database [257]. For specific genera like *Lactobacillus, Prevotella, Sneathia,* and *Mobiluncus*, ASVs were

further classified at the species level using speciateIT (version 1.0, http://ravel-lab.org/speciatIT). The integration of read counts, ASV tables, taxonomy tables, phylogeny, and metadata was managed with the 'phyloseq' R package, resulting in a comprehensive phyloseq object. Community state types (CSTs) were assigned using VALENCIA (VAginaL community state typE Nearest Centroid classification algorithm) [258], with reference centroids available at github.com/ravel-lab/VALENCIA

3.2.3 HPV DNA detection and genotyping in cervicovaginal specimens

DNA was extracted from cervicovaginal SoftCup pellets using an automated MagNA pure instrument (Roche Diagnostics, Indianapolis, IN, USA) following the manufacturer's guidelines. HPV DNA was then amplified by PCR with the Roche Linear Array® HPV Genotyping Kit (Roche Diagnostics, Indianapolis, IN, USA), adhering to the provided instructions. In brief, a pool of biotinylated primers was used to amplify HPV DNA from the CVL pellet by targeting capture sequences within the polymorphic L1 region of the HPV genome. This assay identifies 37 HPV genotypes, including those associated with a significant risk of progression to cervical cancer (oncogenic HPV types). The HPV genotypes detected in this study include HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39, and CP6108. Notably, CP6108 is a subgroup of HPV type 89, IS39 is a subgroup of HPV type 82, HPV type 55 is a subgroup of type 44, and HPV type 64 is a subgroup of type 34. Additionally, the assay includes a cross-reactive probe that hybridizes with HPV genotypes 33, 35, 52, and 58 from the Alpha 9 genus, as well as single probes for each specific type to distinguish these closely related HPV types [51, 259]. Additional primer pairs were used to amplify the human β-globin gene as an internal control to ensure the adequacy of the DNA extraction and amplification.

3.2.4 Measurement of cytokine and chemokine biomarkers of genital inflammation

Cervicovaginal SoftCup supernatants from the CAPRISA 083 cohort were collected at matching time points. To measure cytokine concentrations, vaginal swabs were analyzed using a 48-cytokine panel, which included chemokines, pro-inflammatory cytokines, adaptive cytokines, growth

factors, and anti-inflammatory cytokines. The analysis was conducted with Bio-Plex Pro-Human Cytokine Group I (27-Plex Panel) (Bio-Rad Laboratories, USA), as previously described [256]. The cytokine panel covered a broad range of factors, including: chemokines (CTACK, Eotaxin, IP-10, MCP-1, MCP-3, MIG, MIF, RANTES), pro-inflammatory cytokines (IL-1β, IL-1Rα, IL-6, IL-8, IL-12p70, IL-12p40, IL-16, IL-18, IL-1α, IL-2RA, IL-15, IL-17, G-CSF, GM-CSF, GRO-α, IFN-γ, IFN-α2, LIF, M-CSF, MIP-1α, MIP-1β, TNF-α, TNF-β, TRAIL), adaptive cytokines (IL-2, IL-4, IL-5, IL-7, IL-9, IL-10, IL-13), growth factors [basic fibroblast growth factor (FGF-basic), hepatocyte growth factor (HGF), nerve growth factor beta (NGF-β), platelet-derived growth factor (PDGF-β), stem cell factor (SCF), stem cell growth factor beta (SCGF-β), stromal cell-derived factor 1-alpha (SDF-1α), VEGF], and anti-inflammatory cytokines (IL-10). Cytokine concentrations were determined using a standard curve and a 5-parameter logistic regression model. Inter- and intra-assay variability were assessed by comparing cytokine concentrations in replicate samples, with Spearman r values >0.8 considered acceptable for each cytokine. Cytokine levels below the assay's lower limit of detection were reported as the mid-point between zero and the lowest detectable concentration. Data were log10-transformed to normalize distributions.

3.2.5 Assessment of immune cell phenotype and activation in endocervical cytobrushes

Endocervical cytobrushes were collected by inserting the brush into the endocervical os and performing a 360° rotation, as previously described [260]. To release cervical mononuclear cells from the brush, the samples were vortexed, washed, filtered, and then processed for flow cytometry ex vivo [261]. The cells (fixed number of 50,000 cells per staining reaction) were first stained with LIVE/DEAD Aqua fluorescence (Invitrogen by Thermo Fisher Scientific™) for 20 minutes at 4°C in the dark. Following this, they were washed with FSC phosphate-buffered saline and surface-stained for 20 minutes at room temperature with a cocktail of antibodies, including Alexa Fluor 700 (AF) labeled anti-HLA-DR, APC labeled anti-CD86, Brilliant Violet 785 (BV785) labeled anti-CD16, Peridinin-chlorophyll-protein CY5.5 (PerCP-CY5.5) labeled anti-CD123, BV650 labeled anti-CD11c (Biolegend, CA, USA), Phycoerythrin (PE) labeled anti-CD4, Allophycocynin-H7 labeled (APC-H7) anti-CD3, Fluorescein Isothiocyanate (FITC) labeled anti-CD38, BV605 labeled anti-CCR6, BV421 labeled anti-CCR5, PECy7 labeled anti-CD14, V510 labeled ant-CD19, and V510 labeled anti-CD56 (BD, NJ, USA), respectively. Data acquisition

was carried out using a FACSDiva Flow Cytometer (Becton and Dickinson Immunocytometry Systems) and analyzed with FlowJo Software version 10 (Tree Star, CA, USA). Fluorescence minus one (FMO) controls were used to set gates for distinguishing negative and positive populations [262]. All data were adjusted for background based on unstimulated conditions. As previously described, the gating strategy for characterizing cervical T and dendritic cells from cytobrush specimens were performed [263].

3.2.6 Statistical analysis

All statistical calculations were performed using R software version 4.3.1 (www.r-project.org). To compare characteristics between the HPV negative and HPV positive groups, Fisher's exact test and Pearson's chi-squared test were used for categorical variables. For continuous variables, including age and number of sexual partners, the Wilcoxon rank sum test was applied. Medians and interquartile ranges were used to summarize descriptive data of continuous variables, and categorical data were summarized with both frequency and percentages. A linear regression model was used to estimate the unadjusted and adjusted effects of HPV types on cell frequency and cytokine concentration. The multivariable model adjusted for *lactobacillus* dominance status, age, and sexually transmitted infections. Alpha diversity measures, Dominance, Shannon's, and Simpson's indices were calculated using the R-vegan package. Bray-Curtis distances of species relative abundance were used to determine beta-diversity measures between samples. The distances were visualized using a Non-metric Multidimensional scaling (NMDS) to assess the overall spatial segregation of samples of various CST groups, the number of HPVs (single or multiple), and the type of HPV (oncogenic, or non-oncogenic). Permutational multivariate analysis of variance (PERMANOVA) was used to assess the extent of the segregation observed between groups. This investigation used a significance level of 0.05 as a signal for potential association.

3.3 Results

Of the 54 HIV-negative women with a median age of 24 years (IQR 21-27) included in this study, 40.7% (22/54) tested positive for at least one HPV genotype. The majority of women (70.3%, 38/54) reported using condoms and the rate of use was comparable between women with

detectable HPV genotypes and those without, at 72.2% and 68%, respectively (p = 0.753). All participants had symptomatic BV, with Nugent scores of 4-6 (41%, or 22/54, indicating intermediate BV) and NS>7 (59%, or 32/54, indicating severe BV), p = 0.269. The overall prevalence of discharge-associated STIs in this cohort was relatively high, with *C. trachomatis* detected in 31.5% (17/54), *N. gonorrhoeae* in 11.1% (6/54), and *T. vaginalis* in 7.4% (4/54), and the prevalence was similar between the groups. Overall, 30% (16/54) of the participants who tested positive for any HPV had at least one high-risk genotype (**Table 3.1**).

Table 3.1: Participant characteristics by any HPV status

Variable	Level	Overall (N=54)	any HPV positive (N=22)	HPV negative (N=32)	p-value
		% (n/N)			
Age (years)	Median (IQR)	24 (21, 27)	23 (20, 26)	26 (22, 27)	0.150
Highest level of education	*Secondary*	35 (65%)	13 (59%)	22 (69%)	0.465
	Tertiary	19 (35%)	9 (41%)	10 (31%)	
Condom use	Yes	38 (70.3%)	16 (72.2%)	22 (68.7)	0.753
	No	16 (29.6%)	6 (27.2%)	10 (31.2%)	
Frequency of condom use	Always	3 (5.6%)	1 (4.5%)	2 (6.3%)	0.898
	Never	16 (30%)	6 (27%)	10 (31%)	
	Sometimes	35 (65%)	15 (68%)	20 (63%)	
Contraceptive use	Yes	20 (37%)	6 (27%)	14 (43.7%)	0.218
	No	34 (62.9%)	16 (72.2%)	18 (56.2%)	
Type of contraception	Injection	11 (55%)	3 (50%)	8 (57%)	>0.999
	Oral	3 (15%)	1 (17%)	2 (14%)	
	Subdermal implant	6 (30%)	2 (33%)	4 (29%)	
	None	34 (63%)	16 (72.2)	18 (56.2%)	
Number of sex partners	Median (IQR)	1.00 (1.00, 2.00)	1.00 (1.00, 2.00)	1.00 (1.00, 1.00)	0.179
	No partner	1 (1.9%)	0 (0%)	1 (3.1%)	0.434

Number of partners (categorical)	One partner	38 (70%)	14 (64%)	24 (75%)	
	More than one partner	15 (28%)	8 (36%)	7 (22%)	
Candida infection	Positive	8 (15%)	2 (9%)	6 (18.8)	0.449
	Negative	46 (85.2)	20 (91%)	26 (81.2)	
Mycoplasma	Yes	5 (9.3%)	2 (13%)	3 (7.9%)	>0.999
	No	49 (90%)	20 (90.1%)	29 (90.6%)	
Bacterial vaginosis Level	Intermediate BV (4-6)	22 (41%)	7 (32%)	15 (47%)	0.269
	BV (7-10)	32 (59%)	15 (68%)	17 (53%)	
At least one STI	Yes	26 (58%)	9 (41%)	17 (53%)	0.377
	No	28 (52%)	13 (59.1%)	15 (47%)	
Chlamydia trachomatis	Yes	17 (31.5%)	7 (31.8%)	10 (31.2%)	0.965
	No	37 (68.5%)	15 (68.2%)	22 (68.7%)	
Neisseria gonorrhoeae	Yes	6 (11.1%)	3 (13.6%)	3 (9.4%)	0.678
	No	48 (88.8%)	19 (86.3%)	29 (90.6%)	
Trichomonas vaginalis	Yes	4 (7.4%)	0	4 (12.5%)	0.137
	No	50 (92.6%)	22 (100%)	28 (87.5%)	

Abbreviations: BV, bacterial vaginosis; IQR, interquartile range; IUD, Intrauterine device; STI, sexually transmitted infection. Any STI includes *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis*.

3.3.1 Frequency and Distribution of HPV Types and Genotypes

Among the 22/54 women (40.7%) with detectable HPV genotypes, 40.9% (9/22) had infections with only oncogenic (high-risk) HPV types, 31.8% (7/22) had infections with only non-oncogenic (low-risk) HPV types, and 27.3% (6/22) had infections with both oncogenic and non-oncogenic

HPV types. (**Figure 3.1**). Twelve out of the 22 (54.5%) HPV types were more commonly detected in cases of single infections compared to multiple infections (10/22, 45.5%). The most common oncogenic HPV genotypes were HPV-16, HPV-58, and HPV-59, each accounting for approximately 13.6% (3/22) of the cases. HPV-39, HPV-45, HPV-51, HPV-52, HPV-66, and HPV-68 each accounted for approximately 9.1% (2/22) of the cases. None of the participants tested positive for HPV-18, an oncogenic type. The most frequently detected non-oncogenic HPV genotypes were HPV-CO6108, HPV-11, HPV-54, HPV-6, HPV-62, HPV-82, and HPV-83, each accounting for approximately 9.1% (2/22) of the cases. HPV-55, HPV-61, HPV-70, and HPV-81, each accounted for approximately 4.5% (1/22) of the cases.

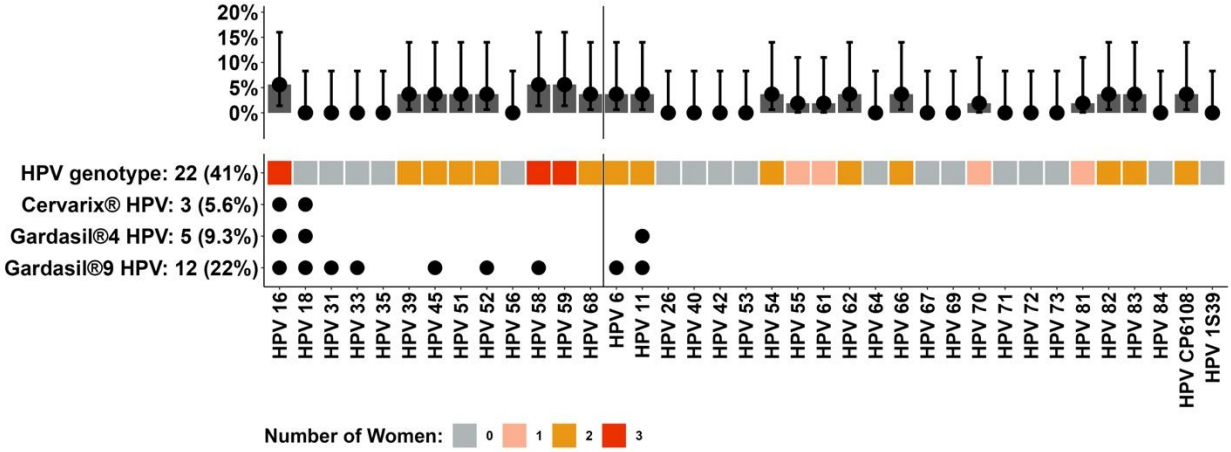


Fig 3.1. Distribution of HPV Genotypes by Risk Type Among Women with HPV Infection (N = 22). Solid gray bars indicate frequency with minimum and maximum values while coloured boxes represent the prevalence (%) of oncogenic HPV and non-oncogenic HPV genotypes. Dots indicate strains covered by vaccines.

3.3.2 The relationship between HPV status and genital cytokines

A linear regression analysis was used to assess the impact of HPV infection on cytokine profiles in the genital mucosa of HPV-positive women. Genital cytokine concentrations of women infected with different oncogenic or any HPV types were compared to those of HPV-negative

women, adjusting for age, *Lactobacillus* dominance, and at least one STI. In univariate analysis, having any HPV strains was inversely associated with the concentrations of the TRAIL [β = -0.97, 95%CI: -1.8 to -0.13, p=0.028], CTACK [β = -1.0, 95%CI: -1.9 to -0.19, p=0.020], MIG [β = -0.07, 95%CI: -1.3 to -0.23, p=0.007], and IFN-α2 [β = -0.89, 95%CI: -1.74 to – (-0.04), p= 0.045] but this significance was lost after adjusting for confounding factors. After adjusting for confounding factors, women with any-HPV strain had decreased concentrations of the MIG [β = -0.72, 95%CI: -1.3 to -0.18, p=0.012] and CTACK [β = -0.89, 95%CI: -1.74 to – (-0.04), p = 0.047]. There were no significant differences in cytokine concentration of women with oncogenic HPV strain compared to women without oncogenic HPV infection (**Figure 3.2**).

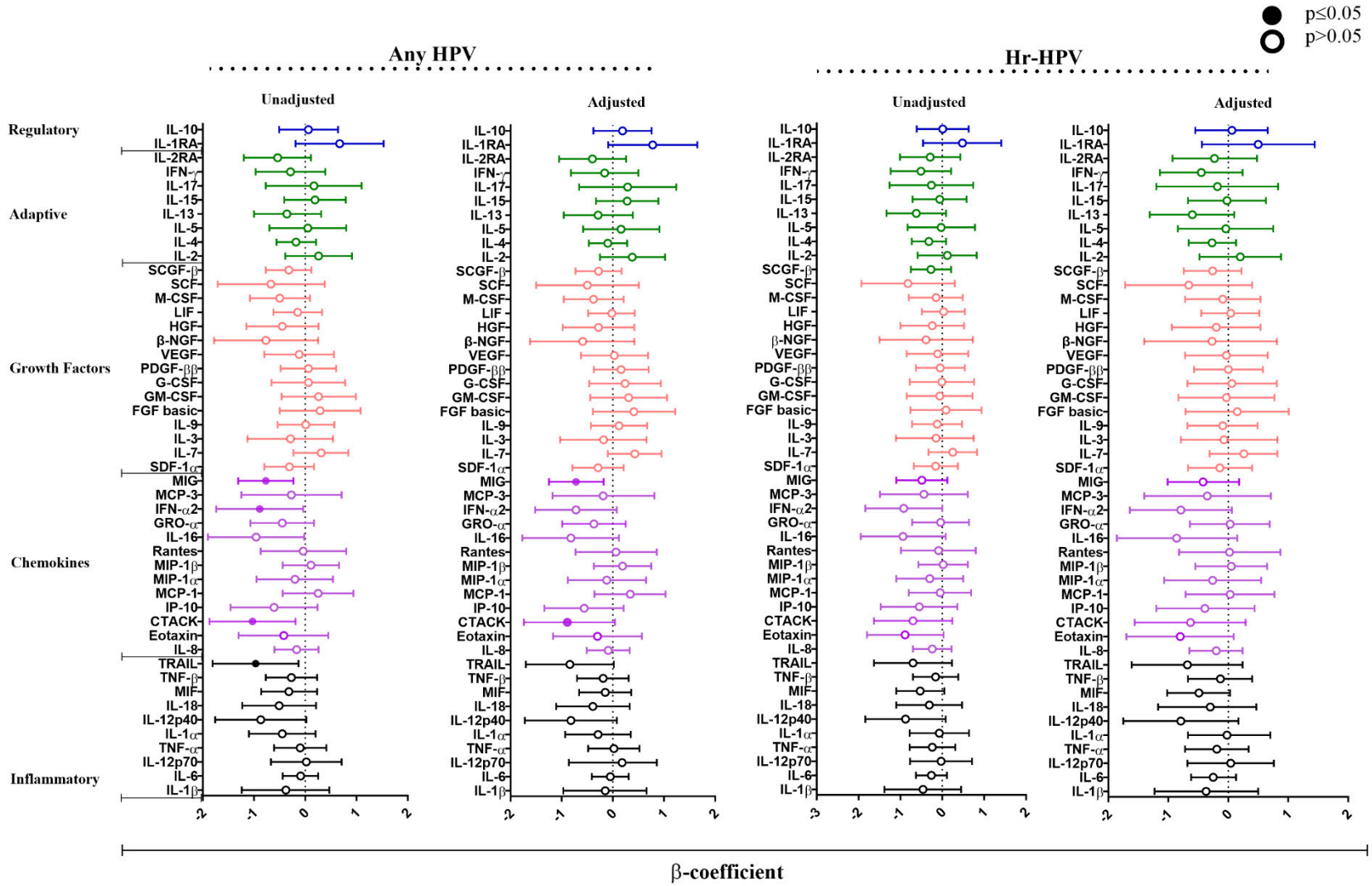


Figure 3.2: The relationship between HPV status and genital cytokines at baseline. Individual associations are demonstrated between HPV status and genital proinflammatory cytokines, chemokines, growth factors, adaptive response, and anti-inflammatory cytokines,

with error bars representing standard error. Significant relationships are indicated by the blue color, following False Discovery Rate (FDR) adjustment using a threshold of 0.05 multivariable models controlling for baseline age, at least one sexually transmitted infection, and *lactobacillus* dominance. The dotted line at $\beta = 0$ marks the point of no difference; cytokines positioned to the right of the line are higher, and those to the left are lower, indicating differences in cytokine levels based on HPV status at baseline.

3.3.3 Assessment of immune cell subsets by HPV status

Next, we compared the frequency of cervical T cell and dendritic cell (DC) subsets in women infected with different oncogenic HPV or any HPV types to those in HPV-negative women. In multivariable analysis, women with any HPV infection exhibited reduced frequencies of CCR5+ CD4+ T cells [β = -11, 95%CI: -20 to -2.0, p = 0.024], In univariate, any HPV strain infection was significantly associated with reduced frequencies of CCR6+ CD4+ cells [β = -12, 95%CI: -21 to -2.7, p = 0.017], whereas, an increase in pDCs [HLADR+CD14-CD123+, β = 10, 95%CI: 1.2 to 19, p =0.033] was observed. In multivariable analysis, oncogenic HPV infection was associated with increased frequencies of pDCs [HLADR+CD14-CD123+, β = 12, 95%CI: 3.4 - to 20, p = 0.011], while mDCs [HLADR+CD14-CD11C+, β = -16, 95%CI: -34 to -2.2, p = 0.098] had a trend towards a decrease (**Table 3.2**).

Table: **3.2** Association between cervical mucosal cell subsets and HPV status

Variable	Any HPV strain infection				oncogenic HPV strain infection			
	Unadjusted	**p-value**	**Adjusted**	**p-value**	**Unadjusted**	**p-value**	**Adjusted**	**p-value**
CD4+ T cells	-6.19(-13.6 - 1.25)	0.114	-6.11(-13.8 - 1.60)	0.132	-5.74(-13.7 - 2.20)	0.167	-6.44(-14.53-1.65)	0.131
CD38+ CD4+ T cells	-7.33(-17.9 - 3.31)	0.187	-9.52(-19.7 - 0.68)	0.079	-3.65(-15.1 - 7.86)	0.539	-5.23(-16.4- 5.97)	0.368
HLADR+CD4+Tcells	-8.31(-20.2 - 3.60)	0.182	-11.1(-22.3 - 0.12)	0.063	-8.80(-21.4 - 3.77)	0.181	-9.82(-21.8 - 2.21)	0.122
CCR5+ CD4+ T cells	-10.2(-20.0 - (-0.37)	0.051	-11.2(-20.4 - (2.04)	**0.024**	-9.00(-19.6 - 1.59)	0.107	-8.31(-18.4 - 1.87)	0.122
CCR6+ CD4+ T cells	-11.7(-20.8 - (-2.68)	**0.017**	-10.7(-20.2 - (1.24)	**0.036**	-8.52(-18.6 - 1.59)	0.109	-7.98(-18.4 - 2.45)	0.146
CD14− HLADR+	2.19(-7.5 - 12.0)	0.663	1.97(-8.20- 12.1)	0.707	6.22 (-3.88 - 16.3)	0.237	6.91(-3.47 - 17.3)	0.203
HLADR++ CD14+	-11.3(-23.9 - 1.35)	0.091	-11.0(-24.1 - 2.16)	0.114	-12.7(-26.0- 0.50)	0.069	-13.4(-26.9 - 0.09)	0.062
CD86+HLADR++CD14+	-1.74(-10.0 - 6.53)	0.683	-1.99(-10.5 - 6.53)	0.650	-1.76(-10.5 - 6.98)	0.696	-2.74(-11.7 - 6.20)	0.553
HLADR+CD14−CD11c+	2.19(-7.58 – 12.0)	0.663	1.97(-8.20 - 12.1)	0.707	-15.8(-34.1 - 2.37)	0.099	-15.8(-33.9 - 2.23)	0.098
CD86+ CD11c+	-5.38(-20.0 - 9.29)	0.478	-2.85(-17.6 - 11.9)	0.708	-5.51(-21.0 - 9.99)	0.492	-4.13(-19.6 - 11.3)	0.605
HLADR+CD14−CD123+	9.96(1.24- 18.7)	**0.033**	8.60(0.10 - 17.0)	0.058	12.6(3.84 - 21.5)	**0.009**	11.8(3.39 - 20.2)	**0.011**
CD86+ CD123+	-2.59(-10.9 - 5.71)	0.545	-1.73(-10.3 - 6.84)	0.695	-4.90(-13.5 - 3.74)	0.276	-4.72(-13.5 - 4.13)	0.306

Beta-coefficient (β). The β-coefficient and corresponding p-values were determined using linear regression analysis: adjusted in a multivariable model controlling for age, at least one STI, and *Lactobacillus dominance*. p- values < 0.05 were considered statistically significant.

3.3.4 Community state types within the vaginal microbiome

The vaginal bacterial communities were categorized into five major groups, known as community state types (CST) (**Figure 3.3**). The majority of communities were classified as CST IV, distinguished by a higher prevalence of anaerobic and strictly anaerobic bacteria, which accounted for over 70.4% of all sequences. Within CST IV, CST IV-A, characterized by a high relative abundance of *BVAB1*, was found in 29.6% (16/54) of the participants, while CST IV-B, marked by dominance of *Gardnerella vaginalis*, was observed in 40.7% (22/54). CST I, found in 1.9% (1/54) of the women, was dominated by *Lactobacillus crispatus*, whereas CST III, present in 27.8% (15/54) of women, was primarily colonized by *Lactobacillus iners*.

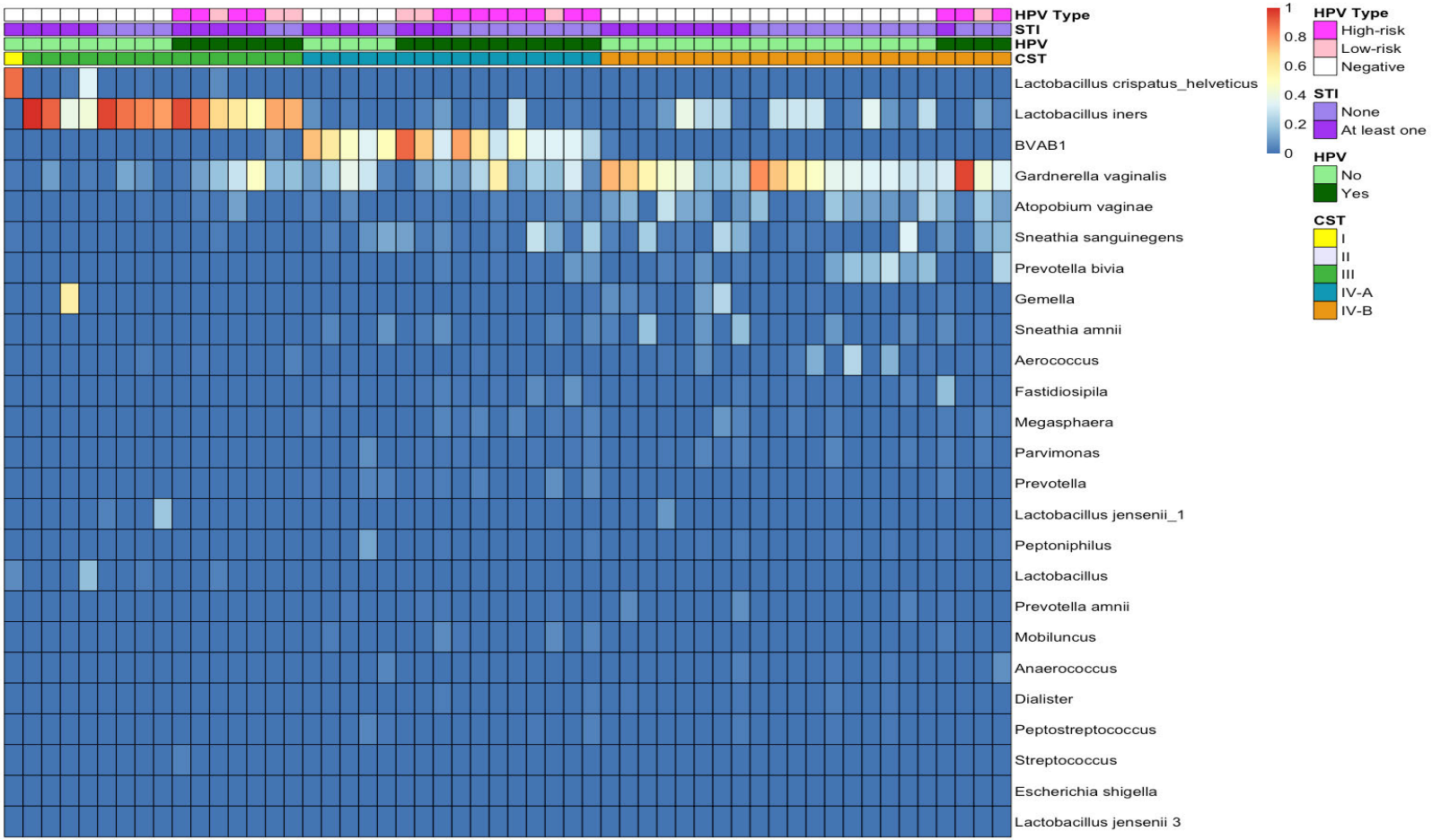


Figure 3.3. Heatmap of the relative abundances of bacterial taxa in the 54 vaginal microbiota from HPV-positive and HPV-negative women. Taxa names of bacteria are shown on the right of the heatmap while rows represent the bacterial taxa and columns the samples. The colour key for the relative abundances is indicated in the upper right corner. Bacterial community state types were

classified based on the dominant species: CST I dominated by *Lactobacillus crispatus*, CST III by *Lactobacillus iners,* CST IV-A by *BVAB1* and CST IV-B by *Gardnerella vaginalis.*

Next, we investigated whether the composition of the vaginal microbiota was influenced by HPV infection status. *Lactobacillus iners*, *BVAB1*, and *Gardnerella vaginalis* were the predominant bacterial species across the oncogenic, non-oncogenic, and no HPV-infection groups. However, *Lactobacillus iners* and *BVAB1* were more prevalent in the non-oncogenic HPV-infection group compared to the no HPV infection group. Additionally, the abundance of *Lactobacillus iners* was lower in women infected with oncogenic HPV genotypes than in those with non-oncogenic HPV infections or no HPV infection. Women without HPV infection also had a higher relative abundance of *Mobiluncus* compared to those with either oncogenic or non-oncogenic HPV infections. *BVAB1* communities were higher in single HPV genotype–infected women compared with multiple HPV genotype–infected women (**Figure 3.4**).

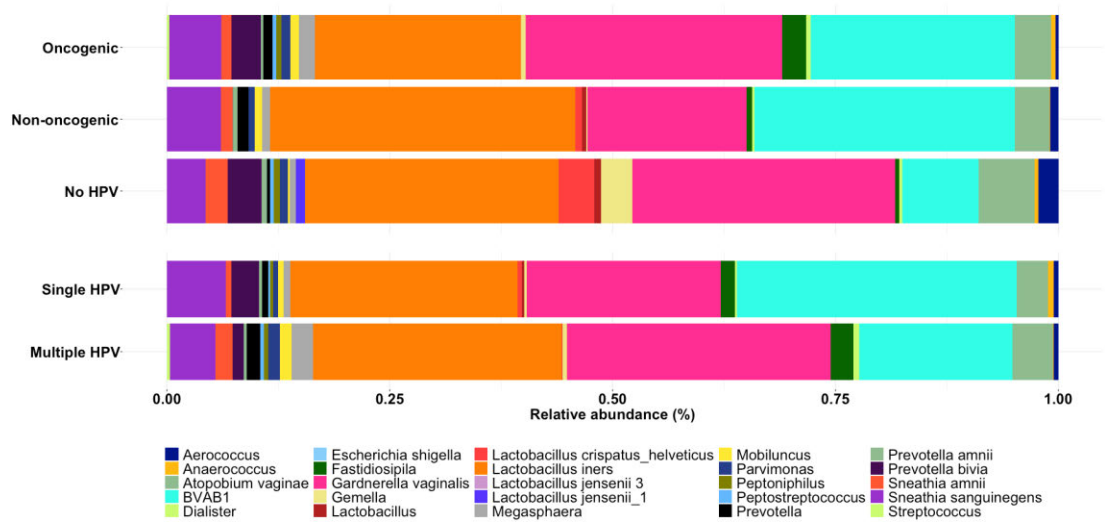


Figure 3.4. The composition of vaginal microbiota is dependent on the HPV infection status. Community barplot analysis on genus level among oncogenic and non-oncogenic, single infection, multiple and no HPV genotype infections.

3.3.5 Impact of HPV infection on vaginal microbiota diversity

We also investigated whether HPV infection affected the diversity of the vaginal microbiota. Shannon diversity analyses revealed that women infected with CST IV-A and IV-B had significantly higher microbial alpha diversity (median 0.46, IQR 0.37–0.54) compared to those with CST I and III (median 0.19, IQR 0.11–0.23, $p < 0.001$) (**Figure 3.5**). However, alpha diversity analyses of the vaginal microbiota showed no significant differences between women with oncogenic HPV and those with any HPV infection compared to women with non-oncogenic HPV or those without HPV infection. The same holds true when comparing women with single HPV infection and those with multiple HPV infection, respectively.

We further compared beta diversity of the vaginal microbiota across CSTs, HPV, and oncogenic HPV groups using non-metric multidimensional scaling (NMDS), with Bray-Curtis dissimilarities as the foundation for comparison [57]. Beta diversity analyses revealed that cervical microbiota dominated by *Lactobacillus* species were spatially segregated from those dominated by non-*Lactobacillus* species, indicating distinct compositional differences between the two groups (pseudo-F statistic = 14.3, $p = 0.001$) (**Figure 3.6**). Women with *Lactobacillus*-dominant communities, particularly those with *L. iners*-dominant CST III (pseudo-F statistic = 26.86, $p = 0.001$), clustered together. Similarly, women with BV-associated CSTs IV-A and IV-B (pseudo-F statistic = 29.08, $p = 0.001$) formed a distinct cluster in the NMDS2 region. However, there was no distinct segregation by HPV status or number of genotypes (single or multiple HPV infections) within these clusters. Furthermore, there was no distinct segregation in the cervical microbiota between women with oncogenic HPV and those with non-oncogenic HPV (pseudo-F statistic = 1.245, $p = 0.26$).

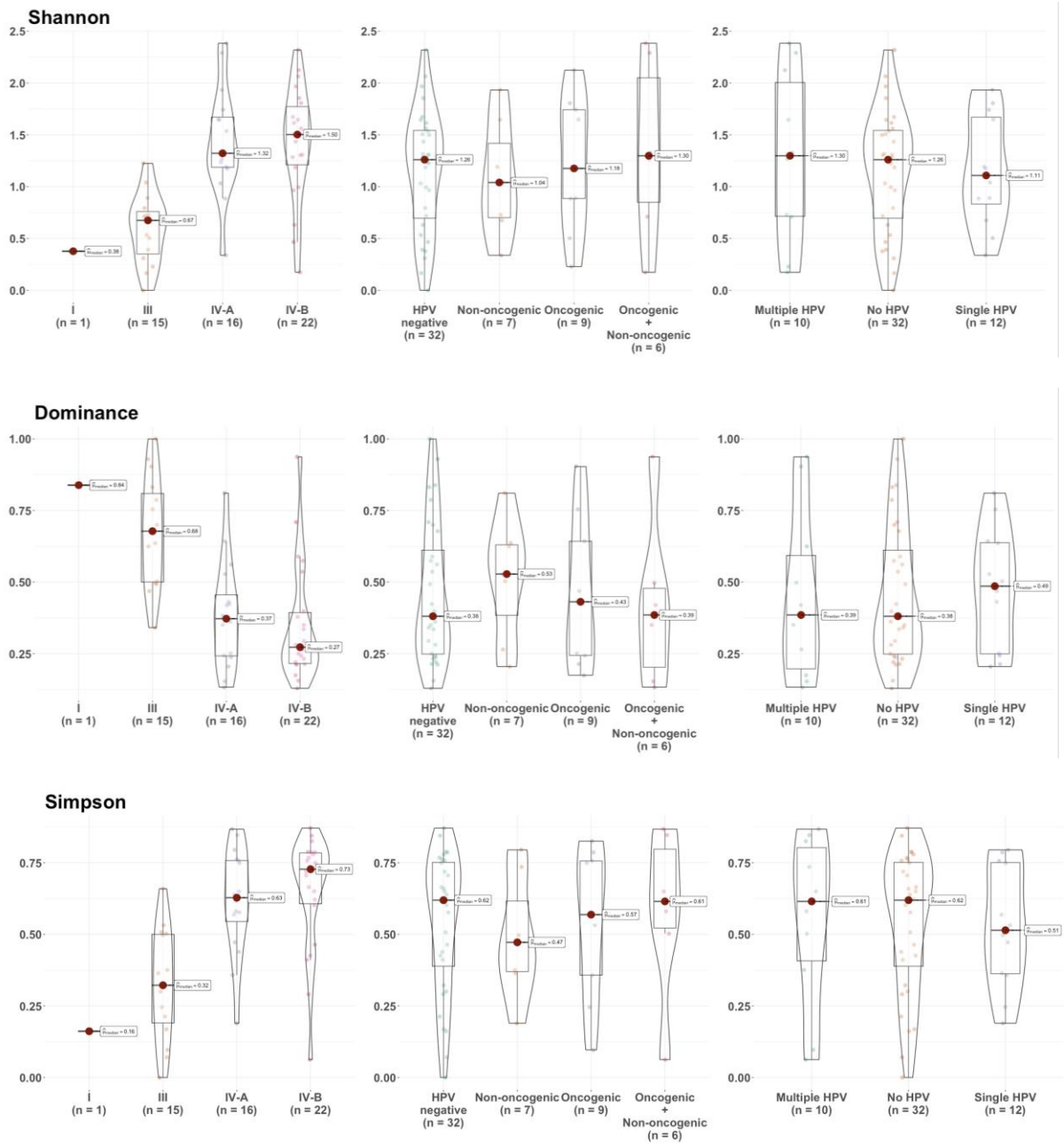


Figure 3.5: Microbial alpha diversity measured by Shannon, Dominance, and Simpson index according to CSTs, HPV type, and number of HPV types. Microbiota diversity of the group of uninfected women, and the groups infected with oncogenic plus non-oncogenic HPVs, oncogenic HPVs, and non-oncogenic HPVs were compared. Microbiota diversity comparison of HPV uninfected group, and groups infected with single and multiple HPV types. Statistical significance between the groups was tested by Wilcoxon signed-rank test (*$q < 0.05$). Data was presented as mean values with standard deviations.

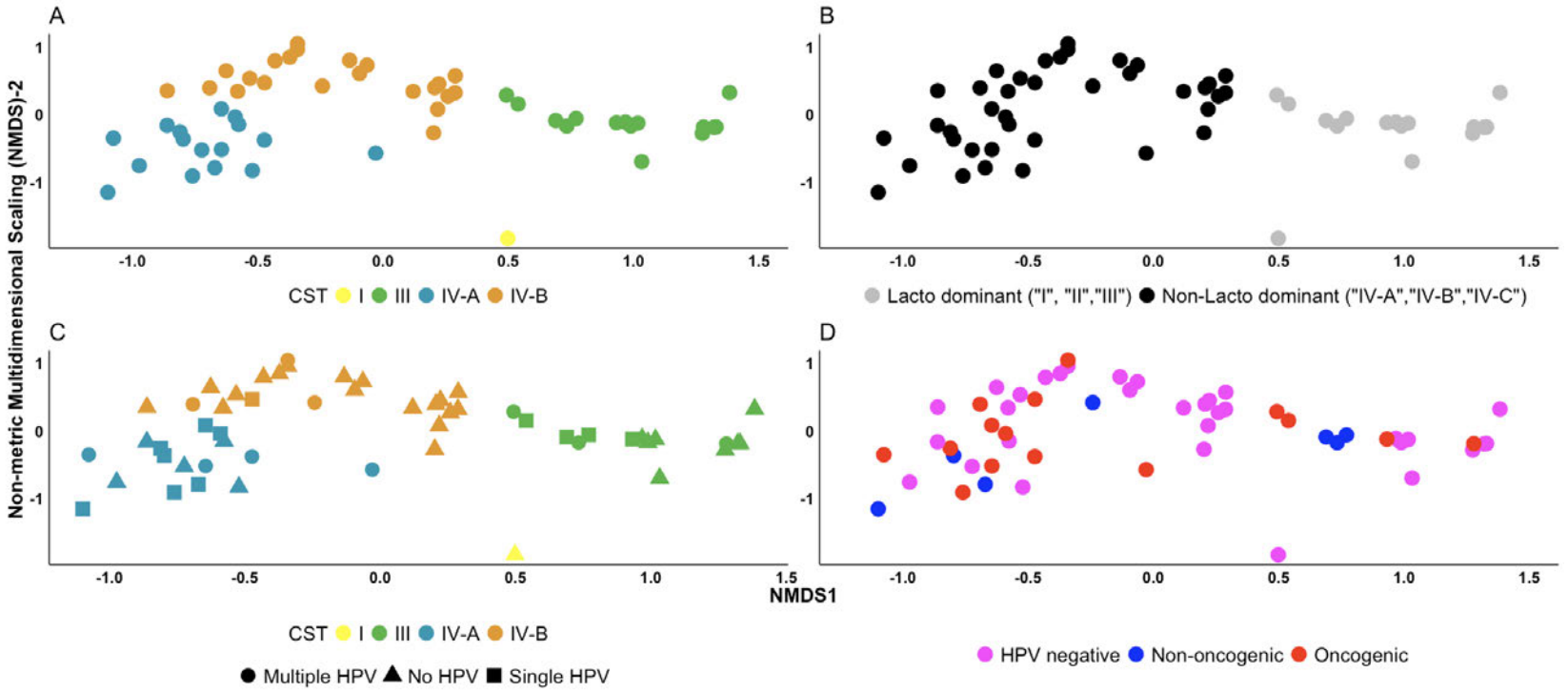


Figure 3.6: Non-metric Multi-dimensional Scaling (NMDS) plots showing beta diversity of the vaginal microbiome by groups: (**A**) CSTs (CST III = green, CST IV-A = light blue, CST IV-B = mustard), B) *lactobacillus* (grey), and non-*lactobacillus* dominant (black) community state type (CSTs), C) CSTs (CST III = green, CST IV-A = light blue, CST IV-B = mustard), and number of HPV strains(multiple HPV strains= circle, negative = triangle, single HPV strain= square), and D) High-risk (Oncogenic) and Low-risk (Non-oncogenic) HPV infection (negative=pink, non- oncogenic=blue, oncogenic= red).

3.3.6 Immune mediators and HPV status in *lactobacillus* and non-*lactobacillus* dominance.

We investigated how HPV status influences cytokine profiles in women with *Lactobacillus*-dominant vaginal communities. In multivariate analyses controlling for age and at least one STI, we found that concentrations of the chemokines MIP-1β (β = 1.4, 95% CI: 0.26 to – 2.5, p = 0.033), RANTES (β = 1.4, 95% CI: 0.23 to 2.6, p = 0.037), and the growth factor IL-7 (β = 1.9, 95% CI: 0.51 to 3.2, p = 0.019) were significantly higher in women with *Lactobacillus* dominance who had any HPV infection compared to those with *Lactobacillus* dominance but no HPV infection (**Table 3.3**). Conversely, concentrations of CTACK were significantly lower in women with *Lactobacillus* dominance and any HPV infection (β = -2.9, 95% CI: -4.3 to – (-1.5), p = 0.002). Similarly, women with *Lactobacillus* dominance and oncogenic HPV infection had reduced CTACK levels (β = -2.8, 95% CI: -4.8 to – (-0.92), p = 0.013) compared to those with *Lactobacillus* dominance and no oncogenic HPV infection.

Table 3.3: Genital cytokines concentrations by any HPV status in *lactobacillus* dominance

Variables	*Lactobacillus*-dominant							
	Any HPV				Oncogenic HPV			
	Unadjusted β (95% CI)	p-value	Adjusted β (95% CI)	p-value	Unadjusted β (95% CI)	p-value	Adjusted β (95% CI)	p-value
Proinflammatory								
IL-1α	-0.95(-2.61 - 0.71)	0.282	-0.99(-2.58 - 0.60)	0.247	0.39(-1.58 - 2.37)	0.701	-0.49(-2.45 - 1.47)	0.633
IL-1β	0.20(-1.53 - 1.93)	0.823	0.81(-1.01 - 2.64)	0.399	0.31(-1.67 - 2.29)	0.762	0.41(-1.78 - 2.60)	0.720
IL-6	0.31(-0.36 - 0.99)	0.373	0.31(-0.48 - 1.10)	0.461	-0.05(-0.84 - 0.74)	0.898	-0.15(-1.09 - 0.80)	0.767
IL-12p70	0.69(-0.69 - 2.07)	0.345	1.20(-0.27 - 2.66)	0.135	0.69(-0.90 - 2.28)	0.411	1.08(-0.71 - 2.87)	0.258
IL-12p40	-1.07(-2.85 - 0.71)	0.258	-1.02(-2.86 - 0.81)	0.295	-0.71(-2.81 - 1.40)	0.521	-1.69(-3.73 - 0.34)	0.129
IL-18	-0.58(-2.06 - 0.89)	0.450	-0.34(-1.70 - 1.02)	0.635	0.46(-1.25 - 2.17)	0.606	-0.18(-1.78 - 1.43)	0.831
MIF	-0.06(-1.01 - 0.89)	0.910	-0.02(-1.05 - 1.02)	0.974	-0.31(-1.38 - 0.77)	0.587	-0.75(-1.89 - 0.38)	0.217
TNF-α	0.33(-0.66 - 1.33)	0.522	0.67(-0.40 - 1.75)	0.244	0.23(-0.93 - 1.38)	0.704	0.40(-0.92 - 1.72)	0.567
TNF-β	-0.20(-1.48 - 1.08)	0.766	-0.01(-1.38 - 1.36)	0.985	0.39(-1.07 - 1.85)	0.610	0.06(-1.54 - 1.66)	0.944
TRAIL	-0.81(-2.72 - 1.10)	0.419	-0.57(-2.41 - 1.26)	0.550	-0.08(-2.32 - 2.17)	0.947	-1.06(-3.15 - 1.03)	0.340
Chemokine								
CTACK	-2.68(-3.91- (-1.45)	**<0.001**	-2.89(-4.28 - (1.49)	**0.002**	-2.20(-4.01- (-0.40)	**0.031**	-2.85(-4.78 - (0.92)	**0.013**

Eotaxin	0.78(-0.81 - 2.37)	0.355	1.40(-0.26 - 3.05)	0.125	0.02(-1.85 - 1.90)	0.980	0.46(-1.67 - 2.60)	0.678
GRO-α	-1.13(-2.81 - 0.54)	0.206	-0.55(-2.24 - 1.14)	0.535	-0.15(-2.18 - 1.89)	0.889	-0.31(-2.31 - 1.70)	0.770
IL-16	-0.82(-2.57 - 0.94)	0.378	-0.49(-2.29 - 1.32)	0.607	0.04(-2.03 - 2.11)	0.970	-0.51(-2.63 - 1.61)	0.644
IL-8	0.43(-0.32 - 1.19)	0.279	0.72(-0.07 - 1.51)	0.101	0.36(-0.52 - 1.24)	0.439	0.56(-0.44 - 1.56)	0.293
IP-10	0.22(-1.18 - 1.62)	0.762	0.87(-0.48 - 2.21)	0.231	0.29(-1.32 - 1.89)	0.732	0.41(-1.26 - 2.07)	0.641
MCP-1	0.39(-0.59 - 1.37)	0.449	0.79(-0.23 - 1.80)	0.155	-0.02(-1.17 - 1.13)	0.972	0.23(-1.06 - 1.52)	0.734
MCP-3	-0.67(-2.57 - 1.22)	0.498	-0.36(-2.32 - 1.59)	0.721	-0.57(-2.76 - 1.62)	0.618	-1.39(-3.55 - 0.77)	0.232
MIG	-1.14(-2.59 - 0.31)	0.145	-1.08(-2.41- 0.26)	0.141	0.03(-1.77 - 1.83)	0.974	-0.79(-2.45 - 0.87)	0.371
MIP-1α	0.88(-0.38 - 2.15)	0.192	1.31(-0.04 - 2.66)	0.082	0.37(-1.16 - 1.90)	0.644	0.80(-0.95 - 2.55)	0.388
MIP-1β	0.87(-0.27 - 2.02)	0.156	1.40(0.26 - 2.53)	**0.033**	0.48(-0.91 - 1.87)	0.510	0.89(-0.65 - 2.42)	0.281
RANTES	0.82(-0.45 - 2.10)	0.226	1.43(0.23 - 2.62)	**0.037**	0.73(-0.76 - 2.23)	0.352	0.83(-0.79 - 2.45)	0.335
IFN-α2	-0.88(-2.17 - 0.40)	0.201	-0.66(-1.98 - 0.66)	0.344	-0.47(-2.01 - 1.08)	0.563	-1.00(-2.50 - 0.50)	0.216
SDF-1α	0.32(-0.68 - 1.31)	0.543	0.43(-0.72 - 1.59)	0.477	0.28(-0.86 - 1.42)	0.638	0.49(-0.87 - 1.84)	0.495
Growth Factor								
β-NGF	-0.46(-2.54 - 1.62)	0.669	-0.52(-2.01 - 0.97)	0.505	1.13(-1.19 - 3.46)	0.357	-0.39(-2.15 - 1.38)	0.675
FGF-basic	1.28(0.03 - 2.54)	0.065	1.37(-0.09 - 2.84)	0.091	0.81(-0.76 - 2.38)	0.330	0.75(-1.15 - 2.64)	0.454
G-CSF	1.02(-0.62 - 2.66)	0.244	1.56(-0.22 - 3.33)	0.112	0.90(-1.02 - 2.82)	0.372	1.18(-1.05 - 3.40)	0.321
GM-CSF	0.83(-0.34 - 2.01)	0.186	0.58(-0.75 - 1.92)	0.410	0.59(-0.81 - 1.99)	0.424	0.63(-0.94 - 2.20)	0.447

HGF	-0.37(-2.27 - 1.53)	0.708	-0.07(-2.10 - 1.96)	0.944	0.69(-1.46 - 2.85)	0.539	0.28(-2.09 - 2.65)	0.822
IL-3	-0.48(-1.74 - 0.78)	0.467	-0.24(-1.60 - 1.11)	0.730	-0.02(-1.50 - 1.45)	0.975	-0.32(-1.91 - 1.26)	0.696
IL-7	1.35(0.04 - 2.66)	0.063	1.87(0.51- 3.24)	**0.019**	1.11(-0.50 - 2.71)	0.198	1.45(-0.40 - 3.30)	0.150
IL-9	0.87(-0.35 - 2.10)	0.185	1.37(0.12 - 2.62)	0.053	0.62(-0.84 - 2.09)	0.418	1.18(-0.41 - 2.77)	0.170
LIF	-0.13(-1.43 - 1.18)	0.854	0.04(-1.08 - 1.17)	0.943	0.62(-0.84 - 2.09)	0.418	-0.07(-1.39 - 1.25)	0.921
M-CSF	-0.87(-2.23 - 0.49)	0.231	-0.65(-1.93 - 0.63)	0.340	0.47(-1.16 - 2.09)	0.581	-0.04(-1.61 - 1.52)	0.956
PDGF-BB	0.56(-0.54 - 1.66)	0.334	0.95(-0.23 - 2.13)	0.140	0.39(-0.90 - 1.68)	0.561	0.57(-0.91 - 2.05)	0.465
SCF	-0.48(-2.40 - 1.45)	0.635	-0.06(-2.08 - 1.96)	0.955	-0.19(-2.41- 2.02)	0.868	-0.73(-3.07 - 1.60)	0.550
SCGF-β	-0.26(-0.85 - 0.33)	0.396	-0.49(-1.10 - 0.13)	0.149	-0.20(-0.88 - 0.49)	0.582	-0.40(-1.16 - 0.36)	0.320
VEGF	0.47(-0.94 - 1.88)	0.522	1.04(-0.41 - 2.49)	0.186	0.69(-0.91 - 2.28)	0.414	0.97(-0.78 - 2.72)	0.298
Adaptive response								
IFN-γ	0.37(-0.85 - 1.59)	0.558	0.78(-0.55 - 2.10)	0.274	0.19(-1.22 - 1.60)	0.793	0.43(-1.19 - 2.05)	0.615
IL-2	0.65(-0.88 - 2.18)	0.418	0.73(-0.93 - 2.38)	0.406	0.12(-1.68 - 1.92)	0.897	-0.55(-2.52 - 1.43)	0.597
IL-4	0.13(-0.23 - 0.48)	0.501	0.21(-0.19 - 0.61)	0.324	-0.02(-0.43 - 0.39)	0.921	0.01(-0.48 - 0.50)	0.963
IL-5	0.41(-0.87 - 1.70)	0.537	0.88(-0.47 - 2.22)	0.226	0.98(-0.42 - 2.38)	0.191	1.20(-0.34 - 2.74)	0.152
IL-13	-0.02(-1.55 - 1.51)	0.982	-0.08(-1.84 - 1.67)	0.928	-0.54(-2.27 - 1.19)	0.553	-0.31(-2.36 - 1.74)	0.771
IL-15	0.48(-0.62 - 1.58)	0.408	0.57(-0.69 - 1.82)	0.393	0.10(-1.20 - 1.39)	0.884	-0.17(-1.69 - 1.34)	0.826
IL-17	1.12(-0.41 - 2.65)	0.173	1.01(-0.77 - 2.79)	0.290	0.74(-1.09 - 2.58)	0.440	0.52(-1.65 - 2.69)	0.648

IL-2Ra	-0.26(-1.74 - 1.22)	0.734	-0.00(-1.34 - 1.33)	0.997	0.38(-1.32 - 2.07)	0.670	-0.35(-1.90 - 1.21)	0.668
Anti-inflammatory								
IL-10	0.60(-0.54 - 1.74)	0.320	1.01(-0.22 - 2.23)	0.134	0.56(-0.77 - 1.89)	0.423	0.83(-0.68- 2.34)	0.302
IL-1ra	1.38(-0.33 - 3.10)	0.137	1.89(-0.01 - 3.78)	0.076	0.75(-1.35 - 2.84)	0.497	1.08(-1.39 - 3.56)	0.407

CI, confidence interval, Beta-coefficient (β), Any HPV includes High-risk (Hr) HPV, Low-risk (LR) HPV, and single and multiple HPV infections. The effect coefficient and corresponding p-values were determined using linear regression analysis. a(β): adjusted β-coefficient in a multivariable model controlling for age, at least one STI, and *Lactobacillus* dominance. P-values less than 0.05 were considered statistically significant.

We further evaluated how HPV status affects the cytokine profiles in women with non-*Lactobacillus*-dominant vaginal communities. In this study, we observed decreased concentrations of pro-inflammatory cytokines and chemokines in women with non-*Lactobacillus* dominance who had any HPV infection compared to those with non-*Lactobacillus* dominance but no HPV infection. Specifically, TRAIL (β = -1.0, 95% CI: -2.0 – 0.10, p = 0.038) and several chemokines, including MIG (β = -0.66, 95% CI: -1.1 to – 0.19, p = 0.010), and SDF-1α (β = -0.62, 95% CI: -1.18 – 0.06, p = 0.037) in multivariate analyses, as well as IP-10 (β = -1.1, 95% CI: -1.9 – 0.16, p = 0.026) in univariate analyses (**Table 3.4**). Multivariate analyses showed that concentrations of pro-inflammatory cytokine TNF-β (β = -0.37, 95%CI: -0.95 – 0.02, p = 0.049); chemokines eotaxin (β = -1.2, 95%CI: -2.3 – 0.17, p = 0.030), IL-16 (β = - 1.4, 95%CI: -2.6 to – 0.23, p = 0.025), and MIG (β = -0.66, 95%CI: -1.2 to 0.16, p = 0.014) were significantly lower, while IFN-α2 (β = -1.1, 95%CI: -2.1 to 0.01, p=0.061) trended towards a decrease in women with non-*Lactobacillus* dominance and oncogenic HPV infection compared to those with non-*Lactobacillus* dominance and without oncogenic HPV infection.

Table 3.4: Genital cytokines concentrations by any HPV status in non-*lactobacillus* dominance

	***non Lactobacillus*-dominant**							
Variables	**Any HPV**				**Oncogenic**			
	Unadjusted	**p-value**	**Adjusted**	**p-value**	**Unadjusted**	**p-value**	**Adjusted**	**p-value**
	β (95% CI)		**β (95% CI)**		**β (95% CI)**		**β (95% CI)**	
Proinflammatory								
IL-1α	-0.22(-0.85 - 0.40)	0.487	-0.16(-0.77 - 0.45)	0.611	-0.26(-0.91 - 0.40)	0.447	-0.24(-0.87 - 0.40)	0.471
IL-1β	-0.64(-1.63 - 0.35)	0.215	-0.46(-1.40 - 0.49)	0.352	-0.75(-1.79 - 0.28)	0.161	-0.65(-1.63 - 0.34)	0.206
IL-6	-0.27(-0.67 - 0.14)	0.203	-0.22(-0.64 - 0.20)	0.319	-0.34(-0.76 - 0.08)	0.119	-0.31(-0.74 - 0.13)	0.176
IL-12p70	-0.27(-1.07 - 0.53)	0.512	-0.11(-0.90 - 0.67)	0.776	-0.28(-1.12 - 0.56)	0.513	-0.17(-0.99 - 0.64)	0.678
IL-12p40	-0.83(-1.83 - 0.18)	0.116	-0.90(-1.95 - 0.15)	0.101	-0.88(-1.93 - 0.18)	0.112	-0.98(-2.07 - 0.11)	0.088
IL-18	-0.45(-1.27 - 0.37)	0.284	-0.54(-1.36 - 0.28)	0.208	-0.64(-1.49 - 0.21)	0.149	-0.77(-1.61 - 0.07)	0.081
MIF	-0.41(-1.07 - 0.25)	0.231	-0.30(-0.92 - 0.32)	0.346	-0.65(-1.33 - 0.03)	0.068	-0.61(-1.23 - 0.02)	0.067
TNF-α	-0.29(-0.89 - 0.32)	0.357	-0.18(-0.78 - 0.41)	0.553	-0.41(-1.04 - 0.22)	0.209	-0.35(-0.96 - 0.27)	0.279
TNF-β	-0.30(-0.79 - 0.20)	0.246	-0.37(-0.83 - 0.08)	0.114	-0.37(-0.88 - 0.15)	0.170	-0.48(-0.95 - 0.02)	**0.049**
TRAIL	-1.01(-1.92 - (0.11)	**0.035**	-1.05(-2.00 - (0.10)	**0.038**	-0.98(-1.94 - (0.01)	0.055	-1.03(-2.03 - 0.02)	0.053
Chemokine								
CTACK	-0.32(-1.33 - 0.70)	0.544	-0.03(-1.03 - 0.98)	0.961	-0.15(-1.23 - 0.92)	0.783	0.13(-0.92 - 1.18)	0.811

Eotaxin	-0.97(-1.98 - 0.05)	0.071	-0.92(-1.95 - 0.10)	0.086	-1.19(-2.24 - (0.14)	**0.033**	-1.21(-2.25 - 0.17)	**0.030**
GRO-α	-0.18(-0.70 - 0.35)	0.510	-0.16(-0.71 - 0.38)	0.561	0.03(-0.52 - 0.59)	0.914	0.04 (-0.53 - 0.61)	0.894
IL-16	-1.03(-2.17 - 0.10)	0.083	-1.09(-2.23 - 0.06)	0.072	-1.28(-2.45 - (0.10)	**0.040**	-1.40(-2.57 - 0.23)	**0.025**
IL-8	-0.43(-0.95 - 0.09)	0.111	-0.42(-0.92 - 0.08)	0.107	-0.46(-1.00 - 0.09)	0.108	-0.48(-1.01 - 0.04)	0.077
IP-10	-1.05(-1.94 - (0.16)	**0.026**	-0.96(-1.89 - (0.02)	0.052	-0.72(-1.70 - 0.25)	0.153	-0.62(-1.63 - 0.39)	0.239
MCP-1	0.17(-0.72 - 1.06)	0.716	0.32(-0.60 - 1.24)	0.496	-0.02(-0.96 - 0.92)	0.965	0.10(-0.87 - 1.07)	0.837
MCP-3	-0.13(-1.28 - 1.01)	0.822	-0.16(-1.36 - 1.04)	0.799	-0.33(-1.53 - 0.87)	0.594	-0.40(-1.65 - 0.85)	0.537
MIG	-0.64(-1.09 - (0.19)	**0.009**	-0.66(-1.13 - (0.19)	**0.010**	-0.63(-1.12 - (0.15)	**0.014**	-0.66(-1.16 - 0.16)	**0.014**
MIP-1α	-0.68(-1.58 - 0.22)	0.149	-0.63(-1.57 - 0.30)	0.193	-0.54(-1.51 - 0.42)	0.274	-0.52(-1.51- 0.47)	0.307
MIP-1β	-0.22(-0.83 - 0.39)	0.479	-0.11(-0.74 - 0.51)	0.721	-0.15(-0.79 - 0.49)	0.646	-0.05(-0.71 - 0.61)	0.878
RANTES	-0.46(-1.46 - 0.55)	0.377	-0.49(-1.49 - 0.52)	0.351	-0.31(-1.38 - 0.75)	0.565	-0.39(-1.45 - 0.67)	0.480
IFN-α2	-0.93(-2.00 - 0.13)	0.095	-0.89(-1.92 - 0.15)	0.101	-1.03(-2.14 - 0.09)	0.080	-1.06(-2.13 - 0.01)	0.061
SDF-1α	-0.59(-1.13 - (0.05)	**0.040**	-0.62(-1.18 - (0.06)	**0.037**	-0.31(-0.91 - 0.28)	0.312	-0.34(-0.96 - 0.27)	0.279
Growth Factor								
β-NGF	-0.91(-2.09 - 0.28)	0.143	-0.96(-2.17 - 0.24)	0.127	-0.93(-2.18 - 0.32)	0.154	-1.04(-2.30 - 0.22)	0.116
FGF-basic	-0.14(-1.12 - 0.83)	0.773	-0.05(-1.06 - 0.96)	0.923	-0.16(-1.18 - 0.87)	0.769	-0.10(-1.15 - 0.95)	0.852
G-CSF	-0.36(-1.10 - 0.39)	0.352	-0.18(-0.90 - 0.53)	0.614	-0.34(-1.12 - 0.44)	0.402	-0.21(-0.95 - 0.53)	0.584
GM-CSF	0.00(-0.91 - 0.91)	0.995	-0.01(-0.94 - 0.92)	0.988	-0.28(-1.23 - 0.67)	0.567	-0.35(-1.32 - 0.62)	0.486

HGF	-0.47(-1.11 - 0.18)	0.162	-0.41(-1.04 - 0.22)	0.208	-0.61(-1.28 - 0.05)	0.079	-0.61(-1.25 - 0.03)	0.072
IL-3	-0.23(-1.31 - 0.85)	0.676	-0.23(-1.33 - 0.87)	0.685	-0.17(-1.30 - 0.97)	0.776	-0.21(-1.37 - 0.95)	0.726
IL-7	-0.12(-0.56 - 0.33)	0.611	-0.03(-0.47 - 0.41)	0.895	-0.10(-0.57- 0.36)	0.665	-0.04(-0.50 - 0.43)	0.875
IL-9	-0.36(-0.93 - 0.22)	0.235	-0.26(-0.83 - 0.32)	0.386	-0.41(-1.02 - 0.19)	0.189	-0.35(-0.94 - 0.25)	0.262
LIF	-0.13(-0.51 - 0.26)	0.516	-0.12(-0.50 - 0.26)	0.548	-0.24(-0.64 - 0.16)	0.252	-0.26(-0.65 - 0.14)	0.211
M-CSF	-0.34(-0.96 - 0.28)	0.292	-0.30(-0.93 - 0.33)	0.361	-0.37(-1.02 - 0.29)	0.276	-0.36(-1.02 - 0.30)	0.294
PDGF-BB	-0.17(-0.79 - 0.45)	0.596	-0.07(-0.69 - 0.56)	0.835	-0.20(-0.84 - 0.45)	0.558	-0.12(-0.77 - 0.53)	0.712
SCF	-0.81(-2.02 - 0.40)	0.200	-0.68(-1.91 - 0.56)	0.290	-0.96(-2.22 - 0.31)	0.146	-0.89(-2.17 - 0.39)	0.181
SCGF-β	-0.35(-0.94 - 0.24)	0.253	-0.24(-0.85 - 0.37)	0.453	-0.31(-0.93 - 0.31)	0.338	-0.20(-0.84 - 0.44)	0.551
VEGF	-0.40(-1.16 - 0.37)	0.321	-0.29(-1.03 - 0.46)	0.457	-0.39(-1.20 - 0.43)	0.358	-0.33(-1.11 - 0.46)	0.419
Adaptive response								
IFN-γ	-0.59(-1.41- 0.23)	0.169	-0.56(-1.35- 0.23)	0.176	-0.76(-1.61- 0.09)	0.087	-0.80(-1.61- 0.01)	0.061
IL-2	0.07(-0.60- 0.73)	0.846	0.11(-0.54- 0.76)	0.741	0.16(-0.54- 0.85)	0.658	0.16(-0.51- 0.84)	0.641
IL-4	-0.32(-0.85- 0.21)	0.242	-0.24(-0.76- 0.28)	0.372	-0.41(-0.96- 0.14)	0.153	-0.36(-0.90- 0.17)	0.194
IL-5	-0.07(-0.96- 0.82)	0.881	0.07(-0.86- 1.00)	0.886	-0.46(-1.39- 0.46)	0.333	-0.35(-1.32- 0.62)	0.483
IL-13	-0.50 (-1.22- 0.22)	0.184	-0.51 (-1.21- 0.19)	0.160	-0.67(-1.42- 0.08)	0.088	-0.74(-1.45- (-0.03)	**0.049**
IL-15	0.06 (-0.68- 0.79)	0.883	0.09 (-0.65- 0.84)	0.808	-0.12(-0.89- 0.65)	0.766	-0.13(-0.91- 0.65)	0.751
IL-17	-0.27 (-1.42- 0.89)	0.654	-0.21 (-1.38- 0.97)	0.732	-0.59(-1.80- 0.61)	0.339	-0.61(-1.83- 0.60)	0.331

IL-2Ra	-0.67 (-1.39- 0.05)	0.078	-0.65 (-1.37- 0.08)	0.091	-0.53 (-1.31- 0.24)	0.186	-0.54(-1.31- 0.23)	0.181
Anti-inflammatory								
IL-10	-0.17 (-0.84- 0.49)	0.616	-0.06 (-0.72- 0.61)	0.865	-0.19(-0.89- 0.51)	0.595	-0.11(-0.81- 0.58)	0.752
IL-1ra	0.37 (-0.63- 1.37)	0.472	0.36 (-0.66- 1.38)	0.492	0.37(-0.68- 1.42)	0.496	0.31(-0.76- 1.38)	0.571

Beta-coefficient (β), Any HPV includes High-risk (Hr) HPV, Low-risk (LR) HPV, and single and multiple HPV infections. The effect coefficient and corresponding p-values were determined using linear regression analysis. a(β): adjusted β-coefficient in a multivariable model controlling for age, at least one STI, and *Lactobacillus dominance*. P-values less than 0.05 were considered statistically significant.

3.3.7 The Relationship Between HPV Status and Endocervical Cell Frequency in *Lactobacillus*-Dominant communities

The mechanism through which elevated genital cytokines increase the risk of HPV infection and related diseases in the context of vaginal microbiota is indeed complex and not entirely understood. For this reason, we compared the frequencies of T cell and DC subsets in women predominantly colonized by *Lactobacillus* communities and tested positive for any type of HPV or hr-HPV. There were no significant differences in the frequencies of cervical T cells or DC subsets between *Lactobacillus*-dominant women with any HPV infection and those dominated by *Lactobacillus* without any HPV infection (**Table 3.5**). In women with *lactobacillus*-dominance, oncogenic HPV infection was associated with an increase in DC subsets [HLADR+CD14-CD123+; β = 24.0, 95% CI: 9.47 to – 38.6, p = 0.014**;** CD14- HLADR+, β = 29.6, 95%CI: 9.72 to – 49.6, p = 0.022], and a reduced frequency CD86+HLADR++CD14+ cells [β = -10.9, 95%CI: -17.5 to – 4.47, p = 0.013] compared to women with non *lactobacillus*-dominance without oncogenic HPV infection. In addition, women with dominant non-*Lactobacillus* communities who tested positive for any HPV infection had a reduced frequency of cervical T cell subset [CD38+ CD4+, β = -15, 95% CI: -25 to – 5.4, p = 0.005, while CCR5+ CD4+ T cells [β = -13.9, 95% CI: -26.7 to –1.07, p = 0.050] trended towards a reduced frequency, compared to those dominated by *Lactobacillus* without any HPV infection. Furthermore, the frequencies of cervical T cells [CD38+ CD4+, β = -14.9, 95% CI: -25.5 to – 4.21, p = 0.015] and DC subsets [HLADR++CD14+, β = -21.9, 95%CI: -39.1 to – 4.78, p = 0.023] were reduced in women with oncogenic HPV, in multivariable analysis.

Table 3.5: Endocervical Cell Prevalence and HPV Status in *Lactobacillus*-Dominant Vaginal Environments

Cells	*Lactobacillus* dominant				Non-*Lactobacillus* dominant			
	Any HPV		oncogenic HPV		Any HPV		oncogenic HPV	
	Adjusted β (95%CI)	p-value	Adjusted β (95%CI)	p-value	Adjusted β (95%CI)	p-value	Adjusted β (95%CI)	p-value
CD4+	-11.0(-29.3- 7.27)	0.277	-20.2(-38.6 - (-1.84)	0.068	-3.24(-11.0 - 4.56)	0.428	-2.48(-10.5 - 5.62)	0.558
CD38+ CD4+	-0.06(-20.1- 19.9)	0.995	7.17(-15.9 - 30.3)	0.563	-16.1(-25.8- (-6.41)	**0.005**	-14.8(-25.4 - (-4.21)	**0.015**
HLADR+ CD4+	-13.35(-32.4- 5.73)	0.213	-14.4(-37.6 - 8.74)	0.262	-12.2(-26.1- 1.73)	0.105	-11.4(-26.0 - 3.09)	0.142
CCR5+ CD4+	-6.98(-20.4 - 6.46)	0.342	9.33(-24.9 - 6.30)	0.280	-13.9(-26.7- (-1.07)	**0.050**	-8.94(-23.2 - 5.36)	0.238
CCR6+ CD4+	-10.62(-23.3- 2.05)	0.144	-8.60(-25.1 - 7.92)	0.341	-10.8(-24.3 - 2.74)	0.138	-9.26(-23.5 - 5.01)	0.222
HLADR+CD14-CD123+	7.65(-10.9 - 26.2)	0.445	24.0(9.47 - 38.6)	**0.014**	8.67(-1.34 - 18.6)	0.109	6.74(-3.96 - 17.4)	0.235
CD86+ CD123+	5.60(-5.33 - 16.5)	0.348	0.12(-13.7 - 14.0)	0.987	-6.01(-17.9 - 5.96)	0.340	-7.51(-19.6 - 4.64)	0.243
HLADR+CD14-CD11C+	22.2(-7.22- 51.7)	0.183	-16.5(-54.6 - 21.7)	0.425	-12.1(-33.9 - 9.52)	0.288	-14.2(-36.3 - 7.84)	0.224
CD86+ CD11c+	0.62(-32.5- 33.8)	0.972	9.01(-29.8 - 47.8)	0.663	-6.33(-22.9 - 10.3)	0.465	-11.7(-28.1- 4.67)	0.180
CD14- HLADR+	15.8(-6.20- 37.9)	0.202	29.6(9.72 - 49.6)	**0.022**	-4.16(-11.5 - 3.22)	0.285	-3.85(-11.5 - 3.80)	0.338
CD86+HLADR++CD14+	-3.45(-11.8- 4.96)	0.448	-10.9(-17.5- (-4.47)	**0.013**	-2.16(-14.7 - 10.5)	0.741	-0.76 (-13.8 - 12.3)	0.911
HLADR++ CD14+	-1.89(-21.3- 17.5)	0.854	7.20(-15.2 - 29.6)	0.550	-15.2(-33.4 - 2.97)	0.120	-21.9(-39.1 - (-4.78)	**0.023**

3.4 Discussion

HPV infection is linked to cervical cancer through changes in the cervical microbiota, which can influence the persistence of the virus and the associated inflammatory response[117]. However, the precise effects of HPV on immune responses and the impact of vaginal microbiota changes on these responses are still not fully understood. This study examined how HPV infection affects the vaginal microbiome, endocervical cell subsets, and genital cytokine profiles in 54 women with a Nugent score >4. Among women with detectable HPV genotypes, 40.9% had only oncogenic HPV types, 31.8% had only non-oncogenic types, and 27.3% had both. HPV types were more commonly detected in single infections compared to multiple infections. In multivariable analysis, HPV infection was linked to reduced frequencies of certain immune cell subsets and altered cytokine levels, with specific effects for oncogenic HPV types. Women with *Lactobacillus* dominance and any HPV infection had higher concentrations of certain cytokines but lower levels of others, while those with non-*Lactobacillus* dominance and HPV had diminished immune cell frequencies and cytokine levels.

It has been hypothesized that cytokine dysregulation may impact immune balance and influence the susceptibility of cervical epithelial cells to HPV infections [29, 163]. However, there is a limited amount of data on the relationship between local immune marker profiles and infections caused by oncogenic and non-oncogenic HPV infections. Several studies have observed that HPV-positive women might exhibit lower levels of specific cytokines in cervicovaginal samples [55, 264-266], supporting the findings of this study. Our study reveals several key findings related to HPV infection and its impact on immune function. HPV-positive women exhibited significantly decreased levels of TRAIL, a cytokine critical for inducing apoptosis [267-269], and a trend towards a decrease in IFN-α2, which plays a crucial role in antiviral defense to control HPV infections [270, 271]. Additionally, there were lower concentrations of CTACK and MIG, both essential for immune cell trafficking and response [271-274]. The presence of any HPV and oncogenic HPV infections was also linked to reduced frequencies of CCR5+ CD4+ T cells, which are important for viral responses [275, 276], CD86+ HLADR++ CD14+ cells, crucial for antigen presentation [277, 278], and HLADR+ CD14-CD123+ cells, which are involved in innate immunity and inflammation [279, 280]. These findings indicate that reduced levels of key

cytokines and immune cells could weaken the immune system's ability to fight HPV infection. They also highlight the need to investigate if HPV early proteins interfere with immune responses, potentially leading to persistent infection and cancer progression.

Understanding the interplay between host immune response, HPV status, and vaginal microbiota changes is crucial for unraveling the complex mechanisms that influence susceptibility to infections and disease progression. Here, we investigated the alterations in host immune response in relation to HPV status and variations in vaginal microbiota. Our findings reveal distinct immune responses associated with HPV infection in the context of vaginal microbiota. Women with *Lactobacillus* dominance and HPV infection showed increased levels of MIP-1β, RANTES, and IL-7, reflecting heightened inflammation and immune activation [29, 46]. In contrast, women with non-*Lactobacillus* dominance and high-risk HPV infection had significantly reduced levels of TNF-β, eotaxin, IL-16, MIG, and IFN-α, suggesting impaired immune regulation, compromised antiviral defense [270, 281, 282]. and potential disruptions in inflammatory responses. Additionally, these women exhibited fewer cervical T cell subsets (CD38+ CD4+ and CCR5+CD4+) and activated monocytes CD86+ HLADR++ CD14+. These results suggest that HPV infection may result in a less effective immune response and pose challenges to antigen presentation and immune activation [276]. Taken together, these results highlight the intricate relationship between vaginal microbiota, immune response, and HPV infection, underscoring the need for further research to understand these interactions and their implications for disease management.

Several limitations were identified in this study. Firstly, the relatively small sample size may have impacted our findings' statistical power and generalizability. Additionally, the absence of longitudinal cytokine data prevented us from distinguishing between persistent and transient signatures. We also tested only 37 HPV types, so other untested types could potentially affect the observed cytokine patterns. Another limitation was the lack of documentation regarding participants' HPV vaccination status. While this study focused on women with laboratory-confirmed STIs and/or BV to address translationally relevant questions, inclusion of a healthy control group with a Lactobacillus-dominated vaginal microbiome could have strengthened

comparative interpretation of cytokine, immune cell, and microbiota profiles. Moreover, we did not explore other potential confounding factors such as sexually transmitted infections (HSV), use of hormonal contraceptives, dietary habits, hormonal status, or vaginal disorders (e.g., aerobic vaginitis), which could influence the vaginal microbiota and immune environment. Understanding the impact of these factors is crucial for a thorough assessment of the associations observed in this study.

The study concludes that HPV infection, especially oncogenic types, is linked to significant changes in immune cell frequencies and cytokine/chemokine levels. HPV-positive women showed reduced frequencies of specific immune cell subsets and altered levels of inflammatory markers. Women with *Lactobacillus*-dominant vaginal flora had elevated concentrations of certain inflammatory markers in the presence of HPV, whereas those with non-*Lactobacillus* dominance had lower marker levels and reduced immune cell frequencies. These findings highlight the intricate interplay between HPV infection, vaginal microbiota, and immune responses, emphasizing the need to consider these factors for a comprehensive understanding of HPV-related immunological changes. Understanding the specific cytokines and cells involved in detecting and eliminating cells infected or transformed by HPV, along with how HPV proteins evade the immune system, will enable the development of new strategies to boost the immune response against HPV. While this study did not investigate cervical cancer cases or progression, the findings have implications for HPV infection management and cervical cancer prevention strategies.

3.5 Declarations:

3.5.1 Ethics Approval

The protocol for this study was approved by the Ethics Review Committee of the University of KwaZulu-Natal (BREC number: BE303/17). Written informed consent was obtained from all CAPRISA 083 participants.

3.6 Consent for publication:

Not applicable

3.7 Availability of Data and Materials:

The raw 16S rRNA gene sequence, along with all the experimental and procedural metadata such as unrarefied OTU tables, and R scripts used in this study data will be deposited in the sequenced Read archive (SRA) at NCBI and GitHub (https://github.com)

3.8 Conflict of interest:

The authors declare no conflict of interest.

3.9 Funding:

The study was funded by the Poliomyelitis Research Foundation (PRF grant number: 19/108) and the DST-NRF Center of Excellence in HIV Prevention, which is supported by the Department of Science and Innovation and the National Research Foundation (NRF). The CAPRISA 083 cohort study was funded by a United States–South African Program for Collaborative Biomedical Research grant through the South African Medical Research Council and the National Institute of Health (AI116759).

3.10 Authors Contributions:

The authors confirm their contribution to the paper as follows: Conceptualization, W.P.Q., G.M., A.M. (Andile Mtshali) and S.N.; methodology, W.P.Q., A.M. (Andile Mtshali) and A.S.; formal analysis, M.P.L., F.O., J.E.S. and A.O.K.; parent study PIs, N.G., A.R. and A.M. (Adrian Mindel); writing—original draft preparation, W.P.Q., G.M., A.M. (Andile Mtshali), M.P.L., F.O., J.E.S., A.O.K. and S.N.; writing—review and editing, W.P.Q., G.M., A.M. (Andile Mtshali), M.P.L., F.O., J.E.S., A.O.K., N.G., A.R., A.M. (Adrian Mindel), L.J.P.L., D.A., A.S. and S.N.; supervision, G.M., A.M. (Andile Mtshali) and S.N.; project funding acquisition, S.N. All authors have read and agreed to the published version of the manuscript.

Acknowledgment:

We would like to thank the CAPRISA 083 study participants for their contribution to the research and the clinical and laboratory teams for the collection of clinical data and specimens.

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CHAPTER 4

Metronidazole Treatment Failure and Persistent BV Lead to Increased Frequencies of Activated T- and Dendritic-Cell Subsets

Recurrence and identifying women with BV are key challenges, as a large proportion of cases are asymptomatic. Furthermore, antibiotic treatment of BV, the current standard of care, is often ineffective and recurrence rates following treatment are high [214]. The effects of BV recurrence on normal immune function may become apparent over time, as persistent or repeated episodes of bacterial imbalance can gradually alter immune responses. Chronic or recurrent BV may lead to sustained inflammation, which can disrupt the delicate equilibrium of the immune system. This disruption may result in the gradual development of immune dysfunctions, such as altered immune cell frequencies and inflammatory responses [256, 283, 284]. Although research has shown that vaginal microbiota influences cytokine and cellular immune response patterns related to HIV risk, it is still uncertain whether MDZ treatment affects the mucosal immune environment. The connection between BV resolution, including instances of treatment failure, and changes in the mucosal immune response remains unclear. Further investigation is needed to determine how MDZ treatment impacts immune responses and to clarify the relationship between successful or unsuccessful BV treatment and subsequent immune function.

In Chapter 4, we investigated the immune parameters linked to treatment response by analyzing vaginal microbiota and immune cell frequencies in endocervical cytobrush samples from 32 South African women with symptomatic BV, both before and after MDZ treatment. It addresses critical gaps in understanding the long-term effects of MDZ on mucosal immune responses and their connection to BV recurrence. The findings could enhance clinical diagnosis, therapeutic strategies, and preventive measures for BV-related complications, including increased HIV susceptibility. Although MDZ treatment led to a modest reduction in BV-associated bacteria, *Lactobacillus* colonization remained insufficient. Notable increases in CCR5+ CD4+ T cells and plasmacytoid dendritic cells (pDCs) were observed six and twelve weeks post-treatment. MDZ non-responders showed higher frequencies of activated CD4+ T cells and monocytes compared to responders. These results suggest that MDZ treatment failure is associated with elevated levels of activated T cells and dendritic cells, potentially increasing HIV susceptibility. Clinically, these findings underscore the importance of monitoring immune parameters in addition to traditional BV

treatments to better tailor interventions for individuals at higher risk of recurrence and associated complications.

Dr. Ngcapu, Dr Mtshali, Dr. Mzobe, and I conceptualized and designed the study. Dr Mtshali and I were responsible for measuring flow cytometry and vaginal microbiota in vaginal specimens. I analyzed and interpreted the data, which was validated by the study statisticians, Mrs. Osman, and Marothi Peter Letsoalo. Bioinformatics was conducted by Dr James Emmanuel San and Miss Asavela Olona Kama. I also wrote the manuscript, which was reviewed by my supervisors and approved by all coauthors for publication.

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Metronidazole Treatment Failure and Persistent BV Lead to Increased Frequencies of Activated T- and Dendritic-Cell Subsets

Wenkosi Perez Qulu [1,2,†], Gugulethu Mzobe [1,2,†], Andile Mtshali [1,2], Marothi Peter Letsoalo [1], Farzana Osman [1], James Emmanuel San [3], Asavela Olona Kama [1], Nigel Garrett [1,4], Adrian Mindel [1], Anne Rompalo [5], Lenine J. P. Liebenberg [1,2,6], Derseree Archary [1,2], Aida Sivro [1,2,7,8] and Sinaye Ngcapu [1,2,*]

1 Centre for the AIDS Programme of Research in South Africa (CAPRISA), Durban 4001, South Africa; wenkosi.qulu@caprisa.org (W.P.Q.); gugulethu.mzobe@caprisa.org (G.M.); andile.mtshali@caprisa.org (A.M.); marothi.letsoalo@caprisa.org (M.P.L.); farzana.osman@caprisa.org (F.O.); asavela.kama@caprisa.org (A.O.K.); nigel.garrett@caprisa.org (N.G.); adrian.mindel@gmail.com (A.M.); lenine.liebenberg@caprisa.org (L.J.P.L.); desh.archary@caprisa.org (D.A.); aida.sivro@caprisa.org (A.S.)
2 Department of Medical Microbiology, University of KwaZulu-Natal, Durban 4001, South Africa
3 KwaZulu-Natal Research Innovation and Sequencing Platform, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban 4001, South Africa; sanemmanueljames@gmail.com
4 Discipline of Public Health Medicine, University of KwaZulu-Natal, Durban 4001, South Africa
5 Department of Gynecology and Obstetrics, Johns Hopkins University, Baltimore, MD 21287, USA; arompalo@jhmi.edu
6 Centre for Epidemic Response and Innovation (CERI), Stellenbosch 7600, South Africa
7 JC Wilt Infectious Disease Research Centre, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB R3E 3L5, Canada
8 Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, MB R3E 3L5, Canada
* Correspondence: sinaye.ngcapu@caprisa.org; Tel.: +27-31-260-4694; Fax: +27-31-260-4566
† These authors contributed equally to this work.

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Citation: Qulu, W.P.; Mzobe, G.; Mtshali, A.; Letsoalo, M.P.; Osman, F.; San, J.E.; Kama, A.O.; Garrett, N.; Mindel, A.; Rompalo, A.; et al. Metronidazole Treatment Failure and Persistent BV Lead to Increased Frequencies of Activated T- and Dendritic-Cell Subsets. *Microorganisms* **2023**, *11*, 2643. https://doi.org/10.3390/microorganisms11112643

Academic Editors: Sara Baptista da Silva and Sandra F. Borges

Received: 21 September 2023
Revised: 21 October 2023
Accepted: 23 October 2023
Published: 27 October 2023

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Abstract: Metronidazole (MDZ) treatment failure and bacterial vaginosis (BV) recurrence rates are high among African women. This cohort study identified genital immune parameters associated with treatment response by comparing vaginal microbiota and immune cell frequencies in endocervical cytobrushes obtained from 32 South African women with symptomatic BV pre- and post-metronidazole treatment. Cervical T- and dendritic-cell subsets were phenotyped using multiparameter flow cytometry and the composition of vaginal microbial communities was characterized using 16S rRNA gene sequencing. MDZ treatment led to a modest decrease in the relative abundance of BV-associated bacteria, but colonization with *Lactobacillus* species (other than *L. iners*) was rare. At 6 and 12 weeks, MDZ-treated women had a significant increase in the frequencies of CCR5+ CD4+ T cells and plasmacytoid dendritic cells compared to the pre-treatment timepoint. In addition, MDZ non-responders had significantly higher frequencies of activated CD4 T cells and monocytes compared to MDZ responders. We conclude that MDZ treatment failure was characterized by an increased expression of activated T- and dendritic-cell subsets that may enhance HIV susceptibility. These data suggest the need to further assess the long-term impact of MDZ treatment on mucosal immune response and the vaginal microbiota.

Keywords: vaginal microbiota; genital immune cells; BV treatment

1. Introduction

Bacterial vaginosis (BV) is a polymicrobial condition in the female genital tract (FGT) that can present clinically with vaginal discharge, a fish-like odor, vaginal discomfort, and/or urinary symptoms, and is microbiologically characterized by a lack of beneficial *Lactobacillus* species and by colonization with a diverse spectrum of primarily anaerobic bacteria [1]. Symptomatic BV is usually treated with either oral or topical metronidazole

(MDZ) or clindamycin as the standard of care, or with emerging, experimental alternatives including antiseptic treatments, pro- and pre-biotics, and vaginal microbiome transplantation [2–6]. BV treatment leads to a modest reduction in bacterial abundances of BV-associated bacteria (BVAB), but re-colonization with *Lactobacillus* species (other than *L. iners*) is often slow or improbable [7–9]. BV recurrence rates following treatment are high and can reach up to 60% within six months of treatment [5,10–13], suggesting that the effects of BV on normal immune function may manifest over time.

BV has been associated with increased levels of genital inflammation, an important contributing factor to a wide variety of adverse sexual and reproductive outcomes, including an increased risk of preterm birth, cervical dysplasia, miscarriage, and sexually transmitted infections (STIs, including HIV) [14–17]. In addition, diverse microbial communities reminiscent of bacterial communities found to be associated with BV are closely associated with increased inflammatory mediators and immune cells in the FGT [9,14,15,18]. *Gardnerella vaginalis* increases the concentrations of toll-like receptor (TLR) ligands and pro-inflammatory cytokines, including Interleukin (IL)-1α, IL-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, and TNF-β, in the FGT [14,18–21]. Diverse microbial communities dominated by *G. vaginalis* were closely associated with increased levels of IL-17-inducing cytokines (IL-23 and IL-1β) and an increased HIV risk, likely by increasing the mucosal CD4+ T helper 17 (Th17)-cell frequency and immune cell activation [14,15]. Anahtar et al. (2015) detected increased numbers of mucosal CCR5+ CD4+ T cells in mice that received *Prevotella bivia* compared to those intravaginally inoculated with *L. crispatus*. Furthermore, highly diverse microbial communities were directly correlated with increased genital pro-inflammatory cytokine concentrations. This mechanism is likely through an enhanced stimulation of cognate TLR4 and activation of the nuclear factor kappa B (NF-κB) signaling pathway, leading to an increased activation of CCR5+ CD4+ T cells in the female genital tract [14]. Studies suggest that BVAB induces an inflammatory response through the increased production of lipopolysaccharides (LPS), resulting in the activation of the NF–κB pathway by binding to TLR4 and CD14 on genital epithelial cells, monocytes, and macrophages [22–24]. While pro-inflammatory cytokine classes are generally upregulated in BV, some studies have shown that certain chemokines (IP10 and monokine induced by gamma-interferon (MIG), in particular) may be downregulated [9,18,25,26]. The hypothesis is that BVAB specifically downregulates chemokines (e.g., interferon-γ-inducible protein (IP)-10 and MIG) that bind to CXCR3 to avoid immune responses from T and other immune cells that express the CXCR3 receptor. In contrast, *Lactobacillus* spp. have been shown to modulate cytokine release by monocyte-macrophages through enhancing the negative regulators of the NF–κB pathway in vitro [27]. *Lactobacillus* spp. have been associated with decreased inflammatory mediator concentrations in the FGT and a reduced inflammatory response to bacterial pathogens in vitro [20,28]. Similarly, *Lactobacillus* spp. significantly suppressed IL-6 and IL-8 production in vaginal epithelial (VK2) cells stimulated with *G. vaginalis* [29].

Although studies have demonstrated that vaginal microbiota modulates cytokine and cellular immune response signatures of HIV risk, it remains unclear whether MDZ treatment induces changes in the mucosal immune environment. The relationship between the BV resolution (including treatment failure) and the mucosal immune response remains undefined. To address this research gap, we assessed the composition of vaginal microbiota and cell phenotypes in endocervical cytobrushes of women pre- and post-treatment for BV, including those of women with BV resolution or persistence. We hypothesized that an MDZ treatment and an increase in the dominance of *Lactobacillus* species reduces the mucosal immune response frequency and activation. We further posited that treatment failure or recurrent BV correlates with a more diverse vaginal microbiota that leads to a high number of activated HIV-targeted cells in the FGT.

2. Materials and Methods

2.1. Study Design, Participants and Specimen Collection

For this ex vivo study, we used endocervical cytobrushes from 32 women with a laboratory-diagnosed STI and/or BV-intermediate or -positive enrolled in the CAPRISA 083 cohort study. The study aimed at reducing STIs in women by enhancing management packages for targeted STI care, ensuring that the individual is cured, and reducing the risk of reinfection using expedited partner therapy [9,30]. Women who were pregnant, living with HIV, or who had received antibiotic treatment within the last 7 days were excluded from the study. Participants diagnosed with *Chlamydia trachomatis* (treatment: azithromycin 1 g oral), *Neisseria gonorrhoeae* (ceftriaxone 250 mg intramuscular and azithromycin 1 g oral), *Trichomonas vaginalis* (metronidazole 2 g oral), Nugent score $\geq$ 4 (metronidazole 2 g oral), or candidiasis (clotrimazole 500 mg pessary and clotrimazole 1% cream) were asked to return after 6-weeks and 12-weeks post-treatment for follow-up examinations and provided genital specimens for further assays. The protocol for this study was approved by the Ethics Review Committee of the University of KwaZulu-Natal (BREC number: BE303/17).

2.2. BV Classification Using Nugent Score

Vaginal swabs were scored using Nugent criteria and classified as normal (<4), intermediate (4–6), and Nugent-BV ($\geq$7). For the purpose of this study, BV was classified as Nugent score $\geq$ 4. Women who had Nugent score $\geq$ 4 at baseline and 6-weeks post-treatment, but had a Nugent score < 4 at 12-weeks post-treatment visit were considered "BV cleared"; while women who had Nugent score $\geq$ 4 at baseline and all consecutive visits were considered "BV persistence". Those who had Nugent score < 4 at 6-weeks post-treatment, but a Nugent score that had increased to >4 at 12-weeks post-treatment were classified as having "BV recurrence".

2.3. 16S rRNA Gene Amplicon Sequencing and Upstream Analysis

Genomic DNA was extracted from each vaginal swab using the PowerSoil DNA Isolation kit (Qiagen, Hilden, Germany), and a fragment of the 16S rRNA gene spanning the V3–V4 variable region was PCR amplified using primers 319F (forward) and 806R (reverse), as described previously [9]. Libraries were constructed using ~120 pooled samples that were sequenced on the Illumina MiSeq platform (paired-end sequence reads with v3 chemistry). The 'DADA2' R package was used to perform quality-based filtering and trimming, inference of amplicon sequence variants (ASVs), chimera removal, and taxonomy assignment. Taxonomic classification was performed using the SILVA ribosomal RNA database. The ASVs for several key genera (e.g., *Lactobacillus*, *Prevotella*, *Sneathia*, *Mobiluncus*) were classified to the species level using speciateIT (version 1.0, http://ravel-lab.org/speciateIT, accessed on 10 December 2020). Samples were assigned to 4 community state types using VALENCIA (VAginaL community state typE Nearest CentroId clAssifier), a nearest centroid classification algorithm [31]. The reference centroids are available at github.com/ravel-lab/VALENCIA (accessed on 10 December 2020). The ASV table, taxonomy table, and metadata were integrated into a single phyloseq object using the 'phyloseq' R package for further downstream analysis and visualization.

2.4. Cytokine and Chemokine Measurement

CAPRISA 083 cervicovaginal SoftCup supernatants collected at matching time points with vaginal swabs were used to measure concentrations of 48 cytokines/chemokines using Bio-Plex Pro-Human Cytokine Group I (27-Plex Panel) and Group II (21-Plex Panel) kits (Bio-Rad Laboratories, Hercules, CA, USA), as previously reported [9]. The panel included chemokines, pro-inflammatory cytokines, adaptive, growth factors, and the following anti-inflammatory cytokines: IL-1β, IL-1Rα, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-12p40, IL-16, IL-18, IL-1α, IL-2RA, IL-3, IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF-basic), cutaneous T-cell attracting chemokine (CTACK), Eotaxin, granulocyte colony-stimulating factor (G-CSF), GM–CSF, GRO-α, hepatocyte growth factor (HGF), Interferon

(IFN)-γ, IFN-α2, IP-10, leukemia inhibitory factor (LIF), monocyte chemoattractant protein (MCP)-1, MCP-3, macrophage colony-stimulating factor (M-CSF), MIG, macrophage migration inhibitory factor (MIF), macrophage inhibitory protein (MIP)-1α, MIP-1β, nerve growth factor-beta (NGF-β), platelet-derived growth factor (PDGF-β), regulated-upon-activation normal T-cell expressed and presumably secreted (RANTES), stem cell factor (SCF), stem cell growth factor-beta (SCGF-β), stromal cell-derived factors 1-alpha (SDF-1α), TNF-α, TNF-β, TNF-related apoptosis-inducing ligand (TRAIL), and vascular endothelial growth factor (VEGF). A standard curve was used to determine cytokine concentrations in the samples using a 5-parameter logistic regression model. The inter- and intra-assay variability was performed by comparing cytokine concentrations in replicates plated on the same or across plates, with Spearman r values > 0.8 considered acceptable per cytokine. Cytokine levels below the lower limit of detection of the assay were reported as the mid-point between zero and the lowest concentrations measured for each cytokine. Data were presented as log10-transformed values to normalize distributions.

2.5. Assessment of Immune Cell Phenotype and Activation in Endocervical Cytobrushes

Endocervical cytobrushes were collected as previously described using insertion and 360° rotation of a cytobrush in the endocervical os [32]. Cervical mononuclear cells were liberated from the brush through a combination of vortexing and washing; they were then filtered and processed for flow cytometry ex vivo, as previously described [33]. The cells were incubated with LIVE/DEAD Aqua fluorescence (Invitrogen from Thermo fisher scientific™, Waltham, MA, USA) stain for 20 min at 4 °C in the dark, washed with FSC phosphate-buffer saline, and surface stained for 20 min at room temperature with following antibody cocktail: Alexa Fluor 700 (AF)-labelled anti-HLA-DR, APC-labelled anti-CD86, Brilliant Violet 785 (BV785)-labelled anti-CD16, Peridinin-chlorophyll-protein CY5.5 (PerCP-CY5.5)-labelled anti-CD123, BV650-labelled anti-CD11c (Biolegend, San Diego, CA, USA), Phycoerythrin (PE)-labelled anti-CD4, Allophycocynin-H7 labelled (APC-H7)-anti-CD3, Fluorescein Isothiocyanate (FITC)-labelled anti-CD38, BV605-labelled anti-CCR6, BV421-labelled anti-CCR5, PECy7-labelled anti-CD14, V510-labelled ant-CD19, and V510-labelled anti-CD56 (Becton Dickinson, Franklin Lakes, NJ, USA). Data acquisition was conducted using a FACSDiva Flow cytometer (Becton and Dickinson Immunocytometry Systems) and data were analyzed using FlowJo Software version 10 (Tree Star, Woodburn, OR, USA). Fluorescence minus one (FMO) staining was used to set gates to differentiate negative and positive populations [34]. All data were corrected for background, using the unstimulated condition. Figure 1 shows the gating strategy used to characterize cervical T and dendritic cells from cytobrush specimens.

2.6. Statistical Analysis

R software version 4.3.1 was used for statistical analysis. Continuous data were described using median and interquartile range (IQR), while categorical data were described using the number of participants (*n*) and percentage of participants with non-missing data. Wilcoxon rank-sum and Chi-square (or Fisher's exact) tests were used to compare continuous and categorical data, respectively, in bivariate analysis. For paired data, the Wilcoxon signed-rank test was used. The baseline characteristics, clinical assessments, and sexual behaviors of the participants were compared across the outcome of BV infection after treatment. In addition, the clinical assessments, and sexual behaviors that varied over time were also compared across the outcomes at 6 and 12 weeks. Genital inflammation was classified as having 5 or more of 9 pro-inflammatory cytokines and chemokines (IL-1α, IL-6, IL-8, interferon gamma-induced protein (IP)-10, macrophage inflammatory protein (MIP)-1α, MIP-1β, monocyte chemotactic protein (MCP)-1, tumour necrosis factor (TNF)-α, and IL-1β) above the 75th percentile [35,36]. Cell population percentages between time-points (baseline, 6 weeks, and 12 weeks) were compared using a Wilcoxon signed-rank test. A Wilcoxon rank-sum test was used to determine whether at 12 weeks the outcomes of BV after treatment were associated with cell population percentages. In this investigation,

a significance level of 0.05 was employed to determine the statistical significance of the results. Given the exploratory nature of the analyses conducted in this paper, no adjustments were made for multiple comparisons. The findings presented herein are intended to generate hypotheses rather than provide confirmatory evidence.

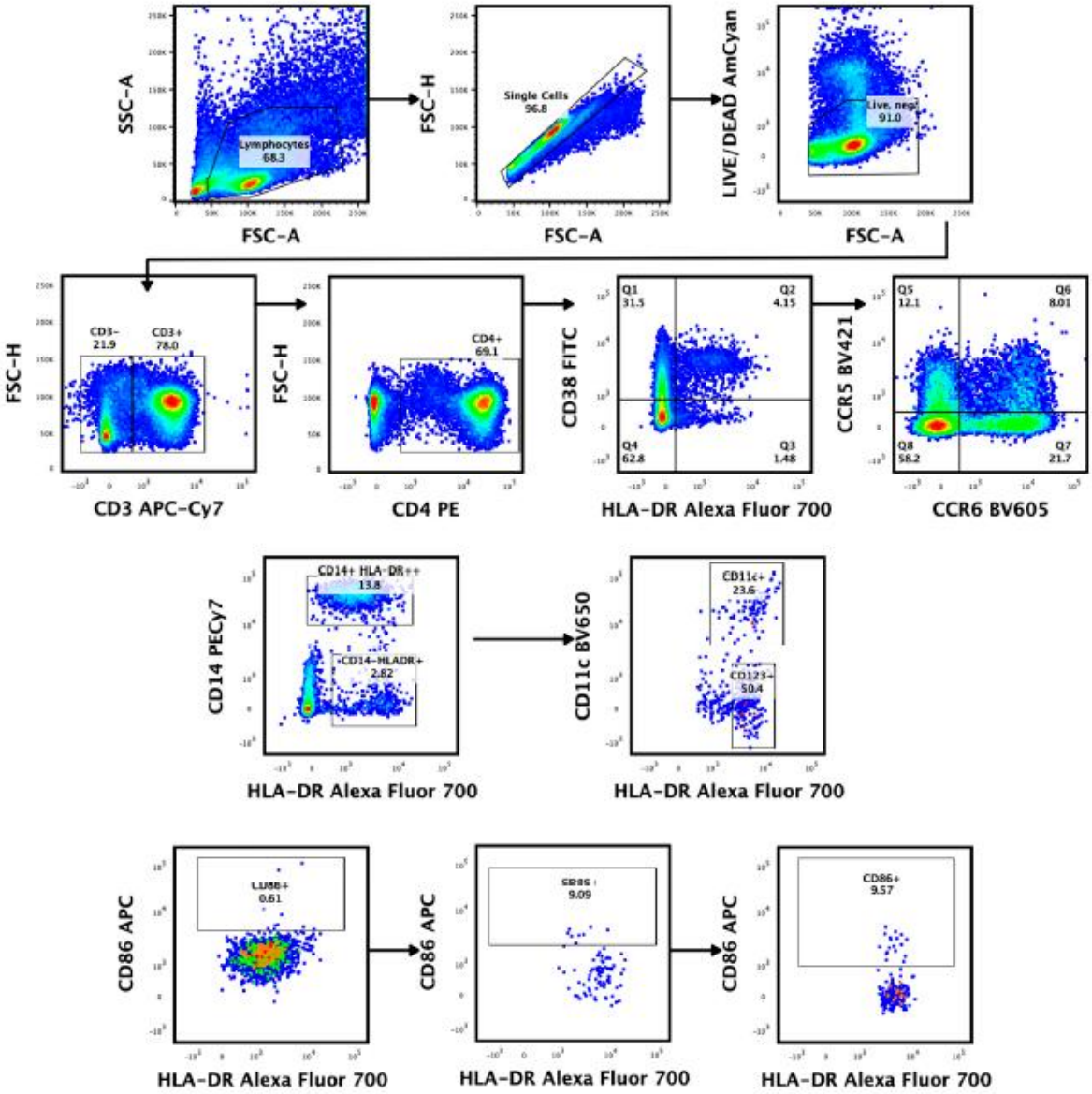


Figure 1. Identification of cervical T- and monocyte/dendritic-cell subsets from endocervical cytobrush specimens at three time points. Representative gating plot shows the exclusion of non-lymphocytes, doublets, dead (LIVE/DEAD™), CD19, and CD56 B lymphoid cells. The resulting cells were separated into CD3+ and CD3− subsets. The CD3+ T cells were further classified into CD4+ T cells. The expression of CD38, HLA-DR, CCR5, and CCR6 on CD4+ CD3+ lymphocytes was examined. The CD3- subset was separated into dendritic cell subsets: mDCs (HLADR+ CD14− CD11c+), pDCs (HLADR+ CD123+), and monocytes (HLADR+ CD14+ CD11c+). Activated DCs were identified using their CD86 expression, while monocytes were classified as CD14+ HLADR++.

3. Results

3.1. Participant Characteristics

Thirty-two women had 16S rRNA gene sequences, cytokine, and cellular data for all three visits. The cohort characteristics are presented in Table 1. The median age was 24 years (interquartile range (IQR) 21–28 years), and more than two-thirds reported condom use during the study (72%; 23/32). Only 6% (2/32) of these women reported consistent condom use, with the majority reporting that they used condoms occasionally (63%; 20/32). Only 47% (15/32) of study participants reported the use of any form of contraception, with the majority of these reporting the use of progesterone-based injectable contraceptives (32%; 10/32).

Table 1. Demographic, clinical, and behavioral data of study population.

Variable	Level	Baseline	6 Weeks	12 Weeks
N		32	-	-
Age, median [IQR]		24 [21, 28]	-	-
Condom use, *n* (%)	No	9 (28.1)	-	-
	Yes	23 (71.9)	-	-
Frequent use of condom, *n* (%)	Always	2 (6.2)	-	-
	Sometimes	20 (62.5)	-	-
	Never	10 (31.2)	-	-
Contraceptive use, *n* (%)	No	17 (53.1)	-	-
	Yes	15 (46.9)	-	-
Contraception type, *n* (%)	Oral	1 (3.1)	-	-
	Injectable	10 (31.2)	-	-
	Implant	4 (12.5)	-	-
	None	17 (53.1)	-	-
BV score category, *n* (%)	Positive	20 (62.5)	13 (40.6)	12 (37.5)
	Intermediate	12 (37.5)	11 (34.4)	12 (37.5)
	Negative	0 (0.0)	8 (25.0)	8 (25.0)
BV community state type, *n* (%)	I	0 (0.0)	1 (3.1)	2 (6.2)
	III	11 (34.4)	13 (40.6)	14 (43.8)
	IV-B	11 (34.4)	11 (34.4)	11 (34.4)
	IV-A	10 (31.2)	7 (21.9)	5 (15.6)
Genital inflammation, *n* (%)	No	23 (71.9)	25 (78.1)	26 (81.2)
	Yes	9 (28.1)	7 (21.9)	6 (18.8)
Previous sexual activity, *n* (%)	No	25 (78.1)	22 (71.0)	20 (74.1)
	Yes	7 (21.9)	9 (29.0)	7 (25.9)
Candidiasis, *n* (%)	No	27 (84.4)	28 (87.5)	26 (81.2)
	Yes	5 (15.6)	4 (12.5)	6 (18.8)
Any STI, *n* (%)	No	12 (37.5)	26 (81.2)	24 (75.0)
	Yes	20 (62.5)	6 (18.8)	8 (25.0)
Trichomoniasis, *n* (%)	Negative	28 (87.5)	31 (96.9)	32 (100.0)
	Positive	4 (12.5)	1 (3.1)	0 (0.0)
Gonorrhea, *n* (%)	Negative	28 (87.5)	32 (100.0)	31 (96.9)
	Positive	4 (12.5)	0 (0.0)	1 (3.1)
Chlamydia, *n* (%)	Negative	21 (65.6)	31 (96.9)	32 (100.0)
	Positive	11 (34.4)	1 (3.1)	0 (0.0)
Herpes simplex virus type 2, *n* (%)	Negative	31 (96.9)	31 (96.9)	31 (96.9)
	Positive	1 (3.1)	1 (3.1)	1 (3.1)

At baseline, 28% (9/32) of the women had evidence of genital inflammation, defined by having at least 5/9 inflammatory cytokines above the 75th percentile, as described previously [36,37]. Evidence of genital inflammation reduced slightly after treatment for BV, with 22% (7/32) of women having inflammation at 6 weeks and 19% (6/32) having inflammation at 12 weeks. All 32 women had Nugent scores ≥ 4 at baseline; the majority (63%; 20/32) were considered BV positive (Nugent score ≥ 7) and 38% (12/32) had intermediate Nugent scores (4–6). In addition, 78% (25/32) of women had any STI or vaginal candidiasis at baseline, with *C. trachomatis* being the most prevalent bacterial STI (34%,

11/32), while 16% (5/32) of women had Candidiasis evident. All women who had Nugent scores ≥ 4 at baseline and/or an STI received targeted antibiotic or antifungal treatment and were then tested for cure at their 6-week visit, and again at 3 months. At 6 weeks, only 25% (8/37) of women were BV negative and this proportion remained unchanged at 12 weeks.

3.2. Shifts in Cell Phenotypes Following MDZ Treatment

Here, we investigated changes in cervical immune cell profiles following metronidazole treatment ex vivo. The frequency of CCR5+ CD4+ T cells was significantly increased in cytobrush specimens collected at 6- (5%, $p = 0.034$) and 12-weeks (7%, $p = 0.0096$) post-treatment compared to the pre-treatment time point (Figure 2). In addition, the frequency of pDCs (HLADR+ CD14− CD123) was significantly increased at 6 (16%, $p = 0.0036$) and 12 weeks (22%, $p = 0.0002$), while mDCs (HLRDR+ CD14− CD11c+) were only increased at 6-weeks (13%, $p = 0.0410$) post-treatment compared to the pre-treatment timepoint. The frequency of CD14− HLDR+ was increased at 12-weeks (8%, $p = 0.029$) post-treatment. In contrast, the MDZ treatment reduced the frequencies of activated mDCs (CD86+ CD11c+, $p = 0.00016$; at 6 weeks: −21%, $p = 0.0002$; 12 weeks: −22%, $p = 0.0004$) and pDCs (CD86+ CD123+, at 6 weeks: −7%, $p = 0.0023$; 12 weeks: −7%, $p = 0.0003$) at the post-treatment compared to the pre-treatment timepoint. Furthermore, we observed a trend towards an increase in the frequency of monocytes (HLADR++ CD14+) at 6 (10%, $p = 0.058$) and 12 weeks (10%, $p = 0.053$), while contrasting trends in the frequency of activated monocytes (CD86+ HLRDR++ CD14+) were observed at 6-weeks (−2%, $p = 0.06$) post-treatment.

We further tested whether genital inflammation increases the number of activated cells within the mucosa. Women with genital inflammation had increased frequencies of pDCs and mDCs compared to the non-inflamed group, after controlling for age and time in the study (Supplementary Table S1). We observed no significant differences in proportions of other T-cell and DC subsets among women with genital inflammation and the non-inflamed group.

3.3. Vaginal Microbiota Characteristics Pre- and Post-Metronidazole

Based on the composition and relative abundance of bacterial species, bacterial communities were assigned into four community state types: CST I dominated by *L. crispatus*, CST III dominated by *L. iners*, CST IV-A representing samples with high relative abundance of BVAB1, and CST IV-B characterized by *G. vaginalis* dominance. Among the vaginal samples of women with BV (Nugent score ≥ 4) at baseline, most were dominated by *G. vaginalis* (CST IV-B; 34%, 11/32) while *L. iners* (CST III, 34%, 11/32) was the most common lactobacilli.

Next, we modelled the movement of bacterial communities between BV-related CSTs (CST IV-A or IV-B) and *Lactobacillus*-related state types (CST I and III) in MDZ-treated women over time. More than half of the participants (53%, 17/32) remained in the same profile of BV-associated CSTs [CST IV-A (50%, 5/10) or CST IV-B (55%, 6/11)] at 6-weeks post MDZ treatment, but only 16% (5/32) remained in their baseline CST at 12 weeks (Figure 3). The proportions of participants transitioning from a profile of BV-associated CSTs IV-B into *Lactobacillus*-dominant CSTs III increased from 13% (4/32) at week 6 to 28% (9/32) at week 12. Similarly, the proportions of participants transitioning from *Lactobacillus*-dominated communities into BV-associated CSTs also increased from 3% (1/32) at 6 weeks to 13% (4/32) at 12 weeks. Although *L. crispatus* was rare pre-MDZ, only 1/32 (3%) woman had *L. crispatus*-dominant communities in week 6 and 12 post-treatment, while others only achieved this dominance at 12-weeks post-MDZ visits.

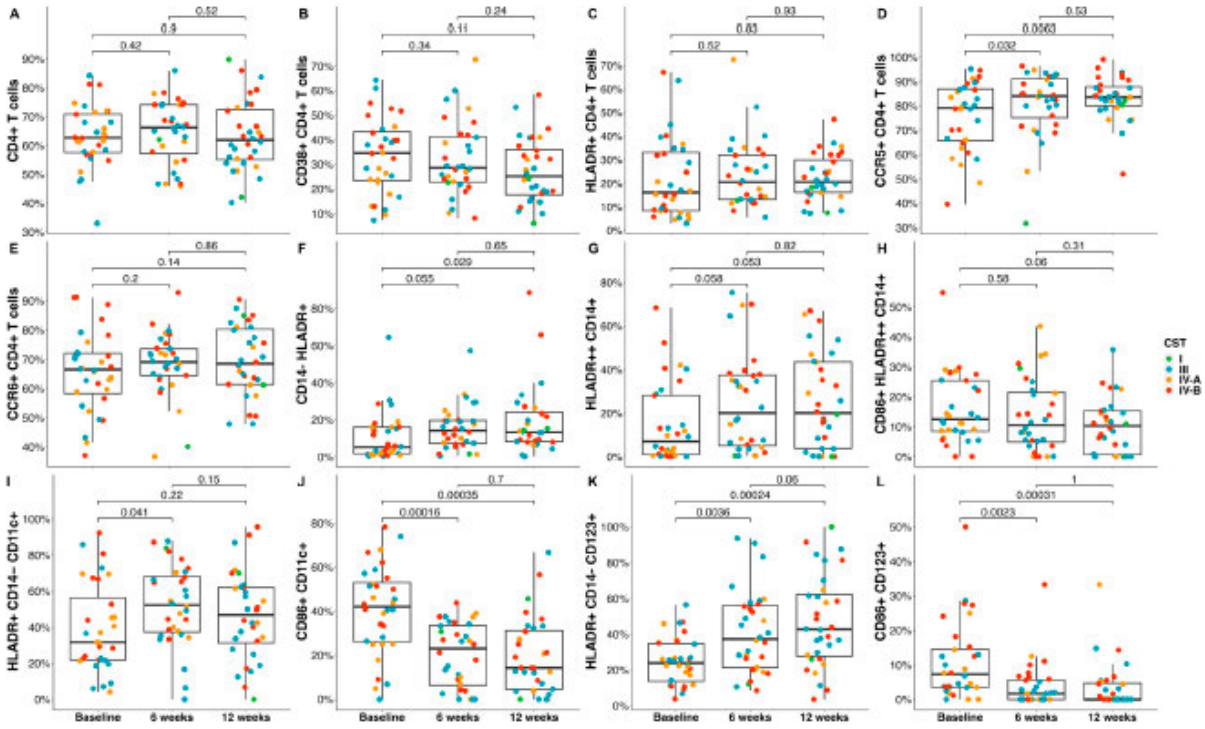


Figure 2. Frequency distribution of T- and dendritic-cell subsets in cytobrush specimens at baseline compared to 6- and 12-weeks post-treatment. Graph shows the differences over time for the frequencies of cervical (**A**) CD4+ T cells, (**B**) CD38+ CD4+ T cells, (**C**) HLADR+ CD4+ T cells, (**D**) CCR5+ CD4+ T cells, (**E**) CCR6+ CD4+ T cells, (**F**) CD14− HLADR+, (**G**) HLADR++ CD14+, (**H**) CD86+ HLADR++ CD14+, (**I**) HLDR+ CD14− CD11c+, (**J**) CD86+ CD11c+, (**K**) HLADR+ CD14− CD123+, and (**L**) CD86+ CD123+. Green color depicts CST I, blue CST III, orange CST IV-A, and red CST IV-B. The Mann–Whitney U test was used to compare the frequency distribution of T- and dendritic-cell subsets at pre- and post-treatment timepoints.

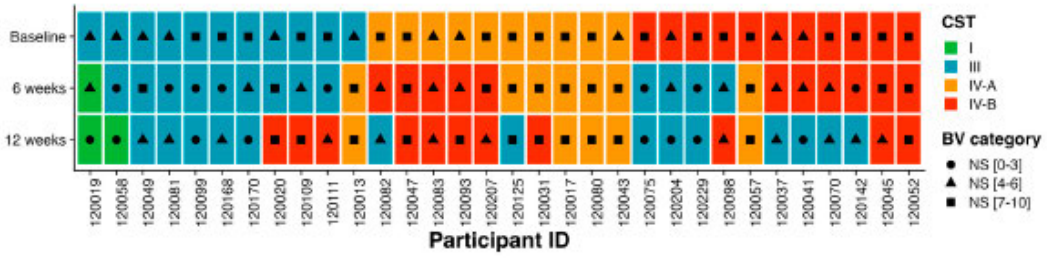


Figure 3. Composition of bacterial species and transitions within bacterial communities over the 12–week period. Box plots showing CSTs assigned to individual participants over 3 time points at baseline, 6 weeks, and 12 weeks. Green color depicts CST I, blue CST III, orange CST IV–A, and red CST IV–B.

3.4. Effect of Bacterial Community Transition Status on Cell Percentage Difference

We hypothesized that shifts in the vaginal microbiota are key regulators of mucosal inflammatory responses, causing immune activation that increases the risk for HIV acquisition. A Mann–Whitney U test was used to examine the relationship between the vaginal microbiome and HIV-susceptible cell number by comparing cell percentage differences between *Lactobacillus*-dominant CSTs (combination of CST I and III) and BV-associated

CSTs (CST IVA and IVB) over the three timepoints. The frequency of activated CD4+ T cells (HLADR+ CD4+, $p = 0.0364$) and mDCs (HLADR+ CD14− CD11c+, $p = 0.0321$) was significantly higher in women with highly diverse bacterial communities compared to those with a cervicovaginal microbiota dominated by *Lactobacillus* spp. (predominantly *L. iners*). We observed no significant changes in the frequency of any of the other cell phenotypes between women with a cervicovaginal microbiota dominated by *Lactobacillus* spp. compared to those with highly diverse bacterial communities (reminiscent of bacterial communities found in BV, Figure 4).

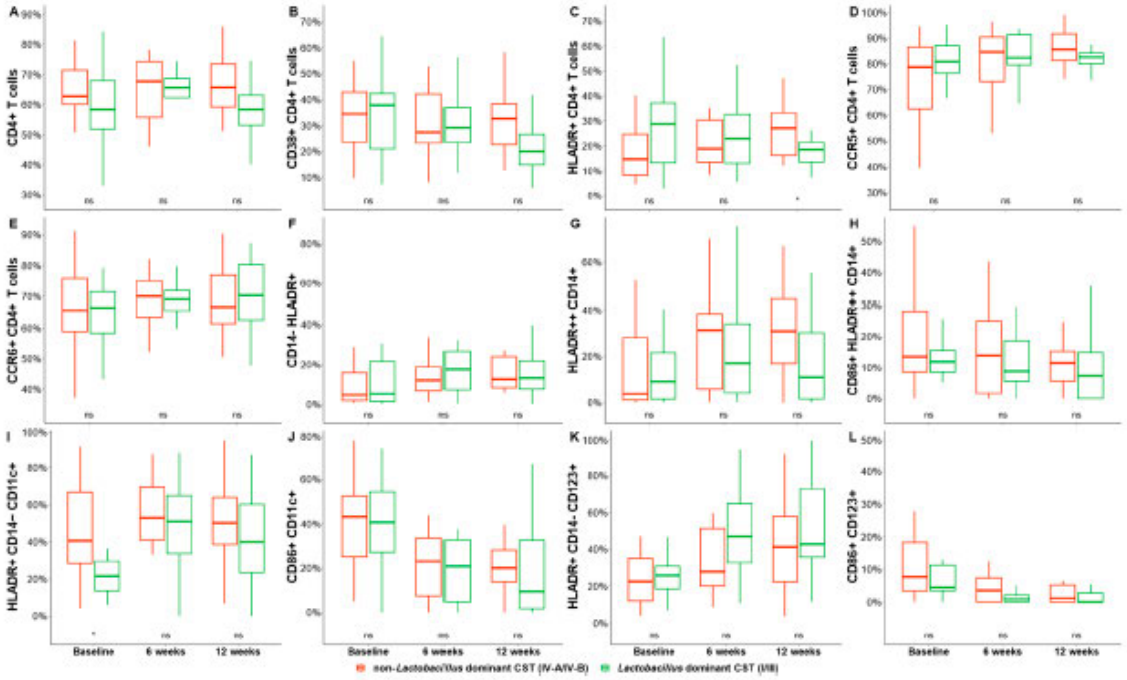


Figure 4. Frequencies of cervical T-cell and DC subsets in women with a cervicovaginal microbiota dominated by *Lactobacillus* spp. (predominantly *L. iners*, red) and non-*Lactobacillus* at baseline, 6 weeks, and 12 weeks after initiating MDZ. Graphs show the differences between pre- and post-MDZ initiation for the frequencies of cervical: Graph shows the differences over time for the frequencies of cervical (**A**) CD4+ T cells, (**B**) CD38+ CD4+ T cells, (**C**) HLADR+ CD4+ T cells, (**D**) CCR5+ CD4+ T cells, (**E**) CCR6+ CD4+ T cells, (**F**) CD14− HLADR+, (**G**) HLADR++ CD14+, (**H**) CD86+ HLADR++ CD14+, (**I**) HLDR+ CD14− CD11c+, (**J**) CD86+ CD11c+, (**K**) HLADR+ CD14− CD123+, and (**L**) CD86+ CD123+. Red color depicts non-*Lactobacillus* dominant CST (IV-A/IV-B) and red depicts *Lactobacillus* dominant CST (I/III). Statistical comparisons were performed using Mann–Whitney U tests for unmatched data or the Kruskal–Wallis test with false discovery rate correction for cross-sectional data. * Indicates significant differences ($p < 0.05$), while *ns* indicates non–significant difference ($p > 0.05$).

Next, a Mann–Whitney U test was used to compare the frequencies of cervical T-cell and DC subsets in women who cleared BV (at 12-weeks post-MDZ) to those who had persistent BV (at 12-weeks post-MDZ). We observed significantly higher frequencies of activated CD4 T cells (HLA-DR+ CD4+, $p = 0.021$, mean difference of 7%) and monocytes (HLADR++ CD14+, $p = 0.027$) in endocervical cytobrush specimens of women experiencing persistent BV relative to those who cleared BV (Figure 5). Although not significant, frequencies of CD4+ T ($p = 0.060$, mean difference of 9%) in women who experienced persistent BV trended higher compared to those who cleared BV. Furthermore, the frequency of CD38+ CD4+ T cells ($p = 0.093$, mean difference of 7%) showed a trend towards being elevated in women experiencing persistent BV compared to women who experienced BV recurrence.

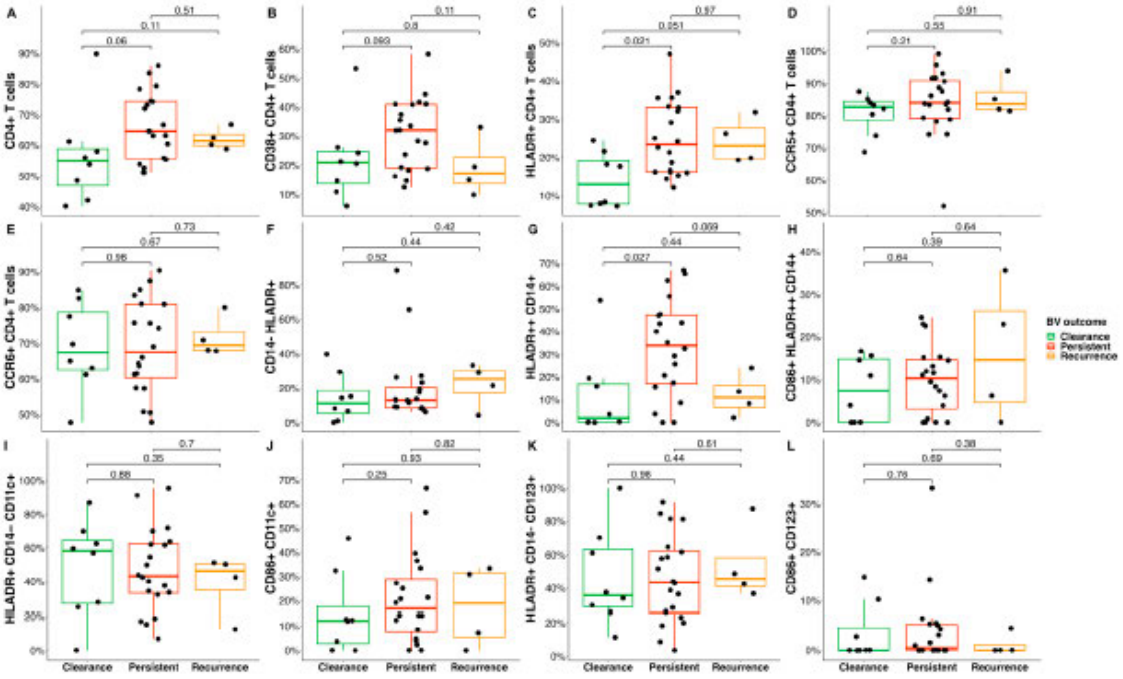


Figure 5. Frequencies of cervical T-cell and DC subsets in women who cleared BV (green) and compared to women with persistent or recurrent BV after initiating MDZ. Graph shows the differences over time for the frequencies of cervical (**A**) CD4+ T cells, (**B**) CD38+ CD4+ T cells, (**C**) HLADR+ CD4+ T cells, (**D**) CCR5+ CD4+ T cells, (**E**) CCR6+ CD4+ T cells, (**F**) CD14− HLADR+, (**G**) HLADR++ CD14+, (**H**) CD86+ HLADR++ CD14+, (**I**) HLDR+ CD14− CD11c+, (**J**) CD86+ CD11c+, (**K**) HLADR+ CD14− CD123+, and (**L**) CD86+ CD123+. Clearance is depicted in green, persistent in red, and recurrence in orange. Individual associations are shown between BV status and immune subsets including CD3+, CD4+ T cells, CD38+ CD4+ T cells, HLA-DR+ CD4+ T cells, CCR5+ CD4+ T cells, CCR6+ CD4+ T cells, CD14+, CD11c+ CD14+, and CD123+ CD14+ cells.

We further assessed whether the frequency of T-cell and DC subsets was stable over time in women who had BV resolution or persistent at 12 weeks post-MDZ. There were no significant differences between the frequency of T-cell and DC subsets among women who cleared BV over time. The frequency of CCR5+ CD4+ T cells was significantly higher in women with persistent BV at 6- ($p = 0.0058$, mean difference of 2%) and 12-weeks ($p = 0.0095$, mean difference of 2%) post-MDZ compared to baseline (Supplementary Figure S1). Similarly, the frequency of HLADR (CD14− HLADR+) was significantly increased in women with persistent BV at 6- ($p = 0.0034$, mean difference of 2%) and 12-weeks ($p = 0.0030$, mean difference of 2%) post-MDZ compared to baseline. Monocytes (6 weeks: $p = 0.0160$, mean difference of 2%; 12 weeks: $p = 0.0034$, mean difference of 2%), pDCs (6 weeks: $p = 0.0105$, mean difference of 2%; 12-weeks: $p = 0.0042$, mean difference of 2%) and activated mDCs (6 weeks: $p = 0.0084$, mean difference of 2%; 12 weeks: $p = 0.0106$, mean difference of 2%) were also increased in women with persistent BV post-MDZ compared to pre-MDZ timepoint. Women with persistent BV had higher frequencies of activated pDCs ($p = 0.0069$, mean difference of 2%) and monocytes ($p = 0.0239$, mean difference of 2%) at 12-weeks post-MDZ compared to baseline. Women who cleared BV had decreased frequencies of CD38+ CD4+ T cells ($p = 0.0423$, mean difference of 1%) and HLADR+ CD4+ T cells ($p = 0.0294$, mean difference of 1%) at 12-weeks post-MDZ compared to baseline. The frequencies of activated mDCs ($p = 0.0299$, mean difference of 1%) were decreased at 6-weeks post-MDZ compared to baseline.

4. Discussion

The diverse non-*Lactobacillus* dominant communities are closely associated with elevated genital inflammation and an increased HIV risk, likely due to increasing the mucosal HIV target-cell frequency and T-cell activation [14,15]. However, less is known about the mucosal immune milieu pre- and post-MDZ treatment, including in those who cleared or had persistent BV. The current study determined the effects of MDZ treatment on the frequency of mucosal immune cell subsets in endocervical cytobrushes from women with a laboratory diagnosed STI and/or BV. We observed a significantly higher number of the activation marker HLA-DR and activated CCR5+ CD4+ T cells in cytobrush specimens post-MDZ treatment, and this was specifically observed in the BV-persistent subgroup. Similarly, the frequencies of DC subsets (pDCs and mDCs) were increased post-MDZ treatment. *L. iners* was the most common *Lactobacillus* species pre- and post-MDZ. Women classified as having persistent BV (Nugent score > 4) had significantly increased frequencies of activated CD4 T cells (HLA-DR+ CD4+ T) and monocytes (HLADR++ CD14+) compared to those who cleared BV (Nugent score < 3) at 12-weeks post-MDZ.

While recent studies have demonstrated that MDZ has multiple effects on different components of immunity [9,15], very few have evaluated the association between the mucosal immune milieu pre- and post-BV treatment [38]. We observed a significantly increased frequency of endocervical CCR5+ CD4+ T cells and pDCs (HLADR+ CD14− CD123+) in cytobrush specimens collected at 6- and 12-weeks post-treatment compared to the pre-treatment timepoint. CCR5-expressing T lymphocytes are densely populated in the subepithelial stromal tissues in the vagina and CCR5 is a key cellular co-receptor required for HIV entry into CD4 T lymphocytes, including cell-to-cell spread [38]. DCs are known to play an important role in the host response to infection and are the first cells to sense and respond to microbes [39]. DCs interact with the microbes via pathogen-associated molecular patterns to trigger specific T- and B-cell responses [40–42]. The increased levels of CCR5+ cells and pDCs may be attributed to the persistence of BV-associated bacteria and this is confirmed through increased levels over time in women who had persistent BV. These findings suggest that the putative mechanism mediating HIV susceptibility in women with persistent BV is through a DC–microbe interaction and the recruitment of activated CCR5+ cells, which are known to facilitate HIV acquisition by allowing the virus to attach to the cell and establish an infection.

L. iners is normally the first *Lactobacillus* spp. to emerge after treatment of BV with metronidazole. Although *L. crispatus* has been associated with reproductive health benefits (including a decrease in pre-term births, STIs, genital inflammation, and HIV risk), mounting evidence suggests that *L. iners* may contribute to the onset and maintenance of BV or intermediate-BV status [43,44]. Furthermore, using a Markov model, we [9] and others [45,46] have previously demonstrated that *L. iners* dominant communities have a high probability of transitioning to BV-associated states. Consistent with previous studies, the current study also observed a high prevalence of *L. iners* and near-pan absence of *L. crispatus* after MDZ treatment. It has been reported that the standard BV treatment tends to promote dominance by *L. iners* rather than *L. crispatus*, which may partially explain the high recurrence rates and long-term vaginal dysbiosis, especially after repeated treatment cycles [13,47,48]. There are many knowledge gaps remaining regarding the activity and role of *L. iners* in the vaginal microbiota, including whether this enigmatic bacterium is only a predictive biomarker of the transition in vaginal ecology or a risk factor for BV and its impact on the mucosal immune microenvironment. Due to the important role non-*iners Lactobacillus* spp. play in maintaining the optimal mucosal environment, the discovery of interventions that shift *L. iners*-dominant communities to *L. crispatus*-dominant communities, along with novel approaches to selectively inhibit *L. iners* while sparing protective vaginal lactobacilli, is needed and may potentially provide insights into bacterial community vulnerabilities that can translate into new therapeutic approaches.

Although multiple studies have explored the association between vaginal microbiota and mucosal HIV target-cell frequency and activation [9,14,15,18], very few have evalu-

ated the relative effects of BV eradication or recurrence on the mucosal immune milieu. We previously showed that BV clearance reduced levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-8, and LIF), while BV persistence was associated with the increased concentrations of cytokines (IL-1α, IL-18, MIF, IL-7, and LIF) linked to an increased risk of acquiring HIV infection. These findings, however, were in the absence of testing the effect on frequency and activation of HIV-susceptible cells [9]. In the current study, BV persistence was associated with a significant increase in frequencies of activated CD4 T cells and monocytes compared to women who cleared BV at 12 weeks. These findings are in agreement with previous studies that showed an increased frequency of activated CD4+ T cells in women with highly diverse non-*Lactobacillus*-dominant communities [14,15]. In addition, we also found that increased frequencies of activated CD4 T cells and mDCs were significantly associated with the highly diverse bacterial communities usually present in BV. Furthermore, we observed increased levels of HLA-DR over time in women who had persistent BV. HLA-DR is an antigen-presenting molecule that is typically expressed at high levels on professional antigen-presenting cells [49], but is also expressed on chronically activated T cells [50–52]. HLA-DR expression is associated with immune checkpoint molecules expressed on cells susceptible to HIV infection, such as percentages of activated CD4+ T cells [53]. In contrast, women who cleared BV over time had decreased levels of activated CD4 T cells (CD38+ CD4+ T cells and HLADR+ CD4+ T cells) and activated mDCs (CD84+ CD11c+). A highly diverse and strictly anaerobic vaginal microbiota in women of African descent has been associated with increased genital inflammation and HIV risk, likely due to an increased activation and recruitment of HIV-susceptible cells to the vaginal mucosa [7,14–16]. These findings suggest that diverse microbial communities may influence HIV susceptibility by increasing the frequency and differentiation of mucosal HIV target cells within the genital mucosa.

The limitations of our study include a small sample size that restricted the ability to assess in detail the effects of BV clearance, persistence, or recurrence on the mucosal immune milieu. Another limitation was the lack of a control group of BV-negative women to investigate immune subset changes between the MDZ experienced and naïve groups. Additionally, we did not investigate the impact of other potential co-factors such as STIs (bacterial and viral), hormonal contraceptives, diet, hormonal status, and other vaginal disorders (e.g., aerobic vaginitis) on microbiota, although each may affect the immune milieu. A major strength of our study is that we leveraged the existing inflammatory and 16S rRNA data to longitudinally assess the impact of BV treatment outcomes on the frequency of mucosal immune subsets.

5. Conclusions

L. iners was the most predominant *Lactobacillus* spp. before and after treatment with metronidazole. Treatment failure and persistent BV contributed to an increase in the cell-associated inflammatory response, potentially as a result of exposure to persistent bacterial diversity that is consistently associated with elevated genital inflammation and increased HIV risk. The current treatment regimens for BV are less likely to promote the absolute abundance of low-inflammatory *L. crispatus* communities. Therefore, viable alternative strategies such as pre- and pro-biotics, vaginal microbiome transplantation, and phage endolysins to improve *Lactobacillus* representation are proposed as part of the curative or preventative vaginal dysbiosis interventions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms11112643/s1, Figure S1: Comparison of baseline cellular percentages to the preceding weeks within BV outcomes (clearance in green; persistence in red and recurrence in orange); Table S1: Frequency distribution of T and dendritic cell subsets in women who had genital inflammation.

Author Contributions: The authors confirm contribution to the paper as follows: Conceptualization, W.P.Q., G.M., A.M. (Andile Mtshali) and S.N.; methodology, W.P.Q., A.M. (Andile Mtshali) and A.S.; formal analysis, M.P.L., F.O., J.E.S. and A.O.K.; parent study PIs, N.G., A.R. and A.M. (Adrian Mindel); writing—original draft preparation, W.P.Q., G.M., A.M. (Andile Mtshali), M.P.L., F.O., J.E.S., A.O.K. and S.N.; writing—review and editing, W.P.Q., G.M., A.M. (Andile Mtshali), M.P.L., F.O., J.E.S., A.O.K., N.G., A.R., A.M. (Adrian Mindel), L.J.P.L., D.A., A.S. and S.N.; supervision, G.M., A.M. (Andile Mtshali) and S.N.; project funding acquisition, S.N. All authors have read and agreed to the published version of the manuscript.

Funding: The study was funded by the Poliomyelitis Research Foundation (grant number: 19/108) and the DST-NRF Center of Excellence in HIV Prevention, which is supported by the Department of Science and Innovation, and the National Research Foundation (NRF). The CAPRISA 083 cohort study was funded by a United States—South African Program for Collaborative Biomedical Research grant through the South African Medical Research Council and the National Institute of Health (grant number: AI116759). W.P.Q. received support from the CAPRISA Research Administration and Management Training Program (grant number: G11 TW010555-01). W.P.Q. was funded by a DST-NRF CoE in HIV Prevention (grant number: 96354). A.M. was funded by the DST/NRF Innovation Postdoctoral Fellowship (grant number: PDG210309589501). G.M. was funded by a DST/NRF Innovation Postdoctoral Fellowship (grant number: SFP180507326699) and the Poliomyelitis Research Foundation (grant number: 18/16).

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author (S.N.).

Acknowledgments: We would like to thank the CAPRISA 083 study participants for their contribution to the research and the clinical and laboratory teams for the collection of clinical data and specimens.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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CHAPTER 5

5.1 Discussion

5.1.1 Summary:

HPV is the most prevalent sexually transmitted virus globally and a leading cause of cervical cancer, with notably high infection rates in sub-Saharan Africa, especially among young women. In South Africa, HPV prevalence is notably high, particularly among those under 25. This dissertation explored the relationship between HPV infection, the genital microbiome, immune responses, and matrix metalloproteinases (MMPs) in individuals infected with HPV, with findings that have significant implications for the prevention and management of cervical cancer. In **Chapter 1**, the literature review on these interactions is introduced to set the stage. In **Chapter 2**, we presented findings on HPV prevalence and its correlation with STIs and BV, highlighting the variability in high-risk HPV prevalence across studies and its link to increased MMP-10 levels when combined with STIs/BV. In **Chapter 3,** we examined the immune responses to HPV infection, noting decreased levels of key cytokines and immune cells in women with HPV-positive status. We also investigated how variations in vaginal microbiota impact immune responses, finding that *Lactobacillus*-dominant microbiota are associated with increased inflammation, while non-*Lactobacillus*-dominant microbiota correlate with impaired immune responses. The study further explored the effects of metronidazole (MDZ) treatment for BV on the mucosal immune environment, revealing increased frequencies of specific immune cells and suggesting that persistent BV may enhance HIV susceptibility. Overall, this research highlights the intricate relationships between HPV, microbial communities, immune responses, and the necessity for enhanced screening and treatment strategies.

5.1.2 **Epidemiology of HPV and the Link Between High-Risk HPV Infection and Elevated Expression of ECM-Degrading Matrix Metalloproteinases (MMPs) in Cervical Cancer Progression**

HPV, the most common sexually transmitted virus globally, is the primary cause of cervical cancer [22, 285]. While HPV prevalence varies by region, sub-Saharan Africa exhibits the highest rates, with HPV prevalence among women with normal cervical cytology ranging from 8.5% to 74.6%

[286-288]. The risk of HPV infection is notably higher in young women who begin sexual activity early in their reproductive years [10, 289]. In South Africa, women under 25 are particularly affected, with HPV prevalence reaching up to 70% and high-risk HPV (hr-HPV) prevalence at 50% [259, 286, 290]. Additionally, the prevalence of multiple HPV infections and cervical cancer incidence among young women varies significantly across different ethnic groups and geographic regions [276, 291, 292]. In **Chapter 1,** we highlighted the significance of the study and presented a thorough literature review that investigated the complex interplay between the genital microbiome, immune response, and MMP expression in the context of HPV-associated cervical cancer. The chapter also offers a concise overview of the study's overall design and a detailed analysis of the methodologies employed in the research. This foundation is informed by existing literature that emphasizes the potential effects of microbial dysbiosis and immune responses on HPV-related cervical cancer outcomes.

In **Chapter 2** of this dissertation, we explored the epidemiology of HPV in conjunction with the prevalence of STIs and BV among South African women. This dissertation found an overall HPV prevalence of 34% (83 out of 243 women), with 54.2% of these infections being high-risk genotypes. This prevalence is consistent with previous research showing a 54% high-risk HPV prevalence among South African women [55, 122, 259, 293]. However, studies within Southern Africa have reported varying prevalence rates for high-risk HPV, ranging from lower [10, 294, 295] or higher [296, 297] than those observed in our study. The discrepancies in high-risk HPV prevalence across studies in this region may stem from several factors, including differences in the population studied, sample types, and collection methods. Additionally, the transient nature of HPV infection, where the virus often clears on its own in most individuals but persists in some, can contribute to variability in prevalence rates. For instance, McDonald et al. (2014) investigated HPV genotype distribution among both HIV-positive and HIV-negative women [47], whereas other studies focused exclusively on HIV-negative women [55, 122, 259, 293]. It is well-established that HIV infection is associated with a higher prevalence of HPV [298]. Our study utilized menstrual cup pellets for sample collection, while other studies employed different methods such as cervical-vaginal lavage (CVL) pellets [55], cervical biopsies [297], and swabs or brushes [51, 107]. These differences in sample collection methods could also impact HPV

detection rates. Overall, our findings underscore the importance of enhancing and accelerating HPV screening programs to meet the WHO's 2030 targets for cervical cancer prevention and control. We further investigated whether high-risk HPV infection, including its potential progression to cancer, was linked to elevated expression of specific extracellular matrix (ECM)-degrading matrix metalloproteinases (MMPs) in cervical cancer. Our analysis revealed that women with STIs and/or BV who also had concurrent HPV infection exhibited higher concentrations of MMP-10 compared to those with STIs and/or BV but without HPV. This indicates that HPV alone may not trigger increased MMP expression; rather, it is the combination of HPV with STI/BV that is associated with higher levels of MMPs.

5.1.3 **Interactions Between Host Immune Responses, HPV Status, and Vaginal Microbiota: Influences on Immune Responses in HPV Infection**

While Chapter 2 suggests that HPV does not directly influence MMP levels, we also examined whether HPV affects immune responses and how changes in vaginal microbiota might impact these immune responses. Our research in **Chapter 3** identified key impacts of HPV infection on immune function: HPV-positive women showed significantly reduced levels of TRAIL, a cytokine essential for inducing apoptosis, and IFN-α2, which is crucial for antiviral defense against HPV [267-271]. Additionally, there were lower concentrations of CTACK and MIG, both important for immune cell trafficking and response [271-274]. HPV and oncogenic HPV infections were also associated with decreased frequencies of CCR5+ CD4+ T cells and HLADR+ CD14-CD123+ cells, which are vital for viral responses, antigen presentation, and innate immunity, respectively [275-278]. To understand how host immune responses, HPV status, and vaginal microbiota interact, we investigated how these interactions influence immune responses in the context of HPV infection and variations in vaginal microbiota. We found distinct immune responses associated with HPV infection depending on the vaginal microbiota. Women with *Lactobacillus*-dominant microbiota and HPV infection had increased levels of MIP-1β, RANTES, and IL-7, indicating heightened inflammation and immune activation [29, 46]. Conversely, women with non-*Lactobacillus*-dominant microbiota and high-risk HPV infections had significantly reduced levels of TNF-β, eotaxin, IL-16, MIG, and IFN-α, suggesting impaired immune regulation and compromised antiviral defense [270, 281, 282]. These women also had fewer cervical T cells

(CD38+ CD4+) and specific dendritic cell subsets (HLADR+ CD14- CD123+). These findings suggested that HPV infection, combined with variations in vaginal microbiota, may lead to a less effective immune response, affecting antigen presentation and immune activation [276]. These findings highlighted the complex interplay between vaginal microbiota, immune responses, and HPV infection, emphasizing to the need for further research to better understand these interactions and their implications for disease management.

5.1.4 **Immune Parameters and MDZ Treatment Response: Effects on Microbiota and Immune Cell Frequencies During BV Resolution and Recurrence**

In Chapter 2, we demonstrated that while HPV alone was not linked to changes in MMP concentrations across the general population, the combination of HPV infection with STI and/or BV was associated with increased levels of MMP-10 in these women. Given the confounding effect of BV, we then stratified the cohort to focus specifically on women with positive BV results. The high prevalence of BV and multiple infections with sexually transmitted pathogens (such as HPV and other STIs has been associated with increased HIV acquisition risk in sub-Saharan African women [299-301]. In addition, diverse microbial communities reminiscent of bacterial communities found to be associated with BV are closely associated with increased inflammatory mediators and immune cells in the FGT [59, 302-304]. Although studies have shown that vaginal microbiota can influence cytokine and cellular immune response signatures related to HIV risk, it remains unclear whether MDZ treatment induces changes in the mucosal immune environment. Consequently, the relationship between BV resolution (including treatment failure) and the mucosal immune response is not well defined.

To address this research gap, Chapter 4 examined immune parameters associated with MDZ treatment response, focusing on changes in microbiota and immune cell frequencies during BV resolution and recurrence in women, both before and after treatment. MDZ treatment resulted in a modest reduction in the relative abundance of BV-associated bacteria, but colonization by *Lactobacillus* species (other than *L. iners*) remained rare. *L. iners* is typically the first *Lactobacillus* species to emerge after treating BV with metronidazole. Growing evidence suggests that *L. iners* may play a role in the onset and maintenance of BV or intermediate BV states [305, 306].

Additionally, using the Markov model, we [304] and others [307, 308] have shown that communities dominated by *L. iners* have a high likelihood of transitioning to BV-associated states. Consistent with previous research, this study also found a high prevalence of *L. iners* and a near absence of *L. crispatus* after MDZ treatment. It has been reported that standard BV treatments often favor the dominance of *L. iners* over *L. crispatus*, which may partially explain the high recurrence rates and persistent vaginal dysbiosis, particularly after repeated treatment cycles [309-311]. There are significant gaps in understanding the role of *L. iners* in the vaginal microbiota. It is unclear whether *L. iners* is just a marker for changes in vaginal ecology or if it contributes to BV risk and affects the mucosal immune environment. Research into methods for shifting *L. iners*-dominated communities to those dominated by *L. crispatus*, and developing strategies to selectively inhibit *L. iners* while preserving beneficial *lactobacilli*, could offer new insights and lead to improved therapeutic strategies.

Although recent studies have highlighted that MDZ impacts various aspects of immunity [302, 304], there has been limited evaluation of how the mucosal immune environment changes before and after BV treatment. At weeks 6 and 12, women treated with MDZ exhibited a significant increase in the frequencies of CCR5+ CD4+ T cells and plasmacytoid dendritic cells compared to pre-treatment levels. Additionally, MDZ non-responders had significantly higher frequencies of activated CD4+ T cells and monocytes compared to MDZ responders. CCR5-expressing T lymphocytes are densely located in the subepithelial stromal tissues of the vagina, with CCR5 serving as a crucial co-receptor for HIV entry into CD4+ T lymphocytes, including facilitating cell-to-cell spread [312]. DCs engage with pathogens through pathogen-associated molecular patterns to initiate specific T and B cell responses[314-316]. Increased levels of CCR5+ T cells and plasmacytoid dendritic cells (pDCs) have been observed in women with persistent BV. This suggests that persistent BV may enhance HIV susceptibility through interactions between DCs and microbes, leading to the recruitment of activated CCR5+ cells that facilitate HIV acquisition and infection.

Many studies have explored the connection between vaginal microbiota and the frequency and activation of mucosal HIV target cells [59, 302-304], but few have examined the specific effects

of BV eradication or recurrence on the mucosal immune environment. Our previous research showed that clearing BV led to reduced levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-8, and LIF), while persistent BV was associated with increased levels of cytokines (IL-1α, IL-18, MIF, IL-7, and LIF) linked to a higher risk of HIV infection. However, these studies did not assess the impact on the frequency and activation of HIV-susceptible cells [304]. The study found that persistent BV was linked to a significant increase in activated CD4+ T cells and monocytes compared to women who cleared BV after 12 weeks. This supports earlier research showing higher levels of activated CD4+ T cells in women with diverse, non-*Lactobacillus*-dominant microbial communities [59, 302]. Additionally, increased frequencies of activated CD4+ T cells and myeloid dendritic cells (mDCs) were associated with the varied bacterial communities typical of BV. Women with persistent BV also had elevated levels of HLA-DR, an antigen-presenting molecule associated with immune checkpoint molecules on HIV-susceptible cells. In contrast, women who cleared BV had lower levels of activated CD4+ T cells and mDCs. These results suggest that diverse microbial communities in persistent BV may enhance HIV susceptibility by increasing the frequency and differentiation of mucosal HIV target cells in the genital mucosa.

5.2 **Limitations**

Several limitations were identified in this study that could affect the reliability and applicability of its findings. First, the small sample size, determined primarily by the availability of samples in the repository, may have limited the statistical power and generalizability of the results, making it challenging to draw broad conclusions. Additionally, the study lacked longitudinal cytokine data, which would have been useful in distinguishing between persistent and transient immune responses. The research focused on only 37 HPV types, leaving the possibility that other untested HPV types might influence the observed cytokine patterns. The absence of documentation on participants' HPV vaccination status also poses a limitation, as vaccination could impact HPV infection and associated immune responses. This study focused on women with laboratory-confirmed STIs and/or BV to address translationally relevant questions, but the absence of a healthy control group with a Lactobacillus-dominated vaginal microbiome limits the ability to fully contextualize and compare cytokine, immune cell, and microbiota profiles against a baseline representative of optimal genital tract health. Furthermore, the study did not consider other

potential confounding factors, such as sexually transmitted infections (both bacterial and viral), use of hormonal contraceptives, dietary habits, hormonal status, or vaginal disorders like aerobic vaginitis. These factors could significantly affect the vaginal microbiota and immune environment, and their omission highlights the need for further research to fully understand the associations observed. Despite these limitations, the cross-sectional and longitudinal assessments of HPV status, vaginal microbiota, and host immune responses provide valuable insights for future research. Addressing these limitations in subsequent studies will enhance our understanding of the intricate relationships between HPV status, BV, immune responses, and other associated factors, ultimately contributing to more refined and comprehensive knowledge in this field.

5.3 **Future directions**

Future research should prioritize longitudinal studies to better understand the dynamics between HPV status, host immune responses, and vaginal microbiota over time. Tracking these interactions over extended periods would help differentiate between persistent and transient effects and provide a more comprehensive view of how these factors evolve. Another important area for exploration is the inclusion of a broader range of HPV types in research. The current focus on only 37 HPV types may overlook other variants that could influence immune responses and microbiota composition. Expanding HPV typing could reveal additional associations and enhance our understanding of HPV-related pathogenesis. Incorporating HPV vaccination status into studies is crucial for understanding its impact on immune responses and interactions with vaginal microbiota. The effects of vaccination on the progression and management of HPV infections, as well as its influence on the immune environment, remain areas that need further investigation.

A thorough analysis of potential confounding factors is also necessary. Factors such as sexually transmitted infections (both bacterial and viral), use of hormonal contraceptives, dietary habits, hormonal status, and vaginal disorders like aerobic vaginitis could significantly affect the immune environment and microbiota. Addressing these variables will help clarify their role in the observed interactions. Detailed examination of microbiota dynamics is essential to understanding how changes in vaginal microbiota influence or are influenced by HPV infection and immune responses. Investigating these dynamic interactions could provide valuable insights into the

mechanisms underlying HPV-related processes. Functional immune assessments should be prioritized to explore cytokine profiles, immune cell activity, and the impact of microbiota on immune functions. Such studies would provide deeper insights into the functional aspects of the immune response in relation to HPV and vaginal microbiota. Research should also consider the interplay between HPV and other infections or diseases, such as HIV or other STIs. Understanding how these co-infections affect or are affected by HPV, immune responses, and microbiota could uncover additional layers of complexity in disease progression and susceptibility. Finally, interventional studies that test the effects of treatments, such as antibiotics for BV or HPV vaccines, on immune responses and microbiota are needed. These studies could offer actionable insights for managing HPV-related health issues and improving prevention and treatment strategies.

Addressing these gaps will contribute to a more nuanced understanding of the complex interactions between HPV, vaginal microbiota, and host immune responses, ultimately leading to more effective strategies for managing HPV-related conditions.

5.4 **Conclusions**

In conclusion, this study underscores the multifaceted nature of HPV infection and its interactions with the vaginal microbiota and host immune responses. HPV remains a critical global health issue, particularly in sub-Saharan Africa, where its prevalence is alarmingly high among young women. The research highlights significant regional variations in HPV prevalence and associated high-risk genotypes, pointing to the need for tailored screening and prevention strategies. By examining the relationship between HPV infection, immune responses, and vaginal microbiota, the study reveals that while HPV alone may not directly influence matrix metalloproteinase (MMP) levels, the combination of HPV with other factors like BV does contribute to elevated MMP levels, which are linked to cancer progression. Furthermore, the study demonstrates that HPV infection impacts immune function, with variations in vaginal microbiota further influencing immune responses. Specifically, *Lactobacillus*-dominant microbiota associated with HPV infection showed increased inflammation, whereas non-*Lactobacillus*-dominant microbiota correlated with impaired immune regulation and decreased antiviral defenses. The findings

emphasize the intricate interplay between vaginal microbiota and immune responses, suggesting that variations in microbiota composition can significantly affect the body's ability to respond to HPV infection.

The research also highlights the complex dynamics of BV and its treatment with metronidazole (MDZ), revealing that persistent BV and the presence of specific *Lactobacillus* species can affect immune cell frequencies and susceptibility to HIV. Persistent BV was associated with increased levels of activated HIV target cells, indicating a potential pathway for enhanced HIV acquisition risk. These insights call for further investigation into how different vaginal microbiota profiles influence immune responses and disease susceptibility. Overall, addressing the identified research gaps (such as the need for longitudinal studies, broader HPV type inclusion, and the consideration of confounding factors) will be crucial for advancing our understanding of HPV-related health issues and developing more effective preventive and therapeutic strategies.

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APPENDICES

Appendix A

1.1. STUDY METHODOLOGIES

1.1.1 Study design and sample collection

Participant recruitment for this study took place at the Prince Cyril Zulu Communicable Disease Clinic in Durban, South Africa. The study involved 56 sexually active, HIV-negative women aged 18 to 40 who consented to participate in the CAPRISA 083 STI study before sample collection [317]. Ethical approval was granted by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Ref: BE 303/17). Exclusion criteria included being HIV-positive, pregnant, or undergoing 7-day antibiotic treatment. On-site HIV and pregnancy testing determined eligibility. STI screening was performed using the CT/GC Xpert® (Cepheid, Sunnyvale, CA) assay for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in vulvovaginal swabs, and the OSOM Trichomonas rapid test for *Trichomonas vaginalis*. Genital specimens, including menstrual cups, genital swabs, and cytobrushes, were collected, and microscopy was used to assess bacterial vaginosis (BV).

STI treatment followed international and national guidelines: Women who tested positive for *Chlamydia trachomatis* were treated with 1g oral azithromycin, *Neisseria gonorrhoeae* with 250mg intramuscular ceftriaxone, and *Trichomonas vaginalis* with 2g metronidazole. Participants were classified as BV positive or negative using the Nugent scoring system, with 2g metronidazole administered for BV scores of 4-6 and 7-10. Vulvovaginal candidiasis was treated with 500mg clotrimazole pessary and 1% clotrimazole cream. Participants with STI diagnoses were offered an Expedited Partner Therapy (EPT) pack containing treatment, male condoms, and an information sheet. The EPT included 400mg oral cefixime instead of 250mg intramuscular ceftriaxone for *Neisseria gonorrhoeae* treatment. Women with BV and/or STI were monitored over 3 visits (baseline, 6 weeks, and 12 weeks), and genital specimens were collected at all visits for processing.

1.2 Methodology

1.2.1 Processing of genital specimens

a) *Swab collection and processing*

Sterile Dacron swabs were utilized for collecting genital specimens for STI and BV testing. Using moistened cotton swabs, the vulva was cleaned by wiping from back to front and examined for external lesions or genital warts. The labia were softly parted, and the swab was inserted into the vagina. Vaginal swabs were placed in 1.5ml cryovials for storage, while one swab was smeared onto a glass slide for BV Gram staining. Another swab was used for STI screening, employing the CT/GC Xpert® assay (Cepheid, Sunnyvale, CA) for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, and the OSOM Trichomonas Rapid Test for *Trichomonas vaginalis*. The swab stem was trimmed to fit into the cryovials, with the remaining swab stored at -85°C for future analysis of the vaginal microbiome [318].

b) *Collection and processing of SoftCup menstrual cups*

Cervicovaginal fluid, required for detecting genital tract cytokines, was gathered using SoftCup menstrual cups (SoftCup, San Diego, CA). Specimens were collected into 50 mL conical tubes and promptly processed upon arrival at the laboratory. Briefly, the tube underwent centrifugation at 2000 rpm for 10 minutes at 22°C to remove all cervicovaginal lavage (CVL) content, after which the menstrual cup was carefully discarded using forceps. A second centrifugation was then performed at the same speed and temperature. Should supernatant separation occur, 50µl of the supernatant was carefully aspirated into a cryovial, followed by the addition of 200µl of PBS. The sample was vortexed and aliquoted into four cryovials, while the pellet was transferred into the fifth cryovial. Finally, the specimens were stored at -85°C for subsequent analysis.

c) *Cervical Cytobrush Collection, Isolation, and Processing*

Cytobrush specimens were collected and processed in the laboratory within 4 hours of collection using the DMSO pellet method, as outlined in the protocol by Nkwanyana et al. (2009) [261]. Briefly, participants were positioned on their backs at the edge of the exam table. A vaginal

speculum was inserted, and its blades were adjusted for optimal cervix visualization. The Cytobrush was then inserted into the vagina and gently rotated 360° twice before being detached. The detached Cytobrush was placed into a conical centrifuge tube containing 3 ml of collection medium. The tube was labeled, sealed, and transported on ice to the CAPRISA Laboratory within 2 hours. Upon arrival, the sample was rinsed with sterile PBS using a pasture pipette and centrifuged at 400 x g for 10 minutes.

To isolate cervical cytobrush cells, any cervical mononuclear cells (CMC) with visible blood contamination were excluded to avoid including PBMCs in the final sample. The Cytobrush and scraper were vortexed in Falcon tubes for 45 seconds to dissociate cells, with the brush used to scrape any remaining material from the scraper, which was then discarded into a bleach solution. To collect residual cells, a fresh glove was used to squeeze the scraper between the thumb and forefinger, allowing cells and PBS to collect in a 50 ml Falcon tube. The PBS-cell suspension was filtered through a 100µm nylon cell strainer into a new Falcon tube. The original sample tube was rinsed with 5 ml of RPMI 1640, and the filter and rinse were transferred to the new collection tube. The sample was centrifuged at 514 x g with no brakes to prevent pellet dislodgement. The supernatant was carefully removed, and the pellet was resuspended with gentle agitation and washed with 5 ml of PBS. The sample was centrifuged again at 514 x g with no brakes. Cells were then either cryopreserved following the PBMC cryopreservation protocol or prepared for flow cytometry staining. After processing, the cells were stored at -80 °C overnight before being transferred to liquid nitrogen for long-term storage and downstream analysis [319].

1.2.2 Flow Cytometric analysis of human *Cervical Cytobrush*

The thawed cells were removed from the liquid nitrogen tank and transported in dry ice to the lab. Upon arrival, the cells were thawed up to 75% in a water bath until they began to separate from the tube. The cryovial was then wiped with 70% ethanol, and warm R10 medium was added dropwise to the cells. All cells were transferred to a 15 ml tube, with the cryovial being washed with R10, and the residual cells added to the 15 ml tube. The tube was filled to 10 ml with warm R10, also added dropwise. The tube was centrifuged at 1250 rpm for 7 minutes. After centrifugation, the supernatant was removed, and the pellet was gently resuspended in the

remaining liquid. The cells were incubated at 37°C in a CO2 incubator for 3 hours, followed by a second centrifugation at 1600 rpm for 10 minutes. Following the second centrifugation, cell pellets were transferred to a V-bottom plate and centrifuged at 2100 rpm for 3 minutes at 4°C. The cells were washed with 200 µl of PBS-1 (1% FBS), and the plate was centrifuged again at 2100 rpm for 3 minutes at 4°C, with the supernatant decanted.

Total cell counts were then performed using a hemocytometer. Due to the inherent variability in cell recovery from mucosal cytobrush sampling, the entire available cell suspension was used for staining. All samples were stained with identical volumes of antibody master mix, consistent incubation times, and temperature-controlled conditions to ensure uniformity in the procedure. Viable cells were stained using the Aqua Live/Dead Cell Stain kit (Aqua, Molecular Probes, Life Technologies, USA). The Live/Dead stain was prepared by resuspending 2 µl of the dye in 80 µl of dH_2O. An antibody cocktail, as detailed in **Table 1.2**, was prepared, with 50 µl of the cocktail added to each FACS tube containing cells, followed by 3 µl of the Live/Dead dye. The V-bottom plate was incubated in the dark at room temperature for 20 minutes. After incubation, 150 µl of FACS wash buffer was added, and the plate was centrifuged at 2100 rpm for 3 minutes at 4°C. The supernatant was removed, and the cells were rewashed with 200 µl of FACS wash buffer (PBS-1), followed by another centrifugation. The supernatant was discarded, and 200 µl of BD Cell Fix (1×) was added to resuspend the cells. The cells were then transferred to a FACS tube, with an additional 100 µl of Cell Fix (1×) added. The samples were analyzed on a BD LSRFortessa flow cytometer. The frequency of various cell markers was assessed using Alexa Fluor 700 (AF) labelled anti-HLA-DR, APC labelled anti-CD86, Brilliant Violet 785 (BV785) labelled anti-CD16, Peridinin-chlorophyll-protein CY5.5 (PerCP-CY5.5) labelled anti-CD123, BV650 labelled anti-CD11c (Biolegend, CA, USA), Phycoerythrin (PE) labelled anti-CD4, Allophycocyanin-H7 labelled (APC-H7) anti-CD3, Fluorescein Isothiocyanate (FITC) labelled anti-CD38, BV605 labelled anti-CCR6, BV421 labelled anti-CCR5, PECy7 labelled anti-CD14, V510 labelled anti-CD19, and V510 labelled anti-CD56 (BD, NJ, USA). The fluorescence was measured using a FACSDiva Flow cytometer (Becton and Dickinson Immunocytometry Systems). Flow cytometry data analysis, including gating strategies and frequencies, was performed using FlowJo V10 software (Tree Star, CA, USA). Specimens with a CMC CD3 T cell event count of fewer than 100

were excluded from the analysis. Below is the representative gating strategy used: (**Figure 1.7**).

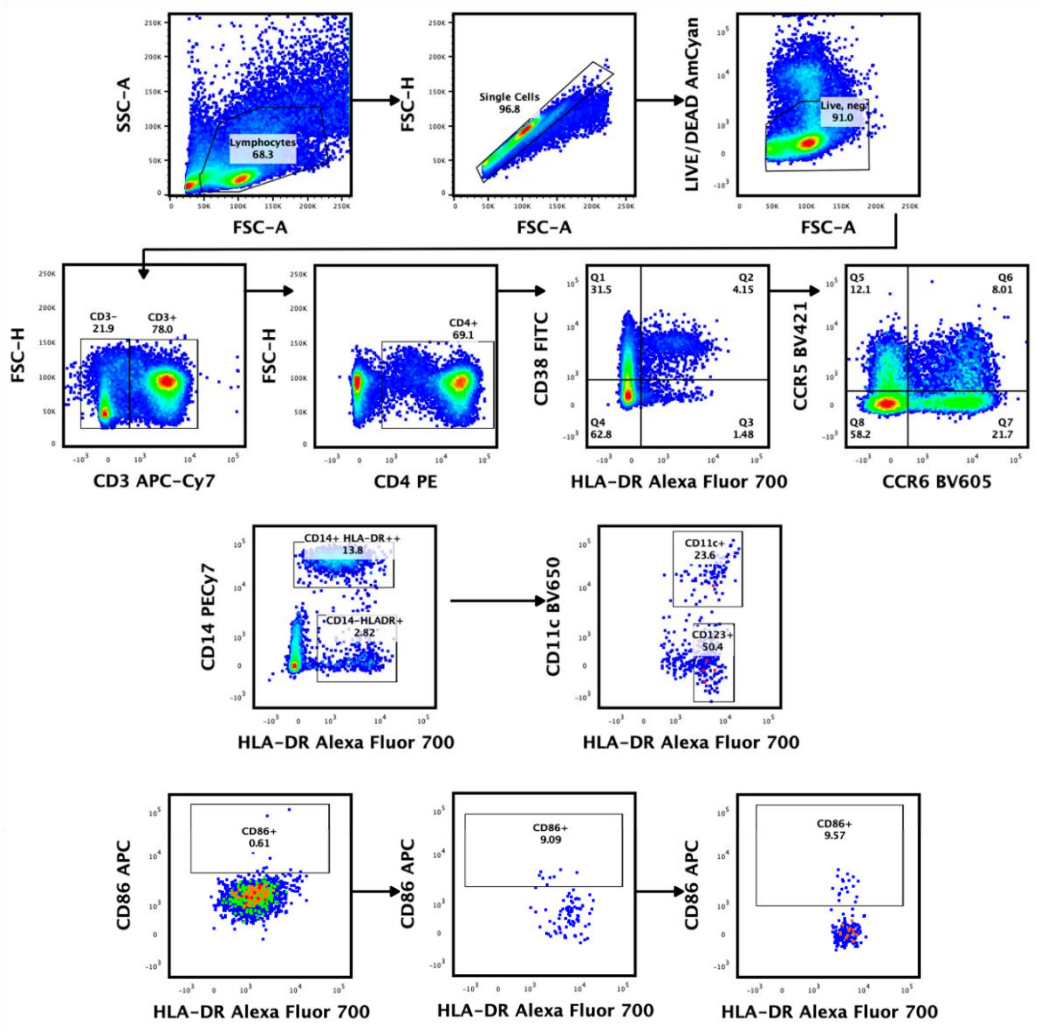


Figure 1.7. Representative flow cytometry gating strategy for assessing cervical T – and monocyte/dendritic cell subsets. The exclusion of non-lymphocytes, doublets, dead cells (LIVEDEAD^{TM}), CD19, and CD56 B lymphoid cells. The resulting cells were divided into CD3+ and CD3- subsets. CD3+ T cells were further classified into CD4+ T cells, with the expression of CD38, HLADR, CCR5, and CCR6 being examined on CD4+CD3+ T lymphocytes. The CD3- subset was further separated into dendritic cell subsets: mDCs (HLADR+CD14–CD11c+), pDCs (HLADR+CD123+), and monocytes (HLADR+CD14+CD11c+). Activated DCs were identified by CD86 expression in both CD14+HLADR+ monocytes and CD14–CD11c+ mDCs. Monocytes were categorized as CD14+HLADR++, where "++" indicates a relatively higher median fluorescence intensity (MFI) of HLADR compared to other subsets, as previously published. All gating was conducted using fluorescence minus one (FMO) controls, following a previously peer-reviewed and published strategy.

1.2.3 Compensation controls

Single-stained controls were employed to calculate fluorescent spillover between channels. Compensation beads (BD Biosciences) were used for this purpose, with each bead set stained with a single fluorochrome-conjugated antibody to serve as single-color compensation controls. The preparation involved labeling 8 FACS tubes, each corresponding to a different fluorochrome. The compensation beads were vortexed at high speed, and 2 ml of FACS wash buffer was added to each FACS tube. Six drops of negative (white cap) and positive (blue cap) beads were added, with 120 µl of each type placed into their respective labeled tubes. Next, 2 µl of the specific antibody was added to each tube, and the tubes were incubated for 5 minutes at room temperature. Each tube was then filled to 350 µl with FACS wash buffer. Compensation controls were acquired on the flow cytometer with 5000 events per tube, and compensation was calculated accordingly.

1.2.4 Preparation of Rainbow beads

To set and maintain the target median fluorescence intensities (MFIs) for the different channels, we employed 8-peak Rainbow Calibration Beads (BD Bioscience) as a reproducible fluorescence standard. Initially, these beads were run on a fully compensated BD LSRFortessa flow cytometer to establish baseline MFIs for each fluorescence channel. From these baseline data, a fixed reference peak was selected for every channel, which then served as the daily MFI target to ensure consistent fluorescence detection. Before each acquisition session, the rainbow beads were rerun to verify the instrument's performance. If the observed MFI for any channel deviated by more than ±10% from the preset target, the photomultiplier tube (PMT) voltages were carefully adjusted to bring the fluorescence intensities back within the acceptable range. This procedure ensured that data collected across different days were comparable and not confounded by instrument variability. All adjustments, instrument settings, and calibration values were systematically documented to maintain traceability and reproducibility throughout the study. For compensation controls, a small aliquot (one drop) of well-mixed rainbow beads was diluted in 350 µL of FACS wash buffer and added individually to FACS tubes. These were incubated at room temperature for 5 minutes to allow for adequate staining and fluorescence stabilization. Initial data acquisition was performed with compensation turned off to record the full spectrum of raw fluorescence emission for each fluorochrome. Subsequently, compensation was enabled to correct for spectral overlap and

spillover between adjacent fluorescence channels, ensuring accurate assignment of fluorescence signals. This rigorous calibration and compensation protocol allowed for precise and reproducible measurement of fluorescence signals, minimizing technical variability and enabling confident interpretation of flow cytometry data.

Table 1.2: Antibody staining for immune cell activation

Marker	**Colour**	**Volume**	**Number of samples + 1**	**Total volume Ab**
CD3	APC-H7	1 µl	5	5
CD4	PE	1 µl	5	5
HLA-DR	AF700	1 µl	5	5
CD38	FITC	1 µl	5	5
CCR6	BV605	1 µl	5	5
CCR5	BV421	1 µl	5	5
CD123	PerCpCy5.5	2 µl	5	10
CD11c	BV650	2 µl	5	10
CD14	PECy7	4 µl	5	20
CD86	APC	2 µl	5	10
CD16	BV785	1 µl	5	5
CD19	V510	0.5 µl	5	2.5
CD56	V510	1 µl	5	5
LD	Aqua	3 µl	5	15
Ab total		21.5		
PBS-1		(top up to 50 µl)		

1.2.5 Fluorescence Minus One (FMO) Control

The Fluorescence Minus One (FMO) control is essential for the accurate interpretation of flow cytometry data. It includes all fluorochromes in a panel except for the one being measured. This control helps identify cells and determine the appropriate placement of gates, particularly useful when distinguishing positive from negative populations with low expression levels (Maecker and Trotter, 2006). To prepare FMO controls, FACS tubes were labeled with each fluorochrome in the panel.: Alexa Fluor 700–labelled anti-HLA-DR, FITC labelled anti-CD38, BV605–labelled anti-CCR6, BV421–labelled anti-CCR5, PerCpCy5.5–labelled anti-CD123, BV650–labelled anti-CD11c, PECy7–labelled anti-CD14, APC–labelled anti-CD86, and BV785–labelled anti-CD16. Approximately 1×10^6 previously isolated peripheral blood mononuclear cells (PBMCs) were added to each tube. The PBMCs were centrifuged at 800 × g for 10 minutes. After decanting the supernatant, the tubes were flicked to resuspend the cells. An appropriate volume of each antibody was added, excluding the one corresponding to the fluorochrome labeled in the tube, based on antibody titrations. The tubes were then centrifuged at 800 × g for 3 minutes at 4ºC. Following centrifugation, the tubes were vortexed and resuspended. The samples were incubated for 20 minutes in the dark at room temperature. After incubation, 1 ml of PBS was added to each tube, followed by a second centrifugation at 800 × g for 3 minutes at 4ºC. The supernatant was decanted, and 150 µl of 1× cell fix (prepared by adding 1 ml of 10× cell fix to 9 ml distilled water) was added to each tube and vortexed briefly.

1.2.6 Investigation of Soluble biomarkers of genital inflammation in genital fluid

Before performing the multiplex array assay, the SoftCup supernatants were thawed overnight at 4°C to ensure gradual thawing and minimise protein degradation prior to cytokine quantification. Each cryovial contained 300 µL of sample. On the following day, standards, detection antibodies, streptavidin-phycoerythrin (SA-PE), and other reagents were prepared according to the manufacturer's protocol. The concentration of 48 cytokines, including pro-inflammatory, chemokines, anti-inflammatory, adaptive, and growth related cytokines were measured using the multiplex array assay, including IL-1β, IL-1Rα, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-12p40, IL-16, IL-18, IL-1α, IL-2Rα, IL-3, IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF), cutaneous T-cell attraction chemokines (CTACK), eotaxin, granulocyte colony

stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), growth regulated (GRO)-α, hepatocyte growth factor (HGF), interferon gamma induced protein (IP)-10, interferon (IFN)- γ, IFN-2α, leukemia inhibitory factor (LIF), monocyte chemotactic protein (MCP)-1, (MCP)-3, macrophage colony stimulating factor (M-CSF), monokine induced by gamma-interferon (MIG), macrophage migration inhibitory factor (MIF), macrophage inflammatory protein (MIP)-1, (MIP)-1β, nerve growth factor (NGF)-β, platelet derived growth factor (PDGF)-ββ, regulated upon activation normal T cell expressed and presumably secreted (RANTES), stem cell factor (SCF), stem cell growth factor (SCGF)-β, stromal derived factor (SDF)-1α, tumor necrosis factor (TNF)-α, TNF- β, TNF-related apoptosis inducing ligand (TRAIL), vascular endothelial growth factor (VEGF), and markers of epithelial barrier integrity (MMP-1, MMP2, MMP3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, and MMP-13) in cervicovaginal lavage fluid. To examine cytokine and MMP concentrations, respectively, the Bio-Plex Human Cytokine 27 plex and MMP kits (Bio-Rad, Hercules, CA) were utilized.

The Bio-Plex Manager software version 6 and 5-parameter logistic (5 PL) regression formulae were used to calculate sample concentrations from standard curves. Each cytokine or MMP standard was expected to have a recovery range of 70% to 130% to generate a valid 5-point standard curve. Standards outside this range were discarded. Standards with coefficients of variation (CV) higher than 20% were either fully or partially excluded based on their impact on the standard curve [320]. For validation, concentrations observed for each plate were combined. Values extrapolated were accepted, and any value falling below or above the detectable limits was adjusted to half the dataset's lowest value or twice the maximum value, respectively. Interplate and intraplate variability were assessed to detect significant differences between duplicate or interpolated wells, serving as a quality control measure for pipetting accuracy. Spearman correlation coefficients (r) greater than 0.8 and Wilcoxon T-test p-values less than 0.05 were considered indicative of strong agreement between replicate measurements

1.3 Genital microbiome characterization

1.3.1 DNA Extraction

Dry vaginal swabs were removed from the -80°C freezer and thawed at room temperature for 10 minutes before being resuspended in 500 µl of PBS. After a 10-minute incubation at room temperature, DNA extraction was performed using the PowerSoil DNA Isolation Kit (MO BIO Inc, USA). In brief, the sample was vortexed at high speed for 15 seconds in a 2 ml DNase-free tube (ThermoFisher, South Africa). Then, 50 µl of lysozyme, 6 µl of Metanolysin, and 3 µl of lysostaphin (Sigma-Aldrich, USA) were added. The mixture was incubated on a dry heating block at 37°C for one hour, with periodic vortexing every 10 to 20 minutes. Following incubation, the contents were transferred to a PowerBead tube (MO BIO Inc, USA) containing 0.25 g of soil particles. DNA extraction proceeded according to the manufacturer's instructions. Solution C1 (60 µl) was added to the PowerBead tube and mixed by inversion. The tube was then placed on a bead beater and spun twice at 4000 × g for 30 seconds. The supernatant was transferred to a 2 ml DNase-free tube, and 250 µl of solution C2 was added. The mixture was vortexed for 5 seconds and incubated for 5 minutes at 4°C. The sample was centrifuged at 25°C (room temperature) for 1 minute at 10,000 × g, and 750 µl of the supernatant was transferred to a 2 ml cryotube. This supernatant was mixed with 1000 µl of solution C4 and briefly vortexed. A spin filter was loaded with 675 µl of the mixture and centrifuged at 10,000 × g for 1 minute at 25°C. After adding 500 µl of solution C5, the sample was centrifuged at 10,000 × g for 30 seconds at room temperature, and the pellet was discarded. The spin filter was then placed in a 2 ml collection tube. Finally, 100 µl of solution C6 was added, and the tube was centrifuged at 10,000 × g for 30 seconds at 25°C. The extracted DNA was quantified and stored at -80°C. The Qubit 2.0 Fluorometer (Invitrogen, USA) was used for DNA quantification, with the Qubit quantification kit (Invitrogen, USA) to determine DNA concentration. To prepare the Qubit working solution, 1 µl of Qubit reagent was mixed with 199 µl of Qubit buffer. For standards, 10 µl of each standard was added to 190 µl of Qubit working solution. For sample preparation, 2 µl of each sample was combined with 198 µl of Qubit working solution. Both samples and standards were vortexed and incubated for 2 minutes at room temperature. The fluorometer was then used to measure the concentration of the DNA in the standards and samples.

1.3.2 Illumina 16S iTag library preparation

DNA samples were thawed on ice before PCR preparation. The mastermix was prepared for the required number of samples in duplicate, consisting of 1 µl of V4R primer (5 µM), 12.5 µl of KAPA Hotstart mix (Sigma-Aldrich, St Louis, USA), and 0.5 µl of PCR-grade water. This mastermix was added to a PCR plate, followed by 10 µl of DNA template. The contents were mixed gently, and the PCR plate was sealed and transferred to a preheated thermocycler. PCR was performed using the following cycling conditions: an initial denaturation at 95°C for 30 seconds, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension step at 72°C for 30 seconds was included, and the samples were held at 4°C. Amplicons were stored at -20°C for up to one week. For gel electrophoresis, 300 ml of 10x TRIS-Borate-EDTA (TBE) buffer was diluted with 2700 ml of deionized water to prepare the TBE solution. A 1% agarose gel was made by dissolving 0.5 g of molecular agarose (Sigma-Aldrich, USA) tablets in 300 ml of 1x TBE buffer in a 400 ml conical flask. The tablets were allowed to dissolve at room temperature, and the solution was then boiled in a microwave until fully dissolved. After cooling to room temperature, the agarose solution was poured into a gel tray with combs, leaving a gap of 2-4 mm from the top of the comb's teeth. Once set, the combs were removed. Each PCR product was mixed with gel loading dye (Sigma-Aldrich, USA) containing GelRed (Anatech, South Africa), and 5 µl of this mixture was loaded into the electrophoresis wells. The gel was run at 100V until the dye migrated 75-80% down the gel. The gel was then visualized using a GelDoc instrument (Bio-Rad Laboratories, South Africa) to check for specific PCR products.

1.3.3 Amplicon 1ST PCR clean-up

PCR samples were pooled in duplicate to achieve a total volume of 50 µl per well, and the plate was briefly centrifuged to collect condensation. AMPure beads were vortexed for 30 seconds to ensure even dispersion, and 50 µl of beads were added to each well. The mixture was pipetted up and down 10 times and incubated for 10 minutes without shaking. After incubation, the plate was placed on a magnetic separation device until the supernatant cleared. The supernatant was then discarded. Next, 200 µl of ethanol was added to the plate while it remained on the magnetic stand, and it was incubated for 30 seconds. The ethanol wash step was repeated, and excess ethanol was

removed with a P20 pipette. The beads were then air-dried. The beads were resuspended in 52.5 µl of 10 mM Tris (pH 8.5), pipetted up and down 10 times, and briefly incubated on the magnetic stand until the supernatant cleared. Finally, 50 µl of the supernatant was transferred to a new plate for agarose gel electrophoresis, and the remaining amplicons were stored in the freezer for the second PCR.

1.3.4 Index (2nd) PCR

This step involves attaching dual indices and Illumina sequencing adapters with the Nextera XT Index Kit. First, the amplicon from the initial PCR, along with the Nextera XT Index primers and KAPA Hotstart mix (Sigma-Aldrich, St Louis, USA), was thawed from the freezer. To prepare the Mastermix for the required number of samples, combine 5 µl of Index Primer 1, 1.5 µl of Index Primer 2, 25 µl of KAPA Hotstart mix, and 10 µl of PCR-grade water. After preparing the Mastermix, add 5 µl of the DNA template. Perform the PCR using a thermal cycler with the following program: 95°C for 3 minutes; 8 cycles of (95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds); then 72°C for 5 minutes, and hold at 4°C.

1.3.5 Index (2nd) PCR cleanup

AMPure beads are used to purify the final library before quantification. Start by briefly centrifuging the plate containing the library to collect any condensation. Vortex the AMPure beads for about 30 seconds to ensure uniform dispersion, then add 50 µl of beads to each well. Mix the entire volume thoroughly by pipetting up and down 10 times, and incubate the plate without shaking for 10 minutes. Next, place the plate on a magnetic stand for 2 minutes or until the supernatant becomes clear. Remove the plate from the magnetic stand and discard the supernatant. Add 200 µl of freshly prepared 80% ethanol to each well while the plate remains on the magnetic stand, and incubate for an additional 30 seconds. Gently remove the supernatant, and repeat the ethanol wash step. Use a P20 pipette to remove any remaining excess ethanol. Resuspend the beads with 27.5 µl of 10 mM Tris pH 8.5 by pipetting up and down approximately 10 times. Incubate the plate on the magnetic stand for 2 minutes or until the supernatant is clear. Finally, transfer 25

µl of the clear supernatant to a new plate. Store the amplicons in the freezer until you proceed with library quantification, normalization, and pooling.

1.3.6 Library quantification, normalization, and pooling

PCR libraries were quantified using the Qubit kit (Life Technologies, USA) and the Qubit 2.0 fluorometer. The libraries were then pooled in equimolar amounts (5ng/µl). Agarose gel electrophoresis was performed on the pooled library to assess its quality. The pooled library was re-quantified with the Qubit 2.0 fluorometer. For sequencing, the purified libraries, comprising 94 pooled samples, were sequenced on the Illumina MiSeq platform (Illumina, Inc, San Diego, California, USA) using 2x150 bp paired-end reads with v3 chemistry.

1.3.7 16S data analysis

Raw sequence data quality control and parameter inference for upstream processing were carried out using FastQC. The Divisive Amplicon Denoising Algorithm (DADA) was employed to infer amplicon sequence variants (ASVs) [321]. Taxonomic assignment was done using the SILVA ribosomal RNA database [322], and species-level annotation was achieved with Mcclassifier (https://github.com/pgajer/MCclassifier). Sequences that lacked species-level data were examined with Mcclassifier, and those matching the DADA2 genus-level assignment were recorded. The resulting phylogenetic tree and metadata were compiled into a phyloseq object using the phyloseq R package for community compositional analysis and ordination. For 16S rRNA gene data analysis, the clusGap function from the cluster R package [323] was used to determine community state types (CSTs) within the sample population, and the Mclust R package [324] was utilized to generate model-based clusters representing dominant bacteria. Distinct community types were defined based on bacterial composition within these clusters [325].

1.3.8 HPV Genotype Detection using HPV Roche Linear Array Assay

HPV status was determined using the Roche Linear Array HPV Genotyping Test, which detects 37 genital HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 56, 58, 59,

61, 62, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, 55, 64, CP6108, and IS39) in CVL pellet specimens collected at baseline. The assay was performed according to the manufacturer's instructions. Briefly, HPV DNA extraction was conducted using the MagNa Pure 96 DNA and Viral Nucleic Acid Small Volume Kit (Roche Applied Science, Mannheim, Germany). Specifically, 200 µl of the SoftCup pellet was added to a MagNa Pure 96 processing cartridge, which was then placed in the automated MagNa Pure 96 Extraction Machine. The resulting HPV target DNA was used for PCR. For PCR preparation, 125 µl of Magnesium Chloride solution was added to a vial of HPV Master Mix provided in the kit. The vial was inverted to mix its contents and then transferred to a reservoir. A volume of 50 µl of this working Master Mix was added to each well of a 96-well PCR plate. The samples and controls were then added to the plate. The 96-well plate with samples and working Master Mix was placed into the GeneAmp® PCR System 9700 (Applied Biosystems, Norwalk, CT). The thermal cycling program was performed as outlined in **Table 1.3**. The ramp rate for the cycling program was set to 50%. After completion, and within 4 hours of the final "Hold" step, the plate was removed and the samples were immediately denatured by adding 100 µl of denaturation solution.

Table 1.3. Thermal cycler conditions for amplification (Table adapted from Roche Linear Array instrument manual).

Phase	**Condition**	**Number of Cycles**
Hold	50 º C, 2 minutes	1
Hold	95 º C, 9 minutes	1
	95 º C, 30 minutes	40
Cycle	55 º C, 1 minute	40
	72 º C, 1 minute	40
Hold	72 º C, 5 minutes	1
	72 º C, continually	1

Using sterile forceps, the Linear Array HPV strips were carefully removed from the pouch, labeled, and placed into the 24-well tray with the probe lines facing upwards. Next, 4 ml of pre-warmed Working Hybridization Buffer was added to each well containing the labeled HPV strips. Following this, 75 µl of denatured PCR product was added to the wells. The tray was covered with a lid, and the contents were mixed by gently rocking the tray back and forth between each addition. The tray was then sealed and placed into a 53°C shaking water bath, weighted with a 0.5 kg lead ring to prevent movement. The hybridization process was carried out for 30 minutes while shaking at 60 RPM on a Mini Orbital SSMI Shaker (Stuart, Staffordshire, UK).

To prepare the working conjugate, 15 µl of Streptavidin-Horseradish Peroxidase was added to 5 ml of Working Ambient Wash Buffer for each strip to be tested. After hybridization, the Working Hybridization Buffer was vacuum-aspirated from the tray. Next, 4 ml of Ambient Wash Buffer was added to each well, gently shaken to rinse the strips, and the wash buffer was removed by vacuum aspiration. Following the ambient wash, 4 ml of Stringent Wash Buffer was added to each well, and the tray was covered and incubated at 53°C in the water bath for 15 minutes. After incubation, the Stringent Wash Buffer was aspirated. Then, 4 ml of Working Conjugate was added to each well, and the tray was incubated at room temperature for 30 minutes at 60 RPM on the shaker.

After the incubation, the Working Conjugate was aspirated, and 4 ml of Working Ambient Wash Buffer was added to each well. The tray was gently shaken to rinse the strips and the wash buffer was vacuum-aspirated. An additional 4 ml of Working Ambient Wash Buffer was added to each well and incubated for 10 minutes at room temperature on a platform shaker. The wash buffer was then removed, and this step was repeated with vacuum aspiration. The Working Citrate Buffer was added to each well and incubated at 60 RPM for 5 minutes at room temperature on the platform shaker. For substrate preparation, 4 ml of Substrate A was mixed with 1 ml of Substrate B per strip. After mixing, the Working Citrate Buffer was aspirated, and 4 ml of Working Substrate was added to each well. The tray was incubated for 5 minutes at room temperature on the platform shaker. After incubation, the Working Substrate was aspirated, and 4 ml of dH_2O was added to each well. Finally, the strips were removed from the tray using forceps and allowed to air dry at

room temperature for 72 hours. An HPV reference guide was used to interpret the results by aligning the reference line on the guide with the ink lines on the strips. Beta globulin detection served as an internal positive control to confirm successful DNA extraction.

Appendix B: A Detailed Methodology for Assessment of Immune Cell Activation and Phenotype in Endocervical Cytobrush Specimen.

CAP 083 - CYTOBRUSH STAINING PROTOCOL

1. **Purpose**

Assessment of immune cell activation and Phenotype in endocervical cytobrushes.

1.2 **Study Staff**

Trained laboratory staff

1.3 **Principle**:

This protocol describes the procedures for measuring immune cell phenotype and activation in endocervical cytobrush cells, such as cervical T- and dendritic cells by flow cytometry.

1.4 **Reagents**

RPMI 1640 (with added Glutamine**)**

Supplier: Whitehead

Cat No: 12-702F

Stock: 500 ML bottles

Store: between 2°C to 8°C out of direct light.

Note: The medium normally has a deep orange colour. If the pH changes, the colour of the medium will change to pink.

Use fresh medium if:

- There is a colour change.
- Use the contents of an opened bottle within a week

Store at 4°C and discard after the expiry date is reached.

Sterile water

Supplier: Lab

Stock: 1 liter sterile bottled water

Store: at room temperature

Note: Open bottles aseptically in the biosafety cabinet. Replace the cap after use and seal it with parafilm before storing it.

BD Perm/wash Buffer

Supplier: Becton and Dickenson (BD) Biosciences

Cat No: 554723

Stock: 100mL bottles.

Store: At 4°C. For use, make a 1:10 dilution in sterile water

BD FACS Comp Beads (Anti-mouse Ig, K/negative control (FBS*) Compensation Particles Set)

Supplier: Becton and Dickenson (BD) Biosciences

Cat No: 552843

Stock: 6mL of each

Store: At 4°C

BD FACS Flow Solution

Supplier: Becton and Dickenson (BD) Biosciences

Cat No: 342003

Stock: 100mL

Store: At room temperature

1.5 **Hardware**

a) Minus 80°C Freezer: Snijders Ultra Low chest freezer

b) Racks for -80°C freezer

c) Disposable pipettes, 5ml and 10ml. (Whitehead Scientific; Cat# PN5E1 and PN10E1)

d) 5ml Falcon tubes (BD, Cat#: 352054)

e) Eppendorf Mult pipette plus (stepper) (MERK; Cat# HK4981000019).

f) 1ml comb-tips plus (for use with stepper) (MERK; Cat# HK0030069234)

g) P20, P200, P1000 Gibson Pipetman (LASEC).

h) Filtered micropipette tips: p20, p200, p1000

g) Polypropylene 15ml and 50ml sterile screw cap tubes (LASEC; Cat# PGRE 188261 and PGRE 227261).

i) Vortex (Snijders).

j) Water-bath (MEMMERT WB22).

k) CO_2 incubator (Thermo Steri Cycle' LABOTECH, Model 371).

l) 10 x 10 cryoboxes (for Eppendorf tubes) (LASEC; Cat# HTBS 5026-1010)

1.6 **Procedure**

1. Pre-warm the water bath to 37°C
2. Prepare the centrifuge to be used
3. Calculate how much R10 is needed and prepare 10% R10 media (10ml FCS in 90ml RPMI/Pen-Strep) . Warm the R10 media in the water bath
4. Remove the cells from liquid nitrogen tank and transport the cells in ice to the lab
5. Thaw the cells up to 75% in the water bath
6. Remove the cryovial from the water bath and wipe it down with 70% ethanol
7. Add the warm R10 to the cells in a dropwise manner
8. Transfer all the cells to a 15 ml tube
9. Wash the cryovial with R10 and transfer the residual to the 15ml tube
10. Top the 15ml tube to 10ml with warm R10 in a dropwise manner
11. Centrifuge the tubes at 1250 rpm for 7 min
12. Decant the supernatant and resuspend the pellet with 200µl of R10
13. Centrifuge the tubes at 2100 rpm for 3 min at 4°C
14. Wash with 200 µl of PBS (1%FBS)
    1%FBS (455Ml PBS in 5mL FBS)
15. Centrifuge the V bottom plate at 2100 rpm for 3 min at 4°C
16. Decant the supernatant
17. Prepare the Aqua dye 1:40 dilution in the dark (1 µl Aqua stain in 40µl dH_20) in a cryovial
18. Prepare Aqua working solution (2.5µl Aqua stock in 47.5µl PBS per tube + 1)

20 µl Aqua stock in 380 µl PBS

19. Resuspend the cells by adding 50µl of the Aqua working solution and cover the tubes with foil
20. Incubate the FACS tubes at 4°C for 20 min in the dark
21. Add 200 µl FACS wash buffer (1% FCS in PBS)
22. Resuspend gently and centrifuge the tubes at 2100 rpm for 3 min at 4°C
23. Flick out the supernatant
24. Determine the number of FACS tubes that will need the Ab cocktail
25. Prepare Ab cocktail as follows:

Table 1: Antibody staining for immune cell activation

Marker	Colour	Volume	Number of samples + 1	Total volume Ab
CD3	APC-H7	1 µl	5	5
CD4	PE	1 µl	5	5
HLA-DR	AF700	1 µl	5	5
CD38	FITC	1 µl	5	5
CCR6	BV605	1 µl	5	5
CCR5	BV421	1 µl	5	5
CD123	PerCpCy5.5	2 µl	5	10
CD11c	BV650	2 µl	5	10
CD14	PECy7	4 µl	5	20
CD86	APC	2 µl	5	10
CD16	BV785	1 µl	5	5
CD19	V510	0.5 µl	5	2.5
CD56	V510	1 µl	5	5
LD	Aqua	3 µl	5	15
Ab total		21.5		
PBS-1		(top up to 50 µl)		

NB! Resuspend the Ab cocktail into the *required* FACS wash buffer

Number of samples × 50µl/sample = X

X - Ab total volume = Z (FACS wash buffer)

26. Add 50 µl of Ab cocktail to each FACS tube containing cells
27. Incubate at 4 °C for 20 min in the dark
28. Add 200 µl of FACS wash buffer

29. Centrifuge at 2100 rpm for 3 min at 4°C

30. Flick out the supernatant

31Add 100 µl BD Cell Fix (1×) and resuspend

1ml BD Cell Fix in 9 ml Deionized water

1.7 **Compensation beads preparation**

a) Label FACS tubes with the name of Ab and colour (one colour per tube)

b) Vortex the rainbow beads at high speed (record the lot number)

c) Vortex the compensation beads at high speed

d) Add 2ml of FACS wash buffer into the FACS tube

e) Add 6 drops of negative (white cap) and positive (blue cap) beads

f) Add 120ul into the respective labelled FACS tubes

g) Add the same volume of Ab as was added to the cells to the individual tubes

h) Incubate for 5 min at room temperature

i) Top up to 350ul with FACS wash buffer

j) Disable compensation

k) Acquire compensation controls at 5000 events on the Flow cytometer

l) Calculate compensation

1.8 **Rainbow beads preparation**

a) Add 350 ul of FACS wash buffer into the FACS tube

b) Add 1-2 drops of rainbow beads

c) Incubate for 5 min at room temperature

d) Acquire single peak Rainbow Beads at 5000 events on the Flow cytometer

e) Enable compensation

f) Acquire samples at 500000 events on the FORTESSA

Appendix C: A Detailed Methodology for DNA Extraction for Genital Microbiome Characterization

2. **DNA EXTRACTION**

2.1 **Purpose**

Genital Microbiome Characterization

2.2 **Scope**

Applicable to all personnel conducting DNA extraction in the laboratory.

2.3 **Responsibilities**

Principal Investigator (PI): Ensure SOP adherence and personnel training.

Laboratory Technicians: Perform DNA extraction according to the SOP.

Quality Assurance (QA): Monitor compliance and maintain documentation.

2.4 **Materials and Equipment**

QIAGEN DNeasy Blood & Tissue Kit

Ethanol (96-100%)

Pipettes and sterile tips

Microcentrifuge tubes (1.5 mL)

Vortex mixer

Microcentrifuge

Water bath or heating block (56°C)

Nuclease-free water, and Disposable gloves

2.5 **Procedure**

1. Spin the PowerBead Pro Tube briefly to ensure that the beads have settled at the bottom. Add up to 250 mg of soil and 800 µl of Solution CD1. Vortex briefly to mix.

2. Secure the PowerBead Pro Tube horizontally on a Vortex Adapter for 1.5–2 ml tubes (Cat. no. 13000-V1-24). Vortex at maximum speed for 10 min. Note: If using the Vortex Adapter for more than 12 preps simultaneously, increase the vortexing time by 5–10 min.

3. Centrifuge the PowerBead Pro Tube at 15,000 x g for 1 min.
4. Transfer the supernatant to a clean 2 ml Microcentrifuge Tube (provided).
Note: Expect 500–600 µl. The supernatant may still contain some soil particles.
5. Add 200 µl of Solution CD2 and vortex for 5 s.
6. Centrifuge at 15,000 x g for 1 min. Avoiding the pellet, transfer up to 700 µl of supernatant to a clean 2 ml Microcentrifuge Tube (provided). Note: Expect 500–600 µl.
7. Add 600 µl of Solution CD3 and vortex for 5 s.
8. Load 650 µl of lysate to an MB Spin Column. Centrifuge at 15,000 x g for 1 min.
9. Discard the flow-through and repeat step 8 to ensure that all of the lysate has passed through the MB Spin Column.
10. Carefully place the MB Spin Column into a clean 2 ml Collection Tube (provided). Avoid splashing any flow-through onto the MB Spin Column.
11. Add 500 µl of Solution EA to the MB Spin Column. Centrifuge at 15,000 x g for 1 min.
12. Discard the flow-through and place the MB Spin Column back into the same 2 ml Collection Tube.
13. Add 500 µl of Solution C5 to the MB Spin Column. Centrifuge at 15,000 x g for 1 min.
5.14 Discard the flow-through and place the MB Spin Column into a new 2 ml Collection Tube (provided).
15. Centrifuge at up to 16,000 x g for 2 min. Carefully place the MB Spin Column into a new 1.5 ml Elution Tube (provided).
16. Add 50–100 µl of Solution C6 to the center of the white filter membrane.
17. Centrifuge at 15,000 x g for 1 min. Discard the MB Spin Column. The DNA is now ready for downstream applications.
Note: We recommend storing the DNA frozen (–30 to –15°C or –90 to –65°C) as Solution C6 does not contain EDTA. To concentrate DNA, refer to the Troubleshooting Guide.

2.6 **Storage and Stability**

PCR reagent to be stored at 2 – 8 °C to maintain stability until the specified expiry date. PCR reagents should be kept in areas free of DNA or PCR products. Once the package containing the tube strip with lyophilized PCR mix is opened, the remaining tubes should be stored up to a

maximum of one week at 2-8°C in the original package.

2.7 **References**

QIAGEN DNeasy Blood & Tissue Kit Handbook. Institutional biosafety protocols. This SOP ensures a consistent and reliable method for extracting high-quality genomic DNA suitable for downstream applications.

Appendix D: Detailed Methodology for Investigation of Soluble Biomarkers of Genital Inflammation in Genital Fluid

3. **The Bio-Plex Pro Human Cytokine Assays**

3.1 **Purpose**

Investigation of Soluble Biomarkers of Genital Inflammation in Genital Fluid

3.2 **Scope**

Cytokines and chemokines are extracellular mediators and regulators within a signaling network between cells and are key regulators of inflammation, participating in acute and chronic conditions via complex network interactions. The Bio-Plex Pro Human Cytokine Assays are designed for the quantification of a panel of 48 cytokines, chemokines, and growth factors in plasma, serum, culture supernatant, and many other sample types.

3.3 **Responsibilities**

Principal Investigator (PI): Ensure SOP adherence, compliance with regulations, and ethical Standards, and personnel training.

3.4 **Material and Reagents**

Component	**1 x 96-well format**	**10 x 96-well format**
Standard diluent*	10ml	100ml
Sample diluent *	40ml	80ml
Assay buffer	50ml	500ml
Wash buffer	250ml	1.5L
Detection antibody diluent	5ml	50ml
Streptavidin-PE (100x)	1 tube	1 tube
Filter plate and/or flat bottom plate (96-well)	1 plate	10 plates
Sealing plate	1 pack of 4	10 packs of 4
Assay quick guide	1 booklet	1 booklet
Standard	1 vial	10 vials
Human and mouse cytokine groups (group I and II)		
Detection antibody diluent (10x)	1 tube	1 tube
Coupled magnetic beads (10x)	1 tube	1 tube
Mouse cytokine (group III) and Rat cytokine (group I)		
Detection antibodies (20x)	1 tube	1 tube
Coupled magnetic beads (20x)	1 tube	1 tube

Bio-Plex Pro high dilution reagent kit, 1 x 96 well, contains 70ml serum-based diluent instead of standard diluent and sample diluent.

3.5 **Recommended Materials**

Bio-Plex Pro™ Human Cytokine Screening Assay Quick Guide

Bio-Plex® Validation Kit 4.0

Note: Run the validation kit monthly to ensure optimal performance of fluidics and optics systems

Bio-Plex Calibration Kit

Note: Run the calibration kit daily to standardize the fluorescence signal

Bio-Plex Pro Wash Station

Bio-Plex Handheld Magnetic Washer

Bio-Plex Pro Flat Bottom Plates (40 x 96-well)

For magnetic separation on the Bio-Plex Pro Wash Station

Titertube® Micro Test Tubes

For preparing replicate standards, samples, and controls before loading the plate

Microtiter Plate Shaker

IKA MTS 2/4 Shaker for 2 or 4 microplates or Barnstead/Lab-Line Titer Plate Shaker Model 4625 (or equivalent capable of 300–1,100 rpm)

BR-2000 Vortexer

Reagent reservoirs, 25 ml

For capture beads and detection antibodies

Reagent reservoir, 50 ml

For reagents and buffers

Acrodisc PF Syringe (25 mm) with Supor Membrane (0.8/0.2 µm), sterile

Other: 5 or 15 ml polypropylene tubes for reagent dilutions, calibrated pipets, pipet tips, sterile distilled water, absorbent paper towels, and 1.5 or 2 ml microcentrifuge tubes

3.6 **Procedure**

1. Add 50 µl 1 x beads to wells
2. wash buffer: 2 x 100µl
3. Add 50 µl of standards, samples, and controls and incubate on shaker at 850rpm for 30min at room temperature
4. wash buffer: 3 x 100 µl
5. Add 25 µl 1x detection antibody, and incubate on a shaker at 850rpm for 30 minutes at room temperature.
6. wash buffer: 3 x 100µl
7. Add 50 µl of 1 x streptavidin-PE, and incubate on a shaker at 850 rpm for 10 minutes at

room temperature

8. wash buffer: 3 x 100µl

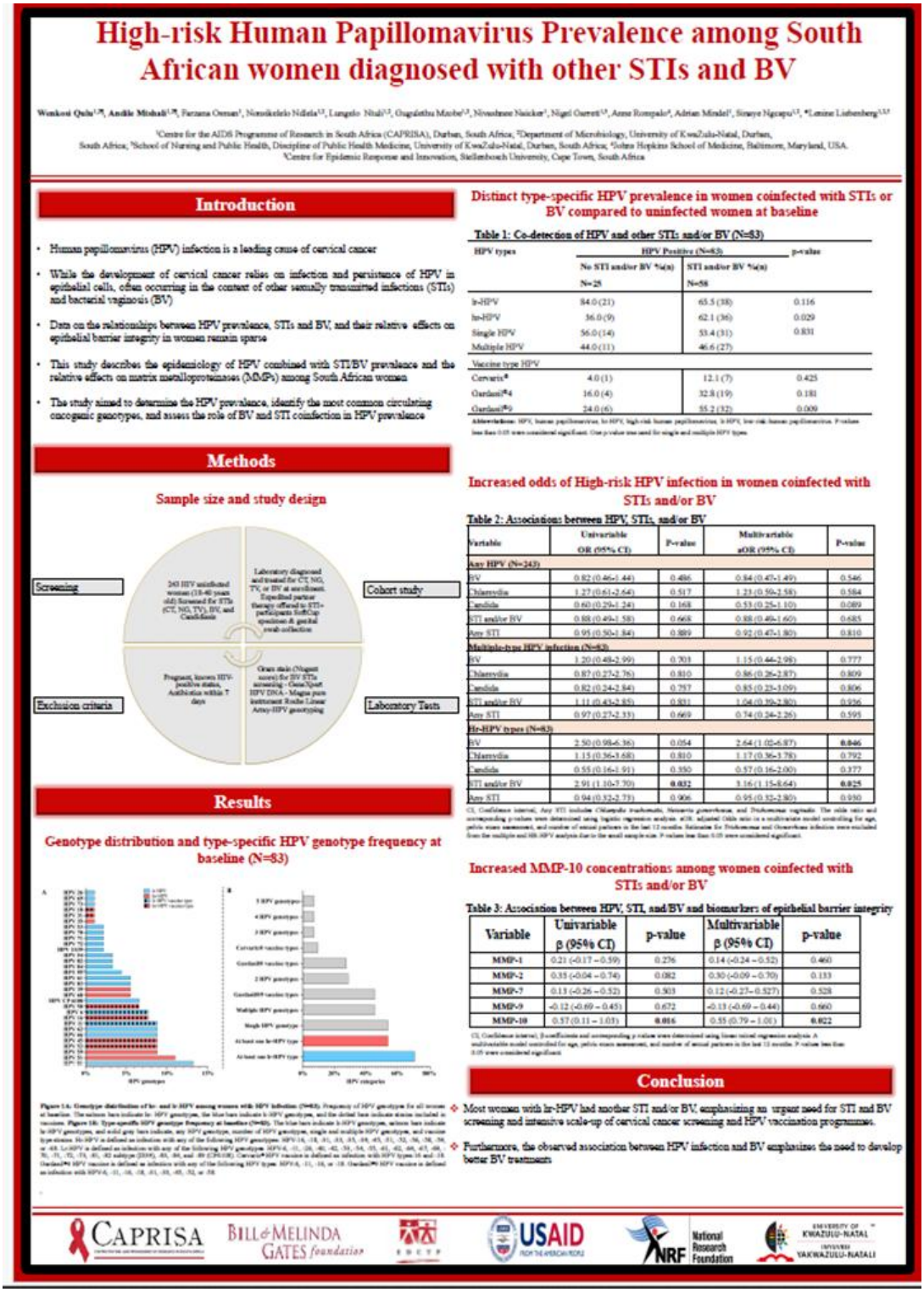
9. Resuspend in 125µl assay buffer, shake at 850rmp for 30 sec

10. Acquire data on the Bio-Plex® system

Storage and Stability

Kit contents are stored at 4°C and not frozen. Coupled magnetic beads and Streptavidin should be kept in the dark. All components of the kit are guaranteed for a maximum of 6 months from the date of purchase, provided all components are stored as instructed.

Appendix E: Poster presentation



High-risk Human Papillomavirus Prevalence among South African women diagnosed with other STIs and BV

Wonkosi Quin[1,2], Andile Mkhabi[1,2], Farzana Osman[1], Nonzikelelo Ndlela[1,2], Lungelo Ntuli[1,2], Ongezithu Mzobe[1,2], Nivashnee Naicker[1], Nigel Garrett[1,3], Anne Rompalo[4], Adrian Mindel[1], Sinaye Ngcapu[1,2], *Lenine Liebenberg[1,2,5]

[1]Centre for the AIDS Programme of Research in South Africa (CAPRISA), Durban, South Africa; [2]Department of Microbiology, University of KwaZulu-Natal, Durban, South Africa; [3]School of Nursing and Public Health, Discipline of Public Health Medicine, University of KwaZulu-Natal, Durban, South Africa; [4]Johns Hopkins School of Medicine, Baltimore, Maryland, USA. [5]Centre for Epidemic Response and Innovation, Stellenbosch University, Cape Town, South Africa

Introduction

- Human papillomavirus (HPV) infection is a leading cause of cervical cancer
- While the development of cervical cancer relies on infection and persistence of HPV in epithelial cells, often occurring in the context of other sexually transmitted infections (STIs) and bacterial vaginosis (BV)
- Data on the relationships between HPV prevalence, STIs and BV, and their relative effects on epithelial barrier integrity in women remain sparse
- This study describes the epidemiology of HPV combined with STI/BV prevalence and the relative effects on matrix metalloproteinases (MMPs) among South African women
- The study aimed to determine the HPV prevalence, identify the most common circulating oncogenic genotypes, and assess the role of BV and STI coinfection in HPV prevalence

Methods

Sample size and study design

Results

Genotype distribution and type-specific HPV genotype frequency at baseline (N=83)

Distinct type-specific HPV prevalence in women coinfected with STIs or BV compared to uninfected women at baseline

Table 1: Co-detection of HPV and other STIs and/or BV (N=83)

HPV types	HPV Positive (N=83)		p-value
	No STI and/or BV %(n) N=25	STI and/or BV %(n) N=58	
lr-HPV	84.0 (21)	65.5 (38)	0.116
hr-HPV	36.0 (9)	62.1 (36)	0.029
Single HPV	56.0 (14)	53.4 (31)	0.831
Multiple HPV	44.0 (11)	46.6 (27)	
Vaccine type HPV			
Cervarix®	4.0 (1)	12.1 (7)	0.425
Gardasil®4	16.0 (4)	32.8 (19)	0.181
Gardasil®9	24.0 (6)	55.2 (32)	0.009

Abbreviations: HPV, human papillomavirus; lr-HPV, high-risk human papillomavirus; lr-HPV, low-risk human papillomavirus. P-values less than 0.05 were considered significant. Cox p-value was used for single and multiple HPV types.

Increased odds of High-risk HPV infection in women coinfected with STIs and/or BV

Table 2: Associations between HPV, STIs, and/or BV

Variable	Univariable OR (95% CI)	P-value	Multivariable aOR (95% CI)	P-value
Any HPV (N=243)				
BV	0.82 (0.46-1.44)	0.486	0.84 (0.47-1.49)	0.546
Chlamydia	1.27 (0.61-2.64)	0.517	1.23 (0.59-2.58)	0.584
Candida	0.60 (0.29-1.24)	0.168	0.53 (0.25-1.10)	0.089
STI and/or BV	0.88 (0.49-1.58)	0.668	0.88 (0.49-1.60)	0.685
Any STI	0.95 (0.50-1.84)	0.889	0.92 (0.47-1.80)	0.810
Multiple-type HPV infection (N=83)				
BV	1.20 (0.48-2.99)	0.703	1.15 (0.44-2.98)	0.777
Chlamydia	0.87 (0.27-2.76)	0.810	0.86 (0.26-2.87)	0.809
Candida	0.82 (0.24-2.84)	0.757	0.85 (0.23-3.09)	0.806
STI and/or BV	1.11 (0.43-2.85)	0.831	1.04 (0.39-2.80)	0.936
Any STI	0.97 (0.27-2.33)	0.669	0.74 (0.24-2.26)	0.595
Hr-HPV types (N=83)				
BV	2.50 (0.98-6.36)	0.054	2.64 (1.02-6.87)	**0.046**
Chlamydia	1.15 (0.36-3.68)	0.810	1.17 (0.36-3.78)	0.792
Candida	0.55 (0.16-1.91)	0.350	0.57 (0.16-2.00)	0.377
STI and/or BV	2.91 (1.10-7.70)	**0.032**	3.16 (1.15-8.64)	**0.025**
Any STI	0.94 (0.32-2.73)	0.906	0.95 (0.32-2.80)	0.930

CI, Confidence interval; Any STI include Chlamydia trachomatis, Neisseria gonorrhoeae, and Trichomonas vaginalis. The odds ratio and corresponding p-values were determined using logistic regression analysis. aOR: adjusted Odds ratio in a multivariate model controlling for age, pelvic exam assessment, and number of sexual partners in the last 12 months. Estimates for Trichomonas and Gonorrhoea infection were excluded from the multiple and Hr-HPV analysis due to the small sample size. P-values less than 0.05 were considered significant.

Increased MMP-10 concentrations among women coinfected with STIs and/or BV

Table 3: Association between HPV, STI, and BV and biomarkers of epithelial barrier integrity

Variable	Univariable β (95% CI)	p-value	Multivariable β (95% CI)	p-value
MMP-1	0.21 (-0.17 – 0.59)	0.276	0.14 (-0.24 – 0.52)	0.460
MMP-2	0.35 (-0.04 – 0.74)	0.082	0.30 (-0.09 – 0.70)	0.133
MMP-7	0.13 (-0.26 – 0.52)	0.503	0.12 (-0.27– 0.527)	0.528
MMP-9	-0.12 (-0.69 – 0.45)	0.672	-0.13 (-0.69 – 0.44)	0.660
MMP-10	0.57 (0.11 – 1.03)	**0.016**	0.55 (0.79 – 1.01)	**0.022**

CI, Confidence interval; β coefficients and corresponding p value were determined using linear mixed regression analysis. A multivariable model controlled for age, pelvic exam assessment, and number of sexual partners in the last 12 months. P value less than 0.05 were considered significant.

Conclusion

- Most women with hr-HPV had another STI and/or BV, emphasizing an urgent need for STI and BV screening and intensive scale-up of cervical cancer screening and HPV vaccination programmes.
- Furthermore, the observed association between HPV infection and BV emphasizes the need to develop better BV treatment.

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Appendix F: UKZN BREC approval letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

31 March 2023

Mr W Qulu (209511420)
School of Laboratory Medicine and Medical Sciences
College of Health Sciences
Wenkosi.qulu@yahoo.com

Dear Mr Qulu

Protocol: The role of genital inflammation and microbiota on HIV susceptibility in the female genital tract: understanding the potential susceptibility factors for HIV acquisition using ex vivo human cervical explants.
Degree: PhD
BREC Ref No: BE303/17

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	**07 June 2023**
Expiration of Ethical Approval:	**06 June 2024**

I wish to advise you that your application for recertification received on 22 February 2023 for the above study has been noted and approved by a subcommittee of the Biomedical Research Ethics Committee (BREC). The start and end dates of this period are indicated above.

Your response to BREC queries dated 07 March 2023 and 27 March 2023 has been noted by a subcommittee of the BREC.

- Amendments are now to be submitted separately for review.

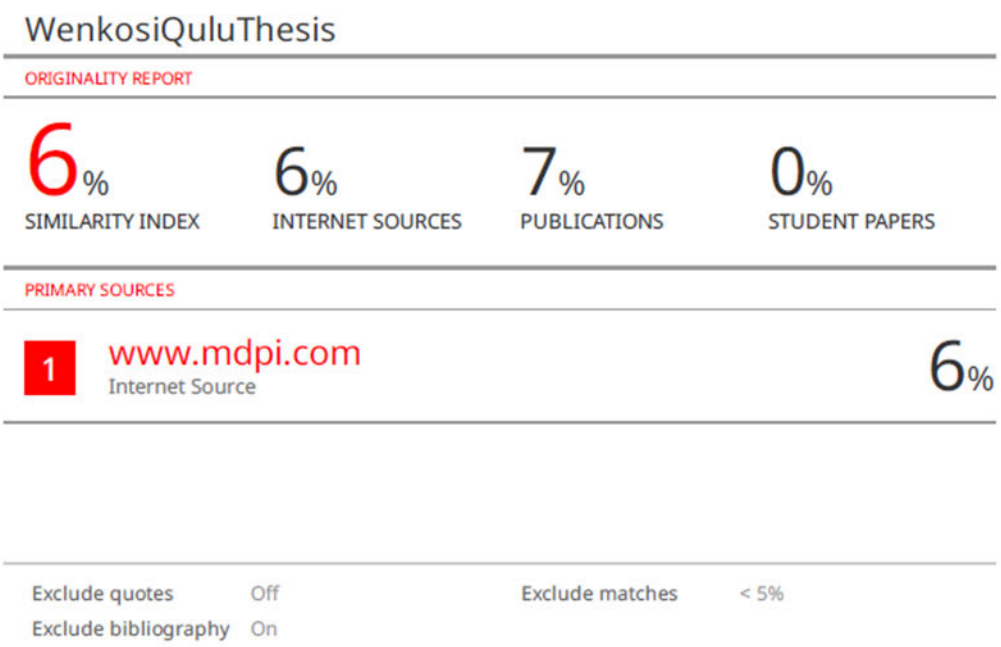
If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 09 May 2023.

Yours sincerely

[redacted]

Ms A Marimuthu
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee


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