The Impact of Vaginal Microbiota on Human Papillomavirus Infection

Submitted by

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Submitted in fulfilment of the requirements for the degree of

Masters in Medical Science

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2022

PREFACE

The experimental work described in this thesis was carried out at the Centre of AIDS Programme of Research in South Africa (CAPRISA) laboratory, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, South Africa, under the supervision of Dr. Sinaye Ngcapu.

This study represents original work by the author and has not been submitted in any other form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

10 May 2022

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PLAGIARISM DECLARATION

I, **Lungelo Ntuli,** declare that:

1) The research reported in this dissertation, except where otherwise indicated is my original work.
2) This dissertation has not been submitted for any degree or examination at any other university.
3) This dissertation does not contain other person's data, pictures, graphs, or other information, unless specifically acknowledged as being sourced from other persons.
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5) Where I have reproduced a publication of which I am an author, co-author, I have indicated in detail which part of the publication was written by myself alone and have fully referenced such publications.
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Name of Student: Lungelo Ntuli Date: 10 May 2022

ACKNOWLEDGEMENTS

- I would like to thank my supervisor Dr Sinaye Ngcapu and my co-supervisors Dr Andile Mtshali and Dr Gugulethu Mzobe for their remarkable scientific support and moulding
- A special thanks to the organization CAPRISA for the fellowship program support
- I would like to thank the Poliomyelitis Research Foundation (PRF) for financial support and DSI-NRF Centre of Excellence in HIV prevention.
- I would like to thank my family (my mother and my siblings) for their support towards my studies
- Lastly, thanks to Mucosal Immunology Laboratory staff and a very special thanks to CAPRISA fellow for their support and encouragement throughout the period.

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LIST OF ABBREVIATIONS

APC - ANTIGEN-PRESENTING CELL

BREC - BIOMEDICAL RESEARCH ETHICS COMMITTEE

BV - BACTERIAL VAGINOSIS

CIN - CERVICAL INTRAEPITHELIAL NEOPLASIA

CST - COMMUNITY STATE TYPE

CT - CHLAMYDIA TRACHOMATIS

CTL - CYTOTOXIC T-LYMPHOCYTE

CVL - CERVICOVAGINAL LAVAGE

DC - DENDRITIC CELLS

DNA - DEOXYRIBOSE NUCLEIC ACID

E GENE- EARLY GENE

FGT - FEMALE GENITAL TRACT

GMCSF- GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR

HIV - HUMAN IMMUNODEFICIENCY VIRUS

HPV - HUMAN PAPILLOMAVIRUS

IFN - INTERFERON

IL - INTERLEUKIN

IQR - INTERQUARTILE RANGE

L GENE- LATE GEN

LC - LANGERHAN CELL

MHC - MAJOR HISTOCOMPATIBILITY COMPLEX

MIP - MACROPHAGE INFLAMMATORY PROTEIN

MMX - MASTER MIX

NG	-	NEISSERIA GONORRHOEA
NLR	-	NODE LIKE RECEPTORS
NMDS	-	NONMETRIC MULTIDIMENSIONAL SCALING
OTU	-	OPERATIONAL TAXONOMIC UNIT
PCOA	-	PRINCIPAL COORDINATE ANALYSIS
PCR	-	POLYMERASE CHAIN REACTION
PSA	-	PROSTATE-SPECIFIC ANTIGEN
RLR	-	RIG-I-LIKE RECEPTORS
SDS	-	SODIUM DODECYL SULPHATE
SHPC	-	STREPTAVIDIN HORSERADISH PEROXIDASE CONJUGATE
SSPE	-	SODIUM CHLORIDE SODIUM PHOSPHATE EDTA
STI	-	SEXUALLY TRANSMITTED INFECTION
TGF	-	TRANSFORMING GROWTH FACTOR
TH...	-	T HELPER CELLS
TLR	-	TOLL-LIKE RECEPTORS
TMB	-	TETRAMETHYLBENZINE
TNF	-	TUMOR NECROSIS FACTOR
TV	-	TRACHOMONAS VAGINALIS
URR	-	UPSTREAM REGULATORY REGION
VIN	-	VULVAR INTRAEPITHELIAL NEOPLASIA
WHO	-	WORLD HEALTH ORGANIZATION
WLWH	-	WOMEN LIVING WITH HIV

ABSTRACT

Background: Cervical human papillomavirus (HPV) infection is the most common sexually transmitted infection (STI) in sub-Saharan African women of reproductive age. While most women clear HPV infection, persistent infection with high-risk HPV is the most common non-system biological risk factor for cervical cancer development. Increased levels of pro-inflammatory cytokines and overgrowth of diverse microbial communities have been implicated in undermining the clearance of the infection and promoting oncogenesis. Here we aimed to evaluate the role of vaginal microbiota composition in the persistence and clearance of HPV infections in women.

Methods: This study included the assessment of 56 women who participated in the CAPRISA 083 cohort. The CAPRISA 083 study evaluated point of care STI testing immediate treatment and expedited partner therapy. Sexually transmitted infections (STIs) and BV were screened using the GeneXpert system or OSOM Trichomonas rapid test and Nugent score, respectively. Vaginal swabs and SoftCup genital secretions were collected at enrolment, 6 weeks, and 13 weeks post-treatment. The Roche Linear Array was used for HPV genotyping, and the vaginal microbiome was characterized using 16S rRNA sequencing.

Results: The study demonstrated a 36/56 (64 %), 28/56 (50 %), and 36/56 (64 %) prevalent of HPV at baseline, 6 weeks and 13 weeks, respectively. The prevalence of high-risk HPV infection at baseline was 58%, 61% at 6 weeks, and 45% at 13 weeks. HPV 16, 45, 58, and 59 were the most dominant high-risk genotypes in all visits, while HPV 6 was the least common. Overall, 46% (26/56) of participants cleared any HPV genotype, while 45% (25/56) acquired and 38% (21/56) had persisted any HPV genotype at follow-up visits. Alpha diversity of the vaginal microbiome of women with HPV (p value= 0.57) and high-risk HPV (p value= 0.6) infection did not differ significantly to that in vaginal microbiome from uninfected women. LEfSe analysis identified *Lactobacillus spp*. (particularly *L. iners*) as potential biomarkers for HPV clearance between visits, whereas HPV persistence was associated with enrichment of *Sneathia amnii* and other BV-associated bacteria.

Conclusion: While our data do not indicate the causal link between the diverse genital microbiome and HPV clearance or persistent, *L. iners* and *Sneathia* abundance were associated with HPV clearance and persistent, respectively. These data suggest the need for longitudinal investigation to confirm a biological mechanism for this relationship, which will likely benefit cervical cancer management.

CHAPTER 1: INTRODUCTION

1.1 Introduction

Human Papillomavirus (HPV), a primary cause of genital warts and cervical cancer, is the most common sexually transmitted infection (Burchell et al., 2006). Despite efforts to implement prophylactic HPV vaccination in young women, cervical cancer is the fourth most common cancer among sexually active women globally (Sung et al., 2021). A majority (~90%) of women clear HPV infections spontaneously within 6–18 months (Myers et al., 2000, Plummer et al., 2007), with very few progressions from precancerous lesion to invasive cervical cancer (Stanley, 2008). Several studies have hypothesized that host defense mechanisms, the genital microbiome, and other factors in the female genital tract play a role in the clearance and persistence of HPV, including the risk of developing cervical cancer (Daud et al., 2011, Łaniewski et al., 2018, Liebenberg et al., 2019b, Moscicki et al., 2001, Onywera et al., 2019).

Innate and adaptive immune responses represent the first line of host defense at the mucosal surface against pathogens such as HPV (Barros et al., 2018). Several studies have demonstrated a relationship between genital mucosal cytokine concentrations and the control or elimination of HPV infection in the cervix (Scott et al., 2013b, Lieberman et al., 2008, Li et al., 2019, Liebenberg et al., 2019b). It is hypothesized that cytokine response occurs within days after the establishment of an HPV infection and is subsequently reversed when HPV clearance has been effectively achieved by appropriate effector cells (Amaral et al., 2006, Scott et al., 2013b, O'Byrne and Dalgleish, 2001, Balkwill and Mantovani, 2001, Hawes and Kiviat, 2002). The initial response against HPV infection includes mature antigen-presenting cells (APCs) that secret cytokines and contribute to the activation and recruitment of other immune cells to the site of infection (Stanley, 2006). Upon interaction with mature APCs, naïve CD4+ and CD8+ T cells differentiate into various T helper effector lineages and cytotoxic T lymphocytes (CTLs), respectively, which are needed for the effective clearance of HPV(Stanley, 2006, Steele et al., 2002). However, these cells do not entirely prevent disease progression. HPV can use various immune evasion mechanisms to limit the anti-viral activity of immune response, resulting in HPV infection tolerance in the host's immune system. HPV infection could affect the differentiation of monocytes into mature DCs and distinctively affect the functionality of CD4+/CD8+, and regulatory T cells (Song et al., 2015).

Increased secretion of anti-inflammatory cytokine IL-10 in Th2 response has been associated with compromised innate and adaptive immune defense and cervical lesion progression during high-risk HPV infection (Scott et al., 2013a, Peghini et al., 2012, Lin et al., 2019). Although studies have demonstrated the anti-viral activities of immune cells, less is known about the cell-mediated mucosal immune response to HPV incidence, persistence, and clearance.

Emerging evidence suggests that women with a low relative abundance of vaginal *Lactobacillus* species and high proportions of the *Gardnerella, Sneathia,* and *Atopobium* genera are less likely to clear HPV infection (Audirac-Chalifour et al., 2016b, Brotman et al., 2014, Critchlow et al., 1995, Gao et al., 2013, Gillet et al., 2011, Łaniewski et al., 2018, Mitra et al., 2015, Norenhag et al., 2020). Two recent meta-analyses summarizing findings from several microscopy or molecular studies have reported that women with *Lactobacillus*-enriched microbiomes were less likely to acquire HPV infection compared to women with overgrowth of bacterial vaginosis (BV)-linked bacteria (*Gardnerella, Atopobium,* and *Prevotella*) (Brusselaers et al., 2019, Norenhag et al., 2020). A *Lactobacillus gasseri*-enriched microbiome was associated with rapid rates of HPV clearance (Brotman et al., 2014, Di Paola et al., 2017a), while a *L. iners* dominant microbiome was commonly reported in women presenting with cervical intraepithelial neoplasia (CIN). Several studies further reported an association between BV-linked microbial communities (*Gardnerella, Prevotella, Dialister, Streptococcus, Ureaplasma, Megasphaera,* and *Mycoplasma*) and persistent infection with high-risk HPV that may result in the development of CIN (Wei et al., 2020, Norenhag et al., 2020). Furthermore, specific vaginal microbiota may modulate host immune responses, including critical antiviral and anti-tumor immunity components in the female genital tract (Anahtar et al., 2015, Doerflinger et al., 2014). Diverse microbial communities have been closely associated with altered innate immune responses, host susceptibility to infection (Anahtar et al., 2015, Gosmann et al., 2017, Mittal et al., 2013), and development of cervical diseases, but proven causality remains unclear.

While there is evidence of a relationship between the vaginal microbiome, host immune responses, and HPV infection, a link between the vaginal microbiome, host responses, and the progression to HPV-associated cervical cancer remains unclear. Therefore, understanding the concept of vaginal

microbiome fluctuations and associated host immune responses during HPV infection stages could shed light on possible mechanisms associated with cervical carcinogenesis.

1.2 RATIONALE AND STUDY AIMS

1.2.1 Rationale of the study

South African women have a high burden of high-risk HPV genotypes that causes cervical cancer. There is evidence of a relationship between the vaginal microbiome and HPV infection. Culture-independent studies have demonstrated that most black South African women have non-Lactobacilli-dominated vaginal microbial communities associated with bacterial vaginosis. However, the role of these vaginal microbiome in HPV persistence and clearance is not yet fully elucidated. Understanding the role of vaginal microbiome fluctuations and associated inflammation during HPV stages could shed light on possible mechanisms associated with cervical carcinogenesis.

1.2.2 Aim

To evaluate the role of vaginal microbiota composition and associated cytokine profiles in the persistence and clearance of HPV infections in women.

1.2.3 Objectives

a. To characterise bacterial diversity and measure cytokine concentrations in longitudinal genital samples from women with HPV infection.

b. To evaluate the relationship between the vaginal microbiome, genital inflammation, and HPV oncogenicity

1.2.4 Hypothesis

Diverse vaginal microbiota and associated inflammation are associated with persistence (including re-infection after clearance) of HPV infections, while a *Lactobacilli* dominated microbiota predicts clearance

CHAPTER 2: LITERATURE REVIEW

2. In accordance with UKZN guidelines, the literature review for this thesis was drafted for publication. The manuscript has been submitted to the *Frontiers in Cellular and Infection Microbiology, section Virus and Host,* and is currently under review (Manuscript ID: 927131, Appendix C).

2.1 HPV genotypes and screening

HPV is a member of the non-enveloped double-stranded DNA papillomaviridae family that infects squamous epithelium found beneath the foreskin of the penis, the scrotum, vulva, vagina, cervix, skin, and anus (Doorbar et al., 2015, Harden and Munger, 2017). The papillomavirus structure is icosahedral with approximately 50–60 nm and contains 8000 base pairs (Harden and Munger, 2017, Gupta and Mania-Pramanik, 2019). HPV is divided into three major regions: upstream regulatory region (URR), the early (E) gene region, and the late (L) gene region (Pal and Kundu, 2019). The URR is a non-coding region with multiple transcription factor binding sites necessary for the regulation of viral transcription and initial replication occurs (Shanmugasundaram and You, 2017). The E gene region encodes E1, E2, E4, E5, E6, and E7 regulatory proteins, each holding important functions in the genome (Hoppe-Seyler et al., 2018). E1 has DNA helicase activity that drives the viral DNA replication process. E2 regulates cellular gene expression and plays an important role in the transfer of the viral genome to daughter cells when the host cell divides. E4 is responsible for viral assembly, while E5 controls cell growth and differentiation (Pal and Kundu, 2019). E6 is an oncoprotein that interacts with E7 to maintain a suitable environment for viral replication and bind p53 to tamper with its tumor-suppressive function and direct it to degradation (Scheffner et al., 1993). The L gene region codes for L1 and L2 proteins of the viral capsid, known to facilitate the formation of complete virions (Taghizadeh et al., 2019).

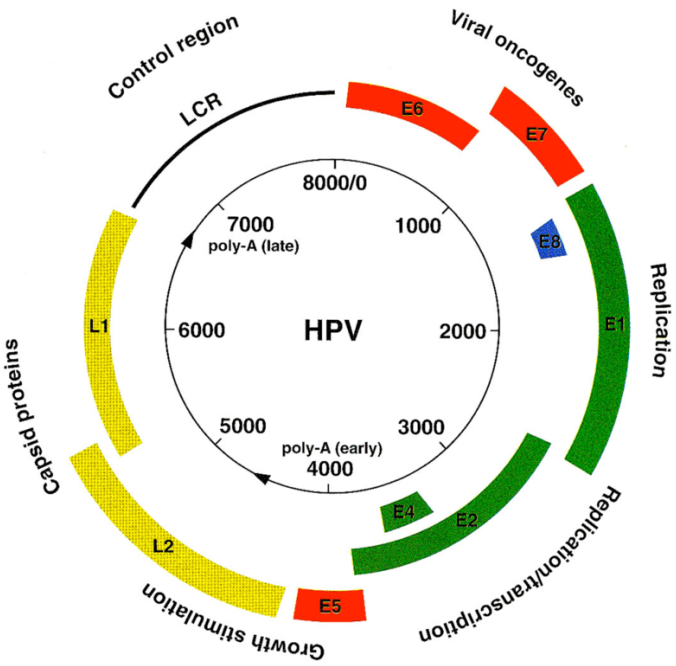


Figure 1. Typical genomic organisation of HPV (figure taken from Prendivillie, 2004). The viral genome contains 8000 base pairs and consists of the upstream regulatory region, the early gene region, and the late gene region. The upstream regulatory region controls early gene transcription and replication, while the early region consists of genes (E2, E1, E6, and E7) that are necessary for the viral cycle, and they play essential roles in cell transformation. The major and minor capsid proteins (L1 and L2, respectively) are responsible for the formation of the capsid during viral assembly.

HPV infection is classified into five major genera, *Alphapapillomavirus, Gammapapillomavirus, Betapapillomavirus, Deltapapillomavirus,* and *Mupapillomavirus*, with *Alphapapillomavirus, Gammapapillomavirus* and *Betapapillomavirus* affecting humans (Harden and Munger, 2017). Of the genera affecting humans, *Alphapapillomavirus* is the most common genus infecting the genital tract (International Agency for Research on Cancer, 2018). More than 200 genital HPV genotypes have been molecularly characterized, with some categorized into low-risk genotypes (causing genital warts) while others were categorized as high-risk genotypes (causing CIN and cervical cancer) (Juckett and Hartman-Adams, 2010). High-risk HPV types include HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 68, and 59, with HPV 16 and 18 being the most prevalent (accounting for 70% of cervical cancer cases) genotypes (Clifford et al., 2017). The most common low-risk HPV

include HPV 6 and 11, accounting for ~90% of genital warts and rarely developing into cancer (Ebrahim et al., 2016). HPV screening uses biopsy, colposcopy and acetic acid test, Pap smear, and nucleic acid-based tests (Agorastos et al., 2010). A Pap smear is the main screening tool to identify precancerous cells in the cervix, and any observed abnormalities are further evaluated with colposcopy, biopsy, and molecular-based tests (Agorastos et al., 2010).

Table 1: Groups of HPV genotypes and clinical associations

HPV group	HPV types	Clinical association
Low risk	6, 11, 42, 43, 44	Genital warts or benign
High risk	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68	All types in cervical cancer

2.2 HPV treatment and prevention strategies

Although there is no effective treatment for the virus itself, cervical cancer can be controlled in three prevention phases: primary, secondary, and tertiary prevention strategies. Primary prevention includes using prophylactic vaccines to prevent the initial acquisition of HPV infection and subsequent HPV-associated lesions (WHO, 2022). There are three commercially available prophylactic vaccines targeting different HPV types, including the bivalent HPV2, quadrivalent HPV4, and 9-valent vaccines (Arbyn and Xu, 2018). The bivalent HPV2 (also known as Cervarix) vaccine protects against the two commonly known high-risk oncogenic types, HPV 16 and HPV 18 (Juckett and Hartman-Adams, 2010). At the same time, the quadrivalent HPV4 (also known as Gardasil) vaccine acts against both cancer-causing types (HPV 16 and 18) as well as the genital wart causing-low-risk types (HPV 6 and 11) (Hancock et al., 2018, Stern et al., 2000). The 9-valent (also known as Gardasil 9) is directed against 9 HPV genotypes, four of which are targeted by quadrivalent vaccine (HPV 16, 18, 6, and 11), as well as five additional oncogenic strains (HPV 31, 33, 45, 52, 58) associated with cervical cancer (Lekoane et al., 2019). The adverse effects of HPV vaccination are normal mild local reactions, and their safety has resulted in high uptake of the vaccine among females and herd immunity among males of similar age (Delany-Moretlwe et al., 2018). HPV vaccination is given to adolescents from 9 to 12 years (Wang et al., 2020) annually

to prevent abnormal cervical cytology, HPV infections, and cervical cancer (Palefsky, 2020). However, these primary prevention strategies cannot eradicate the existing HPV-infected cells, as capsid proteins are established either before viral entry, or in the terminally differentiated epithelium. Furthermore, the secondary prevention strategy includes screening and treating precancerous lesions using cryotherapy or thermal ablation (WHO, 2022). The tertiary prevention strategy involves diagnosing and treating invasive cervical cancer through surgery, chemotherapy, and radiotherapy (WHO, 2022).

Table 2: HPV vaccine types and their targeted HPV genotypes

Vaccines	Coverage (HPV types)	Gender and age range
Cervarix (bivalent HPV vaccine)	HPV 16 and 18	Females, 9-25 years
Gardasil 4 (quadrivalent HPV vaccine)	HPV 6, 11 (genital warts), 16, and 18	Males and Females, 9-26 years
Gardasil 9 (9-valent HPV vaccine	HPV 6, 11 (genital warts), 16, 18, 31, 33, 45, 52, and 58	Males and females, 9-26 years

2.3 HPV infection in young women

Adolescent girls and young women are more susceptible to HPV infection than their male counterparts (Bruni et al., 2010). The unique vulnerability of women to HPV infection comes from several different behavioural and biological factors. While the risk for infection differs from person to person, increased number of sexual partners, early sexual debut, the use of intravaginal insertion products, uncircumcised male partner, and the number of pregnancies is some of the documented behavioural risk factors in women (Sasagawa et al., 2012, Dreyer, 2018). Several biological factors, including vaginal surface, immunosuppression, co-occurrence of STIs, disturbance of vaginal microenvironment and menstruation, defective immune responses associated with genetic variations, and condomless sex, may predispose women to become infected with HPV. It has been reported that HPV prevalence is 5-fold higher amongst young women than their male peers (Ebrahim et al., 2016). Bruni *et al*. (2010) showed that HPV infection peaks in younger women

around the age of sexual debut and declines in the early 30s. Understanding the role of behavioural and biological risk factors in adolescent girls and young women could be crucial to developing effective ways to prevent or treat HPV infection, including management of cervical dysplasia and developing prophylactic vaccines.

2.4 Immune response and evasion mechanisms

Women are particularly vulnerable to HPV and are predominantly infected through heterosexual transmission (Branstetter et al., 2017). HPV infections are not systemic; the protective first line of host defense would be at the mucosal surface. The mucosal epithelium provides the first line of defense against pathogen entry and mediates the initial host immune response against STIs, including HPV (Stanley, 2006). Pathogens, including HPV, are detected by an intricate matrix of innate and adaptive immune responses of the lower and upper female genital tract.

2.4.1 Innate immune response

The innate immune response is the first line of defense against invading pathogens (Stanley, 2006). Innate immunity immune cells like neutrophils, monocytes, macrophages, eosinophils, mast cells, dendritic cells, and other associated cells identify and elicit protecting responses to invading pathogens through pattern recognition receptors such as toll-like receptors (TLR), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) (Bordignon et al., 2017). Antigen-presenting cells (APCs) such as Langerhans cells (LC), macrophages, and dendritic cells play an essential role in connecting the innate immune response to the adaptive immune system (Woodworth, 2002). LCs are the only APCs that can access the HPV proteins in the epithelial cells of the surface layer (Doorbar et al., 2012). HPV transfection of DCs leads to changes in DC migratory pattern and induces cytokine production, while macrophages contribute to the clearance of HPV infection through the production of tumour necrosis factor-alpha and nitric oxide-dependent mechanisms (Bergot et al., 2011). Natural killer cells have been associated with viral clearance, elimination of HPV infected cells, and cancer prevention in HPV-related carcinogenesis (de Freitas et al., 2017). Although less is known about neutrophils and cervical cancer, a study demonstrated that high concentrations of neutrophils were strongly associated with poor prognosis and disease progression in women with cervical cancer (Alvarez et al., 2017).

2.4.2 Adaptive immune response

The adaptive immune response is another protective line of host defense against pathogens. It is differentiated into two pathways, the Th1 cell responses that promote cell-mediated immunity and Th2 cell responses that promote humoral immunity (Stanley, 2006). During HPV infection, the antigen is taken up by DCs and migrates to site lymphoid tissues to activate adaptive immunity via the expression of inflammatory cytokines such as IL-1α, IL-1β, IL-6, TNF-α, and IL-12 (Sasagawa et al., 2012). T lymphocytes (CD4+) recognize HPV antigen presented by mature APCs (Figure 2b) and undergo proliferation/differentiation (Figure 2c), and digest the antigen into shorter peptides or differentiate into effector T cells that interact with naive B cells and produce cytokines to assist in the maturation of B cell responses (Figure 2d) (Steele et al., 2002). An anti-viral immune response needs a Th1 cell response which secretes cytokines such as IL-2, IL-12, and IFN-γ to activate immune cells, including the naïve CD8+ T-lymphocytes (Figure 2e) that later differentiate into cytotoxic T lymphocytes (CTL) and become effector T-cells that can kill CIN or HPV infected cells (Figure 2f) (Sasagawa et al., 2012). CTLs, CD4+ T cells, and other Th1 responses have been associated with effective clearance of HPV 16 and HPV 18 (Torcia, 2019), while the lack of these T lymphocytes lineages has been associated with persistent HPV infection and the development of high-grade disease (Pao et al., 1995). Furthermore, Th2 cells produce cytokines such as IL-4, IL-6, IL-8, and IL-10 that help B cells differentiate into plasma cells that produce HPV-specific antibodies into circulation (Woodworth, 2002). Some naive B and T cells transform into HPV-specific memory B and T cells that migrate to the bone marrow to survive as long-lived memory cells and differentiate into plasma cells or activated T cells upon reinfection (Stern et al., 2000). In contrast, previous studies demonstrated that women with high grade HPV+ cervical lesions had an increased IL-17-associated Th17 response known to be pro-tumorigenic in HPV-associated cancers (Walch-Ruckheim et al., 2015). Activation and recruitment of Th17 cells is mediated by stromal tumour-associated fibroblasts and tumour-derived chemokines (CXCR3) (Walch-Ruckheim et al., 2015). Taken together, these findings suggest that not all innate and adaptive immune lineages are capable of migrating to sites of infection to kill HPV-infected cells; some promote the progression of cervical lesions to invasive cervical cancer.

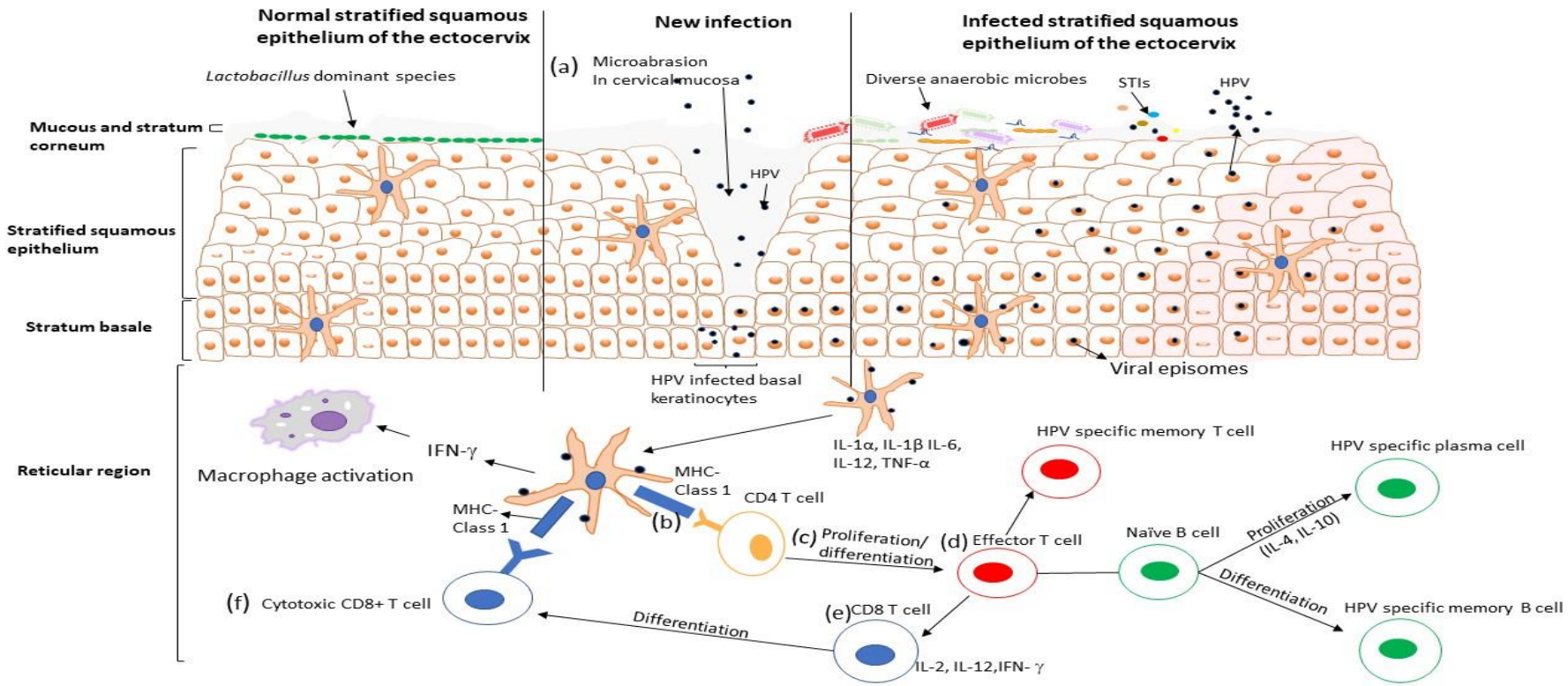


Figure 2: HPV infection cycle in the ectocervix of the female genital tract. (a) the HPV virus particles penetrate the stratified squamous epithelium through microabrasion. The virus particles infect the basal keratinocytes in the basement membrane and establish an infection, viral replication occurs, and the infected cells are transported up the epithelium. (b) viral particles are taken up by dendritic cells that secrete T cells attracting pro-inflammatory cytokines and chemokines (IL-1 α, IL-1β, IL-6, TNF- α, IL-12), which may facilitate activation and recruitment of CD4+ T cells. CD4+ T cells recognize HPV antigen presented by the dendritic cells and (c) undergoes proliferation and differentiation into the effector T cell. (d) The effector T cells [which proliferate into HPV specific memory T cells and (e and f) cytotoxic CD8+ T cell] bind with the naïve B cells, which differentiate to HPV specific plasma cells and HPV specific memory B cells. Activation of cellular and humoral immunity is associated with clearance of HPV infection

2.4.3 Evasion of host immune response by HPV

Although the immune response is a key factor in fighting against HPV infection, the virus can deploy various mechanisms to evade such response and subsequently establish persistence. One of the mechanisms involves altering APCs' function, including pathogen recognition receptors. High-risk HPV has been suggested to inhibit the keratinocytes-derived CCL20 expression, which reduces the ability of LCs to induce a cytotoxic immune response by compromising LCs infiltration (Chandra et al., 2016). In addition, HPV infection has been shown to affect the differentiation of monocytes into mature DCs, leading to altered functionality of DCs (Song et al., 2015). Another evasion mechanism of HPV infection is by inhibiting the recruitment of macrophages and other immune cells into the site of infection by the HPV E6 and E7 proteins. (HPV16 only) (Stern et al., 2000). Hasan *et al*. (2007) demonstrated that HPV-16 downregulated the expression and function of TLR-9 in human epithelial cells (Hasan et al., 2007). Another high-risk-HPV genotype (HPV-18) has been associated with suppression of the cyclic guanosine monophosphate-adenosine monophosphate synthase, which plays a role in activating type I interferon genes and production of inflammatory cytokine response (Lo Cigno et al., 2020). The interferons play a role in inhibiting viral replication in the host cells and activating immune cells that can eradicate the infected cells (Doorbar et al., 2015). More studies on the interaction between HPV proteins and type I IFN response remain critical. In addition, a better understanding of the TLR agonists augment HPV-specific T cell responses by modulating monocyte-derived dendritic cells are vital in the development of new prophylactic and treatment strategies.

Similar to innate immunity, the virus is able to also escape adaptive immune responses. Although the mechanisms by which HPV can evade the host immune system remain unclear, HPV proteins and regulatory cytokines have been suggested as contributors (Westrich et al., 2017). HPV16 E5 and E7 have been reported to reduce expression interferons and HLA class-1 molecules, resulting in a lack of CTL response against HPV (Stern et al., 2000). In addition, studies have reported high concentrations of an anti-inflammatory cytokine (IL-10) in women with persistent HPV infection compared to those who cleared the HPV infection. Similarly, increased levels of immunosuppressive cells such as transforming growth factor-beta (TGF-β) producing Tregs have

been observed in cervical tissues and vulvar intraepithelial neoplasia (VIN) lesions. The increased concentrations of IL-10 and TGF-β in tissues may indirectly undermine T cell function by limiting the ability of APCs to promote the $CD4^+$ T cells differentiation and proliferation, thereby modulating the adaptive immune responses (Sasagawa et al., 2012). Another mechanism by which CD4+ and CD8+ T cell function is inhibited involves the downregulation of surface MHC I expression and the impairment of APC trafficking and maturation (Bontkes et al., 1998, Hilders et al., 1995, Keating et al., 1995). Furthermore, chemokine receptor CXCR3 has been associated with limited efficacy of T cells in rejecting the engraftment of HPV16 E7 transgenic skin in non-transgenic mouse model (Kuo et al., 2018). Collectively, these findings suggest that APC and T cell responses downregulated by immunosuppressive immune response may result in HPV-immune evasion and consequently lead to persistent HPV infection and the development of high-grade disease

2.5 Association between genital inflammation and HPV

Genital inflammation is defined as the natural immune response following injury or infection (Fernandes et al., 2015). It is generally characterised by elevated levels of cellular markers and pro-inflammatory cytokines in the genital tract (Passmore et al., 2016). Host defense mechanisms, including immune mediators in the female genital tract microenvironment, play a role in the clearance and persistence of HPV and the risk of developing cervical cancer (Łaniewski et al., 2018, Daud et al., 2011, Stanley, 2010, Onywera et al., 2019, Moscicki et al., 2020a, Liebenberg et al., 2019b).

Contradicting data have been reported between HPV and altered cytokine milieu profiles (Liebenberg et al., 2019b, Scott et al., 2013b, Łaniewski et al., 2018). Łaniewski *et al*. (2018) showed a positive correlation between elevated concentrations of several cytokines (IL-36γ, MIP-1β, RANTES, IP-10, IL-2, IL-4, Flt-3L, sCD40L) and invasive cervical cancer carcinoma in women with BV (Łaniewski et al., 2018). In addition, a strong association between invasive cervical carcinoma and elevated concentrations of TNF-α, TNF-β, MIP-1α, GMCSF, and IL-10 were reported (Łaniewski et al., 2018). Increased mucosal cytokine profiles in the reproductive tract of women infected with HPV were also associated with HPV prevalence and increased HIV

acquisition risk (Liebenberg et al., 2019b). The apparent association between HPV infection and genital cytokine responses may likely indicate the role of cellular immunity to control HPV infection. Another study showed that women with elevated concentrations of mucosal cytokine interleukin (IL)-10, IL-12, macrophage inflammatory protein (MIP)-1α, and TNF-α were less likely to clear any HPV type, while low levels of these cytokines (including IL-8) correlated with HPV clearance (Scott et al., 2013b). HPV persistence was associated with chemokine MIP-1α and growth factor GM-CSF, which play a significant role in the activation and recruitment of granulocytes (Marks et al., 2011). Moscicki *et al*. (2020) found that 9 of the 13 cytokines (IL-4, IL-5, IL-10, IL-12, IL-13, IFN-γ, IFN-2α, MIP-1α, and TNF-1α) tested were elevated after the clearance of HPV infection compared to prior visits (Moscicki et al., 2020b). In contrast, two studies did not show the relation between increased concentrations of genital inflammation and HPV acquisition or clearance (Shannon et al., 2017, Kriek et al., 2016a). Understandably, while there are no structural conformations on host cells during HPV invasion, HPV also maintains the anti-inflammatory state likely by avoiding the host immunity through disruption of the interplay between infected cells and effector cells (Kriek et al., 2016b). There is a need to better understand the cellular or other factors associated with the cytokines in the different HPV status categories.

2.6 Association between vaginal microbiome and HPV infection

The female genital tract (vagina and ectocervix) is dominated by lactic acid producing bacteria and cervicovaginal fluids acting as a lubricant that traps invading pathogens (Lee et al., 2013, Aldunate et al., 2015). A genital environment dominated by *Lactobacillus* spp. has been associated with optimal pregnancy outcomes, lack of abnormal vaginal symptoms and urogenital disease, and reduced risk for several STIs, including HPV and HIV (Petrova et al., 2013). In contrast, the opposite is observed in the genital tract dominated by non-optimal vaginal microbiota. Several *Lactobacillus spp.* has been described in the non-BV state, although the most frequent and abundant are *L. crispatus, L. gasseri,* and *L. jensenii* (Ravel et al., 2011, Bik et al., 2019). *L. gasseri* enriched microbiome was also associated with rapid rates of HPV clearance (Brotman, 2011). A recent study showed that women with *L. crispatus* enriched microbiome were less likely to have prevalent high-risk HPV infection than women with overgrowth of pathogenic microorganisms such as those linked with BV (*Gardnerella, Atopobium,* and *Prevotella*) (Di Paola et al., 2017b).

Furthermore, *L. crispatus* abundance in the genital tract was also associated with HPV clearance, suggesting the relative association between clearance and *L. crispatus* (Di Paola et al., 2017b).

BV is a condition characterized by a shift in vaginal microbiota from *Lactobacillus* dominant towards more diverse bacteria, including strict and facultative anaerobes such as *Gardnerella, Prevotella,* and *Sneathia,* often resulting in vaginitis and discharge (Cohen et al., 2012, Di Paola et al., 2017b). BV has been associated with elevated levels of cytokines and cellular biomarkers of inflammation associated with increased HIV acquisition risk (Cohen et al., 2012, Eade et al., 2012). Furthermore, numerous studies have suggested a link between BV and other STIs such as *Chlamydia trachomatis, Neisseria gonorrhoea,* and cervical HPV (Brotman, 2011, Cherpes et al., 2003). The ulcerative and highly inflammatory sequelae caused by these infections provide biologically plausible mechanisms supporting a possible increased susceptibility to HPV among co-infected individuals. A high relative abundance of *Gardnerella* and *Atopobium vaginae* was associated with CIN (Berggrund et al., 2020, Godoy-Vitorino et al., 2018). Increased abundance of *Sneathia, Atopobium,* and *Gardnerella* is associated with incident high-risk HPV infection (Onywera et al., 2021). A study by Lee *et al*. (2013) showed that HPV-infected women had lower *Lactobacillus* species and increased *Fusobacteria* and *Sneathia* compared to HPV-uninfected women (Lee et al., 2013). In agreement with these findings, longitudinal analysis from 32 sexually active women showed that a low *Lactobacillus* community with high proportions of the genera *Atopobium* species were associated with a low rate of HPV clearance (Kyrgiou et al., 2017). While existing evidence suggests the association between HPV infection and genital dysbiosis, justifiable concerns that positive associations merely reflect residual confounding by unmeasured sexual risk behaviours (such as engaging in condomless sex and having sex with an uncircumcised partner) still exist. Thus, further research investigating this interplay is warranted. A detailed understanding of the genital microbial composition and structure in women with HPV infection may help identify the causal connections between microbiota, HPV infection, and cervical cancer.

2.7 Association between HPV and HIV

2.7.1 HPV infection and HIV risk

The best biological correlate of HIV risk that has been described to date is mucosal pro-inflammatory cytokines that, if elevated prior to infection, were associated with an approximately three-fold higher risk of acquiring HIV (Yegorov et al., 2019). However, the causes of inflammation are not clear, but STIs and BV have been shown to play an important role. In addition to its role as a biological factor in the development of anogenital cancers, HPV may also be an important co-factor in the increased risk of HIV acquisition in women. Overall, HIV infection risk doubled in women with prevalent HPV infection, with either oncogenic or non-oncogenic HPV genotypes (Liu et al., 2018). Another study showed a significant association between high-risk HPV genotypes and HIV acquisition (Looker et al., 2018). Persistent HPV infection has been associated with increased biomarkers of HIV acquisition, and the causal link is still not well-understood (Chikandiwa et al., 2017). Further research is still needed to explore the association between HPV and HIV and validate HPV as a potential risk factor for HIV acquisition, and if found to be true, this may highlight the importance of decreasing HPV burden in settings with high prevalence to curb HIV infections.

2.7.2 HPV infection in women living with HIV

HIV infection has also been associated with the development of CIN2, CIN3, and invasive cervical carcinoma in HPV infected population (Clifford et al., 2017). Although multiple types of HPV have been associated with HIV infection, HPV16 is the commonest cause of cervical carcinoma in HIV infected population (Dreyer, 2018). Studies have reported that women living with HIV (WLWH) are more likely to be infected with high-risk HPV and multiple HPV genotypes, resulting in the development of pre-invasive lesions that, if left untreated, can develop into invasive cervical cancer (Kriek et al., 2016b, Liu et al., 2018). WLWH have up to five times more cervical cancer than HIV-uninfected (Liu et al., 2018). In two large prospective studies that assessed the prevalence of HPV genotypes in women living in the United States, HPV 6 or 11 was 3.6 and 5.6 times high in WLWH compared to their HIV-uninfected counterparts, respectively (Bogale et al., 2020, Bruni et al., 2010). Furthermore, patients living with HIV were at increased risk of cervical abnormalities and onward HPV transmission due to the high prevalence of high HPV viral load (Looker et al., 2018). Given the growing evidence of an increased risk of cervical cancer in

WLWH, regular HPV screening and possibly treatment for cervical cancer is needed to effectively control HPV and its adverse sequelae.

2.8 Conclusion

While there is substantial progress in increasing vaccine access and immunization coverage, young sub-Saharan African women remain disproportionately infected by HPV. Factors that may render women more vulnerable to HPV infection have not been fully characterized. This review showed that the vaginal microbiome, cellular, and cytokine markers of inflammation are some of the biological markers that are associated with neoplastic disease in cervical carcinogenesis. While significant progress has been made in understanding how HPV evades immunity, mechanistic studies on how risk factors influence host-mucosal microenvironment and viral persistence are warranted. The role of other biological risk factors such as intravaginal practices are less studied and could be important drivers of HPV risk in young women. Preclinical models and clinical investigation to better understand the role of biological risk factors in the development of cervical neoplasm and the progression to invasive disease could shed light on possible underlying mechanisms.

CHAPTER 3: METHODOLOGY

3. MATERIALS AND METHODS

3.1 General study design, cohort, and sample collection

From a prospective cohort study of 267 HIV-uninfected CAPRISA 083 women (BREC number: BE403/16), we selected 56 women with complete laboratory-diagnosed sexually transmitted infection (STI) and BV-intermediate or positive data for inclusion in the analysis (Mtshali et al., 2021b). We excluded pregnant women or those who had received antibiotic treatment within seven days of sampling. A structured questionnaire administered by a trained counsellor was used to collect demographic and clinical data. Laboratory samples, including vaginal swabs and SoftCup genital secretion, were collected from each participant at enrolment, 6 weeks, and 13 weeks post treatment. The Soft Cup samples were collected by placing a menstrual cup for approximately 1 hour. BV and STI screening were performed using Gram stain (Nugent score), GeneXpert, OSMO Trichomonas (Cepheid, CA, US), and standard PCR multiplex assay (Choe et al., 2013), and participants that tested positive were treated with regimens recommended in the South African STI treatment guidelines (Department of Health, 2015). The regimens included 1g oral azithromycin (*Chlamydia trachomatis*), ceftriaxone 250mg intramuscular and azithromycin 1g oral (*Neisseria gonorrhoeae*), metronidazole 2g oral (*Trichomonas vaginalis*), metronidazole 2g oral single dose (Nugent score ≥4) or clotrimazole 500mg pessary and clotrimazole 1% cream (candidiasis). Treated women were asked to return after 6 weeks and 12 weeks for follow-up examinations, further collections of specimens, and retesting for STIs, Nugent-BV, and Candidiasis. Women with Nugent score ≥4 after initial treatment were further offered metronidazole 400mg for 5 days. This study was approved by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal, BREC number: BREC00002792/2021.

3.2 Total DNA extraction

Vaginal fluid from the swab was transferred to clean tubes and centrifuged. The supernatant was transferred to clean tubes and stored for future virome work. Briefly, the pellet was then lysed in buffer solution (consisting of TE50 buffer lyzozyme, metanolysin, and lysostaphin) and incubated at 37^{o}C for 60 minutes. The sample was then transferred to PowerBead tubes, and solution C1 was

added. Solution C1 includes agents that aid in disrupting the cell such as SDS, to ensure that the cell is completely lysed. The tubes were sonicated in the bead beating machine at 10000 x g for 30 seconds in intervals of 3 x 30s. The supernatant was washed 3 times using different solutions (C2, C3, C4, and C5 solutions) provided with the kit. These reagents are used to remove additional materials and substances, purify the DNA, and aid the DNA binding to the column. Thereafter, the spin filters were carefully placed in clean collection tubes, and solution C6 was added to the centre of the white filter membrane to elude the sample. The method was done according to the manufacturer's protocol (MoBio PowerSoil kit, Germany).

All extracted samples were then quantified using the Qubit fluorometer. The Qubit working solution was prepared by adding 1µl concentrated assay reagent into 199µl buffer solution. Kit standards were then prepared and ran on the qubit fluorometer to generate a standard curve. Samples were prepared by adding a working solution (198 µl) and extracted sample (2 µl) in clear tubes and ran on the qubit fluorometer as per the manufacturer's protocol.

3.3 Detection of HPV genotype in cervicovaginal pellet sample

To identify the infecting HPV genotype, DNA was extracted from a SoftCup pellet using an automated MagNA pure instrument (Roche Diagnostics, Indianapolis, IN, USA), followed by HPY genotyping using Roche Linear Array® HPV Genotyping kit (Roche Diagnostics, Indianapolis, IN, USA) according to manufacturer instructions. Briefly, extracted DNA and HPV primers, HPV master mix with magnesium ion (Mg^{2+}) (Roche, USA) were used to amplify a 450 base pair product of the L1 region of more than 37 HPV genotypes and a 268-bp fragment of the human beta-globin gene. PCR was performed under the following conditions: 50°C for 2 min to activate AmpErase, 95°C for 9 min, followed by 40 cycles consisting of 95°C for 30 sec, 55°C for 1 min, 72"C for 1 min, and a final hold at 72°C for 5 min on an AB9700 machine (Applied Biosystems). The reaction was kept at 72°C until an alkaline denaturation solution was added from the Linear Array Detection Kit (Roche Diagnostics, Mannheim, Germany) to denature the amplicons and AmpErase. The amplified product was hybridized to an array of oligonucleotide probes located in the polymorphic region of L1, and the colorimetric reaction was used to detect individual HPV genotypes. Following hybridization, products were further washed with buffers

containing SDS and sodium salts. This was followed by the addition of streptavidin-horseradish peroxidase conjugate (SHPC) to the strip to bind to the biotin-labelled amplicons hybridized to the probes on the strip. The strips are pre-stained with a substrate mixture of hydrogen peroxide (H_2O_2) and 3,3',5,5'-tetramethylbenzine (TMB) and oxidized to a blue colour by catalysis action of streptavidin-horseradish peroxidase in the presence of H_2O_2. The presence of HPV genotype is interpreted as a blue colour that precipitated at probe positions. Negative and positive controls were included in each assay to demonstrate the test's specificity and rule out contamination. The HPV genotypes detected included 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.

Women who tested positive for any of the HPV genotypes were considered to have a current infection, and the HPV results from follow-up visits were grouped and classified into women who "cleared any", those who "persisted," and "recurrent" HPV (Liebenberg et al., 2019a). The term "cleared any" was defined as having a detectable HPV genotype at baseline and cleared at 6 weeks and 12 weeks follow-up visits. The term "persistence" was defined as having any detectable HPV genotype at the baseline visit and remaining so at the next consecutive visit. The term "recurrent" was defined as having any detectable HPV genotype at baseline, cleared at 6 weeks follow-up visit, but had any detectable HPV genotype at 12 weeks follow-up visit.

3.4 Characterization of Microbiome

DNA was extracted using the PowerSoil DNA kit (MoBio) after mechanical and enzymatic disruption using lysozyme, mutanolysin, and bead beating. 16S V4 region was amplified using universal 515F/806R primers and quality checked with Bioanalyzer (Agilent). Pooled triplicate samples were purified with Agencourt AMPure XP beads (Beckman Coulter). Amplicons will be pooled in equal quantities. Purified libraries consisting of ~100 pooled samples were sequenced with the Illumina MiSeq platform (paired end with v3 chemistry).

3.4.1 16S amplification and clean-up

Extracted DNA was used to amplify the V4 region by nested 16S PCR. Briefly, the first round of PCR was amplified using *KAPA* HiFi *HotStart* Mix (Kapa Biosystems, South Africa) with the forward primer V4F (5'TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG NNN NNG TGC CAG CMG CCG CGG TAA3') and reverse primer V4R (5'GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GNN NNN GGA CTA CHV GGG TWT CTA AT3'). The assay was performed in duplicates for each sample. The mixture was then incubated at 95^{o}C for 3 minutes, followed by 30 cycles of 95^{o}C for 30 seconds, 55^{o}C for 30 seconds, and 72^{o}C for 30 seconds, then the final extension of 72^{o}C for 5 minutes and held at 72^{o}C for 4 minutes. Duplicated PCR products were then pooled together (2 x 25 µl) AMPure XP beads were added (50 µl per sample) and mixed by pipetting up and down 10 times. The plates were incubated at room temperature for 10 minutes. The plates were then placed on a magnetic stand for 2 minutes, and the supernatant was then discarded. 200 µl of 80% ethanol was added to each well and incubated on a magnetic stand for 30 seconds. The supernatant was removed, and the ethanol step was repeated. The beads were then allowed to air-dry. 52.5 µl of 10 mM Tris (pH 8.5) was added to each well, and the plate was incubated on the magnetic stand at room temperature for 2 minutes. The supernatant was transferred to a sterile PCR tube and stored at -20^{o}C. Amplicons were electrophoresed in a 1% agarose gel to confirm the presence and intensity of the amplicon.

The second round of PCR was amplified using Hotstart Mix (Kapa Biosystems, South Africa) with the 12 Nextera index primers. This step was not done in duplicates as it uses the products from PCR 1 and the total volume in each well was 50 µl. The mixture was incubated at 95^{o}C for 3 minutes, followed by 8 cycles of 95^{o}C for 30 seconds, 55^{o}C for 30 seconds, and an extension of 72^{o}C for 30 seconds, then the final extension of 72^{o}C for 5 minutes and held at 72^{o}C for 4 minutes. 50 µl of AMPure XP beads were added and mixed by pipetting up and down 10 times. The plates were incubated at room temperature for 10 minutes. The plates were then placed on a magnetic stand for 2 minutes and the supernatant was then discarded. 200 µl of 80% ethanol was added to each well and incubated on a magnetic stand for 30 seconds. The supernatant was removed, and the ethanol step was repeated. The beads were then allowed to air-dry. 27.5 µl of 10 mM Tris (pH 8.5) was added to each well, and the plate was incubated on the magnetic stand at room temperature

for 2 minutes. The supernatant was transferred to a sterile PCR tube and stored at -20^{o}C. Amplicons were electrophoresed in a 1% agarose gel to confirm the presence and intensity of the amplicon.

3.5 Quantification and pooling of PCR products

The PCR 2 products were quantified using the Qubit fluorometer to analyze the final yield of samples after PCR as per the manufacturer's protocol. All PCR products were then normalized (diluted to equal concentrations) and pooled together into a single tube for further microbiome analysis through next generation sequencing in the Jaspan Laboratory, Seattle Children's Hospital, Seattle, USA.

3.5.1 16S rDNA Data Analysis.

All data analyses were performed using UPARSE and QIIME (http://qiime.org) (Edgar, 2013), using vegan and phyloseq R libraries. UPARSE was used to merge paired reads and perform *de-novo* clustering into OTUs at 97% sequence identity. A representative sequence was picked for each OTU and used for taxonomic classification. OTUs were taxonomically classified to genus or higher levels using a Naïve Bayes classification approach (McDonald et al., 2012) and Greengenes taxonomy (McDonald et al., 2012). Phylogenetic trees were built with FastTree (Price et al., 2009, Price et al., 2010) based on the PyNAST (Caporaso et al., 2010) alignment.

3.5.2 Rarefaction Analysis, Diversity Estimates, and Sample Ordinations.

To assess the quality of microbial diversity sampling, multiple rarefactions at different sequencing depths were performed. α-diversity estimates, richness (Chao1), evenness (Simpson's E), and phylogenetic diversity (Faith's PD) were calculated using the R vegan library. β-diversity between samples was estimated by UniFrac distances. An all-by-all pairwise distance matrix based on UniFrac distances was generated and used to hierarchically cluster and ordinate samples. The ordinations were performed using Principal Coordinate Analysis and Nonmetric Multidimensional Scaling (NMDS) and compared. Distinct community types were defined based on hierarchical clustering and examination of species-level classifications (Albertsen et al., 2013, Ravel et al., 2011, Zerbino and Birney, 2008, Trapnell et al., 2013)

3.6 Statistical analysis

Differences in the demographic variables between participants were tested using the analysis of variance model (ANOVA) for continuous variables and Fisher's exact test for categorical variables. Differential abundance testing was performed using the R metagenomeSeq package, with strict criteria for inclusion: coefficient≥2, at least 50% positive in at least one group, and FDR≤ 0.05. The relationship between the participant metadata and identified CSTs was calculated using Fisher's exact/Chi-square tests for categorical variables and the Mann-Whitney unpaired nonparametric test for continuous variables. Mann-Whitney unpaired nonparametric test was used to compare alpha diversity between the CSTs, while the Kruskal-Wallis test was used to compare alpha diversity measures of the CSTs in three or more groups. A P-value less than 0.05 was considered significant. This analysis will be carried out using SAS (version 9.4; SAS Institute Inc., Cary, NC, USA).

RESULTS

4.1 Demographics, behavioral and clinical characteristics of the study population

Two hundred and fifty-two women met the CAP083 study inclusion criteria. Of these, 56 had matching HPV and 16S rRNA sequences for all 3 visits, as previously demonstrated (Mtshali et al., 2021a). The median age was 24 years (interquartile range (IQR) 21-27 years), and more than two-thirds reported condom use during the study (71%, 40/56) (Table 3). Only 5% (3/56) of these women reported consistent condom use, with the majority reporting that they used condoms occasionally (66%; 37/56). Only 38% (21/56) of study participants reported the use of any form of contraception, with the majority of these reporting use of progesterone-based injectable contraceptives (11/21). At baseline, all 56 women have Nugent scores >3 at baseline; with the majority (61%; 34/56) being considered BV positive (Nugent score 7-10) and 39% (22/56) having intermediate Nugent scores (4-6). In addition, 48% (27/56) of women had an STI or vaginal candidiasis at baseline, with *C. trachomatis* being the most prevalent bacterial STI (30%, 17/56), and 14% (8/56) of women had Candidiasis evident.

All women who had Nugent scores of >3 at baseline and an STI received targeted antibiotic or antifungal treatment and were then tested for the cure at their 6-week visit, and again at 12 weeks. Women with Nugent scores of >3 at 6-week visit were further treated with 5 days of oral metronidazole. By 6 weeks, only 30% (17/56) of women had resolved BV but were reduced to 23% (13/56) at 12 weeks ($p<0.0001$; Table 3). The prevalence of both intermediate BV and BV-positivity significantly decreased 6 weeks after treatment and remained reduced even 12 weeks after treatment for the BV-positive group ($p<0.0001$). In addition to improved BV rates after treatment, Table 3 demonstrates that STI rates were also significantly reduced at 6 weeks and remained so at 12 weeks post-treatment ($p<0.0001$), with very few women also having BV.

Table 3: Clinical characteristics of the study population at baseline and follow-up (N= 56)

Variable	Level	Baseline	Week 6	Week 12	p-value
		% (n/N)			
Age (years)	Median (IQR)	24 (21-27)	-	-	-
Condom use	Yes	71.4 (40)	-	-	-
	No	28.6 (16)	-	-	-
Frequency of condom use	Always	5.3 (3)	-	-	-
	Sometimes	66.1 (37)	-	-	-
	Never	28.6 (16)	-	-	-
Contraceptive use	Yes	37.5 (21)	-	-	-
	No	62.5 (35)	-	-	-
Type of contraception	Injection	52.4 (11/21)	-	-	-
	IUD	4.8 (1/21)	-	-	-
	Oral	14.3 (3/21)	-	-	-
	Subdermal implant	28.6 (6/21)	-	-	-
Genital examination	Abnormal	62.5 (35)	-	-	-
	Normal	37.5 (21)	-	-	-
Candidiasis	Yes	14.3 (8)	12.5 (7)	19.6 (11)	0.451
	No	85.7 (48)	87.5 (49)	80.4 (45)	
BV status (Nugent score)	No BV (0-3)	0 (0)	30.4 (17)	23.2 (13)	<0.0001
	Intermediate BV (4-6)	39.3 (22)	33.9 (19)	46.4 (26)	
	BV (7-10)	60.7 (34)	35.7 (20)	30.4 (17)	
Chlamydia trachomatis	Yes	30.4 (17)	5.4 (3)	3.4 (2)	0.001
	No	69.6 (39)	94.6 (53)	96.4 (54)	
Neisseria gonorrhea	Yes	10.7 (6)	0.0 (0)	1.8 (1)	0.113
	No	89.3 (50)	100 (56)	98.2 (55)	
Trichomonas vaginalis	Yes	8.9 (5)	3.6 (2)	0.0 (0)	0.013
	No	91.1 (51)	96.4 (54)	100 (56)	
Any STI	Yes	50.0 (28)	8.9 (5.0)	5.4 (3)	<0.0001
	No	50.0 (28)	91.1 (51)	94.6 (53)	
Co-conditions	BV only	51.8 (29)	60.7 (34)	73.2 (41)	-
	STI only	0.0 (0)	0.0 (0)	1.8 (1)	
	BV and STI	48.2 (27)	8.9 (5)	3.6 (2)	
	No BV or	0 (0)	30.4 (17)	21.4 (12)	
PSA	Yes	19.6 (11)	21.4 (12)	25 (14)	0.785
	No	80.3 (45)	78.6 (44)	75 (42)	

Abbreviations: BV; bacterial vaginosis, IQR; interquartile range, IUD; intrauterine device, PSA; prostate specific antigen, STI; sexually transmitted infection. Any STI includes all STIs tested excluding candidiasis.

4.2 Prevalence of HPV infection in CAPRISA 083 women overtime

Overall, 56 women at 3 time points were included in this study. Among the 56 women, 36 (64 %) had detectable HPV DNA at baseline but decreased to 28 (50 %) at 6 weeks and increased gain at 13 weeks to 36 (64 %) (Table 4). The prevalence of high-risk HPV infection at baseline was 58%, 61% at 6 weeks, and 45% at13 weeks. Gardasil 9 vaccine strains were the most prevalent throughout the visits, followed by Gardasil 4 vaccine strains and Cervarix vaccine strains.

Table 4: Prevalence of HPV infection overtime

HPV infection	Baseline	Visit 2	Visit 3
HPV positive	36 (64%)	28 (50%)	36 (64%)
HPV negative	20 (36%)	28 (50%)	20 (36%)
Low-risk HPV	15 (42%)	11 (39%)	20 (55%)
High-Risk HPV	21 (58%)	17 (61%)	16 (45%)
Cervarix	2 (3.6%)	3 (5.4%)	4 (7.1%)
Gardasil 4	6 (11%)	6 (11%)	9 (16%)
Gardasil 9	10 (18%)	12 (21%)	14 (25%)

4.3 Prevalence of HPV genotype

Among the 37 genotypes of HPV detected by the Roche Linear Array® HPV Genotyping kit, there were 12 high risk HPV genotypes (HPV16, 18, 39, 45, 51, 52, 58, 59, 66, 68, 70, 82) and 10 low-risk genotypes (6, 11, 54, 55, 61, 62, 71, 81, CP83, CP 618) overtime (Table 5). The distributions of positive rates were different in each visit. Although the distribution varies between the visits, HPV16, 45, 58, and 59 (≥4% of all infections, respectively) were the most dominant high-risk genotypes at all visits. HPV6 (≥4% of all infections) was the most common low-risk HPV type observed at all visits, while HPV81 was also dominant at 6weeks and 13 weeks but not at baseline.

Table 5: Distribution of HPV genotypes detected overtime

HPV genotype	High/Low risk	IARC classification#	HPV Vaccine types*	Baseline	Week 6	Week 13
HPV 16	High risk	1	2,4,9	2/56 (3,57%)	2/56 (3,57%)	3/56 (5,35%)
HPV 39	High risk	1		2/56 (3,57%)	0/56 (0,00%)	1/56 (1,78%)
HPV 45	High risk	1	9	2/56 (3,57%)	2/56 (3,57%)	2/56 (3,57%)
HPV 51	High risk	1		2/56 (3,57%)	2/56 (3,57%)	1/56 (1,78%)
HPV 52	High risk	1	9	2/56 (3,57%)	1/56 (1,79%)	1/56 (1,78%)
HPV 58	High risk	1	9	2/56 (3,57%)	3/56 (5,36%)	2/56 (3,57%)
HPV 59	High risk	1		2/56 (3,57%)	3/56 (5,36%)	3/56 (5,35%)
HPV 68	High risk	2A		2/56 (3,57%)	1/56 (1,79%)	0/56 (0,00%)
HPV 66	High risk	2B		2/56 (3,57%)	1/56 (1,79%)	2/56 (3,57%)
HPV 82	High risk	2B		2/56 (3,57%)	1/56 (1,79%)	0/56 (0,00%)
HPV 70	High risk	2B		1/56 (1,78%)	0/56 (0,00%)	0/56 (0,00%)
HPV 18	High risk	1	2,4,9	0/56 (0,00%)	1/56 (1,79%)	2/56 (3,57%)
HPV 6	Low risk	3	4.9	2/56 (3,57%)	3/56 (5,36%)	3/56 (5,35%)
HPV 11	Low risk	3	4.9	2/56 (3,57%)	0/56 (0,00%)	2/56 (3,57%)
HPV 54	Low risk	3		2/56 (3,57%)	1/56 (1,79%)	2/56 (3,57%)
HPV 62	Low risk	3		2/56 (3,57%)	1/56 (1,79%)	2/56 (3,57%)
HPV 83	Low risk	3		2/56 (3,57%)	2/56 (3,57%)	1/56 (1,78%)
HPV CP 618	Low risk	3		2/56 (3,57%)	0/56 (0,00%)	2/56 (3,57%)
HPV 55	Low risk	3		1/56 (1,78%)	1/56 (1,79%)	1/56 (1,78%)
HPV 61	Low risk	3		1/56 (1,78%)	0/56 (0,00%)	1/56 (1,78%)
HPV 81	Low risk	3		1/56 (1,78%)	3/56 (5,36%)	6/56 (10,51%)
HPV 71	Low risk	3		0/56 (0,00%)	0/56 (0,00%)	1/56 (1,78%)

[#]IARC categories refer to group 1 as carcinogenic to humans, group 2A is probably carcinogenic to humans, group 2B is possibly carcinogenic to humans, and group 3 is not classifiable as to its carcinogenicity to humans, respectively. *Vaccine types 2, 4, and 9 refer to bivalent Cervarix®, quadrivalent Gardasil®, and nonavalent Gardasil®9 HPV types, respectively.

4.4 Multiple HPV infections in women

Although the single infection was most common in this study, some participants also observed multiple HPV infections. Figure 3 ranks the frequency of HPV genotype by coinfection status. There were 16% (9/56) women with multiple HPV infections at baseline, 10% (6/56) at 6 weeks, and 18% (10/56) at 13 weeks. The HPV genotypes more frequently identified in participants with multiple infections were HPV16 (7/56, 12.5%), HPV81 (8/56, 14%), HPV61 and 59 (5/56, 9%, respectively), and HPV6, 51, 45 (4/56, 7%, respectively). Majority of HPV genotypes detected were associated with other genotypes than alone, except for HPV39.

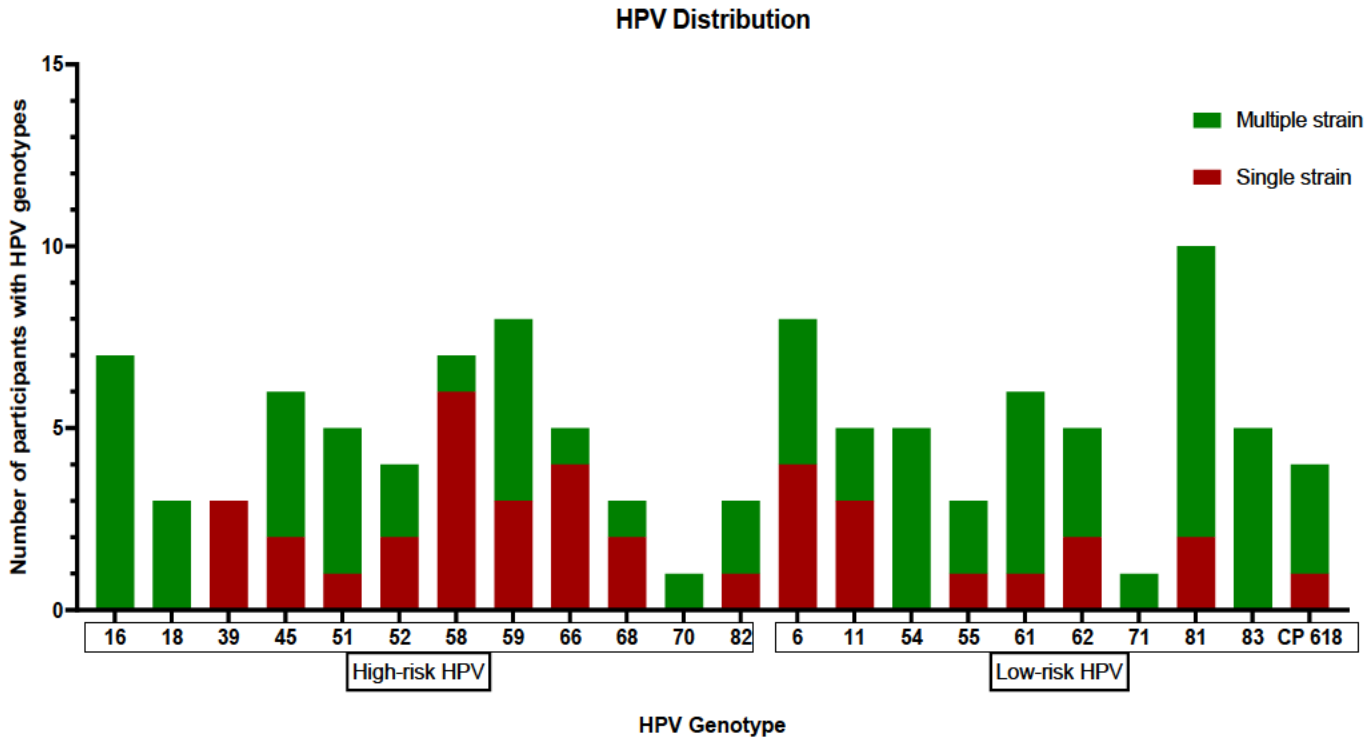


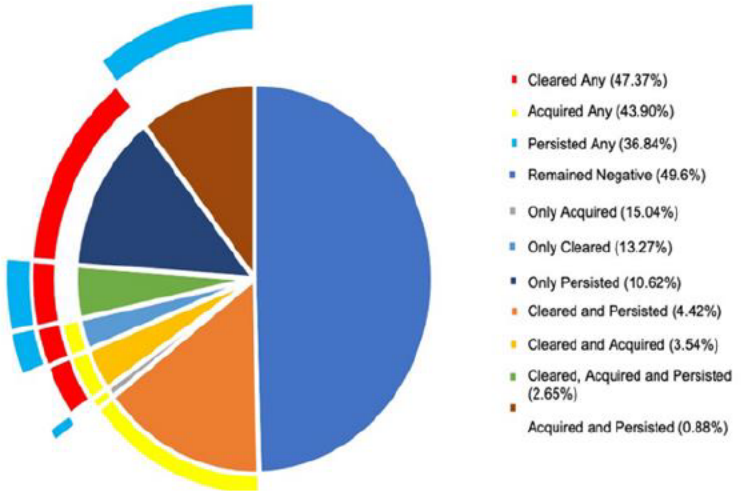
Figure 3. Prevalence of the most common HPV genotypes by single and multiple infections between visits. The green color depicts multiple HPV infections, and a single infection is shown in red.

4.5 Clearance and persistence of HPV infection

Next, we investigated changes in HPV status between visits. As such, there was evidence of women acquiring, clearing, and persisting with any HPV genotypes between visits. Overall, 46%

(26/56) of participants cleared any HPV genotype, while 45% (25/56) acquired and 38% (21/56) had persisted any HPV genotype at follow-up visits (Figure 4A). Of the participants who cleared, 13% (7/56) cleared any HPV genotypes completely, 4% (2/56) were reinfected (acquired) at 13 weeks. Fifteen percent (8/56) of participants that were HPV negative at baseline acquired any HPV genotypes at 6 weeks, and 1% (1/56) of these persisted through 13weeks. Of the 38% who persisted at 6weeks, 26% (15/56) cleared at 13 weeks. Very few participants (3%, 2/56) cleared and acquired some HPV genotype between visits, while other HPV genotypes persisted throughout the visits. As shown in Figure 4B, high-risk (HPV51, 52, HPV58) and low-risk HPV genotypes (HPV6, 66, HPV81) were more likely to cause persistent infections alone or in combination with HPV16 and 62.

A



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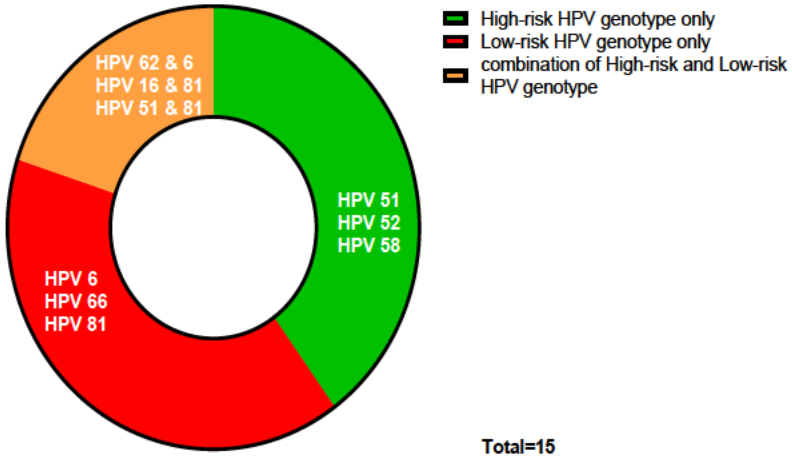


Figure 4: (A) Changes in HPV status between visits. HPV outcomes were classified as follows: "cleared any" as having a detectable HPV genotype at baseline and cleared at 6 weeks and 12 weeks follow-up visits; "persistence" as having any detectable HPV genotype at baseline visit and remain so at the next consecutive visits; and "acquired" as having any detectable HPV genotype at baseline, cleared at 6 weeks follow-up visit, but had any detectable HPV genotype at 12 weeks follow-up visit. Percentages reflect the number of participants who changed HPV status between visits. (B) Persistent HPV Genotypes, High-risk, Low-risk, and Combination of High-risk and Low-risk.

4.6 Association between vaginal microbiome and diverse microbial species in HPV infection.

Here, we longitudinally characterized vaginal microbiome composition in 56 women with HPV infection, including those with oncogenic genotypes. The vaginal microbiota of these women clustered into five major community state types based on the dominant species: CST I, dominated by *L. crispatus*; CST II, dominated by *Gardnerella vaginalis* and *L. crispatus;* CST III, dominated by *L. iners*, and CST IV characterized by a wide array of strict, facultative anaerobes without a consistent dominant species, and CST V dominated by *L. iners and L. jensenii* (Figure 5). CSTIV was further categorized into sub-CSTs: CST IV-A representing samples with a high relative abundance of BVAB1, CST IV-B characterized by *G. vaginalis* dominance, and CST IV-C characterized by an even community with a moderate abundance of *Prevotella bivia*.

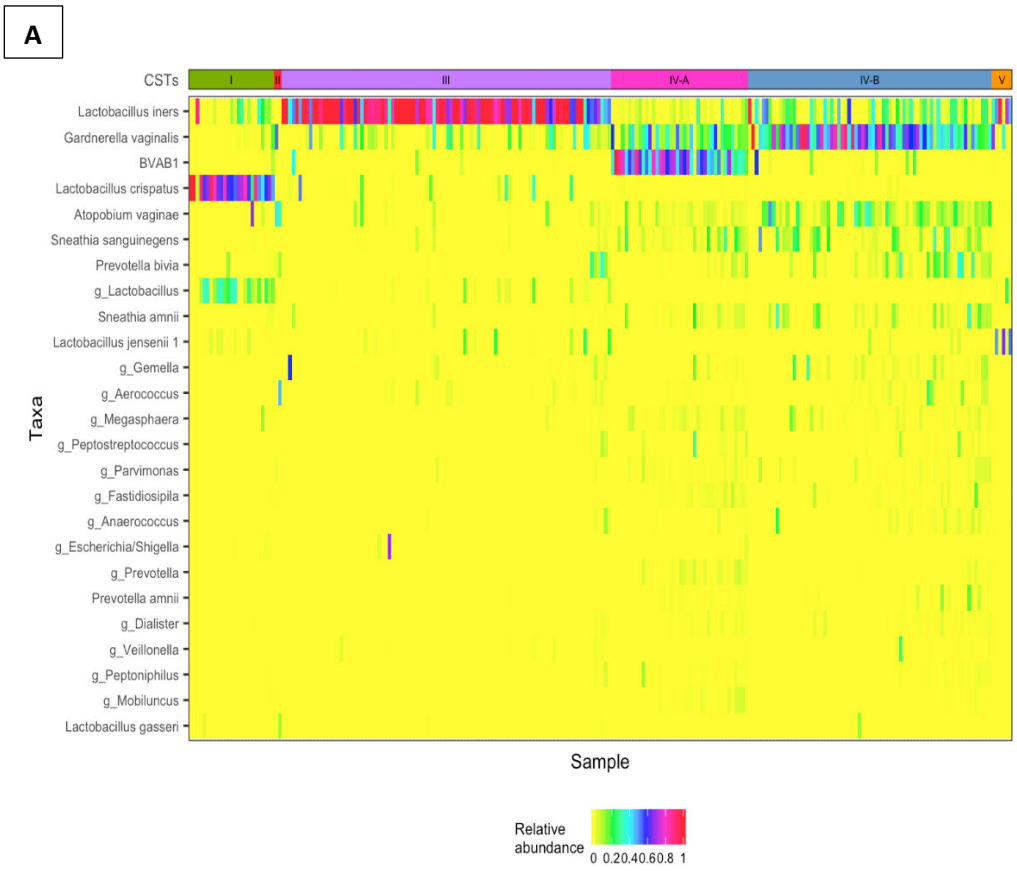


Figure 5: Heatmap showing relative abundances of twenty-five key vaginal microbial taxa of the 56 HIV-uninfected women used to define each of the five subtypes over time. Error bars indicate the standard error of the mean as determined using 100 bootstraps of ten percent of the training dataset.

When we stratified by HPV infection, HPV negative women had a relative abundance of the microbiome community state types (CST) dominated by *L. iners*, *L. crispatus*, and non-Lactobacillus dominated communities such as *Gardnerella vaginalis*, BVAB1, *Atopobium*, *Prevotella*, and *Sneathia* (Figure 6). Similar to the microbiome CST of HPV positive participants, women with oncogenic and non-oncogenic HPV genotypes had *Gardnerella vaginalis* and BVAB1, while *L. crispatus* was absent or poorly represented in both groups.

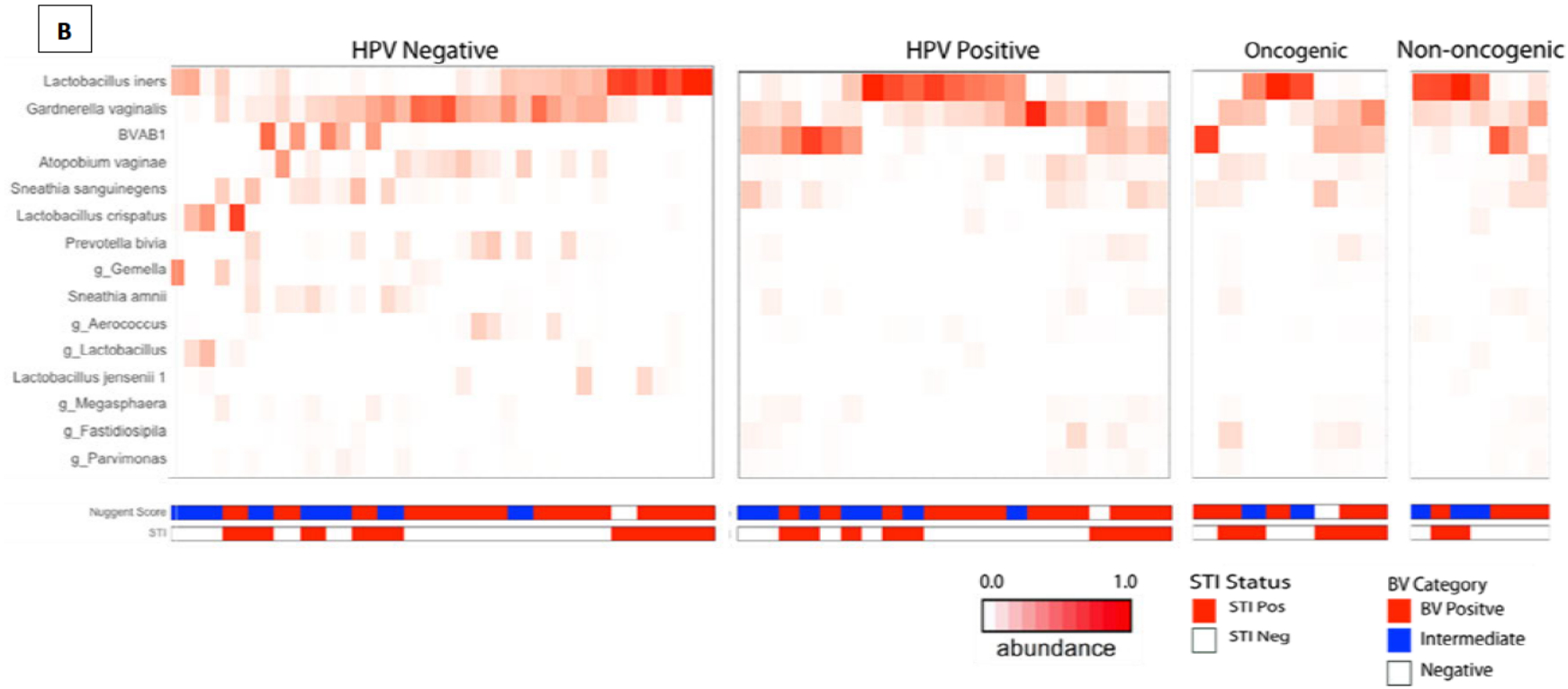


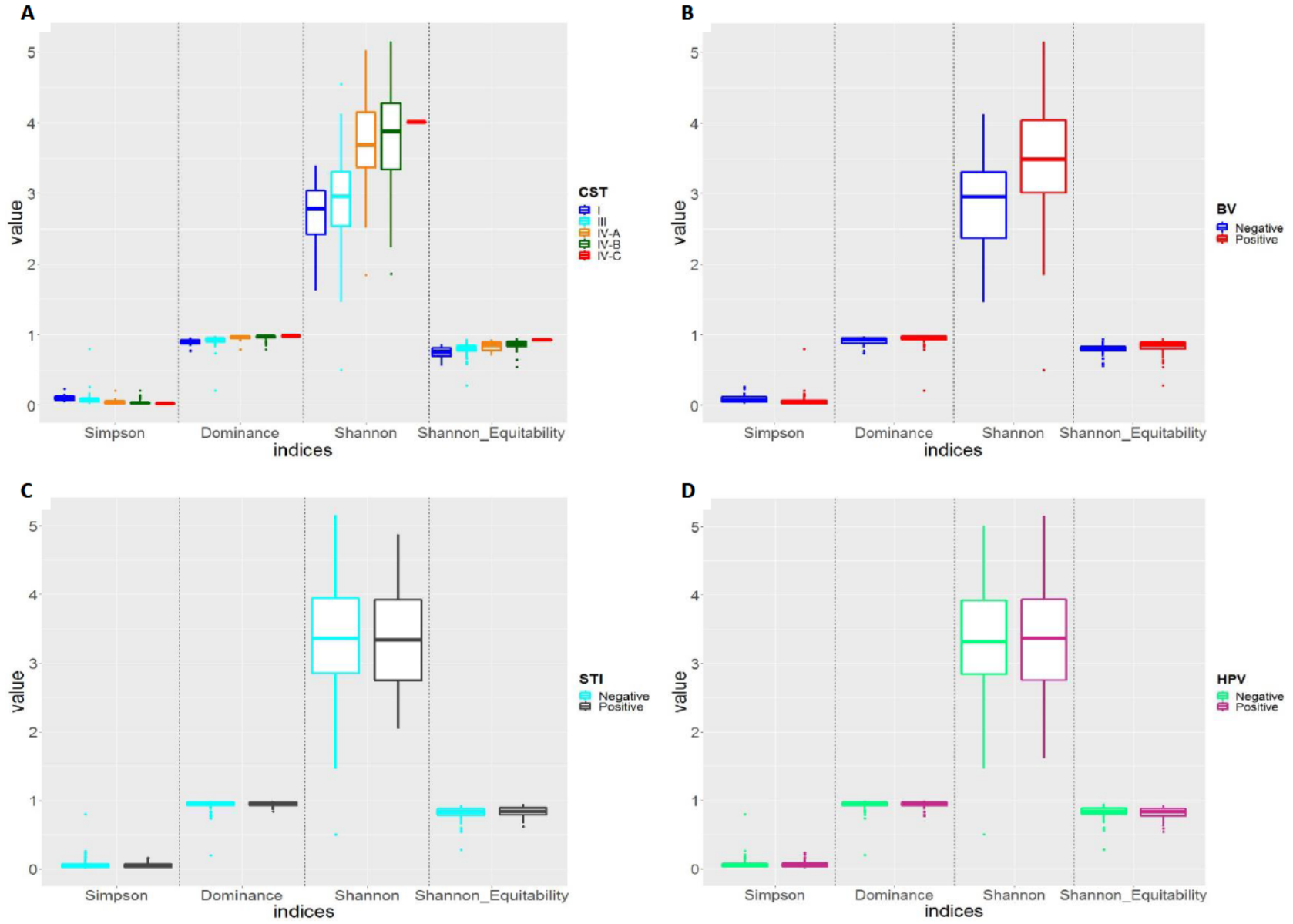
Figure 6: Vaginal microbiome composition of the women across different HPV states. Relative abundances of the dominant bacterial species were detected in the vaginal swabs of women who were HPV positive, HPV negative, oncogenic, and non-oncogenic HPV states.

4.7 Differences in community composition by beta and alpha diversity

First, we determined the differences in vaginal microbiome composition of women using Simpson, Dominance, Shannon Diversity, and Shannon Equitability. The alpha diversity was compared across CSTs, BV status, STIs, HPV infection status, and high-risk HPV infection status. A comparison of bacterial diversity between women with *L. crispatus*-dominated communities (CST I) and non-*Lactobacillus*-dominated (CST IVA and IVB) communities revealed significant differences by Shannon index. Women with CST I (median 2.781, IQR 2.294-3.084) vaginal communities had a significant decrease in diversity compared to women with CST IVA (median 3.679, IQR 3.333-4.155, Shannon index in the Kruskal-Wallis test p-value p= 0.0004) and IVB (median 3.8744, IQR 3.333-4.276, Shannon index in the Kruskal-Wallis test p-value p=0.0006) dominant vaginal microbiome communities (Figure 7A). The median alpha diversities of women diagnosed with BV were significantly increased compared to those without BV (3.4828, IQR 2.986-4.047 vs 2.9564, IQR 2.273-3.308, Shannon index in the Kruskal-Wallis test p-value <0.00001) (Figure 7B). The median alpha diversities were not different among women diagnosed with anySTI compared to those without anySTIs (3.3387, IQR= 2.726-3.926 vs 3.3565, IQR= 2.827-3.942, p value= 0.7) (Figure 7C). Similarly, alpha diversity of the vaginal microbiome of women with HPV (3.3633, IQR= 2.707-3.931 vs 3.3081, IQR= 2.801-3.931, p value= 0.57) (Figure 7D) and high-risk HPV (3.4657, IQR= 2.658-3.988 vs 3.3032, IQR= 2.885- 3.93, p value= 0.6) (Figure 7E) infection did not differ significantly to that in vaginal microbiome from uninfected women, respectively.

Next, we determined the beta diversity using a Principal Coordinate Analysis (PCoA) plot of clustering based on a weighted UniFrac distance matrix. Weighted UniFrac analysis demonstrated that overall vaginal microbiome CSTs were spatially segregated and, therefore, compositionally and phylogenetically distinct from each other (pseudo-F statistic= 31.32, p=0.001) (Figure 7F). Women with *Lactobacillus*-dominated communities (*L. crispatus* dominated CST I and *L. iners* dominated CST III) clustered tightly together, and BV-linked CST IVA, IVB, and IVC communities were found clustering together in the 2D space. Beta analysis with PCoA showed distinct segregation of the vaginal microbiome in women diagnosed with BV compared to those without BV (pseudo-F statistic= 27, p= 0.1) (Figure 7G). There was no distinct separation of the vaginal microbiome on the PCoA plot by STI (pseudo-F statistic= 1.35, p= 0.201) (Figure 7H),

HPV status (pseudo-F statistic= 2.18, p= 0.07) (Figure 7I) and high-risk HPV (pseudo-F statistic= 1.16, p= 0.268) status (Figure 7J).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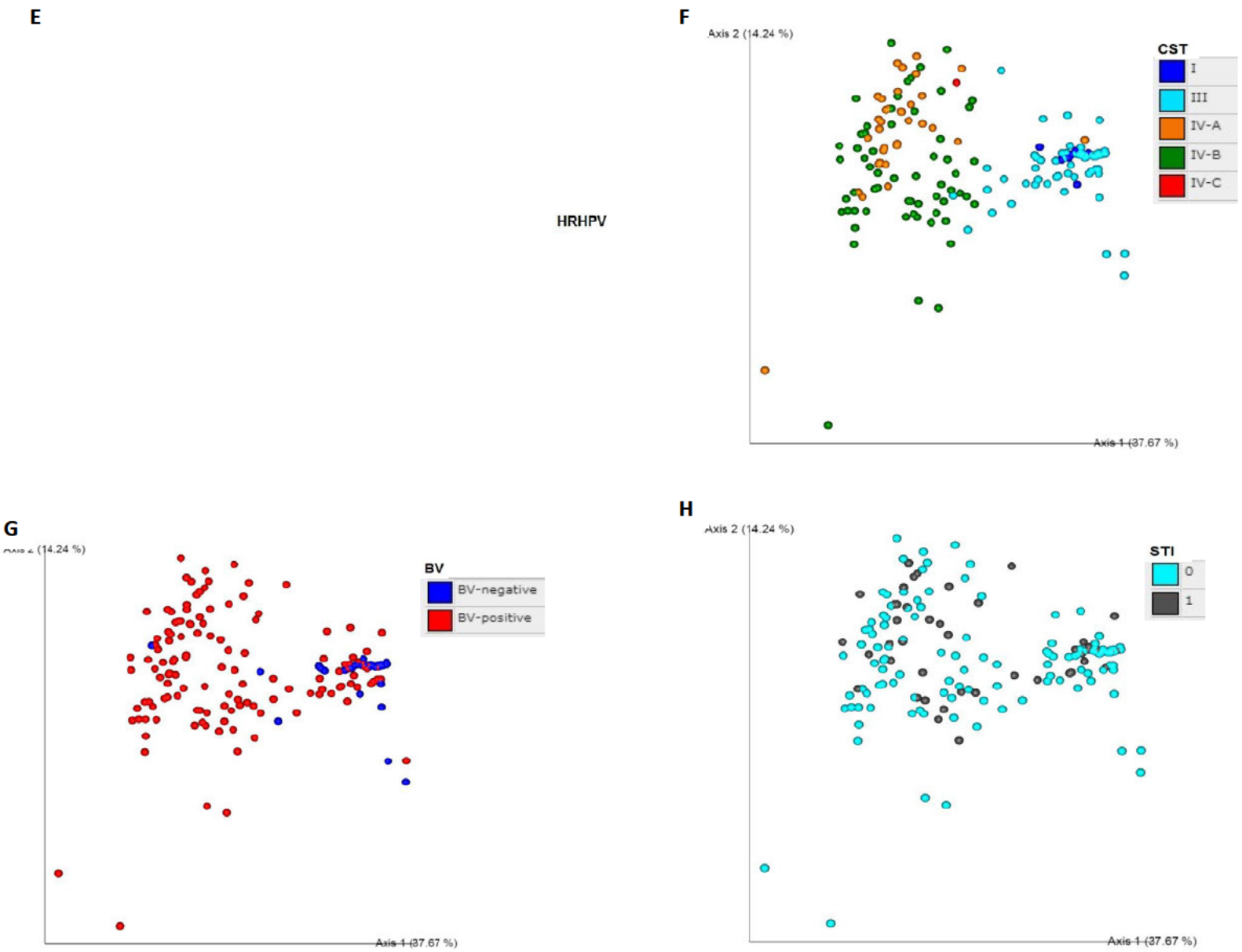
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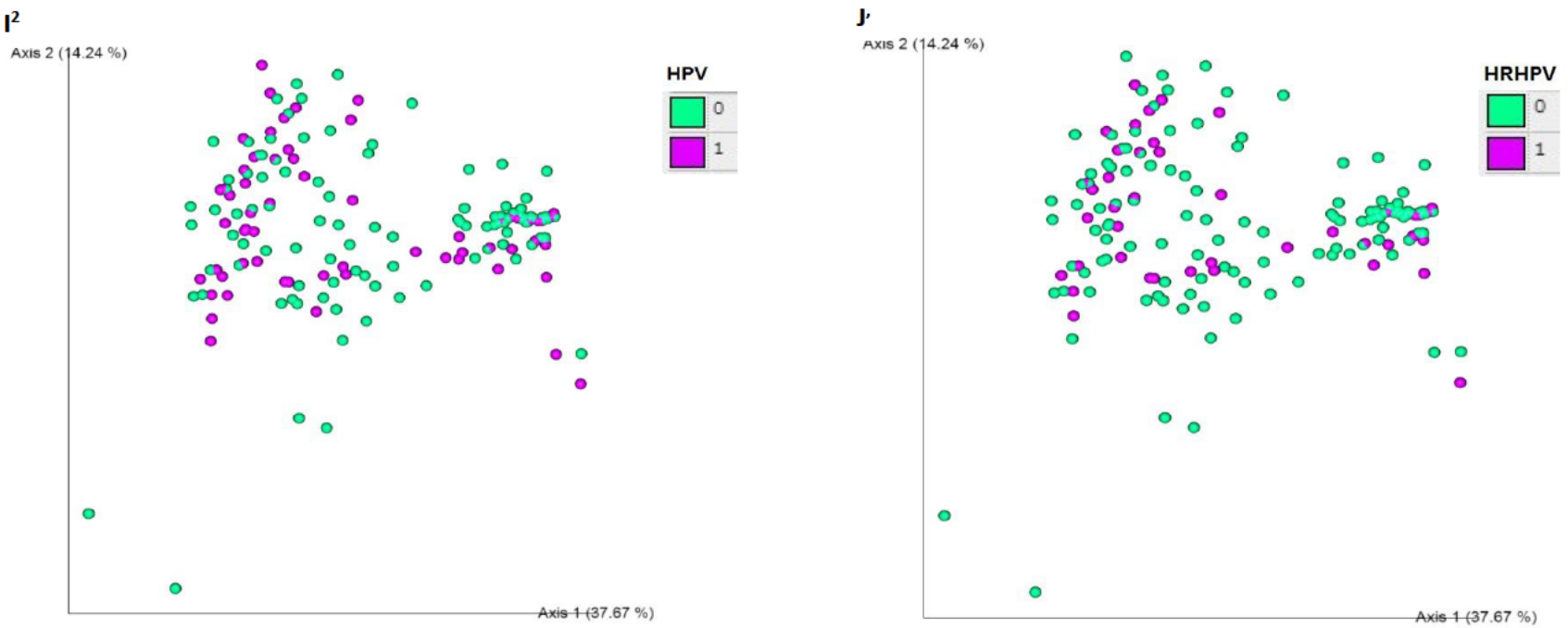

Figure 7: (A-J): Alpha and beta diversity measures. Box plots of observed OTUs, Simpson, Dominance, Shannon Diversity, and Shannon Equitability index grouped by: (A) CSTs; (B) BV status; (C) STIs; (D) HPV infection status, and (E) high-risk HPV infection status. Each box plot is constructed from the first quartile, median (horizontal line), to the third quartile, and the whiskers goes from the smallest to largest non-outliers. Dots represent outliers. (F-J) Principal coordinates analysis using the weighted UniFrac distance metric on 56 samples, colored by (F) CSTs; (G) BV status; (H) STIs; (I) HPV infection status; and (J) high-risk HPV infection status. The first two principal coordinate axes of variations and the percentage variation explained by each (Axis.1: 37.67% and Axis.2: 14.24% (f-j). Each solid point is a bacterial community.

4.8 Potential biomarkers for HPV clearance and HPV persistence

Bacterial taxonomy was differentially abundant in the vaginal microbiome of women who experienced HPV cleared, or persistence overtime were analyzed using the Linear Discriminant Analysis (LDA) effect size (LEfSe) algorithm. A total of 16 bacterial taxa were found to be differentially abundant (LDA score >2.0, $p < 0.05$) between vaginal microbiome of HPV-positive and HPV negative women (Figure 8A). LEfSe analysis identified *Lactobacillus spp*. (particularly *L. iners*) as potential biomarkers for HPV clearance between visits, whereas HPV persistence was associated with enrichment of *Sneathia amnii* and other BV-associated bacteria such as *Mobiluncus, Actinomycetaceae, Mycoplasma, Dialister, Actinomycetales, Prevotella,* and *Bifidobacteriaceae*. Although not significant, the relative abundance of *L. iners* was higher in women who cleared HPV (Figure 8B) whereas women with HPV persistence were associated with a high relative abundance of *Sneathia amnii* (Figure 8C).

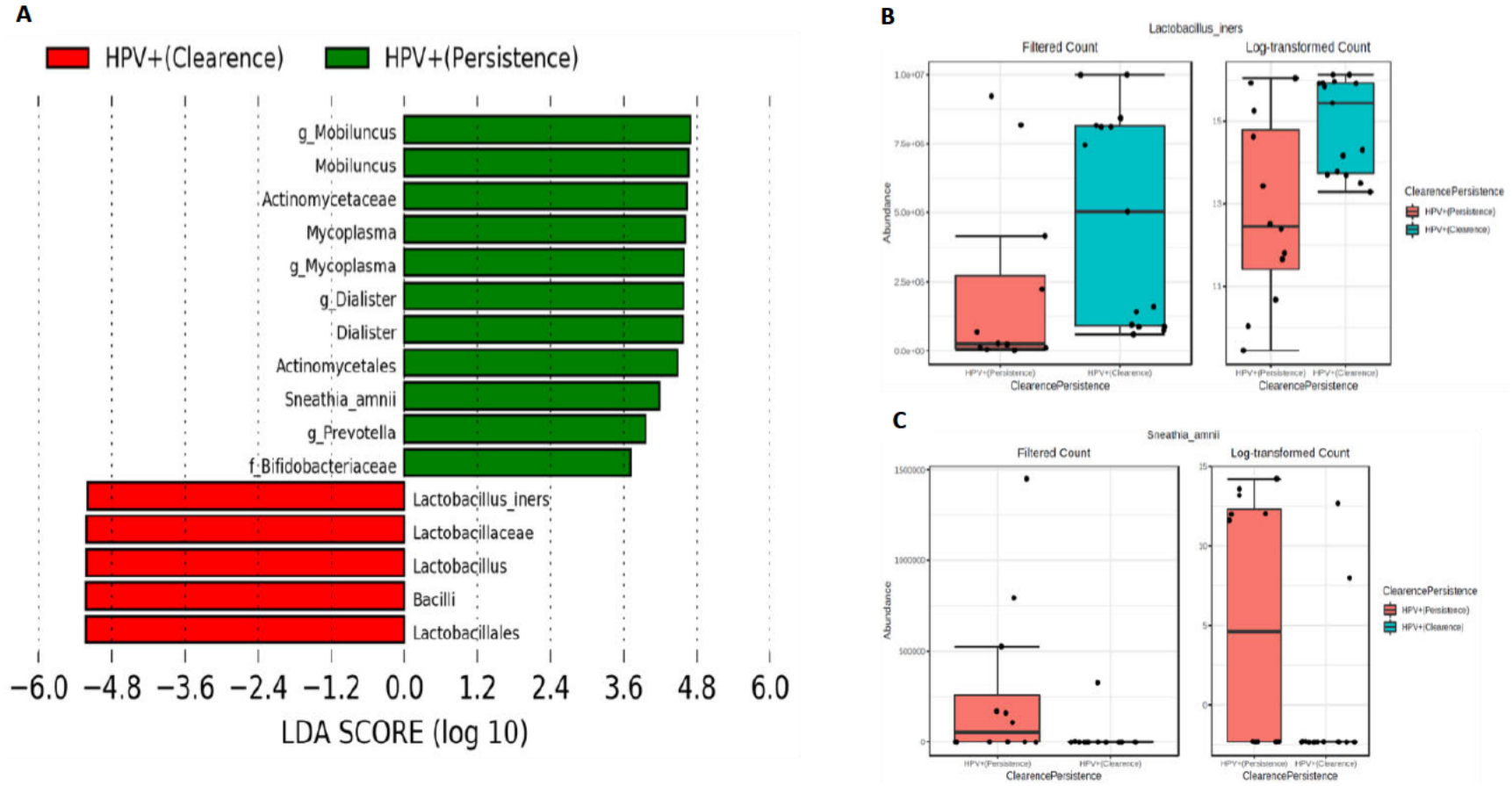

Figure 8: Histogram of differentially abundant taxa in vaginal microbiota of women who cleared or persistent HPV. (a) LefSe identified 16 bacterial taxa that were differentially abundant (LDA score>2.0, $p < 0.05$). (B) Filtered and (C) Log-transformed abundance of *L. iners* and *Sneathia amnii* in vaginal microbiome of women who experienced HPV clearance (blue) or persistence (red)

5. DISCUSSION

Cervical cancer disproportionately affects women of reproductive age, with 80% of cases occurring in low- and middle-income countries. Persistent infection with high-risk human papillomavirus (HPV) genotypes has been described as the most common non-systemic biological risk factor for the development of cervical cancer. Recently, a diverse vaginal microbiome (associated with bacterial vaginosis) has emerged as a potential driver of high-risk HPV positivity and disease severity in women. However, the role of the vaginal microbiome on HPV clearance, recurrence, and persistence remains unclear. In this study, we assessed whether there was an association between vaginal microbiome and HPV infection status in 56 women of reproductive age.

In this cohort of women in KwaZulu-Natal, the overall HPV prevalence was above 50% at all 3 visits, and high-risk HPV genotypes were the most common at baseline and 6 weeks, while the frequency of low-risk HPV genotypes (55%) increased at 13 weeks. Our findings are consistent with previous studies reporting HPV prevalence (low and high-risk) that ranges between 44% and 85% among South African women (Richter et al., 2013, Giuliano et al., 2015, Adler et al., 2014, Ebrahim et al., 2016, Mbulawa et al., 2021). The varying HPV prevalence statistics may be attributed to testing frequency, population, age, and assay used for HPV detection. While single infections were predominant in this study, we also observed infection with multiple HPV genotypes associated with genital warts and cancer (including those targeted by the current vaccines). Gardasil 9 vaccine strains were the most common between visits, followed by Gardasil 4 vaccine strains and Cervarix vaccine strains. Previous studies have also observed a high prevalence of genotypes targeted by the current vaccine (Taku et al., 2021). These vaccine strains' circulation in vaccinated women may have been acquired prior to vaccination. Current Gardasil 9, Gardasil 4, and Cervarix vaccines are unable to eradicate the existing HPV-infected cells, as capsid proteins are established either before viral entry, or in the terminally differentiated epithelium (WHO, 2022). The findings of this study highlight the urgency of vaccinating young girls and developing strategies that will target the existing HPV-infected cells.

Consistent with previous studies (Rosa et al., 2008, Jaisamrarn et al., 2013), almost half (46%) of women in this cleared HPV infection between visits. Although epidemiological data suggest that the meantime for HPV clearance is usually 6–12 months in women HIV non-infected (Travassos et al., 2017, Rosa et al., 2008, Jaisamrarn et al., 2013), our samples were collected 6weeks apart. This is a slightly higher clearance rate than the reported period (weeks and not months), but the clearance mechanism remains unknown. We hypothesize that the rapid clearance may be attributed to an intricate matrix of innate and adaptive immune responses of the lower and upper female genital tract capable of clearing the virus during the early stages of HPV infections. With the same token, we also observed almost half (45%) of women who were HPV negative at baseline and those who cleared at 6 weeks getting reinfected between the visits. Although factors associated with HPV recurrence are unclear in this study, it is possible that sexually active men who are not routinely checked for HPV can transmit the virus to women who are cleared, despite not having any signs or symptoms. In addition, HPV can be transient, a negative result is not a guarantee of total clearance of the virus, and thus less dominant genotype can dominate in the absence of a high fit and susceptible virus (Hogewoning et al., 2003, Bleeker et al., 2003).

Furthermore, we observed that about one-third (38%) of women persisted with HPV infection until the end of the follow-up period without evidence of cervical precancer. In addition, this study showed that women with persistent infection were also likely to be infected with multiple HPV (low and high risk) genotypes between the visits. It has been suggested that certain HPV genotypes can influence persistence. HPV genotypes 16, 18, 31, and 45 have been associated with the highest rate of persistence and cervical cancer (Schiffman et al., 2010, Schiffman et al., 2005, Richardson et al., 2003). This study did not observe these HPV genotypes, except HPV16. It has been suggested that co-infection and persisting infection may be due to an increased viral load which may overcome immune control. Another explanation involves a lower immune response that cannot clear the infection (Schmeink et al., 2011). Our results warrant a need for frequent testing of HPV and to monitor the duration of the infection rather than the number of positive tests.

In this cohort of African women, *L. iners* were found to be the most abundant *Lactobacillus* species, followed by a modest relative abundance of *L. crispatus*, while *L. jensenii* was extremely modest. Lactobacilli (particularly *L. crispatus*) are believed to contribute to reproductive health by

inhibiting the colonization of genital pathogens through the production of lactic acid, H_2O_2, and bacteriocins (Buve et al., 2014, Hayes et al., 2010). Culture-independent 16S rRNA gene sequencing studies have shown that unlike white women in the U.S., the majority of young, healthy, black South African women have vaginal communities with low *L. crispatus* abundance and high diversity (Anahtar et al., 2015, Gosmann et al., 2017, Lennard et al., 2018, Mtshali et al., 2021b, Ravel et al., 2011). Consistent with our findings, previous studies reported *L. iners* as the most prevalent FGT *Lactobacillus* sp. in African women. However, this species is poorly understood and associated with compositional instability and transition to a non-optimal microbiota (Gajer et al., 2012, Kenyon et al., 2013, McKinnon et al., 2019). Munoz et al. (2021) showed that *L. iners*-dominated vaginal community type seems to be less stable or easily transition to other community state types, especially CSTs associated with bacterial vaginosis (Munoz et al., 2021). In our study, the predominance of L. iners may be due to metronidazole treatment given to those symptomatic participants diagnosed with BV using a Nugent score of >4 (Mtshali et al., 2021b). Mtshali et al., (2021) and others demonstrated that BV treatment leads to a decrease in the presence of BV-associated microbes. However, re-colonization with *Lactobacillus* species is often slow or dominated by *L. iners*, and recurrence rates of BV following treatment are high (Barrons and Tassone, 2008, Mtshali et al., 2021b). This is particularly concerning and may explain the high BV occurrence among South African women.

We also observed a high relative abundance of diverse vaginal microbiome throughout the visits. The diverse vaginal microbiome is reminiscent of bacterial communities found associated with the condition known as bacterial vaginosis (BV), which is also characterized by a lack of *Lactobacillus* species, and 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 (Shipitsyna et al., 2013). These diverse communities are closely associated with elevated genital inflammation and increased HIV risk, likely by increasing mucosal HIV target cell frequency and activation (Anahtar et al., 2015, Gosmann et al., 2017, Lennard et al., 2018). Given the specific health benefits afforded by a Lactobacillus-dominant vaginal microbiota against infections and the limited efficacy of antibiotics against BV-associated sequelae, a viable alternative such as probiotics to improve Lactobacillus representation as part of curative or preventive vaginal dysbiosis interventions is needed.

Previous studies have demonstrated an association between certain vaginal microbial community taxa and HPV infection and HPV-related disease (Audirac-Chalifour et al., 2016b, Brotman et al., 2014, Critchlow et al., 1995, Gao et al., 2013, Gillet et al., 2011, Łaniewski et al., 2018, Mitra et al., 2015, Norenhag et al., 2020, Brusselaers et al., 2019, Di Paola et al., 2017a). In agreement with these studies, we found that *Lactobacillus* species (particularly *L. iners*) were potential biomarkers for HPV clearance between visits, whereas BV-linked bacterial taxa were makers for HPV persistence. Although *L. iners* are moderate producers of lactic acid needed to inhibit invading pathogens like HPV, other *Lactobacillus* species are the highest lactic acid producers and can also secrete factors for competitive exclusion of pathogens (Petrova et al., 2017). Previously, BV-linked bacteria such as *G. vaginalis* (Hardy et al., 2015, Di Paola et al., 2017b) and *Sneathia* species (Mitra et al., 2016, Audirac-Chalifour et al., 2016a) have been associated with HPV persistence. Both *G. vaginalis* and *Sneathia* species have been associated with a squamous intraepithelial lesion in HPV-positive women (Achilles and Hillier, 2013), cervical neoplasm (Łaniewski et al., 2018), and increased genital inflammation in women. The hypothesized mechanism by which these BV-linked taxa promote HPV persistence is through the production of the sialidase-encoding gene (Di Paola et al., 2017b) and other genes encoding proteins implicated in adherence to cervical cells and cytotoxicity (Harwich et al., 2012).

Although this study is strengthened by a longitudinal assessment of the relationship between vaginal microbiome and HPV infection, it also has some limitations. This includes a sample size that restricted the ability to investigate in detail the effects of HPV clearance, persistence, or recurrence on the vaginal microbiome. Another limitation is that almost two-thirds of participants had STI/BV and were also treated with azithromycin, ceftriaxone, and metronidazole; this may have confounded the results.

Conclusion

Our findings confirm previous reports that African women continue to be disproportionately affected by HPV infection, and the risk of cervical cancer remains a lingering threat. This

highlights a need for regular HPV testing and better programmes to monitor the duration of the infection rather than the number of positive tests. *Lactobacillus* spp. are associated with increased clearance of HPV, whereas BV-associated bacterial taxa promote HPV persistence. Further studies assessing the role of the vaginal microbiome and other biological risk factors in low-risk and high-risk HPV infection infections remains urgently needed, especially in communities where the HPV prevalence has reached its greatest scale.

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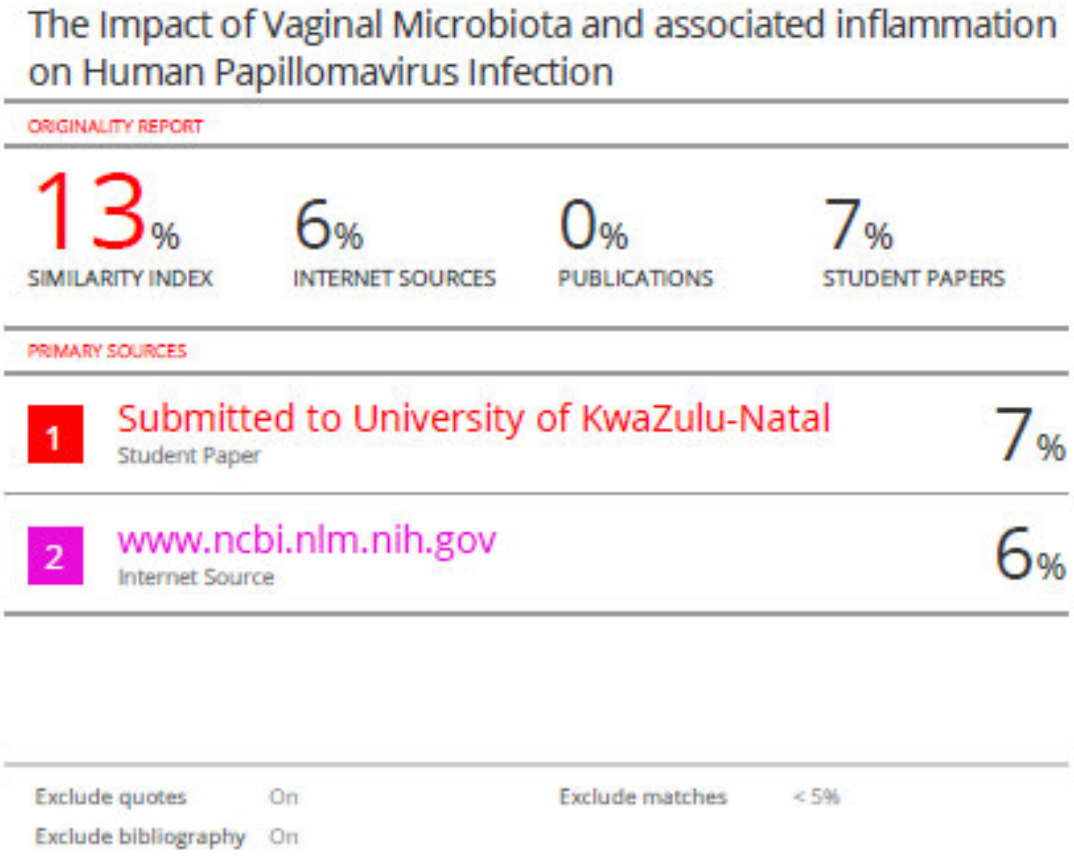
Appendix A: Turn-it in



The Impact of Vaginal Microbiota and associated inflammation on Human Papillomavirus Infection

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Appendix B: Linear array HPV genotyping test SOP

1. **PURPOSE**

The LINEAR ARRAY HPV (Human Papilloma Virus) Genotyping Test is a qualitative *in vitro* test for the detection of Human Papilloma Virus in clinical specimens. The test utilizes amplification of target DNA by the Polymerase Chain Reaction (PCR) and nucleic acid hybridization and detects thirty seven anogenital HPV DNA genotypes [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 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39 and CP6108] in cervical cells collected in PreservCyt® Solution.

2. **SCOPE**

Persistent infection with human papillomavirus (HPV) is the principal cause of cervical cancer and its precursor cervical intraepithelial neoplasia (CIN). The presence of HPV has been implicated in greater than 99% of cervical cancers, worldwide. HPV is a small, non-enveloped, double-stranded DNA virus, with a genome of approximately 8000 nucleotides. There are more than 100 different genotypes of HPV, and approximately 40 different HPV genotypes that can infect the human genital mucosa. However, only a subset of these sexually-transmitted viral genotypes is associated with high-grade cervical dysplasia and cervical cancer. These are termed high-risk HPV genotypes, while the low-risk HPV genotypes are often associated with benign low-grade intraepithelial lesions or condylomas. Sexually transmitted infection with HPV is extremely common, with estimates of up to 75% of all women experiencing exposure to HPV at some point. The majority of HPV infections clear spontaneously, but persistence of a high risk HPV is a significant risk factor for the development of cervical cancer.

The LINEAR ARRAY HPV Genotyping procedure involves five main stages.

1. Specimen preparation
2. PCR amplification of target DNA using HPV primers
3. Hybridization of the amplified products to oligonucleotide probes
4. Detection of the probe-bound amplified products by colorimetric determination

- The specimen preparation using the AmpliLute Liquid Media Extraction Kit or QIAamp DNA Blood Mini Kit (Qiagen) or MagNa Pure automated DNA extraction yields HPV target DNA and human genomic DNA suitable for PCR amplification. The Master Mix reagent contains primers for the amplification of DNA from 37 HPV genotypes and the human ß-globin gene. The detection and genotype determination is performed using the denatured amplified DNA and an array of

oligonucleotide probes that permit independent identification of individual HPV genotypes.

This procedure applies to all samples collected and processed for CAPRISA LINEAR ARRAY HPV Genotyping testing which are shipped to the DDMRI laboratory.

5. RESPONSIBILITY

The Laboratory Manager and/ or designate is responsible for training and compliance of all staff on this SOP.
The Laboratory Manager and/ or Head of Quality Assurance are responsible for reviewing and revision of this SOP.

6. REFERENCES

SOP SPPE003	-	Safety Personal Protective Equipment
SOP SHAZ006	-	Safety Handling of Bio hazardous Waste
SOP SSHP007	-	Safety Use of Sharp safes

7. DEFINITIONS

BSL-2 - biosafety level-2 hood
DN - Denaturation Solution
DNA – deoxyribonucleic acid
DNAse – deoxyribonuclease
HPV Strip - LINEAR ARRAY HPV Genotyping Strip
SA-HRP - Streptavidin-Horseradish Peroxidase Conjugate
CIT - Citrate Concentrate
ETOH – ethanol
HPV - Human Papilloma Virus
HPV MMX - LINEAR ARRAY HPV Master Mix
CAR - Carrier RNA
PK - Proteinase K
AVE – Elution buffer
AW – Wash Buffer
ATL - Tissue Lysis Buffer
AL - Lysis Buffer
ml - millilitre
NB – Pay attention to
PP – Post-processing
PPE - Personal Protective Equipment
RNA - ribonucleic acid

RNAse - ribonuclease
SUB A/B - Substrate A or B
PCR - polymerase chain reaction
SN – serial number
HPV Mg2+ - LINEAR ARRAY HPV Magnesium Solution
HPV (+/-) C - LINEAR ARRAY HPV Positive or Negative Control
V – version
Tech – technician/technologist

8. INSTRUCTIONS AND PROCEDURE

All staff must identify their involvement in sample handling by entering the following information at the indicated time-points:

- Date
- Time
- Name
- Signature

Information must be entered on the appropriate documentation, at each of the following steps:

STEP	FORM	STAFF
• Specimen collection	Laboratory Request Form	-Nurse
• Dispatch to on-site Laboratory	Shipping Manifest	-Nurse
• Receipt at on-site laboratory	Specimen Register	-Tech
• Enter Results	Results Report	-Tech

For details on these aspects, refer to the current version of these SOP's:

- Specimen Tracking: SOP TTRK001
- Shipping Manifest completion: SOP TMAN005
- Specimen Receipt: SOP LREC014

Samples must be taken from the clinic site to the in-house CAPRISA Labs at eThekwini and Vulindlela sites respectively by the study staff, who will be responsible for those samples until they are given to the CAPRISA Lab Staff.

The CAPRISA Lab staff must ensure that the Lab Request Form is filled and signed to the completion and then take the ownership of those samples.

The CAPRISA Lab staff must ensure that the shipping manifest is filled and signed to the completion and then take the ownership of those samples.

For batches that are ≥10 samples, please include a positive control (extracted DNA from serum (male) sample) and negative control (water).

A. SAMPLE PREPARATION

Specimen Requirements:

1. Cervicovaginal lavage specimens are collected and processed according to SOP CCVL007 and SOP LCVL021, respectively.

Procedure:

A. DNA Extraction

RECOMMENDATIONS BEFORE SAMPLE PREPARATION:

Set the centrifuge to 4ºC.

Thaw out reagents needed on ice and keep them on ice Mix thawed reagents gently, vortex (except the enzymes), and quick spin the tube to collect the contents in the bottom of each tube.

Thaw distilled water and store between 18 - 25°C.

Do not have more than one sample tube open at a time.

A positive and negative control must be included with every run with ≥ 10 samples

Wear gloves at all time to avoid RNase or DNase contamination.

Prior to working in any hood or on any work surface and using pipettes, please decontaminate with 10% bleach first, followed by 70% ethanol twice to ensure all traces of bleach are removed.

Reagent and equipment:

- Fume cardboard or BSL-2 hood
- Heating Block
- Pipet-Aid®
- Sterile disposable, polystyrene serological pipets
- Vacuum manifold
- Gold-plated 96-Well GeneAmp PCR System 9700
- Distilled or deionized water
- Orbital shaker
- Forceps, stainless steel
- Vacuum aspiration apparatus
- 1 L, 2 L, 3 L storage bottles or flasks
- 100 mL, 250 mL, 500 mL beakers
- 100 mL, 250 mL, 500 mL, 1 L graduated cylinders
- RBS35 Tray Cleaning Solution
- 50 mL Eppendorf Combitip plus
- 50 mL adapter Eppendorf Biopur
- 1.5 ml RNAse/DNAse free, screw-capped tube.
- Centrifuge, refrigerated (Jouan MR22i)
- Cervicovaginal sample
- Shaking Incubator
- QIAamp DNA Blood Mini Kit (Qiagen) or MagNa Pure automated DNA extraction
- Distilled water
- -80^{o}C freezer
- Permanent maker
- Pipette Tips (DNAse, RNAse Free)
- Absolute Ethanol
- Pipettes (100µl and 1000µl)
- Centrifuge (Heraeus Biofuge)
- Vortex
- Plastic resealable bag
- Eppendorf Multipette® Pipet*
- RBS35 Tray Cleaning Solution (or 10% bleach)

1. The HPV DNA Extraction steps must be carried out in a fume cardboard or BSL-2 hood in the extraction room at CAPRISA Research Laboratory.

a) Set the temperature of a dry heat block at 56ºC ± 2ºC.
b) Set the temperature of another dry heat block at 70ºC ± 2ºC.
c) Equilibrate reagents, specimens and controls to ambient temperature (at least 15 minutes). If a precipitate has formed in ATL or AL, dissolve by heating to 70ºC and agitating gently.

2. For each experimental DNA sample, label **(DNA, PID & Date)** a new 2.0 ml screw-capped tube (RNAse/DNAse free)

3. Transfer 200 µl aliquot of cervical mucous to each marked tube.

4. Resuspend cell pellet in 180 µl buffer ATL.

5. Add 20µl proteinase K, mix by vortexing, and incubate at 56°C until completely lysed (10min – 1hr). Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform. – incubated for 1h.

6. Continue extracting the samples using the AmpliLute Liquid Media Extraction Kit (Roche) or QIAamp Blood DNA Mini Kit (Qiagen) or MagNa Pure automated DNA extraction (Roche), according to the manufacturer's instructions (booklet inside the QIAamp or Roche box).

QIAamp Blood DNA Protocol(Qiagen)

7. Briefly centrifuge the 2.0 ml microcentrifuge tube to remove drops from the inside of the lid.

8. Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

9. Add 200µl ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

10. Carefully apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim.

Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

11. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min.

12. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.

13. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

14. Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover.

15. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube, and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column,

16. Soak the column with the 20 µl distilled water (RNase & DNase Free) for 1 to 5 minutes before continuing. and then centrifuge at 6000 x g (8000 rpm) for 1 min.

17. Store the DNA at -80ºC until further use, with no more than one freeze-thaw cycle.

AmpliLute Liquid Media Extraction Protocol (Roche)

1. Dissolve the lyophilized CAR by adding 310 µL of AVE. Vortex for 10 seconds. Initial and date the vial.

NOTE: Dissolved CAR is stable at 2-8ºC for up to 24 hours or can be aliquoted and frozen at -20ºC. Do not freeze-thaw the dissolved CAR more than 3 times.

2. Add 30 mL of absolute ethanol to AW2. Initial and date the bottle. Mix diluted AW2 by shaking.

NOTE: Diluted AW2 may be stored at room temperature for up to 1 year or until the expiration date, whichever comes first.

3. Prepare Working AL by adding the appropriate volume of dissolved CAR to AL as shown in Table 1. Gently mix by inverting the tube 10 times. To avoid foaming, do not vortex.

Table 1
Preparation of Working AL

e.g ***Add 40µl of carrier RNA into 4000µl of AL buffer***

	Number of Specimens/Controls to be processed						
Reagents	12	24	36	48	60	72	96
CAR (mL)	0.04	0.07	0.10	0.13	0.16	0.20	0.25
AL (mL)	4.0	7.0	10.0	13.0	16.0	20.0	25.0

NOTE: Working AL is stable at 2-8ºC for up to 48 hours.

4. Label one 2 mL screw cap tube for each specimen and control to be processed.

NOTE: DO NOT use 1.5 mL screw cap tubes or any conical-shaped tubes as it will interfere with the heat transfer during incubation and may result in incomplete lysis.

5. Add 80 µL ATL to each tube.
6. Vortex each specimen and control for 10 seconds. Add 250 µL of each specimen and control to the appropriately labeled tube.
7. Add 20 µL PK to each tube. Cap the tubes and vortex for 10 seconds. Incubate the tubes at 56ºC ± 2ºC for 30 minutes in a dry heat block.
8. While the specimens and controls are incubating, assemble the QIAvac 24 (or QIAvac 24 Plus) vacuum manifold according to the QIAGEN vacuum manifold handbook. Remove one CLMfor each specimen and control from the blister packages and label. Save the waste collection tubes for use in Step B.23. Open the lid on the CLM and insert into the VC. Insert the EXT into the CLM.
9. After the incubation at 56ºC ± 2ºC is complete, add 250 µL of Working AL to each tube. Cap the tubes and vortex for 10 seconds at maximum speed.

NOTE: A white precipitate may form when Working AL is added. The precipitate does not interfere with the AmpliLute procedure and will dissolve during the following incubation.

10. Incubate the tubes at 70ºC ± 2ºC for 15 minutes in a dry heat block. Vortex the specimens and controls occasionally throughout the incubation period.
11. After the incubation of specimens and controls at 70ºC ± 2ºC is complete, add 300 µL of absolute ethanol to each tube. Cap the tubes and vortex for 15 seconds at maximum speed.
12. Incubate the tubes at ambient temperature for 5 minutes. Pulse spin the tubes using a tabletop centrifuge at maximum RPM.
13. Transfer the lysate from each tube into the corresponding CLM. Allow to incubate at least 1 minute.
14. Turn on the vacuum pump. Apply the vacuum to the manifold to remove all lysate and continue vacuum application for at least 1 more minute. Release the vacuum from the manifold as described in QIAGEN vacuum manifold handbook.

NOTE: If the lysate from an individual specimen or control has not completely passed through the membrane, place the CLM into a clean 2 mL collection tube, close the cap, and centrifuge at full speed for 1 minute or until the lysate has completely passed through. Additional 2 mL collection tubes can be purchased separately (see "MATERIALS REQUIRED BUT NOT PROVIDED").

15. Add 750 µL AW2 to each CLM. Allow to incubate for at least 1 minute.
16. Turn on the vacuum pump. Apply the vacuum to the manifold to remove all liquid and continue vacuum application for at least 1 more minute. Release the vacuum from the manifold as described in QIAGEN vacuum manifold handbook. [**if lysate is not removed, then spin the manifold at 25000 xg (14000 rpm) for 1minute**]
17. Add 750 µL of absolute ethanol to each CLM. Allow to incubate for at least 1 minute.
18. Turn on the vacuum pump. Apply the vacuum to the manifold to remove all liquid and continue vacuum application for at least 1 more minute. Release the vacuum from the manifold as described in QIAGEN vacuum manifold handbook. [**if lysate is not removed, then spin the manifold at 25000 xg (14000 rpm) for 1minute**]
19. Carefully remove the EXT from each CLM. Discard the EXT.

NOTE: To avoid cross contamination, be careful not to contact the adjacent CLM while EXT are being removed.

20. Place each CLM into a waste collection tube (from Step B.11) and close the lid. Centrifuge the assemblies at 14000 rpm (25000 xg) for 3 minutes.
21. Label one ELT for each specimen and control.
22. Discard the waste collection tubes and place each CLM into the corresponding ELT.
23. Open the lid on each CLM and incubate at room temperature for 15 minutes.
24. Add 120 µL DNase/RNase free distilled Water to each CLM. Close each lid.

NOTE: Ensure that dH_2O (DNase/RNase free) is equilibrated to room temperature prior to adding to each CLM.

25. Incubate the assemblies at ambient temperature for 5 minutes and then centrifuge at maximum RPM for 1 minute.
26. Remove and discard the CLM from the assemblies and cap the tubes.

***NB: Do not store DNA at -20^oC or leave at room temperature for extended periods; avoid multiple freeze thaw cycles.**

B. DNA synthesis and PCR

RECOMMENDATIONS BEFORE MASTER MIX REACTIONS:

Thaw out reagents needed (Working Master Mix and HPV Mg2+) on ice and keep them on ice Mix thawed reagents gently, vortex (except the enzymes), and quick spin the tube to collect the contents in the bottom of each tube.

First, prepare the PCR master mix

Do not have more than one sample tube open at a time.

The PCR mixes (without DNA template) can be kept at 4 - 8 °C if used on the same day or - 20 °C if the next day.

Thaw DNA samples and keep in the fridge (separate from RT-PCR reagent).

A positive and negative control must be included with every run.

Wear gloves at all time to avoid RNase or DNase contamination.

Prior to working in any hood or on any work surface and using pipettes, please decontaminate with 10% bleach first, followed by 70% ethanol twice to ensure all traces of bleach are removed.

Reagent and equipment:

- Working Master Mix
- HPV Mg2+

- 0.2ml RNAse/DNAse free tubes
- 1.5 ml RNAse/DNAse free tubes
- Pipette Tips (DNAse, RNAse Free)
- Distilled water (DNAse, RNAse Free)
- E-centrifuge (Spectrafuge Mini Centrifuge and/or Jouan MR22i)
- Thermocyler: GeneAmp®PCR System 9700 (Amplification Room at CAPRISA Lab)
- Pipettes
- Vortex
- Dead Air Space or BSL-2 hood

PCR steps must be carried out in a dead air space cardboard or BSL-2 hood in the master mix room in CAPRISA Research Laboratory (DDMRI)

HPV master mix:

Reagent Preparation

Performed in: Pre-Amplification – Reagent Preparation Area

1. Determine the appropriate number of reaction tubes needed for specimen and control testing. Place the tubes in the MicroAmp tray and lock in place with retainer.
2. Prepare Working Master Mix by adding 125 µL of HPV Mg2+ to one vial of HPV MMX. It is not necessary to measure the volume of Master Mix. Add 125 µL HPV Mg2+ to the entire vial of HPV MMX. Recap the tube and mix well by inverting the tube 10-15 times. Do not vortex the Working Master Mix. The pink dye in HPV Mg2+ is used for visual confirmation that HPV Mg2+ has been added to HPV MMX. Discard remaining HPV Mg2+.
3. Add 50 µL of Working Master Mix into each reaction tube using a Multipette pipet or a pipettor with an aerosol barrier or positive displacement tip. Do not cap the reaction tubes at this time.
4. Place the tray containing Working Master Mix and the appropriate number of reaction tube caps in a resealable plastic bag and seal the plastic bag securely. Move to the Pre-Amplification – Specimen and Control Preparation Area. Store the tray(s) containing Working Master Mix at 2-8ºC in the Pre-Amplification – Specimen and Control Preparation Area until specimen and control preparation is completed. Working Master Mix is stable for 6 hours at 2-8ºC in the reaction tubes sealed in the plastic bag.

5. Add 50 µL of each processed specimen (HPV DNA) and control to the appropriate amplification tubes containing Working Master Mix. Use a new aerosol barrier or positive displacement tip for each specimen and control. Cap the amplification tubes.
6. Transfer the prepared specimens and controls in the amplification tray to the Amplification/Detection Area.
7. Program the Applied Biosystems Gold-plated 96-Well GeneAmp PCR System 9700 for the LINEAR ARRAY HPV Genotyping Test as follows:

8. Linear Array HPV Genotyping Test cycling conditions:

No. of Cycles	Temperature	Time	Process
1	50ºC	2minutes	Initial denaturation
40 **Set ramp rate to 50%**	95ºC	9minutes	DNA denaturation Primer annealing Primer extension
	95ºC	30 seconds	
	55ºC	1minute	
	72ºC	1minute	
1	72ºC	5minutes	Final extension

9. Remove the tray from the thermal cycler within 4 hours of the start of the final HOLD program, place in the MicroAmp base and continue immediately with Step 5. Do not allow the reaction tubes to remain in the thermal cycler beyond 4 hours. **DO NOT BRING AMPLIFIED SPECIMENS INTO THE PRE-AMPLIFICATION AREA. AMPLIFIED CONTROLS AND SPECIMENS SHOULD BE CONSIDERED A MAJOR SOURCE OF POTENTIAL CONTAMINATION.**
10. Remove the caps from the reaction tubes carefully to avoid creating aerosols of the amplification products. Immediately pipet 100 µL DN to the first column (or row) of reaction tubes using a multichannel pipettor with aerosol barrier tips and mix by pipetting up and down five times. For each column (or row), repeat this procedure using a fresh set of tips.
11. The denatured amplicon can be held at room temperature for no more than 5 hours before proceeding to Detection (Part D). If the detection reaction can not be performed within 5 hours, re-cap the tubes with new caps and store the denatured amplicon at 2-8ºC for up to 7 days.

LINEAR ARRAY Detection

Performed in: Post Amplification – Amplification/Detection Area

NOTE: Throughout the detection procedure, do not allow the specimens to splash from one tray well to another. Do not allow the water from the bath to splash into the tray wells. Do not stack the 24-well trays.

1. Warm all detection reagents to room temperature.
2. Pre-warm a water bath to 53ºC ± 2ºC.
3. Pre-warm the shaking water bath to 53ºC ± 2ºC at a shaking speed of approximately 60 RPM.
Be sure that there is sufficient water in the bath to heat the 24-well tray, but not too much water such that it splashes into the 24-well tray. Water should cover no more than 1/4 of exterior well depth (approximately 0.5 cm) to prevent leaking into tray while shaking.
4. Prepare Working Hybridization Buffer as follows: Examine SSPE and SDS and, if necessary, warm to 53ºC ± 2ºC in a water bath to re-dissolve any precipitate. Add 100 mL SSPE to 388 mL of distilled or deionized water. Mix well. Add 12.5 mL SDS and mix well. The Working Hybridization Buffer is sufficient for 100 LINEAR ARRAY HPV Genotyping Strips. Working Hybridization Buffer should be stored at room temperature in a clean container and is stable for 30 days.
5. Prepare Working Ambient Wash Buffer as follows: Examine SSPE and SDS and, if necessary, warm to 53ºC ± 2ºC in a water bath to re-dissolve any precipitate. Add 133 mL SSPE to 2520 mL of distilled or deionized water. Mix well. Add 13.3 mL SDS and mix well. The Working Ambient Wash Buffer is sufficient for 100 LINEAR ARRAY HPV Genotyping Strips. Working Ambient Wash Buffer should be stored at room temperature in a clean container and is stable for 30 days.
6. Prepare Working Stringent Wash Buffer as follows: For each strip being tested, remove 5 mL of Working Ambient Wash Buffer (prepared in the previous step) and add it to an appropriately sized clean media bottle. (e.g., For a run of 24 strips, remove 120 mL of Working Ambient Wash Buffer and add to the media bottle.) Working Stringent Wash Buffer should be prepared fresh prior to each run.
7. Warm the Working Hybridization Buffer and Working Stringent Wash Buffer in the 53ºC ± 2ºC water bath for a minimum of 15 minutes. Leave the Working Hybridization Buffer and Working Stringent Wash Buffer in the water bath until use.
8. Prepare Working Citrate Buffer as follows: Examine CIT and, if necessary, warm to 53ºC ± 2ºC in a water bath to dissolve any precipitate. Add 25 mL CIT to 475 mL of distilled or deionized water. Mix well. The Working Citrate Buffer is sufficient for 100 LINEAR ARRAY HPV Genotyping Strips. Working Citrate Buffer should be stored at room temperature in a clean container and is stable for 30 days.

9. Remove the required number of LINEAR ARRAY HPV Genotyping Strips from the HPV Strip pouch using clean forceps.
10. With a water/chemical/heat-resistant permanent ink pen, label each HPV Strip with the appropriate specimen or control identification.
11. Place each strip with the probe lines upright into the appropriate well of the 24-well tray. 12. Add 4 mL of pre-warmed Working Hybridization Buffer into each well that contains a labeled strip.
13. Using a pipettor with an aerosol barrier or positive displacement tip, carefully pipet 75 µL of denatured amplicon into the appropriate well containing a labeled strip. Rock the tray gently between each addition. Use a new tip for each amplicon addition.
14. Cover the 24-well tray with the lid and place tray in the 53ºC ± 2ºC shaking water bath **or shaking incubator**. Place a 1 lb. lead ring weight on the tray lid in order to hold the 24-well tray in place in the shaking water bath. Hybridize for 30 minutes at a shaking speed of approximately 60 RPM.
15. During hybridization (Step 14), prepare Working Conjugate as follows: Add 15 µL SA-HRP to 5 mL of Working Ambient Wash Buffer for each strip being tested. Mix well. Working Conjugate should be stored at room temperature in a clean container and is stable for 3 hours.
16. Remove the 24-well tray from the shaking water bath and remove the Working Hybridization Buffer from the wells by vacuum aspiration.
17. Add 4 mL of Working Ambient Wash Buffer to each well containing a strip. Gently rock the 24-well tray 3-4 times to rinse the strips and immediately vacuum aspirate the Working Ambient Wash Buffer from the wells.
18. Add 4 mL of pre-warmed Working Stringent Wash Buffer to each well containing a strip. Wipe any condensation from the tray lid with a clean paper towel, place the lid on the 24-well tray and return the 24-well tray to the 53ºC ± 2ºC shaking water bath. Place a 1 lb. lead ring weight on the tray lid in order to hold the 24-well tray in place in the shaking water bath. Incubate for 15 minutes at a shaking speed of approximately 60 RPM.
19. Remove the 24-well tray from the shaking water bath and remove the Working Stringent Wash Buffer from the wells by vacuum aspiration.
20. Add 4 mL of Working Conjugate to each well containing a strip. Wipe any condensation from the tray lid with a clean paper towel, place the lid on the 24-well tray and place the 24-well tray on the room temperature orbital shaker. Incubate for 30 minutes at room temperature (15-30ºC) at a shaking speed of approximately 60 RPM.
21. Remove the 24-well tray from the orbital shaker and remove the Working Conjugate from the wells by vacuum aspiration.
22. Add 4 mL of Working Ambient Wash Buffer to each well containing a strip. Gently rock the 24-well tray 3-4 times to rinse the strips and immediately vacuum aspirate the Working Ambient Wash Buffer from the wells.

23. Add 4 mL of Working Ambient Wash Buffer to each well containing a strip. Wipe any condensation from the tray lid with a clean paper towel, place the lid on the 24-well tray and place the 24-well tray on the room temperature orbital shaker for 10 minutes at a shaking speed of approximately 60 RPM.
24. Remove the 24-well tray from the orbital shaker and remove the Working Ambient Wash Buffer from the wells by vacuum aspiration.
25. Add 4 mL of Working Ambient Wash Buffer to each well containing a strip. Place the lid on the 24-well tray and place the 24-well tray on the room temperature orbital shaker for 10 minutes at a shaking speed of approximately 60 RPM.
26. Remove the 24-well tray from the orbital shaker and remove the Working Ambient Wash Buffer from the wells by vacuum aspiration.
27. Add 4 mL of Working Citrate Buffer to each well containing a strip. Place the lid on the 24- well tray and place the 24-well tray on the room temperature orbital shaker for 5 minutes at a shaking speed of approximately 60 RPM.
28. Prepare Working Substrate as follows: Add 4 mL SUB A to 1 mL SUB B per strip being tested. Mix well. Working Substrate should be stored at room temperature, protected from exposure to direct light in a clean container and is stable for 3 hours.
29. Remove the 24-well tray from the orbital shaker and remove the Working Citrate Buffer from the wells by vacuum aspiration.
30. Add 4 mL of Working Substrate to each well containing a strip. Place the lid on the 24-well tray and place the 24-well tray on the room temperature orbital shaker for 5 minutes at a shaking speed of approximately 60 RPM.
31. Remove the 24-well tray from the orbital shaker and remove the Working Substrate from the wells by vacuum aspiration.
32. Add 4 mL of distilled or deionized water to each well containing a strip. Strips can be stored for up to one day at room temperature in the distilled or deionized water until interpretation.

24-Well Tray Cleaning

Performed in: Post Amplification – Amplification/Detection Area
NOTE: The 24-well trays are disposable or may be re-used. To re-use the trays, this cleaning procedure must be followed after every use.

1. Prepare a 10% solution of RBS35 by adding 1 part RBS35 to 9 parts distilled or deionized water. Or 10% solutions of bleach by 10µl bleach to 90µl of distilled water.
2. Fill each tray well with the 10% solution and let soak overnight at room temperature.
3. Rinse the tray **thoroughly** with distilled or deionized water.
4. Dry tray completely before use.

RESULTS

Ensure that the control results for the run are valid (see Quality Control Section). If the run is invalid, repeat the entire run (specimen preparation, amplification and LINEAR ARRAY detection). For a valid run, interpret the LINEAR ARRAY HPV Genotyping Strip by removing a strip from the 24-well tray using clean forceps. Place the strip on a clean non-porous surface (e.g. acetate sheet) and align the LINEAR ARRAY HPV Genotyping Test Reference Guide with the ink reference line (REF) at the top of the strip.

Record the positive visible bands and interpret the HPV and ß-globin (BG) results for each strip as follows:

HPV Result	BG Low Result	BG High Result	Interpretation
–	–	–	**Result Invalid.** HPV DNA, if present, could not be detected. The absence of BG High and BG Low results indicates inadequate specimen collection, processing, or the presence of inhibitors. Process another aliquot of the original specimen and repeat the test. If the original specimen is not available, a new specimen must be collected.
–	–	+	**Result Invalid.** HPV DNA, if present, may not have been detected. The absence of BG Low is suggestive of inadequate specimen collection, processing, or the presence of inhibitors. Process another aliquot of the original specimen and repeat the test. If the original specimen is not available, a new specimen must be collected.
–	+	+	**HPV DNA not detected.** A negative result does not preclude the presence of HPV infection because results depend on adequate specimen collection, processing, absence of inhibitors, and sufficient HPV DNA to be detected.
+	–	–	**HPV DNA detected (Report genotypes).** Specimen is positive for the presence of HPV. The absence of BG Low and BG High is suggestive of inadequate specimen collection, processing, presence of inhibitors or competition with a high titer HPV target. Additional HPV genotypes that were not detected may be present.
+	–	+	**HPV DNA detected (Report genotypes).** Specimen is positive for the presence of HPV. The absence of BG Low is suggestive of inadequate specimen collection, processing, presence of inhibitors, or competition with a high titer HPV target. Additional HPV genotypes that were not detected may be present.
+	+	+	**HPV DNA detected (Report genotypes).** Specimen is positive for the presence of HPV. The presence of additional HPV genotypes in the specimen cannot be completely ruled out.

Cross Reactive Probe Interpretation

The LINEAR ARRAY HPV Genotyping Strip contains a cross reactive probe (probe line 14) that hybridizes with HPV genotypes 33, 35, 52 and 58. Positive band results for the probe should be interpreted as follows:

Band Result	Interpretation
33, 52/33/35/58	HPV 33*
35, 52/33/35/58	HPV 35*
58, 52/33/35/58	HPV 58*
52/33/35/58	HPV 52

* co-infection with HPV Genotype 52 cannot be ruled out by these test results.

QUALITY CONTROL

At least one replicate of the LINEAR ARRAY HPV Negative Control and one replicate of the LINEAR ARRAY HPV Positive Control must be processed with each run of up to 22 specimens. As with any new laboratory procedure, new operators should consider the use of additional positive and negative controls each time the test is performed until such time that a high degree of confidence is reached in their ability to perform the test procedure correctly. There are no requirements regarding the position of the controls in the MicroAmp tray. Specimens and controls from separate specimen preparation runs may be amplified and detected at the same time. However, each separate specimen preparation run is validated individually by the set of controls included with the run. Therefore, it is possible to reject one run of specimens from a common amplification and/or detection run while accepting another run based upon the performance of the controls processed with those specimens. All test specimens and controls prepared in the same run should be amplified and detected in adjacent positions in the thermal cycler and in the detection tray. The exact order of placement of these specimens and controls in the thermal cycler or detection tray is not critical.

Negative Control

The assay result of the LINEAR ARRAY HPV Negative Control must be no positive bands. If any positive bands are visible, the entire run is invalid. Repeat the entire process (Specimen and Control Preparation, Amplification and LINEAR ARRAY Detection). If the LINEAR ARRAY HPV Negative Control consistently yields results with positive bands, contact your local Roche office for technical assistance.

Positive Control

The assay result of the LINEAR ARRAY HPV Positive Control must yield a positive result interpreted as HPV GT16, ß-Globin high, and ß-Globin low. The ß-Globin low band will be faint in comparison to the ß-Globin high band but must be visible by eye. If the LINEAR

ARRAY HPV Positive Control does not yield this exact result, the entire run is invalid. Repeat the entire process (Specimen and Control Preparation, Amplification and LINEAR ARRAY Detection). If the LINEAR ARRAY HPV Positive Control consistently yields results with a different pattern of positive bands, contact your local Roche office for technical assistance.

PROCEDURAL LIMITATIONS

1. This test has been validated for use with human cervical cells collected in PreservCyt Solution. Testing of other specimen types may result in false negative or false positive results.
2. This test has been validated for use with the AmpliLute Liquid Media Extraction Kit only. Testing using other specimen extraction procedures may lead to incorrect results.
3. Reliable results are dependent on adequate specimen collection, transport, storage and processing procedures.
4. Detection of HPV is dependent on the number of virus genomes present in the specimen and may be affected by specimen collection methods, patient factors (i.e. age, presence of symptoms), and/or stage of infection.
5. False negative results may occur due to polymerase inhibition or cell inadequacy. The ß-globin amplification has been added to the LINEAR ARRAY HPV Genotyping Test to permit the identification of processed specimens containing substances that may interfere with PCR amplification and or inadequate cell collection.
6. The presence of AmpErase enzyme in the LINEAR ARRAY HPV Master Mix reduces the risk of amplicon contamination. However, contamination from HPV positive controls and specimens can be avoided only by good laboratory practices and careful adherence to the procedures specified in this insert.
7. Use of this product should be limited to personnel trained in the techniques of PCR.
8. Only the Applied Biosystems Gold-plated 96-Well GeneAmp PCR System 9700 has been validated for use with this product. No other thermal cycler, including GeneAmp PCR System 2400, GeneAmp PCR System 9600, or GeneAmp PCR System 9700 thermal cycler with aluminum block, can be used with this product.
9. Specimens containing Advantage-S® Bioadhesive Contraceptive Gel may interfere with the performance of the LINEAR ARRAY HPV Genotyping Test and may give false negative or invalid results.
10. Specimens containing greater than 3.5% (v/v) blood have been shown to inhibit PCR amplification and may give false negative results.
11. Identification of HPV genotypes 64 and 69 are based on LINEAR ARRAY HPV Genotyping Test results for HPV genotypes 64 and 69 plasmid DNA. HPV genotypes 64 and 69 were not detected in a clinical specimen during the performance evaluations.

Appendix C: BREC Ethics

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

17 June 2021

Mr Lungelo Ntuli (219039019)
School of Lab Med & Medical Sc
Medical School

Dear Mr Ntuli,

Protocol reference number: BREC/00002792/2021
Project title: The Impact of Vaginal Microbiota and associated inflammation on Human Papillomavirus Infection
Degree: MMedSc

EXPEDITED APPLICATION: APPROVAL LETTER

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

The conditions have been met and the study is given full ethics approval and may begin as from 17 June 2021. Please ensure that outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is subject to national and UKZN lockdown regulations, see (http://research.ukzn.ac.za/Libraries/BREC/BREC_Lockdown_Level_3_Guidelines.sflb.ashx). Based on feedback from some sites, we urge PIs to show sensitivity and exercise appropriate consideration at sites where personnel and service users appear stressed or overloaded.

This approval is valid for one year from 17 June 2021. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

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

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

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

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 13 July 2021.

Yours sincerely,

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
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UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

26 April 2022

Mr Lungelo Ntuli (219039019)
School of Laboratory Medicine & Medical Science
Medical School

Dear Mr Ntuli,

Protocol reference number: BREC/00002792/2021
Project title: The Impact of Vaginal Microbiota and associated inflammation on Human Papillomavirus Infection
Degree: MMedSci

We wish to advise you that your application for amendments received on 06 April 2022 listed below for the above study has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee.

Amendments noted and approved:

- To use the CAPRISA 083 (BREC: BFC410/15) study cohort samples (cervicovaginal swabs) to investigate vaginal microbiota in HPV infected women

The committee will be advised of the above at its next meeting to be held on 10 May 2022.

Yours sincerely

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

Appendix D: Review paper submitted to Frontiers Oncology

Role of immunity and vaginal microbiome in clearance and persistence of Human Papillomavirus infection

Lungelo Ntuli[1], Andile Mtshali[1], Gugulethu Mzobe[1], Lenine J. Liebenberg[1], SINAYE NGCAPU[1*]

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Submitted to Journal:
Frontiers in Oncology

Specialty Section:
Molecular and Cellular Oncology

Article type:
Review Article

Manuscript ID:
927131

Received on:
23 Apr 2022

Journal website link:
www.frontiersin.org

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

All authors contributed to the article and approved the submitted version

Keywords

Human papillomavirus, cellular, Cytokines, Inflammation, vaginal microbiota Immunity and Genital Microbiome in HPV Infection 2

Abstract

Word count: 169

Cervical cancer disproportionately affects women of reproductive age, with 80% of cases occurring in low- and middle-income countries. Persistent infection with high-risk human papillomavirus (HPV) genotypes has been described as the most common non-systemic biological risk factor for the development of cervical cancer. The mucosal immune system plays a significant role in controlling HPV infection by acting as the first line of host defense at the mucosal surface. However, the virus can evade host immunity using various mechanisms, including inhibition of the antiviral immune response necessary for HPV clearance. Pro-inflammatory cytokines and the vaginal microbiome coordinate cell-mediated immune responses and play a pivotal role in modulating immunity. Recently, diverse vaginal microbiome (associated with bacterial vaginosis) and genital inflammation have emerged as potential drivers of high-risk HPV positivity and disease severity in women. The potential role of these risk factors on HPV recurrence and persistence remains unclear. This article reviews the role of vaginal microbiome dysbiosis and cellular or cytokine response in the clearance, persistence, and recurrence of HPV infection.

Contribution to the field

This article reviews the role of cellular or cytokine response and vaginal microbiome dysbiosis in the clearance, persistence, and recurrence of HPV infection. While there is evidence of a relationship between the host immune responses, vaginal microbiome, and HPV infection, a link between the vaginal microbiome, host responses, and the progression to HPV-associated cervical cancer remains unclear. This review contributes to the understanding of the concept of vaginal host immune and microbiome fluctuations responses during HPV infection stages could shed light on possible mechanisms associated with cervical carcinogenesis.

Funding statement

This review was conducted as part of the DST-NRF Centre of Excellence (CoE) in HIV Prevention, supported by the Department of Science and Innovation and the National Research Foundation (grant 96354). LN was funded by DST-NRF CoE in HIV Prevention (grant 96354) and Poliomyelitis Research Foundation Research Bursary (bursary# 21/73). SN was supported by Columbia University-Southern African Fogarty AITRP Programme (grant# D43TW00231), National Research Fund Thuthuka Research Grant (grant# TTK160510164586), and Poliomyelitis Research Foundation Research Grant (grant# 16/17). GM was funded by Poliomyelitis Research Foundation Research Grant (grant# 18/16) and National Research Foundation Grant (grant# SFP180507326699). AM was supported by National Research Foundation Grant (grant# PDG210309589501).

frontiers

Role of immunity and vaginal microbiome in clearance and persistence of Human Papillomavirus infection

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Keywords: **Human papillomavirus, cellular, cytokines, inflammation, vaginal microbiota**

Immunity and Genital Microbiome in HPV Infection

Abstract

Cervical cancer disproportionately affects women of reproductive age, with 80% of cases occurring in low- and middle-income countries. Persistent infection with high-risk human papillomavirus (HPV) genotypes has been described as the most common non-systemic biological risk factor for the development of cervical cancer. The mucosal immune system plays a significant role in controlling HPV infection by acting as the first line of host defense at the mucosal surface. However, the virus can evade host immunity using various mechanisms, including inhibition of the antiviral immune response necessary for HPV clearance. Pro-inflammatory cytokines and the vaginal microbiome coordinate cell-mediated immune responses and play a pivotal role in modulating immunity. Recently, diverse vaginal microbiome (associated with bacterial vaginosis) and genital inflammation have emerged as potential drivers of high-risk HPV positivity and disease severity in women. The potential role of these risk factors on HPV recurrence and persistence remains unclear. This article reviews the role of vaginal microbiome dysbiosis and cellular or cytokine response in the clearance, persistence, and recurrence of HPV infection.

1. Introduction

Human Papillomavirus (HPV), a primary cause of genital warts and cervical cancer, is the most common sexually transmitted infection (1). Despite efforts to implement prophylactic HPV vaccination in young women, cervical cancer is the fourth most common cancer among sexually active women globally (2). A majority (~90%) of women clear HPV infections spontaneously within 6–18 months (3, 4), with very few progressions from precancerous lesion to invasive cervical cancer (5). Several studies have hypothesized that host defense mechanisms, the genital microbiome, and other factors in the female genital tract play a role in the clearance and persistence of HPV, including the risk of developing cervical cancer (6-10).

Innate and adaptive immune responses represent the first line of host defense at the mucosal surface against pathogens such as HPV (11). Several studies have demonstrated a relationship between genital mucosal cytokine concentrations and the control or elimination of HPV infection in the cervix (8, 12-14). It is hypothesized that cytokine response occurs within days after the establishment of an HPV infection, and is subsequently reversed when HPV clearance has been effectively achieved by appropriate effector cells (12, 15-18). The initial response against HPV infection includes mature

antigen presenting cells (APCs) that secret cytokines and contribute in activation and recruitment of other immune cells to the site of infection (19). Upon interaction with mature APCs, naïve CD4+ and CD8+ T cells differentiate into various T helper effector lineages and cytotoxic T lymphocytes (CTLs), respectively, which needed for the effective clearance of HPV(19, 20). However, these cells do not entirely prevent disease progression. HPV can use various immune evasion mechanisms to limit anti-viral activity of immune response, resulting in HPV infection tolerance in the host's immune system. HPV infection could affect the differentiation of monocytes into mature DCs and distinctively affect the functionality of CD4+/CD8+, and regulatory T cells (21). Increased secretion of anti-inflammatory cytokine IL-10 in Th2 response has been associated with compromised innate and adaptive immune defense and cervical lesion progression during high-risk HPV infection (22-24). Although studies have demonstrated the anti-viral activies of immune cells, less is known about the cell-mediated mucosal immune response to HPV incidence, persistence, and clearance.

Emerging evidence suggests that women with a low relative abundance of vaginal *Lactobacillus* species and high proportions of the *Gardnerella*, *Sneathia*, and *Atopobium* genera are less likely to clear HPV infection (7, 25-31). Two recent meta-analyses summarizing findings from several microscopy or molecular studies have reported that women with *Lactobacillus*-enriched microbiomes were less likely to acquire HPV infection compared to women with overgrowth of bacterial vaginosis (BV)-linked bacteria (*Gardnerella*, *Atopobium,* and *Prevotella*) (31, 32). A *Lactobacillus gasseri*-enriched microbiome was associated with rapid rates of HPV clearance (26, 33), while a *L. iners* dominant microbiome was commonly reported in women presenting with cervical intraepithelial neoplasia (CIN). Several studies further reported an association between BV-linked microbial communities (*Gardnerella, Prevotella, Dialister, Streptococcus, Ureaplasma, Megasphaera,* and *Mycoplasma*) and persistent infection with high-risk HPV that may result in the development of CIN (31, 34). Furthermore, specific vaginal microbiota may modulate host immune responses, including critical antiviral and anti-tumor immunity components in the female genital tract (35, 36). Diverse microbial communities have been closely associated with altered innate immune responses, host susceptibility to infection (35, 37, 38), and development of cervical diseases, but proven causality remains unclear.

While there is evidence of a relationship between the vaginal microbiome, host immune responses, and HPV infection, a link between the vaginal microbiome, host responses, and the progression to HPV-associated cervical cancer remains unclear. Therefore, understanding the concept of vaginal microbiome fluctuations and associated host immune responses during HPV infection stages could shed light on possible mechanisms associated with cervical carcinogenesis. This review summarizes the role of vaginal microbiome fluctuations, cytokine and cellular immune response during HPV infection, persistent and clearance.

2. HPV genotypes and screening

HPV is a member of the non-enveloped double-stranded DNA papillomaviridae family that infects squamous epithelium found beneath the foreskin of the penis, the scrotum, vulva, vagina, cervix, skin, and anus (39, 40). The papillomavirus structure is icosahedral with approximately 50–60 nm and contains 8000 base pairs (40, 41). HPV infection is classified into five major genera, *Alphapapillomavirus, Gammapapillomavirus, Betapapillomavirus, Deltapapillomavirus,* and *Mupapillomavirus,* with only *Alphapapillomavirus, Gammapapillomavirus* and *Betapapillomavirus* affecting humans (40). Of the genera affecting humans, *Alphapapillomavirus* is the most common genus infecting the genital tract (International Agency for Research on Cancer, 2018).

More than 200 genital HPV genotypes have been molecularly characterized, with some categorized into low-risk genotypes (causing genital warts) while others are categorized as high-risk genotypes (causing CIN and cervical cancer) (42). High-risk HPV types include HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 68, and 59, with HPV 16 and 18 being the most prevalent (accounting for 70% of cervical cancer cases) genotypes (43). The most common low-risk HPV include HPV 6 and 11, accounting for ~90% of genital warts and rarely developing into cancer (44). HPV screening uses biopsy, colposcopy and acetic acid test, Pap smear, and nucleic acid-based tests (45). A Pap smear is the main screening tool to identify precancerous cells in the cervix, and any observed abnormalities are further evaluated with colposcopy, biopsy, and molecular-based tests (45).

3. HPV treatment and prevention strategies

Although there is no effective treatment for the virus itself, oncogenic HPV infections and subsequent HPV-associated lesions can be prevented by vaccination (46). There are three commercially available prophylactic vaccines targeting different HPV types, including the bivalent (Cervarix®), quadrivalent (Gardasil®), and nonavalent (Gardasil®9) vaccines (47). The bivalent vaccine protects against the two commonly known high-risk oncogenic types, HPV 16 and HPV 18 (42). The quadrivalent vaccine acts against these and two genital wart-causing low-risk types (HPV 6 and 11) (48, 49). The nonavalent vaccine is directed against 9 HPV genotypes, the four collectively targeted by the bivalent and quadrivalent vaccines (HPV 16, 18, 6, and 11) and five additional strains associated with cervical cancer (HPV 31, 33, 45, 52, 58) (50). The adverse effects of HPV vaccination are normal mild local reactions and their safety has resulted in effective HPV vaccination program in majority of developed countries, but the uptake of these vaccinations by native populations is low (51). HPV vaccination is provided to adolescents from 9 to 12 years (52) to prevent HPV infections, abnormal cervical cytology, and cervical cancer (53). However, these primary prevention strategies are unable to protect against all types of HPVs or eradicate the existing HPV-infected cells, as capsid proteins are established either before viral entry, or in the terminally differentiated epithelium (54). Women identified with precancerous lesions can be treated using cryotherapy or thermal ablation, and invasive cervical cancer can be treated by surgery, chemotherapy, and radiotherapy (46). Collectively, the partial protection prophylactic vaccines has against certain HPV types that are associated with cervical cancer justifies the need for continual screening and development of additional therapeutic options to resolve cases post-infection.

4. HPV infection in young women

Young women in sub-Saharan Africa continue to bear a disproportionate burden of HPV (55), where the HIV epidemic has reached its greatest scale (56). Adolescent girls and young women are more susceptible to HPV infection than their male counterparts (55, 57, 58). Bruni *et al*. (2010) showed that HPV infection peaks in younger women around the age of sexual debut and declines during later decades of life (55). The unique vulnerability of women to HPV infection comes from several different behavioural and biological factors. While the risk for infection differs from person to person, increased number of sexual partners, early sexual debut, the use of intravaginal insertion products, uncircumcised male partners, and the number of prior pregnancies are some of the documented behavioral risk factors in women (59, 60). Several biological factors, including vaginal surface, the relatively larger area of cervical epithelium undergoing squamous metaplasia, immunosuppression, co-occurrence of STIs,

disturbance of vaginal microenvironment and menstruation, defective immune responses associated with genetic variations, and condomless sex, may predispose women to become infected with HPV (58, 61). Despite evidence for an etiological role for HPV, these risk factors remain largely unexplored. Understanding the role of behavioural and biological risk factors for HPV infection in adolescent girls and young women could be crucial to developing effective ways to prevent HPV infection, including management of cervical dysplasia and developing prophylactic vaccines.

5. Immune response and evasion mechanisms

Women are particularly vulnerable to HPV and are predominantly infected through heterosexual transmission (55, 62, 63). HPV infections are localised to keratinocytes. The mucosal epithelium provides the first line of defense against pathogen entry and mediates the initial host immune response against HPV (19). Pathogens like HPV are detected by an intricate matrix of innate and adaptive immune responses of the lower and upper female genital tract.

5.1 Innate immune response

Innate immune cells like neutrophils, monocytes, macrophages, eosinophils, mast cells, dendritic cells, and other associated cells identify and elicit protecting responses to invading pathogens through pattern recognition receptors such as toll-like receptors (TLR), nucleotide oligomerization domain (NOD)-like receptors (NLRs), and retinoic acid-inducible gene I (RIG-I)-I-like receptors (RLRs) (64). Antigen-presenting cells (APCs) such as Langerhans cells (LC), macrophages, and dendritic cells play an essential role in connecting the innate immune response to the adaptive immune system (65). LCs are the only APCs that can access the HPV proteins in the epithelial cells of the surface layer (66). HPV transfection of DCs leads to changes in DC migratory pattern and induces cytokine production, which may suppress immune response to viral antigens (67). Macrophages contribute to the clearance of HPV infection through the production of tumor necrosis factor-alpha and nitric oxide-dependent mechanisms (68, 69). Natural killer cells have been associated with viral clearance, elimination of HPV infected cells, and cancer prevention in HPV-related carcinogenesis (70). Although less is known about neutrophils and cervical cancer, recent data demonstrates that high concentrations of neutrophils are strongly associated with poor prognosis and disease progression in women with cervical cancer (71).

5.2 Adaptive immune response

The adaptive immune response is another protective line of host defense against pathogens. It is differentiated into two pathways, the T-helper (Th)1 cell responses that promote cell-mediated immunity and Th2 cell responses that promote humoral immunity (19). During HPV infection, the antigen is taken up by DCs through phagocytosis and migrates to site lymphoid tissues to activate adaptive immunity via the expression of inflammatory cytokines such as IL-1α, IL-1β, IL-6, TNF-α, and IL-12 (59). T lymphocytes (CD4+) recognized HPV antigen presented by mature APCs (Figure 1b) and undergo proliferation/differentiation (Figure 1c), and digest the antigen into shorter peptides or differentiate into effector T cells that interact with naive B cells and produce cytokines to assist in the maturation of B cell responses (Figure 1d) (20). An anti-viral immune response needs a Th1 cell response which secrete cytokines such as IL-2, IL-12, and IFN-γ to activate immune cells, including the naïve CD8+ T-lymphocytes (Figure 1e) that later differentiate into cytotoxic T lymphocytes (CTL) and become effector T-cells that can kill CIN or HPV infected cells (Figure 1f) (59). CTLs, CD4+ T cells and other Th1 responses have been associated with effective clearance of HPV 16 and HPV 18 (72), while the lack of these T lymphocyte lineages has been associated with persistent HPV infection and the development of high-grade disease (73). Furthermore, Th2 cells produce cytokines such as IL-4, IL-6, IL-8 and IL-10 that help B cells differentiate into plasma cells that produce HPV-specific antibodies into circulation (65). Some naive B and T cells transform into HPV-specific memory B and T cells that migrate to the bone marrow to survive as long-lived memory cells and differentiate into plasma cells or activated T cells upon reinfection (49). In contrast, previous studies demonstrated that women with high grade HPV+ cervical lesions had an increased IL-17-associated (Th17) response known to be pro-tumorigenic in HPV-associated cancers (74). Activation and recruitment of Th17 cells is mediated by stromal tumor-associated fibroblasts and tumor-derived chemokines (CXCR3) (74). Taken together, these findings suggest that not all innate and adaptive immune lineages are capable of migrating to sites of infection to kill HPV-infected cells, some actually promote progression of cervical lesions to invasive cervical cancer.

5.3 Evasion of host immune responses by HPV

HPV can deploy various mechanisms to evade host immune responses to establish persistent infections. One of the mechanisms involves altering APC function, including pathogen recognition receptors. High-risk HPV has been suggested to inhibit keratinocyte CCL20 expression, which reduces

the ability of LCs to induce a cytotoxic immune response by compromising LCs infiltration (75). In addition, HPV infection has been shown to affect the differentiation of monocytes into mature DCs, leading to altered functionality of DCs (21). Another evasion mechanism of HPV infection is by inhibiting the recruitment of macrophages and other immune cells into the site of infection by the HPV E6 and E7 proteins. (HPV16 only) (49). Hasan *et al*. (2007) demonstrated that HPV-16 downregulated the expression and function of TLR-9 in human epithelial cells (76). Another high-risk-HPV genotype (HPV-18) has been associated with suppression of the cyclic guanosine monophosphate-adenosine monophosphate synthase, which plays a role in activating type I interferon genes and production of inflammatory cytokine response (77). The interferons play a role in inhibiting viral replication in the host cells and activating immune cells that can eradicate the infected cells (39). More studies on the interaction between HPV proteins and type I IFN response remain critical.

The virus is also able to escape adaptive immune responses. Although the mechanisms by which HPV can evade the host immune system remain unclear, HPV proteins and regulatory cytokines have been suggested as contributors (78). HPV16 E5 and E7 have been reported to reduce expression of interferons and HLA class-1 molecules, resulting in a lack of CTL response against HPV (49). In addition, studies have reported high concentrations of an anti-inflammatory cytokine (IL-10) in women with persistent HPV infection compared to those who cleared the HPV infection. Similarly, increased levels of immunosuppressive cells such as transforming growth factor-beta (TGF-β)-producing Tregs have been observed in cervical tissues and vulval intraepithelial neoplasia (VIN) lesions. The increased concentrations of IL-10 and TGF-β in tissues may indirectly undermine T cell function by limiting the ability of APCs to promote $CD4^+$ T cell differentiation and proliferation, thereby modulating the adaptive immune responses (59). Another mechanism by which CD4+ and CD8+ T cell function is inhibited involves downregulation of surface MHC I expression and the impairment of APC trafficking and maturation (79-81). Collectively, these finding suggest that APC and T cell response downregulated by immunosuppressive immune response may result in HPV-immune evasion, and consequently lead to persistent HPV infection and the development of high-grade disease

6. Association between genital inflammation and HPV

Inflammation is defined as the natural immune response following injury or infection (82, 83). It is generally characterised by elevated levels of cellular markers and pro-inflammatory cytokines in the

231 genital tract (84). Host defense mechanisms, including immune mediators in the female genital tract
232 microenvironment, play a role in the clearance and persistence of HPV and the risk of developing
233 cervical cancer (6-8, 10, 85, 86).

234

235 Contradicting data have been reported between HPV and altered cytokine milieu profiles (7, 8, 12).
236 Łaniewski *et al*. (2018) showed a strong association between invasive cervical carcinoma and elevated
237 concentrations of TNF-α, TNF-β, MIP-1α, GMCSF, and IL-10 (7). In addition, HPV persistence was
238 associated with chemokine MIP-1α and growth factor GM-CSF that play a significant role in activation
239 and recruitment granulocytes (87). Another study showed that women with elevated concentrations of
240 mucosal cytokine interleukin (IL)-10, IL-12, macrophage inflammatory protein (MIP)-1α and TNF-α
241 were less likely to clear any HPV type while low levels of these cytokines (including IL-8) correlated
242 with HPV clearance (12). A positive correlation between elevated concentrations of several cytokines
243 (IL-36γ, MIP-1β, RANTES, IP-10, IL-2, IL-4, Flt-3L, sCD40L) and invasive cervical cancer
244 carcinoma in women with BV were reported (7). Increased mucosal cytokine profiles in the
245 reproductive tract of women infected with HPV was also associated with HPV prevalence, clearance,
246 acquisition, persistence and increased HIV acquisition risk (8). The apparent association between HPV
247 infection and genital cytokine responses may likely indicate the role of cellular immunity to control
248 HPV infection. Moscicki *et al*. (2020) found that 9 of the 13 cytokines (IL-4, IL-5, IL-10, IL-12, IL-
249 13, IFN-γ, IFN-2α, MIP-1α, and TNF-1α) tested were elevated after the clearance of HPV infection
250 compared to prior visits (88). In contrast, two studies did not show the relation between increased
251 concentrations of pro inflammatory cytokines and HPV acquisition or clearance (89, 90).
252 Understandably, while there are no structural conformations on host cells during HPV invasion, HPV
253 also maintains the anti-inflammatory state likely by avoiding the host immunity through disruption of
254 the interplay between infected cells and effector cells (91). There is a need to better understand the
255 cellular or other factors associated with the cytokines in the different HPV status categories.

256

257 **7. Association between the vaginal microbiome and HPV infection**

258 The vagina and ectocervix are dominated by lactic-acid producing bacteria and cervicovaginal fluids
259 that act as a lubricant that traps invading pathogens (92, 93). A genital environment dominated by
260 *Lactobacillus* spp. has been associated with optimal pregnancy outcomes, lack of abnormal vaginal
261 symptoms and urogenital disease, and reduced risk for several STIs, including HPV and HIV (94). In

contrast, the opposite is observed in the genital tract dominated by non-optimal vaginal microbiota. Several *Lactobacillus spp.* have been described in the non-BV state, although the most frequent and abundant are *L. crispatus, L. gasseri,*and *L. jensenii,* (95, 96). *L. gasseri* enriched microbiome was also associated with rapid rates of HPV clearance (97). A recent study showed that women with *L.crispatus* enriched microbiome were less likely to have prevalent high-risk HPV infection than women with overgrowth of pathogenic microorganisms such as those linked with BV (*Gardnerella, Atopobium,* and *Prevotella*) (98). Furthermore, *L.crispatus* abundance in the genital tract was also associated with HPV clearance, suggesting the relative association between clearance and *L. crispatus* (98).

BV is a condition characterised by a shift in vaginal microbiota from *Lactobacillus* dominant towards more diverse bacteria, including strict and facultative anaerobes such as *Gardnerella, Prevotella*, and *Sneathia*, often resulting in vaginitis and discharge (98, 99). BV has been associated with elevated levels of cytokines and cellular biomarkers of inflammation associated with increased HIV acquisition risk (99, 100). Furthermore, numerous studies have suggested a link between BV and other STIs such as *Chlamydia trachomatis, Neisseria gonorrhea*, and cervical HPV (97, 101). The ulcerative and highly inflammatory sequelae caused by these infections provide biologically plausible mechanisms supporting a possible increased susceptibility to HPV among co-infected individuals. A high relative abundance of *Gardnerella* and *Atopobium vaginae* were associated with CIN (102, 103). Increased abundance of *Sneathia*, *Atopobium,* and *Gardnerella* is associated with incident high-risk HPV infection (104). A study by Lee *et al*. (2013) showed that HPV-infected women had lower *Lactobacillus* species and increased *Fusobacteria* and *Sneathia* compared to HPV-uninfected women (92). In agreement with these findings, longitudinal analysis from 32 sexually active women showed that a low *Lactobacillus* community with high proportions of the genera *Atopobium* species were associated with the low rate of HPV clearance (105). While existing evidence suggests the association between HPV infection and genital dysbiosis, justifiable concerns that positive associations merely reflect residual confounding by unmeasured sexual risk behaviours (such as engaging in condomless sex and having sex with uncircumcised partner) still exists (106). Thus, further research investigating this interplay is warranted. Detailed understanding of the genital microbial composition and structure in women with HPV infection may help identify the causal connections between microbiota, HPV infection, and cervical cancer.

8. Association between HPV and HIV

8.1 HPV infection and HIV risk

Epidemiological and meta-analyses data suggest that sexually transmitted infections like herpes, gonorrhoea, chlamydia, and syphilis can increase the risk of HIV acquisition (107, 108). Other biological correlates of HIV risk that have been described to date is mucosal pro-inflammatory cytokines and BV-associated bacteria that, if altered prior to infection, were associated with higher risk of acquiring HIV (35, 109, 110). However, less is known about the mechanism by which HPV infection might increase HIV acquisition risk.

In addition to its role as a biological factor in the development of anogenital cancers, HPV may also be an important co-factor in the increased risk of HIV acquisition in women. Overall, HIV infection risk doubled in women with prevalent HPV infection, with either oncogenic or non-oncogenic HPV genotypes (111). Another study showed a significant association between high-risk HPV genotypes and HIV acquisition (112). Persistent HPV infection has been associated with an increased biomarkers of HIV acquisition, and the causal link is still not well-understood (113). Higher frequency of the CD4+ T cells in the stroma and epithelium are closely associated with HPV lesion regression (114) and consequently increasing mucosal HIV target cell frequency and activation (115). In addition, pro-inflammatory cytokines essential for HPV clearance are also known to increased risk of HIV acquisition (8, 110). Further research is still needed to explore the association between HPV and HIV, and validate HPV as a potential risk factor for HIV acquisition, and if found to be true, these may highlight the importance of decreasing HPV burden in settings with high prevalence to curb HIV infections.

8.2 HPV infection in women living with HIV

HIV infection has also been associated with the development of CIN2, CIN3, and invasive cervical carcinoma in HPV infected population (43). Although multiple types of HPV have been associated with HIV infection, HPV16 is the commonest cause of cervical carcinoma in HIV infected population (60). Studies have reported that women living with HIV (WLWH) are more likely to be infected with high-risk HPV and multiple HPV genotypes, resulting in the development of pre-invasive lesions that, if left untreated, can develop into invasive cervical cancer (91, 111). WLWH have up to five times more cervical cancer than HIV-uninfected (111). In two large prospective studies that assessed the

prevalence of HPV genotypes in women living in the United States, HPV 6 or 11 was 3.6 and 5.6 times high in WLWH compared to their HIV-uninfected counterparts, respectively (55, 116). Furthermore, patients living with HIV were at increased risk of cervical abnormalities and onward HPV transmission due to high prevalence of high HPV viral load (112). Given the growing evidence of an increased risk of cervical cancer in WLWH, regular HPV screening and possibly treatment for cervical cancer is needed to effectively control HPV and its adverse sequelae.

9. Conclusion

While there is substantial progress in increasing vaccine access and immunization coverage, young women, particularly in sub-Saharan African remain disproportionately infected by HPV. Factors that may render women more vulnerable to HPV infection have not been fully characterized. This review showed that the vaginal microbiome, cellular, and cytokine markers of inflammation are some of the biological markers that are associated with neoplastic disease in cervical carcinogenesis. While significant progress has been made in understanding how HPV evades immunity, mechanistic studies on how risk factors influence host-mucosal microenvironment and viral persistence are warranted. The role of other biological risk factors such as intravaginal practices are less studied and could be important drivers of HPV risk in young women. There remains a need to conduct more preclinical models to understand how biological risk factors might block efficient HPV clearance from the mucosa and pave the way for cervical cancer.

Conflict of interest: The authors have declared that no conflict of interests exists.

Author contribution: All authors contributed to the article and approved the submitted version.

Ethics statement: No ethical approval was required as this is a review article with no original research data.

Funding

This review was conducted as part of the DST-NRF Centre of Excellence (CoE) in HIV Prevention, supported by the Department of Science and Innovation and the National Research Foundation (grant 96354). LN was funded by DST-NRF CoE in HIV Prevention (grant 96354) and Poliomyelitis Research Foundation Research Bursary (bursary# 21/73). SN was supported by Columbia University-Southern African Fogarty AITRP Programme (grant# D43TW00231), National Research Fund Thuthuka Research Grant (grant# TTK160510164586), and Poliomyelitis Research Foundation Research Grant (grant# 16/17). GM was funded by Poliomyelitis Research Foundation Research Grant (grant# 18/16) and National Research Foundation Grant (grant# SFP180507326699). AM was supported by National Research Foundation Grant (grant# PDG210309589501)

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