UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

Characterisation of genital and systemic biomarkers of cervical HPV viral load in women at risk of cervical intra-epithelial neoplasia.

By

YANGA MDLELENI (210517129)

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (Medicine) in the Discipline of Virology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences

University of KwaZulu-Natal

South Africa

UNIVERSITY OF KWAZULU-NATAL ™
INYUVESI YAKWAZULU-NATALI

HPP
HIV Pathogenesis Programme
Doris Duke Medical Research Institute
Nelson R Mandela School of Medicine

DECLARATION

I, YANGA MDLELENI, declare that;

1. The research reported in this thesis is my original research, except where indicated otherwise.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs, or other information, unless specifically acknowledged and referenced as being sourced from other persons.
4. This thesis does not contain other persons' writing unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

    a. Their words have been rewritten, but the general information attributed to them has been referenced.

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

5. This thesis does not contain text, graphics, or tables copied and pasted from the Internet, unless specifically acknowledged, and the source is detailed in the thesis and in the References sections.

YANGA MDLELENI (Candidate)

[redacted]

.....

Dated this, 18th day July of 2024

As the candidate's supervisor(s) we agree to the submission of this thesis

DR. BONGIWE NDLOVU (Supervisor)

[redacted]

.....

DR DANIEL MUEMA (Co-supervisor)

[redacted]

.....

DR. WENDY DHLOMO (Co-supervisor)

[redacted]

CONFERENCE CONTRIBUTIONS

Presentation details

Yanga Mdleleni, Wendy Dhlomo, Sam Rasehlo, Thumbi Ndung'u, Bongiwe Goodness Ndlovu, Daniel Muema. **The relationship between vaginal and peripheral cytokine profiles and HPV viral load in women living with HIV before and after treatment for Cervical Intra-epithelial Neoplasia**. International Center for Genetic Engineering and Biotechnology, Oral Presentation, Trieste, Italy, 2024

Yanga Mdleleni, Wendy Dhlomo, Mosa Moshabela, Bongiwe Goodness Ndlovu, Alltalents T Murahwa, Anna-Lise Williamson, Polycarp Mogeni, Daniel Muema, and Thumbi Ndung'u. **The role of high-risk HPV viral load in women with CIN recurrence after excisional treatment.** 35th International Papillomavirus Conference, Washington, D.C, USA. April 2023

Yanga Mdleleni, Wendy Dhlomo, Sam Rasehlo, Thumbi Ndung'u, Bongiwe Goodness Ndlovu, and Daniel Muema. **The relationship between vaginal and peripheral cytokine profiles and HPV viral load in women living with HIV before and after treatment for Cervical Intra-epithelial Neoplasia.** Bill & Melinda Gates Foundation's sponsored CAVD program, 9th Norman L. Letvin Early Career Investigator Meeting. Oral presentation, March 2023

Yanga Mdleleni, Wendy Dhlomo, Mosa Moshabela, Murahwa, Polycarp Mogeni, Thumbi Ndung'u, Bongiwe Goodness Ndlovu, and Daniel Muema. **The role of high-risk HPV infection in the recurrence of CIN in women living with HIV.** Sub-Saharan African Network for TB/HIV Research Excellence (SANTHE) South African research day-Oral presentation, June 2022

Yanga Mdleleni, Wendy Dhlomo , Sam Rasehlo, Thumbi Ndung'u, Bongiwe Goodness Ndlovu and Daniel Muema. **Differences in CD4+ and CD8+ T cell responses to HPV 16 and HPV 18 E6 and E7 peptides in HIV-HPV co-infected women treated for cervical intraepithelial neoplasia.** Sub-Saharan African Network for TB/HIV Research Excellence (SANTHE) Annual consortium meeting. Nairobi- Oral presentation, October 2022

ABSTRACT

Cervical cancer is the fourth most common cancer worldwide and the second most common among South African women. Over 90% of all cervical cancer cases and mortalities occur in low- and middle-income countries (LMICs). The persistence of infection with high-risk Human papillomaviruses (HPV) drives the development and progression of cervical intraepithelial neoplasia (CIN), a precursor for cervical cancer. Women living with HIV (WLWH) have a higher incidence of CIN and cervical cancer and experience higher rates of CIN recurrence after treatment when compared with HIV-negative women. Effective intervention against HPV and CIN in WLWH will require a thorough understanding of how virological parameters and host immune responses, which comprises the role of T-cell responses and the resultant cytokines profiles, impact their viral clearance and CIN regression, progression, and recurrence. Such information will also guide on the biomarkers that can be used to predict or monitor their treatment outcomes. However, there is a paucity of information on virological and immunological basis for CIN recurrence in this population. Therefore, we aimed to characterize the virological and immune biomarkers of HPV-associated abnormalities and determine their relationship with CIN treatment outcomes in 96 women living with HIV. The study participants were between the ages of 18-65 years old and were recruited from the King Edward's Hospital in Durban, South Africa, from April 2012 to April 2015. All participants presented with abnormal pap smears at enrolment, required colposcopy, and were treated for CIN by Large Loop Excision of the Transformation Zone (LLETZ). Participants were subsequently followed up at six-month intervals for a year after treatment. For these analyses, they were then stratified into “No-recurrence” and “Recurrence” groups based on clinical CIN detection at either six months or one year post-treatment, with 69 participants being in the “No-recurrence” group and 27 being in the “Recurrence” group. Genital and blood samples were collected at study entry, 6 months, and 1 year after treatment. Virological assays on the number of HPV types and HPV viral loads were done on the cervicovaginal lavage (CVL) and cytobrush samples, respectively, by conducting HPV genotyping at baseline using Roche Linear array and then quantifying high-risk HPV viral load at all study visits using Hybrid Capture II (HCII) assay. Immune correlates of CIN recurrence were assessed by measuring anti-HPV IFN-γ+ CD4+ and CD8+ T cell

responses in peripheral blood mononuclear cells of 28 women (16 in the "No-recurrence" group and 12 in the "Recurrence" group) using intracellular cytokine staining at baseline and 1-year post-treatment. Genital and peripheral cytokine responses were also measured in serum and CVL of 39 women (20 in the "No-recurrence" group and 19 in the "Recurrence" group) using a multiplexed Enzyme-Linked Immunosorbent Assay (ELISA).

Women who experienced CIN recurrence after excisional surgical treatment had higher numbers of high-risk HPV types at enrolment ($p=0.008$) and higher high-risk HPV viral loads at all time points ($p<0.001$). Notably, the combined presence of both high-risk HPV types and high HPV viral load was the best predictor for CIN recurrence in a multivariable regression model (odds ratio 1.985, 95% CI 1.020 to 3.865, $p=0.044$).

There were no differences in T cell responses between the two groups, suggesting minimal impact of naturally elicited systemic anti-HPV T-cell responses on treatment success. Interestingly, the frequencies of IFN-γ+ producing CD8 T cells to the E7 oncoprotein at the pre-treatment time point were significantly higher in women with CIN 3 when compared to those with CIN 2 ($p=0.033$) and had a trend for a positive correlation with HPV viral loads (Rho= 0.38, $p=0.08$), suggesting the dependence of naturally elicited pre-treatment anti-HPV CD8 T-cell responses on antigen load. Our study also revealed stable kinetics of the anti-HPV16 and anti-HPV18 E6- and E7-specific T cell immunity post-treatment in blood for both "No-recurrence" and "Recurrence" groups.

Cytokine profiles were mostly similar between the two groups with a few exceptions. The Recurrence group had higher baseline levels for CVL Eotaxin as well as lower baseline plasma levels of IL-2, IL-12, IL-13 and IL-17 when compared to the No-recurrence group. The kinetics of the plasma cytokines also differed between the two groups, with the Recurrence group showing an increase in IL-2, IL-13, IL-7 and IL-17 whereas the No-recurrence group had a decrease in IL-13 and IL-12. There were notable inverse associations between HR-HPV viral loads and some CVL Th2 cytokines at the post-treatment timepoint, namely IL-4 and IL-10, suggesting that absence of Th2 skewing could be associated with successful post-treatment viral control. On the other hand, in plasma, positive correlations were observed between HR-HPV viral loads and cytokines from all classes, including IL-2, IL-4, and IL-16, suggesting association of

viral persistence with general systemic inflammation regardless of Th1/Th2 skewing. Notably, the kinetics of cytokines after treatment differed between treatment outcomes. IL-15 and VEGF in CVL declined exclusively in the Recurrence group, while IL-2, IL-13, IL-17, and IL-7 levels in plasma increased in the Recurrence group and not the No-recurrence group.

These data reinforce the potential role of combining HPV genotyping, HPV viral load quantification, and holistic immune response assessment, comprising both systemic and local immunity, as prognostic tools in identifying women at the highest risk of CIN recurrence after treatment, especially for women living with HIV. The study also sheds light on the potential role of viral persistence and immune modulation in CIN recurrence.

DEDICATION

I dedicate this work to all the Black women who have paved a way for me to be here.
I also dedicate this journey to my support system:

To my late uncle Xolani Mdleleni, you made me feel like the smartest girl in the whole world! Thank you for having such big dreams and speaking success into my life with so much pride and love. Your words linger in my heart and have carried me through the hardest of times. Your journey brought me to this field- this became our journey together, and I carry you with me to all the places around the world that you said I would go.

To my late great-grandmother Manguta, Lulama Rose Mdleleni, the angel who taught me the importance of education. Your prayers and love carried me through this journey; I feel your presence in everything I do. Thank you for the letters you wrote me. They are my fuel in dark times. Ndiyabulela Nguta wam ngemithandazo yakho.

A special thank you to my mother, Bulelwa Mdleleni. Thank you for walking this journey with me. For wiping my tears, praying for me, and always encouraging me to press on. I appreciate you and you have raised a Dr. This is all you momma, ndiyabulela!

My ML*, none of this would have happened without you. Thank you for being patient with me throughout the ups and downs. This was a journey of transformation, one about me reconnecting with my true self, trusting my voice and standing firmly in what in believe. Thank you for bringing me back home, to myself. Thank you for always having difficult conversations with me, for always holding me to account, and most importantly teaching me to trust myself as a scientist and to carve my scientific path, while guiding others to do the same. We did it.

To Dr Nonhlanhla Kaula, you have been my pillar of strength throughout these years. I will never cease to thank God for our friendship/sisterhood.

To my siblings, Nwabisa and Sisipho Mdleleni, my niece Liyema Zoey Mdleleni, my grandmother Nozipho Malinzi, My great grandma Thandiwe Magobhiyane, rest in peace my angel. To my entire family and friends, thank you for your love and support.

ACKNOWLEDGEMENT

Firstly, I would like to thank the Lord almighty for the blessing, opportunity, and courage to pursue and complete this degree. My journey was met with great challenges but through God's grace, I grew both as an individual and a passionate scientist.

Secondly, I would like to thank my supervisor, Dr Wendy Dhlomo. Thank you for always believing in me and for affording me this wonderful opportunity to learn from you. Thank you for taking me in as your younger sister and friend. I could not have done this without you. To Dr Daniel Muema, thank you for always encouraging me, for all you have taught me, and for your patience throughout this journey. You have carried me through so many dark times in this journey and I thank the Lord for you. You are a wonderful supervisor! Thank you to Dr Bongiwe Ndlovu for all you have done to help me complete this degree. I appreciate you. Thank you for support.

I would like to also acknowledge Prof Thumbi Ndung'u for the opportunity to work in this cohort. I am grateful for guidance, support, career advice, and opportunities.

A special thank you to Prof Mosa Moshabela. Thank you for the opportunities you have given me, for always coaching me, for your patience, and for all you teach me. I know no better mentor.

Distinctively, I treasure my wonderful mother Bulelwa Mdleleni, late-great grandmother Manguta Lulama Rose Mdleleni, grandmother Nozipho Malinzi, my siblings Sisipho and Nwabisa Mdleleni, and my lovely niece Zoey Liyema Mdleleni and my friends. Thank you for all your prayers and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

THESIS STRUCTURE

The thesis is presented in the following order:

CHAPTER 1: Introduction and Literature Review

This chapter provides a framework for the thesis by giving an overview of the Human Papillomavirus (HPV) prevalence, pathogenesis, and understanding of the role of HPV in the formation and progression of precancerous lesions. It also gives information on the global and regional HPV-disease burden in women living with HIV, especially those residing in low- and middle-income countries. It also provides the hypotheses, aims, and objectives of the work.

CHAPTER 2: Materials and Methods

This chapter described the demographic information of the study participants, treatment of women with CIN, sample collection and processing, as well as detailed laboratory experiments.

CHAPTER 3: High viral load for high-risk HPV genotypes is associated with the recurrence of cervical intraepithelial neoplasia after excisional treatment in women living with HIV.

This chapter provides an understanding of the role that different HPV types and HPV viral load play in the development of precancerous lesions in women living with HIV. This chapter is the main focus of the thesis, written as a developing manuscript that shows how high-risk HPV and their viral quantity are strong drivers of cervical intraepithelial neoplasia development and recurrence after excisional treatment in South African women living with HIV. This was done by genotyping HPV types using linear array HPV genotyping assay and also quantifying high-risk HPV genotypes using hybrid capture II at baseline, 6 months, and 1 year after CIN excisional treatment.

CHAPTER 4: The Relationship between anti-HPV T cell responses, high-risk HPV viral load, and treatment outcome in women living with HIV undergoing excisional treatment for cervical intraepithelial neoplasia

This chapter is a developing manuscript that aims to provide insight into the role of cellular immunity in the recurrence of CIN. Furthermore, it explains the role of CD4+ and CD8+ T cells in the recurrence of CIN after excisional treatment in women living with HIV. This was achieved through intra-cytokine stimulation of HPV 16 and HPV 18 E6 and E7 in the plasma of women living with HIV before and 1 year after CIN excisional treatment.

CHAPTER 5: The relationship between cytokine profiles, treatment outcome, and high-risk HPV viral load in women living with HIV undergoing excisional treatment for cervical intraepithelial neoplasia

This chapter explores the role of cytokines as biomarkers of CIN recurrence in women before and 1 year after CIN excisional treatment. This was done by measuring 27 cytokines in the genital tract and plasma using a commercially available multiplexed ELISA assay at baseline and 1 year after CIN excisional treatment.

CHAPTER 6: Synthesis and Conclusion

This chapter provides an overall summary of the thesis chapters and explains how high-risk HPV types and their viral loads influence cellular immunity and the cytokine released in order to drive successful CIN treatment. This chapter also sheds light on future research interests that are inspired by our study findings.

CHAPTER 7: Appendix

This chapter describes additional information on the preparation of buffers, including tables showing how reagents were prepared.

ABBREVIATIONS

Description	Abbreviation
Acquired Immune Deficiency Syndrome	AIDS
Antiretroviral Therapy	ART
Adenosine Triphosphate	ATP
Antigen Presenting Cells	APCs
Biomedical Research Ethics Committee	BREC
Brefeldin A	BFA
Caspase 8	CASP8
Cervical Intraepithelial Neoplasia	CIN
Coefficients of variation	%CV
Cell Mediated Immunity	CMI
Cervicovaginal Lavage	CVL
Cluster of Differentiation 4	CD4
Cluster of Differentiation 8	CD8
cyclin-dependent kinase inhibitor	p16INK
Cytotoxic T lymphocytes	CTLs
Deoxyribonucleic Acid	DNA
Dendritic Cells	DC
Dimethyl sulfoxide	DMSO
Early region 6	E6
Early region 7	E7
Early region 2	E2
Early region 4	E4
Early region 5	E5
Enzyme-Linked Immunosorbent Assay	ELISA
Epidermal Growth Factor	EGF
Epidermodysplasia verruciformis	EV
Fas-associated via Death Domain	FADD
Fluorescence-activated cell sorting	FACS
Heparin Sulfate Proteoglycans	HSPGs
High-grade Squamous Intra-epithelial lesions	HGSIL
High-risk Human Papillomavirus	HR-HPV
High Income Countries	HICs
HPV Clearance and Treatment outcomes of Cervical High-grade Squamous Intra-epithelial lesions cohort	HATCH cohort
Human immunodeficiency virus	HIV
Human Papillomavirus	HPV
Human telomerase reverse transcriptase	hTERT
Hybrid Capture II	HCII
Immunoglobulin	Ig
International Agency for Research on Cancer	IARC
Intra-Cytokine Staining	ICS
Interferon	IFN
Interleukin	IL

Laboratory Information Management System	LIMS
Late region	L region
Large Loop Excision of the Transformation Zone	LLETZ
Langerhans cells	LCs
Long control region	LCR
Loop Electrosurgical Procedure	LEEP
Low and Middle-Income Countries	LMICs
Low-grade Squamous Intra-epithelial lesions	LGSIL
Low-risk Human Papillomavirus	LR-HPV
Master Mix	MMX
Major Histocompatibility Complex	MHC
Monocyte Chemotactic Protein-1	MCP-1
Multiplexed Enzyme-Linked Immunosorbent Assay	ELISA
Natural Killer Cells	NKCs
Nuclear factor-κB	NF-κB
Open-Reading Frames (ORFs)	ORFs
Peripheral blood mononuclear cell	PBMC
Platelet-derived growth factor	PDGF
Polymerase chain reaction	PCR
Receiver operator characteristic	ROC
Relative light units per cut-off	RLU/CO
Regulatory T cells	Tregs
Revolutions Per Minute	RPM
Ribonucleic Acid	RNA
Roswell Park Memorial Institute	RPMI
Sexually Transmitted Infection	STI
Streptavidin-PE	SA-PE
Sub-Saharan Africa	SSA
Toll-Like Receptor	TLR
Trans-activator of transcription	Tat
Transformation Zone	TZ
Tumour protein P53	p53
Tumour Suppressor Protein Retinoblastoma	Prb
Tumour necrosis Factor	TNF
Virus-Like Particles	VLPs
World Health Organization	WHO
Women living with HIV	WLWH

Chapter 1: Introduction and Literature Review

1.1. Human Papillomavirus

Amongst all cancers observed in humans, 15% are caused by viral infections, including Human Papillomavirus (HPV) [1]. HPV is a common sexually transmitted infection (STI) that is classified into carcinogenic or High-risk (HR-HPV) as well as non-malignant Low-risk (LR-HPV) types, with a tropism for squamous epithelial cells of the skin, vaginal, penile, anal, and oropharyngeal region [2]. The causal role of HPV in the development of oropharyngeal and anogenital cancers has been firmly established both epidemiologically and biologically [3]**.** Moreover, the prevalence of HPV and the HPV-associated burden of the disease vary significantly across the globe. Currently, the global HPV prevalence is approximately 12%, while Sub-Saharan Africa (SSA) has the highest prevalence (24%), followed by Eastern Europe (21%), which contrasts with the low prevalence observed in North Africa (9%), and Western Asia (2%) [4, 5].

These variations are attributed to numerous factors, including geographic location, cultural practices, behaviour, socioeconomic status, genetic factors related to the variability of the HPV genome, age, gender, site of infection, as well as individual health status [6, 7]. Young women ≤25 years of age bear a disproportionately higher prevalence of HPV infection soon after sexual debut, with over 24 million active cases and 5.5 million new cases each year globally [8-10]. In South Africa, the prevalence of HR-HPV and LR-HPV among adolescents and young women with normal cytology ranges between 85% and 44%, respectively [11]. This decreases with increasing age in women [12]. A consequence of HR-HPV infection, especially if they are persistent, is the later development of pre-cancerous lesions and even cervical cancer, whose incidences increase among women ≥30 years of age [12-14]. The prevalence of HPV is also high and varied among men, ranging between 12.5% among those aged 14-19 years and over 48% in males >25 years of age [15, 16]. Men serve as reservoirs for HPV transmission to women, therefore indirectly contributing to the incidence of cervical cancer in women [17-19].

Approximately 80% of all sexually active people will become infected by a type of HPV at least once in their lifetime [20, 21]. Up to 90% of all HPV infections are transient and spontaneously clear within

18-24 months [22]. Although this phase is referred to as clearance, which implies that infection is undetectable by HPV genotyping analyses, it could also be that HPV remains in its latent phase [23] (Figure 1.1). In some individuals, persistence of HPV infection leads to the development of low-grade squamous epithelial lesions that are known as cervical intraepithelial neoplasia (CIN) 1, which can regress spontaneously without treatment. In instances where CIN1 does not regress, the infection progresses to CIN2 and subsequently CNI3. If the infection is not treated, it can progress to invasive cervical cancer (Figure 1.1). The duration of the process of HPV infection to the onset of cervical cancer can take up to 10 years. The persistence of HR-HPV types, particularly HPV-16 and HPV-18, is the etiological agent of over 90% of all cervical cancer cases, 70% of vulvovaginal, 60% of penile, 90% of anal, and 70% of oropharyngeal cancers, and an etiological agent of benign tumours in both men and women worldwide [24-26].

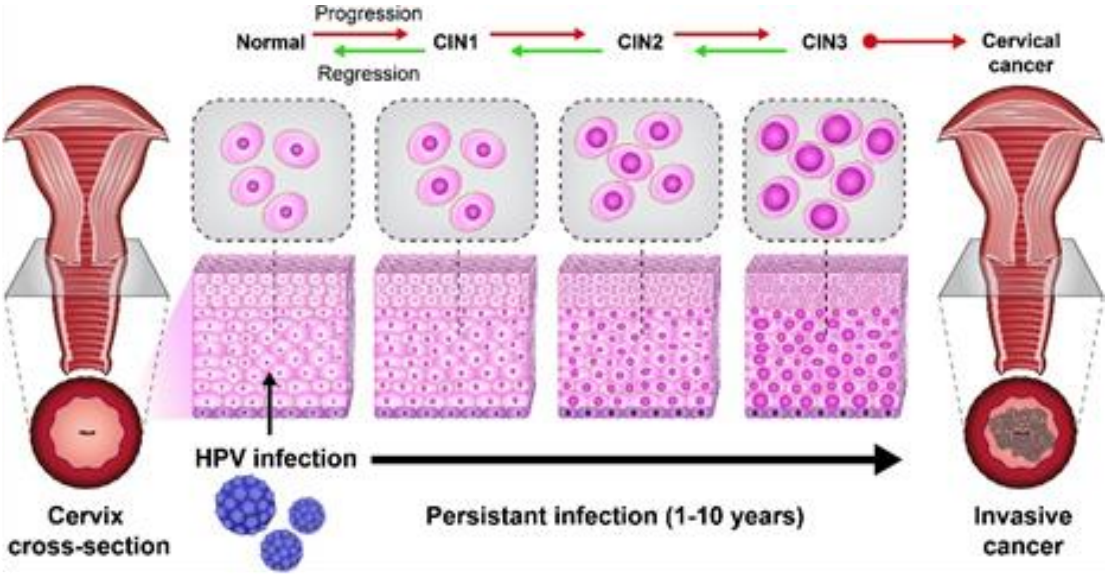

Figure 1.1. Schematic model of the natural history of high-risk Human papillomavirus infection [27]. The diagram shows progression of HPV infection from CIN1 to the cervical cancer development, or regression from various CIN levels to the normal cytology.

1.2. Cervical cancer and HIV

Cervical cancer is the fourth most common cancer worldwide and the second most common among South African women [28]. In 2021, there were over 604,000 cases of cervical cancer and 342,000 cervical cancer-related deaths reported globally, over 90% of which occurred in LMICs [29, 30]. In the African region, approximately 117,316 new cases and 76,645 deaths were reported in 2020, of which 10,702 cases and 5,870 cervical cancer-related deaths were reported in South Africa alone [31-33]. Also, a major public health concern, especially in LMICs, is the Human Immunodeficiency virus (HIV). Globally in 2020, an estimated 38 million people were living with HIV, with approximately 1.8 million new infections occurring annually. Sub-Saharan Africa (SSA) contributes roughly 71% of the total global HIV epidemic [34-36]. Young women and adolescent girls aged 18-24 years have an eight-fold higher HIV prevalence compared to their male counterparts [34, 37-39]. Similarly, this is also the age group in which HPV infection is the highest in SSA countries, particularly South Africa [40]. HPV and HIV are both sexually transmitted infections that result in complex interactions during co-infection. Previous studies reported that the risk of HPV infection, infection with multiple HPV types, and persistent HR-HPV infections are significantly higher in WLWH) [41-46]. This may be explained by HIV-induced immune suppression, especially among women who are not on antiretroviral therapy (ART), which further permits uncontrolled papillomaviral replication and multiple-site infections [47, 48].

Both cervical cancer and HIV infection present a significant public health burden in SSA. The incidence of cervical cancer is approximately six-fold higher among WLWH compared to healthy HIV-negative women [49, 50]. SSA countries such as South Africa, Eswatini, Zimbabwe, Malawi, Tanzania, and Uganda account for the highest cervical cancer cases reported among WLWH [50, 51]. In South Africa alone, the incidence of cervical cancer is estimated to be 506 per 10,000 person-years [11, 41]. Hence, cervical cancer is classified as an Acquired immunodeficiency syndrome (AIDS)-defining illness [52, 53]. Notably, CIN risk among HIV infected women increases with decreasing CD4+ T cell count and an upsurge in HIV viral load [54]. These lesions are treated by surgical excision; nevertheless, recurrence and treatment failure occur 2.7-fold more commonly in WLWH when compared to HIV

negative women [55]. Furthermore, there are controversial reports on the effects of ART on HPV infections and disease progression, particularly on HPV 16 [55, 56]. A report by Kelly et al indicated that early ART could reduce incidence of HPV-associated cervical lesions and cancer, possibly as a result of immune restoration and optimal HIV control [57]. However, although ART increases the lifespan of WLWH, their risk of cervical abnormalities still remains exacerbated, especially in LMICs [58, 59]. This, therefore, highlights the question of whether ART fully restores the cellular immunity required for the regression of HPV-induced malignancy in WLWH. Data in this area warrants further exploration.

1.3. Definition of Human Papillomavirus Genome

Human papillomaviruses are heterogeneous, non-encapsulated double-stranded circular DNA viruses of about 8kb in size [60, 61]. HPVs encompass over 180 genotypes, 40 of which cause mucosal epithelial infections. These viruses are further classified into high-risk, probable, or low-risk types based on their oncogenic potential. Despite their heterogeneity, papillomaviruses all consist of an analogous genome structure and organization, with eight open-reading frames (ORFs) across three main regions: the early region (E), late region (L), and the long control region (LCR), as demonstrated in Figure 1.2 [62, 63]. The E region consists of 6 viral proteins: E1, E2, E4, E5, E6, and E7, while the late region encodes the major capsid (L1) and the minor capsid (L2) structural proteins, whereas the long control region contains the origin and transcription factor-binding sites [64, 65]. The HPV genome is highly conserved throughout Papillomaviridae, with mutations occurring subsequent to viral DNA integration within the host genome [66, 67].

HPV E1 and E2 are regulatory proteins that bind to the replication origin site and initiate viral replication and transcription [68, 69]. Additionally, E1 proteins function as Adenosine Triphosphate (ATP)-dependent helicases, responsible for the separation of double-stranded viral DNA strands in preparation for viral replication, while E2 is an important regulator of viral transcription and maintains genome replication [70]. The E4 protein plays an important role in genome amplification, genome packaging, and viral synthesis through interaction with Ribonucleic acid (RNA) Helicase, while also facilitating viral release and transmission [64]. Viral proteins E5, E6, and E7 are important instigators

of immune evasion, cellular transformation, and proliferation [71]. The viral E5 protein facilitates uncontrolled, continuous host cell proliferation while inhibiting differentiation [64]. Furthermore, the E5 protein interacts with the Epidermal Growth Factor (EGF) receptors and activates Platelet-derived growth factor (PDGF) receptor, resulting in modification of signalling pathways, immune evasion, and impaired apoptosis [72-74]. E6 and E7 are classified as oncoproteins due to their role in the disruption of cell cycle regulation in the upper epithelial cells, resulting in malignant transformations in high-risk HPV types, while causing benign lesions during low-risk HPV infections [75]. The E7 protein is responsible for the degradation of the tumour suppressor protein Retinoblastoma (pRB) while on the other hand, E6 is responsible for the degradation of tumour suppressor protein p53, overexpression of tumour suppressor protein p16, as well as activation of telomerase by means of Human telomerase reverse transcriptase (hTERT) expression [64, 76, 77]. Consequently, the collaborative effects of E6 and E7 oncoproteins override cell cycle modulation, allow chromosomal instability and dysregulated DNA replication in non-cycling cells, while also promoting apoptosis resistance, which may result in the development of pre-cancerous lesions and even cancer [70, 71, 78].

Both the major capsid (L1) and the minor capsid (L2) proteins are expressed on terminally differentiated squamous epithelial cells [79, 80]. The L1 region forms the majority of the capsid and can self-assemble into empty virus-like particles (VLPs), and it plays an important role in viral assembly [79, 80]. Additionally, the L1 capsid is the most conserved region of the HPV genome, and it contains pairwise nucleotide sequences used to classify different HPV genotypes. A new HPV type can be identified as such if the complete genome has been sequenced and its L1 ORF nucleotide sequence differs by more than 10% from the closest known type [67, 79]. L2 alone lacks the capacity to form VLPs and co-assembles with L1 into VLPs, enhancing HPV viral assembly. L2 is not expressed in malignant and

pre-malignant cells [80-82]. This is crucial in the HPV life cycle and oncogenesis, as described in section 1.5.

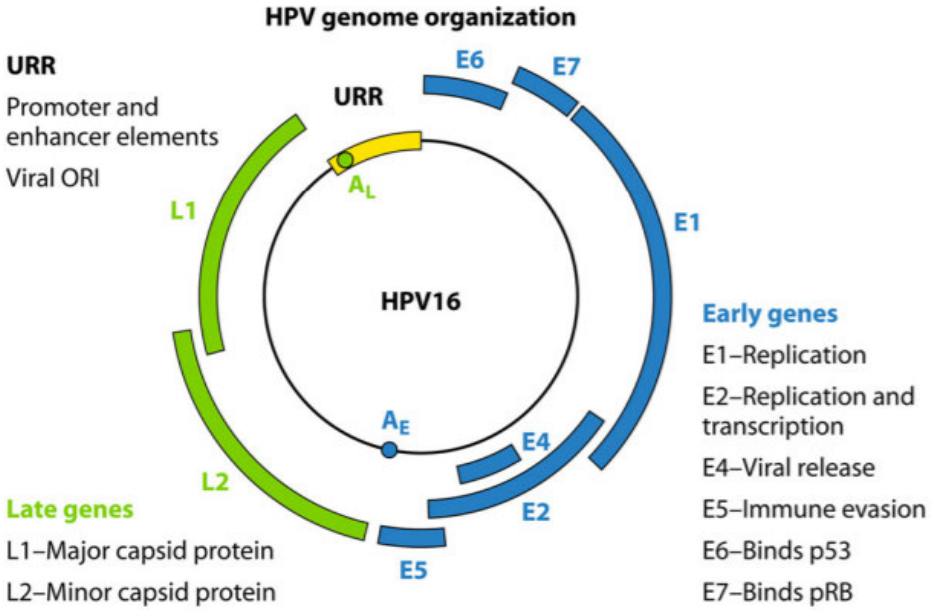

Figure 1.2. Schematic diagram of the HPV genome [83]. The open reading frames (ORFs), the early (E) in blue, comprise the 6 early genes: E1, E2, E4, E5, E6, and E7. These genes are distinguished based on their various functions. Mainly, E1 for viral replication, E2 for viral replication and transcription, E4 for viral release, E5 for immune evasion, E6 and E7 for transforming host cells and facilitating oncogenesis, with E6 Binding and hindering p53, while E7 binds and inhibits pRB protein. Moreover, the late (L) viral proteins and the non-coding upstream regulatory region (URR) are shown, with both the L1 and L2 regions highlighted in green.

1.4. Classification of Human Papillomaviruses

Human Papilloma Viruses are classified into five genera: Alpha (∝)-papillomavirus, Beta (β)-papillomavirus, Gamma (γ)-papillomavirus, mu-papillomavirus, and nu-papillomavirus based on their genomic structure, particularly the L1 gene, as well as their tropism to epithelial tissues [67, 83-85]. Furthermore, HPVs infect differentiating squamous epithelium cells, and therefore, are allocated into three groups, mainly mucocutaneous, cutaneous, and those associated with rare autosomal recessive disorder *epidermodysplasia verruciformis (EV)*, depending on the infection site [86]. According to the International Agency for Research on Cancer (IARC), HPV types of the ∝genus have a predilection for either mucosal or cutaneous epithelial surfaces and are further classified as HR or LR, depending on their carcinogenicity [87]. From these, a subgroup of Twelve ∝-HPV types, namely HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 are categorised as high-risk, HPV 68 and 73 are categorised as

possible high risk types, while HPV types 2, 3, 6, 10, 11, 27, 40, 42, 43, 44, 54, 57, 62, 72, 73, 81 are categorised as low-risk types [88]. High-risk HPVs are the etiological agents of a variety of cancers, such as cancer of the cervix, vulva, vagina, penis, anus and a subset of head and oropharyngeal cancer, whereas low-risk types such as HPV 6, 11, 42, 43 and 44 commonly result in benign tumours. HPV 6 and 11 are commonly associated with genital warts [83, 89, 90]. Alpha-papillomaviruses are further categorised into clades of five species: Alpha-5 (26, 51, 69 and 82), Alpha-6 (30,53,56 and 66), Alpha-7 (18, 39, 45, 59, 68, 70, 85, 97), Alpha-9 (16, 31, 33,35, 52, 58, 67) and Alpha-11 (34, 73), with Apha-7 and Alpha-9 consisting of the most ubiquitous oncogenic types [88]. Additionally, ∝-HPV types: HPV26, 66, 68, 73, and 82, whose carcinogenicity remains to be classified, are referred to as probable carcinogens (IARC Groups 2A and 2B) [85]. These HPVs seldomly exist in cancer cases and/or are associated with additional risk factors. Moreover, the majority of HPV types from the β-genus, with a few types from the γ, mu, and nu genera, result in asymptomatic infections but can be triggered by an immunosuppressive state, therefore, increasing predisposition to cutaneous papilloma and/or skin cancer [84, 91].

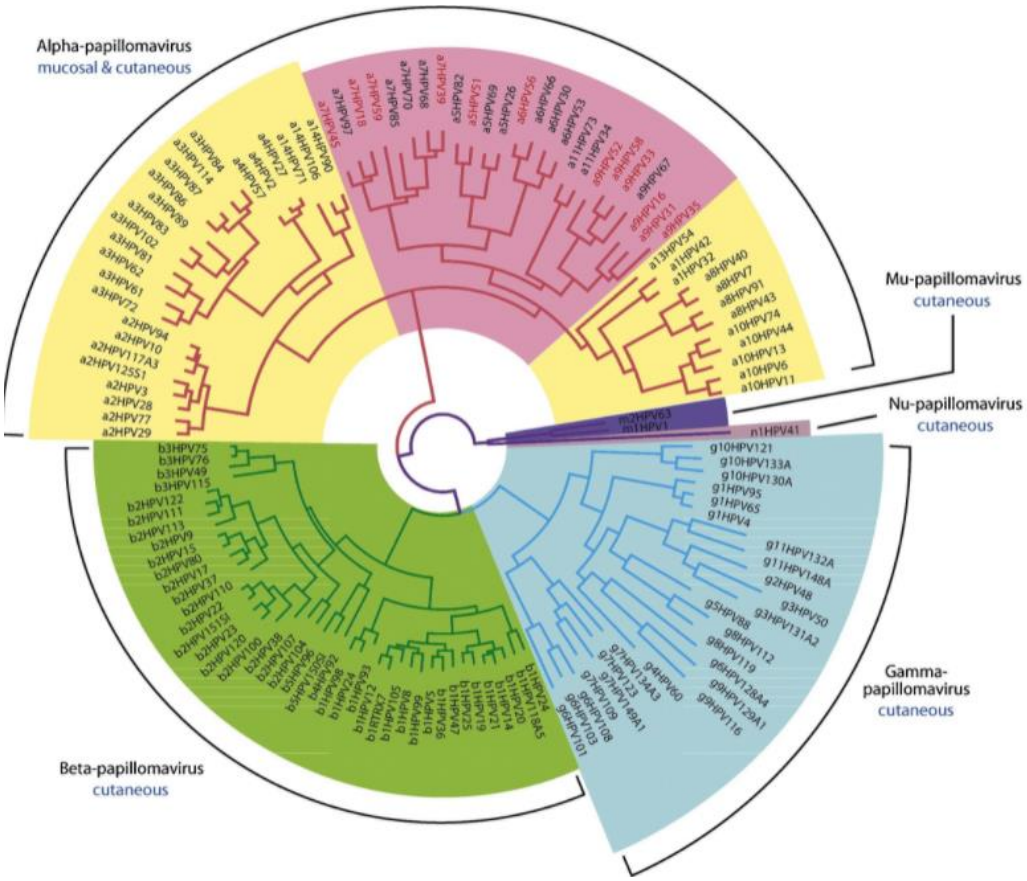

Figure 1.3. Schematic diagram depicting Human papillomavirus types classified into five genera [92]. Alpha (∝)-papillomavirus, Beta (β)-papillomavirus, Gamma (γ)-papillomavirus, mu-papillomavirus, and nu-papillomavirus. Alpha-genus consists of High-risk mucosal and cutaneous types that are highlighted in red in the dusty pink shade, as well as low-risk mucosal and cutaneous types in the yellow-shade. Beta-genus consists of low-risk cutaneous types as shown in the green shade. Gamma-genus consists of low-risk cutaneous types as shown in the blue shade. Mu- and nu-papillomaviruses consists of low-risk cutaneous types as shown in light and dark purple shades respectively. Carcinogenic HPV types and possible carcinogens are shown in red text.

1.5. Life cycle of HPV

Human papilloma Viruses are exclusively epitheliotropic, and infections occur within stratified epithelial cells of the oral cavity, anogenital tract, and skin [93]. The productive life cycle of HPV takes place in the upper layers of the epithelium and is closely linked to keratinocyte differentiation once the virus has infected the cells [76]. The life cycle of HPV is divided into four distinct phases, including entry, the establishment of a non-productive infectious state, the productive expansion stage, and virus assembly and release, as shown in Figure 1.4. During entry, viral particles interact with heparin sulfate proteoglycan receptors (HSPGs) through the L1 capsid protein and gain access to the basal lamina through epithelial wounding or micro-abrasions [94]. Viral particles then migrate to the nucleus, together with the L2 proteins.

This is followed by the non-productive infectious stage, which involves viral DNA replication. The viral copy number is amplified to 50-100 copies per cell, and this initial amplification leads to the establishment of an infection [76, 95]. The infected host cell then enters the proliferative compartment of the epithelium, where the viral copy number remains unchanged and gene expression is low [96, 97]. At this stage of the life cycle of HR-HPV, there is expression of E1 and E2 proteins, while the expression of the E6 and E7 oncogenes is under stringent control. This leads to persistent latent HPV infections as shown in Figure. 1.4 [98, 99]. The HIV genome continues to replicate in the infected basal keratinocytes. This is followed by the distribution of the HPV genome into new keratinocytes during cell division. Some infected keratinocytes remain in the basal layer, while other cells migrate to the stratum spinosum layer for further differentiation [100].

The third stage is the productive expansion stage; during this stage, viral gene expression is upregulated and the viral copy number is amplified to thousands of copies per cell [101-103]. At this stage, there is high expression of the E6 and E7 genes, which are responsible for oncogenesis [76, 94, 104]. This is followed by an expression of late genes, L1 and L2 proteins, which are under the control of a viral

promoter. The final step is viral assembly and release that leads to shedding of virions with dead keratinocytes and further transmission of HPV into the new cells. The new virions then go on to infect new keratinocytes. The time taken from infection to the generation of infectious virus is at least 3 weeks [105].

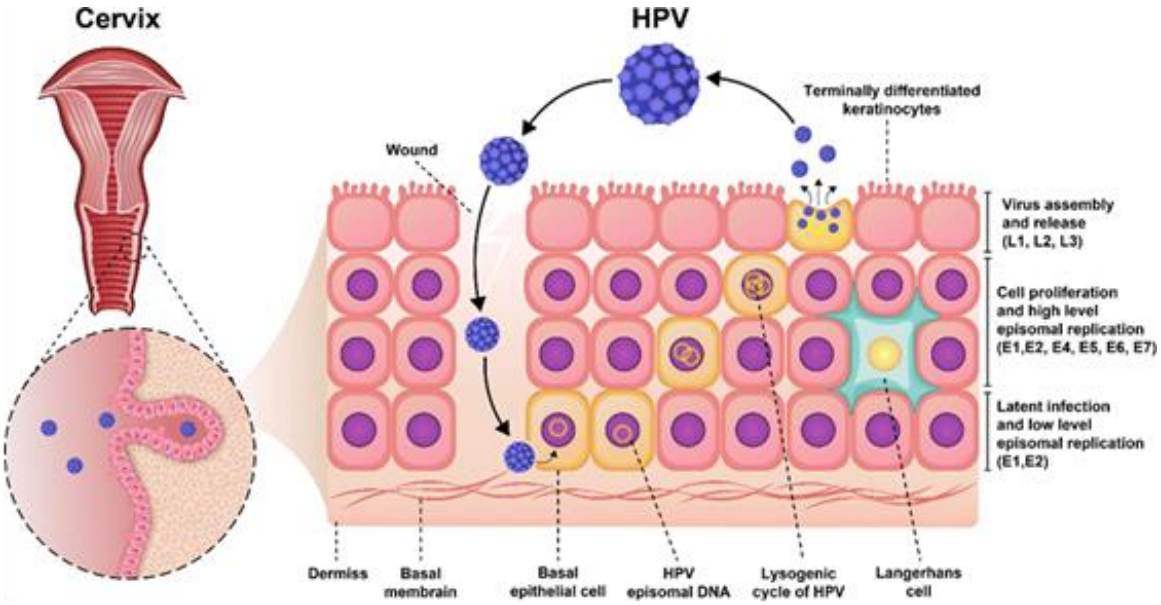

Figure 1.4. Life Cycle of Human Papilloma Virus [106]. Initially**,** HPV infects the basal epithelium upon micro-abrasions, wounds, or injury. Infected epithelial cells divide, giving rise to transit-amplifying cells that differentiate. HPV genome then segregates into daughter cells, which migrate to the upper epithelium. Upon differentiation of keratinocytes, viral gene expression occurs, following which cell proliferation and viral assembly and release occur. Finally, the viruses are assembled and secreted from keratinocytes to repeat the infection cycle.

1.6. HPV induced carcinogenesis

HPV replication concludes once viral DNA integrates into the host's genome, a key characteristic of HPV-induced carcinogenesis [88, 107]. Integration and maintenance of a transformed phenotype may be triggered by genomic instability due to elevated expression of high-risk HPV oncoproteins E6 and E7. This leads to increased denaturation of both host and viral DNA, inducing chromosomal rearrangements, as well as interfering with the duplication of centromeres during mitosis [108-110]. Elevated levels of E6 and E7 oncoproteins have been directly proportional to oncogenic HPV viral load [111-113]. Furthermore, both E6 and E7 oncoproteins disrupt central cellular tumour suppressor pathways: E6 promotes proteasomal degradation of p53 tumour suppressor, binds and accelerates the

rate of degradation of the vital pro-apoptotic protein procaspase 8 (CASP8) [99, 114, 115]. On the other hand, E7 interferes with the retinoblastoma pathway, leading to the disruption of the feedback loop by inducing a cyclin-dependent kinase inhibitor (p16INK4A) and uncontrolled activation of the cell cycle [116, 117]. These two HPV oncoproteins can avert apoptosis, cause genomic instabilities that restrict cell death, therefore, permitting uncontrolled proliferation of cells, as well as leading to the formation of CIN [118-121].

CIN are cellular abnormalities that occur in the lower third of the epithelium, and they are stratified according to the grade of the lesions as explained in section 1.1 [122-124]. Consequently, a productive infection by HR-HPV types may develop into non-malignant low-grade squamous intraepithelial lesions (LGSIL), also known as CIN1, of which approximately 90% regress spontaneously within 2 years due to natural immunity [125-127]. Persistence of HR-HPV infections results in the formation of high-grade histological abnormalities categorized as high-grade squamous intra-epithelial lesions (HGSIL) or CIN2/3 that show a higher risk of progression to cervical disease as described in section 1.1 [61, 123].

1.7. The role of HPV genotypes and viral load in HPV infection and the development of cervical intra-epithelial neoplasia

Of late, HPV genotyping has emerged as an important molecular assay used in conjunction with cytology for the detection of HPV infection, identifying women with definitive persistent infections, for primary cervical cancer screening as well as triage of patients [128, 129]. Some genotyping techniques include Nucleic Acid Hybridization, which uses sets of pooled probes, while others use Polymerase chain reaction (PCR) assays, using consensus primers to target the conserved L1 region of the viral genome to accurately test for HPV infection [130-133]. These HPV genotyping technologies demonstrate higher sensitivity than cytology in predicting CIN in some cases, but have poor specificity in terms of predicting the presence of CIN, its severity, or the risk of progression thereof [134-136]. Additionally, the specificity and positive predictive value vary depending on the HPV type and the group being tested. A large number of women test positive for HPV DNA with transient infections that do not lead to cervical intra-epithelial neoplasia [137-139]. Therefore, considering the established

relationship between the persistence of high-risk HPV and CIN diagnosis, type-specific HPV genotyping is a more nuanced option for diagnosis and prediction of CIN. Moreover, persistent infection with HPV 16 and 18 is much more likely to be associated with high-grade lesions and the recurrence /persistence of CIN than other high-risk HPV types [140-143]. Additionally, types 31, 33, 35, 45, 52, and 58 are among the other few genotypes commonly associated with malignancy [144-147]. Although the role of high-risk HPV persistence in carcinogenesis is well documented, there are conflicting data on the role of HPV viral load, especially type-specific HPV viral load, in HPV-induced carcinogenesis.

HPV viral load is a proxy measure of the number of infected cervical epithelial cells and the viral copies in each cell. This is often measured from cytobrush samples. It has been reported that HPV 16 viral load may potentially be a marker of CIN progression, as higher cervical HPV 16 viral load is associated with the development of CIN2+ [148-150]. This association has also been observed among other non-HPV 16 types, within the Alpha species group 9, particularly types 31, 35, 52, and 58 [151, 152]. However, semi-qualitative HPV viral load reports, using HCII demonstrated that higher viral loads are associated with the presence of CIN2/3 but not with the severity of these lesions [153]. While other studies have reported a significant association between HPV 16 viral load and risk of carcinogenesis, no association has been found with other high-risk HPV types [154, 155]. On the contrary, other studies have not found an association between HPV viral load and CIN severity.

The discrepancies in the current literature on HPV viral load and CIN may be due to various limiting factors such as the type of HPV infection, whether one is infected with single or multiple HPV types, the method of viral load quantification, the unit of reporting, and the sample type. Viral load quantities vary among numerous HPV types, with HPV 16 contributing the highest quantities per cell equivalent, followed by other oncogenic group 9 HPV types [156, 157]. Yet, lower viral loads have been observed during multiple HPV infections compared to single-type infections, with HPV-16 contributing the highest viral load and a further severe risk of disease [158]. Furthermore, HPV 16 is the most virulent high-risk type due to its several variants and mutations in the long control region: the most variable

region of the HPV genome responsible for viral transcription, replication, and consequently promoting HPV 16 persistence, elevated viral load levels, and therefore, the progression of cervical disease [159].

The method of viral quantification also affects viral load reports, as semi-quantitative studies, such as those using HCII, report on relative light units of a combination of HR-HPV types without discriminating among the various oncogenic HPV types influencing viral load, whereas real-time PCR evaluates absolute HPV copy numbers [153]. The type of sampling also plays a role; a previous study reported that viral load measured in the combination of endo- and ectocervical sampling was significantly higher than simple endocervical sampling, which in turn may predict the site in which the lesion is most likely to develop [160]. Moreover, the sampling technique is also a significant factor; for instance, the Digene cervical sampler is a common tool in HPV testing, however due to its small radius of 0.5 cm, it restricts the collection of cells to the area external to the orifice of the uterus. Therefore, lesions that exists beyond this point cannot be sampled leading to inaccurate and the underestimation of viral load quantification [54]. Other controversies in HPV viral load results may also be due to a lack of normalization with an externally validated method of the number of studied cells in the samples [161, 162]. Nevertheless, these studies suggest that HPV viral load is a promising biomarker for assessing the risk of development and severity of cervical lesions. However, additional studies are required to address the numerous limiting factors, including the patient's age, different study populations, and the impact of other concurrent infections, including HIV infection.

1.8. HPV viral load in Women living with HIV

Persistent infection with HR-HPV types is a precursor for the development of CIN and ultimately cervical cancer, and this is exacerbated in WLWH [43, 163, 164]. Additionally, the prevalence of HPV is generally much higher in WLWH, with concurrent and multiple type infections than in HIV-negative women of comparable sociodemographic characteristics [165]. This may be explained by an HIV immune suppression, especially among women who are not on antiretroviral therapy, which permits high HPV viral load and persistent infection leading to increased risk of reactivation of latent infections, increased susceptibility to new HPV acquisition, and cervical neoplasia [166]. At a molecular level, the HIV Trans-activator of transcription (Tat) viral protein disrupts epithelial tight junctions, which

subsequently promote paracellular penetration and entry of HPV virions into the target cells, therefore increasing HPV-infected cells and viral load [167]. Furthermore, an association between HPV viral and host immunity has been established, with viral load variations reported according to Cluster of Differentiation (CD) 4 counts [168]. An inverse relationship between HPV viral load of HPV Alpha-7 and 9 species and CD4 count was observed among women living with HIV, and this was a predictor of abnormal cytology [163, 169].

High viral loads of HR-HPV types such as HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 58 and 59, were associated with severe disease, even though these were not significantly different between CIN grades and carcinoma in HIV-positive women [170]. Some studies in women living with HIV have found that elevated HPV16 and 18 viral loads were associated with abnormal histology outcomes, and a high HPV16 viral load at baseline was associated with incident CIN2+, even though HPV 18 quantities were lower and less performant in identifying the risk of high-grade lesions. It is important to note that these women were not profoundly immunosuppressed; therefore, no significant difference was observed between HPV 16 and 18 viral loads and CD4 counts greater or lower than 350 cells/μL [163]. Essentially, a high HPV16 viral load represents a useful marker of high-grade cervical lesions and might be considered as a triage test for women identified as HPV16-positive, especially for women living with HIV who are at a disproportional risk of HPV acquisition and carcinogenesis.

1.9. Host immune response to HPV infection

A competent antiviral immune response plays a pivotal role in the control and elimination of HPV infection. The immune response prevents CIN progression and cervical cancer. Importantly, both the innate and adaptive immune responses, comprising mucosal immunity, humoral immunity, and cellular immunity, are central to the prevention of HPV infection and HPV-induced malignancy [171]. Although the immune response to HPV cervical infections is not fully understood, it is known that both local and systemic immunity play a key role in eliminating infection and dysplasia. However, there are various strategies that HPV employs to evade host immunity, consequently exacerbating the risk of viral persistence as well as oncogenicity, as described in section 1.10.

1.9.1. Innate immunity

The skin and mucosal surfaces are key physical barriers that provide some innate immunity, and their damage or injury allows HPV entry into the basal layer of the epithelium as described in section 1.5 [172]. Moreover, keratinocytes in the basal layer of the epithelium are the target cells for HPV infection. During the early stages of an HPV infection, the host innate immune response becomes the first line of defense against the infection. The vaginal mucosal epithelium consists of two major groups of antigen-presenting cells (APCs) that present antigens to the T cells to initiate a specific immune response, mainly, Dendritic (DC), Langerhans (LC), natural killer (NK), natural killer T (NKT) cells and keratinocytes, among others [173]. DCs are the most abundant APCs in the cervical mucosa [174]. Moreover, NK cells are able to directly eliminate HPV infected cells [173]. However, evasion of the immune response by HPV is critical for a successful infection as described in section 1.10. In addition, the epithelial layer of the transformation zone (TZ) of the endocervix is highly susceptible to successful pathogen invasion due to its low number of LCs compared to the exocervix [175]. Most of these cell types can promote a cytokine-mediated pro-inflammatory process during early infection, which links the innate with the adaptive immune response.

Keratinocytes can function as non-professional APCs, and are able to induce the expression of Th1 and Th2 types cytokines and cytotoxic responses in CD4+ and CD8+ memory T cells, respectively. In the female genital tract keratinocytes express several Toll-like receptors (TLRs), located either on the cell surface (TLR-1, TLR-2, TLR-4, TLR-5 and TLR-6) or in the endosomes (TLR-3 and TLR-9) [176]. The TLRs are a family of immunological receptors that recognize pathogen-associated molecular patterns (PAMPs), their activation initiates signaling pathways that result in innate and adaptive immune responses [177]. Endosomal TLRs play an important role in combating viral infections and in the recognition of viral nucleic acids. The activation of these receptors promotes the production of cytokines and creates a powerful pro-inflammatory environment [178]. Nevertheless, HPV immune evasion mechanisms result in persistent HPV infections.

According to Wu et al.[179], persistent infections by HPVs 16 and 18 reduce the production of TNF-α as well as TNF-γ, subsequently decreasing the presentation capacity of Langerhans cells. This is a consequence of local immune dysfunction, leading to the disruption of local immune surveillance mechanisms, both in the induction phase (antigen presentation) as well as activation of adaptive immune responses. Persistent untreated HPV infection further increases the risk of developing high-grade squamous intraepithelial lesions and subsequently cervical cancers over a period of 5-14 years [96, 180]. In the absence of co-stimulatory molecules, keratinocytes also tend to show antigen tolerance and lesion progression; however, lesion regression and effective immunity are induced in the presence of co-stimulatory molecules such as B7 [181, 182].

1.9.2. Adaptive immunity

A functional adaptive immune system, on the other hand, is specific and comprises a delayed response following infection, with long-term protection. Adaptive immune responses consist of cellular and humoral immunity [183]. A signal cascade is initiated once a pathogen is recognized by the host immune machinery, and a response is elicited to eliminate the pathogen and stimulate acquired immunity, a consequence of the adaptive immune system [184].

1.9.2.1. Humoral immunity: the role of antibodies in HPV infection and CIN

As with other viral infections, neutralizing antibodies are crucial in eliminating invading pathogens. The role of B cells and antibodies has been extensively explored in vaccine studies [185, 186]. During primary HPV infection, antibodies against HPV major capsid L1 and L2 proteins bind the virion to prevent viral attachment to the basal epithelium [187, 188]. Notably, the course and magnitude of antibody responses vary considerably depending on the type of infecting HPV and the target HPV antigen used. It is well known that the lamina propria of the endocervix consists of an extensive amount of Immunoglobulin (Ig) IgA- and IgG-secreting plasma cells, and approximately half of women infected with HPV show weak IgA and/or IgG antibodies, six months post-infection [189]. Women that have persistent HR-HPV frequently elicit weak IgA and IgG antibodies against the L1 virus-like proteins (VLPs) [185]. This suggests that persistent HPV infection is generally a sign of immune failure, not a

prerequisite for a strong antibody response, a phenomenon highly observed among WLWH [185, 190]. Furthermore, HPV types with similar biological and pathological properties to HPV16 tend to behave similarly. Studies on HPV16 VLPs have further demonstrated that cervicovaginal viral clearance is associated with higher titres of IgA, while persistence is associated with higher titres of IgG [191].

Additionally, the risk of subsequent HPV infections is reduced by 53% among women showing persistence of both IgA and IgG, compared to those with persistent IgG alone [191, 192]. Women with a higher risk of developing high-grade lesions/CIN3 and cervical cancer also tend to show high IgG reactivity to HPV16 L1 VLPs, while systemic IgA HPV16 VLP-specific antibodies seem to be associated with viral clearance [193, 194]. Women with onset cervical cancer have been shown to have high reactivity to anti-E7 antibodies, suggesting that anti-E7 antibodies may be a useful marker for HPV-induced cervical malignancy [195, 196]. While humoral immune responses have important preventive utility, several lines of evidence suggest that cell-mediated immune responses play a crucial role in the regression of precancerous lesions and the clearance of infection.

1.9.2.2. The role of cell-mediated immunity in HPV infection

Amongst the various host factors influencing HPV infection, cell-mediated immunity (CMI) plays a central role in HPV elimination and lesion regression [197]. Particularly, CD4 and CD8 T cells are the most important components of CMI, both systemically and locally (in the genital tract). Once HPV infection has been established, APCs present antigens to naïve T cells within draining lymph nodes [198]. Upon exposure to the antigen, naïve CD4+ T cells differentiate into Type-1 helper (Th1), Type-2 helper (Th2), Th17 cells, and regulatory T cells (Treg cells), while naïve CD8+ T cells differentiate into cytotoxic and memory T cells [199]. Subsequently, activated CD4+ T cells secrete Th1 and Th2 cytokines, which activate various immune responses to eliminate the infection [171, 200]. The Th1 cytokines that are produced by the various differentiated CD4+ T cells include IFN-γ, TNF-α, IL-2, IL-12, and IL-15, whereas the Th2 cytokines include IL-13, IL-10, IL-6, IL-5, and IL-4. Tregs/Th3 cytokines include IL-10 and TGFβ, and the Th17 responses include the production of IL-17, IL-23, and IL-32.

On the other hand, activated CD8+T-cells differentiate into cytotoxic T lymphocytes (CTL), which secrete granzyme, proteolytic enzymes, and perforin, and facilitate the clearance of HPV infection as depicted in Figure 1.5 [201]. Notably, a Th1 dominant environment is associated with regression of HPV-induced genital warts and clearance of HPV infections, while persistence is highly associated with a skewing of the cytokine milieu to a Th2 response [202-204].

Current literature has shown discrepancies in the frequency and quality of circulating CD4+ and CD8+ T cells in participants with HPV infection. During primary HPV infection, higher CD8+ T cell responses compared to CD4+ T cell responses have been observed in participants with CIN clearance [197]. This evidence suggests a strong impact of CD8+ T cells in eliminating HPV-infected cells, promoting regression of cervical dysplasia, and sustaining anti-tumour immune responses [205]. Similarly, spontaneous regression of HPV-induced anogenital warts has been linked to elevated infiltration of IFN-γ producing CD4+ and CD8+ T cells compared to non-regressing warts, while a reduction in CD8+ T cell frequency is associated with poor disease outcome and the persistence of anogenital warts [187]. Furthermore, there is evidence to suggest that these infiltrating lymphocytes associated with wart regression and HPV clearance are activated memory cells, thereby supporting evidence that CTL responses confer protection and last for over 20 months among women who spontaneously clear HPV infections [206, 207].

Historically, in individuals living with HIV, the reduction of absolute CD4+ T cells and having high HIV viral loads have been important biomarkers of HIV immune suppression. However, in recent years, ART has successfully lowered HIV viraemia and restored CD4 T cell counts [208]. Even though absolute CD4+ T cell counts are restored to normal levels, immune dysfunction may persist, as denoted by the elevated HPV persistence and risk of cervical cancer among WLWH[209]. Moreover, the natural progression of HIV infection results in contradicting levels of circulating CD4+ and CD8+ T cells; prior to the reduction of CD4+ T cells by HIV, circulating CD8+ T cells become elevated in response to infection, therefore leading to a low CD4/CD8 ratio that generally becomes restored upon treatment uptake. Furthermore, there is evidence to suggest that an immune-compromised state in WLWH, despite treatment and viral suppression, may be a consequence of an unrestored CD4/CD8 ratio and

functionality among other factors, which increases cervical cancer incidence among WLWH [78]. For this reason, WLWH should be prioritized for cervical cancer screening and prevention services, especially in LMICs where HIV is rampant.

1.9.2.3. The role of cytokines in HPV infection

Inflammation is a process facilitated by cytokines and chemokines whereby the immune system recognizes and eliminates invading pathogens and damaged host cells, subsequently initiating the healing process [210]. Additionally, inflammation plays a multifaceted role in HPV pathogenesis and clearance of current infections, yet persistent HPV infections result in chronic inflammation. This is due to excessive production of pro-inflammatory cytokines and prostaglandins, which intensifies inflammation, leading to accelerated cell division and increased risk of malignant transformation [211-213].

Cytokines and chemokines are soluble immune mediators that play a role in the activation and regulation of inflammation, as observed in both innate and adaptive immunity [140]. As demonstrated in figure1.6, during initial HPV infection, there is secretion of Type-1 INF, TNF-α, IL-8, CCL2, CXCL9, and CCL20. High levels of interferon-1 (IFN-∝ and IFN- β) leads to activation of Th-1 cytotoxic responses [212]. In contrast, type-II IFNs include IFN-γ, which is limited to T lymphocytes and natural killer (NK) cells [21]. Moreover, by upregulating MHC molecules on APCs, IFN-γ enhances the ability of the immune system to recognize and target HPV-infected cells, thereby indirectly promoting an antiviral response [76]. The activation of Type 1 IFNs during HPV infection results in the upregulation of pro-inflammatory responses. Persistent HPV infection is associated with chronic inflammation and expression of various effector molecules [171]. The relevance of inflammation in HPV infection outcomes on cell lines is vastly emphasised *in vitro* studies; IFN-α, IFN-β, IL-1 and TNF-α inhibit the transcription of HPV oncogenes and activate NK cells, which eliminates infected cells [211]. High-risk HPV types adopt various immune evasion strategies, which include down-regulating IFN responses, ultimately reducing pro-inflammatory cytokines responses in cervical keratinocytes [214].

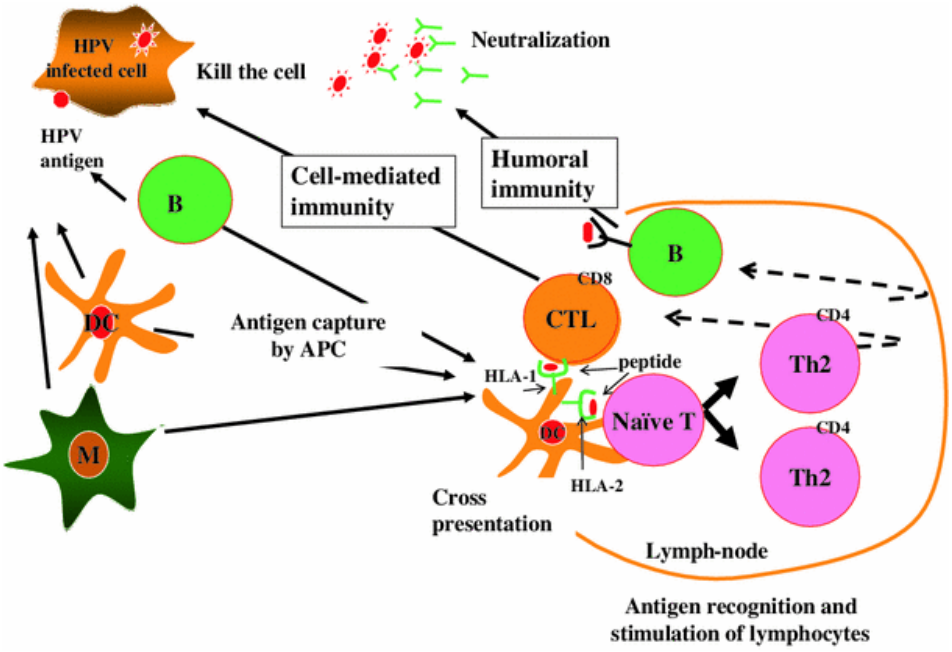

Figure 1.5. Immune responses to HPV infection [215]. Where B=lymphocytes, DC=Dendritic cells, M=monocytes, APC antigen-presenting cells, and CTL=cytotoxic T lymphocytes. HPV infects keratinocytes in the basal epithelial layer. This results in the release of HPV antigens, which subsequently activate the innate immune response, which involves the activation of immune cells and the release of cytokines. Consequently, the adaptive immune response comprises both the humoral immunity, which involves the release of antibodies following HPV infection, and cell-mediated immunity. In the lymph nodes, naïve T cells differentiate into Th2 and Th1 cells, while naïve CD8 T cells differentiate into cytotoxic T cells that facilitate viral clearance.

1.10. HPV immune evasion

HPVs have adapted various mechanisms to evade both innate and adaptive immune responses, and this leads to the progression of pre-cancerous lesions, as well as establishment of cervical cancer. These strategies are described below.

1.10.1. Low gene expression levels

Upon infection, HPV maintains a low viral gene expression in the basal epithelia and lower epithelial layers, thus hindering detection by APCs and preventing the induction of potent adaptive immunity against HPV-infected cells [180]. Moreover, HPV proteins are not secreted and this process is controlled by E2 viral regulatory proteins. Notably, disruption of E2 in the loci occurs during disease

progression when the HPV genome becomes integrated into host cells. Throughout this process, the HPV E6 and E7 oncoprotein expression increases, resulting in the onset of pre-malignancy [216, 217].

1.10.2. No cytolytic cell death

HPV infection and vegetative growth are dependent on the differentiation program of keratinocytes [76]. Naturally, keratinocytes take about three weeks to undergo differentiation and move up to the upper epithelial layer and desquamate [180]. During HPV infection, immunogenic virions and viral proteins are expressed in the upper layers of the epithelial cells. However, viral replication and assembly does not result in virus-induced cytolysis or cytopathic death because these differentiating keratinocytes are already programmed to die, and therefore cannot trigger an extensive inflammatory response [218]. Also, HPV degrades pro-apoptotic molecules procaspase-8 and Fas-associated via Death Domain (FADD). Thus, there is poor activation of DCs, resulting in little to no release of proinflammatory cytokines and migration into the local milieu, and the essential signals required for immune responses in squamous epithelia are absent [90, 180]. Naturally, the period between infection and development of lesions is highly variable and can range from weeks to months, suggesting that the virus does effectively evade host defences.

1.10.3. HPV Compromises Innate Defences in Keratinocytes

Keratinocytes form the protective skin barrier, and also play a crucial role in antigen presentation; they functionally induce the expression of Th1 and Th2 cytokines as well as cytotoxic responses in CD4+ and CD8+ memory T cells [212]. As an immune evasion strategy, HPVs downregulate keratinocytes' innate immune signalling pathways by downregulating antigen-presenting pathways, degrading the expression of TLR-9, which subsequently reduces IFN secretion and culminates in the downregulation of inflammatory cytokines.

1.10.4. HPV interferes with Interferon responses

HPV may cause chronic asymptomatic infections followed by long-term production of virions with limited viral expression through downregulating IFN synthesis, HR-HPV E6 proteins bind to the Tyrosine Kinase 2 (TYK2) enzyme, and prevent the IFN signalling cascades and subsequently reduction in type I and type II IFN production [219]. HPV 16 and HPV 18 E6 and E7 oncoproteins prevent IFN synthesis and activation by blocking nuclear factor-kappa light chain enhancer of activated B cells (NF-κB), a transcription factor, this process subsequently promotes viral persistence [220].

1.10.5. Modulation of antigen presentation

HPV interferes with antigen processing machinery, resulting in the downregulation of peptide-MHC complexes on the surface of the infected cells and enabling them to escape host immune attack. This process is mediated by the E5 oncoproteins and results in the dysregulation of Human Leukocyte Antigen (HLA) class I molecules [221]. In addition to disrupting the HLA class I pathway, HPV16 E5 has also been shown to interfere with HLA Class II antigen presentation, which results in the inhibition of CD4+ T-cell recognition, most likely via a similar mechanism [222, 223].

1.10.6. Skewing Cytokine Profiles

During immune evasion, HPV inhibits Th1 responses and upregulates Th2 responses that promote viral persistence and oncogenicity [76]. As such, elevated levels of IL-10 and IL-6, with low to no detectable levels of IFN-γ, have been observed in HPV-induced lesions [224, 225]. A Predominately Th2 immune response inhibits anti-HPV immune response and promotes viral persistence and the onset of pre-malignancy and malignancy. It has therefore been noted that skewing of cytokine profiles to favour an immunosuppressive state may be induced by HPV and occur prior to the development of cancerous lesions, although the mechanism by which this occurs is yet to be fully elucidated [226].

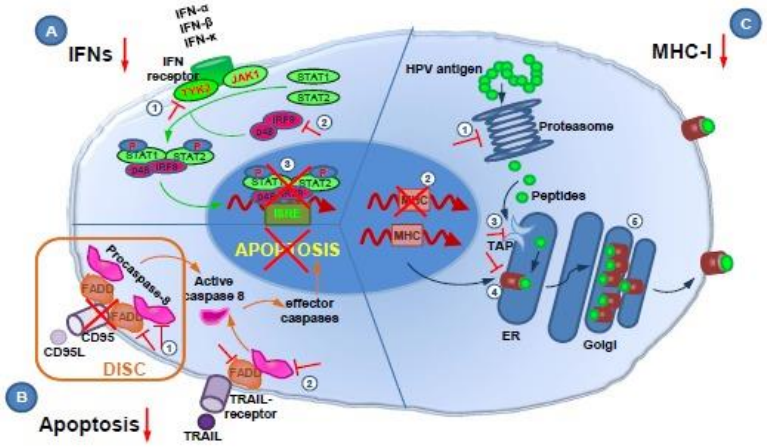

Figure 1.6. HPV immune evasion strategies [212]. (A) HPV downregulates IFN responses by blocking tyrosine kinase signalling (B) HPV prevents cytolytic cell death through dysregulation of CD95 receptors and degradation of procapase-8, molecules that modulate apoptosis of infected cells. (C) HPV alters APCs by downregulating the expression of human leukocyte antigen class I (HLA class I) molecules and arresting HLA class I molecules in the Golgi apparatus.

1.11. HPV prevention and vaccination

The primary goal for the World Health Organization (WHO)is to eliminate cervical cancer as a public health problem by the end of the century, the interim 2030 targets is known as the "90-70-90" goal. These targets are: 90% of girls must be fully vaccinated with the HPV vaccine by age 15, 70% of women screened with a high-performance test at ages 35 and 45, and 90% of women with cervical disease must be receiving treatment [227]. Currently, **t**here are six licensed prophylactic HPV vaccines, these include Gardasil® (quadrivalent), Cervavac® (quadrivalent), Gardasil®9 **(**nonavalent), Cervarix® (bivalent), Cecolin® (bivalent)**,** and Walrinvax® (bivalent) [228]**.** The bivalent vaccines protect from HPV types 16 and 18, which are associated with more than 70% of cervical cancers, while the quadrivalent vaccines protect from HPV 16, 18, 6 and 11, and the nonavalent vaccine against HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58 [229]. Prophylactic vaccination targets children before sexual debut, but there are now catch-up campaigns, which have also been shown to be beneficial in reducing HPV infection and disease [230, 231].

While the HPV vaccine has been available since 2006, its introduction in LMICs has lagged, with Bhutan being the first LMIC to introduce the vaccine in 2010, followed by Rwanda in 2011 [232]. Additionally, within the first 10 years of the vaccine's introduction, 80% of the high income countries (HICs) introduced the HPV vaccine, compared to <20% of HPV vaccinations in LMICs [233]. This is a major problem as cervical cancer poses a disproportionately larger burden in these regions when compared to HICs. Furthermore, the low vaccine update in LMICs, especially in sub-Saharan Africa is a consequence of various factors such as a lack of knowledge about the vaccine, vaccine hesitancy, cultural practices, misinformation, health system barriers like inadequate funding and poor logistics, as well as the high influence of parents and community members on vaccination decisions [234-236]. Therefore, there is a critical need for efficient context-specific interventions including awareness campaigns to increase HPV vaccination rates, early detection and treatment of HPV infections and elimination of HPV related cancers, especially in LMICs.

1.11. Justification for the study

Cervical cancer is one of the leading causes of death among women in SSA, with the highest disease burden observed in WLWH. The role of HR-HPV types and viral persistence has been well documented in the carcinogenic process. However, much less is known about the biology of CIN recurrence and persistence of HR-HPV viral loads after treatment, especially in WLWH. In particular, the relationship between CIN recurrence and HR-HPV viral loads, and the associations with immune responses in WLWH are not clear, yet understanding these would determine potential biomarkers of risk for CIN recurrence and cervical cancer in this population. Therefore, this study aids in understanding the relationship between CIN recurrence and HR-HPV viral load and their associations with the profiles of genital and systemic immunity in WLWH following excisional treatment of cervical intra-epithelial neoplasia. This has implications for understanding the mechanisms of CIN clearance and viral persistence, as well as identifying potential biomarkers for recurrence in WLWH.

1.12. Specific Aim

To characterize genital and systemic immune biomarkers of cervical HPV viral load and determine their relationship with CIN treatment outcomes in women living with HIV.

1.13. Objectives

a. To determine HPV virological predictors of CIN recurrence after surgical excisional treatment in WLWH.
b. To determine the associations of anti-HPV T cell responses with the recurrence of CIN and HR-HPV viral loads in WLWH undergoing surgical excisional CIN treatment.
c. To determine the associations of systemic and cervical cytokine profiles with the recurrence of CIN and HR-HPV viral loads in WLWH undergoing surgical excisional CIN treatment.

1.14. Hypothesis

There is an association between HPV viral load and the profile of genital and systemic immunity in WLWH who are undergoing surgical excisional CIN treatment, and these associations have implications for understanding the clearance of cervical intra-epithelial neoplasia after treatment.

Chapter 2: Materials and Methods

2.1. Study population/Cohort description

Ninety-six WLWH, who presented with abnormal pap smears showing high-grade cervical lesions, were recruited into a prospective longitudinal study called the HPV Clearance and Treatment Outcomes of Cervical High-grade Squamous Intra-epithelial Lesions (HATCH) cohort, from April 2012 to April 2015, at the King Edward Hospital, Durban, in KwaZulu Natal, South Africa. These women were between the ages of 18 and 65 years; they received surgical excision of SIL by Large loop excision of the transformation zone (LLETZ), cold knife conization of the cervix, or cone biopsy as per standard treatment protocol [237]. Sixty-nine women cleared the HPV infection following treatment (No-Recurrence group), while 27 women had recurring infections (Recurrence group). They were followed up at 6- and 12-months post-treatment. Written informed consent was obtained from each participant, and ethical approval was obtained from the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (BE070/16 and BREC/00000815/2019). Women who were willing to participate and provide the required specimen, participants with available pre-treatment cytology data, as well as those with available histology results of excision, were enrolled in the study. Pregnant women, those with invasive cancer of the cervix pre-colposcopy or excision, and those with in-situ carcinoma, with chronic or acute inflammatory disease were excluded. Their demographic data, such as race, age, socioeconomic status, as well as clinical data, including HIV status, histology results, and grade of cervical lesions, were captured for retrospective analysis of clinical outcomes for all participants. Notably, all participants that were included in this study were on ART for more than 6 months, and were virally suppressed.

2.2. Study procedures and specimen collection

At enrolment, all women underwent a colposcopy examination and surgical excision of CIN. Repeat cytology was performed, cytobrush, cervicovaginal lavage (CLV), and Peripheral blood (35ml) were collected at baseline before treatment, 6- and 12 months after treatment. Peripheral blood was used for immunological profiling, while CVL samples were used for immunological profiling, HPV testing, and HPV viral load quantification. CD4+ T cell count was measured using Trucount technology and

analysed further with flow cytometry. HIV viral load was measured using Nuclisens HIV-1 RNA PCR for all women. Sexual, reproductive, and clinical history was obtained using a structured questionnaire. Cytobrush and Cervicovaginal lavage fluid (CVL) samples were used for HPV genotyping and HR-HPV viral load quantification. Both blood and genital specimens (CVL and cytobrush) were used to characterise cytokines and cellular immune responses.

2.2.1 Collection and processing of cervicovaginal fluid samples

CVL was collected by introducing 5m of normal saline to the ectocervix and vaginal walls. The lavage fluid from the posterior fornix was consecutively aspirated with a syringe and aliquoted into 15 ml Falcon tubes. The recovered samples were transported to the laboratory within 6 hours after collection, where they were centrifuged and separated into CVL supernatant and pellet segments and stored at -80 °C for HPV genotyping and cytokine measurement analyses, as described in Chapter 3 and Chapter 5, respectively.

2.2.2 Collection and processing of blood samples

At least 35 ml of blood was collected into acid citrate dextrose tubes (BD, Franklin Lakes, NJ, USA) from each participant at baseline, 6 months, and 12 months after treatment. These samples were centrifuged at 500 x g for 10 minutes at room temperature within 6 hours after collection. The cellular and plasma components were separated. Plasma was then stored at -80 °C for future use. The cellular component was then diluted in an equal volume of PBS and layered onto Leucosep medium, followed by centrifugation for 30 minutes at 500 × g. The buffy layer of PBMCs was then harvested and resuspended in PBS. The cells were counted under a microscope using the Neubauer chamber, pelleted, and resuspended in freezing media (10% DMSO in fetal calf serum) at 1×10^7 cells per ml and stored in a Nalgene Mr. Frosty Cryo 1°C Freezing Container (Thermo Fisher Scientific). Afterwards, the cells were stored in liquid nitrogen. Plasma freeze-thaw cycles that could undesirably affect cytokines were avoided, and samples were used as described in Chapter 4. Likewise, temperature escalations during storage of cells in liquid nitrogen were avoided to maintain cell integrity.

2.3. Laboratory Procedures

2.3.1. HPV Genotyping

2.3.1.1. DNA Extraction and Quantification

CVL pellet samples from all participants collected at baseline, before treatment, were used to extract DNA for HPV genotyping. Briefly, 200 µl of the CVL pellet was centrifuged for 5 minutes at 300 × g (190 RPM). CVL pellet samples were then resuspended in PBS, and DNA was isolated using QIAgen DNeasy Blood and Tissue Kit according to the manufacturer's instructions (QIAGEN, Gaithersburg, USA). Briefly, 20 µl proteinase K and 200 µl Buffer AL were added, and the microcentrifuge tubes were then mixed thoroughly by vortexing. The mixture was added to a Dneasy Mini spin column and placed in a 2 ml collection tube, which was then centrifuged at ≥6000 x g (8000 RPM) for 1 minute. The collection tube and supernatant were discarded, and 500 µl of the wash Buffer AW1 was added to the columns. The tubes were centrifuged for 1 min at ≥6000 x g, and the supernatant was discarded, and the cycle was repeated three times with buffer AW2. The genomic DNA was further eluted in 200 µL of elution buffer AE. The concentration of gDNA was analysed using 2 µL of each sample on the GloMax®-Multi Microplate Multimode Reader using the fluorescence-based QuantiFluor® dsDNA System (Promega, Madison, Wisconsin, United States).

2.3.1.2. Detection of PCR inhibitors

The presence of inhibitory substances in the gDNA samples was evaluated on a subset of samples by means of the SPUD-assay as described by Nolan et al [238]. Briefly, SPUD template (IDT) was diluted 1/1000 in nuclease-free water, added to the extracted gDNA then amplified using forward and reverse SPUD primers (IDT) and 1× PowerUp SYBR Green Master Mix (Thermo Fisher) using the following conditions on a QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher): 95 °C, 10 minutes; followed by 40 cycles of denaturation at 95°C for 15 seconds; and annealing/elongation at 60°C for 1 minute. Data were analysed using the QuantStudio 12k Flex software to determine the Cq values.

2.3.1.3. β-globin quantitative PCR

Prior to PCR amplification, the DNA integrity and cellular adequacy were assessed by measuring the βeta-globin (β-globin) gene. The β-globin gene was quantified in the genomic DNA using quantitative PCR (qPCR) in triplicates. Quantitative PCR was carried out using 1× PowerUp SYBR Green Master Mix (Thermo Fisher), β-globin primers 500nM GH20 (5′-GTG CAC CTG ACT CC TGA GGA GA-3′), and 500nM PC04 (5′-CCT TGA TAC CAA CCT GCC CAG -3′) and gDNA template (Integrated DNA Technologies). A standard curve was generated using data from PCR amplicons of human genomic DNA (Promega) using a 10-fold titration series, starting with 10 pg/µl to 100 ng/µl. Three DNA samples obtained from Promega or Intergrated technologies were included as positive controls and they were used to generate a standard curve. Nuclease-free water was used as a negative quality control. Participant samples, standards and controls were also analysed on the same MicroAmp® Optical 384-well Reaction plate. All qPCR reactions were conducted on the QuantStudio 12K Flex real-time PCR machine (Thermo Fisher Scientific) with the following cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Quantitative amplification was followed by a melting curve analysis.

2..3.1.4 HPV Genotyping using Roche Linear array assay

HPV genotyping was performed using a commercial Roche linear array kit (Roche Diagnostics), which uses biotinylated PGMY09/11 consensus primers to amplify the L1 gene for detection of 37 HPV genotypes as previously described [239]. Briefly, HPV L1 gene was amplified using quantitative PCR; master mix was prepared using 2.5nM $MgCl_2$, KCl, Ampli*Taq* Gold DNA polymerase, uracil-*N*-glycosylase, dATP, dCTP, dGTP, dUTP, dTTP, and biotinylated PGMY L1 PGMY11 (CGTCCCAAAGGAAACTGATC) and PGMY09: (GCACAGGGACATAACAATGG) primers (Roche Molecular Systems, Inc.). PCR was performed in a 100-µl volume, using 50 µl of the Master mix (Roche Molecular Systems) and 50 µl DNA the template DNA. The PCR plate was then incubated in the GeneAmp® PCR System 9700 (Applied Biosystems, Norwalk, CT) using the conditions listed in Table 2.1, the ramp rate was set to 50%.

Table 2.1. Thermal cycler specification for amplification (Table adapted from Roche Linear Array Instruction Manual)

Program Type	*Description*	*Number of Cycles*
Hold	50ºC, 2 minutes	1
Hold	95ºC, 9 minutes	1
	95ºC, 30 seconds	40
Cycle	55ºC, 1 minute	40
	72ºC, 1 minute	40
Hold	72ºC, 5 minutes	1
Hold	72ºC, forever	1

After amplification, the PCR product was denatured by adding denaturation solution. The denatured PCR product was then hybridized to an array strip that had immobilized oligonucleotide probes. Briefly, the Working Hybridization Buffer, Working Ambient Wash Buffer, Stringent Wash Buffer, and Working Citrate Buffer (all supplied with the kit) were prepared as shown in Table 2.2. The Linear Array HPV strips were labelled and placed into the 24-well tray with the probe lines facing upwards. Thereafter, pre-warmed Working Hybridization Buffer was added into each well containing the labelled strip to facilitate hybridization, denatured DNA was added into the wells, and the plate was covered with the lid. The contents were mixed by shaking the plate back and forth, and the plate was incubated at 53ºC in a water bath on a Mini Orbital SSM1 Shaker for 30 minutes (Stuart, Staffordshire, UK). After incubation, the tray was removed from the water bath, and the Working Hybridization Buffer was drained using vacuum aspiration. The enzyme substrate was diluted by adding 15 µl of Streptavidin-Horseradish Peroxidase (SA-HRP) into 5 ml of the working ambient Wash Buffer (Table 2.2) for each strip.

Table 2.2. Preparation of Hybridization, Wash, and Citrate Buffers for the HPV genotyping (Table adapted from Roche Linear Array Instruction Manual)

Buffer	*Contents*	*Volume (ml)*
Working Hybridization Buffer	Distilled Water (dH_2O)	388
	Sodium Chloride-Sodium Phosphate-EDTA (SSPE)	100
	20% Sodium Dodecyl Sulfate (SDS)	12
Working Ambient Wash Buffer	dH_2O	2520
	20x SSPE	133
	20% SDS	13.3
Stringent Wash Buffer	Wash Buffer per strip was warmed to 53ºC for 15 minutes.	5ml per strip
Working Citrate Buffer	20x Citrate Buffer	25
	dH_2O	475

Following hybridization, the 24-well trays were washed with the Ambient Wash Buffer (4ml), the trays were gently shaken to rinse the strips, and the wash buffer was removed using vacuum aspiration. Afterwards, the trays were washed with Stringent Wash Buffer (4ml) and incubated in the 53ºC shaking water bath for 15 minutes. After incubation, the trays were washed again with Wash buffer and the supernatant was removed using vacuum aspiration. After washing, the Working Conjugate containing Streptavidin-Horseradish Peroxidase was added into each well and incubated at room temperature on a shaker shaking at 60 RPM for 30 minutes. After incubation, the Working Conjugate was aspirated, and the plate was washed again with the Working Ambient Wash Buffer, then gently shaken to rinse the strips and vacuum aspirated once more. Subsequently, the trays were washed with Working Ambient Wash Buffer (4ml) and incubated for 10 minutes at room temperature on a platform shaker. The wash buffer was removed, then Citrate Buffer was added, incubated on a platform shaker shaking at 60 RPM for 5 minutes at room temperature, and the wash step was repeated three times with distilled water, followed by vaccum aspiration. The linear array HPV genotyping strips were removed from the 96-well plate using forceps and left on absorbent paper to air dry for up to 2 hours at room temperature. The strips were analyzed and interpreted manually by visually comparing the pattern of ink lines to the linear array reference guide HPV Genotyping test. This assay detected 37 HPV genotypes, comprising of 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 56, 57, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39, and CP6108) which are classified as high risk, possible high risk, as well as low-risk types according to their carcinogenic potential.

2.3.2. HPV Viral load quantification using Hybrid Capture II assay

HR-HPV viral load was quantified from DNA extracted from cervical cytobrush samples collected from baseline, 6- and 12-months post CIN excisional treatment. This quantification was carried out using the Hybrid Capture II commercial kit (QIAGEN, Gaithersburg, USA) following the manufacturer's instructions. HCII is a full-length hybridisation commercial assay with signal amplification using a microplate luminescent detection technique. This assay was used to detect the viral load of thirteen high-risk HPV types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68).

2.3.2.1. Reagents preparation and layout

Reagent composition was described in section 1 of the appendix; the calibrators, quality controls, and specimens were prepared in an 8-microplate well column configuration as shown in Figure. 2.1. The negative calibrator was a carrier DNA (Herring Sperm) in Specimen Transport Medium (STM) with 0.05% (weight per volume in sodium azide). This was added in three replicates in wells A1, B1, and C1. The HR-HPV calibrator was added in microplate wells D1, E1, and F1, the LR-HPV Quality control (QC1-LR) was added in microplate G1, and the High-risk HPV Quality control (QC1-HR) was added in microplate H1. Participants' plasma samples were added in columns 2-12 as shown below.

2.3.2.2. Denaturation and hybridization

CVL samples, Negative calibrator, HR-HPV calibrator, LR-HPV Quality control, and HR-HPV Quality control were denatured using the denaturation reagent to produce single-stranded DNA. Briefly, 100µl of the denaturation reagent and 200µl of control/calibrator were added and thoroughly mixed by vortexing individually at high speed for 5 seconds. In addition, CVL samples were denatured by adding 30µl of denaturation reagent and 60µl of the sample. The samples were incubated at 65^{o}C on a heating block for 45 ± 5 minutes. After incubation, 75 µl of this mixture was added to the bottom of a hybridisation plate, covered with a microplate lid, and incubated for 10 minutes at room temperature.

Following the DNA denaturation process, samples were incubated with the RNA probes that recognize the 13 HR-HPV types. The HR-HPV probe was prepared as shown in Table 2 of the Appendix. Briefly, 25 µl of HR-HPV probe was added to each well of the microplate. The microplate was incubated on a shaking incubator (Bio-Rad Laboratories, Inc., USA) at 1100 ± 100 RPM for 3-5 minutes until the colour in each well changed from purple to yellow. Samples that did not show colour change received an additional 25 µl of the probe mix. After this, the hybridization microplate was carefully placed in the Microplate Heater I (Bio-Rad Laboratories, Inc., USA), equilibrated to 65 ± 2°C. The hybridization microplate was incubated for 65 ± 5 minutes in the heater.

2.3.2.3. Hybrid capture

After incubation, the microplate was removed from the heater, and the contents were carefully transferred from the Hybridization microplate to the Capture microplate (provided in the kit). The capture plate was securely covered with a plate lid and shaken on the orbital plate shaker at 1100 RPM for 60 minutes at room temperature. After incubation, the supernatant was discarded from the microplate wells by fully inverting the plate over a sink and carefully blotting 3-4 times over a dry, clean paper towel.

2.3.2.4. Hybrid detection

Hybrids that bound to the RNA probes were captured on the sides of the microplate using detection antibodies that recognize DNA: RNA hybrids. Briefly, the detection antibody (reagent 1) was added to the wells. This antibody contains alkaline phosphatase-conjugated murine monoclonal antibodies to RNA: DNA hybrids in buffered solution with 0.05% sodium azide (Table 7.1, Appendix). The plate was covered and incubated for 30-45 minutes at room temperature. Following incubation, the plate was then washed using the Automated Plate Washer I (QIAGEN, Gaithersburg, USA), followed by washing 6 times with deionised water and the liquid was decanted, then blot-dried 3-4 times on a clean paper towel. The plate was left inverted for 5 minutes to allow complete water drainage from the wells, while ensuring a complete white colour on the wells.

2.3.2.5. Signal amplification

The signal was amplified by adding detection reagent 2, containing CPD-Star with Emerald II Chemiluminescent substrate in wash buffer concentrate and 1.5% sodium azide (described in Table 7.1, Appendix). Afterwards, the plate was covered with a foil to avoid direct sunlight and incubated for 15-20 minutes at room temperature in the dark. After incubation, the chemiluminescent signal was measured on the DML 3000 Luminometer (QIAGEN, Gaithersburg, USA).

2.3.2.6. Analysis and Interpretation of the results

Chemiluminescent signals from each sample were compared to a standard consisting of 1.0 pg/ml (approximately 5000 viral copies of HPV per test); the intensity of light emitted after a signal amplification denoted the presence or absence of the HPV genome, which was then quantified using relative light units (RLU). Specimens with RLU/Cut-off value ratio ≥ 1.0 with HR-HPV Probe were considered "Positive" for 1 or more of HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. However, specimens with RLU/Cut-off ratio<1.0) were considered negative, if the RLU/Cut-off ratio of a specimen was close to but less than <1.0, and HR-HPV infection was suspected, then alternate HPV testing methods, such as linear array, were repeated for validation of infection, and the viral load quantification was repeated. Results were further grouped into 3 thresholds, respectively: <10 pg/ml=low viral load, medium 10 to <100 pg/ml, and high viral load was >100 pg/ml.

2.3.3. Bio-plex Multiplex ELISA Assay

Cervicovaginal lavage (CVL) supernatant and plasma samples collected from all participants at baseline and 12 months post-treatment were used to measure peripheral and genital tract cytokines using the Bio-Plex Pro Human Cytokine 27-plex (Bio-Rad Laboratories, Inc., USA). In principle, the Bio-Plex multiplex ELISA utilises fluorescently labelled magnetic beads, each with distinct colour codes, which are then coupled to a capture antibody to distinguish between individual analytes within a multiplex suspension. These beads are incubated with the analytes, followed by incubation with a cocktail of biotinylated detection antibodies that target each cytokine. Streptavidin Phycoerythrin (SA-PE) substrate is usually added to the wells to bind to the biotin followed by determination of fluorescence

which corresponds to the amount of analyte. Beads were washed between incubations to avoid carry-over of excess reagents.

For this study, NUNC Maxisorp® 96-well ELISA flat-bottom plates were labelled and marked. Magnetic beads that were 6.5 µl in size, coated with magnetite, were vortexed for 30 seconds, and 50µl of coupled beads were added to the 96-well plates. The plate was then washed twice with 100 µl of Bio-Plex Wash Buffer using an automated magnetic washer. Afterwards, CVL and plasma samples, standards, blanks, and controls were vortexed, and 50 µl was added into corresponding wells. The plate was covered with sealing tape and aluminum foil and incubated on the plate shaker for 30 minutes. After incubation, the plate was washed three times with 100 µl of Wash Buffer. Detection antibodies were briefly vortexed and 25 µl was added to each well, the plate was covered with sealing tape and aluminium foil, and incubated for 30 minutes on the plate shaker. After incubation, the plate was washed three times with 100 µl of Wash Buffer. The Streptavidin-Phycoerythrin (SA-PE) was vortexed for 30 seconds, and 50 µl was added to each well of the 96-well microtiter plate. The plate was covered with sealing tape and aluminium foil and incubated on the plate shaker for 10 minutes. The plate was then washed three times with 100 µl wash buffer. Afterwards, Assay Buffer was added into each well with a multichannel pipette, and the plate was covered with sealing tape and foil and incubated on the plate shaker for 1 minute, and the plate was read on the Bio-Plex 200 system (Bio-Rad Laboratories, Inc., USA).

The Bio plex multiplex assay measured 27 cytokines comprising anti-inflammatory, pro-inflammatory, regulatory, hematopoietic as well as growth-related cytokines; Interleukin; IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, Eotaxin, Basic Fibroblast Growth Factor (FGF-basic), Granulocyte Colony Stimulating Factor (GCSF), GM-CSF, Interferon (IFN-γ), Interferon gamma-induced Protein (IP-10), Monocyte Chemotactic Protein (MCP-1), Macrophage Inflammatory Protein (MIP-1α, MIP-1β), Platelet Derived Growth Factor (PDGF-BB), Regulated upon Activation Normal T cell Expressed and presumably Secreted (RANTES), Tumor necrosis factor (TNF-α) and Vascular Endothelial Growth Factor (VEGF).

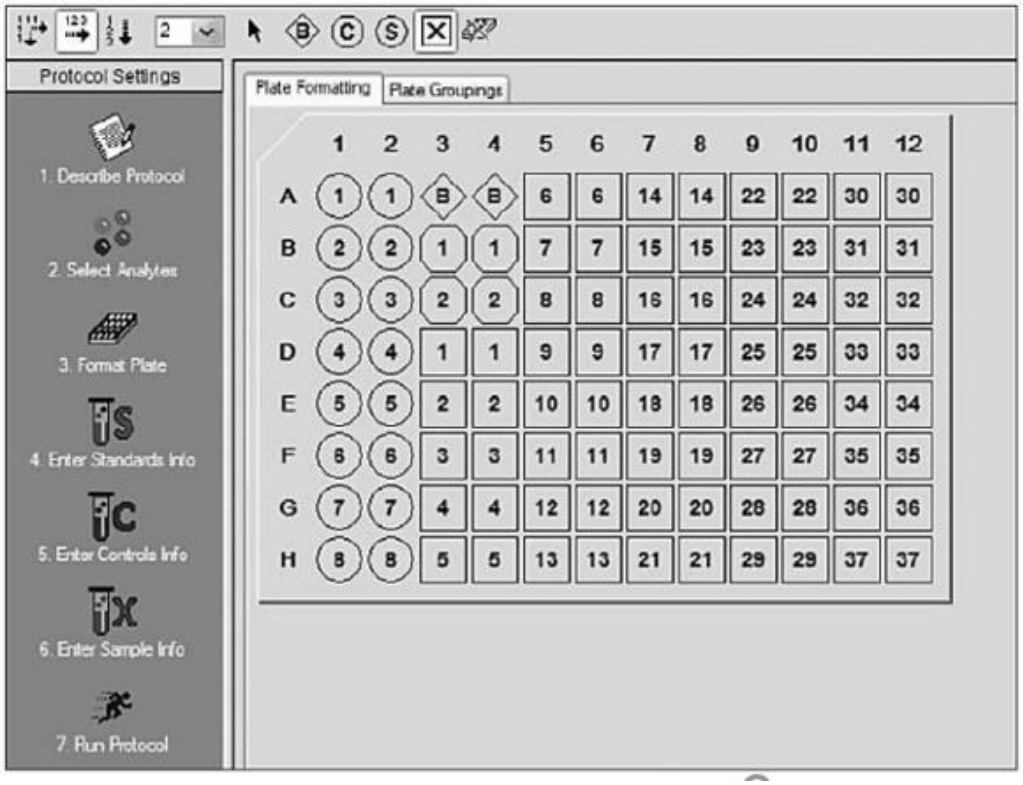

Figure 2.1. A depiction of a plate layout on the Bio-Plex 200 (figure adapted from the Bio-Plex Pro instruction manual). Standards were added from wells A1-H8, Controls B1-C2, Blanks (B) were added in wells 3A and 4A, while Samples as represented in number 1-37 were added in duplicates from wells D11-H37.

Quality Control

To ensure data integrity and traceability, the kits used all had a uniform product number. Bio-Plex Manager software (version 6) was used to acquire data, to extrapolate sample concentrations from the standard curves. For each cytokine, an acceptable recovery range of 70%-130% was considered for each standard, and standards outside this range were excluded to obtain an appropriate 5-point curve. Moreover, standards with a coefficient of variation (% CV) greater than 20% were excluded, depending on how they affected the standard curve. The observed concentrations of replicate plates were combined for validation. All extrapolated values were accepted, and all values below or above the detectable limit were replaced with either half the lowest value in the dataset or double the highest value in the dataset, respectively. Intraplate and interplate variability were assessed to determine whether there were significant differences between duplicate wells or interplate wells, for both plasma and the genital tract, respectively. This was conducted as quality control to assess any possible pipetting errors.

2.3.4. Determination of HPV-specific T cell responses

Determination of frequencies of HPV-specific CD4 and CD8 T cells was done as per the schema in Figure. 2.2.

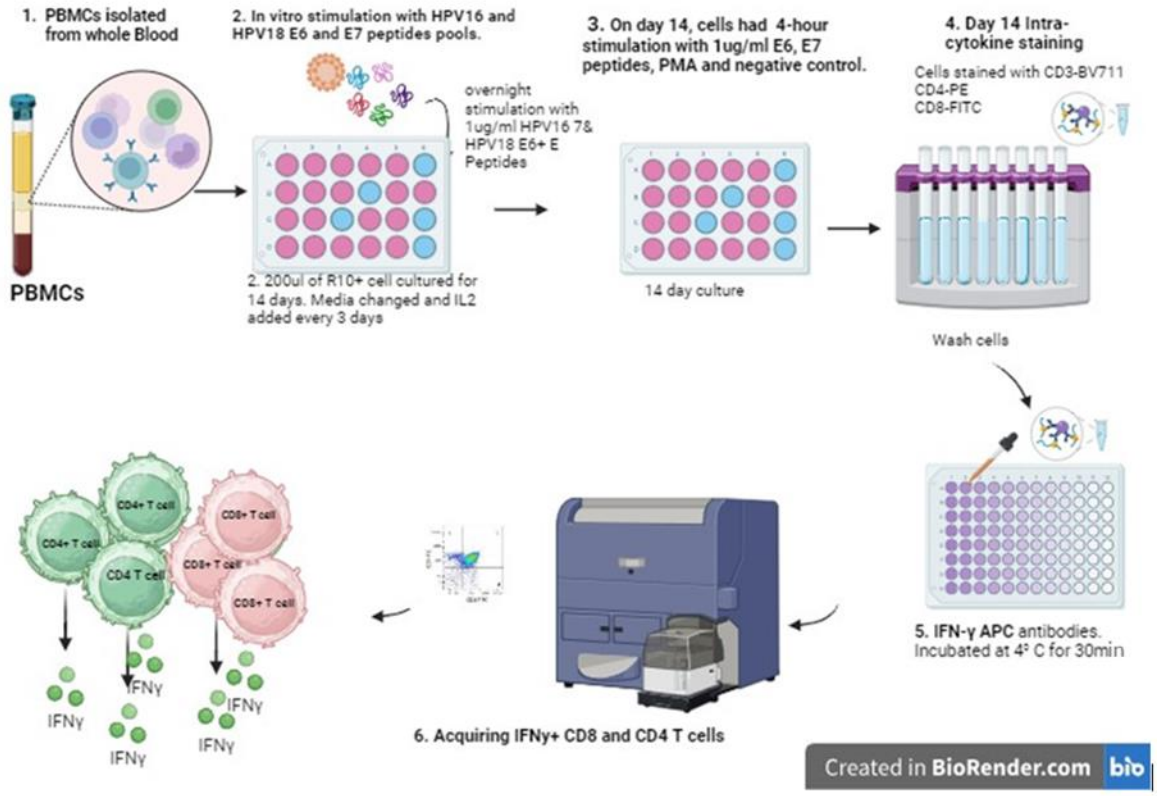

Figure 2.2. In vitro stimulation of PBMCs with HPV16 and HPV18 E6 and E7 peptides and intra-cytokine staining (Image produced in Biorender.com).

2.3.4.1. In vitro stimulation with HPV16 and HPV18 E6 and E7 peptides

Frozen PBMCs were thawed in a 37°C water bath, rested for 2 hours at 37°C in 6.5% CO_2, then stimulated overnight with HPV-16 and HPV-18 E6 and E7 Peptide p,ools (JPT Peptide Technologies GmbH) at a concentration of 1 µg/ml per peptide. The peptide pools included four peptides, namely, PepMix HPV 16 (E6) - a mix of 37 peptides (15mers, overlap 11), PepMix HPV 16 (E7) - which was a mix of 22 peptides (15mers, overlap 11), PepMix HPV 18 (E6) - which was a mix of 37 peptides (15mers, overlap 11), as well as PepMix HPV 18 (E7) - which was a mix of 24 peptides (15mers, overlap 11). The negative controls were unstimulated cells, supplemented with an equal volume of 1%

dimethyl sulfoxide (DMSO) (Sigma-Aldrich). Stimulated and unstimulated cells were incubated overnight at 37°C in 6.5% CO_2. On the following day, cells were then washed and cultured for 14 days in 24-well plates at 37°C in 6.5% CO_2. During incubation, the culture was maintained with the Roswell Park Memorial Institute (RPMI)-1640 medium (Life Technologies) containing 10% fetal bovine serum and 200 IU/ml of recombinant IL-2 (BioLegend), and the growth media was changed every three days. On day 14, approximately 5 million cells from each participant were evenly distributed into 5 wells in a 24-well plate. The cells were distributed into well-labelled wells that contains the following stimulants; (1) unstimulated- cell suspension in 1 µg/ml of DMSO on day 14, (2) negative control- cells not exposed to peptides on day 0, but were maintained in growth media over the culture period, and were not re-stimulated on day 14. (3) Positive control- cells stimulated with PMA (10ng/ml) and Ionomycin (200ng/ml), (4) cells stimulated with 1 µg/ml of HPV 16 and HPV 18 E6 peptides, and (5) cells stimulated with 1 µg/ml of HPV 16 and HPV 18 E7 peptides. The cells were stimulated for 1 hour at 37 °C, 6.5% CO_2, then 1µl of Brefeldin A (BFA) and 1 µl Monensin were added into each well to inhibit protein secretion. BFA primarily blocks transport from the endoplasmic reticulum to the Golgi apparatus, while monensin disrupts the function of the trans-Golgi network by interfering with ion gradients, effectively trapping proteins within the cell. The cells were then incubated at 37°C in 6.5% CO_2 for 4 hours. After incubation, the plate was kept at 4°C in preparation for intracytoplasmic staining.

2.3.4.2. Intra-cytokine staining and analyses

PBMCs from each stimulation well were transferred into labelled Fluorescence-activated Cell Sorting (FACS) tubes, pelleted, and incubated in 1ml of 1:1000 Aqua viability dye (Invitrogen) at room temperature for 30 minutes. They were then pelleted and resuspended in 50µL of anti-CD3-BV711 (BD Biosciences), anti-CD4-PE (BD Biosciences), and anti-CD8-FITC (BioLegend) antibody cocktail and incubated at room temperature for 30 minutes, followed by washing with FACS buffer (PBS with 2% fetal calf serum and 5mM EDTA). Subsequently, all cells were thoroughly resuspended in 100 µL of the fixation/permeabilization solution (BD Biosciences Cytofix/Cytoperm kit) and incubated for 20 minutes at 4°C, then washed twice with Perm/wash buffer (BD Biosciences Cytofix/Cytoperm kit).

Afterward, the cells were intracellularly stained with IFN-γ APC (BioLegend) antibodies and incubated at 4 °C for 30 minutes. The cells were then washed with R10 and acquired using an LSRFortessa cytometer (BD Biosciences) with FACSDiva™ software. Fluorescence minus one control was used to define gates for the different cell subsets. Data was analysed using the FlowJo version 10.0.8 (Flowjo, LLC). Frequencies of anti-HPV CD4+ and CD8+ T cells were determined as shown in the gating strategy (Figure 2.6). Specifically, anti-HPV specific CD4 T cells were defined as viability dye$^-$ CD56$^-$ CD19$^-$ CD3$^+$ CD8$^-$ CD4$^+$ IFN-γ$^+$ cells and anti-HPV specific CD8 T cells were defined as viability dye$^-$ CD56$^-$ CD19$^-$ CD3$^+$ CD4$^-$ CD8$^+$ IFN-γ$^+$ cells.

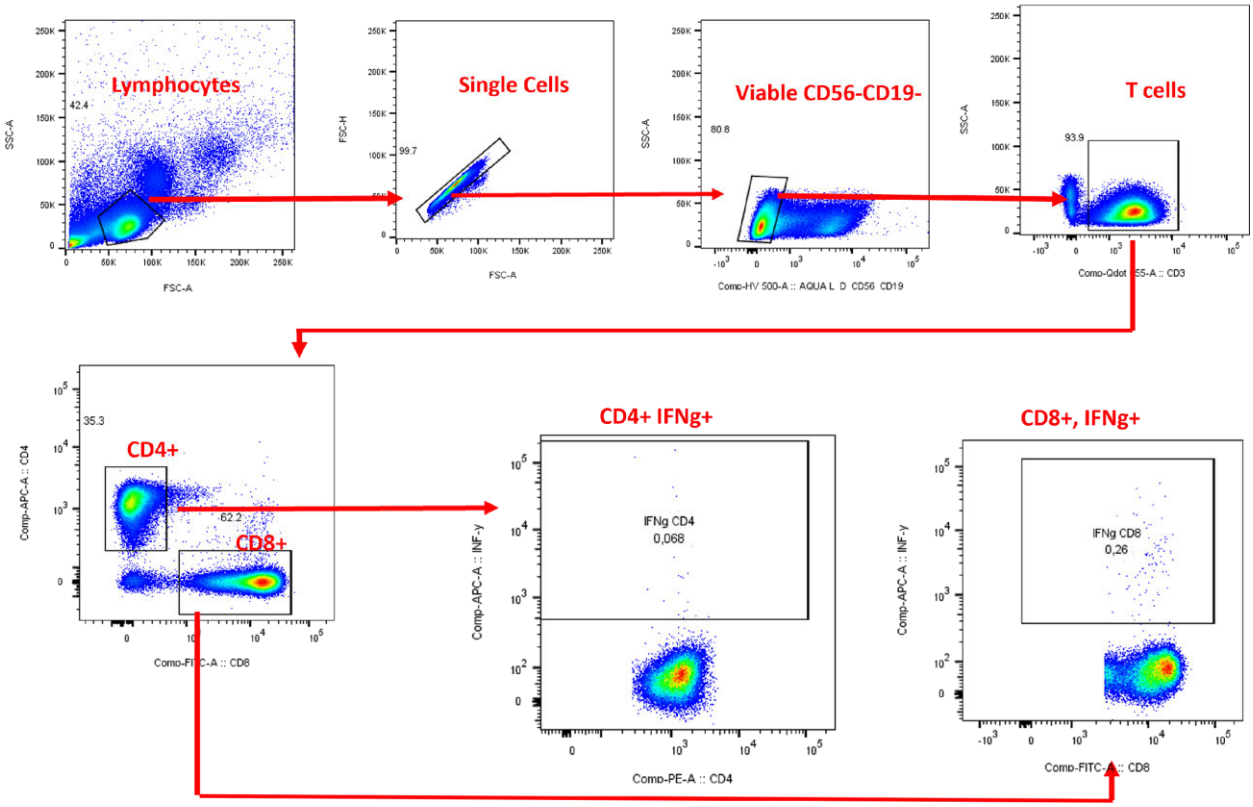

Figure 2.3. Gating strategy for determination of anti-HPV CD4 and CD8 T cells. Anti-HPV specific CD4 T cells were defined as Viability dye$^-$ CD56$^-$ CD19$^-$ CD3$^+$ CD8$^-$ CD4$^+$ IFN-γ$^+$ cells. Anti-HPV specific CD8 T cells were defined as Viability dye$^-$ CD56$^-$ CD19$^-$ CD3$^+$ CD4$^-$ CD8$^+$ IFN-γ$^+$ cells.

Chapter 3: The relationship between high-risk HPV genotypes, high-risk HPV viral loads and treatment outcomes in women living with HIV undergoing excisional treatment for cervical intraepithelial neoplasia

Yanga Mdleleni[1], Wendy Dhlomo[2], Alltalents T Murahwa[3,4], Anna-Lise Williamson[3,5], Polycarp Mogeni[6,7], Mosa Moshabela[8], Thumbi Ndung'u[1,9], Bongiwe Ndlovu[1], Daniel Muema[9,10]

[1]*HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R. Mandela.*

[2]Discipline of Obstetrics & Gynaecology, *University of KwaZulu-Natal, Durban, South Africa*

[3] *Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, Observatory, Cape Town, South Africa.*

[4] *Wellcome Centre for Infectious Diseases Research in Africa (CIDRI-Africa) Faculty of Health Sciences.*

[5] *SAMRC Gynaecological Cancer Research Centre, Faculty of Health Sciences, University of Cape Town, Cape Town 925, South Africa*

[6]*Faculty of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, London, UK*

[7] *Kenya Medical Research Institute, Nairobi, Kenya.*

[8] *University of Cape Town, Cape Town 7925, South Africa*

[9] *Africa Health Research Institute (AHRI) Durban, South Africa.*

[10] *Kenya AIDS Vaccine Initiative (KAVI) Institute of Clinical Research - University of Nairobi*

Abstract

The persistence of high-risk HPV drives the development of cervical intraepithelial neoplasia (CIN) and progression to cervical cancer. Furthermore, women living with HIV have a higher incidence of CIN and experience higher rates of CIN recurrence after treatment when compared with HIV-negative women, thus increasing their risk of cervical cancer. Effective biomarkers for predicting CIN recurrence in this population will enable the design of treatment programs that achieve timely diagnosis and treatment upon CIN recurrence. We assessed virological predictors of CIN recurrence after treatment in a case-control study of 96 South African women living with HIV who had high-grade cervical lesions at enrolment, received treatment by surgical excision of CIN by Loop Electrosurgical Excision

Procedure (LEEP), and were monitored by colposcopy, histology, and cytological screening for CIN recurrence. Using cervicovaginal lavage (CVL) and cytobrush samples, we determined the infecting HPV genotypes at enrolment and the HPV viral loads for 13 high-risk HPV types longitudinally at enrolment, 6 months, and 12 months. Women who experienced CIN recurrence after excisional surgical treatment had higher numbers of high-risk HPV types at enrolment ($p=0.008$) and higher high-risk HPV viral loads at both time points ($p<0.001$). Notably, the combined presence of both high-risk HPV types and high HPV viral load was the best predictor for CIN recurrence in a multivariable regression model (odds ratio 1.985, 95% CI 1.020 to 3.865, $p=0.044$). These data reinforce the potential role of combining HPV genotyping and HPV viral load quantification as prognostic tools in identifying women at the highest risk of CIN recurrence after treatment.

Keywords: HPV; viral load; cervical intra-epithelial neoplasia; cervical cancer

3.1. Introduction

Despite being preventable, cervical cancer is one of the leading causes of death among women globally. Approximately 604,000 cases of cervical cancer and 342,000 deaths were reported globally in 2020 [29, 30]. Over 87% of cervical cancer cases and 90% of deaths occur in Low- and Middle-income countries (LMICs) [240]. Notably, the same LMICs, especially Sub-Saharan Africa (SSA), also bear a disproportionate burden of Human Immunodeficiency Virus (HIV) [34-36]. The SSA region is home to approximately 71% of the 38 million people who are living with HIV (PLHIV) globally [34-36]. Even though antiretroviral therapy (ART) has successfully increased the lifespan of PLHIV, comorbidities such as cervical cancer still pose a huge burden. As such, cervical cancer is classified as an AIDS-defining ailment as its incidence is approximately six-fold higher among WLWH when compared to HIV-negative women [52, 53].

The development of both precancerous cervical intraepithelial neoplasia (CIN) and cervical cancer has been linked to persistent infections with particular oncogenic Human Papillomavirus (HPV) genotypes, which are classified as high-risk types [87]. These include HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68; HPV 16 and 18 are associated with approximately 70% of all cervical cancer cases

[88]. More than 60% of low-grade CIN lesions (classified as CIN1) regress spontaneously due to effective natural immune response. However, some low-grade CIN lesions progress to high-grade CIN lesions (classified as CIN2+), which are recognised as true pre-invasive precursors and may further progress to cervical cancer [241, 242]. Conization of the cervix, through either cryotherapy, LLETZ, loop electrical procedure (LEEP), or laser ablation, is a therapeutic procedure that can effectively eradicate high-grade CIN lesions (CIN2 and CIN3, referred to as CIN2+) [243, 244]. Notably, WLWH have a higher prevalence of HPV infection and are more likely to be infected with multiple HPV types, especially high-risk types that tend to persist, subsequently leading to elevated prevalence of CIN and increased incidence of cervical cancer [245-247].

Cervical cancer screening has successfully reduced the incidence and mortality related to cervical cancer, especially in high-income countries. However, there are enormous disparities in SSA where CIN recurrence ranges between 20% and 75% among WLWH [248, 249]. Immune suppression due to HIV infection, HPV latency, infection with high-risk HPV types, and inadequate removal of lesions have been described as risk factors for residual and/or recurring CIN lesions after treatment [179, 250-252]. Considering that early diagnosis of CIN recurrence is a fundamental determinant of prognosis, there is an urgent need to best identify women who are at risk of CIN recurrence. HPV viral load has been associated with persistent CIN [253-257]; however, there are very few studies that have assessed its link with CIN recurrence, and their findings are contradictory [255, 258-260]. We, therefore, aimed to determine the HPV virological predictors of CIN recurrence after surgical excisional treatment in WLWH. We show that infection with high-risk HPV types, multiple HPV infections, as well as high-risk HPV viral loads at baseline and after treatment, are associated with CIN recurrence. This work could potentially impact prevention of HPV-related disease, clinical care decisions, as well as post-treatment follow-up guidelines for WLWH.

3.2. Materials and Methods

3.2.1 Study population

A subset of Ninety-six participants was selected from the parent HPV Clearance and Treatment Outcomes of Cervical High-grade Squamous Intra-epithelial Lesions (HATCH) cohort from the King Edward Hospital, Durban, in KwaZulu Natal, South Africa, as described in section 2.1. Participants were characterized for the determination of HPV virological predictors of CIN recurrence after surgical excisional treatment by Loop Electrosurgical Excision Procedure (LEEP) in WLWH, twenty-seven of whom had experienced CIN recurrence and sixty-nine of whom had not experienced CIN recurrence during the 1-year follow-up. All participants had archived cervicovaginal lavage fluid (CVL) and cytobrush specimens that were used for HPV genotyping and HR-HPV viral load quantification as described in detail in section 2.3. Written informed consent was obtained from each participant, and ethical approval was obtained from the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (BE070/16 and BREC/00000815/2019).

3.2.2. HPV Genotyping

HPV Genotyping was conducted from CVL pellet samples collected at baseline, using the Roche Linear Array assay (Roche Molecular Systems, Inc.). This assay distinguished and detected 37 high-risk, possible high-risk, as well as low-risk HPV genotypes as described in chapter 2.3.

3.2.3. HPV Viral load quantification

HR-HPV viral load was quantified from DNA extracted from cervical cytobrush samples collected from baseline, 6- and 12-months post CIN excisional treatment. This quantification was carried out using the Hybrid Capture II commercial kit (QIAGEN, Gaithersburg, USA) following the manufacturer's instructions. Hybrid Capture II is a full-length hybridisation commercial assay with signal amplification using a microplate luminescent detection technique. This assay was used to quantify the viral load of

thirteen high-risk HPV types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) as described in section 2.3.2.

3.2.4. Statistical analyses

A chi-square test was used to compare the two groups of participants with regard to categorical variables, including age, age at sexual debut, relationship status, number of sexual partners, highest level of education attained, employment status, history of smoking, baseline pap smear, and histology. The Mann-Whitney test was used to compare the number of HPV types, HR-HPV viral load, the expression levels of β-globin, and CD4+ T-cell counts between the two groups. The Wilcoxon signed-rank test was used for intragroup paired comparison between time points for HPV viral load and β-globin. A logistic regression model was used to assess the prediction of CIN recurrence by HPV viral load upon adjustment for the impact of the presence of HR-HPV types, number of HR-HPV types, age, and HIV viral load. Receiver operating curves (ROC) were generated to determine if HPV viral load is a reliable predictive tool for CIN recurrence. The p-values less than 0.05 from two-sided tests were considered significant. Data was analyzed using GraphPad Prism version 9 (GraphPad Software, La Jolla, CA, USA) and STATA version 17 (StataCorp, College Station, TX, USA).

3.3. Results

The median age of the participants who were selected for this sub-study was 35 years. There was no difference in median age at enrolment and age at sexual debut between the Recurrence and No-recurrence groups (Table 3.1). Furthermore, there were no significant differences in the number of sexual partners, the histology appearance of the baseline pap smear, and CD4+ T cell counts between the two groups. Over 92% of women had high-grade lesions (HGSIL), and there was no significant difference between the two groups (96% in the Recurrence group and 90% in the No-recurrence group, $p= 0.30$). Notably, women in the Recurrence group had significantly lower education levels and lower employment rates when compared to the No-recurrence group ($p<0.0001$ and $p=0.0073$, respectively) (Table 3.1).

Table 3.1. Baseline characteristics of study participants

Demographic and Clinical Information	characteristics	Recurrence n (%)	No-recurrence n (%)	P values
		N=27	N=69	
Age (years at enrolment)	<30	0(0)	6(8.7)	0.114
	30+	27 (100)	63 (91.3%)	
Age at first sexual experience	<18 years	7 (25.9)	26 (37.7)	0.276
	>=18 years	20 (74.1)	43 (62.3)	
Sexual partners	None	6 (22.2)	13 (18.8)	0.641
	1	21 (77.8)	55(79.7)	
	>1	0	1 (1.4)	
Baseline papsmear	HGSIL	26 (96.3)	62 (89.9)	0.305
	LGSILx2	1 (3.7)	7 (10.1)	
CD4+ T cell counts (cells/mm^3) [a]	<=350	8(29.6)	18(30.5)	>0.999
	>350	19 (70.4)	41(69.5)	
Relationship status	Single	7 (25.9)	14 (20.3)	0.591
	Married	20 (74.1)	55 (79.7)	
Highest level of education attained	Passed grade 12	7 (25.9)	50 (72.5)	**<0.0001**
	Did not pass grade 12	20 (74.1)	19(27.5)	
Smoking status[b]	Never	23 (85.2)	63 (92.6)	0.268
	Ex-smoker	4 (14.8)	5 (7.4)	
Employment status	Employed	7(29.1)	43 (62.3)	**0.007**
	Unemployed	20 (74.1)	26 (37.7)	
HIV viral load	<20	20 (74.1)	52 (75.4)	>0.999
	>20	7(29.1)	17 (25.1)	

[a] 10 participants in the No-recurrence group did not have data on CD4 counts.

[b] 1 participant in the No-recurrence group did not have data on smoking status.

All values are represented as whole numbers or percentages (%). A chi-square test was used to compare the two groups of participants with regard to age group, age group at sexual debut, relationship status, number of sexual partners, highest level of education attained, employment status, whether the participants were smokers or had any history of smoking, and a baseline pap smear. The Mann–Whitney test was used to compare the CD4+ T cell count between the two groups. LGIL: low-grade Squamous intraepithelial lesions, HGSIL: high-grade Squamous intraepithelial lesions.

3.3.1. Relationship between multiplicity of infections with high-risk HPV types and recurrence of CIN in women living with HIV undergoing excisional treatment

We first determined the overall frequencies and the numbers of the most prevalent HPV types in the women who were included in this sub-study, then conducted comparisons between the Recurrence and the No-recurrence groups. Interestingly, women in the Recurrence group had more detectable HR-HPV types at baseline when compared to those in the No-recurrence group (p=0.008) (Figure. 3.1A and B). In addition, 74% of women in the Recurrence group had detectable HR-HPV types when compared to 43% of women in the No-recurrence group (Figure. 3.1A and B). HPV 16 was the most prevalent HPV type in the cohort, followed by HPV 18, HPV 35, HPV 58, HPV 72, HPV 61, HPV 62, and HPV 71. However, there were no significant differences in the frequency of each of the HPV types between the Recurrence and No-recurrence groups.

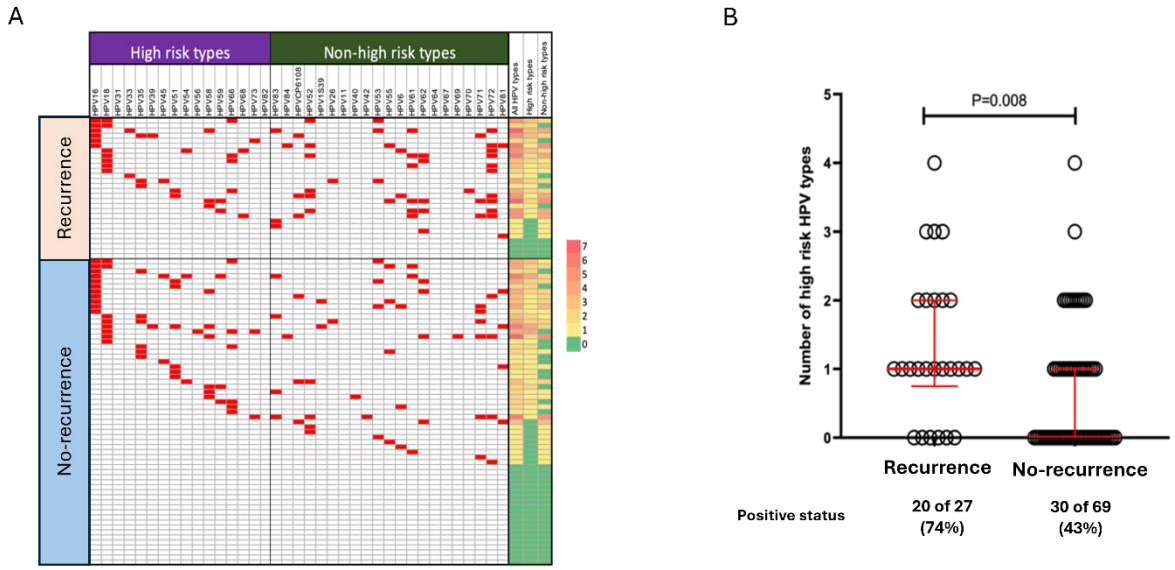

Figure 3.1. Recurrence of CIN is associated with a high prevalence of high-risk HPV types. (A) Heatmap showing detectable HPV genotypes in each participant measured at baseline. The red squares indicate a positive result, while clear squares indicate a negative result for a particular HPV type. (B) Comparison of the number of HPV types in the Recurrence and No-recurrence groups at baseline. Horizontal lines and error bars represent median, 25th, and 75th percentiles. Statistical test used: Mann-Whitney test. P values < 0.05 were considered significant.

3.3.2. Relationship between high-risk HPV viral loads and recurrence of CIN in women living with HIV undergoing excisional treatment

To understand the role of HPV viral load on CIN recurrence, we measured the viral load of 13 HR-HPV types, mainly 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, using the Digene Hybrid Capture II assay (Qiagen Inc.). Viral loads were measured at baseline, 6 months, and 1-year post-CIN treatment in women with and without CIN recurrence after excisional treatment. Women in the Recurrence group started off with a significantly higher HPV median viral load at baseline when compared to those in the No-recurrence group (p<0,001) (Figure. 3.2A). Notably, in both the Recurrence and No-recurrence groups, the viral loads decreased significantly at 6 months and 1 year after the surgical excision of squamous intra-epithelial lesions (P<0.05 in all comparisons between post-treatment timepoints and baseline). However, the viral load remained significantly higher among the Recurrence group (p<0,001), while the viral loads of the No-recurrence group were mostly below the level of detection at 6 months and 1 year after treatment (Figure. 3.2A). To confirm that the viral load results were not driven by differences in DNA integrity and numbers of cells recovered during specimen collection, for each of the DNA samples used for HPV viral load quantification, we measured the human housekeeping gene, β -globin, across all time points. There were no differences between groups and time points in β-globin gene quantities, suggesting that the DNA in all samples was of good quality and comparable quantities (Figure. 3.2B).

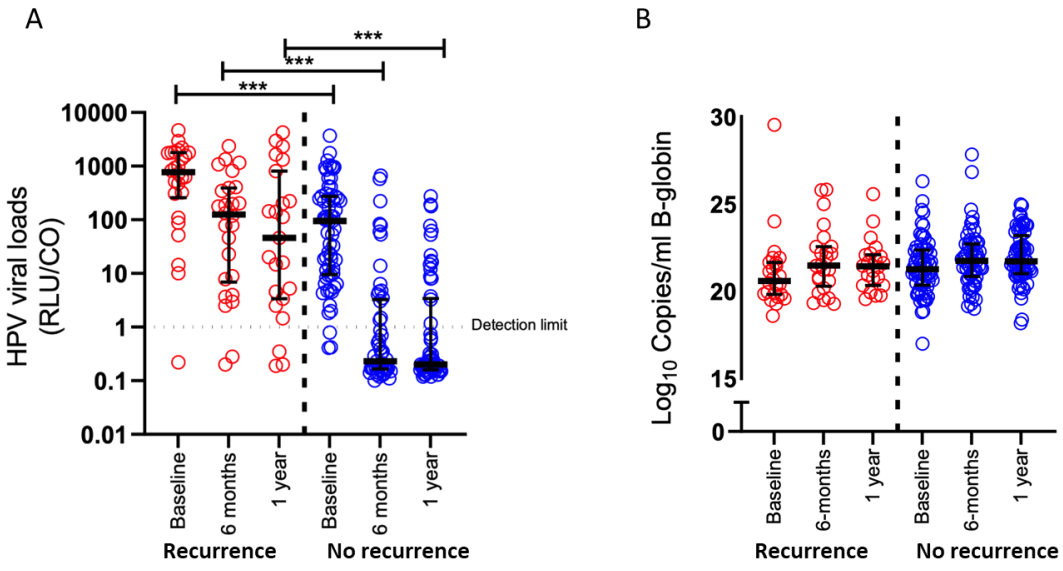

Figure 3.2. Recurrence of CIN lesions after treatment is associated with high and persisting HPV viremia. (A) Changes in high-risk HPV viral load over time before and after CIN treatment in women with no CIN recurrence and those with recurrence. (B) Longitudinal quantification of β-globin gene before and after treatment in women with CIN recurrence and those with No-recurrence. The Recurrence group is shown in red circles, while the No-recurrence group is shown in blue circles. Horizontal lines and error bars represent median, $25^{th,}$ and 75^{th} percentiles. HPV viral load is represented as relative light units of the number of HPV copies/ml. Statistical test used: Mann-Whitney test. *** indicates $p<0.001$.

3.3.3. Multivariable regression analyses to determine the predictors of recurrence of CIN after excisional treatment in women living with HIV

We then used simple and multivariable logistic regression models to assess the risk factors that are associated with CIN recurrence after excisional treatment. Covariates such as the HR-HPV viral load, presence of HR-HPV types, presence of LR-HPV types, number of HR-HPV types, number of LR-HPV types, age, and HIV viral load were first analysed in simple logistic regression models for the prediction of recurrence. We noted that the HR-HPV viral load (OR 1.6, CI 1.2 - 2.2, p=0.001), the presence of HR-HPV types (OR 2.8, CI 1.0-7.957, p=0.043), and the number of HR-HPV types (OR 1.9, CI 1.1 – 3.3, p=0.019) were significantly associated with CIN recurrence in the simple logistic regression. Notably, the interaction between the HR-HPV viral loads and presence of HR-HPV types had a significant association in the multivariable logistic regression (OR 2.0, CI 1.0 – 3.9, p=0.044). While, the age at enrolment, HIV viral load, and the presence of LR-HPV types were not associated with CIN recurrence (Table 3.2).

Table 3.2. Simple and Multivariable logistic regression model to assess risk factors associated with CIN recurrence after excisional treatment

	Simple Logistic Regression			Multivariable Logistic Regression		
Covariate	**OR**	**95% CI**	**P-value**	**OR**	**95% CI**	**P-value**
Log HPV viral load	1.6	1.2 -2.2	**0.001**	1.1	0.8 - 1.7	0.568
Presence of high-risk serotype	2.8	1.0 - 7.6	**0.043**	0.0	0.0 – 2.0	0.107
Log HPV Viral *Presence of HR-HPV	ND	ND	ND	2.0	1.0 - 3.9	0.044
Presence of low-risk serotype	1.5	0.6 - 3.8	0.389	ND	ND	ND
Age	1.0	0.9 - 1.1	0.651	ND	ND	ND
Log HIV Viral Load	1.1	0.8 - 1.3	0.625	ND	ND	ND
Number of high-risk serotypes	1.9	1.1 - 3.3	**0.019**	ND	ND	ND
Number of low-risk serotypes	1.2	0.9 - 1.7	0.224	ND	ND	ND

ND: Not done

We also used receiver operator characteristic (ROC) curves to further assess if baseline HR-HPV viral load can be used as a tool for predicting CIN recurrence. The ROC curves were constructed by computing the true positive rate against the false positive rate for various cut-offs of HR-HPV viral loads. The accuracy of the test for discriminating women with CIN recurrence from those without CIN recurrence was assessed by the area under the ROC curve that was computed using maximum likelihood estimates to fit a smooth curve to all data points. We observed that baseline HR-HPV has a predictive potential for CIN recurrence, with 0.7829 as the area under the ROC curve (Figure. 3.3A). Expectedly, higher baseline HR-HPV viral load was associated with higher specificity but lower sensitivity for differentiating women who would have CIN recurrence from those who would not have recurrence after treatment (Figure. 3.3B).

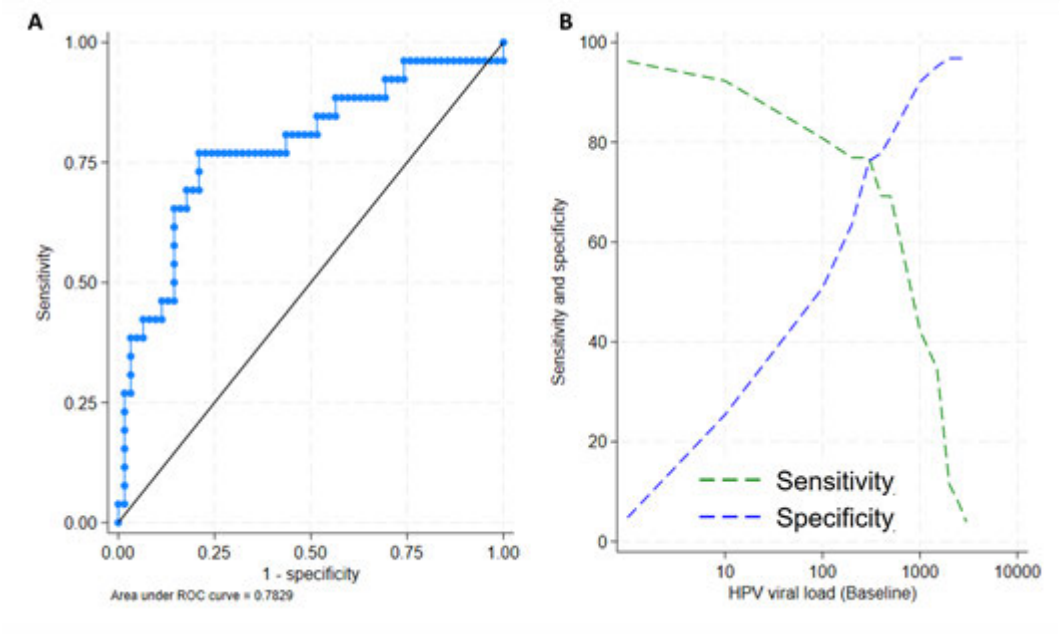

Figure 3.3. Specificity and sensitivity of baseline high-risk HPV viral load as a tool for predicting recurrence of cervical intraepithelial neoplasia after excisional treatment. (A) Receiver operator characteristic (ROC) curve showing predictive potential of high-risk HPV viral load for recurrence of cervical intraepithelial neoplasia. (B) Sensitivity and specificity of baseline high-risk HPV viral load in predicting recurrence of cervical intraepithelial neoplasia at different cut-offs.

3.4. Discussion

This study aimed to investigate the role of HR-HPV viral load on the risk of CIN recurrence in WLWH following CIN excisional treatment. We examined differences in combined HPV viral load for thirteen high-risk HPV types at enrolment, 6 months, and 12 months after treatment. We also assessed the number of infecting HPV types, for both HR-HPV and LR-HPV types, as a covariate at enrolment. We hypothesized that HR-HPV viral loads, in addition to numbers of infecting HPV types, would be associated with CIN recurrence in these women. It is important to note that the women enrolled in our study were HIV virally suppressed and had been on ART for more than 6 months. Even though some women had CD4+ T cell counts below 350 cells/mm^3, there were no differences between the two comparator groups.

WLWH with CIN recurrence had a significantly higher number of HPV types at baseline, the majority of which were oncogenic HPV types, when compared to the No-recurrence group. This is indirectly in keeping with other studies that have reported that multiple HR-HPV infections are associated with an increased risk of high-grade CIN and invasive cervical cancer among African WLWH of similar age groups as our study participants [12, 261, 262]. Moreover, a meta-analysis of women with histologically proven high-grade lesions suggested that multiple HR-HPV infections detected before or at the time of treatment were associated with a minor but significant increase in the risk of persistence and/or recurrence of high-grade cervical lesions [263]. It is, however, still unclear whether the association between multiple HR-HPV types and CIN recurrence is due to higher cumulative HR-HPV viral loads, due to the contribution by each infecting genotype.

In this study, using an assay that reports combined readings of HPV viral loads for 13 HR-HPV types, we further observed higher HR-HPV viral loads in recurrence compared to the No-recurrence group. Notably, viral load declined in both groups after treatment but still remained consistently higher in the recurrence group across all timepoints, suggesting a sustained positive association between persistence of HR-HPV viral load and CIN recurrence. These findings have been corroborated by previous studies that reported higher HR-HPV viral load as a predictor of HPV persistence as well as CIN persistence [153, 255, 264, 265]. Our findings extend this association to WLWH. WLWH have previously been reported to have higher HPV viral loads and a higher likelihood of multiple HPV infections, and it is likely that the combined impact of these two occurrences accounted for treatment outcomes in our study [42, 266, 267]. There is, however, still no consensus on whether HR-HPV viral loads across all HR-HPV types contribute to severe disease and poor treatment outcomes as opposed to particular HR-HPV infections [156, 268].

The majority of studies have only reported on HPV 16 and/or 18 viral loads, while our study explored the role of combined viral loads for multiple HR-HPV types in CIN recurrence [54, 109, 158, 269]. Future studies could explore the associations between viral loads for individual HPV types and CIN recurrence. Interestingly, we also found a significant association between CIN recurrence and the level of education and employment status of women. It has previously been reported that women with a low

education status are likely to engage in unprotected sex and are likely not to present for cervical cancer screening or follow-up [270, 271]. Although the lack of education and unemployment may not be considered as a direct and well-defined exposure to CIN recurrence, it could be a combination of characteristics that predominate among women in LMICs and, therefore, exacerbate their risk of multiple HPV infections, high HR-HPV viral loads, and higher risk of cervical cancer. This highlights the role of socioeconomic factors in the progression of HPV infection, and underscores an urgent need for women empowerment to reduce the impact of infectious diseases.

The limitations of our study included, firstly, the measurement of total HR-HPV viral load instead of type-specific quantification. The Hybrid Capture II assay is widely used and an acceptable genotyping and viral load quantification test due to its ability to quantify multiple HPV types in a single reaction. It also has a good turnaround time, allowing it to be used in resource-constrained settings; hence, it was chosen for our study. To introduce type-specific resolution into these analyses, future studies could consider assays that quantify high-risk type-specific HPV viral load, especially HPV 16 and HPV 18. Secondly, the HPV genotyping was only performed on baseline samples. However, there is evidence that baseline genotyping is sufficient when studying recurrence after CIN treatment when follow-up timepoints are within 1 year after. Nonetheless, in our future work, we will consider genotyping across all timepoints to assess if there are changes in HPV infections at post-treatment timepoints.

A major strength of this study is that WLWH were followed up post-treatment of CIN with longitudinal determinations of HR-HPV viral loads, therefore allowing us to study HPV viral loads dynamics, and their association with CIN recurrence, in the context of HIV-HPV coinfection. Considering that both the prevalence of HIV and the incidence of cervical cancer are high in the sub-Saharan African population, this study provides valuable insights into the dynamics of HPV infections among WLWH who constitute a significant population in the region. In summary, our study shows that CIN recurrence in WLWH is strongly associated with both higher HR-HPV viral loads and the presence of multiple HR HPV infections. As such, HR-HPV viral load could be added as a biomarker for predicting CIN recurrence in WLWH who undergo excisional CIN treatment, to inform monitoring at follow-up timepoints and prevent progression to invasive cervical cancer.

Chapter 4: The Relationship between anti-HPV T cell responses, high-risk HPV viral load and treatment outcome in women living with HIV undergoing excisional treatment for cervical intraepithelial neoplasia

Yanga Mdleleni[1], Wendy Dhlomo[2], Sam Rasehlo[3], Thumbi Ndung'u[1,3], Bongiwe Ndlovu[1] and Daniel Muema[1, 3, 4]

[1]*HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa*

[2]Discipline of Obstetrics & Gynaecology, *University of KwaZulu-Natal, Durban, South Africa*

[3]*Africa Health Research Institute (AHRI) Durban, South Africa*

[4]*KAVI-Institute of Clinical Research - University of Nairobi*

Abstract

The immunological basis for HPV-associated cervical intraepithelial neoplasia (CIN) recurrence in treated women living with HIV (WLWH) are not fully understood. Yet, immune responses, such as cytotoxic T cell responses, are believed to play a major role in the clearance of HPV infections. In this study, WLWH with CIN who were treated using loop electrosurgical procedure (LEEP) were investigated for the associations between CIN recurrence and magnitudes of naturally elicited anti-HPV T cell responses to E6 and E7 oncoproteins of high-risk HPV types 16 and 18. HPV genotyping had been conducted at baseline using the Roche Linear array. A total of 28 participants (12 from the Recurrence and 16 participants from the No-Recurrence groups) who were positive for HR-HPV 16 and HPV 18 were included in this sub study. Naturally elicited anti-HPV IFN-γ+ CD4+ and CD8+ T cell responses were measured in peripheral blood mononuclear cells using intracellular cytokine staining at baseline and 1-year post-treatment. Participants were stratified into "No-recurrence" and "Recurrence" groups based on clinical CIN detection at either six months or one-year post-treatment. The study revealed stable kinetics of anti-HPV16 and anti-HPV18 E6- and E7-specific T cell immunity

post-treatment in blood for both “No-recurrence” and “Recurrence” groups. Notably, there were no differences in responses between the “Recurrence” and “No-recurrence” groups, suggesting minimal impact of naturally elicited systemic anti-HPV T-cell responses on treatment success. Interestingly, the frequencies of IFN-γ+ producing CD8 T cells to the E7 oncoprotein were significantly higher in women with CIN 3 when compared to those with CIN 2 (p=0.033) at the pre-treatment time point and had a trend for a positive correlation with HPV viral loads at 1 year time point (Rho= 0.38, p=0.08), suggesting modest dependence on antigen load. We conclude that the naturally elicited systemic HPV-specific T-cell responses are largely stable in WLWH even after CIN treatment and do not predict the success of excisional treatment. Their possible contribution to CIN clearance in WLWH needs to be further investigated to guide HPV therapeutic vaccine strategies.

Key words: HPV; T cells; CD8 T cells; CD4 T cells, CIN; disease grade; HPV16; HPV18; E6; E7; ICS

4.1. Introduction

There are six licensed prophylactic HPV vaccines that have shown high efficacy in preventing HPV infections [272, 273]; however, sub-Saharan Africa has made limited progress in the implementation of nationwide HPV vaccination programmes, with current rollouts reported to be below 1% [274-276]. The majority of women in sub-Saharan Africa, including women living with HIV (WLWH), will therefore miss these prophylactic HPV vaccines and remain susceptible to high risk HPV (HR-HPV) infection, cervical intraepithelial neoplasia (CIN), and consequently, cervical cancer into the foreseeable future. Thus, there continues to be an urgent need for a therapeutic vaccine that is effective across all strata of the population, including WLWH who comprise a significant proportion of the sub-Saharan Africa population and are at an elevated risk of CIN and cervical cancer.

The majority of low-grade CIN (CIN1) regress spontaneously, possibly due to immune factors [277, 278]. On the other hand, women diagnosed with high-grade CIN (CIN2/3) are treated using conservative surgical modalities such as Large Loop Excision of the Transformation zone (LETZ) or Loop electrosurgical excision procedure (LEEP), cryotherapy, or cone biopsy to eliminate abnormal

cervical cells [279-283]. These procedures have 70-99% effectiveness in eliminating precancerous lesions and preventing progression to cervical cancer [284-286]. Considering that WLWH often have the highest cases of CIN recurrence, ranging between 20-75%, it is highly likely that the immune system synergizes with the excisional treatment to prevent residual or recurrent CIN [248, 249, 287, 288].

Indeed, a robust immune response, involving T-cell responses, is believed to be responsible for the spontaneous clearance of HPV infection and the regression of approximately 50-90% of CIN 1, 40% of CIN 2, and 30% of CIN 3 [126, 278, 289, 290]. This is particularly clear for local CD4+ and CD8+ T cells in cervical lesions that have been associated with regression of lesions [197, 291, 292]. However, there is contradicting data regarding naturally elicited IFN-γ T cell responses to HPV E6 and E7 in peripheral blood, which have been reported to be weak, thus requiring ex vivo sensitization to detect [293-295]. This is further compounded by the possible immune suppression in WLWH. The ability of blood responses to predict or facilitate the regression of CIN in WLWH, and their possible interaction with excisional therapy, is also unclear. This study aims to characterize the associations between blood HPV16 and HPV18 E6 and E7 specific CD4+ and CD8+ T cell responses and recurrence and/or No-recurrence of CIN in WLWH who are undergoing excisional treatment.

4.2. Materials and Methods

4.2.1. Study Design

Twenty-eight (16 in the "No-recurrence" group and 12 in the "Recurrence" group) women living with HIV, with undetectable HIV viremia and confirmed high-risk HPV 16 and HPV18 infection, were included in this sub-study. HPV-16 and HPV-18 E6 and E7 specific CD4+ and CD8+ T cell responses were measured from PBMCs at baseline and 1 year following LEEP treatment. The CD4+ and CD8+ T cell responses were then assessed for associations with treatment outcomes, HR-HPV viral loads, time points and CIN grades.

4.2.2 In-vitro stimulation of PBMCs with HPV 16 and 18 E6 and E7 peptides

Frozen PBMCs were thawed, stimulated with HPV-16 and HPV-18 E6 and E7 Peptide pools (JPT Peptide Technologies GmbH, Berlin, Germany), and incubated for 14 days in culture medium supplemented with 200 IU/ml of recombinant IL-2 (BioLegend). At the end of the culture period, the cells were restimulated with HPV-16 and HPV-18 E6 and E7 Peptide pools in the presence of Brefeldin A (BFA) and Monensin (Sigma-Aldrich) in preparation for intracellular cytokine staining as described in Chapter 2. Unstimulated negative controls were included in all assays.

4.2.3 Intracellular-cytokine staining

Intracellular-cytokine staining was done as described in Chapter 2. Briefly, re-stimulated cells were surface-stained with Aqua viability dye (Invitrogen) and a cocktail of anti-CD3-BV711 (BD Biosciences), anti-CD4-PE (BD Biosciences), and anti-CD8-FITC (BioLegend) antibodies, followed by permeabilization and intracellular staining with IFN-γ APC (BioLegend) antibodies. The cells were then acquired using an LSRFortessa cytometer (BD Biosciences) with FACSDiva™ software. Data were analysed using the FlowJo version 10.0.8 (FlowJo, LLC). Frequencies of anti-HPV CD4 and CD8 T cells were determined as shown in the gating strategy (Figure. 2.6 in Chapter 2).

4.2.4 Statistical analyses

The Mann-Whitney test (Wilcoxon rank sum test) was used to compare frequencies of IFN-γ+ CD4+ and CD8+ T cell responses to HPV 16/18 E6 and E7 peptides between the CIN "Recurrence" and "No-recurrence" groups. Similar comparisons were done between the CIN2 and CIN3 groups. Wilcoxon signed-rank test was used for comparisons between timepoints. Correlations between HPV viral load and the percentage IFN-γ CD4+ and CD8+ T cell responses to HPV 16/18 E6 and E7 peptides were carried out using Spearman's rank-order correlation test. P values were considered significant if they were less than 0.05. Data was analysed using GraphPad Prism version 9 (GraphPad Software, La Jolla, CA, USA).

4.3: Results

4.3.1. Relationship between blood anti-HPV T cell responses and recurrence of CIN in women living with HIV undergoing excisional treatment

To assess the possible association between anti-HPV T cell responses and treatment outcomes after excisional treatment in WLWH, we compared the IFN-γ responses in both CD4+ T cells and CD8+ T cells against HPV16/18 E6 and E7 oncoproteins, before treatment (baseline) and 1 year after CIN excisional treatment, between the Recurrence group and the No-recurrence group. There were no significant differences in the frequency of HPV 16/18 E6 and E7 CD4+ and CD8+ IFN-γ producing T cell responses between the two groups at either of the two timepoints, suggesting that IFN-γ responses do not contribute to outcomes of excisional treatment ($P>0.05$ in all comparisons) (Figure. 4.1 A-D). Notably, there were also no significant differences when we compared baseline responses against those at 1 year within each group ($P>0.05$ in all comparisons), suggesting that the T cell responses were generally stable over 1 year even after reduction of antigen load upon excisional treatment, regardless of treatment outcome. The only exception was a trend that was observed in the recurrence group, where the proportion of IFN-γ+ CD8 T cells to HPV 16/18 E6 was modestly higher at baseline when compared to 1 year after CIN excisional treatment ($p=0.054$) (Figure. 4.1C).

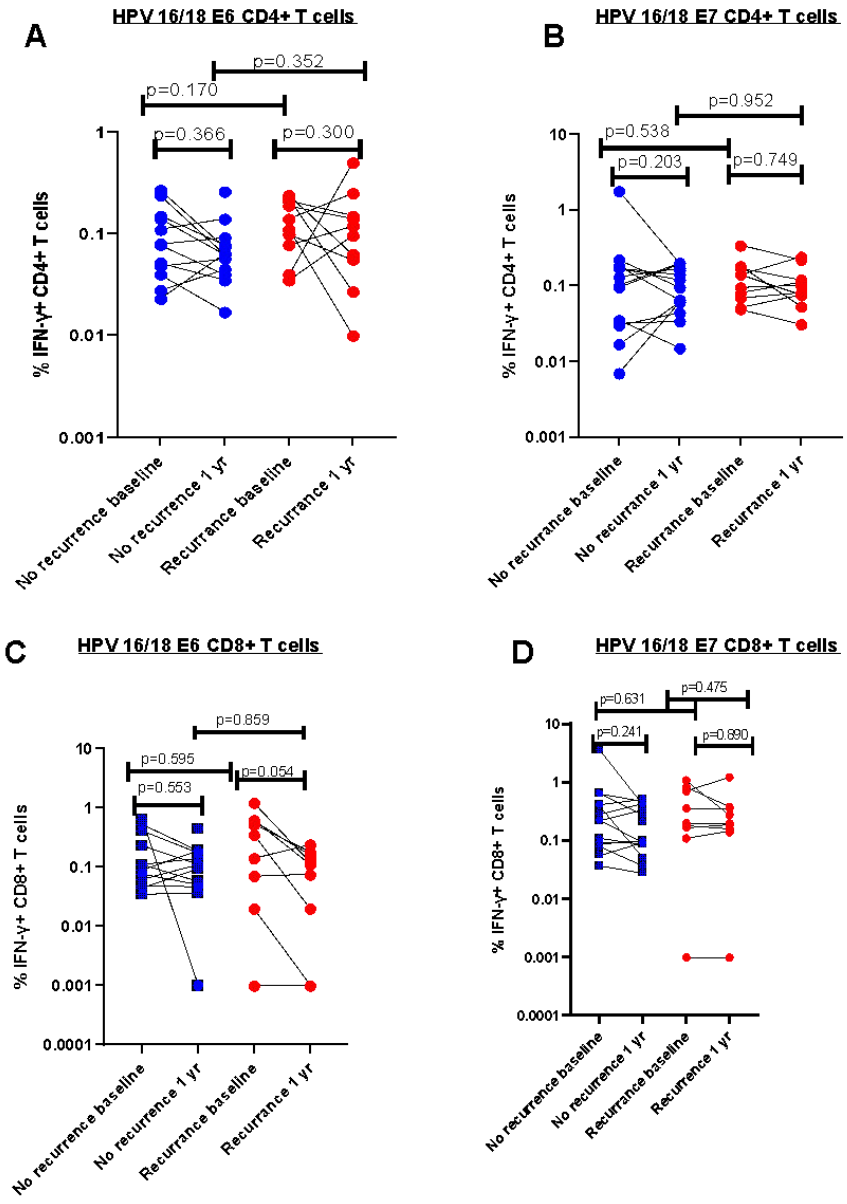

Figure 4.1. The percentages of IFN-γ+ CD8+ and CD4+ T cell response to HPV16/18 E6 and E7 antigens at baseline (before treatment) and 1 year after CIN excisional treatment in women living with HIV. (A) HPV16/18 E6-specific CD4+ T cell responses, (B) HPV16/18 E7-specific CD4+ T cell responses, (C) HPV16/18 E6-specific CD8+ T cell responses and (D) HPV16 and HPV18 E7-specific CD8+ T cell responses. Each symbol represents a participant; women with CIN recurrence are represented in red, while those with No-recurrence are represented in blue. Statistical tests used: Mann-Whitney test for comparisons between groups and Wilcoxon signed-rank test for comparisons between timepoints. P values < 0.05 were considered significant.

4.3.2. Relationship between blood anti-HPV T cell responses and CIN lesion grade in women living with HIV undergoing excisional treatment

To further explore the drivers of anti-HPV T cell responses in the cohort, we then assessed if there were associations between frequencies of anti-HPV T cells and lesion grades as well as HPV viral loads. Data on CIN grade were available at baseline, enabling re-stratification of the study participants into baseline CIN2 and CIN3 groups at baseline. The frequency of E6 IFN-γ+ CD4+ T cells, E7 IFN-γ+CD4+ T cells and E6 IFN-γ+ CD8 T cells did not differ between the CIN2 and CIN3 groups at baseline ($P>0.05$ in all comparisons) (Figure 4.2A-C). However, the frequency of E7 IFN-γ CD8+ T cells was significantly higher in CIN3 group when compared to CIN2 group ($p=0.03$) (Figure 4.2D). It is likely that these comparisons were impacted by samples size since we had only seven participants in the CIN2 group. Future studies with larger sample sizes could enable robust determination on whether CIN3 lesions are also associated with higher E6 IFN-γ+ CD4 T cells, E7 IFN-γ+CD4 T cells and E6 IFN-γ+ CD8 T cells responses.

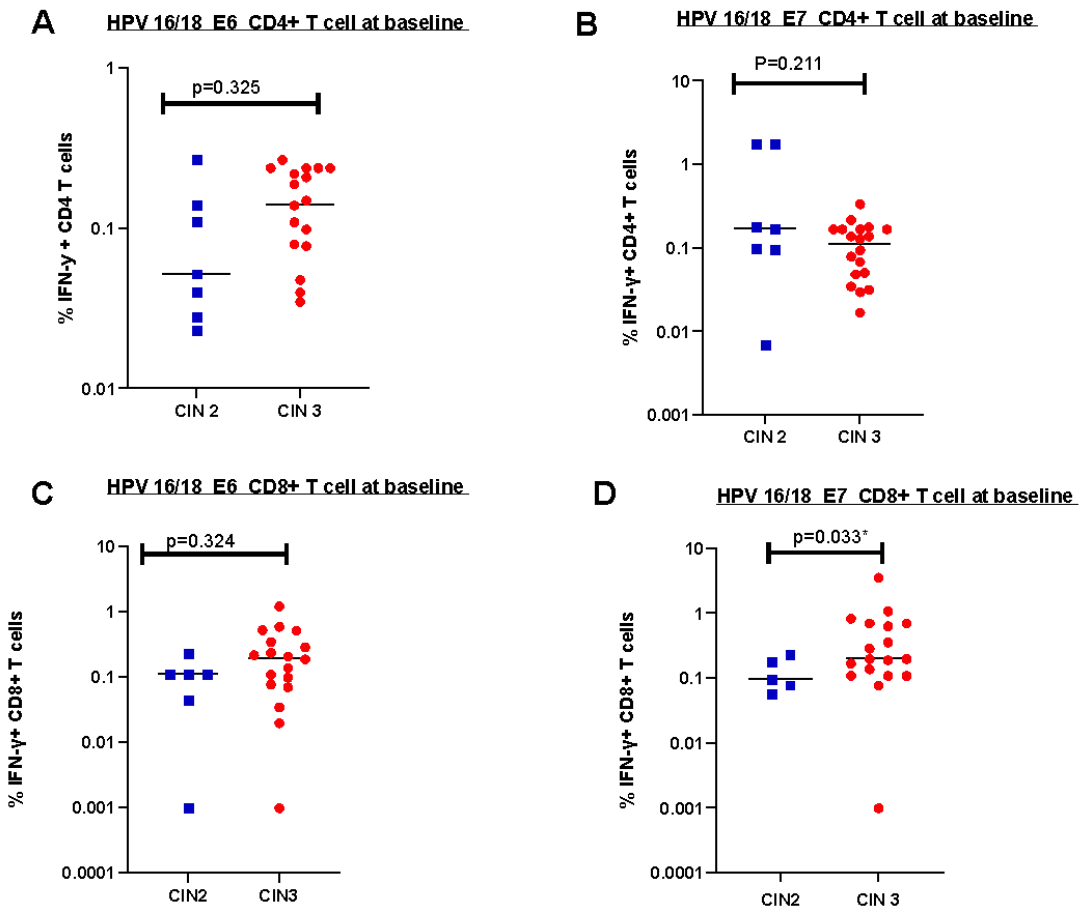

Figure 4.2. IFN-γ+ CD4+ and CD8+ T cell responses to HPV 16/18 E6 and E7 antigens, according to the grade of the lesion at the baseline timepoint; CIN 2 and CIN 3 in women living with HIV. Each symbol represents a participant; women with CIN 2 are represented in blue, while those with CIN 3 are represented in red. (A) IFN-γ+ CD4+ T cell responses to HPV16/18 E6 antigens at baseline, (B) IFN-γ+ CD4+ T cell responses to HPV16/18 E7 antigens at baseline, (C) IFN-γ+ CD8+ T cell responses to HPV16/18 E6 antigens at baseline. (D) IFN-γ+ CD8+ T cell responses to HPV16/18 E7 antigens at baseline. Statistical test used: Mann-Whitney test. P values < 0.05 were considered significant.

4.3.3. Relationship between blood anti-HPV T cell responses and high-risk HPV viral load in women living with HIV undergoing CIN excisional treatment

To directly assess if HPV antigen loads drive anti-HPV T cell responses, we further checked if there were correlations between high-risk HPV viral loads and the frequencies of anti-HPV T cells at both baseline and 1-year timepoints after excisional treatment. Similar to the above-reported lack of associations between anti-HPV CD4+ T cells and lesion grades, there were no significant correlations between the HR-HPV viral loads and the frequencies of anti-HPV CD4+ T cells at both timepoints

(p>0.05 in all comparisons) (Figure. 4.3 A-D). Likewise, there were no significant correlations between the HR-HPV viral loads and the frequencies of anti-HPV CD8+ T cells at both timepoints (p>0.05 in all comparisons) (Figure 4.4 A-D), even though a trend for a positive correlation was observed for anti-HPV E7 IFN-γ+ CD8 T cell responses at the 1-year timepoint (Rho=0.38, p=0.08) (Figure 4.4D).

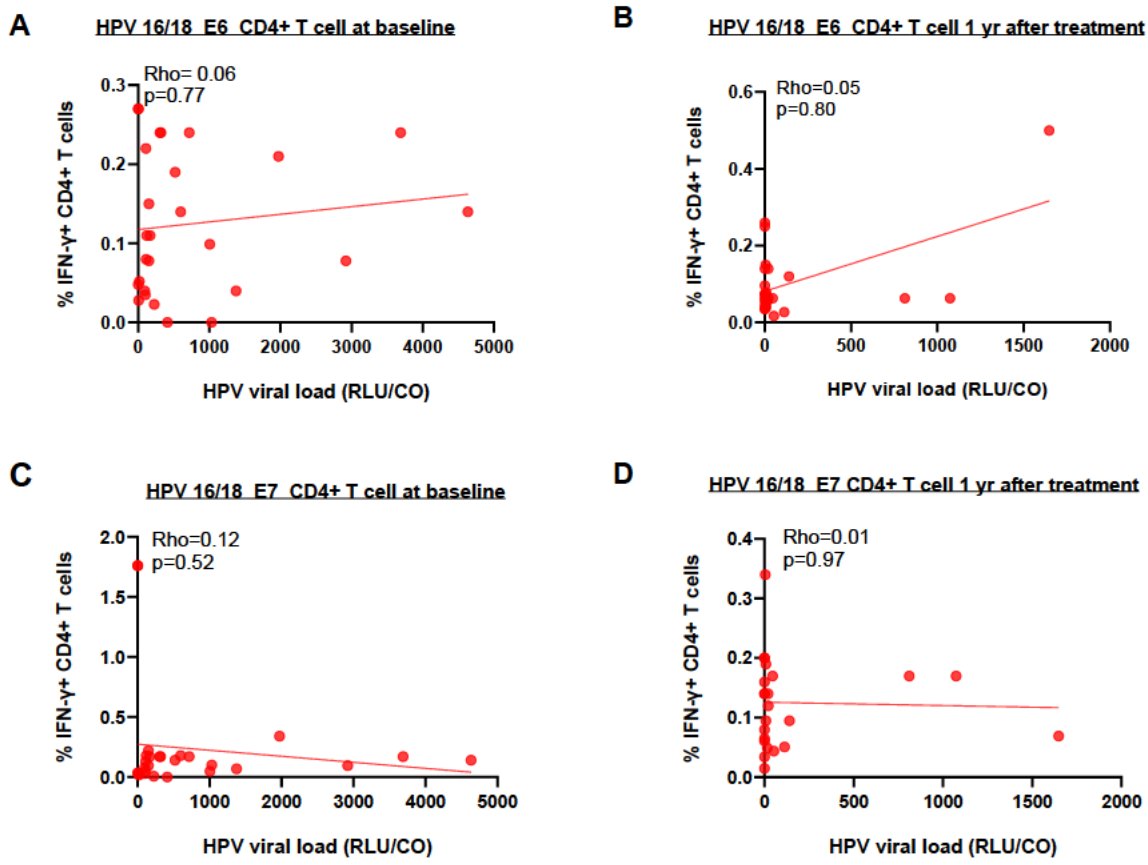

Figure 4.3. Correlation between IFN-γ+ CD4+ T cell responses to HPV 16/18 E6- and E7 antigens and high-risk HPV viral load at baseline and 1-year post CIN treatment in women living with HIV. (A) correlation between the percentage of IFN-γ+ CD4+ T cell responses to HPV 16/18 E6 and high-risk HPV viral load at baseline, (B) correlation between IFN-γ+ CD4+ T cell responses to HPV 16/18 E6 and high-risk HPV viral load 1 year after CIN excisional treatment, (C) correlation between IFN-γ+ CD4+ T cell responses to HPV 16/18 E7 and high-risk HPV viral load at baseline, (D) Correlation between the percentage IFN-γ+ CD4+ T cell responses to HPV 16/18 E7 and high-risk HPV viral load 1 year after CIN excisional treatment. Statistical test: Spearman's rank-order correlation. P values < 0.05 were considered significant.

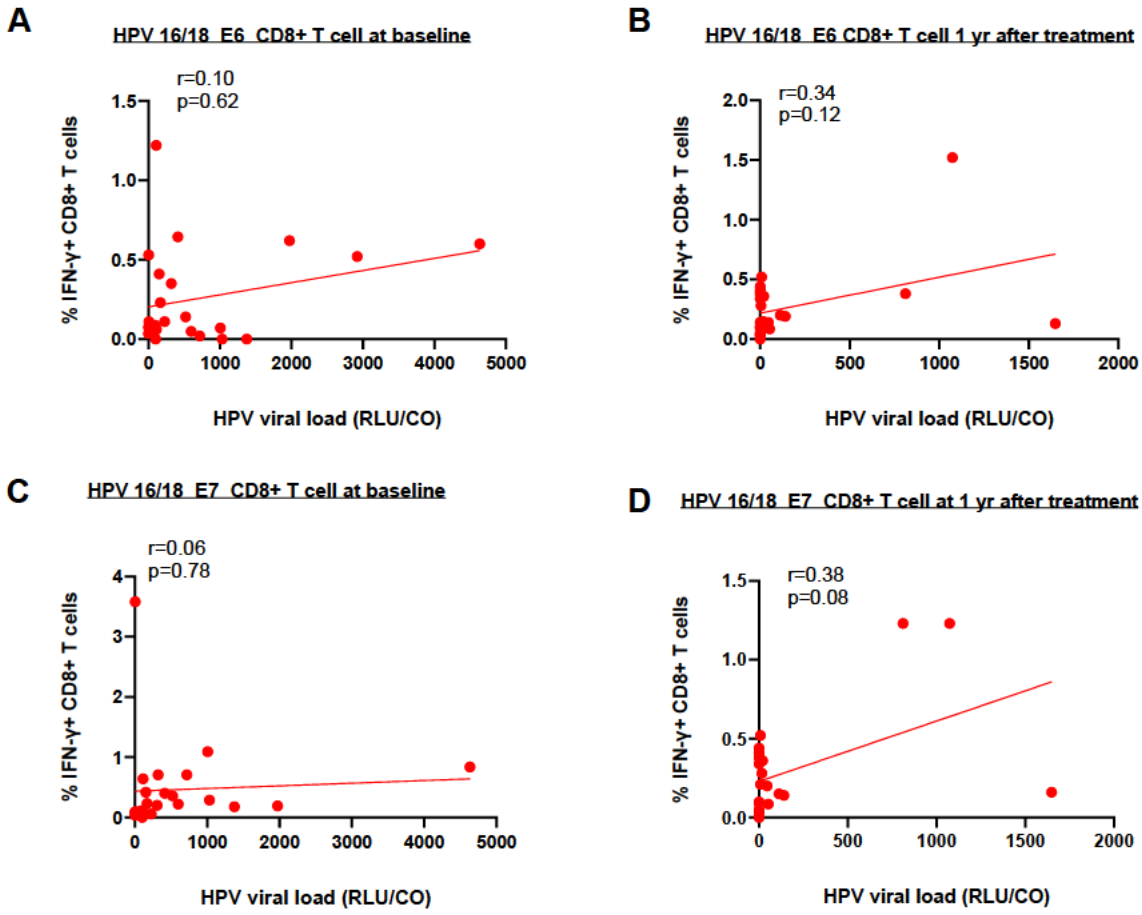

Figure 4.4. Correlation between IFN-γ+ CD8+ T cell response to HPV 16/18 E6- and E7 antigens and HPV viral load at baseline and 1-year post-CIN treatment in women living with HIV. (A) correlation between the percentage of IFN-γ+ CD8+ T cell responses to HPV 16 and HPV 18 E6 and high-risk HPV viral load at baseline, (B) correlation between IFN-γ+ CD8+ T cell responses to HPV 16 and HPV 18 E6 and high-risk HPV viral load 1 year after CIN excisional treatment, (C) correlation between IFN-γ+ CD8+ T cell responses to HPV 16 and HPV 18 E7 and high-risk HPV viral load at baseline, (D) Correlation between the percentage IFN-γ+ CD8+ T cell responses to HPV 16 and HPV 18 E7 and high-risk HPV viral load 1 year after CIN excisional treatment. Statistical test: Spearman's rank-order correlation. P values < 0.05 were considered significant.

4.4. Discussion

Numerous studies have assessed the role of CD4+ and CD8+ T cells in HPV clearance. However, there is limited information on the role of these cell subsets in CIN recurrence after excisional treatment, especially in WLHIV. Moreover, existing data are somewhat discordant. Our study aimed to characterize the associations between HPV16 /18 E6 and E7 specific CD4+ and CD8+ T cell responses and clearance and/or recurrence of CIN in WLWH who received excisional treatment. We targeted HPV16 and HPV18 as these high-risk HPV types are responsible for the vast majority of cervical cancer cases globally [296-298]. We compared the frequencies of HPV16/18 E6 and E7-specific CD4+ and CD8+ T cell responses in blood from the WLWH who had post-treatment CIN recurrence and those who did not have recurrence. We also assessed associations of the T cell responses with CIN lesion grades and HR-HPV viral loads.

We found that HPV 16/18 E6- and E7-oncoproteins had systemic immunogenicity as shown by detection of frequencies of anti-HPV16/18 E6 and E7 CD4 T cells and CD8 T cells in blood. These findings support previous reports that have utilized peripheral blood specimens to study anti-HPV cellular immune responses, despite the fact that HPVs are known to only cause localized mucosal infections with no systemic phase in their infection cycles [299-301]. HPV-specific T cell responses would be more pronounced at the site of pathology than in peripheral blood [302-304]. Additionally, the non-cytolytic nature of Human papillomaviruses, as well as the extremely confined expression of small E6 and E7 viral proteins to the parabasal and basal epithelium cells, may also limit the ability to study anti-HPV cellular immunity in blood [76, 305-309]. Nevertheless, the detection of anti-HPV T cell responses, as shown in our study and in previous studies, justifies the use of blood specimens for comparative analyses of anti-HPV cellular immunity between patient groups, especially because blood is easily accessible.

Our results showed that there were no significant differences in the percentages of IFN-γ+ CD8+ and CD4+ T cell responses to HPV16/18 E6 and E7 antigens at baseline and at 1 year after CIN excisional treatment between the CIN Recurrence and the No-recurrence groups in our cohort of WLWH. Even though this observation suggests that circulating T cells do not play a major role in preventing CIN

recurrence after excisional treatment in WLWH, other studies have suggested a role for T cells in HPV clearance or HIV-associated lesion regression in other groups of individuals [306, 310, 311]. For instance, one study showed an association between blood anti-HPV 16/18 cytotoxic T cell responses and absence of CIN [312], while another study showed better blood anti-HPV 16/18 cytotoxic T cell responses in patients who did not get CIN recurrence after treatment [313, 314]. Furthermore, high levels of HPV16/18 E6 Th1/Th2 CD4+ T cell responses were also associated with CIN clearance after treatment, a phenomenon also observed in cervical cancer, where a reduction in HPV16 E6 CD4+ T cell responses was associated with a poor clinical outcome [315-317]. Recent studies have also compared these responses in women living with and without HIV infection; HPV16/18 E6 and E7 specific T cell responses were lower in WLWH compared to healthy women [318, 319]. These observations imply that T cells, including those in blood, are associated with the successful spontaneous regression of HR-HPV disease or prevention of post-treatment recurrence in non-HIV cohorts [320, 321]. HIV-induced T cell defects could have dampened the beneficial effects of T cells in our cohort, hence leading to the differing observation in our study. Therefore, future studies could assess the impact of HIV infection on the quality and magnitude of anti-HPV T cells by directly comparing WLWH against HIV non-infected women.

In our study, T cell responses in blood remained generally stable after excisional treatment regardless of the treatment outcomes. However, after treatment, there was a modest statistically non-significant decay in IFN-γ+ CD8+ T cell responses to E6 in women with CIN recurrence. This decay in E6-specific CD8+ T cell responses has also been corroborated by other studies [322, 323]. Even though the precise reason may be unknown, it is possible that the resolution of cervical lesions following excisional treatment decreased the source of antigen required to maintain HPV-specific CD8 T cell immunity. A similar possible association between magnitudes of CD8 T cell responses and antigen loads were implied by the observation of higher frequencies on IFN-γ+ CD8+ T cell responses to E7 in CIN3 group when compared to the CIN2 group at baseline, and the trend of positive correlation between HPV viral loads and IFN-γ+ CD8+ T cell responses to E7 at the 1-year timepoint. Overall, T cell responses were stable after excisional treatment, even though occasional and modest possible associations with antigen

loads were seen, especially for CD8+ T cell responses. These observations suggest a weak link between the highly localized HPV infections and systemic T cell immune responses in WLWH. Future studies could investigate if similar trends are seen in cervical tissues of WLWH, and if that is associated with HIV immune suppression.

Our findings suggest that anti-HPV CD4+ and CD8+ T cell responses were not associated with clinical outcomes after excisional CIN treatment in WLWH. Other factors, such as high-risk HPV viral load, may have been the main determinants of treatment outcomes in this cohort, as reported in Chapter 3. Considering that blood anti-HPV T cell responses have been associated with CIN outcomes in other patient groups in other studies, it is likely that HIV infection modulated the blood T cell responses in this cohort, thus dampening their impact on treatment outcomes. Our study had the limitation of not including an HIV-negative group. As such, further studies are needed to directly confirm this by comparing immune responses between treatment outcomes across different HIV status groups. Such studies could include measurement of T cell responses in the cervix since clinically relevant immune responses may not be sufficiently reflected in the peripheral blood in HPV infection and related diseases. Investigations are also needed on how T cell immunity can be harnessed to manage HPV-associated disease in WLWH. This is particularly important because there is a great need for a globally effective therapeutic HPV vaccine that also benefits WLWH. Ideally, such a vaccine would need to induce robust HPV-specific T-cell responses that can mediate the clearance of HPV-infected cells and thereby prevent the progression to cervical cancer regardless of HIV status.

Chapter 5: Relationship between cytokine profiles, treatment outcome, and high-risk HPV viral load in women living with HIV undergoing excisional treatment for cervical intraepithelial neoplasia

Yanga Mdleleni[1], Wendy Dhlomo[2], Lenine. J Liebenberg[4], Thumbi Ndung'u[1,3] Bongiwe Ndlovu[1] and Daniel Muema[1,3,5]

[1]*HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa*

[2]*Discipline of Obstetrics & Gynaecology, University of KwaZulu-Natal, Durban, South Africa*

[3]*Africa Health Research Institute (AHRI) Durban, South Africa*

[4]*Centre for the AIDS Programme of Research in South Africa (CAPRISA), University of KwaZulu-Natal, Durban, South Africa*

[5]*Kenya AIDS Vaccine Initiative (KAVI) Institute of Clinical Research - University of Nairobi*

Abstract

Host factors are critical in regulating HPV-induced pre-malignancy, and cytokines that modulate immunologic control may be of particular importance. This study characterized genital cytokines in cervicovaginal lavage (CVL) and systemic cytokines in plasma among South African women aged 18-65 years who were prospectively followed up after excisional treatment for cervical intraepithelial neoplasia (CIN), to determine associations with treatment outcomes. Participants had presented with abnormal pap smears, received surgical excision of CIN, and were followed up for 12 months post-treatment to determine if CIN recurrence had taken place. HPV genotyping had been conducted at baseline using the Roche Linear array. A total of 39 participants (19 from the Recurrence and 20 from the No-Recurrence group) who were positive for HR-HPV 16 and HPV 18 were included in this sub study. Furthermore, High-risk HPV (HR-HPV) viral loads were determined using a hybrid capture method, while cytokines were measured using a multiplexed bead-based ELISA assay. The cytokines profiles were mostly similar across treatment outcomes (Recurrence versus No-recurrence groups) and

across timepoints (baseline versus 1 year after excisional treatment). The Recurrence group had higher baseline levels for CVL Eotaxin as well as lower baseline plasma levels for IL-2, IL-12, IL-13 and IL-17 when compared to the No-recurrence group. The kinetics of the plasma cytokines also differed between the two groups, with the Recurrence group showing an increase in IL-2, IL-13, IL-7 and IL-17 whereas the No-recurrence group had a decrease in IL-13 and IL-12. There were notable inverse associations between some CVL Th2 cytokines and HR-HPV viral loads at the post-treatment time point, suggesting that absence of Th2 skewing could be associated with successful post-treatment viral control. On the other hand, in plasma, the Recurrence group had higher levels of some Th1 and Th2 cytokines at both timepoints, suggesting association of recurrence with general systemic inflammation, which is not necessarily attributable to Th1/Th2 skewing. The Recurrence group also had higher baseline levels for CVL Eotaxin as well as higher post-treatment plasma levels of IL-7, IL-2, IL-8, and IP-10. These data reinforce the need for detailed analyses of immune dysregulation in CIN patients for better interventions and to develop biomarkers for predicting or monitoring CIN treatment outcomes.

Key words: HPV; CIN; Cytokines; ELISA

5. 1. Introduction

The persistence of high risk Human Papilloma Virus (HR-HPV) infections that occurs in about 10% of HPV infected women is thought to be as a result of compromised immunity or host immune evasion mechanisms [324, 325]. Notably, the cycle of HPV infection is a mechanism of immunological avoidance that impairs the host's viral detection apparatus. As such, HPV infection virtually has no systemic sequelae as they have a strict epithelial tropism, suppress interferon responses, and there is no cell death as a result of viral replication since these viruses hijack differentiated keratinocytes that are already programmed to die and desquamate, thus resulting in natural cell death that does not alarm the immune system [121, 326, 327].

Several lines of evidence show that the course of HPV infection and disease outcomes highly depends on robust local and systemic host immune responses, with varying degrees of immunity across compartments [212, 328, 329]. In support of this view, firstly, more rapid HPV malignancies have been observed in immunosuppressed individuals, while only a minority are noted in immunocompetent

individuals, and these often lead to the development of CIN and, consequently, cervical cancer [330-333]. Secondly, evidence from animal models has demonstrated that immunized mice are protected from HPV infection and from the development of neoplasia [334, 335]. During HPV infection, both $CD4^+$ T-helper cells and Cytotoxic T lymphocytes (CTLs) are dependent on antigen presentation by MHC-class II and class I molecules that are expressed by the Langerhans cells (LCs) and dendritic cells, therefore, leading to the production of cytokines [336, 337]. Successful clearance of HPV infection and disease management is regulated by $CD4^+$ T-helper cells, cytotoxic T lymphocytes, and cytotoxic natural killer cells [197, 291, 338]. The cytotoxic lymphocytes destroy virus-infected cells, while $CD4^+$ T-helper cells release cytokines upon antigen presentation [299, 339, 340]. Cytokines are pleiotropic glycoproteins responsible for regulating cell differentiation and proliferation, as well as the activation of systemic and local immunity [341, 342]. Overexpression of proinflammatory cytokines, however, could be counterproductive, leading to pathogenicity and chronicity of HPV infections. Importantly, CD4+ T helper cells are classified into Th1 (immunostimulatory) and Th2 (immunoinhibitory) cells and are functionally polarized based on the cytokines they produce [343-345]. Th1 cells secrete interleukin (IL) 2 (IL-2), interferon-gamma (IFN-γ), and tumour necrosis factor (TNF)-β, which activate macrophages and play a role in a phagocyte-dependent immunostimulatory and protective antiviral response [346, 347]. On the other hand, Th2 cells secrete IL-4, IL-5, IL-8, IL-10, and IL-13, which are associated with anti-inflammatory responses and eosinophil promotion in atopy as observed in human helminthic infections and allergic syndromes [348, 349].

Cytokines and their receptors significantly contribute to the development and progression of cancers, and their dysregulation can be observed both locally and systemically in cancer patients, with variations observed across the different anatomical compartments [350-352]. Moreover, alterations in the quantity and functionality of cytokines have been associated with the activation of effector immunity, which may result in intricate and severe immune weakening, therefore, promoting carcinogenesis [345, 353, 354]. Infiltrating Natural Killer (NK) cells, CD4+ T helper, and cytotoxic T cells have previously been observed in spontaneously regressing HPV-induced genital warts, and their ability to secrete cytokines has been reported as independent predictors of survival of cervical cancer patients [355, 356]. The

imbalance between Th1 and Th2 cytokine secretion plays a pivotal role in the incidence and progression of various autoimmune and tumour diseases, as also observed in HPV-infected and cervical cancer women [357-359]. It has been reported that women with a Th1 cytokine profile tend to have better clinical outcomes compared with those exhibiting a Th2 cytokine profile [347, 360, 361]. Furthermore, a predominance of Th2 cytokines, either locally or in the peripheral circulation, a phenomenon highly observed in WLWH, may endorse the development of CIN and neoplastic changes [159, 362, 363]. Paradoxically, an association between persistent infection, chronic inflammation, and increased neoplastic transformation has been noted in HPV infections [213, 364, 365].

Excisional treatment for CIN has led to highly successful outcomes for CIN cases; however, treatment failure is still rampant among WHLIV, especially those from LMICs. The limited studies of immune profiles in WLWH with cervical dysplasia are very scarce, and most have focused on one or a few cytokines and are limited to either peripheral or genital cytokine responses. This, therefore, emphasizes the need to further characterize the immune profiles that are associated with CIN treatment outcomes in WLWH. Such studies will also identify biomarkers for the diagnosis of CIN recurrence and the prediction of CIN treatment outcomes. To address this gap, our study aimed to characterize changes in vaginal and peripheral cytokine responses before and after CIN excisional treatment, and to further understand how these changes influence the risk of CIN recurrence and HPV viral loads in WLWH.

5.2. Materials and Methods

5.2.1. Study Design

Thirty-nine women living with HIV (20 in the "No-recurrence" group and 19 in the "Recurrence" group), with undetectable HIV viremia and confirmed high-risk HPV infection, were included in this sub-study. Genital and systemic cytokine responses were measured in cervicovaginal fluids and plasma samples, respectively, before and 1 year after excisional treatment as described in chapter 2. Plasma and genital cytokine levels were compared between in Recurrence and No-recurrence groups before and after treatment. Associations were also determined between cytokine profiles and HPV viral loads.

5.2.2. Determination of genital and systemic cytokine concentrations

A commercially available bead-based multiplexed ELISA assay comprising the Bio-Plex Pro Human Cytokine 27-plex (#M500KCAF0Y) 96-well plate kits was used to detect the presence of peripheral and genital tract cytokines at baseline and 1 year post excisional treatment from CVL supernatant and plasma samples (Figure. 2.2 in Chapter 2). Twenty-seven cytokines were measured comprising of anti-inflammatory, pro-inflammatory, regulatory, hematopoietic as well as growth-related cytokines, namely IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, Eotaxin, Basic Fibroblast Growth Factor (FGF-basic), Granulocyte Colony Stimulating Factor (GCSF), GM-CSF, Interferon (IFN-γ), Interferon gamma-induced Protein (IP-10), Monocyte Chemotactic Protein (MCP-1), Macrophage Inflammatory Protein alpha (MIP-1α), Macrophage Inflammatory Protein beta (MIP-1β), Platelet Derived Growth Factor (PDGF-BB), Regulated upon Activation Normal T cell Expressed and presumably Secreted (RANTES), Tumour necrosis factor (TNF-α) and Vascular Endothelial Growth Factor (VEGF) as per the manufacturer's instructions. The fluorescence of labelled analytes was acquired using the Bio-Plex Manager software (version 6) on a Bio-Plex Array Reader. A 5-parameters' logistic regression curve of standards was used to extrapolate sample concentrations. A limit of detection was assigned as the lowest level of cytokine detected. Intra-plate variability was assessed by including duplicates within each plate, while inter-plate variability was assessed by including duplicates between plates.

5.2.3. Statistical analyses

Cytokine concentrations were compared between anatomical compartments (plasma and cervicovaginal lavage specimens), between timepoints (baseline and 1 year after CIN excisional treatment) for each chosen anatomical compartment as well as between groups (Recurrence and No-recurrence groups) for each chosen timepoint and anatomical compartment. Paired comparisons between compartments and between timepoints were done using the Wilcoxon matched-pairs signed-rank test. Comparisons between groups were done using the Mann-Whitney U test. The Spearman's rank-order correlation test was used to assess the relationship of concentrations for each cytokine in plasma and CVL, as well as the relationship between various cytokines and HPV high-risk viral loads. Unsupervised hierarchical clustering was used to assess clustering of participants' cytokines profiles in heat maps. Two-sided P values < 0.05 were considered statistically significant. All analyses were completed on GraphPad Prism version 7 (GraphPad Software, Inc.).

5.3. Results

5.3.1. Relationship between cytokine levels in the cervicovaginal lavage (CVL) and recurrence of CIN in women living with HIV undergoing excisional treatment

We examined the changes in CVL cytokine concentrations in women living with HIV after CIN excisional treatment. CVL cytokine responses were measured at two time points: baseline and 1 year after CIN excisional treatment. Demographic information of study participants was described in Chapter 3.1 of the thesis. CVL cytokines levels were mostly similar between the groups and between timepoints, with a few exceptions (Figure. 5.1A). Some cytokines showed a general trend of decline in CVL levels upon excisional treatment. This decline after treatment was statistically significant for CVL IL-6 in both groups ($P<0.05$) (Figure. 5.1 B). On the other hand, the kinetics differed by treatment outcome for CVL IL-15 and CVL VEGF, both of which showed statistically significant declines in the Recurrence group but not the No-recurrence group ($P<0.05$ for each cytokine) (Figure. 5.1 C-D). Notably, Eotaxin levels at baseline were higher in the CVL of the Recurrence group when compared to the CVL of the No-recurrence group, suggesting that it could be used as a biomarker for prospectively predicting CIN recurrence ($P<0.05$) (Figure. 5.1E). Overall, there was higher inflammation at baseline, especially in the Recurrence group, and treatment was associated with the resolution of some of the CVL cytokines even in cases where CIN recurred, probably due to a reduction in disease activity and antigen loads.

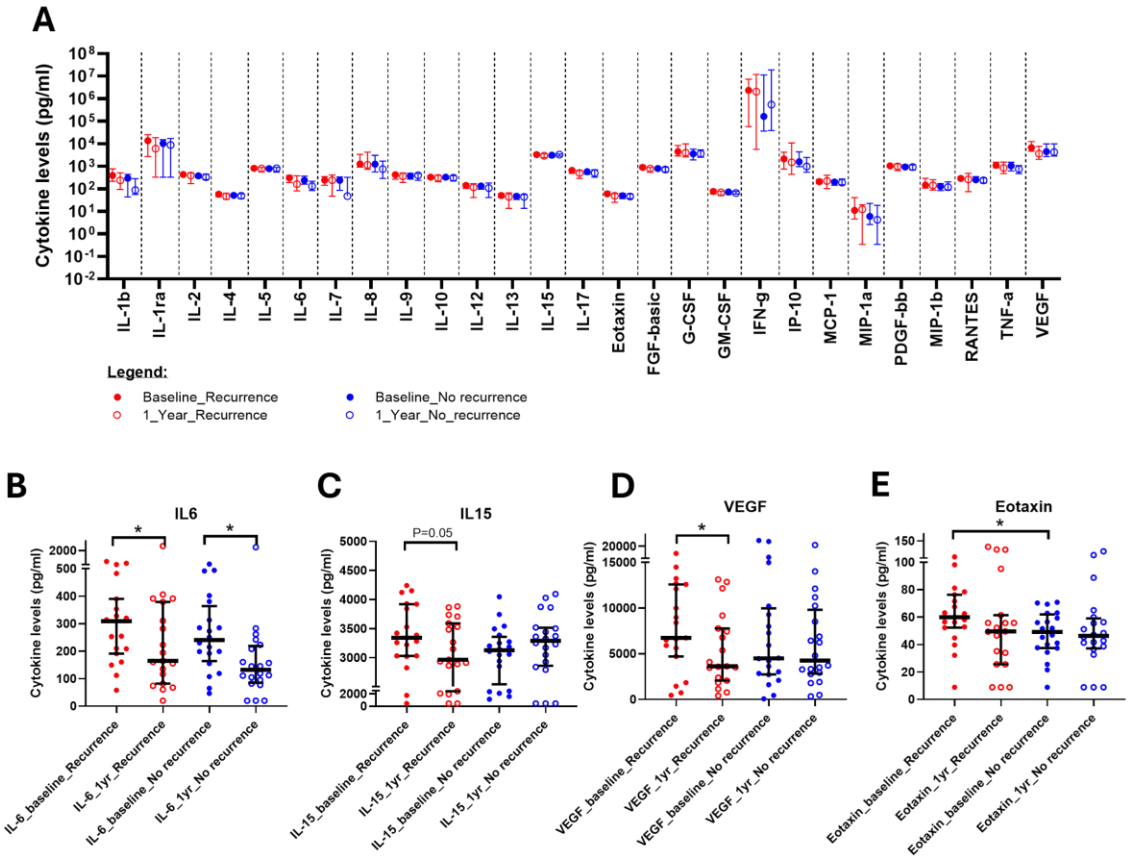

Figure 5.1. Cytokine profiles from Cervical lavage (CVL) samples measured before and 1 year after CIN excisional treatment in women living with HIV. (A) Cytokine levels in CVL across all 27 cytokines in the recurrence and No-recurrence groups at baseline and 1 year after CIN excisional treatment. (B) Interleukin-6 (IL-6) levels in CVL. (C) Interleukin-15 (IL-15) levels in CVL. (D) Vascular endothelial growth factor (VEGF) levels in CVL. (E) Eotaxin levels in CVL. Red coloured symbols represent women in the Recurrence group, while Blue represents women in the No-recurrence group. Statistical test used: Wilcoxon matched-pairs signed-rank test for comparisons of matched samples within a group across time points, and Mann-Whitney U test for comparisons between groups of participants within a time point. *p < 0.05 was considered significant.

5.3.2. Relationship between cytokine levels in the plasma and recurrence of CIN in women living with HIV undergoing excisional treatment

We examined the changes in plasma cytokine concentrations in women living with HIV after CIN excisional treatment. Plasma cytokine responses were measured at two timepoints: baseline and 1 year after CIN excisional treatment. Women in the Recurrence group (red) showed lower concentrations of IL-2, IL-7, IL-13 and IL-17 at baseline when compared to their matched 1 year timepoint. In contrast, the No-recurrence group had higher concentrations of IL-13 and IL-12 at baseline when compared to the matched 1-year timepoint. In inter-group comparisons, at baseline, the Recurrence group had lower

levels of IL-2, IL-12, IL-13 and IL-17 when compared to the No-recurrence group. On the other hand, at 1-year timepoint, the Recurrence group had higher levels of IL-7, IL-12, IL-8 and IP-10 when compared to the No-recurrence group. Overall, treatment was associated with opposite effects on plasma cytokine levels in the two groups, with the Recurrence group having a general increase and the No-recurrence group having a general decrease when the cytokine levels were compared at baseline with the 1 year timepoint.

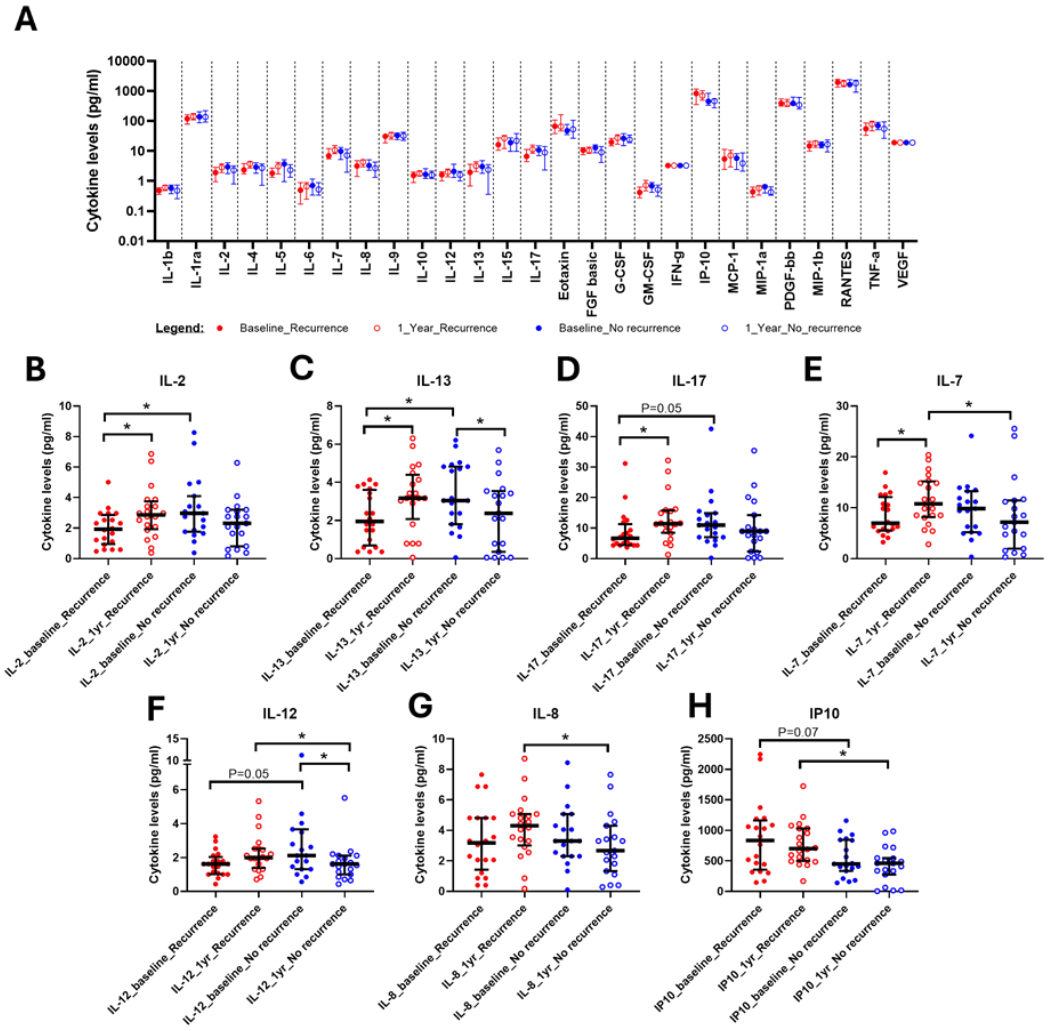

Figure 5.2. Plasma cytokine concentrations measured at baseline and 1 year after CIN excisional treatment in women living with HIV. (A) Plasma cytokine levels across all 27 cytokines in the Recurrence and No-recurrence groups at baseline and 1 year after CIN excisional treatment. (B) Interleukin-2 (IL-2) plasma levels at baseline and 1 year after treatment in both the Recurrence and No-recurrence group in plasma. (C) Interleukin-12 (IL-12)8 plasma levels in plasma. at baseline and 1 year after treatment in both the Recurrence and No-recurrence groups. (D) Interleukin-13 (IL-13)2 plasma levels in plasma at baseline and 1 year after treatment in both the Recurrence and No-recurrence group. (E**)** Interleukin-17 (IL-17) levels in plasma. levels at baseline and 1 year after treatment in both the recurrence and no-recurrence groups. (F**)** Interleukin-7 (IL-7) levels in plasma. (G**)** Interleukin-8 (IL-8) levels in plasma. (H**)** Interferon-gamma Inducible Protein 10KDa (IP10) levels in plasma.

Red coloured symbols represent women in the CIN Recurrence group, while Blue represents women out of the CIN No-recurrence group. Statistical test used: Wilcoxon matched-pairs signed-rank test for comparisons of matched samples within a group across time points, and Mann-Whitney U test for comparisons between groups of participants within a time point. *p < 0.05 was considered significant.

5.3.3. Relationship between cytokine levels and high-risk HPV viral load in women living with HIV undergoing CIN excisional treatment

The aim of this study was to assess if there were associations between cytokine profiles and HPV viral loads in WLWH who were undergoing excisional treatment for CIN. We therefore checked for correlations between the cytokines and HPV viral loads (reported in chapter 3) using the Spearman rank order test. In the CVL, at baseline, significant positive correlations were observed between HR-HPV viral load and IL-1b (Rho=0.45, P<0.05), IL-17 (Rho=0.37, P<0.05), and FGF-basic (Rho=0.35, P<0.05), suggesting that HR-HPV viral loads could have been driving some components of CVL inflammation. Notably, Eotaxin, which had a statistically significant association with the recurrence of CIN lesions in earlier analyses, only had a non-significant trend of correlation with baseline HR-HPV viral load here (Rho=0.30, P=0.07). On the other hand, at 1 year, even though we didn't observe associations between treatment outcome and CVL cytokine levels in earlier analyses, we observed significant inverse correlations between HR-HPV viral load and IL-4 (Rho= -0.38, P<0.05), IL-10 (Rho= -0.37, P<0.05), IL-13 (Rho= -0.41, P<0.05), FGF-basic (Rho= -0.35, P<0.05), GM-CSF (Rho= -0.36, P<0.05), MCP-1 (Rho= -0.34, P<0.05), PDGF-bb (Rho= -0.38, P<0.05), and RANTES (Rho= -0.33, P<0.05). These inverse associations were unexpected and are suggestive of a role of local immune response in synergizing with excisional treatment to maintain HR-HPV clearance. In plasma, there were no correlations between HR-HPV viral load and cytokines at baseline. However, positive correlations were observed between HR-HPV viral loads and IL-2 (Rho= 0.34, P<0.05), IL-4 (Rho= 0.35, P<0.05), IL-7 (Rho= 0.44, P<0.05), IL-8 (Rho= 0.48, P<0.05), IL-10 (Rho= -0.36, P<0.05), IL-12 (Rho= 0.35, P<0.05), IL-17 (Rho= 0.35, P<0.05), GM-CSF (Rho= 0.43, P<0.05), IP-10 (Rho= 0.37, P<0.05), MCP-1 (Rho= 0.38, P<0.05), and TNF-a (Rho= 0.38, P<0.05) at the 1-year timepoint. Thus, there was some concordance in associations of post-treatment IL-7, IL-8, IL-12, and IP-10 with both treatment outcomes and post-treatment HR-HPV viral loads. The associations of post-treatment plasma

cytokines with post-treatment HR-HPV viral loads suggest that systemic inflammation could be driven by persistent HR-HPV viral loads, contrary to observations in the CVL, where cytokines could have been driving viral clearance.

Table 5.1: Correlations between cytokines and high-risk HPV viral loads

	Correlation between baseline cytokines				**Correlation between 1-year cytokines HPV**			
	CVL		**Plasma**		**CVL**		**Plasma**	
Cytokine	**Rho**	**P value**	**Rho**	**P value**	**Rho**	**P value**	**Rho**	**P value**
IL-1b	**0.45**	**0.0048**	0.00	0.9927	-0.09	0.5769	0.23	0.1730
IL-1ra	0.11	0.5045	-0.09	0.5706	-0.18	0.2830	0.12	0.4555
IL-2	0.14	0.4145	-0.03	0.8691	-0.24	0.1386	**0.34**	**0.0373**
IL-4	0.26	0.1132	0.00	0.9922	**-0.38**	**0.0188**	**0.35**	**0.0290**
IL-5	0.22	0.1902	-0.05	0.7439	-0.29	0.0734	0.18	0.2916
IL-6	0.06	0.7306	0.00	0.9800	-0.26	0.1132	0.21	0.2073
IL-7	0.19	0.2514	0.07	0.6608	-0.26	0.1127	**0.44**	**0.0058**
IL-8	0.32	0.0542	0.00	0.9848	-0.03	0.8469	**0.48**	**0.0025**
IL-9	0.27	0.1114	0.05	0.7674	-0.29	0.0766	0.29	0.0775
IL-10	0.20	0.2375	0.08	0.6193	**-0.37**	**0.0208**	**0.36**	**0.0252**
IL-12	0.22	0.1997	-0.08	0.6121	-0.23	0.1604	**0.35**	**0.0326**
IL-13	0.08	0.6527	-0.04	0.7989	**-0.41**	**0.0098**	0.32	0.0512
IL-15	0.28	0.0895	0.14	0.3791	-0.26	0.1094	0.06	0.7150
IL-17	**0.37**	**0.0236**	0.04	0.7936	-0.27	0.1047	**0.35**	**0.0302**
Eotaxin	0.30	0.0728	0.26	0.1132	-0.27	0.1067	0.18	0.2700
FGF	**0.35**	**0.0358**	0.10	0.5632	**-0.35**	**0.0297**	0.24	0.1451
G-CSF	0.07	0.6932	0.05	0.7543	-0.18	0.2668	0.17	0.3003
GM-CSF	0.27	0.1108	0.05	0.7593	**-0.36**	**0.0251**	**0.43**	**0.0077**
IFN-g	0.17	0.3029	0.14	0.3813	-0.22	0.1912	-0.27	0.0990
IP-10	0.06	0.7327	0.18	0.2605	-0.21	0.2021	**0.37**	**0.0213**
MCP-1	0.07	0.6798	-0.06	0.7143	**-0.34**	**0.0394**	**0.38**	**0.0196**
MIP-1a	0.15	0.3768	0.01	0.9332	-0.27	0.1020	0.27	0.0959
PDGF-bb	0.31	0.0637	-0.03	0.8423	**-0.38**	**0.0178**	0.29	0.0785
MIP-1b	0.29	0.0852	0.02	0.8925	-0.32	0.0527	0.09	0.5949
RANTES	0.17	0.3286	0.08	0.6189	**-0.33**	**0.0425**	0.02	0.8823
TNF-a	0.31	0.0625	-0.06	0.7288	-0.30	0.0688	**0.38**	**0.0190**
VEGF	0.30	0.0709	-0.11	0.5086	-0.05	0.7466	-0.20	0.2266

Statistically significant correlations ($P<0.05$) are shown in bold text.

5.3.4. Relationship between CIN recurrence and overall cytokine profiles in women living with HIV

To further explore if there were differences between participants with regard to treatment outcome and time of specimen collection (baseline versus 1-year post-treatment), we performed unsupervised hierarchical clustering across all specimens, based on Spearman correlation analyses (Figure. 5.3). In line with earlier observations that most CVL cytokines levels did not differ between No-recurrence and Recurrence groups, as well as between baseline and 1-year timepoints, we did not see clustering of the CVL cytokines profiles based on the 2 stratifications (Figure. 5.3A). Similarly, the plasma cytokine profiles did not show clustering based on group or time point of sampling (Figure. 5.3 B). Thus, in as much as significant differences had been seen between groups and timepoints for some cytokines in earlier analyses, the overall cytokine profiles were not adequately different to enable distinguishing participants based on treatment outcome or timepoint of specimen collection.

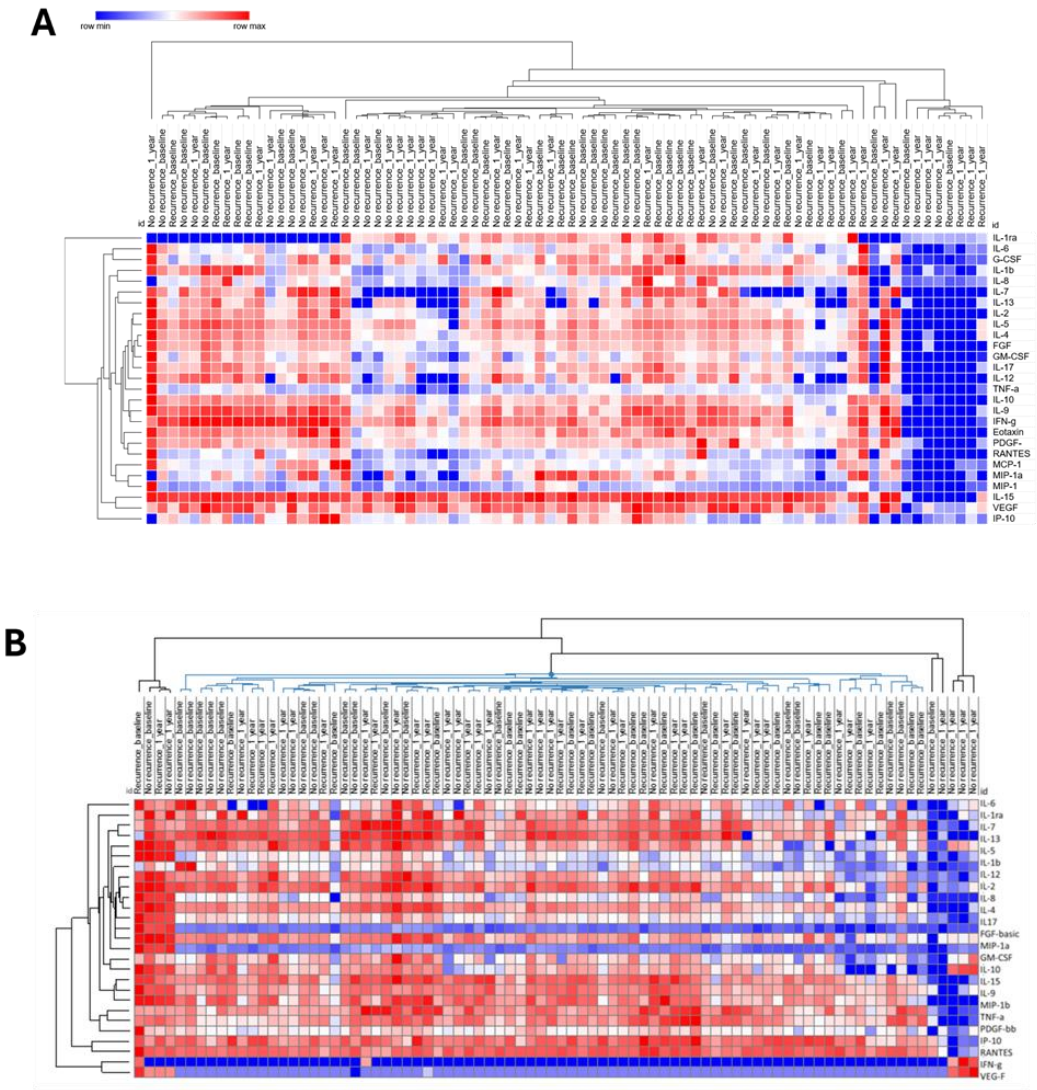

Figure 5.3. Unsupervised hierarchal clustering of plasma and CVL cytokines at baseline and 1 year in women treated for CIN. (A) Unsupervised hierarchal clustering of cytokine levels in CVL for women in different treatment outcome groups at baseline and 1 year after excisional treatment. (B) Unsupervised hierarchal clustering of Plasma cytokine levels for women in different treatment outcome groups at baseline and 1 year after excisional treatment. Clustering was based on Spearman correlation across both cytokines and study participants.

5.4. Discussion

Exploring the interplay between local and systemic immunity is an important factor in HPV immunopathogenesis, CIN development, and progression to cervical cancer [366, 367]. This study aimed to characterize the association between plasma and cervicovaginal lavage (CVL) cytokine responses with HR-HPV viral loads and clearance of cervical intraepithelial neoplasia (CIN) after loop electrosurgical excision (LEETZ) excisional treatment among WLHIV. All women in our study were living with HIV and had high-risk HPV infection.

Generally, the cytokine profiles in the CVL and blood of the WLWH were mostly similar in the CIN Recurrence and No-recurrence groups for the assessed timepoints, i.e., at baseline and at 1 year after treatment. However, some cytokines had notably different levels between groups and/or timepoints. In particular, CVL IL-6 showed a decline in both groups following treatment, suggesting that its levels were sensitive to treatment of the CIN lesions. CVL IL-15 and VEGF also showed declines in the Recurrence group after treatment. These associations with cervical pathology might be independent of antigen load, as shown by the lack of correlations between the same CVL cytokines and HR-HPV viral loads. On the other hand, IL-1b, IL-17, and FGF-basic levels were positively correlated with HR-HPV viral loads at baseline, suggesting their dependence on HPV antigen loads. There were notable inverse correlations between HR-HPV viral loads and several Th2 cytokines, such as IL-4, IL-10, and IL-13 at the 1-year timepoint. This suggests that lower HPV viral load was associated with Th2 responses, including anti-inflammatory and antibody responses. However, considering that Th2 cytokines can dampen local anti-viral Th1 responses, the expectation was for them to be positively associated with HPV viral persistence. Previous reports have shown that HPV progression and recurrence are associated with reduced Th1/ Th2 balance, resulting in the relative dominance of a Th2 cytokine milieu through the suppression of cellular immunity [360, 361, 368, 369]. In addition, an inverse relationship between IL-2 expression and CIN progression in patients with cervical dysplasia has been reported, strengthening the claim that Th1 cytokines, such as IL-2, play a key role in anti-HPV immune responses [370-373]. In our cohort, the inverse correlations of Th2 cytokines with HPV viral loads suggest Th2 responses could still have a beneficial role in CIN-treated WLWH. Considering that similar inverse

correlations were also seen for other cytokines, it is also likely that the inverse relationship with the cytokines is a function of a general inflammatory response that mediates the desired viral suppression. The lack of distinct correlations of HR-HPV viral loads with Th1 cytokines could have been due to their active suppression by the co-infection with HIV, which has been shown to cause Th2 skewing independent of HPV infection. Moreover, it has been reported that the E6 and E7 viral oncogenes from high-risk HPV can directly suppress Th1 cytokines expressed in the genital tract, subsequently leading to progressive disease [294, 374, 375]. Thus, the suppression of Th1 responses in WLWH who are infected with HR-HPV could have dampened the impact of Th1 responses on HR-HPV viral loads in our study.

In addition to local immune profiles, systemic immune profiles can also impact CIN clearance by modulating the induction of effective adaptive immune responses. Here, following treatment, there were increases in plasma levels of both Th1 (IL-2 and IL-12) and Th2 (IL-13) cytokines in the Recurrence group and not in the No-recurrence group. This is indicative of an increase in general chronic inflammation in the Recurrence group, with a possible association with persistence of HR-HPV viral load. Indeed, at the post-treatment 1-year timepoint, positive correlations were seen between HR-HPV viral loads and several cytokines, namely IL-2, IL-4, IL-10, IL-12, IP-10, and TNF-alpha, among others, suggesting that HPV antigen loads may be driving a systemic general inflammatory state. Concentrations increased significantly after 1 year of treatment within the Recurrence group, and not in the No-recurrence group. No differences were observed. This could be indicative of chronic inflammation. Chronic inflammation has been reported to predispose individuals to a condition for tumour development through the suppression of antitumour immunity, favouring tumour progression and CIN recurrence [213, 376]. Additionally, elevated levels of IL-15 and IL-17 have been observed in women with cervical cancer [377]. There is also evidence that the expression of IL-15, IL-10, and GM-CSF in invasive cervical cancer tissues may promote an immunosuppressive environment that permits a disease-progressive state [377]. As such, systemic inflammation, as detected in plasma, could have contributed to the persistence of HR-HPV viremia and the recurrence of CIN. On the other hand, a role for HR-HPV viremia causatively driving the systemic inflammation cannot be ruled out. In accord with

these findings, our study also showed elevated levels of genital IL-10, GM-CSF, IL-13, and IL-15 after 1 year of treatment, although decreased levels of GM-CSF were associated with a good clinical outcome as noted in women with no CIN recurrence. Additionally, conflicting roles of IL-10 in cervical cancer development have been stated, where Wang et al. [378] reported on the dual functionality of IL-10 as an anti-angiogenic (potential cancer inhibitor) and anti-inflammatory (potential cancer promoter) agent. Notably, the role of Th1 cytokines in HPV carcinogenesis has been well documented. Previous reports have shown that HPV progression reduces the Th1/ Th2 balance, resulting in the relative dominance of a Th2 cytokine milieu through the suppression of cellular immunity [362, 379]. Moreover, a shift from a Th1 dominant cytokine to a Th2 dominant cytokine profile has been reported to be strongly associated with CIN progression and recurrence [360, 361, 380, 381]. This phenomenon is highly prevalent among women living with HIV, as also observed in our cohort. Moreover, it has also been reported that the E6 and E7 viral oncogenes from high-risk HPV, individually and together, suppressed Th1 cytokines expressed in the genital tract, subsequently leading to progressive disease [382-384]. The expression of Th1-type cytokines may be the key strategy in stimulating antigen presentation and CD8+ T cell responses in the cervical mucosa, whereas a Th2-type response may be ineffective at preventing the progression to a high-grade lesion. In this study, at baseline, there were notable differences between the Recurrence and the No-recurrence groups with regard to CVL Eotaxin, plasma IL-2, plasma IL-13, plasma IL-17, and plasma IL-12 levels. We also observed differences between the Recurrence and the No-recurrence groups with regard to plasma IL-7, plasma IL-12, plasma IL-8, and plasma IP-10 at the 1-year timepoint. As such, the cytokine levels, especially for the cytokines in CVL where high levels were detected, could serve as biomarkers for predicting or monitoring CIN treatment outcomes. Reliable biomarkers could be employed to identify patients who need intensive follow-up or adjunct treatments following excisional treatment, thus enabling optimized post-treatment care and monitoring. Additional confirmatory studies will be needed to determine the reliability of these biomarkers in WLWH and other populations.

Local and systemic immune response plays a pivotal role in understanding the course of HPV-induced disease. In this study, we observed an association between CVL cytokines and HPV viral load clearance

after excisional CIN treatment. In plasma, however, the cytokine response was associated with HPV viral persistence. We also observed differences in cytokine levels and cytokine kinetics based on treatment outcomes, suggesting that immune responses at the time of CIN treatment could affect disease trajectories. Our study adds to the knowledge on the immunopathogenesis of CIN recurrence upon excisional treatment. The study also reveals potential biomarkers that can be utilized for prospectively predicting CIN recurrence or monitoring patients after treatment. Considering that our sample size was limited, further research with follow-up visits and large numbers of subjects will be needed to gain a better understanding of the immune mechanisms of CIN recurrence and to validate the identified potential biomarkers for predicting/monitoring treatment outcomes, especially among WLWH who are most affected by HPV-associated diseases in LMICs settings such as South Africa.

Chapter 6: Synthesis, Conclusions, and Future Directions

Persistent high-risk HPV infection leads to progression of cervical epithelial neoplasia (CIN), and subsequently cancer. This progression disproportionally affects women living with HIV, particularly in Sub-Saharan Africa. Notably, the recurrence of CIN after treatment is also high in this region, due to various factors including the high burden of HIV infection. Identifying reliable biomarkers for post-treatment CIN recurrence may significantly improve care and monitoring to minimize the risk of progression to cervical cancer. HPV viral load and immune factors are possible biomarkers that could be used to predict post-treatment outcomes. However, their associations with treatment outcomes remains obscure, especially in WLHIV. We therefore sought to assess the association between CIN treatment outcome and HPV viral load, T cell immune responses as well as soluble cytokines among women living with HIV who were treated for CIN using a Large Loop excision procedure.

To explore this, we followed women who had high-grade lesions, requiring colposcopy and excisional treatment using LEEP. Study participants were followed up at baseline, 6 months, and 1 year after treatment. At each of these time points, study participants had gynaecological examinations to assess treatment success. Those with recurrent lesions were classified as the “Recurrence group” while those with successful treatment were the “No-recurrence group”. In the first sub-study in this cohort, we aimed to characterize the ability of high-risk HPV types and HR- HPV viral load to predict post-treatment CIN recurrence. Our study showed that CIN recurrence in WLWH is strongly associated with both higher HR-HPV viral loads and the presence of multiple HR HPV infections. Thus, HR-HPV viral load and the number of HR-HPV types could be added as a biomarker for predicting CIN recurrence in WLWH who undergo excisional CIN treatment, to inform monitoring at follow-up timepoints and prevent progression to invasive cervical cancer.

Cellular immunity, as mediated by CD4+ and CD8+ T cells, plays a crucial role in managing HPV infections. In previous reports, women with cervical lesions and cervical cancer have higher frequencies of local CD4+ and CD8+ T cells that recognize HPV- E6 and E7 epitopes, and elicit Th1 and/or Th2 cytokines in HPV-infected keratinocytes, possibly facilitating the regression of lesions. However, there is contradicting data regarding naturally occurring IFN-γ T cell responses to HPV E6 and E7 in peripheral blood. There is also very little knowledge on the association of CD4+ and CD8+ T cell responses with CIN treatment outcomes among WLWH. Thus, the ability of systemic T cell responses to predict or facilitate the regression of CIN, especially in WLWH after excisional therapy, remains unclear. Our second sub-study, therefore, aimed to characterize the associations between IFN-γ secreting CD4+ and CD8+ T cells to HPV 16 and HPV 18 E6 and E7 with clearance and/or recurrence of CIN in women living with HIV. We found that anti-HPV CD4 and CD8 T cell responses were not associated with clinical outcomes after excisional CIN treatment in WLWH. Other factors, such as high-risk HPV viral load, may have been the main determinants of treatment outcomes in this cohort, as reported in Chapter 3. Considering that blood anti-HPV T cell responses have been associated with CIN outcomes in other patient groups in other studies, it is likely that HIV infection modulated the blood T cell responses in this cohort, thus dampening their impact and association with treatment outcomes.

It has been previously reported that inflammation, as mediated by cytokines, plays a role in HPV infection and its progression, especially among women living with HIV. Moreover, host factors are critical in regulating HPV-induced pre-malignancy, and cytokines that modulate immunologic control may be of particular importance, and these may vary based on the site of infection or the type of sample used. An imbalance between Th1 and Th2 cytokine secretion plays a pivotal role in the occurrence and progression of HPV and CIN progression. A predominantly Th2 cytokine profile favours CIN progression and poor clinical outcome over a Th1 response. Moreover, it has been reported that local immune responses are more robust than peripheral responses, especially in HPV immunopathogenesis. In our third sub-study, we therefore aimed to characterize the role of genital and systemic cytokine profiles in women living with HIV, before and after CIN excisional treatment. We observed an association between CVL cytokines and HPV viral load clearance after excisional CIN treatment. In

plasma, however, the cytokine response was associated with HPV viral persistence. We also observed differences in cytokine levels and cytokine kinetics based on treatment outcomes, suggesting that immune responses at the time of CIN treatment could affect disease trajectories.

Our work adds to the knowledge on the immunopathogenesis of CIN recurrence upon excisional treatment in women living with HIV. The study also reveals baseline/pre-treatment HR-HPV viral load and the number of HR-HPV types as potential biomarkers that can be utilized for prospectively predicting CIN recurrence. Additional pre-treatment/baseline biomarkers that were revealed by the study with potential usefulness in predicting CIN recurrence after excisional treatment include high levels of Eotaxin in CVL and low levels of IL-2, IL-12, IL-13 and IL-17. Further, the study revealed that post-treatment HR-HPV viral loads and high IL-7, IL-12, IL-8 and IP-10 could be used as post-treatment biomarkers to monitor outcomes.

Considering that our sample size was limited, further research with follow-up visits and large numbers of subjects will be needed to gain a better understanding of the immune mechanisms of CIN recurrence and to validate the identified potential biomarkers for predicting/monitoring treatment outcomes, especially among WLWH who are most affected by HPV-associated diseases in LMICs settings such as South Africa.

Our study had the limitation of not including an HIV-negative group. As such, further studies are needed to directly confirm this by comparing immune responses between treatment outcomes across different HIV status groups. Such studies could include measurement of T cell responses in the cervix since clinically relevant immune responses may not be sufficiently reflected in the peripheral blood in HPV infection and related diseases. Investigations are also needed on how T cell immunity can be harnessed to manage HPV-associated disease in WLWH. This is particularly important because there is a great need for a globally effective therapeutic HPV vaccine that also benefits WLWH. Ideally, such a vaccine would need to induce robust HPV-specific T-cell responses that can mediate the clearance of HPV-infected cells and thereby prevent the progression to cervical cancer regardless of HIV status. In conclusion, our findings suggest that HR-HPV viral load, the number of HR-HPV types, as well high levels of Eotaxin in CVL, or low levels of IL-2, IL-12, IL-13 and IL-17 are potential biomarkers for

CIN recurrence in WLWH. These findings have important implications in the treatment or prevention of CIN recurrence, particularly in low-resource settings due to the high rate of CIN recurrence.

References

1. Schiffman, M., et al., *Carcinogenic human papillomavirus infection.* Nature reviews Disease primers, 2016. **2**(1): p. 1-20.
2. Moscicki, A.-B., M. Schiffman, and S. Franceschi, *The natural history of human papillomavirus infection in relation to cervical cancer*, in *Human papillomavirus*. 2020, Elsevier. p. 149-160.
3. Oyouni, A.A.A., *Human papillomavirus in cancer: Infection, disease transmission, and progress in vaccines.* Journal of infection and public health, 2023. **16**(4): p. 626-631.
4. Seyoum, A., et al., *Prevalence and genotype distribution of high-risk human papillomavirus infection among Sub-Saharan African women: a systematic review and meta-analysis.* Frontiers in Public Health, 2022. **10**: p. 890880.
5. Tchouaket, M.C.T., et al., *Variability of high-risk human papillomavirus and associated factors among women in sub-Saharan Africa: a systematic review and meta-analysis.* Pathogens, 2023. **12**(8): p. 1032.
6. Kombe, A.J.K., et al., *Epidemiology and Burden of Human Papillomavirus and Related Diseases, Molecular Pathogenesis, and Vaccine Evaluation.* Frontiers in Public Health, 2020. **8**.
7. De Martel, C., et al., *Worldwide burden of cancer attributable to HPV by site, country and HPV type.* International journal of cancer, 2017. **141**(4): p. 664-670.
8. Duan, R., et al., *Cancer burden attributable to human papillomavirus infection by sex, cancer site, age, and geographical area in China.* Cancer medicine, 2020. **9**(1): p. 374-384.
9. Zhang, M., et al., *Analysis of the global burden of cervical cancer in young women aged 15–44 years old.* European Journal of Public Health, 2024. **34**(4): p. 839-846.
10. Bruni, L., et al., *Global and regional estimates of genital human papillomavirus prevalence among men: a systematic review and meta-analysis.* The Lancet Global Health, 2023. **11**(9): p. e1345-e1362.
11. Rohner, E., et al., *Cervical cancer risk and impact of Pap-based screening in HIV-positive women on antiretroviral therapy in Johannesburg, South Africa.* International journal of cancer, 2017. **141**(3): p. 488-496.
12. Ebrahim, S., et al., *High burden of human papillomavirus (HPV) infection among young women in KwaZulu-Natal, South Africa.* PloS one, 2016. **11**(1): p. e0146603.
13. Abera, G.B., et al., *Epidemiology of pre-cancerous cervical lesion and risk factors among adult women in Tigray, Ethiopia.* PloS one, 2023. **18**(1): p. e0280191.
14. Kiros, M., et al., *Prevalence and determinants of pre-cancerous cervical lesion and human papillomavirus among HIV-infected and HIV-uninfected women in North-West Ethiopia: a comparative retrospective cross-sectional study.* HIV/AIDS-Research and Palliative Care, 2021: p. 719-725.
15. Gargano, J.W., et al., *Prevalence of Genital Human Papillomavirus in Males, United States, 2013-2014.* J Infect Dis, 2017. **215**(7): p. 1070-1079.
16. Tota, J.E., et al., *Anogenital human papillomavirus (HPV) infection, seroprevalence, and risk factors for HPV seropositivity among sexually active men enrolled in a global HPV vaccine trial.* Clinical Infectious Diseases, 2022. **74**(7): p. 1247-1256.
17. Han, J.J., et al., *Prevalence of Genital Human Papillomavirus Infection and Human Papillomavirus Vaccination Rates Among US Adult Men: National Health and Nutrition Examination Survey (NHANES) 2013-2014.* JAMA Oncol, 2017. **3**(6): p. 810-816.
18. Patra, S., et al., *HPV and male cancer: pathogenesis, prevention and impact.* Journal of the Oman Medical Association, 2025. **2**(1): p. 4.
19. Wang, W., S. Sawleshwarkar, and M. Piraveenan, *Computational approaches of modelling human papillomavirus transmission and prevention strategies: a systematic review.* Journal of biological dynamics, 2025. **19**(1): p. 2436376.

20. Weaver, B.A., *Epidemiology and natural history of genital human papillomavirus infection.* Journal of Osteopathic Medicine, 2006. **106**(s1): p. 2-8.
21. Kudela, E., et al., *The interplay between the vaginal microbiome and innate immunity in the focus of predictive, preventive, and personalized medical approach to combat HPV-induced cervical cancer.* EPMA Journal, 2021. **12**(2): p. 199-220.
22. Giuliano, A.R., et al., *E UROGIN 2014 roadmap: Differences in human papillomavirus infection natural history, transmission and human papillomavirus-related cancer incidence by gender and anatomic site of infection.* International journal of cancer, 2015. **136**(12): p. 2752-2760.
23. Gravitt, P.E. and R.L. Winer, *Natural history of HPV infection across the lifespan: role of viral latency.* Viruses, 2017. **9**(10): p. 267.
24. Petry, K.U., *HPV and cervical cancer.* Scandinavian Journal of Clinical and Laboratory Investigation, 2014. **74**(sup244): p. 59-62.
25. Kombe Kombe, A.J., et al., *Epidemiology and burden of human papillomavirus and related diseases, molecular pathogenesis, and vaccine evaluation.* Frontiers in Public Health, 2021: p. 1003.
26. Silverberg, B., et al., *2021 CDC Update: Treatment and Complications of Sexually Transmitted Infections (STIs).* Venereology, 2022. **1**(1): p. 23-46.
27. Yousefi, Z., et al., *An update on human papilloma virus vaccines: history, types, protection, and efficacy.* Frontiers in Immunology, 2022. **12**: p. 805695.
28. Arbyn, M., et al., *Estimates of incidence and mortality of cervical cancer in 2018: a worldwide analysis.* The Lancet Global Health, 2020. **8**(2): p. e191-e203.
29. Shin, M.B., et al., *A framework for cervical cancer elimination in low-and-middle-income countries: a scoping review and roadmap for interventions and research priorities.* Frontiers in public health, 2021. **9**: p. 670032.
30. Singh, D., et al., *Global estimates of incidence and mortality of cervical cancer in 2020: a baseline analysis of the WHO Global Cervical Cancer Elimination Initiative.* The Lancet Global Health, 2023. **11**(2): p. e197-e206.
31. Drokow, E.K., et al., *Cervical cancer survival times in Africa.* Frontiers in Public Health, 2022. **10**: p. 981383.
32. Burt, L.M., et al., *Cervix cancer in sub-Saharan Africa: an assessment of cervical cancer management.* JCO Global Oncology, 2021. **5**(1): p. 173-182.
33. Ibrahim Khalil, A., et al., *Age-specific burden of cervical cancer associated with HIV: a global analysis with a focus on sub-Saharan Africa.* International journal of cancer, 2022. **150**(5): p. 761-772.
34. Justman, J.E., O. Mugurungi, and W.M. El-Sadr, *HIV Population Surveys - Bringing Precision to the Global Response.* N Engl J Med, 2018. **378**(20): p. 1859-1861.
35. Kharsany, A.B. and Q.A. Karim, *HIV Infection and AIDS in Sub-Saharan Africa: Current Status, Challenges and Opportunities.* Open AIDS J, 2016. **10**: p. 34-48.
36. UNAIDS, D., *Report.* Retrieved February, 2021.
37. Zuma, K., et al., *The HIV epidemic in South Africa: Key findings from 2017 national population-based survey.* International Journal of Environmental Research and Public Health, 2022. **19**(13): p. 8125.
38. Kharsany, A.B. and Q.A. Karim, *HIV infection and AIDS in sub-Saharan Africa: current status, challenges and opportunities.* The open AIDS journal, 2016. **10**: p. 34.
39. Makusha, T., et al., *HIV prevalence and associated factors among adolescent boys and young men in South Africa, 2017.* medRxiv, 2023: p. 2023.10. 17.23297141.
40. Mbulawa, Z.Z.A., et al., *High human papillomavirus (HPV) prevalence in South African adolescents and young women encourages expanded HPV vaccination campaigns.* PLoS One, 2018. **13**(1): p. e0190166.

41. Taku, O., et al., *Distribution of human papillomavirus (HPV) genotypes in HIV-negative and HIV-positive women with cervical intraepithelial lesions in the Eastern Cape Province, South Africa.* Viruses, 2021. **13**(2): p. 280.
42. Camargo, M., et al., *Association of HIV status with infection by multiple HPV types.* Tropical Medicine & International Health, 2018. **23**(11): p. 1259-1268.
43. Adebamowo, S.N., et al., *Persistent low-risk and high-risk human papillomavirus infections of the uterine cervix in HIV-negative and HIV-positive women.* Frontiers in Public Health, 2017. **5**: p. 178.
44. Kriek, J.-M., et al., *Female genital tract inflammation, HIV co-infection and persistent mucosal Human Papillomavirus (HPV) infections.* Virology, 2016. **493**: p. 247-254.
45. Williamson, A.-L., *The interaction between human immunodeficiency virus and human papillomaviruses in heterosexuals in Africa.* Journal of clinical medicine, 2015. **4**(4): p. 579-592.
46. Pavone, G., et al., *Entangled connections: HIV and HPV interplay in cervical cancer—a comprehensive review.* International journal of molecular sciences, 2024. **25**(19): p. 10358.
47. Levi, J.E., et al., *Presence of multiple human papillomavirus types in cervical samples from HIV-infected women.* Gynecologic oncology, 2004. **92**(1): p. 225-231.
48. Justiz-Vaillant, A., et al., *Challenges and Advances in Cervical Cancer Prevention for HIV-Positive Individuals: A Review of ART Efficacy, HPV Screening, and Vaccination Strategies Challenges and Advances in Cervical Cancer A Review of ART Efficacy, HPV Screening.* 2024.
49. Lim, S.T. and A.M. Levine, *Non-AIDS-defining cancers and HIV infection.* Curr HIV/AIDS Rep, 2005. **2**(3): p. 146-53.
50. Stelzle, D., et al., *Estimates of the global burden of cervical cancer associated with HIV.* The lancet global health, 2021. **9**(2): p. e161-e169.
51. Dzinamarira, T., et al., *Cervical cancer in sub-Saharan Africa: an urgent call for improving accessibility and use of preventive services.* International Journal of Gynecologic Cancer, 2023. **33**(4).
52. Maiman, M., et al., *Cervical cancer as an AIDS-defining illness.* Obstetrics & Gynecology, 1997. **89**(1): p. 76-80.
53. Blattner, W.A. and R.G. Nowak, *Epidemiology of AIDS-defining malignancies*, in *Cancers in People with HIV and AIDS: Progress and Challenges*. 2014, Springer. p. 17-30.
54. Manawapat-Klopfer, A., et al., *HPV16 viral load and physical state measurement as a potential immediate triage strategy for HR-HPV-infected women: a study in 644 women with single HPV16 infections.* Am J Cancer Res, 2018. **8**(4): p. 715-722.
55. Debeaudrap, P., et al., *Residual or Recurrent Precancerous Lesions After Treatment of Cervical Lesions in Human Immunodeficiency Virus-infected Women: A Systematic Review and Meta-analysis of Treatment Failure.* Clin Infect Dis, 2019. **69**(9): p. 1555-1565.
56. Hidalgo-Tenorio, C., et al., *ART is key to clearing oncogenic HPV genotypes (HR-HPV) in anal mucosa of HIV-positive MSM.* PloS one, 2019. **14**(10): p. e0224183.
57. Kelly, H., et al., *Association of antiretroviral therapy with high-risk human papillomavirus, cervical intraepithelial neoplasia, and invasive cervical cancer in women living with HIV: a systematic review and meta-analysis.* Lancet HIV, 2018. **5**(1): p. e45-e58.
58. Palefsky, J.M., M.L. Gillison, and H.D. Strickler, *Chapter 16: HPV vaccines in immunocompromised women and men.* Vaccine, 2006. **24 Suppl 3**: p. S3/140-6.
59. Wentzensen, N., M.A. Clarke, and R.B. Perkins, *Impact of COVID-19 on cervical cancer screening: Challenges and opportunities to improving resilience and reduce disparities.* Preventive Medicine, 2021. **151**: p. 106596.
60. Sohail, F., et al., *Topic: Genomic characterization of Human Papillomavirus and pathogenesis leading to cervical cancer.* GSJ, 2021. **9**(10).

61. Wu, M., et al., *Unveiling the multifaceted realm of human papillomavirus: a comprehensive exploration of biology, interactions, and advances in cancer management.* Frontiers in Immunology, 2024. **15**: p. 1430544.
62. de Villiers, E.-M., *Cross-roads in the classification of papillomaviruses.* Virology, 2013. **445**(1-2): p. 2-10.
63. Nelson, C.W. and L. Mirabello, *Human papillomavirus genomics: Understanding carcinogenicity.* Tumour virus research, 2023. **15**: p. 200258.
64. Graham, S.V., *Human papillomavirus: gene expression, regulation and prospects for novel diagnostic methods and antiviral therapies.* Future microbiology, 2010. **5**(10): p. 1493-1506.
65. Yu, L., V. Majerciak, and Z.-M. Zheng, *HPV16 and HPV18 genome structure, expression, and post-transcriptional regulation.* International journal of molecular sciences, 2022. **23**(9): p. 4943.
66. Gabriel, J., D. de Figueiredo, and R. de Farias, *Revealing highly conserved regions in the E6 protein among distinct human papillomavirus types using comparative analysis of multiple sequence alignments.* Brazilian Journal of Biology, 2013. **73**: p. 449-450.
67. De Villiers, E.-M., et al., *Classification of papillomaviruses.* Virology, 2004. **324**(1): p. 17-27.
68. Chiang, C.-M., et al., *Control of human papillomavirus type 11 origin of replication by the E2 family of transcription regulatory proteins.* Journal of virology, 1992. **66**(9): p. 5224-5231.
69. Dreer, M., S. van de Poel, and F. Stubenrauch, *Control of viral replication and transcription by the papillomavirus E8^ E2 protein.* Virus Research, 2017. **231**: p. 96-102.
70. McBride, A.A., *Replication and partitioning of papillomavirus genomes.* Advances in virus research, 2008. **72**: p. 155-205.
71. Pinidis, P., et al., *Human papilloma virus' life cycle and carcinogenesis.* Maedica, 2016. **11**(1): p. 48.
72. de Freitas, A.C., et al., *hrHPV E5 oncoprotein: immune evasion and related immunotherapies.* Journal of Experimental & Clinical Cancer Research, 2017. **36**(1): p. 1-15.
73. Zhang, B., D.F. Spandau, and A. Roman, *E5 protein of human papillomavirus type 16 protects human foreskin keratinocytes from UV B-irradiation-induced apoptosis.* Journal of Virology, 2002. **76**(1): p. 220-231.
74. Auvinen, E., A. Alonso, and P. Auvinen, *Human papillomavirus type 16 E5 protein colocalizes with the antiapoptotic Bcl-2 protein.* Archives of virology, 2004. **149**(9): p. 1745-1759.
75. Fontecha, N., et al., *Assessment of human papillomavirus E6/E7 oncogene expression as cervical disease biomarker.* BMC cancer, 2016. **16**: p. 1-7.
76. Stanley, M.A., *Epithelial cell responses to infection with human papillomavirus.* Clinical microbiology reviews, 2012. **25**(2): p. 215-222.
77. Scheffner, M., et al., *The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53.* Cell, 1990. **63**(6): p. 1129-1136.
78. McBride, A.A., *Mechanisms and strategies of papillomavirus replication.* Biological chemistry, 2017. **398**(8): p. 919-927.
79. Varsani, A., et al., *Chimeric human papillomavirus type 16 (HPV-16) L1 particles presenting the common neutralizing epitope for the L2 minor capsid protein of HPV-6 and HPV-16.* Journal of virology, 2003. **77**(15): p. 8386-8393.
80. Evans, M.R., et al., *An oral keratinocyte life cycle model identifies novel host genome regulation by human papillomavirus 16 relevant to HPV positive head and neck cancer.* Oncotarget, 2017. **8**(47): p. 81892.
81. Harari, A., Z. Chen, and R.D. Burk, *Human papillomavirus genomics: past, present and future*, in *Human papillomavirus*. 2014, Karger Publishers. p. 1-18.
82. Zur Hausen, H., *Papillomaviruses and cancer: from basic studies to clinical application.* Nature reviews cancer, 2002. **2**(5): p. 342-350.
83. de Sanjose, S., M. Brotons, and M.A. Pavon, *The natural history of human papillomavirus infection.* Best practice & research Clinical obstetrics & gynaecology, 2018. **47**: p. 2-13.

84. Bernard, H.-U., et al., *Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments.* Virology, 2010. **401**(1): p. 70-79.
85. Bzhalava, D., C. Eklund, and J. Dillner, *International standardization and classification of human papillomavirus types.* Virology, 2015. **476**: p. 341-344.
86. Cubie, H.A., et al., *HPV prevalence in women attending cervical screening in rural Malawi using the cartridge-based Xpert® HPV assay.* Journal of Clinical Virology, 2017. **87**: p. 1-4.
87. Papillomaviruses, H., *IARC monographs on the evaluation of carcinogenic risks to humans.* Lyon, France: IARC, 2011.
88. Chen, Z., et al., *Classification and evolution of human papillomavirus genome variants: Alpha-5 (HPV26, 51, 69, 82), Alpha-6 (HPV30, 53, 56, 66), Alpha-11 (HPV34, 73), Alpha-13 (HPV54) and Alpha-3 (HPV61).* Virology, 2018. **516**: p. 86-101.
89. Gheit, T., *Mucosal and cutaneous human papillomavirus infections and cancer biology.* Frontiers in Oncology, 2019. **9**: p. 355.
90. Doorbar, J., et al., *The biology and life-cycle of human papillomaviruses.* Vaccine, 2012. **30**: p. F55-F70.
91. Bernard, H.U., I.E. Calleja-Macias, and S.T. Dunn, *Genome variation of human papillomavirus types: phylogenetic and medical implications.* International journal of cancer, 2006. **118**(5): p. 1071-1076.
92. Egawa, N. and J. Doorbar, *The low-risk papillomaviruses.* Virus research, 2017. **231**: p. 119-127.
93. De Gregorio, V., et al., *In vitro organotypic systems to model tumor microenvironment in human papillomavirus (HPV)-related cancers.* Cancers, 2020. **12**(5): p. 1150.
94. Harden, M.E. and K. Munger, *Human papillomavirus molecular biology.* Mutation Research/Reviews in Mutation Research, 2017. **772**: p. 3-12.
95. Bienkowska-Haba, M., et al., *Human papillomavirus genome copy number is maintained by S-phase amplification, genome loss to the cytosol during mitosis, and degradation in G1 phase.* Journal of virology, 2023. **97**(2): p. e01879-22.
96. Bienkowska-Haba, M., et al., *A new cell culture model to genetically dissect the complete human papillomavirus life cycle.* PLoS pathogens, 2018. **14**(3): p. e1006846.
97. Doorbar, J., et al., *Human papillomavirus molecular biology and disease association.* Reviews in medical virology, 2015. **25**: p. 2-23.
98. Skelin, J. and V. Tomaić, *Comparative analysis of alpha and beta HPV E6 oncoproteins: Insights into functional distinctions and divergent mechanisms of pathogenesis.* Viruses, 2023. **15**(11): p. 2253.
99. Zhang, Y., et al., *Roles of human papillomavirus in cancers: oncogenic mechanisms and clinical use.* Signal transduction and targeted therapy, 2025. **10**(1): p. 44.
100. Albert, E. and L. Laimins, *Regulation of the Human Papillomavirus Life Cycle by DNA Damage Repair Pathways and Epigenetic Factors.* Viruses, 2020. **12**(7).
101. Bronnimann, M.P., et al., *Furin cleavage of L2 during papillomavirus infection: minimal dependence on cyclophilins.* Journal of virology, 2016. **90**(14): p. 6224-6234.
102. Kirk, A. and S.V. Graham, *The human papillomavirus late life cycle and links to keratinocyte differentiation.* Journal of Medical Virology, 2024. **96**(2): p. e29461.
103. Quinlan, S.F., *The Impact of Cutaneous Papillomavirus and Interferon Regulatory Factors on Interferon Expression in Canine and Human Keratinocytes*. 2022: North Carolina State University.
104. Gao, L., et al., *Anal HPV infection in HIV-positive men who have sex with men from China.* PloS one, 2010. **5**(12): p. e15256.
105. Wang, W., et al., *Association between viral loads of different oncogenic human papillomavirus types and the degree of cervical lesions in the progression of cervical Cancer.* Clinica Chimica Acta, 2018. **483**: p. 249-255.

106. Yousefi, Z., et al., *An Update on Human Papilloma Virus Vaccines: History, Types, Protection, and Efficacy.* Front Immunol, 2021. **12**: p. 805695.
107. Williams, V.M., et al., *HPV-DNA integration and carcinogenesis: putative roles for inflammation and oxidative stress.* Future virology, 2011. **6**(1): p. 45-57.
108. Gupta, S., P. Kumar, and B.C. Das, *HPV: Molecular pathways and targets.* Current problems in cancer, 2018. **42**(2): p. 161-174.
109. Khamseh, A., et al., *Analysis of HPV-16 viral load, integration status, and p16 expression in relation to EBV co-infection and cervical lesion severity.* Scientific Reports, 2025. **15**(1): p. 8329.
110. Akagi, K., et al., *Genome-wide analysis of HPV integration in human cancers reveals recurrent, focal genomic instability.* Genome research, 2014. **24**(2): p. 185-199.
111. Mir, B.A., et al., *Increased expression of HPV-E7 oncoprotein correlates with a reduced level of pRb proteins via high viral load in cervical cancer.* Scientific Reports, 2023. **13**(1): p. 15075.
112. Basukala, O. and L. Banks, *The not-so-good, the bad and the ugly: HPV E5, E6 and E7 oncoproteins in the orchestration of carcinogenesis.* Viruses, 2021. **13**(10): p. 1892.
113. Janiszewska, J., et al., *HPV-driven oncogenesis—much more than the E6 and E7 oncoproteins.* Journal of Applied Genetics, 2025. **66**(1): p. 63-71.
114. Downham, L., et al., *Accuracy of HPV E6/E7 oncoprotein tests to detect high-grade cervical lesions: a systematic literature review and meta-analysis.* British Journal of Cancer, 2024. **130**(4): p. 517-525.
115. Skelin, J., I. Sabol, and V. Tomaić, *Do or die: HPV E5, E6 and E7 in cell death evasion.* Pathogens, 2022. **11**(9): p. 1027.
116. Vats, A., et al., *Regulation of HPV E7 stability by E6-associated protein (E6AP).* Journal of Virology, 2022. **96**(16): p. e00663-22.
117. Xia, C., *Investigate the Role of USP7 in HPV16E7-mediated Carcinogenesis*. 2022, The Chinese University of Hong Kong (Hong Kong).
118. Porter, V.L. and M.A. Marra, *The drivers, mechanisms, and consequences of genome instability in HPV-driven cancers.* Cancers, 2022. **14**(19): p. 4623.
119. Yadav, C., et al., *Overview of genetic and epigenetic regulation of human papillomavirus and apoptosis in cervical cancer.* Apoptosis, 2023. **28**(5): p. 683-701.
120. Ojha, P.S., et al., *Human papillomavirus and cervical cancer: an insight highlighting pathogenesis and targeting strategies.* Virusdisease, 2022. **33**(2): p. 132-154.
121. Pavelescu, L.A., et al., *Molecular Insights into HPV-Driven Cervical Cancer: Oncoproteins, Immune Evasion, and Epigenetic Modifications.* Microorganisms, 2025. **13**(5): p. 1000.
122. Swain, M., *Update in pathological classification of cervical intraepithelial neoplasia and cervical cancer.* Journal of Colposcopy and Lower Genital Tract Pathology, 2023. **1**(2): p. 56-63.
123. Aina, O.E., et al., *Classification of cervical intraepithelial neoplasia (cin) using fine-tuned convolutional neural networks.* Intelligence-Based Medicine, 2021. **5**: p. 100031.
124. Regauer, S. and O. Reich, *The histologic and molecular spectrum of highly differentiated HPV-independent cervical intraepithelial neoplasia.* The American Journal of Surgical Pathology, 2023. **47**(8): p. 942-949.
125. Ehret, A., et al., *Regression rate of high-grade cervical intraepithelial lesions in women younger than 25 years.* Archives of gynecology and obstetrics, 2023. **307**(3): p. 981-990.
126. Muntinga, C.L., et al., *Importance of the immune microenvironment in the spontaneous regression of cervical squamous intraepithelial lesions (cSIL) and implications for immunotherapy.* Journal of Clinical Medicine, 2022. **11**(5): p. 1432.
127. Loopik, D.L., et al., *The natural history of cervical intraepithelial neoplasia grades 1, 2, and 3: a systematic review and meta-analysis.* Journal of Lower Genital Tract Disease, 2021. **25**(3): p. 221-231.

128. Goldstein, A., et al., *The future of cervical cancer screening.* International journal of women's health, 2024: p. 1715-1731.
129. Ni, H., et al., *Targeting HPV for the prevention, diagnosis, and treatment of Cervical Cancer.* Journal of Molecular Cell Biology, 2024. **16**(10): p. mjae046.
130. Han, K.H., *Evaluation of human papillomavirus (HPV) genotyping assays using type-specific HPV L1 reference DNA.* Genes & Genomics, 2021. **43**(7): p. 775-781.
131. Siqueira, J.D., et al., *Comparison of four different human papillomavirus genotyping methods in cervical samples: addressing method-specific advantages and limitations.* Heliyon, 2024. **10**(3).
132. Santos, F.L., et al., *Comparative analysis of different PCR-based strategies for HPV detection and genotyping from cervical samples.* Journal of Medical Virology, 2021. **93**(11): p. 6347-6354.
133. Bandeira, I.C., et al., *Comparative evaluation of HPV genotyping: A study on the performance concordance between Anyplex II HPV28 detection and Linear Array genotyping tests in nationwide studies in Brazil.* PLoS One, 2024. **19**(6): p. e0305122.
134. Güzel, C., et al., *Molecular markers for cervical cancer screening.* Expert review of proteomics, 2021. **18**(8): p. 675-691.
135. Haile, E.L., *Evaluation of cervical cancer screening uptake, HPV genotyping and self-sampling collection techniques in Ethiopia*. 2022, University of Antwerp.
136. Lv, N., et al., *Development of a sensitive droplet digital PCR according to the HPV infection specificity in Chinese population.* BMC cancer, 2023. **23**(1): p. 1022.
137. Shah, U.J., et al., *Emerging biomarkers and clinical significance of HPV genotyping in prevention and management of cervical cancer.* Microbial pathogenesis, 2020. **143**: p. 104131.
138. Banila, C., *Investigation of DNA methylation and microbial biomarkers to improve cervical cancer triage and early diagnosis.* 2021.
139. Sierra-Rojas, J.C., et al., *Modeling and Mathematical Analysis of the Dynamics of HPV in Cervical Epithelial Cells: Transient, Acute, Latency, and Chronic Infections.* Computational and Mathematical Methods in Medicine, 2022. **2022**.
140. Torres-Ibarra, L., et al., *Adjunctive testing by cytology, p16/Ki-67 dual-stained cytology or HPV16/18 E6 oncoprotein for the management of HPV16/18 screen-positive women.* International Journal of Cancer, 2021. **148**(9): p. 2264-2273.
141. Levi, J.E., et al., *Human papillomavirus prevalence, viral load and cervical intraepithelial neoplasia in HIV-infected women.* Brazilian Journal of Infectious Diseases, 2002. **6**: p. 129-134.
142. Saunier, M., et al., *Analysis of human papillomavirus type 16 (HPV16) DNA load and physical state for identification of HPV16-infected women with high-grade lesions or cervical carcinoma.* Journal of clinical microbiology, 2008. **46**(11): p. 3678-3685.
143. Gök, M., et al., *HPV16 and increased risk of recurrence after treatment for CIN.* Gynecol Oncol, 2007. **104**(2): p. 273-5.
144. Fu, Y., et al., *Residual disease and risk factors in patients with high-grade cervical intraepithelial neoplasia and positive margins after initial conization.* Therapeutics and Clinical Risk Management, 2015: p. 851-856.
145. Singhal, D., et al., *Targeted gene panels identify a high frequency of pathogenic germline variants in patients diagnosed with a hematological malignancy and at least one other independent cancer.* Leukemia, 2021. **35**(11): p. 3245-3256.
146. Viveros-Carreño, D., A. Fernandes, and R. Pareja, *Updates on cervical cancer prevention.* International Journal of Gynecological Cancer, 2023. **33**(3): p. 394-402.
147. Wei, F., et al., *Causal attribution of human papillomavirus genotypes to invasive cervical cancer worldwide: a systematic analysis of the global literature.* The Lancet, 2024. **404**(10451): p. 435-444.

148. Dalstein, V., et al., *Persistence and load of high-risk HPV are predictors for development of high-grade cervical lesions: a longitudinal French cohort study.* International journal of cancer, 2003. **106**(3): p. 396-403.
149. Josefsson, A.M., et al., *Viral load of human papilloma virus 16 as a determinant for development of cervical carcinoma in situ: a nested case-control study.* The Lancet, 2000. **355**(9222): p. 2189-2193.
150. Zhou, J., et al., *HPV16/HPV58 viral load is non-linearly correlated with cervical lesions and can be used as a triage marker.* Infectious Agents and Cancer, 2025. **20**: p. 48.
151. Fu Xi, L., et al., *Type-dependent association between risk of cervical intraepithelial neoplasia and viral load of oncogenic human papillomavirus types other than types 16 and 18.* International journal of cancer, 2017. **140**(8): p. 1747-1756.
152. Zhang, Y., et al., *Verification of the association of the cycle threshold (Ct) values from HPV testing on Cobas4800 with the histologic grades of cervical lesions using data from two population-based cervical cancer screening trials.* Infectious Agents and Cancer, 2022. **17**(1): p. 27.
153. Lee, S., et al., *Semi-quantitative HPV viral load in patients with ASC-US cytology: viral load correlates strongly with the presence of CIN but only weakly with its severity.* Cytopathology, 2015. **26**(1): p. 19-25.
154. LAI, H.C., et al., *Differential viral loads of human papillomavirus 16 and 58 infections in the spectrum of cervical carcinogenesis.* International Journal of Gynecological Cancer, 2006. **16**(2): p. 730-735.
155. Baumann, A., et al., *HPV16 load is a potential biomarker to predict risk of high-grade cervical lesions in high-risk HPV-infected women: a large longitudinal French hospital-based cohort study.* Cancers, 2021. **13**(16): p. 4149.
156. Adcock, R., et al., *Role of HPV genotype, multiple infections, and viral load on the risk of high-grade cervical neoplasia.* Cancer Epidemiology, Biomarkers & Prevention, 2019. **28**(11): p. 1816-1824.
157. Oyervides-Muñoz, M.A., et al., *Multiple HPV infections and viral load association in persistent cervical lesions in Mexican women.* Viruses, 2020. **12**(4): p. 380.
158. Flores, R., et al., *Cross-sectional analysis of oncogenic HPV viral load and cervical intraepithelial neoplasia.* International journal of cancer, 2006. **118**(5): p. 1187-1193.
159. Chaberek, K., et al., *The Creation of the Suppressive Cancer Microenvironment in Patients with HPV-Positive Cervical Cancer.* Diagnostics, 2022. **12**(8): p. 1906.
160. Lu, X., et al., *Analysis of influencing factors of viral load in patients with high-risk human papillomavirus.* Virology Journal, 2021. **18**(1): p. 1-8.
161. Bencomo-Álvarez, A., et al., *Human papillomavirus viral load in cervical intraepithelial neoplasia as a prognostic factor in a Mexican population.* Genetics and Molecular Research, 2012. **11**(4): p. 4720-4727.
162. van Eer, K., et al., *The association between viral load and concurrent human papillomavirus infection at the genital and anal sites of young women and the impact of vaccination.* Tumour virus research, 2022. **13**: p. 200233.
163. Mbulawa, Z.Z., et al., *Increased alpha-9 human papillomavirus species viral load in human immunodeficiency virus positive women.* BMC Infectious Diseases, 2014. **14**: p. 1-7.
164. Liu, G., et al., *HIV-positive women have higher risk of human papilloma virus infection, precancerous lesions, and cervical cancer.* Aids, 2018. **32**(6): p. 795-808.
165. Adler, D.H., et al., *Cumulative impact of HIV and multiple concurrent human papillomavirus infections on the risk of cervical dysplasia.* Advances in Virology, 2016. **2016**.
166. Levi, J.E., et al., *High prevalence of human papillomavirus (HPV) infections and high frequency of multiple HPV genotypes in human immunodeficiency virus-infected women in Brazil.* Journal of clinical microbiology, 2002. **40**(9): p. 3341-3345.

167. Tugizov, S.M., et al., *HIV-associated disruption of mucosal epithelium facilitates paracellular penetration by human papillomavirus.* Virology, 2013. **446**(1-2): p. 378-388.
168. Strickler, H.D., et al., *Natural history and possible reactivation of human papillomavirus in human immunodeficiency virus–positive women.* Journal of the National Cancer Institute, 2005. **97**(8): p. 577-586.
169. Viscidi, R.P., et al., *Human papillomavirus capsid antibody response to natural infection and risk of subsequent HPV infection in HIV-positive and HIV-negative women.* Cancer Epidemiology Biomarkers & Prevention, 2005. **14**(1): p. 283-288.
170. Guo, M., et al., *Distribution and viral load of eight oncogenic types of human papillomavirus (HPV) and HPV 16 integration status in cervical intraepithelial neoplasia and carcinoma.* Modern Pathology, 2007. **20**(2): p. 256-266.
171. Stanley, M., *Immune responses to human papillomavirus.* Vaccine, 2006. **24**: p. S16-S22.
172. Lei, V., et al., *Skin viral infections: Host antiviral innate immunity and viral immune evasion.* Frontiers in Immunology, 2020. **11**: p. 593901.
173. Hilligan, K.L. and F. Ronchese, *Antigen presentation by dendritic cells and their instruction of CD4+ T helper cell responses.* Cellular & molecular immunology, 2020. **17**(6): p. 587-599.
174. Sato, K., et al., *Regulatory dendritic cells.* Emerging Concepts Targeting Immune Checkpoints in Cancer and Autoimmunity, 2017: p. 47-71.
175. Fernandes, J.V., J.d. Araujo, and T. Fernandes, *Biology and natural history of human papillomavirus infection.* Open Access J Clin Trials, 2013. **5**(1): p. 1-12.
176. Barros Jr, M.R., et al., *Viral modulation of TLRs and cytokines and the related immunotherapies for HPV-associated cancers.* Journal of immunology research, 2018. **2018**(1): p. 2912671.
177. Taghavi, M., et al., *Role of pathogen-associated molecular patterns (PAMPS) in immune responses to fungal infections.* European journal of pharmacology, 2017. **808**: p. 8-13.
178. Wicherska-Pawłowska, K., T. Wróbel, and J. Rybka, *Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) in innate immunity. TLRs, NLRs, and RLRs ligands as immunotherapeutic agents for hematopoietic diseases.* International journal of molecular sciences, 2021. **22**(24): p. 13397.
179. Wu, J., et al., *Analysis of residual/recurrent disease and its risk factors after loop electrosurgical excision procedure for high-grade cervical intraepithelial neoplasia.* Gynecologic and obstetric investigation, 2016. **81**(4): p. 296-301.
180. Steinbach, A. and A.B. Riemer, *Immune evasion mechanisms of human papillomavirus: An update.* International journal of cancer, 2018. **142**(2): p. 224-229.
181. Murakami, N. and L.V. Riella, *Co-inhibitory pathways and their importance in immune regulation.* Transplantation, 2014. **98**(1): p. 3-14.
182. Zitvogel, L., A. Tesniere, and G. Kroemer, *Cancer despite immunosurveillance: immunoselection and immunosubversion.* Nature reviews immunology, 2006. **6**(10): p. 715-727.
183. Blok, B.A., et al., *Trained innate immunity as underlying mechanism for the long-term, nonspecific effects of vaccines.* Journal of leukocyte biology, 2015. **98**(3): p. 347-356.
184. Amarante-Mendes, G.P., et al., *Pattern recognition receptors and the host cell death molecular machinery.* Frontiers in immunology, 2018. **9**: p. 2379.
185. Mboumba Bouassa, R.-S., et al., *Natural and vaccine-induced B cell-derived systemic and mucosal humoral immunity to human papillomavirus.* Expert Review of Anti-infective Therapy, 2020. **18**(6): p. 579-607.
186. Mollers, M., *Monitoring the impact of HPV vaccination.* Utrecht: Vrije Universiteit Amsterdam, 2014.
187. Frazer, I., *Correlating immunity with protection for HPV infection.* International Journal of Infectious Diseases, 2007. **11**: p. S10-S16.

188. Zhang, T., et al., *A rationally designed flagellin-L2 fusion protein induced serum and mucosal neutralizing antibodies against multiple HPV types.* Vaccine, 2019. **37**(30): p. 4022-4030.
189. Hickey, D.K., et al., *Regulation of mucosal immunity in the genital tract: Balancing reproduction and protective immunity*, in *Mucosal Vaccines*. 2020, Elsevier. p. 255-297.
190. Kann, H., *Vaccine Effectiveness Against HPV Infections*. 2020, Karolinska Institutet (Sweden).
191. Ho, G.Y., et al., *Risk factors for subsequent cervicovaginal human papillomavirus (HPV) infection and the protective role of antibodies to HPV-16 virus-like particles.* The Journal of infectious diseases, 2002. **186**(6): p. 737-742.
192. Schwarz, T.F. and O. Leo, *Immune response to human papillomavirus after prophylactic vaccination with AS04-adjuvanted HPV-16/18 vaccine: improving upon nature.* Gynecologic oncology, 2008. **110**(3): p. S1-S10.
193. Satyaprakash, A. and C. Mansur, *12 Human Papillomaviruses.* Mucocutaneous Manifestations of Viral Diseases: An Illustrated Guide to Diagnosis and Management, 2016: p. 207.
194. Mbuya, W., *Dissecting the influence of HIV on HPV infection and cervical cancer.* Tanzania Journal of Health Research, 2022. **23**.
195. Ramezani, A., et al., *Significance of serum antibodies against HPV E7, Hsp27, Hsp20 and Hp91 in Iranian HPV-exposed women.* BMC infectious diseases, 2019. **19**: p. 1-8.
196. Jasrotia, R., et al., *Nanotechnology based vaccines: Cervical cancer management and perspectives.* Journal of Drug Delivery Science and Technology, 2022. **71**: p. 103351.
197. Maskey, N., et al., *Infiltrating CD4 and CD8 lymphocytes in HPV infected uterine cervical milieu.* Cancer management and research, 2019: p. 7647-7655.
198. Bashaw, A.A., et al., *Modulation of antigen presenting cell functions during chronic HPV infection.* Papillomavirus Research, 2017. **4**: p. 58-65.
199. Walsh, K.P. and K.H. Mills, *Dendritic cells and other innate determinants of T helper cell polarisation.* Trends in immunology, 2013. **34**(11): p. 521-530.
200. de Visser, K.E. and L.M. Coussens, *The inflammatory tumor microenvironment and its impact on cancer development.* Infection and Inflammation: Impacts on Oncogenesis, 2006. **13**: p. 118-137.
201. Chen, X.-Z., et al., *Toll like receptor agonists augment HPV 11 E7-specific T cell responses by modulating monocyte-derived dendritic cells.* Archives of dermatological research, 2010. **302**(1): p. 57-65.
202. Leiben, G., M.P. Velders, and W.M. Kast, *The cell-mediated immune response to human papillomavirus-induced cervical cancer: implications for immunotherapy.* 2002.
203. Einstein, M.H., et al., *Clinician's guide to human papillomavirus immunology: knowns and unknowns.* The Lancet infectious diseases, 2009. **9**(6): p. 347-356.
204. Lin, W., et al., *Imbalance of Th1/Th2 and Th17/Treg during the development of uterine cervical cancer.* International journal of clinical and experimental pathology, 2019. **12**(9): p. 3604.
205. Stern, P.L., *Harnessing immunity for therapy in human papillomavirus driven cancers.* Tumour virus research, 2021. **11**: p. 200212.
206. Nakagawa, M., et al., *Persistence of human papillomavirus type 16 infection is associated with lack of cytotoxic T lymphocyte response to the E6 antigens.* The Journal of infectious diseases, 2000. **182**(2): p. 595-598.
207. Farhat, S., M. Nakagawa, and A.-B. Moscicki, *Cell-mediated immune responses to human papillomavirus 16 E6 and E7 antigens as measured by interferon gamma enzyme-linked immunospot in women with cleared or persistent human papillomavirus infection.* International Journal of Gynecological Cancer, 2009. **19**(4): p. 508-512-508-512.
208. Falcinelli, S.D., et al., *New frontiers in measuring and characterizing the HIV reservoir.* Frontiers in microbiology, 2019. **10**: p. 2878.

209. Meys, R., F. Gotch, and C. Bunker, *Human papillomavirus in the era of highly active antiretroviral therapy for human immunodeficiency virus: an immune reconstitution-associated disease?* British Journal of Dermatology, 2010. **162**(1): p. 6-11.
210. Otani, S., et al., *Cytokine expression profiles in cervical mucus from patients with cervical cancer and its precursor lesions.* Cytokine, 2019. **120**: p. 210-219.
211. Boccardo, E., A.P. Lepique, and L.L. Villa, *The role of inflammation in HPV carcinogenesis.* Carcinogenesis, 2010. **31**(11): p. 1905-1912.
212. Amador-Molina, A., et al., *Role of innate immunity against human papillomavirus (HPV) infections and effect of adjuvants in promoting specific immune response.* Viruses, 2013. **5**(11): p. 2624-2642.
213. Fernandes, J.V., et al., *Link between chronic inflammation and human papillomavirus-induced carcinogenesis.* Oncology letters, 2015. **9**(3): p. 1015-1026.
214. Bordignon, V., et al., *How human papillomavirus replication and immune evasion strategies take advantage of the host DNA damage repair machinery.* Viruses, 2017. **9**(12): p. 390.
215. Stanley, M., *Host defence and persistent human papillomavirus infection.* Current Opinion in Virology, 2021. **51**: p. 106-110.
216. Groves, I.J. and N. Coleman, *Human papillomavirus genome integration in squamous carcinogenesis: what have next-generation sequencing studies taught us?* The Journal of pathology, 2018. **245**(1): p. 9-18.
217. Cordeiro, M.N., et al., *Current research into novel therapeutic vaccines against cervical cancer.* Expert review of anticancer therapy, 2018. **18**(4): p. 365-376.
218. Crosbie, E.J., et al., *Human papillomavirus and cervical cancer.* The Lancet, 2013. **382**(9895): p. 889-899.
219. Ronco, L.V., et al., *Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity.* Genes Dev, 1998. **12**(13): p. 2061-72.
220. Nees, M., et al., *Papillomavirus type 16 oncogenes downregulate expression of interferon-responsive genes and upregulate proliferation-associated and NF-kappaB-responsive genes in cervical keratinocytes.* J Virol, 2001. **75**(9): p. 4283-96.
221. Gensterblum-Miller, E. and J.C. Brenner, *Protecting tumors by preventing human papilloma virus antigen presentation: insights from emerging bioinformatics algorithms.* Cancers, 2019. **11**(10): p. 1543.
222. Senba, M. and N. Mori, *Oncogenesis of human papillomavirus (HPV) associated with cancers and host immune responses.* International Journal of Cancer Research and Prevention, 2016. **9**(1): p. 1.
223. Kaliamurthi, S., et al., *Designing of CD8+ and CD8+-overlapped CD4+ epitope vaccine by targeting late and early proteins of human papillomavirus.* Biologics: Targets and Therapy, 2018: p. 107-125.
224. Song, S.-H., et al., *Interferon-γ (IFN-γ): a possible prognostic marker for clearance of high-risk human papillomavirus (HPV).* Gynecologic oncology, 2008. **108**(3): p. 543-548.
225. Kasashima, S., et al., *Upregulated interleukins (IL-6, IL-10, and IL-13) in immunoglobulin G4-related aortic aneurysm patients.* Journal of vascular surgery, 2018. **67**(4): p. 1248-1262.
226. Zhou, C., Z.K. Tuong, and I.H. Frazer, *Papillomavirus immune evasion strategies target the infected cell and the local immune system.* Frontiers in oncology, 2019. **9**: p. 682.
227. Organization, W.H., *Accelerating the elimination of cervical cancer as a public health problem: Towards achieving 90–70–90 targets by 2030*, in *Accelerating the elimination of cervical cancer as a public health problem: Towards achieving 90–70–90 targets by 2030*. 2022.
228. Ghosh, A., et al., *HPV Vaccines–An Overview.* Indian Journal of Dermatology, 2025. **70**(4): p. 188-200.
229. Williamson, A.-L., *Recent developments in human papillomavirus (HPV) vaccinology.* Viruses, 2023. **15**(7): p. 1440.

230. Kusters, J.M., et al., *Effectiveness of bivalent HPV vaccination against genital HPV DNA-positivity of a catch-up campaign at age 13–16 years compared to routine vaccination at age 12 years: a biennial repeated cross-sectional study.* BMC medicine, 2024. **22**(1): p. 469.
231. Pfingstag, C.S., *A Multi-Component Intervention to Increase Catch-Up HPV Vaccination in a Sexual/Reproductive Health Setting*. 2020, Southeastern Louisiana University.
232. Wigle, J., H.B. Fontenot, and G.D. Zimet, *Global delivery of human papillomavirus vaccines.* Pediatric Clinics, 2016. **63**(1): p. 81-95.
233. Spayne, J. and T. Hesketh, *Estimate of global human papillomavirus vaccination coverage: analysis of country-level indicators.* BMJ open, 2021. **11**(9): p. e052016.
234. Asempah, E. and E. Ikpebe, *Accelerating HPV vaccination in Africa for health equity.* Global Health Research and Policy, 2024. **9**(1): p. 37.
235. Akandinda, M., et al., *A Review on Factors Associated with HPV Vaccination in Africa.* Madonna University journal of Medicine and Health Sciences ISSN: 2814-3035, 2022. **2**(3): p. 1-5.
236. Katz, I.T., et al., *A qualitative analysis of factors influencing HPV vaccine uptake in Soweto, South Africa among adolescents and their caregivers.* PloS one, 2013. **8**(8): p. e72094.
237. Adams, R. and M. Botha, *Cervical cancer prevention in Southern Africa: a review of national cervical cancer screening guidelines in the Southern African development community.* Journal of Cancer Policy, 2024. **40**: p. 100477.
238. Nolan, T., et al., *SPUD: a quantitative PCR assay for the detection of inhibitors in nucleic acid preparations.* Anal Biochem, 2006. **351**(2): p. 308-10.
239. Liu, S.S., et al., *Evaluation of a newly developed GenoArray human papillomavirus (HPV) genotyping assay and comparison with the Roche Linear Array HPV genotyping assay.* J Clin Microbiol, 2010. **48**(3): p. 758-64.
240. Hull, R., et al., *Cervical cancer in low and middle-income countries.* Oncology letters, 2020. **20**(3): p. 2058-2074.
241. Tainio, K., et al., *Clinical course of untreated cervical intraepithelial neoplasia grade 2 under active surveillance: systematic review and meta-analysis.* bmj, 2018. **360**.
242. Koeneman, M.M., et al., *Natural history of high-grade cervical intraepithelial neoplasia: a review of prognostic biomarkers.* Expert review of molecular diagnostics, 2015. **15**(4): p. 527-546.
243. Athanasiou, A., et al., *Comparative efficacy and complication rates after local treatment for cervical intraepithelial neoplasia and stage 1a1 cervical cancer: protocol for a systematic review and network meta-analysis from the CIRCLE Group.* BMJ open, 2019. **9**(8): p. e028008.
244. Saraiya, U.B., *Current trends in the management of cervical intraepithelial neoplasia.* Journal of South Asian Federation of Obstetrics and Gynaecology, 2022. **14**(4): p. 458-464.
245. Thorsteinsson, K., et al., *Persistence of cervical high-risk human papillomavirus in women living with HIV in Denmark–the SHADE.* BMC Infectious Diseases, 2019. **19**(1): p. 1-10.
246. Rohner, E., et al., *Cervical cancer risk in women living with HIV across four continents: a multicohort study.* International journal of cancer, 2020. **146**(3): p. 601-609.
247. Kelly, H., et al., *Diagnostic accuracy of cervical cancer screening strategies for high-grade cervical intraepithelial neoplasia (CIN2+/CIN3+) among women living with HIV: A systematic review and meta-analysis.* EClinicalMedicine, 2022. **53**.
248. Bazzett-Matabele, L., et al., *Outcomes of Loop Electrosurgical Excision Procedures Performed for Severe Cervical Dysplasia in Botswana.* JCO Global Oncology, 2025. **11**: p. e2400472.
249. Tebeu, P.M., et al., *The recurrence of cervical intraepithelial neoplasia in HIV-positive women: a review of the literature.* International journal of STD & AIDS, 2006. **17**(8): p. 507-511.
250. Baloglu, A., et al., *Residual and recurrent disease rates following LEEP treatment in high-grade cervical intraepithelial lesions.* Archives of gynecology and obstetrics, 2010. **282**(1): p. 69-73.

251. Debeaudrap, P., et al., *Residual or recurrent precancerous lesions after treatment of cervical lesions in human immunodeficiency virus–infected women: a systematic review and meta-analysis of treatment failure.* Clinical Infectious Diseases, 2019. **69**(9): p. 1555-1565.
252. Zhang, J., et al., *Human papillomavirus infection and disease recurrence/persistence after treatment for women of high-grade cervical intraepithelial neoplasia with coexisting vaginal intraepithelial neoplasia.* Frontiers in Cellular and Infection Microbiology, 2025. **15**: p. 1602216.
253. Fontaine, J., et al., *Human papillomavirus type 16 (HPV-16) viral load and persistence of HPV-16 infection in women infected or at risk for HIV.* Journal of clinical virology, 2008. **43**(3): p. 307-312.
254. Manawapat, A., et al., *Physical state and viral load as predictive biomarkers for persistence and progression of HPV16-positive cervical lesions: results from a population based long-term prospective cohort study.* American journal of cancer research, 2012. **2**(2): p. 192.
255. Park, J.-Y., et al., *The association of pre-conization high-risk HPV load and the persistence of HPV infection and persistence/recurrence of cervical intraepithelial neoplasia after conization.* Gynecologic oncology, 2008. **108**(3): p. 549-554.
256. Lorincz, A.T., et al., *Viral load of human papillomavirus and risk of CIN3 or cervical cancer.* The Lancet, 2002. **360**(9328): p. 228-229.
257. Alonso, I., et al., *Pre-and post-conization high-risk HPV testing predicts residual/recurrent disease in patients treated for CIN 2–3.* Gynecologic oncology, 2006. **103**(2): p. 631-636.
258. Mongelós, P., et al., *Detection of high risk human papillomavirus by hybrid capture II (r) according cytological findings in women treated for squamous intraepithelial lesions of the cervix, period 2006/2010.* Revista Brasileira de Epidemiologia, 2013. **16**: p. 40-48.
259. Gosvig, C.F., et al., *Persistence and reappearance of high-risk human papillomavirus after conization.* Gynecologic Oncology, 2013. **131**(3): p. 661-666.
260. Sherman, M.E., et al., *Determinants of human papillomavirus load among women with histological cervical intraepithelial neoplasia 3: dominant impact of surrounding low-grade lesions.* Cancer Epidemiology Biomarkers & Prevention, 2003. **12**(10): p. 1038-1044.
261. Kelly, H.A., et al., *Associations of Human Papillomavirus (HPV) genotypes with high-grade cervical neoplasia (CIN2+) in a cohort of women living with HIV in Burkina Faso and South Africa.* PloS one, 2017. **12**(3): p. e0174117.
262. Hawes, S.E., et al., *Increased risk of high-grade cervical squamous intraepithelial lesions and invasive cervical cancer among African women with human immunodeficiency virus type 1 and 2 infections.* The Journal of infectious diseases, 2003. **188**(4): p. 555-563.
263. Cassani, C., et al., *The role of multiple high-risk human papillomavirus infection on the persistence recurrence of high-grade cervical lesions after standard treatment: A systematic review and a meta-analysis.* Acta Obstetricia et Gynecologica Scandinavica, 2024. **103**(6): p. 1028-1035.
264. Mittal, S., et al., *Risk of high-grade precancerous lesions and invasive cancers in high-risk HPV-positive women with normal cervix or CIN 1 at baseline—A population-based cohort study.* International Journal of cáncer, 2017. **140**(8): p. 1850-1859.
265. Schlecht, N.F., et al., *Viral load as a predictor of the risk of cervical intraepithelial neoplasia.* International journal of cancer, 2003. **103**(4): p. 519-524.
266. Luchters, S.M., et al., *Association of HIV infection with distribution and viral load of HPV types in Kenya: a survey with 820 female sex workers.* BMC infectious diseases, 2010. **10**(1): p. 1-10.
267. Tadese, B.K., et al., *The Burden of HPV Infections and HPV-Related Diseases Among People With HIV: A Systematic Literature Review.* Journal of medical virology, 2025. **97**(4): p. e70274.

268. Depuydt, C.E., et al., *Linear viral load increase of a single HPV-type in women with multiple HPV infections predicts progression to cervical cancer.* International Journal of Cancer, 2016. **139**(9): p. 2021-2032.
269. Segondy, M., et al., *Diagnostic value of human papillomavirus (HPV) 16 and HPV18 viral loads for the detection of high-grade cervical intraepithelial neoplasia (CIN2+) in a cohort of African women living with HIV.* Journal of clinical virology, 2018. **99**: p. 79-83.
270. Franceschi, S., et al., *Differences in the risk of cervical cancer and human papillomavirus infection by education level.* British journal of cancer, 2009. **101**(5): p. 865-870.
271. Mitchell, S.M., et al., *Factors associated with high-risk HPV positivity in a low-resource setting in sub-Saharan Africa.* American journal of obstetrics and gynecology, 2014. **210**(1): p. 81. e1-81. e7.
272. Bonanni, P., S. Boccalini, and A. Bechini, *Efficacy, duration of immunity and cross protection after HPV vaccination: a review of the evidence.* Vaccine, 2009. **27**: p. A46-A53.
273. Kjaer, S.K., et al., *Real-world effectiveness of human papillomavirus vaccination against cervical cancer.* JNCI: Journal of the National Cancer Institute, 2021. **113**(10): p. 1329-1335.
274. Adeyanju, G.C., et al., *Human papillomavirus vaccination acceleration and introduction in Sub-Saharan Africa: a multi-country cohort analysis.* Vaccines, 2024. **12**(5): p. 489.
275. Amponsah-Dacosta, E., B.M. Kagina, and J. Olivier, *Health systems constraints and facilitators of human papillomavirus immunization programmes in sub-Saharan Africa: a systematic review.* Health policy and planning, 2020. **35**(6): p. 701-717.
276. Asempah, E., *Cervical Cancer prevalence in sub-Saharan Africa and HPV vaccination policy: a public health grand challenge?* Journal of Cancer Immunology, 2021. **3**(2): p. 87-97.
277. Kadish, A.S., et al., *Regression of cervical intraepithelial neoplasia and loss of human papillomavirus (HPV) infection is associated with cell-mediated immune responses to an HPV type 16 E7 peptide.* Cancer Epidemiology Biomarkers & Prevention, 2002. **11**(5): p. 483-488.
278. Hibma, M.H., *The immune response to papillomavirus during infection persistence and regression.* The open virology journal, 2012. **6**: p. 241.
279. Stern, P.L., et al., *Therapy of human papillomavirus-related disease.* Vaccine, 2012. **30 Suppl 5**(0 5): p. F71-82.
280. Prendiville, W., *The treatment of CIN: what are the risks?* Cytopathology, 2009. **20**(3): p. 145-153.
281. Kalliala, I., et al., *Cancer free survival after CIN treatment: comparisons of treatment methods and histology.* Gynecologic oncology, 2007. **105**(1): p. 228-233.
282. Gupta, S., et al., *From diagnosis to treatment: exploring the latest management trends in cervical intraepithelial neoplasia.* Cureus, 2023. **15**(12).
283. Basu, P., et al., *Management of cervical premalignant lesions.* Current problems in cancer, 2018. **42**(2): p. 129-136.
284. Organization, W.H., *WHO guideline for screening and treatment of cervical pre-cancer lesions for cervical cancer prevention.* 2021.
285. Xu, L., Y. Jiang, and R. Zhao, *Advances in ablative treatment for human papillomavirus related cervical pre-cancer lesions.* Gynecology and Obstetrics Clinical Medicine, 2023. **3**(4): p. 213-219.
286. Inada, N.M., et al., *Long term effectiveness of photodynamic therapy for CIN treatment.* Pharmaceuticals, 2019. **12**(3): p. 107.
287. Heard, I., et al., *High rate of recurrence of cervical intraepithelial neoplasia after surgery in HIV-positive women.* JAIDS Journal of Acquired Immune Deficiency Syndromes, 2005. **39**(4): p. 412-418.
288. Fruchter, R.G., et al., *Multiple recurrences of cervical intraepithelial neoplasia in women with the human immunodeficiency virus.* Obstetrics & Gynecology, 1996. **87**(3): p. 338-344.

289. Avila, J.P., B.M. Carvalho, and E.C. Coimbra, *A comprehensive view of the cancer-immunity cycle (CIC) in HPV-mediated cervical cancer and prospects for emerging therapeutic opportunities.* Cancers, 2023. **15**(4): p. 1333.
290. Øvestad, I.T., et al., *Local immune response in the microenvironment of CIN2–3 with and without spontaneous regression.* Modern pathology, 2010. **23**(9): p. 1231-1240.
291. Monnier-Benoit, S., et al., *Immunohistochemical analysis of CD4+ and CD8+ T-cell subsets in high risk human papillomavirus-associated pre-malignant and malignant lesions of the uterine cervix.* Gynecologic oncology, 2006. **102**(1): p. 22-31.
292. Kojima, S., et al., *The prevalence of cervical regulatory T cells in HPV-related cervical intraepithelial neoplasia (CIN) correlates inversely with spontaneous regression of CIN.* American Journal of Reproductive Immunology, 2013. **69**(2): p. 134-141.
293. Farhat, S., M. Nakagawa, and A.-B. Moscicki, *Cell-mediated immune responses to human papillomavirus 16 E6 and E7 antigens as measured by IFN gamma ELISpot in women with cleared or persistent HPV infection.* International journal of gynecological cancer: official journal of the International Gynecological Cancer Society, 2009. **19**(4): p. 508.
294. Lo Cigno, I., et al., *High-risk HPV oncoproteins E6 and E7 and their interplay with the innate immune response: Uncovering mechanisms of immune evasion and therapeutic prospects.* Journal of Medical Virology, 2024. **96**(6): p. e29685.
295. Trimble, C.L., et al., *Naturally occurring systemic immune responses to HPV antigens do not predict regression of CIN2/3.* Cancer Immunology, Immunotherapy, 2010. **59**(5): p. 799-803.
296. Castellsagué, X., *Natural history and epidemiology of HPV infection and cervical cancer.* Gynecologic oncology, 2008. **110**(3): p. S4-S7.
297. Okunade, K.S., *Human papillomavirus and cervical cancer.* Journal of Obstetrics and Gynaecology, 2020. **40**(5): p. 602-608.
298. Ramakrishnan, S., S. Partricia, and G. Mathan, *Overview of high-risk HPV's 16 and 18 infected cervical cancer: pathogenesis to prevention.* Biomedicine & pharmacotherapy, 2015. **70**: p. 103-110.
299. Passmore, J.A.S., et al., *Comparison of cervical and blood T-cell responses to human papillomavirus-16 in women with human papillomavirus-associated cervical intraepithelial neoplasia.* Immunology, 2006. **119**(4): p. 507-514.
300. Singh, M., et al., *Functional characterization of CD4 and CD8 T cell responses among human papillomavirus infected patients with ano-genital warts.* Virusdisease, 2017. **28**(2): p. 133-140.
301. Nakagawa, M., et al., *CD4-positive and CD8-positive cytotoxic T lymphocytes contribute to human papillomavirus type 16 E6 and E7 responses.* Clinical Diagnostic Laboratory Immunology, 1999. **6**(4): p. 494-498.
302. Shibata, T., et al., *Expansion of human papillomavirus-specific T cells in periphery and cervix in a therapeutic vaccine recipient whose cervical high-grade squamous intraepithelial lesion regressed.* Frontiers in immunology, 2021. **12**: p. 645299.
303. Stanley, M., *Immune responses to genital HPV*. 2006: Basel, Karger.
304. Stanley, M., *Immune responses to human papillomavirus and the development of human papillomavirus vaccines*, in *Human Papillomavirus*. 2020, Elsevier. p. 283-297.
305. Stanley, M., M. Pett, and N. Coleman, *HPV: from infection to cancer.* Biochemical Society Transactions, 2007. **35**(6): p. 1456-1460.
306. Sasagawa, T., H. Takagi, and S. Makinoda, *Immune responses against human papillomavirus (HPV) infection and evasion of host defense in cervical cancer.* Journal of Infection and Chemotherapy, 2012. **18**(6): p. 807-815.
307. Schiller, J.T., P.M. Day, and R.C. Kines, *Current understanding of the mechanism of HPV infection.* Gynecologic oncology, 2010. **118**(1): p. S12-S17.
308. Saunders-Wood, T., et al., *Role of E6 in maintaining the basal cell reservoir during productive papillomavirus infection.* Journal of Virology, 2022. **96**(5): p. e01181-21.

309. Crish, J.F., et al., *Suprabasal expression of the human papillomavirus type 16 oncoproteins in mouse epidermis alters expression of cell cycle regulatory proteins.* Carcinogenesis, 2000. **21**(5): p. 1031-1037.
310. de Gruijl, T.D., et al., *Differential T helper cell responses to human papillomavirus type 16 E7 related to viral clearance or persistence in patients with cervical neoplasia: a longitudinal study.* Cancer research, 1998. **58**(8): p. 1700-1706.
311. Kim, T.J., et al., *Clearance of persistent HPV infection and cervical lesion by therapeutic DNA vaccine in CIN3 patients.* Nature communications, 2014. **5**(1): p. 5317.
312. Steele, J., et al., *T-cell responses to human papillomavirus type 16 among women with different grades of cervical neoplasia.* British journal of cancer, 2005. **93**(2): p. 248-259.
313. Kiamba, E.W., M.R. Goodier, and E. Clarke, *Immune responses to human papillomavirus infection and vaccination.* Frontiers in Immunology, 2025. **16**: p. 1591297.
314. Bourgault Villada, I., et al., *Human papillomavirus 16-specific T cell responses in classic HPV-related vulvar intra-epithelial neoplasia. Determination of strongly immunogenic regions from E6 and E7 proteins.* Clinical & Experimental Immunology, 2010. **159**(1): p. 45-56.
315. de Jong, A., et al., *Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6.* Cancer research, 2004. **64**(15): p. 5449-5455.
316. Welters, M.J., et al., *Induction of tumor-specific CD4+ and CD8+ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine.* Clinical Cancer Research, 2008. **14**(1): p. 178-187.
317. Kim, K.H., et al., *CD4+ T-cell response against human papillomavirus type 16 E6 protein is associated with a favorable clinical trend.* Cancer Immunology, Immunotherapy, 2012. **61**: p. 63-70.
318. Mbuya, W., et al., *Depletion of human papilloma virus E6-and E7-oncoprotein-specific T-cell responses in women living with HIV.* Frontiers in Immunology, 2021: p. 4473.
319. Swase, T.D., et al., *The impact of HPV/HIV co-infection on immunosuppression, HPV genotype, and cervical cancer biomarkers.* BMC cancer, 2025. **25**(1): p. 202.
320. Valdespino, V., et al., *HPV16-specific cytotoxic T lymphocyte responses are detected in all HPV16-positive cervical cancer patients.* Gynecologic oncology, 2005. **96**(1): p. 92-102.
321. Ma, M., et al., *Human papilloma virus E1-specific T cell immune response is associated with the prognosis of cervical cancer patients with squamous cell carcinoma.* Infectious Agents and Cancer, 2018. **13**(1): p. 35.
322. Guberman, M., et al., *Mouse B cells engineered to express an anti-HPV antibody elicit anti-tumor T cell responses.* Frontiers in Immunology, 2025. **16**: p. 1613879.
323. Scholten, K.B.J., *Adoptive transfer of tumor specific T-cells for the treatment of cervical cancer: Generation of HPV specific T-cells.* 2010.
324. Della Fera, A.N., et al., *Persistent human papillomavirus infection.* Viruses, 2021. **13**(2): p. 321.
325. Jee, B., et al., *Immunology of HPV-mediated cervical cancer: current understanding.* International Reviews of Immunology, 2021. **40**(5): p. 359-378.
326. Vanajothi, R., et al., *HPV-mediated cervical cancer: a systematic review on immunological basis, molecular biology, and immune evasion mechanisms.* Current Drug Targets, 2022. **23**(8): p. 782-801.
327. Senba, M. and N. Mori, *Mechanisms of virus immune evasion lead to development from chronic inflammation to cancer formation associated with human papillomavirus infection.* Oncology reviews, 2012. **6**(2).
328. Doorbar, J., *Host control of human papillomavirus infection and disease.* Best practice & research Clinical obstetrics & gynaecology, 2018. **47**: p. 27-41.

329. Heusinkveld, M., et al., *Systemic and local human papillomavirus 16-specific T-cell immunity in patients with head and neck cancer.* International journal of cancer, 2012. **131**(2): p. E74-E85.
330. Reusser, N.M., et al., *HPV carcinomas in immunocompromised patients.* Journal of clinical medicine, 2015. **4**(2): p. 260-281.
331. Schulz, T.F., *Cancer and viral infections in immunocompromised individuals.* International journal of cancer, 2009. **125**(8): p. 1755-1763.
332. Palefsky, J.M., M.L. Gillison, and H.D. Strickler, *HPV vaccines in immunocompromised women and men.* Vaccine, 2006. **24**: p. S140-S146.
333. Wielgos, A.A. and B. Pietrzak, *Human papilloma virus-related premalignant and malignant lesions of the cervix and anogenital tract in immunocompromised women.* Ginekologia Polska, 2020. **91**(1): p. 32-37.
334. Santos, C., et al., *HPV-transgenic mouse models: Tools for studying the cancer-associated immune response.* Virus research, 2017. **235**: p. 49-57.
335. Zottnick, S., A.L. Voß, and A.B. Riemer, *Inducing immunity where it matters: orthotopic HPV tumor models and therapeutic vaccinations.* Frontiers in Immunology, 2020. **11**: p. 1750.
336. Paaso, A., et al., *From HPV infection to lesion progression: the role of HLA alleles and host immunity.* Acta cytologica, 2019. **63**(2): p. 148-158.
337. Lehain, S., *Molecular mechanisms governing CD4+ T cell recognition of human papilloma virus (HPV) E6 in cervical carcinoma*. 2009: Cardiff University (United Kingdom).
338. Chang, W.-C., et al., *Regulatory T cells suppress natural killer cell immunity in patients with human cervical carcinoma.* International Journal of Gynecological Cancer, 2016. **26**(1): p. 156-162.
339. Evans, E.M.-l., et al., *Infiltration of cervical cancer tissue with human papillomavirus-specific cytotoxic T-lymphocytes.* Cancer Research, 1997. **57**(14): p. 2943-2950.
340. Stanley, M.A. and J.C. Sterling, *Host responses to infection with human papillomavirus.* Human Papillomavirus, 2014. **45**: p. 58-74.
341. O'Garra, A. and K. Murphy, *Role of Cytokines in Development of.* Th1 and Th2 cells in Health and Disease, 1996. **63**: p. 1-13.
342. Gulati, K., et al., *Cytokines and their role in health and disease: a brief overview.* Moj Immunol, 2016. **4**(2): p. 00121.
343. Kumar, A. and S. Lata, *Cytokine response against human papillomavirus infection*, in *Cytokine Response Against Major Human Viral Infections*. 2025, Elsevier. p. 79-95.
344. Barros, M.R., et al., *Viral modulation of TLRs and cytokines and the related immunotherapies for HPV-associated cancers.* Journal of Immunology Research, 2018. **2018**.
345. Paradkar, P.H., et al., *Role of cytokines in genesis, progression and prognosis of cervical cancer.* Asian Pacific Journal of Cancer Prevention, 2014. **15**(9): p. 3851-3864.
346. Romani, L., P. Puccetti, and F. Bistoni, *Interleukin-12 in infectious diseases.* Clinical microbiology reviews, 1997. **10**(4): p. 611-636.
347. da Costa, F.A.M., et al., *The Th1-th2 profile in immune responses to Human papillomavirus (HPV) in vitro in men from the city of São Paulo, Brazil.* Brazilian Journal of Sexually Transmitted Diseases, 2019. **31**(1): p. 7-13.
348. Brombacher, F., *The role of interleukin-13 in infectious diseases and allergy.* Bioessays, 2000. **22**(7): p. 646-656.
349. Ogulur, I., et al., *Type 2 immunity in allergic diseases.* Cellular & Molecular Immunology, 2025: p. 1-32.
350. Lippitz, B.E., *Cytokine patterns in patients with cancer: a systematic review.* The lancet oncology, 2013. **14**(6): p. e218-e228.
351. Paul, D., *The systemic hallmarks of cancer.* Journal of Cancer Metastasis and Treatment, 2020. **6**: p. N/A-N/A.

352. Culig, Z., *Cytokine disbalance in common human cancers.* Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2011. **1813**(2): p. 308-314.
353. Lambrou, G.I., K. Hatziagapiou, and S. Vlahopoulos, *Inflammation and tissue homeostasis: The NF-κB system in physiology and malignant progression.* Molecular Biology Reports, 2020. **47**(5): p. 4047-4063.
354. Obeagu, E.I., *Modulation of Chronic Cytokine Dysregulation in Cervical Cancer: Potential Biomarkers and Therapeutic Targets.* Cancer Management and Research, 2025: p. 1113-1126.
355. Coleman, N., et al., *Immunological events in regressing genital warts.* American journal of clinical pathology, 1994. **102**(6): p. 768-774.
356. Singh, M., et al., *Functional characterization of CD4 and CD8 T cell responses among human papillomavirus infected patients with ano-genital warts.* Virusdisease, 2017. **28**: p. 133-140.
357. Kidd, P., *Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease.* Alternative medicine review, 2003. **8**(3): p. 223-246.
358. Butcher, M.J. and J. Zhu, *Recent advances in understanding the Th1/Th2 effector choice.* Faculty reviews, 2021. **10**.
359. Zhang, Y., et al., *Th1/Th2 cell's function in immune system.* T helper cell differentiation and their function, 2014: p. 45-65.
360. Zeng, Y., et al., *Expression and clinical significance of Th1/Th2 cells in different HPV types of cervical cancer.* Int J Clin Exp Med, 2019. **12**(8): p. 9953-9960.
361. Lin, W., et al., *The disease stage-associated imbalance of Th1/Th2 and Th17/Treg in uterine cervical cancer patients and their recovery with the reduction of tumor burden.* BMC women's Health, 2020. **20**(1): p. 1-7.
362. Bais, A., et al., *A shift to a peripheral Th2-type cytokine pattern during the carcinogenesis of cervical cancer becomes manifest in CIN III lesions.* Journal of clinical pathology, 2005. **58**(10): p. 1096-1100.
363. Kobayashi, A., et al., *Evolving immunosuppressive microenvironment during human cervical carcinogenesis.* Mucosal immunology, 2008. **1**(5): p. 412-420.
364. Schottenfeld, D. and J. Beebe-Dimmer, *Chronic inflammation: a common and important factor in the pathogenesis of neoplasia.* CA: a cancer journal for clinicians, 2006. **56**(2): p. 69-83.
365. Anuja, K., et al., *Prolonged inflammatory microenvironment is crucial for pro-neoplastic growth and genome instability: a detailed review.* Inflammation Research, 2017. **66**: p. 119-128.
366. Carrero, Y.N., D.E. Callejas, and J.A. Mosquera, *In situ immunopathological events in human cervical intraepithelial neoplasia and cervical cancer.* Translational Oncology, 2021. **14**(5): p. 101058.
367. Zheng, J.-J., et al., *Difference in vaginal microecology, local immunity and HPV infection among childbearing-age women with different degrees of cervical lesions in Inner Mongolia.* BMC women's health, 2019. **19**(1): p. 109.
368. Shen, L., P.-L. Xue, and Y.-N. Zhu, *Relationship between HPV infection and its genotypes and Th1/Th2 balance and disease progression in lesion micro-environment of patients with cervical lesions.* 2022.
369. Scott, M., D.P. Stites, and A.-B. Moscicki, *Th1 cytokine patterns in cervical human papillomavirus infection.* Clinical Diagnostic Laboratory Immunology, 1999. **6**(5): p. 751-755.
370. Bais, A.G., et al., *Cytokine release in HR-HPV (+) women without and with cervical dysplasia (CIN II and III) or carcinoma, compared with HR-HPV (−) controls.* Mediators of inflammation, 2007. **2007**(1): p. 024147.
371. Cai, Y., et al., *The role of IL-2, IL-4, IL-10 and IFN-γ cytokines expression in the microenvironment of cervical intraepithelial neoplasia.* Advances in Reproductive Sciences, 2022. **10**(4): p. 106-114.

372. Misson, D.R., et al., *Cytokine serum levels in patients with cervical intraepithelial neoplasia grade II-III treated with intralesional interferon-α 2b.* Tumori Journal, 2011. **97**(5): p. 578-584.
373. Zhu, R., et al., *Interactions between vaginal local cytokine IL-2 and high-risk human papillomavirus infection with cervical intraepithelial neoplasia in a Chinese population-based study.* Frontiers in Cellular and Infection Microbiology, 2023. **13**: p. 544.
374. Song, S.-H., et al., *The relationship between cytokines and HPV-16, HPV-16 E6, E7, and high-risk HPV viral load in the uterine cervix.* Gynecologic oncology, 2007. **104**(3): p. 732-738.
375. Lee, S.-J., et al., *Both E6 and E7 oncoproteins of human papillomavirus 16 inhibit IL-18-induced IFN-γ production in human peripheral blood mononuclear and NK cells.* The journal of immunology, 2001. **167**(1): p. 497-504.
376. Afify, S.M., et al., *Cancer-inducing niche: the force of chronic inflammation.* British Journal of Cancer, 2022. **127**(2): p. 193-201.
377. Vidal, A.C., et al., *IL-10, IL-15, IL-17, and GMCSF levels in cervical cancer tissue of Tanzanian women infected with HPV16/18 vs. non-HPV16/18 genotypes.* Infectious Agents and Cancer, 2015. **10**: p. 1-7.
378. Wang, Y., et al., *The paradox of IL-10-mediated modulation in cervical cancer.* Biomedical Reports, 2013. **1**(3): p. 347-351.
379. Feng, Q., et al., *Th2 type inflammation promotes the gradual progression of HPV-infected cervical cells to cervical carcinoma.* Gynecologic oncology, 2012. **127**(2): p. 412-419.
380. Commins, S.P., L. Borish, and J.W. Steinke, *Immunologic messenger molecules: cytokines, interferons, and chemokines.* Journal of Allergy and Clinical immunology, 2010. **125**(2): p. S53-S72.
381. Lee, H.L., et al., *Inflammatory cytokines and change of Th1/Th2 balance as prognostic indicators for hepatocellular carcinoma in patients treated with transarterial chemoembolization.* Scientific reports, 2019. **9**(1): p. 3260.
382. Rs, J., *The Immune Microenvironment in Human Papilloma Virus-Induced Cervical Lesions—Evidence for Estrogen as an Immunomodulator.* Frontiers in Cellular and Infection Microbiology, 2021. **11**: p. 649815.
383. Seresini, S., et al., *IFN-γ produced by human papilloma virus-18 E6-specific CD4+ T cells predicts the clinical outcome after surgery in patients with high-grade cervical lesions.* The Journal of Immunology, 2007. **179**(10): p. 7176-7183.
384. Paaso, A., et al., *Interferon-γ and IL-5 associated cell-mediated immune responses to HPV16 E2 and E6 distinguish between persistent oral HPV16 infections and noninfected mucosa.* Clinical and Experimental Dental Research, 2021. **7**(5): p. 903-913.

Chapter 7: Appendix

Table 7.1 Appendix: Reagent composition for Hybrid Capture 2 Assay

Reagent	Composition
Indicator Dye	Contains Sodium Azide
Denaturation Reagent	Dilute Sodium Hydroxide (NaOH) solution
Probe Diluent	Buffered solution with 0.05%(w/v) sodium azide
High-Risk HPV Probe	HPV 16/18/31/33/35/39/45/51/52/66/58/59/68 RNA probe cocktail in buffered solution (red cap
Low-Risk HPV Probe	HPV 6/11/42/43/44 RNA probe cocktail in buffered solution (green cap)
Negative Calibrator	Carrier DNA (Herring Sperm) in Specimen Transport Medium (STM) with 0.05%(w/v) sodium azide
Low Risk Calibrator	Carrier DNA (Herring Sperm) in Specimen Transport Medium (STM) with 0.05%(w/v) sodium azide
Low Risk Calibrator	1.0 pg/ml cloned HPV 11 DNA and carrier DNA in STM with 0.05%(w/v) sodium azide
High Risk Calibrator	1.0 pg/ml cloned HPV 16 DNA and carrier DNA in STM with 0.05%(w/v) sodium azide
Low-risk HPV Quality Contr	5pg/ml cloned HPV 6 and carrier DNA in STM (serves as additional negative calibrator) with 0.05%(w/v) sodium azide
High-risk HPV Quality Contr	5pg/ml cloned HPV 16 and carrier DNA in STM with 0.05%(w/v) sodium azide
1 Capture Microplate	Coated with goat polyclonal anti-RNA:DNA hybrid antibodies
Detection Reagent 1	Alkaline phosphatase-conjugated murine monoclonal antibodies to RNA:DNA hybrids in buffered solution with 0.05%(w/v) sodium azide
Detection Reagent 2	CDP-Star with Emerald II (chemiluminescent substrate) Wash Buffer Concentrate (contains 1.5% sodium azide)

Table 7.2 Appendix: Probe diluent volumes

No. of Wells (Tests)/Strips	Volume Probe Diluent*	Volume Probe*
48/6	2.0 ml	80 ul
24/3	1.0 ml	40 ul
1 well	0.045 ml	1.8 ul

*These values include the recommended extra volume

(A) Buffer preparation

The wash buffer was prepared by adding 100 ml of wash buffer concentrate to 2900 ml of distilled water thoroughly and transferred into a wash bottle as per Table 3. Additionally, the denaturation buffer was prepared by adding and thoroughly mixing 5 drops of indicator dye to 50 ml of denaturation reagent and stored between 2-8^{o}C.

Table 7.3 Appendix: Preparation of wash buffer

Volume of Wash Buffer required	Volume of Wash Buffer concentrate	Volume of distilled or deionized water
1 liter	33.3 ml	966.7 ml
2 liters	66.6 ml	1933.4 ml
3 liters	100.0 ml	2900.0 ml
6 liters	200.0 ml	5800.0 ml

(B) Plate layout

- The calibrators, quality controls, and specimens are run in an 8-microplate well column configuration.
- Test the calibrators and quality controls in the following positions on the microplate.
- Negative calibrator (NC) replicates in microplate wells A1, B1, C1.
- High-risk HPV calibrator (HRC) replicates in microplate wells D1, E1, and F1.
- Low-risk HPV Quality control (QC1-LR) in microplate G1.
- High-risk HPV Quality control (QC1-HR) in microplate H1.

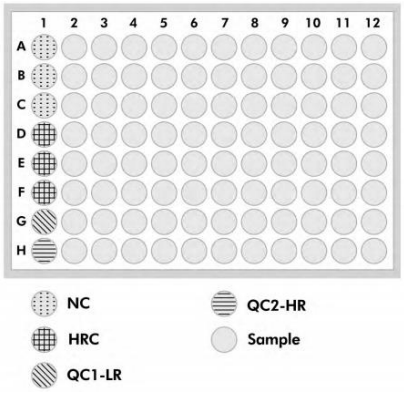

Figure 7.1 Appendix: Plate layout and position of calibrators, quality controls, and specimens on the microplate. **Note that specimens start from A2.**

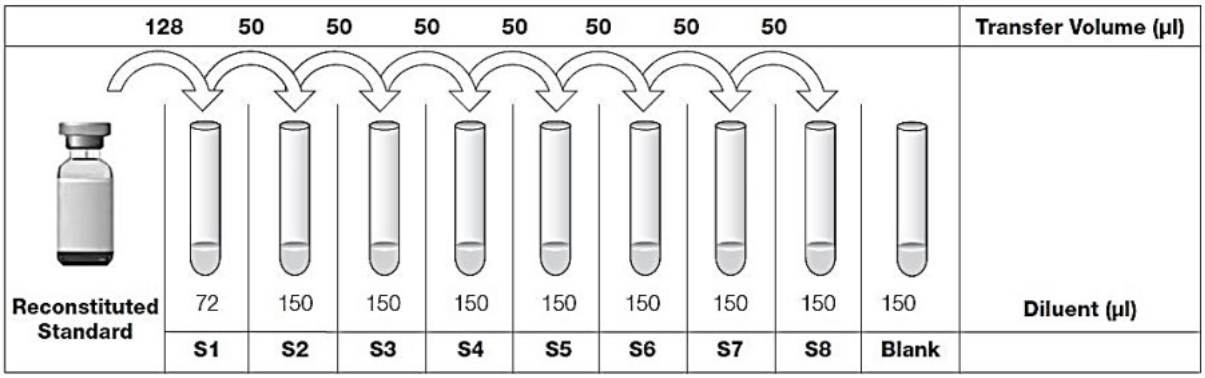

Figure 7.2 Appendix. Preparation of a four-fold standard dilution series (Figure adapted from the Bio-Plex Pro instruction manual).

Ten polypropylene tubes were labeled as standards 1 to 10. A volume of 72 µl of standard diluent was added to standard 1, while 150 µl of the same diluent was added to standards 2 to 10. Additionally, 128 µl of the reconstituted standard was added to the tube labeled Standard 1. Afterwards, 50 µl of the previous standard was added to the subsequent tube, with thorough vortexing between each liquid transfer.