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Investigation of Multiple Concurrent Human Papillomavirus Infections, Oncogenicity, and STI Co-infection as Risk Factors for Human Immunodeficiency Virus Infection.

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Submitted in fulfilment of the requirements for the degree of Master of Medical Science (Medical Microbiology) in the School of Laboratory Medicine and Medical Science, University of KwaZulu-Natal.

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Co-Supervisor: Dr Derseree Archary

2017

PLAGIARISM DECLARATION

I, Janine Jewanraj, declare that the work described in this dissertation has been composed solely by myself except where otherwise stated by reference or acknowledged and has not been submitted to UKZN or any other institution for the purposes of an academic qualification. This work was done under the supervision of Dr Lenine Liebenberg, at the Centre for the AIDS Program of Research in South Africa, Durban.

I, Dr Lenine Liebenberg, hereby declare that I have read this dissertation and believe its contents to be the original work of the candidate, with other work adequately referenced.

Janine Jewanraj
(Candidate)

Dr Lenine Liebenberg
(Supervisor)

DEDICATION

This dissertation is dedicated to my parents, Jane and Rajesh Jewanraj, who from a young age instilled in me the importance of education and striving for greatness in all aspects of life.

POSTER PRESENTATIONS

1. **Human Papillomavirus and Discharge-Associated Sexually Transmitted Infections Increase HIV Risk Through CCR5⁺ T Cell Recruitment.**

   Janine Jewanraj, Anne Rositch, Sanele Mhlungu, Andile Mtshali, Farzana Osman, Leila Mansoor, Salim Abdool Karim, Quarraisha Abdool Karim , Jo-Ann Passmore and Lenine Liebenberg.

   HIV Research for Prevention Conference, Chicago, Illinois, 18 October 2016

CAPRISA

Human Papillomavirus and Discharge-Associated Sexually Transmitted Infections Increase HIV Risk Through CCR5⁺ T Cell Recruitment

Janine Jewanraj [1,6], Anne Rositch [3], Sanele Mhlungu [1], Andile Mtshali [1], Farzana Osman [1], Lyle Mckinnon [1,6,7], Leila Mansoor [1], Salim Abdool Karim [1,2], Quarraisha Abdool Karim [1,2], Jo-Ann Passmore [1,4,5], and Lenine Liebenberg [1,6]

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Introduction

- Sexually transmitted infections (STIs) increase the risk of HIV acquisition likely through its association with inflammation in the female genital tract (FGT).
- Several discharge-associated STIs cause genital inflammation and immune activation, and have been implicated as risk factors for HIV acquisition.
- Human Papillomavirus (HPV), the genital wart and cervical cancer-causing agent, is one of the most common STIs worldwide.
- A coinfection with multiple inflammatory agents could likely impact the level of genital inflammation that promotes HIV acquisition.
- **Here we investigate potential compounding effects of HPV and discharge-associated STI coinfection on targets for HIV infection in the female genital tract.**

Methods

- A Mulitplexed PCR was used to detect the presence of several discharge-associated sexually transmitted microbes *(T.vaginalis, C.trachomatis, N.gonorrhoeae and M.genitalium)* in a study group of 166 participants at baseline visits during the CAPRISA 008 trial.
- The Roche linear array assay was used for genotyping HPV in this population.
- CCR5⁺ expression in cervical cytobrush-derived CD4⁺ and CD8⁺ T cells was assessed by multiparameter flowcytometry.
- Multiplex ELISA assays were conducted on matching cervicovaginal lavage supernatants to detect the presence of 48 cytokines collectively involved in chemotaxis, inflammation, growth, adaptive responses, regulation, and in haematopoeisis.

Results

- Women with a HPV/STI coinfection had greater CCR5⁺ expression in both CD4⁺ and CD8⁺ T cells compared to women with a discharge-associated STI only **(Figure 1)**.
- While cytokine concentrations were similar between the two groups, significant increases in several proinflammatory and chemotactic cytokines were observed predominantly in women infected with discharge-associated STI only, or both HPV and other STIs compared to women with no evidence of HPV or other STIs **(Table 1)** .

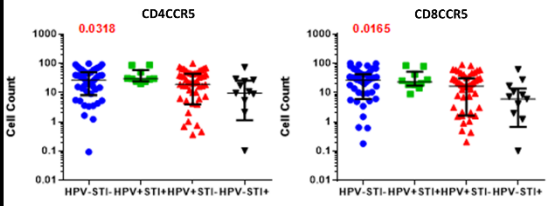

Figure 1. Comparison of cervical cellular event counts among CAPRISA 008 participants at baseline

Table 1. Statistically significant genital cytokine associations among CAPRISA 008 participants at baseline

	HPV-STI-	HPV-STI+	HPV+STI-	HPV+STI+	
Cytokine	Median (IQR) log10 pg/mL	Median (IQR) log_{10} pg/mL	Median (IQR) log_{10} pg/mL	Median (IQR) log_{10} pg/mL	P-value
IL-1α	2.1 (1.6 - 2.5)*	2.7 (2.0 - 3.0)	2.1 (1.8 - 2.4)	2.5 (2.2 - 2.8)*	0,008[a]
IL-1β	1.6 (1.0 - 2.0)*	2.4 (1.7 - 2.7)* **	1.5 (1.0 - 2.2)**	2.1 (1.7 - 2.4)	0,003[a]
IL-6	0.8 (0.5 - 1.3)*	1.2 (1.0 - 1.7)*	1.0 (0.8 - 1.3)	0.9 (0.7 - 1.0)	0,004[a]
IL-12p40	2.4 (2.3 - 2.5)	2.6 (2.4 - 2.7)	2.4 (2.3 - 2.5)	2.5 (2.3 - 2.6)	0,048
TNF-α	1.0 (0.8 - 1.4)	1.4 (1.1 - 1.8)	1.1 (0.9 - 1.3)	1.2 (1.0 - 1.5)	0,028
TNF-β	0.5 (0.4 - 0.7)	0.6 (0.4 - 0.9)	0.5 (0.4 - 0.6)	0.8 (0.5 - 0.9)	0,047
TRAIL	1.4 (1.1 - 1.7)* **	1.9 (1.5 - 2.3)**	1.5 (1.2 - 1.8)	1.9 (1.5 - 2.2)*	0,002[a]
CTACK	1.2 (1.0 - 1.4)* **	1.4 (1.3 - 1.8)**	1.2 (1.1 - 1.4)	1.5 (1.2 - 1.6)*	0,003[a]
IL-8	2.6 (2.2 - 3.0)	3.0 (2.6 - 3.6)	2.6 (2.3 - 3.0)	3.0 (2.7 - 3.2)	0,045
IL-16	1.5 (1.3 - 1.8)*	1.9 (1.7 - 2.3)*	1.6 (1.4 - 1.8)	1.8 (1.5 - 2.1)	0,013[a]
MIG	2.4 (1.8 - 3.0)*	3.2 (2.5 - 3.8)*	2.5 (2.1 - 3.1)	3.3 (2.3 - 3.5)	0,005[a]
MIP-1α	-0.0 (-0.3 - 0.2)*	0.3 (0.0 - 0.7)*	-0.0 (-0.2 - 0.3)	0.2 (-0.0 - 0.4)	0,013[a]
RANTES	0.8 (0.4 - 1.0)*	1.2 (0.9 - 1.5)*	0.8 (0.5 - 1.0)	1.0 (0.7 - 1.2)	0,014[a]
IFN-α2	1.2 (1.1 - 1.3)	1.4 (1.2 - 1.6)	1.2 (1.1 - 1.4)	1.4 (1.2 - 1.6)	0,009
β-NGF	-0.2 (-2.0 - 0.3)	0.2 (-0.1 - 0.7)	-0.1 (-0.5 - 0.2)	0.4 (-0.4 - 0.6)	0,021
HGF	2.1 (1.8 - 2.7)*	2.8 (2.3 - 3.3)*	2.3 (1.9 - 2.8)	2.6 (2.3 - 2.9)	0,004[a]
IL-3	2.0 (1.9 - 2.1)	2.1 (2.0 - 2.5)	2.0 (2.0 - 2.1)	2.3 (2.0 - 2.3)	0,032
LIF	1.1 (0.9 - 1.3)	1.2 (1.1 - 1.6)	1.1 (0.9 - 1.2)	1.3 (1.2 - 1.4)	0,008
PDGF-bb	1.1 (0.9 - 1.3)	1.3 (1.1 - 1.7)	1.1 (1.0 - 1.3)	1.3 (1.0 - 1.4)	0,042
SCF	1.0 (0.7 - 1.2)	1.4 (0.9 - 1.8)	1.0 (0.8 - 1.3)	1.5 (0.9 - 1.7)	0,013
SCGF-β	2.6 (2.1 - 2.9)*	3.1 (2.5 - 3.3)*	2.7 (2.4 - 3.0)	2.8 (2.4 - 3.1)	0,032[a]
IL-4	-0.0 (-0.2 - 0.1)*	0.2 (-0.0 - 0.4)*	0.0 (-0.1 - 0.1)	0.1 (-0.1 - 0.2)	0,039[a]
IL-5	0.0 (-0.9 - 0.4)*	0.5 (0.3 - 0.7)*	0.2 (-0.2 - 0.5)	0.4 (0.3 - 0.6)	0,007[a]
IL-17	1.1 (0.9 - 1.3)	1.2 (1.0 - 1.7)	1.1 (1.0 - 1.2)	1.1 (1.0 - 1.4)	0,040
IFN-γ	1.3 (1.1 - 1.5)	1.6 (1.3 - 1.8)	1.4 (1.2 - 1.5)	1.4 (1.3 - 1.6)	0,052
IL-2Rα	1.3 (1.1 - 1.5)	1.5 (1.4 - 1.9)**	1.2 (1.1 - 1.4)* **	1.6 (1.2 - 1.7)*	0,002[a]

[a] ANOVA p-value statistically significant after multiple comparisons adjustment. * Statistically significant association by Kruskall-Wallace Test. ** Statistically significant association by Kruskall-Wallace Test. P-values <0,05 were considered significant

Conclusion

STI was associated with increased cytokine concentrations. More than discharge-associated STI infection alone, HPV/STI coinfection increases the frequency of CCR5⁺ target cells for HIV infection, rendering these women more susceptible to HIV infection. Further investigation is needed to determine the impact of HPV vaccination, or STI treatment on target cell recruitment.

CAPRISA is an official research institute of the University of KwaZulu-Natal. CAPRISA was established in 2002 through a CIPRA grant from the NIH, as a multi-institutional collaboration, incorporated as an independent non-profit AIDS Research Organization. Registration Number 2002/024027/08

2. Potential Immune Mechanisms for the Relationship between HIV Risk, Multiple Concurrent HPV Infections and Oncogenicity

Janine Jewanraj, Anne Rositch, Sanele Mhlungu, Andile Mtshali, Frazana Osman, Lyle Mckinnon, Leila Mansoor, Salim Abdool Karim, Quarraisha Abdool Karim , Jo-Ann Passmore and Lenine Liebenberg.

8th SAAIDS Conference, Durban, ICC, 13 June 2017

9th IAS Conference on HIV Science, Paris, July 2017

CAPRISA

Potential Immune Mechanisms for the Relationship between HIV Risk, Multiple Concurrent HPV Infections and Oncogenicity

UNIVERSITY OF KWAZULU-NATAL INYUVESI YAKWAZULU-NATALI

Janine Jewanraj [1,6], Anne Rositch [3], Sanele Mhlungu [1], Andile Mtshali [1], Farzana Osman [1], Lyle Mckinnon [1,6,7], Leila Mansoor [1], Salim Abdool Karim [1,2], Quarraisha Abdool Karim [1,2], Jo-Ann Passmore [1,4,5], and Lenine Liebenberg [1,6]

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Introduction

In Sub-Saharan Africa women are disproportionately affected by both human papillomavirus (HPV) and Human Immunodeficiency Virus (HIV). HPV has been shown to increase the risk of HIV infection, but the biological bases for this association remains unclear. Infection with multiple HPV strains and high risk (HR) HPV, has been implicated as possible risk factors for HIV acquisition. **Here we investigated whether pro-inflammatory immune responses associated with multiple HPV infections and HPV oncogenicity, contribute to a genital immune environment conducive to an increased risk of HIV infection.**

Methods

- This study included baseline assessment of 167 women of the CAPRISA 008 trial, aged between 18-40 years, and all without PCR evidence of *Trichomonas vaginalis, Chlamydia trachomatis, Neisseria gonorrhoeae, and Mycoplasma genitalium* (N=139).
- The Roche Linear Array was used to detect the presence of 37 relevant HPV genotypes.
- The frequency of immune cells was assessed on cervical cytobrush-derived specimens by multiparametric flow cytometry.
- Multiplex ELISA was conducted to detect the concentrations of 48 different cytokines and markers of epithelial barrier integrity (Matrix Metalloproteinase and Tissue Inhibitors of Metalloproteinase) in the CVL supernatant.

Results

- Among the 167 women, 85 (51%) were HPV positive and 82 (49%) were HPV negative at baseline (**Table 1**). HPV positive women had a lower mean age, were more likely to have a partner with a higher mean age, were less likely to be living with a regular partner and a lower reported number of live births compared to HPV negative women (**Table 1**).
- HPV positive women had significantly higher frequencies of lymphocytes (**Figure 1a**) and cytokine concentrations of G-CSF, IL-5, IL-6 (**Figure 1b**).
- Women with multiple concurrent HPV infections had a greater frequencies of lymphocytes at the genital tract, compared to HPV negative women (**P=0.035**, data not shown).
- Women with HR HPV had significantly higher levels of growth factor G-CSF compared to HPV negative women and IL-12p70, IL-5 and IL-6 concentrations trended towards women with HR HPV having elevated levels of these cytokines (**Figure 2**).

Conclusion

HPV infection was associated increased cytokines concentrations and lymphocyte frequencies. Having multiple concurrent HPV infections was significantly associated with increased lymphocyte frequencies, and HR HPV was associated with increased concentrations of pro-inflammatory cytokines. These data suggest a potential biological mechanism for the relationship between HIV risk and HPV infection.

Table 1. Participant demographics according to HPV status (N=167)

Demographics	Overall (N=167) % (n)	HPV+ (N=85) % (n)	HPV- (N=82) % (n)	P-value
Age mean (SD)	29.6 (5.5)	28.4 (4.9)	30.9 (5.9)	0.004*
Age (years)				
(20-25)	18.6% (31)	20.0% (17)	17.1% (14)	0.032
(25-29)	41.3% (69)	49.4% (42)	32.9% (27)	
(≥ 30)	40.1% (67)	30.6% (26)	50.0% (41)	
Sexual Behaviour median (IQR)				
Age of Sexual Debut	18 (16 - 19)	18 (16 - 19)	18 (16 - 20)	0.323
Age of regular/stable partner	32 (28 - 37)	34 (30 - 37)	32 (28 - 36)	0.005*
Age of first partner	21 (19 - 24)	20 (19 - 24)	21 (19 - 24)	0.426
Number of live births	1 (1 - 2)	1 (1 - 2)	2 (1 - 3)	0.015*
Age at first pregnancy	19 (17 - 22)	19 (17 - 21)	19.5 (18 - 23)	0.191
Age when started menstruating	14 (13 - 16)	14 (13 - 15)	14 (13 - 16)	0.701
Number of lifetime partners	2 (2 - 4)	3 (2 - 4)	2 (1 - 4)	0.264
Number of vaginal sex acts in the last 30 days	4 (2 – 8)	4 (3 – 8)	4 (2 – 6)	0.110
Living with regular partner				
(Yes)	23.4% (39)	16.5% (14)	30.5% (25)	0.026*
(No)	76.6% (128)	83.5% (71)	69.5% (57)	
Use of Male Condoms				
(Always)	37.7% (63)	34.1% (29)	41.5% (34)	0.638
(Sometimes)	48.5% (81)	45.1% (37)	45.1% (37)	
(Never)	13.8% (23)	13.4% (11)	13.4% (11)	

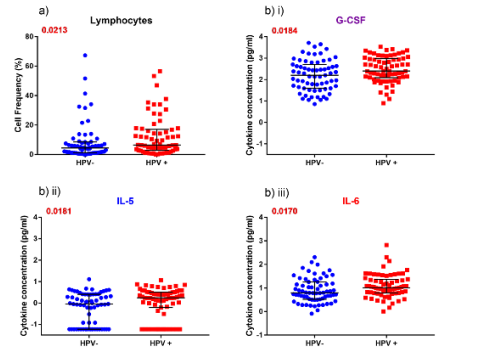

Figure 1. a) Cell frequencies and b) cytokine concentrations among HPV+ (N=68) and HPV- (67) women. *The Mann Whitney-U test was used to compare cell frequencies and cytokine concentrations among HPV positive and HPV negative women. Represented above are the medians and interquartile ranges (IQR).*

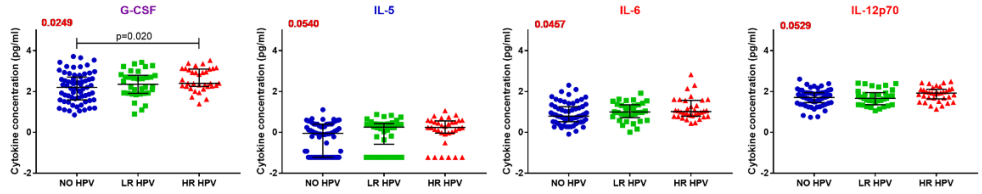

Figure 2. Cytokine concentrations among women with 0 (N=67), LR (N=34) and HR HPV (N=34). *The Kruskal Wallis test was used to compared cytokine concentrations among HPV negative women and women with HR and LR HPV. The Dunn's post test was used to adjust for multiple comparisons. Represented above are the medians and IQR.*

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NRF National Research Foundation

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I would also like to thank my co-supervisor Dr Derseree Archary for her guidance when my supervisor was away on maternity leave and for taking the time to look through my dissertation and offering me detailed and valuable feedback.

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I would like to extend my sincere gratitude to the DST-NRF Centre of Excellence who funded me during this time. Their financial support to young researchers such as myself is both encouraging and admirable.

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Abbreviations

AIDS	Acquired Immunodeficiency Syndromes
APCs	Antigen Presenting Cells
ART	Antiretroviral Therapy
BV	Bacterial Vaginosis
BV700	Brilliant Violet 700
CAPRISA	Centre for the AIDS Program of Research in South Africa
CCR5	C-C Chemokine Receptor 5
CIN	Cervical Intraepithelial Neoplasia
COC	Combined Oral Contraceptives
CTACK	Cutaneous T Cell Attracting Chemokine
CTL	Cytotoxic T Lymphocytes
CV	Coefficient of Variation
CVL	Cervicovaginal Lavage
DCs	Dendritic Cells
dH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
ds	Double-Stranded
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescent-Activated Cell Sorting
FcRn	Neonatal Fc Receptor
FBS	Fetal Bovine Serum
FGF basic	Fibroblast Growth Factor Basic
FGT	Female Genital Tract
FITC	Fluorescein Isothiocyanate
FMO	Fluorescence Minus One
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GRO-α	Growth Regulated Oncogene Alpha

H_2O_2	Hydrogen Peroxide
HGF	Hepatocyte Growth Factor
HIF-1	Hypoxia-Inducible Factor-1
HIV	Human Immunodeficiency Virus
HPV	Human Papillomavirus
HR	High Risk
HSV-2	Herpes Simplex Virus 2
IARC	International Agency for Research on Cancer
ICC	Invasive Cervical Cancer
IFN	Interferon
IL	Interleukin
IP-10	Interferon Gamma Induced Protein 10
KZN	KwaZulu-Natal
LCs	Langerhan Cells
LIF	Leukaemia Inhibitory Factor
LR	Low Risk
M. genitalium	*Mycoplasma Genitalium*
MCP	Monocyte Chemoattractant Protein
M-CSF	Macrophage Colony-Stimulating Factor
MHC	Major Histocompatibility Complex
MIF	Macrophage Migration Inhibitory Factor
MIG	Monokine Induced by Interferon Gamma
MIP	Macrophage Inflammatory Protein
MMP	Matrix Metalloproteinase
MMX	Master Mix
mRNA	Messenger Ribonucleic Acid
N. gonorrhoeae	*Neisseria Gonorrhoeae*
NICD	National Institute for Communicable Diseases
NK	Natural Killer Cell
OC	Oral Contraceptives
OOR	Out of Range

ORF	Open Reading Frame
ORI	Origin of Replication
Pap	Papanicolaou
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF-BB	Platelet Derived Growth Factor BB
PE	Phycoerythrin
PerCP-Cy5.5	Peridinin-Chlorophyll Protein-Cyanine 5.5
pRb	Retinoblastoma Protein
RANTES	Regulated on Activation, Normal T Expressed and Secreted
RPM	Revolutions per Minute
SA-HRP	Streptavidin-Horseradish Peroxidase
SA-PE	Streptavidin Phycoerythrin
SCF	Stem Cell Factor
SCGF-β	Stem Cell Growth Factor Beta
SDF-1α	Stromal Cell-Derived Factor 1 Alpha
SDS	Sodium Dodecyl Sulfate
SSA	Sub-Saharan Africa
SSPE	Sodium Chloride-Sodium Phosphate-EDTA
STIs	Sexually Transmitted Infections
T. vaginalis	*Trichomonas Vaginalis*
TIMP	Tissue Inhibitor of Metalloproteinase
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
TRAIL	Tumour Necrosis Factor Related Apoptosis Inducing Ligand
URR	Upstream Regulatory Region
VEGF	Vascular Endothelial Growth Factor
VLPs	Virus-Like Particles
vs.	Versus
β-NGF	Nerve Growth Factor Beta

ABSTRACT

Background: Human papillomavirus (HPV) is one of the most common sexually transmitted infections (STIs) globally and a necessary factor for cervical cancer development. While HPV infection has been associated with increased Human immunodeficiency virus (HIV) risk, the underlying mechanisms remain unclear. Since STIs upregulate cytokine production and immune cell recruitment, and reduce epithelial barrier integrity, this study investigated whether the immune responses associated with HPV infection contribute to a genital immune environment conducive to an increased risk of HIV infection.

Methods: This study included a baseline assessment of 167 HIV negative women participating in the CAPRISA 008 trial. The Roche Linear Array was used to detect the presence of 37 HPV genotypes in cervicovaginal lavage (CVL) pellets. The concentrations of 48 cytokines and 9 matrix metalloproteinases (MMPs) were assessed in matching CVL supernatants by multiplex ELISA. The frequencies of activated or proliferating T cells, NK cells, and of HIV target cells were assessed on cervical cytobrush-derived specimens by flow cytometry. Multiplex PCR was conducted to determine infection with common discharge-associated STIs.

Results: The study demonstrated a 50.8% HPV prevalence. HPV infection was associated with younger age, older male partners, not living with a regular partner, and higher parity. HPV infection was also associated with greater levels of IL-5, IL-6 and G-CSF, an association otherwise masked by the inflammatory nature of other STI. Concomitant HPV/STI infection resulted in reduced concentrations of IL-6 and IL-1RA relative to HPV-STI+ women. In multivariate analyses controlling for other STI and nugent score, HPV-infected women had increased concentrations of SDF-1α (**β = 0.148 pg/ml**). Women with HR-HPV had higher concentrations of MCP-1 (**β = 0.127 pg/ml**) and IL-13 (**β = 0.117 pg/ml**), and greater frequencies of lymphocytes (**β = 1.987 pg/ml**) relative to those infected with LR-HPV. Having multiple HPV infections was associated with reduced concentrations of IL-5 (**β = -0.170 pg/ml**).

Conclusion: While discharge-related STIs are inflammatory, a more subtle immune profile was associated with HPV infection that did not overtly relate to an increased potential for HIV risk. However, this study demonstrated an association between HR-HPV and biomarkers of inflammation, suggesting the need for longitudinal investigation to confirm a biological mechanism for the relationship between persistent HR-HPV infection and HIV acquisition.

1. CHAPTER 1: INTRODUCTION

The association between STIs and increased HIV risk has been well established (Houlihan *et al.*, 2012). HPV is one of the most common STIs globally, and is related to an increased risk of HIV acquisition (Smith-McCune *et al.*, 2010a; Averbach *et al.*, 2010; Auvert *et al.*, 2011; Low *et al.*, 2011; Konopnicki *et al.*, 2013a). Both HIV and HPV are sexually transmitted, with HPV being more prevalent than HIV, even in areas where HIV is endemic (Lissouba *et al.*, 2013). While the epidemiological link between HPV and HIV risk is well established, the biological mechanisms involved remain to be elucidated.

Since HPV-associated dysplastic cervical lesions are associated with a larger number of $CD4^+$ T cells compared to normal cervical epithelium (Kobayashi *et al.*, 2004), HPV infection could predispose a woman to HIV by exposing susceptible target $CD4^+$ T cells to HIV. Furthermore, dendritic cells (DCs) (van Seters *et al.*, 2008), natural killer (NK) cells (Lee *et al.*, 2001) and T cells are also associated with genital wart regression; and genital warts are successfully treated with Imiquimod, a Toll-like receptor (TLR) 7 agonist that prompts an inflammatory response (van Seters *et al.*, 2008). HPV, an epithelial virus, may also increase the risk of HIV infection and facilitate HIV dissemination through disruption of the epithelial barrier and upregulation of pro-inflammatory cytokines (Herfs *et al.*, 2011; Nicol *et al.*, 2005). Further, studies have reported that the risk of acquiring HIV is greatly increased if there are two or more types of HPV isolated from the same individual (Konopnicki *et al.*, 2013a; Averbach *et al.*, 2010). ***It is therefore possible that multiple concurrent HPV infections could lead to an elevated immune response and a greater number of cervical lesions that disrupt the mucosal epithelia integrity, facilitating HIV entry at the genital tract*** (Laga *et al.*, 1993; Freeman *et al.*, 2006).

Low risk (LR), non-oncogenic HPV is generally cleared by the immune system without any permanent damage. Conversely, high risk (HR), oncogenic HPV has the ability to evade the host immune responses and cause persistent infection, leading to the development of pre-cancer lesions and invasive cervical cancer (ICC). Conflicting reports exist for the impact of HPV oncogenicity on HIV infection risk (Averbach *et al.*, 2010; Smith-McCune *et al.*, 2010a; Auvert *et al.*, 2011). Auvert *et al.* showed that HIV seroconversion among female sex workers was associated with genital HR-HPV infection. Whereas, another study reported that the risk of acquiring HIV between women infected with high versus low risk HPV types was similar (Averbach *et al.*, 2010). Thus ***further investigation is required to determine the relationship***

between HPV oncogenicity and HIV risk in KwaZulu-Natal, the heart of South Africa's HIV pandemic.

Several studies have shown that discharge-associated and ulcer-linked STIs contribute to genital inflammation through the recruitment of immune cells, reduced epithelial barrier integrity and upregulation of pro-inflammatory cytokines (Laga *et al.*, 1993; van de Wijgert *et al.*, 2009; Van Der Pol *et al.*, 2008; Petrova *et al.*, 2013; Wira *et al.*, 2015; Rasmussen *et al.*, 1997). HPV coinfection with Chlamydia, Herpes simplex virus type 2 (HSV-2), or even a state of vaginal dysbiosis such as Bacterial Vaginosis (BV), has been associated with the persistence of HPV infection, and increased risk of cervical cancer (Silins *et al.*, 2005; Simonetti *et al.*, 2009; Castle *et al.*, 2003). ***However, it is unclear whether coinfections with HPV and STIs have a compounding effect on genital inflammation, cellular activation and HIV risk.*** Nevertheless, increased concentrations of similar pro-inflammatory cytokines have been observed in genital specimens of patients with persistent HPV infection (Scott *et al*., 2013, Liebenberg *et al*., unpublished), as well as in patients with other STIs, and this implies the possibility of an additional contribution to the genital inflammation (Masson *et al.*, 2015) that promotes HIV acquisition.

Here it was hypothesized that the immune responses associated with multiple concurrent HPV infections, oncogenicity and STI coinfection each contribute to a genital immune environment conducive to an increased risk of HIV infection. The CAPRISA 008 trial provided a unique opportunity to collectively address these unknowns related to HPV infection, genital inflammation and HIV risk among KwaZulu-Natal (KZN) women, since both HPV prevalence and the risk of acquiring HIV is particularly high in KZN. This study attempts to expand on the research questions posed in a current CAPRISA study underway to determine the immune mediators associated with HPV clearance as predictors of HIV acquisition (5R01 AI115983-02; PI: Liebenberg/Celentano). In this study, multiple HPV genotypes and HPV oncogenicity was associated with the frequency of concurrently-infecting discharge-related STIs; and the cytokine, cellular and epithelial integrity biomarkers of genital inflammation.

2. CHAPTER 1: LITERATURE REVIEW

2.1. HPV Epidemiology

HPV is one of the most common STIs globally and a necessary factor for the development of cervical cancer. In 2008, approximately 12.7 million new cancer infections occurred globally and 4.8% of these were attributable to HPV (Bosch *et al.*, 2013). The estimated global HPV prevalence among women with normal and abnormal cervical cytology was 32.1% (Vinodhini *et al.*, 2012). Furthermore, the prevalence of HPV was shown to be higher in less developed countries (42.2%) compared to more developed countries (22.6%) (Vinodhini *et al.*, 2012). A study by Bruni *et al*. showed that globally, young women (<25 years) remain disproportionately affected by HPV, with a prevalence of 43.9% in African countries (Bruni *et al.*, 2010). In Sub-Saharan Africa (SSA) the HPV prevalence, similar to the prevalence of HIV, is among the highest in the world (Bruni *et al.*, 2010; Denny *et al.*, 2014), particularly among women from urban and rural KZN with an alarming prevalence of 76.3% (Ebrahim *et al.*, 2016).

HPV is generally acquired soon after sexual debut, with the greatest prevalence observed among young women (Mbulawa *et al.*, 2015). Although approximately 80% of women will acquire HPV at some point in their life, most women are able to clear the infection (Vinodhini *et al.*, 2012). However, if infection persists it may result in the development of high grade cervical intraepithelial neoplasia (CIN) and eventually, ICC (Vinodhini *et al.*, 2012). There are currently more than 200 HPV types that have been identified and approximately 40 types infect the anogenital region (Fernandes *et al.*, 2013; Bzhalava *et al.*, 2015). HPV is classified as LR or HR based on their relation to the development of benign or malignant lesions (Fernandes *et al.*, 2013). LR, non-oncogenic HPV is associated with the development of anogenital warts, which are benign, hyperproliferative lesions, the most common types being HPV6 and HPV11 (Fernandes *et al.*, 2013). HR, oncogenic HPV is associated with the development of premalignant and malignant cervical lesion and the most common types are HPV16 and HPV18 (Fernandes *et al.*, 2013). Persistent infection with an oncogenic HPV type has been established as the cause of almost all cervical cancers (Denny *et al.*, 2014; McDonald *et al.*, 2014). The International Agency for Research on Cancer (IARC) classifies HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58 and HPV59 as "carcinogenic" and HPV68 as "probably carcinogenic" to humans (Denny *et al.*, 2014; IARC, 2015). The prevalence of HPV in women fluctuates by country, region and population group and is particularly high among women exhibiting high risk sexual behaviour (Smith, 2008).

2.2. HPV Virology

HPV belongs to the Papillomaviridae family and is further categorised into five genera, represented by letters of the Greek alphabet (alpha, beta, gamma, mu and nu) according to their nucleotide sequences and their phylogenetic and pathology features (Bernard *et al.*, 2010; de Villiers *et al.*, 2004). Alphapapillomaviruses are linked to infections of the anogenital tract and the oral mucosa, and consists of a group of oncogenic HPV types that are responsible for the majority of cervical cancers and other cancers of the genital tract (Stanley, 2010). Alphapapillomaviruses consist of three primary clades in which all potentially carcinogenic HPV genotypes are grouped to form one high risk clade, consisting of five species (Alpha 5, Alpha 6, Alpha 7 and Alpha 9) (Fernandes *et al.*, 2013). Alpha 9 is the most important HPV species group as it is composed solely of carcinogenic types, with the main being HPV16 (Schiffman *et al.*, 2010). In 1977, Harald zur Hausen postulated that HPV played a significant role in the development of cervical cancer (zur Hausen, 1977). In 1983 and 1984 zur Hausen and colleagues went on to identify HPV16 and HPV18 in cervical cancer, which has since been identified as a major cause of cancer (zur Hausen, 1977).

Papillomaviruses are non-enveloped viruses consisting of a double-stranded, closed circular DNA genome of approximately 8000 base pairs (Fernandes *et al.*, 2013; de Sanjose *et al.*, 2017; Schiffman *et al.*, 2016; Doorbar *et al.*, 2012) (**Figure 1**). HPV encodes proteins that are able to initiate cellular DNA synthesis, prevent apoptosis and delay differentiation of infected keratinocytes, thereby establishing an environment conducive to viral DNA replication (Stanley, 2012). The viral genome is divided into three main regions: the early region (E) which encodes genes that play role in the viral life cycle and cell transformation (E1, E2, E4, E5, E6 and E7), the late region (L) which encodes the major and minor capsid proteins (L1 and L2, respectively), and the upstream regulatory region (URR) which consists of the origin of replication (ORI) and transcription factor-binding sites that function in the control of early gene transcription and replication (de Sanjose *et al.*, 2017; Stanley, 2012).

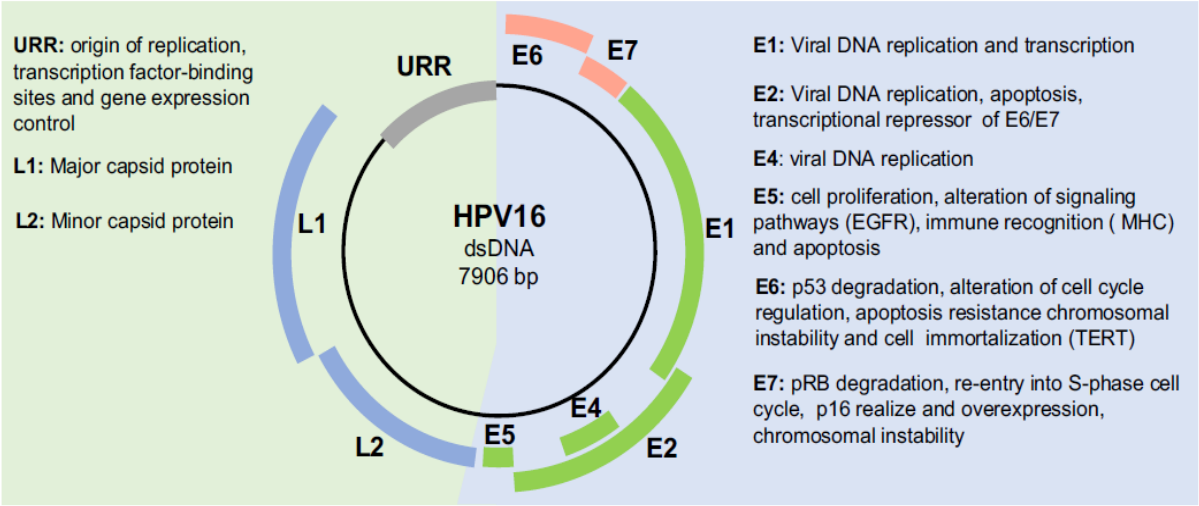

Figure 1. Typical genomic structure of HPV 16 *(figure taken from de Sanjosé, 2017)**. The viral genome consists of early and late regions. The early region consists of genes (E2, E1, E6, and E7) that are necessary for the viral cycle and they play essential roles in cell transformation. The E2 gene encodes a protein involved in viral replication and transcriptional regulation of the viral early genes while the E6 and E7 genes encode proteins that play essential roles in the viral life cycle of HR-HPV. The major and minor capsid proteins (L1 and L2 respectively) are involved in the formation of the capsid during viral assembly. The URR controls early gene transcription and replication and is comprised of promoter and enhancer elements and the ORI.*

These proteins can be further divided into core (E1, E2, L1 and L2) and accessory proteins (E4, E5, E6 and E7) (Schiffman *et al.*, 2016). The core proteins are highly conserved and play important roles in viral genome replication (E1 and E2) and viral assembly (L1 and L2) (Schiffman *et al.*, 2016). In contrast, the expression and functional characteristics of accessory proteins tend to vary (Schiffman *et al.*, 2016). The genes that encode accessory proteins are able to modify the host cell, thereby facilitating viral replication (Wise-Draper *et al.*, 2008). One of the most important roles of the E6 and E7 proteins is to increase viral fitness and virion production by facilitating cell cycle re-entry and genome amplification (Doorbar, 2006; Doorbar, 2005). The E6 and E7 oncogenes of HR-HPV also mediate carcinogenesis through the inhibition of p53 and the retinoblastoma protein (pRb) which are important tumour suppressors (de Sanjose *et al.*, 2017; Stanley, 2012). The E6 protein binds to p53 and degrades it, leading to alteration of cell cycle regulation (Stanley, 2012).

The E7 protein binds to the pRb and deactivates it, resulting in its degradation which leads to the infected cell's loss of control over its cell cycle (Hoffmann *et al.*, 2004; Jefferies *et al.*, 2001; Stanley, 2012). Expression levels of the E6 and E7 proteins is related to the type of cervical lesions: in low grade cervical lesion, these proteins are present at lower levels in the basal cells and greater levels in the upper layers of the epithelium, whilst in high-grade lesions, these proteins are present in high levels throughout the epithelium (Scheurer *et al.*, 2005). The L1 and L2 capsid proteins are crucial in maintaining replication of the virus and synthesis of the genes through differentiation of the epithelium (Morshed *et al.*, 2014). These capsid proteins are also required for the virus to complete replication (Remy-Ziller *et al.*, 2014).

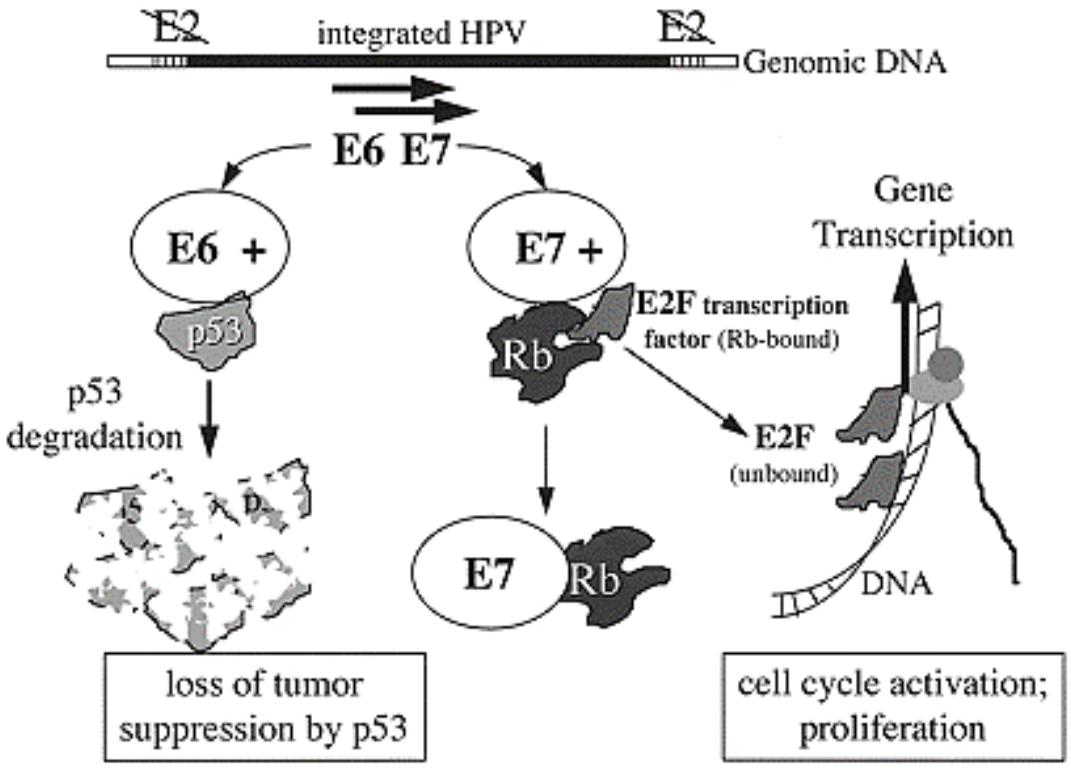

Figure 2. HPV E6 and E7 oncoproteins mechanisms of action *(figure taken from Janicek and Averette, 2001). The E6 protein binds to p53 and induces its degradation. The E7 protein binds to the Rb gene, causing the transcription factor E2F-1 to become unbound and free to activate the cell cycle.*

2.3. HPV Life Cycle

The duration of the HPV life cycle is long and even under optimal conditions, it takes approximately three weeks from the time of initial infection to the release of HPV virions (Stanley, 2012). However, the duration from infection of the host cells to the appearance of lesions is variable, ranging from weeks to months, which suggests that in some cases, HPV is able to effectively evade host immune responses (Stanley, 2012). HPV is separated into two tropism groups: those infecting the keratinized surface of the skin, resulting in the development of warts; and those infecting the mucosa of the mouth, throat, respiratory tract and the anogenital tract (Burd, 2003; Mistry *et al.*, 2008). HPV is highly infectious and may incubate for weeks, to months, or even years. At some point the environmental conditions become permissive and viral growth commences, and infectious virions are produced and released from the cell (Fernandes *et al.*, 2013). The replication phase can continue for variable lengths of time; however, the majority of infected individuals are able to produce an effective immune response and are able to clear the infection (Stanley, 2006). HPV is an intraepithelial virus and infection and vegetative growth are reliant on the expression of the complete cycle of keratinocyte differentiation (Doorbar, 2005). The viral particles enter the basement membrane through microabrasions in the squamous epithelium and infect basal epithelial cells (**Figure 3**) (Passmore *et al.*, 2016). High levels of viral gene expression occurs solely in the upper, more differentiated layers of the stratum spinosum and granulosum of the squamous epithelium (Chow *et al.*, 2010). After the virus has infected the wounded basal cells, viral DNA replication occurs and the viral copy number is amplified to 50-100 copies per cell (Stanley, 2012). The infected host cell then enters the proliferative compartment of the epithelium, where the viral copy number remains unchanged and gene expression is low (Stanley, 2012). At this stage of the life cycle of HR-HPV, the expression of the E6 and E7 oncogenes are under stringent control and the E6 and E7 transcripts are hardly detectable in the proliferative compartment of the epithelium (Stanley, 2012). Once the infected keratinocyte reaches the differentiating compartment in the stratum spinosum, viral gene expression is upregulated and the viral copy number is amplified to thousands of copies per cell (Chow *et al.*, 2010; Doorbar, 2007). At this stage, there is high expression of the E6 and E7 early and late genes from the late promoter (Chow *et al.*, 2010; Doorbar, 2007).

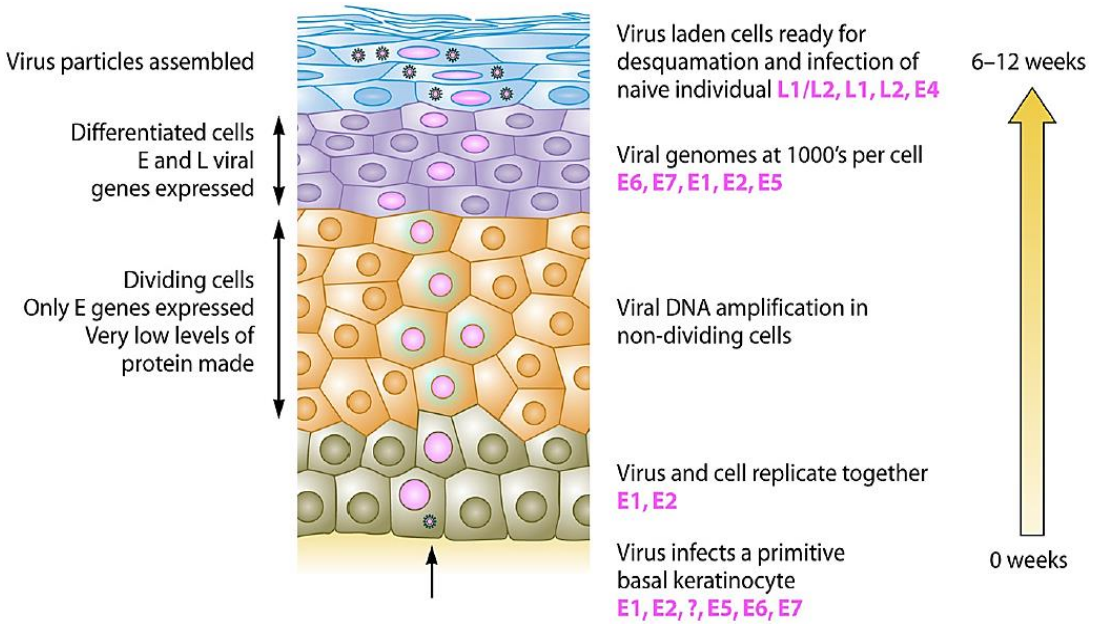

Figure 3. Human Papillomavirus life cycle *(figure taken from Stanley, 2012). HPV is species and tissue specific and only infects and replicates in completely differentiated squamous epithelium. Microtrauma of the genital epithelium provides a portal of entry for HPV to infect keratinocytes of the basal epithelium. In the proliferating compartment of the epithelium, the virus replicates along with the host cell and the viral copy number is approximately 50-100 copies in the daughter cells. In HR-HPV the E6 and E7 genes are under stringent control during cell division and are consequently expressed at low levels. Once the host cell stops dividing and starts to differentiate into a mature keratinocyte, the virus activates all of its genes and the viral genomes is highly expressed. In the upper layers of the epithelium, all viral genes are expressed, viral genomes are encapsidated and the resulting virions exit the cell. The time taken from infection of the basal cell to the production of infectious viral particles is approximately 3 weeks.*

2.4. HPV Immune Evasion Strategies

The life cycle of HPV is suited to the differentiation of the keratinocyte and the virus utilises this differentiation program to evade the host immune responses (Stanley, 2012). HPV infection does not culminate in cytolysis or cytopathic death and important events of the virus life cycle occurs in fully differentiated keratinocytes, which are cells that are destined for death and desquamation at distant locations from immune activation (Stanley, 2012; Sasagawa *et al.*, 2012). Hence, there is no virus-induced cell death, no inflammatory response throughout most of its lifecycle and minimal production of pro-inflammatory cytokines, crucial for activation and recruitment of antigen-presenting cells (APCs), into the local environment (Stanley, 2006; Sasagawa *et al.*, 2012). HPV is exclusively intraepithelial and does not have a viremic phase of the life cycle, thus only small amounts of virus are visible to the host immune defences (**Figure 4**).

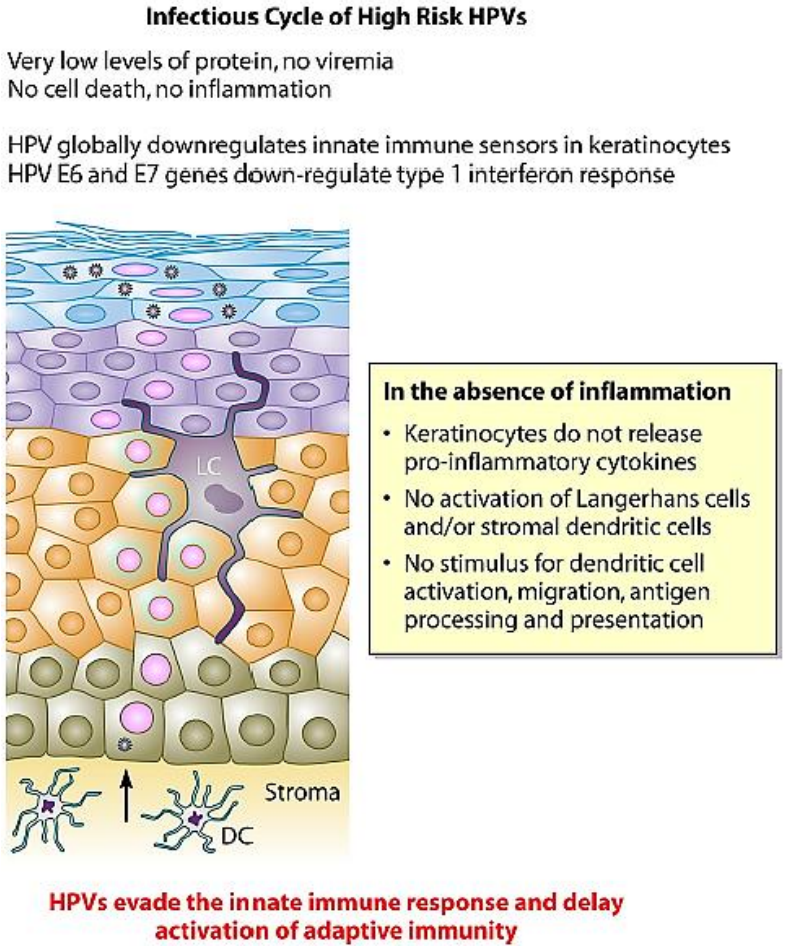

Figure 4. Human Papillomavirus host immune evasion strategies *(figure taken from Stanley, 2012). HPV is capable of downregulating keratinocyte innate immune responses and supressing type I interferon responses, which are crucial for controlling viral infections (Stanley, 2012). HPV does not cause viremia or virus-induced cell death, thus there is no host inflammatory response (Stanley, 2012).*

2.4.1. Evasion of Host Innate Immunity in HPV Infection

HPV has developed immune evasion strategies in order to escape the host innate and adaptive immunity (Senba *et al.*, 2012; Sasagawa *et al.*, 2012; Stanley, 2012). The innate immune response towards HPV infection is non-specific and involves the recruitment of macrophages, NK cells and NK T cells (Sasagawa *et al.*, 2012). NK cells are a group of lymphocytes that destroy virus-infected and tumour cells not expressing major histocompatibility complex (MHC) class I molecules (Sasagawa *et al.*, 2012). Cervical cancer and precursor lesions were found to have low expression of NK cell receptors and decreased cytotoxic activity of NK cells (Garcia-Iglesias *et al.*, 2009), indicative of a reduced ability to destroy infected cells.

Virus-infected cells release type 1 interferons (IFNs), such as IFN-α and IFN-β (Grandvaux *et al.*, 2002) that have antiviral, antiproliferative and antiangiogenic properties, and activate NK cells (Sasagawa *et al.*, 2012; Le Bon *et al.*, 2002). HPV16 E6 and E7 proteins downregulate the transcription of type 1 IFNs (Koromilas *et al.*, 2001) and inhibit its translocation to the nucleus (Arany *et al.*, 1995). Downregulation of co-stimulatory molecules such as IFNs during antigen recognition may induce immune tolerance of HPV-infected cells (Sasagawa *et al.*, 2012). Macrophages are activated by cytokines and are able to destroy HPV infected cells through Tumour necrosis factor-α (TNF-α) (Routes *et al.*, 2005) secretion or antibody-dependent cytotoxicity (Sasagawa *et al.*, 2012). Monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-3α (MIP-3α) are chemokines that recruit macrophages to the site of infection (Sasagawa *et al.*, 2012). These chemokines are downregulated by the HPV16 E6 and E7 proteins, thereby inhibiting translocation of macrophages to the site of HPV infection (Hacke, 2010; Guess *et al.*, 2005).

2.4.2. Evasion of Host Adaptive Immunity in HPV Infection

The adaptive immune response is specific and is largely mediated by cytotoxic T lymphocytes (CTLs) (Sasagawa *et al.*, 2012). MHC class I molecules bind viral peptides and present them at the cell surface to $CD8^+$ CTLs. The HPV16 E5 protein reduces the expression of MHC class I and results in evasion of CTL attack (Sasagawa *et al.*, 2012). HPV is exclusively intraepithelial and HPV antigens are processed and presented by Langerhans Cells (LCs), which are APCs that reside within the epidermis (Stanley, 2012; Sasagawa *et al.*, 2012). Since HPV gene expression is restricted to keratinocytes, cross presentation of viral antigens by LCs and other DCs is crucial for the production of effective T cell responses towards HPV proteins (Stanley, 2012). However, the suboptimal codon usage by HPV (Zhao *et al.*, 2005) results in low levels of protein expression in infected cells and may prevent effective cross presentation by intraepithelial DCs (Stanley, 2012). Furthermore, LCs are not activated upon exposure to the HPV16 L1 protein (Stanley, 2012; Fausch *et al.*, 2002), but rather immune tolerance is induced (Fausch *et al.*, 2005). Most HPV genotypes are also able to decrease the expression of E-cadherins on the epidermal cell surface, thereby decreasing the localization of LCs in the epidermis (Leong *et al.*, 2010). Although the host immune responses are able to clear the majority of HPV infections (Senba *et al.*, 2012), persistent infection may result in the development of lesions that may progress to malignancy and eventually ICC (Moody *et al.*, 2010; Bodily *et al.*, 2011; Richardson *et al.*, 2003).

2.5. HPV Persistence and Clearance

Infection with oncogenic HPV generally lasts for 12-18 months before it is cleared by the host immune responses (Franco *et al.*, 1999). HPV clearance has been linked to an increased risk of HIV infection (Averbach *et al.*, 2010), however, the biological mechanisms involved in the clearance of HPV remains unclear (Fernandes *et al.*, 2013). HPV clearance results in lasting B cell antibody and cellular responses able to prevent reinfection by the same HPV genotype (Gonzalez *et al.*, 2010). In contrast, the persistence of HPV infection, dramatically increases the risk of progression to cancer (Schiffman *et al.*, 2010). HPV is defined as persistent if the same genotype is detectable in two consecutive visits (Fernandes *et al.*, 2013). However, HPV infections that go unrecognised by the host's immunity may be due to viral latency, during which viral gene expression levels are below the limit of detectability of current HPV DNA detection assays (Fernandes *et al.*, 2013). The persistence of HPV, and its integration into the genome of the host cell, leads to an increased possibility of developing high-grade and malignant lesions due to genomic instability (Fernandes *et al.*, 2013). The expression of the E5, E6 and E7 genes can prompt cellular defects, such as fusion of cells, generation of aneuploidy and chromosomal instability (Fernandes *et al.*, 2013). Abnormal centrosome duplication may also result in abnormal numbers of centrosomes (Fernandes *et al.*, 2013). Disruption of the host cell-cycle through the interference of p53 and pRB, allows retention of cells with chromosomal anomalies (Münger *et al.*, 2004). These anomalies culminate in genomic instability in HPV-infected cells, thereby increasing the risk of occurrence and accumulation of genetic mutations that extend over a long period of time (Fernandes *et al.*, 2013). The combination of genetic mutations and lack of an effective host immune response to identify and eradicate infected cells, provide an environment conducive for cancer to develop (Fernandes *et al.*, 2013). Cellular proliferation within benign and malignant HPV lesions increase the need for nutrients, thereby causing competition for nutrients and oxygen (Fernandes *et al.*, 2013). In order to overcome this, both oncogenic and non-oncogenic HPV E7 proteins increase the levels of transcription hypoxia-inducible factor-1 (HIF-1) and HIF-1 target genes under hypoxic conditions (Toussaint-Smith *et al.*, 2004). The heightening of HIF-1 activity results in the upregulation of gene transcription that favours angiogenesis, and this plays a fundamental role in the persistence and progression of HPV-induced lesions, as it provides sufficient nutrients and oxygen in both benign and malignant HPV-induced lesions (Smith *et al.*, 2007; Mukonoweshuro *et al.*, 2005).

2.6. HPV Symptoms and Risk Factors

HPV is predominantly a sexually transmitted infection and can be spread through penetrative vaginal or anal intercourse, oral sex and by direct contact with infected skin or mucosa (Moscicki *et al.*, 2006). HPV infection is generally asymptomatic and is not typically related to symptoms such as itching, burning and vaginal discharge (Mao *et al.*, 2003). The Papanicolaou (Pap) test is utilised to screen for cervical cancer; however, such tests are only recommended for women aged 30 years or older (CDC, 2017). Most HPV infected women are unaware that they are infected until they develop genital warts or when they receive abnormal Pap test results (CDC, 2017). The HPV genotype is the primary factor that determines the progression of persistent HPV infection, however, there are several other risk factors that contribute to HPV acquisition and persistence in women, including sexual behaviour and age, microabrasions in the genital tract, circumcision, smoking, alcohol abuse, long-term use of oral contraceptives and immunosuppression (Zitkute *et al.*, 2016; Burchell *et al.*, 2006; Dunne *et al.*, 2007; Kahn *et al.*, 2007; Manhart *et al.*, 2006; Rosa *et al.*, 2009; da Silva Barros *et al.*, 2012; Vaccarella *et al.*, 2008; Minkoff *et al.*, 2004; Koshiol *et al.*, 2006; Schabath *et al.*, 2015; Appleby *et al.*, 2007; ACS, 2016; Goedert *et al.*, 1998).

2.6.1. Sexual Behaviour and Age

Sexually active women between 15-25 years have the highest prevalence of HPV infection and are at greatest risk of acquiring incident HPV (Zitkute *et al.*, 2016; Burchell *et al.*, 2006; Dunne *et al.*, 2007; Kahn *et al.*, 2007; Manhart *et al.*, 2006). Biological factors and immune responses in young women may increase their susceptibility to HPV infection. Younger women lack sufficient adaptive immune responses (Dempsey, 2008) and have a large area of cervical epithelium undergoing squamous metaplasia, and during this time the cervical cells are more vulnerable to HPV infection and persistence (Trottier *et al.*, 2006; Moscicki *et al.*, 1999; Louie *et al.*, 2009; Moscicki *et al.*, 2006; Castle *et al.*, 2006). Studies have demonstrated an important link between women who had their sexual debut before the age of 16 years and HPV infection (Ribeiro *et al.*, 2015; Baudu *et al.*, 2014). HPV prevalence has been described as bimodal, with a second peak among women 55 years and older, this may be due to weakening immunity, reactivation of latent HPV infection or birth-cohort effects (Herrero *et al.*, 2000; Castle *et al.*, 2005). Sexual intercourse with a new partner or multiple partners has also been linked with increased risk of acquiring HPV infection, progression to pre-cancer lesions and invasive cervical cancer (Zitkute *et al.*, 2016; Burk *et al.*, 1996).

2.6.2. Microabrasions in the Genital Tract

In order to establish infection, HPV requires a portal of entry to the basal cells through microabrasions in the mucosal epithelium (Mammas *et al.*, 2009). Genital warts, HSV and chlamydia cause genital inflammation and lesions in the epithelial barrier, providing HPV direct access to basal cells (Rosa *et al.*, 2009; da Silva Barros *et al.*, 2012). Friction during sexual intercourse may also cause lesions in the epithelium and contribute to HPV acquisition. A study conducted by Norvell *et al*. demonstrated that microabrasions were present in 60% of healthy women after consensual sexual intercourse (Norvell *et al.*, 1984).

2.6.3. Circumcision

Circumcised men are at a reduced risk of developing penile and prostate cancer (Zitkute *et al.*, 2016). According to the Centres for the Disease Control, the foreskin of the penis is more susceptible to infections than the skin on the shaft of the penis. The foreskin is more susceptible to lesions during sexual intercourse, thereby providing a point of entry for HPV infection and the ideal environment for viruses and bacteria to flourish (Zitkute *et al.*, 2016). HPV is highly infectious and is easily transmitted from one sexual partner to another (Lacey, 2005). Studies have shown that HPV is often transmitted from men with penile warts to their female partners (Campion *et al.*, 1985). Circumcision of adolescent men was shown to significantly reduce the prevalence and incidence of HPV infection among their female partners (Wawer *et al.*, 2011). Furthermore, clearance of HR-HPV was more likely among women with circumcised male partners as opposed to those with uncircumcised partners (Wawer *et al.*, 2011). Removal of the foreskin is likely to be associated with a reduction of penile HPV carriage (Wawer *et al.*, 2011). Studies have shown HPV detection differs by the anatomical site and male circumcision is related to a reduction in HPV detection at the urethra, coronal sulcus and shaft of the penis (Giuliano *et al.*, 2009; Weaver *et al.*, 2004; Nielson *et al.*, 2009).

2.6.4. Smoking

Cigarette smoking has been linked to HPV prevalence, persistence and increased risk of progression to ICC among women (Vaccarella *et al.*, 2008; Minkoff *et al.*, 2004; Koshiol *et al.*, 2006; Bauer *et al.*, 1993; Giuliano *et al.*, 2002; Sellors *et al.*, 2003; Sellors *et al.*, 2000; Syrjanen *et al.*, 2007; Winer *et al.*, 2003). Although the biological mechanisms are uncertain, studies have demonstrated that smoking upregulates cellular proliferation and metaplasia in numerous tissues and cell subsets (Harris *et al.*, 2004; Peters *et al.*, 1993; Sekhon *et al.*, 1994; Wright *et al.*, 2001; Wright *et al.*, 1983), which may lead to increased replication of HPV due

to cell proliferation that is induced by smoking (Schabath *et al.*, 2012). Acrolein, an aldehyde in cigarette smoke, was shown to alter neutrophil function (Finkelstein *et al.*, 2001), cause DNA damage (Feng *et al.*, 2006), and suppress resistance to pulmonary infections (Li *et al.*, 1998). Cigarette smoking may also increase viral load by reducing the cellular immune response since it has deleterious effects on systemic and local immunity (Barton *et al.*, 1988; Kalra *et al.*, 2000; Poppe *et al.*, 1995; Sopori, 2002). Smoking leads to the recruitment of cells and the upregulation of cytokines, chemokines, oxygen radicals and protease production, which changes the function of immune cells (Mehta *et al.*, 2008). Additionally, nicotine has been associated with immune suppression in animals (Geng *et al.*, 1996) and humans (Guslandi, 1999).

2.6.5. Alcohol Abuse

Alcohol is a potent modulator of immune function and may result in immune deficiency and increased susceptibility to chronic and infectious diseases (Dunne, 1989; Szabo, 1997; Cook, 1998; Diaz *et al.*, 2002). Chronic alcohol abuse and even moderate alcohol consumption may have an undesirable effect on the immune system (Szabo, 1997; Diaz *et al.*, 2002; Molina *et al.*, 2010; Nelson *et al.*, 2002). Alcohol consumption can hamper both the inflammatory response and development of immunity towards the pathogen (Szabo, 1997). Men and women who have a greater consumption of alcohol are at increased risk of having multiple HPV genotypes, resulting in higher cervical/anal lesions and genital warts (Zitkute *et al.*, 2016). Four alcoholic drinks per week has been shown to double the risk of developing genital warts and five or more drinks is associated with a 2-4 times greater risk of HPV infection (Schabath *et al.*, 2015).

2.6.6. Long Term use of Oral Contraceptives

Studies have reported that long-term use of oral contraceptives (OCs) is associated with progression to cervical cancer in HPV infected women (ACS, 2016). Contraceptive use may affect the clearance or persistence of HPV infection and the progression of pre-cancer lesions (Appleby *et al.*, 2007). The risk of acquiring cervical cancer is significantly increased if OCs are used for more than 5 years (Appleby *et al.*, 2007; Moreno *et al.*, 2002). Marks *et al.* showed that women who reported current combined oral contraceptive (COC) use were less likely to clear HPV infection (Marks *et al.*, 2011a).

2.6.7. Immunosuppression

HIV attacks the immune system leading to an increased risk of incident HPV infection and progression to cervical cancer (Mbulawa *et al.*, 2012; Wang *et al.*, 2011; Massad *et al.*, 2004; Aubin *et al.*, 2011). Individuals with autoimmune or oncological diseases, undergoing immunosuppressive treatments (chemotherapy, monoclonal/polyclonal antibodies and glucocorticoids) are also at greater risk of acquiring HPV (Zitkute *et al.*, 2016). Immunosuppression may facilitate the reactivation of latent HPV infections (Jamieson *et al.*, 2002; Moscicki *et al.*, 2000; Ozsaran *et al.*, 1999; Palefsky *et al.*, 1999; Paternoster *et al.*, 2008; Savani *et al.*, 2009; Savani *et al.*, 2008; Veroux *et al.*, 2009). The dampening of the T cell immune responses through the administration of cyclosporine to renal transplant recipients or due to HIV infection, has been suggested as a cause of HPV reactivation (Jamieson *et al.*, 2002; Moscicki *et al.*, 2000; Ozsaran *et al.*, 1999; Palefsky *et al.*, 1999; Paternoster *et al.*, 2008; Savani *et al.*, 2009; Savani *et al.*, 2008; Veroux *et al.*, 2009). Additionally, among older women lacking signs of cervical disease by cytology, waning T cell immunity may be responsible for the increased prevalence of HPV infection (Maglennon *et al.*, 2014).

2.7. HPV Prevention

Since there is no cure currently available for HPV infection, prevention of HPV infection is of utmost importance (Hathaway, 2012). Current HPV prevention strategies include vaccination, limiting sexual partners, postponing age of sexual debut, avoiding smoking, use of latex or vinyl condoms, and male circumcision (Hathaway, 2012; Diaz, 2008). Vaccines developed to prevent HPV infection were initially developed as the first vaccines to prevent cervical cancer (Fernandes *et al.*, 2013). These vaccines consist of non-infectious virus-like particles (VLPs) that are immunologically similar to HPV and can induce neutralizing antibodies to the specific virus genotype (Frazer *et al.*, 2011). Cervarix™ (GlaxoSmithKline), Gardasil™ (Merck and Co, Inc.) and Gardasil 9™ (Merck and Co, Inc.) are licensed prophylactic HPV vaccines that are accessible to adolescent girls as part of routine immunization schedules in some countries (**Figure 5**) (Frazer *et al.*, 2011). Cervarix, the bivalent vaccine consists of VLPs assembled from the major coat proteins (L1) of HPV16 and HPV18 which have been implicated as the leading cause of cervical cancer (Stanley, 2012). Gardasil, the quadrivalent vaccine incorporates VLPs assembled from the L1 proteins of HPV16, HPV18, HPV6 and HPV11 (Frazer *et al.*, 2011; Stanley, 2012). Both vaccines are administered intramuscularly with three doses and provide rapid access to the local lymph node, thereby escaping the immune evasion strategies of the HPV life cycle (Stanley, 2012). These vaccines confer long-term protection

for up to 6.4 years for the bivalent vaccine (David *et al.*, 2009) and 8.5 years for the quadrivalent vaccine (Villa *et al.*, 2006; Rowhani-Rahbar *et al.*, 2009). Recently, a 9-valent vaccine which additionally incorporates HPV31, HPV33, HPV45, HPV52 and HPV58 has been approved for routine use and has a high rate of efficacy against cervical lesions (Yang *et al.*, 2016). HPV VLPs induce the production of neutralizing antibodies targeting the L1 capsid protein (Harro *et al.*, 2001). The protective efficacy of these vaccines has been defined as the absence of persistent HPV infection and/or diseases related to the HPV genotypes incorporated into vaccines (Frazer *et al.*, 2011). Donovan *et al.* demonstrated a reduction in the appearance of HPV-associated genital warts among women below 28 years old, this provided the first evidence of field efficacy of HPV vaccines in the general population (Donovan *et al.*, 2011). There are currently thirteen HR-HPV types that may cause cervical cancer and some cross protection against other HPV types not included in the current vaccines, has been observed (Frazer *et al.*, 2011).

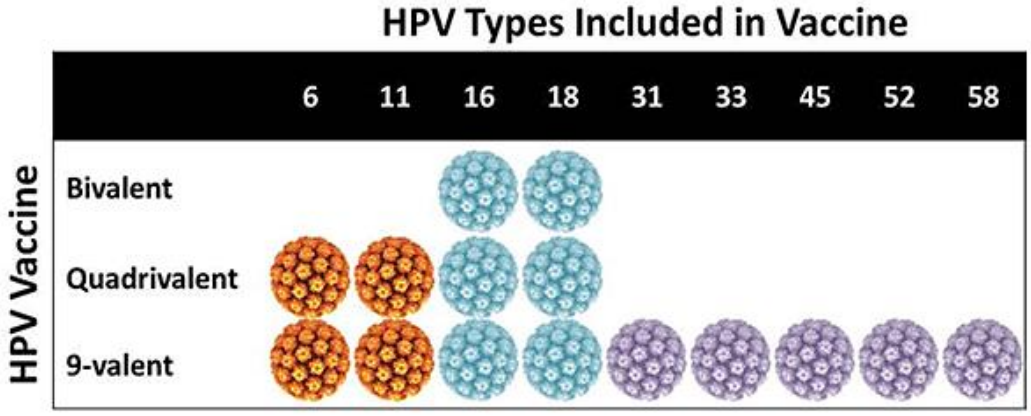

Figure 5. Current HPV vaccines available as routine *(figure taken from elbiruniblogspotcom.blogspot.com). The bivalent vaccine incorporates HPV16 and HPV18. The quadrivalent vaccine also incorporates HPV6 and HPV11. The 9-valent vaccine confers protection against HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV45, HPV52 and HPV58.*

2.8. HPV Treatment

Most HPV-infected women will clear infection spontaneously (Hathaway, 2012). However, some individuals are unable to do so and may undergo treatment. Treatment of HPV-related disease generally involves ablative and/or excisional procedures in order to remove the HPV-infected cells (Fernandes *et al.*, 2013). Chemical therapy for genital warts include trichloroacetic acid, podophyllin compounds, and Imiquimod (Hathaway, 2012; Fernandes *et al.*, 2013).

Trichloroacetic acid is a caustic substance that is applied topically to the lesion (Hathaway, 2012). Podophyllin compounds block cell division and Imiquimod compounds induce IFN and TNF production (Tyring *et al.*, 1998). Cryotherapy is used to freeze the lesion which leads to cell death (Chumworathayi *et al.*, 2010). Common agents used for cryotherapy are liquid nitrogen or a cooled metal probe known as a cryoprobe (Hathaway, 2012). The cryoprobe is not typically suitable for use in the vagina due to fistula formation, however, liquid nitrogen is acceptable (Hathaway, 2012). Extensive HPV lesions may be treated with electrocautery (Hathaway, 2012). The spread and damage caused by vaginal lesions can be control with precise destruction by a laser (Hathaway, 2012). Large and extensive lesions may require surgical removal (Hathaway, 2012). In this case, a scalpel is used to excise the lesion to the level of the skin and the base of the lesion is cauterized with electrocautery or a laser (Hathaway, 2012).

2.9. HPV Infection Increases the Risk of HIV Acquisition

In recent years, the association between genital HPV infection and HIV risk has generated much interest. Several studies have reported an association between HIV infection and increased HPV prevalence (Banura *et al.*, 2008a; Ng'andwe *et al.*, 2007; Baay *et al.*, 2004; Didelot-Rousseau *et al.*, 2006; Safaeian *et al.*, 2008; Marais *et al.*, 2008; Yamada *et al.*, 2008), HR-HPV prevalence (Ng'andwe *et al.*, 2007; Didelot-Rousseau *et al.*, 2006), HPV incidence (Safaeian *et al.*, 2008) and HPV persistence, which is related to increased risk of HPV-related lesions (Safaeian *et al.*, 2008; Banura *et al.*, 2008b) and infection with multiple concurrent HPV genotypes (Ng'andwe *et al.*, 2007; Didelot-Rousseau *et al.*, 2006; Marais *et al.*, 2008). In women, the risk of acquiring HIV was increased in the presence of any HPV genotype compared to those without HPV (Averbach *et al.*, 2010; Low *et al.*, 2011; Smith-McCune *et al.*, 2010a). Averbach *et al.* compared infection with LR-HPV (no HR-HPV) whilst Smith-McCune *et al*. compared infection with LR-HPV (irrespective of concurrent HR-HPV infection) to no HPV, and a doubling of HIV risk was observed in both studies (Averbach *et al.*, 2010; Smith-McCune *et al.*, 2010a). Several studies have shown that infection with HR-HPV was independently associated with an increased risk of HIV seroconversion (Averbach *et al.*, 2010; Auvert *et al.*, 2011; Smith-McCune *et al.*, 2010b). Having 2 concurrent HPV genotypes has been related to a doubling in the odds of acquiring HIV, whilst having 4 or more concomitant HPV genotypes had a odds ratio of 5.6 (Averbach *et al.*, 2010). Moreover, Auvert *et al*. demonstrated that having multiple concurrent HR-HPV infections significantly increases the risk of acquiring HIV (Auvert *et al.*, 2011).

2.10. HIV Epidemiology

In 2015, an estimated 36.7 million people were living with HIV, 2.1 million people became newly infected and 1.1 million people died from Acquired Immunodeficiency Syndrome (AIDS)-related illnesses globally (UNAIDS, 2016). In Africa, more than 1% of the population becomes infected with HIV each year (Wang *et al.*, 2016). SSA is home to merely 12% of the global population, yet accounts for 71% of the global burden of HIV infection (**Figure 6**) (Kharsany *et al.*, 2016; UNAIDS, 2016). It is interesting to note that it is also in SSA that the prevalence of HPV is among the highest in the world (Bruni *et al.*, 2010; Denny *et al.*, 2014).

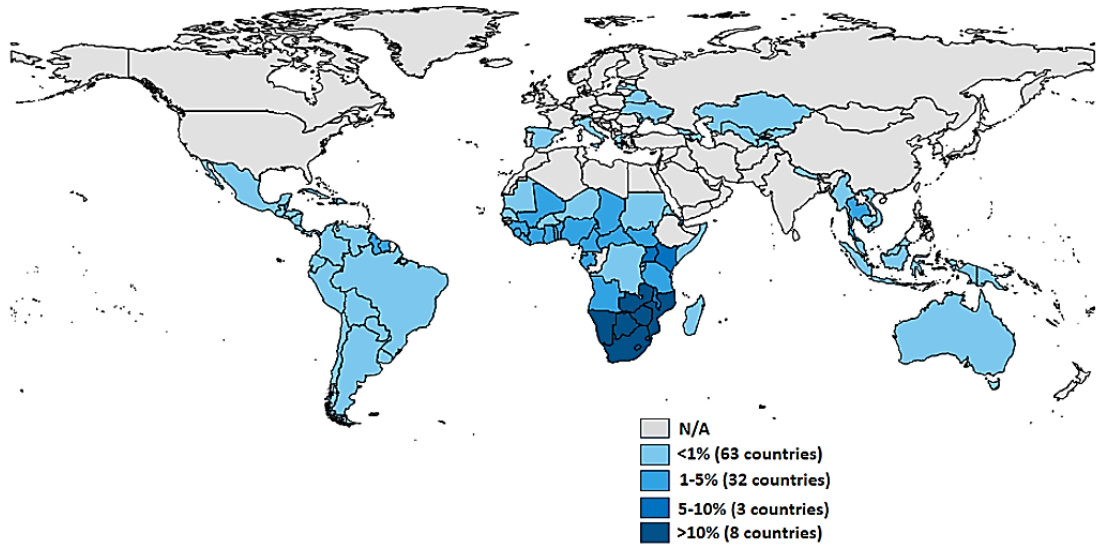

Figure 6. Global HIV prevalence in 2015 *(figure taken from: Kaiser Family Foundation based on UNAIDS). Sub-Saharan Africa had the highest global HIV prevalence >10% in 2015.*

In 2016, an estimated 7.1 million people were living with HIV in South Africa (UNAIDS, 2017). HIV incident rates remain unacceptably high in South Africa, with 270,000 new infections and 110,000 AIDS-related deaths in 2016 (UNAIDS, 2017). The KZN province bares a disproportionate burden of HIV infection, accounting for the highest percentage (16.9%) of prevalent infections in South Africa (Shisana *et al.*, 2014). The introduction of antiretroviral therapy (ART) in 1996 has greatly reduced HIV-related mortality (Ford *et al.*, 2016; Granich *et al.*, 2015). However, maintenance and scale-up of adequately funded AIDS efforts will be imperative to accomplish the goal of an "AIDS free generation" by 2030 (Piot *et al.*, 2015). New prevention strategies which address the biological risk factors associated with HIV acquisition are urgently required.

2.11. HIV Transmission in the Female Genital Tract

In order to develop successful HIV prevention strategies it is important to understand how HIV is transmitted in the female genital tract (FGT). Most female HIV-1 infections are the result of heterosexual intercourse with an infectious male partner (UNAIDS, 2012). The female reproductive tract is comprised of two different mucosal surfaces (Eid *et al.*, 2015). In order for HIV to establish infection in the FGT, HIV-1 in the male ejaculate must evade both innate and adaptive immune responses, cross the epithelial barrier and establish infection in the underlying $CD4^+$ target cells (Carias *et al.*, 2013). The primary receptor for HIV entry into the host cells is CD4, and the major co-receptors are CXCR4 and CCR5 (Sattentau *et al.*, 1986; Deng *et al.*, 1996; Feng *et al.*, 1996). There are three main mechanisms that HIV employs to traverse the epithelial cells of the FGT: through microabrasions in the epithelial barrier, through transcytosis mediated by neonatal Fc receptor (FcRn); or by paracellular movement between the epithelial cells (Rodriguez-Garcia, 2013). The endocervix and upper reproductive tract is made up of a monolayer of columnar epithelial cells (**Figure 7A**) and the ectocervix and vagina which is covered with a multilayer of squamous epithelial cells and tight junctions (**Figure 7B to F**) that HIV-1 must transverse in order to establish infection (Carias *et al.*, 2013; Eid *et al.*, 2015; Kumamoto *et al.*, 2012).

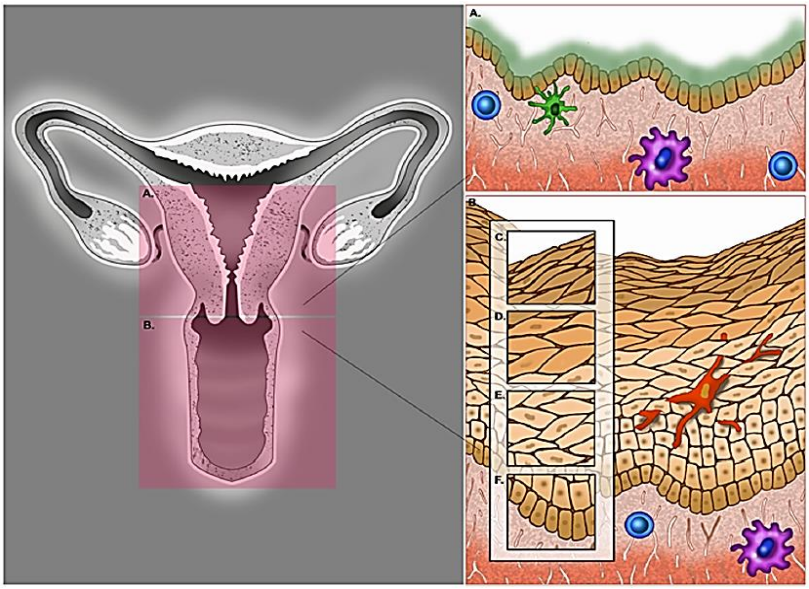

Figure 7. Epithelial composition and HIV targets of the female genital tract *(figure taken from Carias et al., 2013). (A) The upper reproductive tract is primarily composed of a monolayer of columnar epithelial cells. (B) The lower reproductive tract consisting of the ectocervix and vagina is covered with a multilayer of squamous epithelial cells which is made up of the stratum corneum (C), stratum granulosum (D), stratum spinosum (E) and stratum basale (F). Intraepithelial target cells include Langerhans cells (red). HIV target cells in the lamina propria consists of dendritic cells (green), T cells (blue) and macrophages (purple).*

The columnar epithelium of the endocervix and upper FGT may be an easier route of entry for HIV-1 to access target cells since it is composed of a thin, single layer of cells (Shattock *et al.*, 2003). Additionally, the transition between the squamous and columnar epithelial cells that occurs at the transformation zone provides a portal of entry for HIV-1 (Eid *et al.*, 2015).

2.12. Biological Defences of the Female Genital Tract

Globally, females are at greater risk of acquiring HIV than their male counterparts (UNAIDS, 2013; Ramjee *et al.*, 2013; Boily *et al.*, 2009). Although unprotected vaginal sex increases the risk of acquiring HIV infection, the majority of exposures to the virus does not lead to infection (Boily *et al.*, 2009). This is largely due to the mucosal immune responses at the FGT that help to prevent HIV from establishing infection. Mucosal immunity in the FGT consists of innate and adaptive immune responses. Innate immunity includes defences such as the epithelial barrier, mucus, low or acidic pH, the complement system and cells of the immune system (Reis Machado *et al.*, 2014). The layer of mucus produced by the vagina and cervix can trap HIV and prevent it from crossing the epithelial barrier and minimises damage caused by friction during sexual intercourse (Petrova *et al.*, 2013; Wira *et al.*, 2015). The mucus consists of mucins that form a thick gel mesh which acts as a physical barrier to HIV (Gipson, 1997; Vigil, 2009). The aqueous component of the mucus is rich in immunoglobulins and antimicrobial peptides which also provide protection against invading pathogens (Ming, 2007). The squamous epithelium of the vagina and ectocervix are many layers thick and acts as a physical barrier against HIV entry (Shattock *et al.*, 2003). The multi-layered and single cell layer epitheliums are both held together by proteins that form desmosomes, tight junctions and adherens junctions, which reduces its permeability (Carias, 2013). Among women in developed countries, the lower portion of the FGT (vagina and ectocervix) is colonised by commensal bacteria that consists predominantly of *Lactobacillus species* (Ravel *et al.*, 2011; Martin *et al.*, 2008). Lactobacillus maintains an acidic pH in the vagina through the production of lactic acid and hydrogen peroxide (H_2O_2), which has antimicrobicidal activity (Ravel *et al.*, 2011; Martin *et al.*, 2008; Lai *et al.*, 2009). The adaptive immune response in the FGT consists of antibodies and immune cells in the vaginal mucus and epithelial lining that attacks and inactivates HIV (Reis Machado *et al.*, 2014)rkell. Although the immune system offers protection against HIV infection, the immune cells located in the FGT may also increase vulnerability to HIV infection, because HIV can attack these immune cells in the vaginal mucous and epithelial lining (Griesbeck *et al.*, 2016; Adimora *et al.*, 2013). The adaptive immune response is pathogen-specific that occurs after T cell presentation and stimulation by APCs or by B cell

antibody secretions (Reis Machado, 2014). APCs in the FGT include macrophages, DCs, Langerhans cells and epithelial cells of the cervix and endometrium (Reis Machado, 2014). The effector cells are $CD4^+$ T cells including the cytokines they produce, $CD8^+$ T cells and immunoglobulins (Reis Machado, 2014).

2.13. Genital Inflammation Increases Susceptibility to HIV Infection

Inflammation is a natural response of the immune system to fight off infection and invading pathogens, however, inflammation can increase susceptibility to HIV infection. Genital inflammation may render an individual more vulnerable to HIV infection through the recruitment and activation of HIV target cells ($CD4^+CCR5^+$) (McKinnon *et al.*, 2011; Arnold *et al.*, 2016). Infection, irritation and/or epithelial disruption may lead to immune activation in the FGT, resulting in increased expression of soluble immune proteins (Haase, 2010) and recruitment of cells expressing HIV co-receptors, thereby increasing the risk of HIV acquisition (Fichorova *et al.*, 2001b). Although there are several HIV target cells in the FGT, the virus either directly infects $CD4^+CCR5^+$ T cells in the mucosa or binds to DCs with subsequent presentation and infection of $CD4^+$ T cells (Belyakov *et al.*, 2004; Li *et al.*, 2009).

The innate immune response in the FGT is regulated by cytokines and chemokines. Cytokines are small glycoproteins that are synthesised by numerous cell types, primarily leukocytes, they regulate immunity, inflammation and haematopoiesis (Khan, 2008). Cytokines can be categorized as pro-inflammatory, anti-inflammatory, chemokines, growth factors and adaptive cytokines based on their biological activity. Pro-inflammatory cytokines are synthesised predominantly by activated macrophages and helper T cells and are involved in the upregulation of inflammatory responses (Zhang *et al.*, 2007). Chemokines are secreted in order to recruit immune cells to the epithelial surface (Fahey *et al.*, 2005). The levels of inflammatory cytokines and chemokines which recruit activated HIV target cells to the genital mucosa are used as biomarkers of inflammation in the FGT (Fichorova *et al.*, 2004; Schwartz *et al.*, 2006; Fichorova *et al.*, 2001b; Fichorova, 2004; Masson *et al.*, 2015). In the CAPRISA004 1% tenofovir gel trial, a pro-inflammatory cytokine profile predicted HIV acquisition (**Figure 8**) (Masson *et al.*, 2015). Genital inflammation was defined by having at least 5 of 9 pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, TNF-α, IL-8, IP-10, MCP-1, MIP-1α and MIP-1β) concentrations above the 75^{th} percentile (Masson *et al.*, 2015). The risk of acquiring HIV was increased among women with evidence of genital inflammation, and also with increasing concentrations of MIP-1β, IP-10, and IL-8 (Masson *et al.*, 2015). Furthermore, the presence of

chemokines MIP-1α, MIP-1β and IL-8 in the genital tracts of rhesus macaques was shown to be necessary for establishment of SIV infection after vaginal challenge (Li *et al.*, 2009). High levels of chemokines also result in the recruitment of $CD4^+$ T cells to the site of infection thereby facilitating HIV replication (Li *et al.*, 2009). Genital cytokine production has been associated with the frequency of local HIV target cells (Arnold *et al.*, 2016; McKinnon *et al.*, 2011); and a reduced ability of the genital mucosa to function as an effective physical barrier to HIV infection (Arnold *et al.*, 2016).

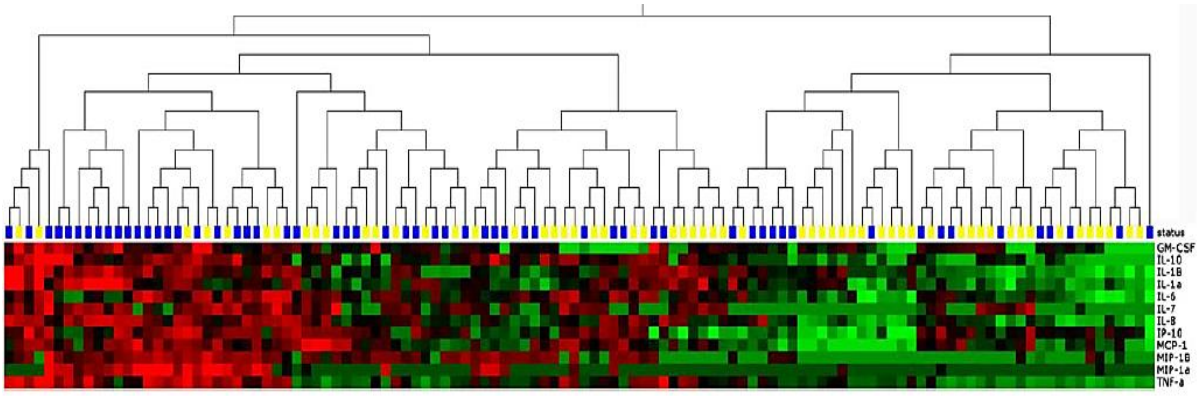

Figure 8. Clustering of HIV positive and HIV negative women based on their cytokine expression profiles *(figure taken from Masson et al., 2015). Women who later became HIV seropositive (n = 58; blue blocks) had greater cytokine concentrations in CVL specimens and clustered together, whilst women who remained HIV seronegative (n = 58; yellow blocks) had lower cytokine concentrations and clustered separately. Cytokine concentrations range from low (green) to high (red). The dendrogram above the heat map demonstrates degrees of relatedness between the genital cytokine profiles.*

The epithelial barrier is one of the most important defences against HIV, since the virus must cross this barrier in order to establish infection. Matrix metalloproteinases (MMPs) are zinc dependent endopeptidases that function in the degradation and remodelling of the epithelial barrier (O'Sullivan *et al.*, 2015). They are grouped according to domain structure and substrate preferences into collagenases, gelatinases, stromelysins and membrane type MMPs (Egeblad *et al.*, 2002). MMPs contribute to inflammation through the regulation of physical barriers, modulating cytokines and chemokines and establishing chemokine gradients in inflamed tissues that control the recruitment of leukocytes at the site of infection (O'Sullivan *et al.*, 2015). MMP concentrations have been used as biomarkers of inflammation and an indicator of reduced epithelial barrier integrity (Ngcapu *et al.*, 2015; Arnold *et al.*, 2016). Selhorst *et al.* reported that reduced epithelial barrier integrity and HIV target cell recruitment facilitates infection by less-infectious HIV variants (Selhorst *et al.*, 2017). Furthermore, among HIV

positive individuals, mucosal ulcerations have been associated with higher genital HIV viral load (Kreiss *et al.*, 1989), indicating that an intact epithelial barrier may reduce secondary sexual HIV transmission from an infected person (Kaul *et al.*, 2008).

2.14. Sexually Transmitted Infections and Genital Inflammation

STIs are able to induce robust mucosal immune responses and genital inflammation in order to clear infection (Patel *et al.*, 2014). Since STIs upregulate the production of pro-inflammatory cytokines and are associated with reduced epithelial barrier integrity and HIV target cell recruitment, they are considered risk factors for HIV acquisition (Laga *et al.*, 1993; van de Wijgert *et al.*, 2009; Van Der Pol *et al.*, 2008; Anahtar *et al.*, 2015; Arnold *et al.*, 2016; Kaul *et al.*, 2008). Ulcerations caused by STIs may facilitate blood to blood contact and HIV transmission, furthermore, HIV infected individuals with concomitant STI infection may have increased viral shedding at the genital tract (Fox *et al.*, 2010). STIs such as genital herpes or syphilis cause ulcerations in the epithelial cell layers of the vagina or cervix, providing a portal of viral entry (Petrova *et al.*, 2013; Wira *et al.*, 2015). Furthermore, independent associations have also been reported between HIV risk and *Chlamydia trachomatis* (*C. trachomatis), Neisseria gonorrhoeae* (*N. gonorrhoeae), Trichomonas vaginalis (T. vaginalis)* and HPV (Ramjee *et al.*, 2005; Losina *et al.*, 2010; Wilkinson *et al.*, 1998; Naidoo *et al.*, 2014). Gonorrhoea and Chlamydia have been shown to cause genital inflammation and increase activated mucosal $CD4^+$ T cells, thereby increasing the risk of HIV acquisition (Levine *et al.*, 1998).

Among HPV positive women, concomitant STI infection has been shown to prolong HPV infection and increase the risk of CIN (Finan *et al.*, 2002; Syrjänen *et al.*, 1985; Silins *et al.*, 2005; Alberico *et al.*, 1988). For instance, *T. vaginalis* infection has been associated with a greater risk of squamous intraepithelial lesions and CIN (Noel *et al.*, 2010). *N. gonorrhoea* was identified as a co-factor for the persistence of HR-HPV infection in cervical carcinogenesis (de Abreu *et al.*, 2016). Studies have even demonstrated that *Mycoplasma genitalium (M. genitalium)* was significantly associated with an increased risk of HR-HPV infection (Biernat-Sudolska *et al.*, 2011). Furthermore, coinfection with *C. trachomatis* has been associated with HPV persistence and cancer (Simonetti *et al.*, 2009). Silins *et al.* demonstrated that among women with HR-HPV, antibodies elicited against *C. trachomatis* were associated with a two-fold increased risk of cervical cancer (Silins *et al.*, 2005). Cervical carcinoma cell lines infected with *C. trachomatis* secrete increased levels of pro-inflammatory cytokines compared to primary uninfected cervical cells and non-cervical epithelial cell lines (Rasmussen *et al.*, 1997),

suggesting that coinfection with HPV and *C. trachomatis* may exacerbate genital inflammation (Castle *et al.*, 2003).

2.15. Genital inflammation as the underlying link between HPV and HIV risk

The biological mechanisms HPV employs to increase HIV risk remains to be elucidated. HPV infection could escalate HIV infection and dissemination via disruption of the epithelial barrier and alteration of the genital mucosa (Clarke *et al.*, 2002), through recruitment and activation of HIV target cells (Nicol *et al.*, 2005) and upregulation of pro-inflammatory cytokines (Herfs *et al.*, 2011; Nicol *et al.*, 2005). Both HIV and HPV use common portals of entry such as the squamo-columnar junctions which are primary sites of metaplastic conversion and viral establishment in order to gain access to susceptible cells (Herfs *et al.*, 2011). Adhesion molecules are less dense at squamo-columnar junctions which allows for paracellular permeation (Herfs *et al.*, 2011). Microabrasions which permit HPV access to the basal epithelial layers may also favour HIV acquisition (Herfs *et al.*, 2011). The host immune response to HPV is mediated by T-lymphocytes, which are also primary target cells for HIV (Houlihan *et al.*, 2012; Stanley, 2001). Recruitment of HIV target cells ($CD4^+CCR5^+$) to the FGT to clear HPV infection may also provide an immune environment conducive to HIV acquisition. An abundance of the pro-inflammatory cytokine IL-1β, which activates a promoter region in the HIV genome (Osborn *et al.*, 1989) has been observed in women with HPV-associated abnormal cervical cytology (Behbakht *et al.*, 2002).

Combining HIV treatment with adequate prevention strategies is crucial in continuing the path to altering the trajectory of the AIDS epidemic (Kharsany *et al.*, 2016). HPV is one of the most common STIs globally, and prior infection with HPV is associated with an increased risk of HPV persistence and HIV acquisition in both women and men (Mbulawa *et al.*, 2012; Smits *et al.*, 2005; Heard *et al.*, 2000; Lissouba *et al.*, 2013; Rositch *et al.*, 2013; Chin-Hong *et al.*, 2009). The HPV vaccine is highly effective in preventing HPV-associated cervical cancers and could have potential use as an additional HIV prevention strategy, particularly in KZN where both HPV and HIV prevalence are unacceptably high. Understanding the biological mechanisms for the relationship between HPV and increased risk of HIV acquisition is critical to the design of effective methods to limit the spread of both HPV and HIV. This study investigated whether the immune responses associated with HPV infection contribute to a genital immune environment conducive to an increased risk of HIV infection.

3. HYPOTHESIS

The immune responses associated with HPV infection (including multiple HPV infections, oncogenicity, and STI coinfection) each contribute to a genital immune environment conducive to an increased risk of HIV acquisition.

4. OBJECTIVES

1) To compare genital inflammation **(**as measured by cytokine biomarkers, HIV target cell frequency, and/or epithelial barrier integrity) in HPV positive and HPV negative women.
2) To determine the contribution of multiple concurrent HPV infections on genital inflammation.
3) To compare genital inflammation in women with oncogenic and non-oncogenic HPV infection.
4) To compare genital inflammation in women with HPV/STI coinfection and those without.

5. METHODOLOGY

5.1. Study Design

This cross-sectional study was conducted to compare biomarkers of genital inflammation among women with HPV infection and those without, women with multiple concurrent HPV infections and those without, women with HR-HPV infection and LR-HPV infection, and among women with an HPV/STI coinfection and those without. This study was approved by the Biomedical Research Ethics Committee at the University of KwaZulu-Natal under the ethics number BE248/16 (**Appendix A**). All laboratory assays were conducted at the CAPRISA Mucosal Immunology Laboratory in Durban, South Africa.

5.2. Study Population

The study population included 167 HIV negative women from the CAPRISA 008 trial, an open-label randomized controlled trial to assess the effectiveness of delivering tenofovir 1% gel in the context of routine family planning. The women enrolled in this study were aged 18-40 years old, were from urban and rural KwaZulu-Natal, and had previously participated in the CAPRISA 004 trial. At the time of sampling, all participants had not used 1% Tenofovir gel for a minimum of 3 years since exiting the CAPRISA 004 trial.

5.3. Specimen Collection and Processing

5.3.1. Cervical Cytobrush Collection and Processing

The study participants were positioned with their buttocks at the edge of the exam table. The speculum was inserted into the vagina and the blades were locked into position wide enough to allow complete visualisation of the cervix. The cytobrush was inserted into the endocervical canal, gently rotated 360º twice, and removed. The cytobrush was then immediately placed into a conical centrifuge tube containing 3ml of collection medium. The tube was labelled, sealed and transported on ice to the CAPRISA laboratory within 2 hours of sample collection. Immediately upon receipt of the sample, the cytobrushes were irrigated with a Pasteur pipette containing sterile PBS and thereafter, centrifuged at 400 xg. The cytobrush pellet was used for *ex vivo* cellular phenotyping by flow cytometry.

5.3.2. Cervicovaginal Lavage Collection and Processing

The tip of a plastic bulb pipette was cut off and the bulb was affixed to a 10 ml syringe. A volume of 5 ml of sterile saline was drawn up, a speculum was lubricated and inserted into the vagina and the pipette was inserted into the vagina through the speculum. The saline was squeezed out of the pipette and allowed to bathe the cervix. The fluid was then aspirated into the same pipette and this procedure was repeated 2 to 3 times and then dispensed into a collection container. The cervicovaginal lavage (CVL) sample was kept on ice and transported to the laboratory along with the cytobrush. Immediately upon receipt at the CAPRISA laboratory, the sample was processed by: spinning at 800 g for 10 minutes and the supernatant was stored in 1 ml cryovials without disturbing the pellet. The cryovials containing the cell pellet and supernatant were stored at -85ºC degrees in the cryofreezer.

5.3.3. Swab Collection and Processing

Moistened cotton wool swabs were used to clean the vulva, wiping from front to back. The vulva and perineum were examined for external lesions or warts. The labia were then gently parted and a swab was inserted into the vagina. Two vaginal swabs were collected and each was placed into a 1.5 ml cryovials containing 0.4 ml PBS. The stem of the swab was cut off, allowing the swab to fit into the container. The cryovials were sealed and shipped to the CAPRISA laboratory. One swab was sent to the National Institute for Communicable Diseases (NICD) where Multiplex PCR assays were conducted as a service, to identify infection with discharge-associated agents: *T. vaginalis, C. trachomatis, N. gonorrhoeae, and M. genitalium*, as described by Mhlongo *et al*., 2010.

5.4. Detection of HPV Genotypes in Cervicovaginal Lavage Pellet Specimens

The Roche linear array assay was conducted to detect the presence of 37 relevant HPV types (HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, 55, 64, CP6108, and IS39) in CVL pellet specimens that were collected at baseline.

5.4.1. HPV Roche Linear Array Assay

The Linear Array assay (Roche Diagnostics, Indianapolis, IN, USA) was conducted according to the manufacturer's instructions. These are briefly detailed here.

5.4.1.1. Specimen Preparation

HPV DNA extraction was conducted using the MagNA Pure 96 DNA and Viral Nucleic Acid Small Volume Kit (Roche Applied Science, Manheim, Germany). A volume of 200 µl of the

CVL pellet was placed into a MagNA Pure 96 Processing Cartridge, which was inserted into the MagNA Pure 96 Extraction Machine. The specimen preparation yielded HPV target DNA which was subsequently used for polymerase chain reaction (PCR) amplification.

5.4.1.2. Amplification Reagent Preparation

The Master Mix (MMX) was prepared by adding 125 µl of Magnesium Chloride solution to a vial of HPV MMX, included in the kit. The vial was inverted to mix its contents, which were then transferred to a reservoir. A multichannel pipette was used to add 50 µl of Working MMX into a 96 well PCR plate. A volume of 50 µl of the samples and controls were added to the microtiter plate.

5.4.1.3. Amplification Procedure

The 96 well plate containing samples and working MMX was placed into the GeneAmp® PCR System 9700 (Applied Biosystems, Norwalk, CT). The thermal cycler program was set according to **Table 1**. In the cycle programs, the ramp rate was set to 50%. The plate was removed within 4 hours of the start of the final "Hold" programme, and samples were immediately denatured by the addition of 100 µl of the denaturation solution.

Table 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

5.4.1.4. HPV Genotyping

The Working Hybridization Buffer, Working Ambient Wash Buffer, Stringent Wash Buffer and the Working Citrate Buffer (all supplied with kit) were prepared as shown in **Table 2**. The Linear Array HPV strips were removed from the pouch using sterile forceps and labelled. Each labelled HPV strip was placed into the 24 well tray with the probe lines facing upwards. Thereafter, 4 ml of pre-warmed Working Hybridization Buffer was added into each well containing the labelled strip. A volume of 75 µl of denatured PCR product was added to the wells and the tray was covered with the lid. The tray contents were mixed by rocking back and forth between each sample addition. The covered tray was placed into a 53ºC shaking water

bath with a 0.5 kg lead ring on top to prevent the tray from moving. Hybridization occurred for 30 minutes while shaking at 60 revolutions per minute (RPM) on a Mini Orbital SSM1 Shaker (Stuart, Staffordshire, UK). A Working Conjugate was prepared by adding 15 µl of Streptavidin-Horseradish Peroxidase (SA-HRP) to 5 ml of Working Ambient Wash Buffer for each strip to be tested. The tray was removed from the water bath and the Working Hybridization Buffer was vacuum aspirated.

Table 2. Preparation of Wash Buffers for HPV genotyping.

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

A volume of 4 ml of Working Ambient Wash Buffer was added to each well of the tray which was gently shaken to rinse the strips and the wash buffer was removed using vacuum aspiration. A volume of 4 ml of Stringent Wash Buffer was added to each well and the lid was placed onto the tray which was incubated in the 53ºC shaking water bath for 15 minutes. Afterwards, the tray was taken out of the water bath and the wash buffer was aspirated. A volume of 4 ml of Working Conjugate was pipetted into each well and the lid was placed back onto the tray and incubated at 60 RPM for 30 minutes at room temperature on the shaker. The Working Conjugate was aspirated and 4 ml of Working Ambient Wash Buffer was added to the tray which was then gently shaken to rinse the strips and vacuum aspirated once again. A volume of 4 ml of the Working Ambient Wash Buffer was added to each well and the tray was incubated for 10 minutes at room temperature on a platform shaker. The wash buffer was aspirated and the wash step was repeated with aspiration. A volume of 4 ml of Working Citrate Buffer was added to each well and incubated at 60 RPM for 5 minutes at room temperature on a platform shaker. The Working Substrate was prepared by adding 4 ml of Substrate A to 1 ml of Substrate B per strip and mixed well. The Working Citrate Buffer was aspirated and 4 ml of Working Substrate was added to each well and incubated for 5 minutes on a platform shaker at room temperature. The Working Substrate was aspirated and 4 ml of dH_2O was added to each well. The strips were removed from the tray using forceps and left to air dry for up to 72 hours at room temperature. The strips were interpreted manually using the HPV Reference Guide by

aligning the reference line on the guide with the ink line of the strip. Detection of the endogenous beta-globin sequence was used as an internal positive control to determine whether DNA extraction was successful.

5.5. Investigation of Immune Cell Frequency Cervical Cytobrush Pellet Specimens

Multiparametric flow cytometry was conducted to investigate the dynamics and the frequency of activated ($CD38^+$ or $HLA\text{-}DR^+$) or proliferating ($Ki67^+$) T cells ($CD3^+$ $CD4^+$ or $CD8^+$), NK cells ($CD56^+CD16^+$) and activated $CCR5^+CD4^+$ targets for HIV replication in cervical cytobrush pellet specimens.

5.5.1. Surface Staining of Cellular Immune Markers

After processing, cells were isolated from cytobrushes (~100 µl) and placed into a 96 well plate for staining. The cytobrush samples were washed with 150 µl Phosphate Buffered Saline (PBS) and added to the respective wells. The cells were pelleted by centrifugation at 2100rpm for 3 minutes (833 xg) at 4ºC. The supernatant was discarded and the pellets were resuspended in 200 µl PBS and centrifuged at 2100rpm for 3 minutes (833 xg) at 4ºC. The live/dead stain Fixable Violet Dead Cell Stain kit (VIVID, Molecular probes, Life technologies, USA) was used to identify viable cells. The Vivid dye was prepared by making a 1:40 dilution with dH_2O. This solution was further diluted 1:20 with PBS and 50 µl of this working stock solution was added to each well containing cell pellets. The plate was incubated at 4ºC for 20 minutes in darkness. The pellets were resuspended in 200 µl Wash Buffer (1% FBS in PBS) to each well. The plate was centrifuged at 2100rpm (833 xg) for 3 minute at 4°C in an Eppendorf 5810 Centrifuge (Eppendorf AG, Hamburg, Germany) and the supernatant was discarded. The mononuclear cells were stained extracellularly with a concoction of antibody-conjugated fluorochromes: Allophycocyanin Tandem Dye (APC-H7)-labelled anti-CD3 (BD Bioscience), Pacific Blue–labelled anti-CD14 (BD Bioscience), Pacific Blue labelled anti-CD19 (BD Bioscience), eF605-labelled anti-CD16 (eBioscience), Fluorescein Isothiocyanate (FITC)-labelled anti-CD8 (BioLegend) , Peridinin-Chlorophyll Protein-Cyanine 5.5 (PerCP-Cy5.5)-labelled anti-CD4 (BD Bioscience) and Phycoerythrin Cyanine 7 labelled anti-CD56 (BD Bioscience). These antibodies were pre-titrated and 2 µl of each was added to the wells, with the exception of anti-CD4, of which only 1 µl was added into each well. The plate was incubated at 4ºC for 20 minutes in darkness to facilitate antibody binding.

5.5.2. Intracellular Staining of Cellular Immune Markers

Once incubation was completed, the cells were gently resuspended in 200 µl Wash Buffer (1% FBS in PBS) and centrifuged at 2100rpm for 3 minutes (833 xg) at 4ºC. The supernatant was removed and the pellets were treated with 100 µl Cytofix/Cytoperm (BD Bioscience) and the plate was incubated at 4ºC for 20 minutes in darkness. A volume of 150 µl of Perm Wash Buffer (BD Biosciences) was added to each well and the plate was centrifuged at 2100rpm for 3 minutes at 4ºC. The supernatant was removed and 10 µl Allophycocyanin-labelled anti-CCR5, along with 2 µl of the following antibodies were added into each well: eF655-labelled anti-CD38 (eBioscence) and Phycoerythrin (PE)-labelled anti-HLA-DR (eBioscience), and Brilliant Violet 700 (BV700)-labelled anti-Ki67 (BioLegend). The plate was then incubated for 20 minutes at 4ºC in darkness. After incubation, 200 µl of Perm Wash Buffer was added to each well and the plate was centrifuged at 2100rpm for 3 minutes (833 xg) at 4ºC. The supernatant was removed and 100 µl of BD Cell Fix was added to each well to resuspend the pellet. The cells were then transferred to a sterile, fluorescence-activated cell sorting (FACS) tube. The plate wells were washed with an additional 50 µl of BD Cell Fix and were transferred to FACS tubes. Acquisition was conducted using an LSRII flow cytometer (BD Immunocytometry Systems).

5.5.3. Preparation of Compensation Beads

The compensation beads were vortexed thoroughly at high speed and 1 drop each of the negative and positive beads were added into each FACS tube. A volume of 2 µl of antibodies was added into labelled FACS tubes.

5.5.4. Data Acquisition

Data acquisition was conducted on a LSRII flow cytometer (BD Immunocytometry Systems), and the data was analysed using FlowJo Software version 9.9 (Tree Star). Fluorescence minus one (FMO) controls were prepared using thawed peripheral blood mononuclear cells (PBMC). FMOs consist of all the markers excluding the one of interest, and are perfect for showing gating boundaries in flow cytometry. Specimens with a CMC CD3 T cell event count below 100 were excluded from analysis. Below is a representative of the gating strategy used (**Figure 9**).

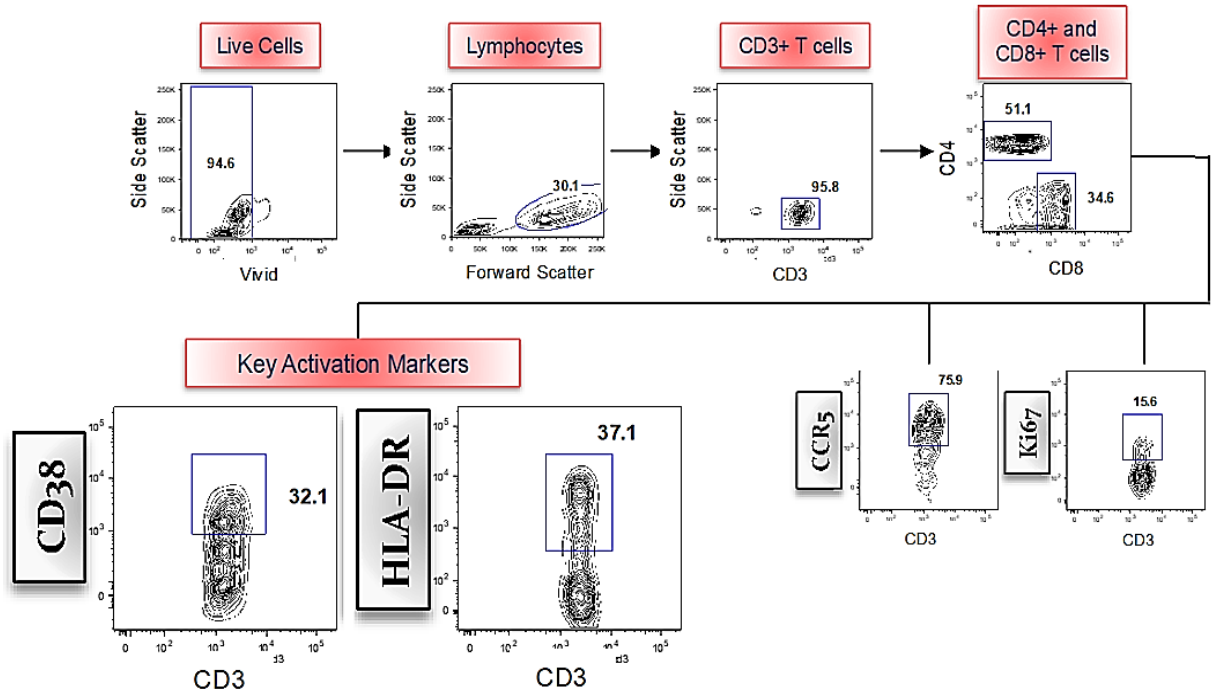

Figure 9. Representative flow cytometry gating plot for the assessment of T cell activation. *Live cells were identified, followed by lymphocytes, and CD3$^+$ T cells. The expression of activation markers (CD38$^+$, HLA-DR$^+$), the marker of proliferation (Ki67$^+$), and the HIV co-receptor for entry (CCR5$^+$) was assessed on CD4$^+$ and CD8$^+$ T cell subsets. NK cells were assessed by measuring CD16 and CD56 co-expression.*

5.6. Investigation of Epithelial Barrier Integrity and Cytokine Biomarkers of Genital Inflammation in CVL Supernatant Specimens

A multiplexed Enzyme-Linked Immunosorbent Assay (ELISA) was used to detect the presence of 48 cytokines (IL-1α, IL-2Rα, IL-3, IL-12 (p40), IL-16, IL-18, CTACK, GRO-α, HGF, IFN-α2, LIF, MCP-3, M-CSF, MIF, MIG, β-NGF, SCF, SCGF-β, SDF-1α, TNF-β, TRAIL, FGF basic, Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1β, IL1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL12 (p70), IL-13, IL-15, IL-17, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α and VEGF), and to assess markers of epithelial barrier integrity (MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12 and MMP-13) in CVL supernatant specimens. The Bio-Plex Pro Human Cytokine 21 plex (#MF0005KMII), 27 plex (#M500KCAF0Y) kits and MMP (#171-AM001M) kits (Bio-Rad, Hercules, CA) were used to investigate cytokine and MMP concentrations respectively.

5.6.1. Multiplex ELISA Assay

The Bio-Plex multiplex ELISA utilises fluorescently dyed beads, each with distinct colour codes to distinguish between individual analytes within a multiplex suspension. In this study, this allowed for simultaneous detection of 48 cytokines and 9 MMPs in a single well of a 96 well microplate. Lasers and associated optics measure the various molecules bound to the surface of the beads and a high speed digital signal processor efficiently manages the fluorescent data (**Figure 10**).

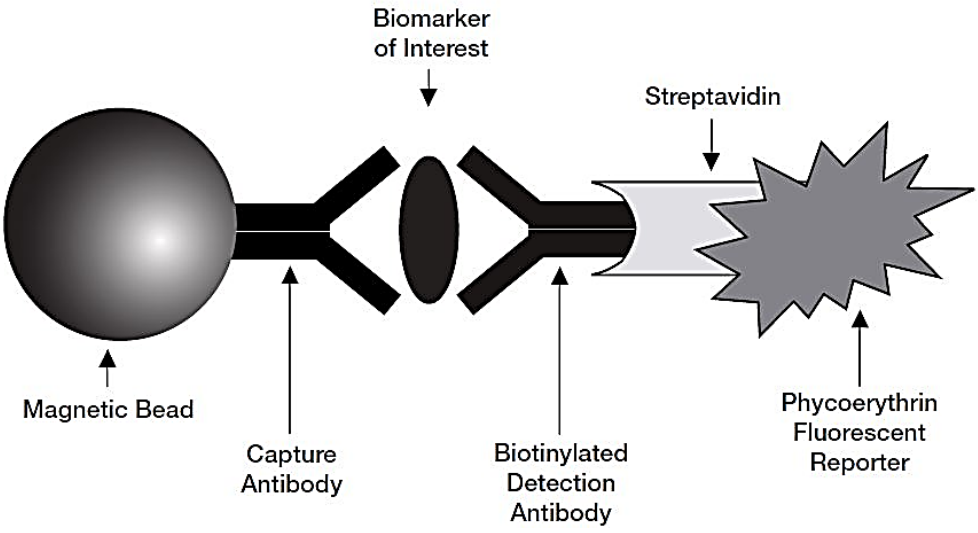

Figure 10. Graphical representation of multiplex ELISA assay *(figure taken from the Bio-Plex Pro instruction manual). The magnetic bead is coupled to a capture antibody that will bind specifically to the analyte of interest. A biotinylated detection antibody is bound to the biomarker of interest, sandwiching the analyte between the capture antibody and the detection antibody. Streptavidin Phycoerythrin (SA-PE) is added to bind to the biotin. The laser will detect the fluorescent phycoerythrin and the mean fluorescence intensity of the analyte of interest is determined.*

5.6.1.1. Preparation of the Bio-Plex 200 Suspension Array System and Software for Data Acquisition

The Bio-Plex system was switched on and allowed to warm up for approximately 30 minutes. In the accompanying Bio-Plex Manager software, the appropriate analytes to be quantified were selected, and the assay standard information was loaded. The plate layout was formatted similarly to that depicted in **Figure 11**. On each plate, 8 biological samples in addition to 10 standards, were run in duplicate to assess intraplate variability. Once the Bio-Plex system had been warmed up, calibration was conducted using the Bio-Plex Pro Calibration Kit.

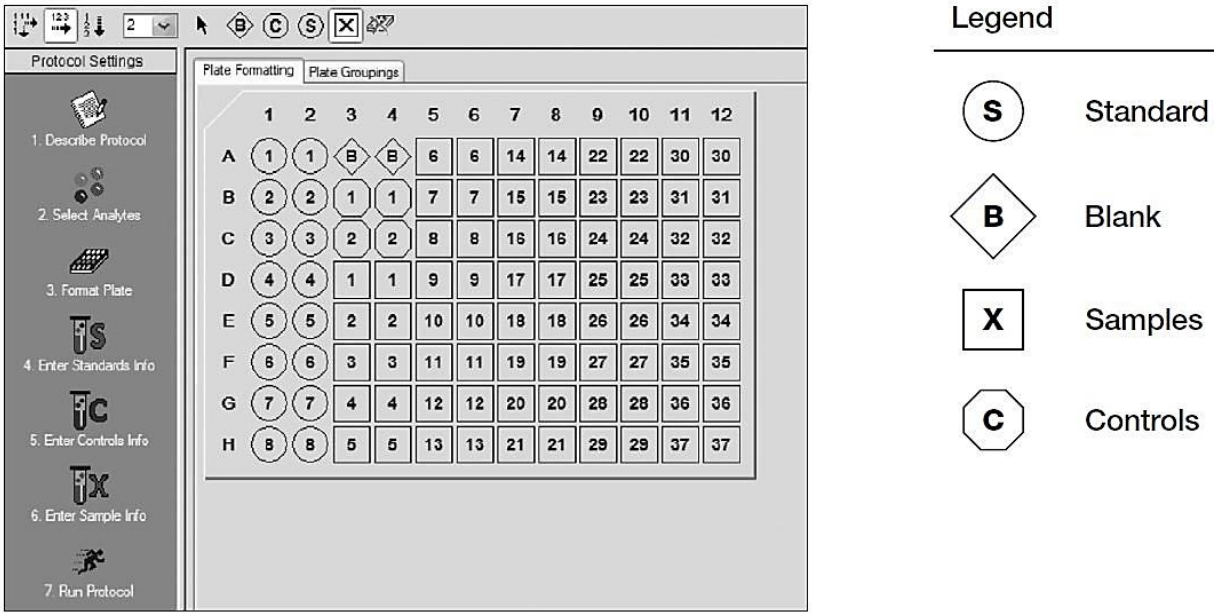

Figure 11. Example of a plate layout on the Bio-Plex 200 *(figure taken from the Bio-Plex Pro instruction manual). The plate layout corresponded with the plating of Standards, Blanks, Samples and Controls on the Bio-Plex Pro Magnetic 96 well microtitre plate. Standards were encoded by circles, Blanks were diamond shape, Unknown Samples were the square blocks and Controls were indicated with octagons.*

5.6.1.2. Preparation of samples, standards, beads, controls, the detection antibody and SA-PE for the Bio-Plex Pro Human Cytokine Assays

The biological controls were prepared by combining excess CVL samples, vortexing thoroughly, and dispensing into cryovials for impending use. The standards were reconstituted with 500 µl of Standard Diluent, vortexed and incubated on ice for 30 minutes. A fourfold standard dilution series was prepared as shown in **Figure 12**. A 15 ml Falcon tube was labelled as 10x coupled beads and 5175 µl of Assay Buffer was added to the tube. The beads were stored on ice, in darkness until ready for use. The beads were vortexed for 30 seconds and 575 µl was added to the tube containing Assay Buffer. The Detection Antibody was prepared by adding 300 µl of 10x Detection Antibody to a polypropylene tube containing 2700 µl of Detection Antibody Diluent. The SA-PE was prepared by adding 60 µl of 100x SA-PE to a 15 ml Falcon tube containing 5940 µl of Assay Buffer. Undiluted samples were assessed.

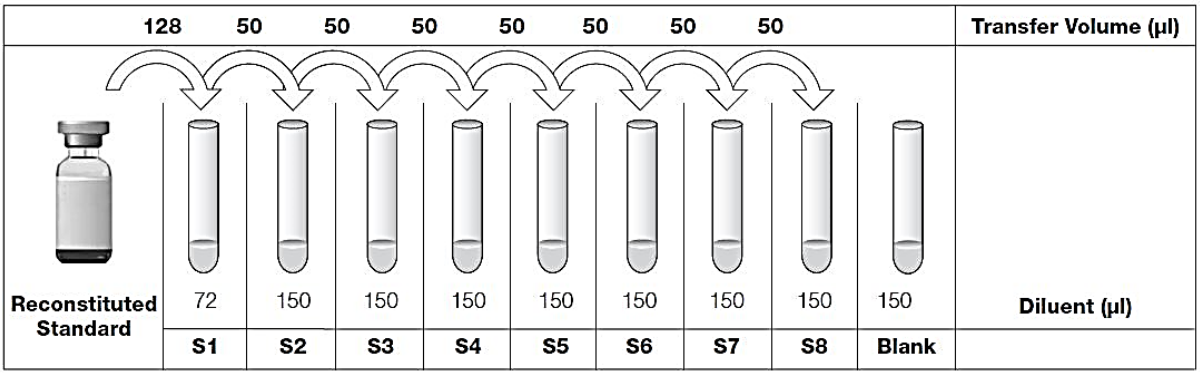

Figure 12. Preparation of a fourfold standard dilution series *(figure taken from the Bio-Plex Pro instruction manual). Ten polypropylene tubes were labelled as standards 1 to 10. A volume of 72 µl of standard diluent was added to standard 1, while standards 2 to 10 contained 150 µl each of the same diluent. A volume of 128 µl of the reconstituted standard was added to the tube labelled Standard 1. Thereafter, 50 µl of the previous standard was added to the subsequent tube, with thorough vortexing between each liquid transfer.*

5.6.1.3.Preparation of sample, standards, beads, controls and detection antibody and SA-PE for the Bio-Plex Pro Human MMP Assays

The biological controls were prepared by combining excess CVL samples, vortexing thoroughly, and dispensing into cryovials for impending use. A 1x Wash Buffer was prepared by diluting 60 ml of 10x Wash Buffer in 540 ml of dH_2O. The 10% Serum-Based Standard Diluent was prepared by adding 4 ml Standard Diluent HB to 6 ml Sample Diluent HB. The standards were reconstituted with 781 µl of Standard Diluent, vortexed and incubated on ice for 30 minutes. A threefold standard dilution series was prepared as shown in **Figure 13**. The beads were kept on ice, in darkness till until ready for use. The beads were vortexed for 30 seconds and 288 µl was added to a 15 ml Falcon tube containing 5472 µl Assay Buffer. The Detection Antibody was prepared by adding 150 µl of 20x Detection Antibody to a polypropylene tube containing 2850 µl of Detection Antibody Diluent. The SA-PE was prepared by adding 60 µl of 100x SA-PE to a 15 ml Falcon tube containing 5940 µl of Assay Buffer. Samples were diluted 1:4 by adding 50 µl of sample to 150 µl Sample Diluent.

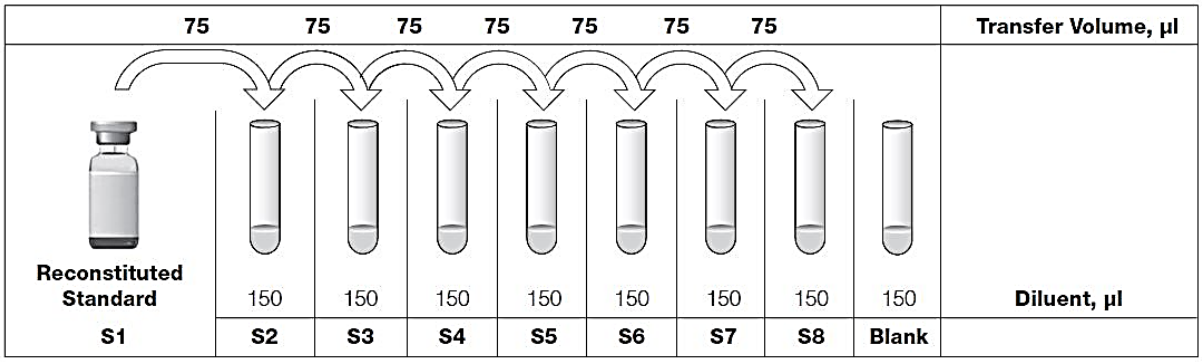

Figure 13. Preparation of a threefold standard dilution series *(figure taken from the Bio-Plex Pro instruction manual). Ten polypropylene tubes were labelled as standards 1 to 10. A volume of 150 µl of sample diluent was added to each tube. A volume of 75 µl of the reconstituted standard was added to the tube labelled standard 1. Thereafter, 75 µl of the previous standard was added to the subsequent tube, with thorough vortexing between each liquid transfer.*

5.6.1.4. Conducting the Multiplex ELISA Assays (Cytokines and MMPs)

A volume of 50 µl of the Coupled Beads was added to each well of the 96 well microtiter plate using a multichannel pipette. The plate was washed twice with 100 µl of Bio-Plex Wash Buffer. Once the plate was washed, the samples, standards, blanks and controls were vortexed and 50 µl was added to each well. The plate was covered with sealing tape and aluminium foil and incubated on the plate shaker for 30 minutes (cytokine assays) or 1 hour for (MMP assays). After incubation the plate was washed 3 times with 100 µl of Wash Buffer. The Detection Antibodies were vortexed for 30 seconds and 25 µl was added to each well of the microtiter plate using a multichannel pipette. 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 3 times with 100 µl of Wash Buffer. The 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 3 times with 100 µl wash buffer. A volume of 125 µl of Assay Buffer was added to each well with a multichannel pipette. The plate was covered with sealing tape and foil and incubated on the plate shaker for 1 minute. Finally, the sealing tape and foil were removed and the plate was read on the Bio-Plex 200 system (Bio-Rad Laboratories, Inc., USA). **Figure 14** depicts a summarised version of the Multiplex ELISA protocol.

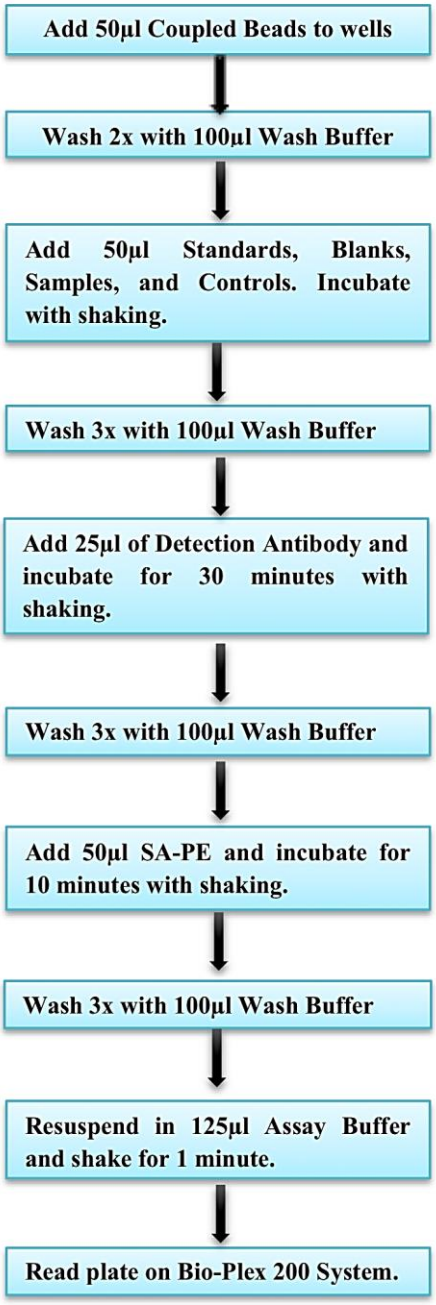

Figure 14. Multiplex ELISA assay protocol summary.

5.6.1.5. Data Quality Control

The standard curves were automatically optimised by Bio-Plex Manager software. For each cytokine or MMP, each standard had an acceptable recovery range of between 70% and 130%, and those standards outside this range were excluded to obtain an appropriate 5-point standard curve. Additionally, standards with coefficients of variation (%CV) greater than 20% were either removed partially or completely, depending on its effect on the standard curve. The observed concentrations of all the 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 was assessed to determine whether there were significant differences between duplicate wells or interplate wells, respectively. This was conducted as quality control to assess any possible pipetting errors. Significant ($P<0.05$) Spearman r values >8, and Wilcoxon T test P-values <0.05 were considered acceptable when conducting intraplate and interplate variability.

5.7. Statistical Considerations

Statistical analyses were performed using GraphPad Prism version 7 (GraphPad Software, San Diego, CA), STATA version 11 (StataCorp, College Station, TX), SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) and Microsoft Excel (2013). The output variables were cytokine concentrations (pg/ml), MMP concentrations (pg/ml), cellular frequency (%) and genital inflammation status. Cytokine and MMP concentrations were log10-transformed. The immune exposure variables were analysed as either dichotomous or continuous variables as appropriate based on their presence or level of detection in the population. Genital inflammation was defined as having 5 of the 9 pro-inflammatory cytokines above the 75^{th} percentile in the FGT and analysed as binary variables using the Fisher's exact test. Since the cytokine, MMP and cellular data were unpaired and followed a non-gaussian distribution, the Mann-Whitney *U* test was used when comparing biomarkers of inflammation between two groups. The Dunn's post-test was used to adjust for multiple comparisons. Linear regression models were used to compare the levels of immune markers between HPV+ versus (vs.) HPV- women; women with HR-HPV vs. LR-HPV; those with single vs. multiple HPV infections; and those with an HPV/STI coinfection and those without. Multivariate regression analyses adjusted for discharge-associated STIs and nugent score, which was significantly associated with genital inflammation in this cohort of women.

6. RESULTS

6.1. HPV prevalence and genotype distribution

Among the 167 women who participated in this study, 50.8% had detectable HPV DNA at baseline (**Table 3**). The prevalence of HR-HPV was 26.9% (**Table 3**), and HR-HPV was detected in 53% of HPV-infected participants. HPV52 was the most prevalent HR HPV genotype; while the most prevalent LR HPV genotype was HPV62 (**Figure 15).**

Table 3. Prevalence of HPV at Baseline (N = 167).

HPV Infection	***% (n)***
HPV Negative	49.2 (82)
HPV Positive	50.8 (85)
Low Risk HPV strains	35.3 (59)
High Risk HPV strains	26.9 (45)
Cervarix strains	7.8 (13)
Gardasil strains	10.8 (18)
Gardasil 9 strains	24.6 (41)

Cervarix: *HPV16, HPV18;* ***Gardasil:*** *HPV16, HPV18, HPV6, HPV11;* ***Gardasil 9:*** *HPV16, HPV18, HPV6, HPV11, HPV31, HPV33, HPV45, HPV52, HPV58*

6.2. Baseline demographics, behavioural and clinical characteristics of the study population

As expected, HPV positive women had a significantly lower mean age than their HPV negative counterparts at baseline [mean±standard deviation (SD) 28.4±4.9 years vs 30.9±5.9 years, respectively, **P = 0.004**; **Table 4**]. HPV positive women were more likely to have a partner with a higher median age [median 34 (interquartile range (IQR) 30 – 37) years vs 32 (IQR 28 – 36) years, **P = 0.005**; **Table 4**], and were less likely to be living with a regular partner than HPV negative women (16.5% and 30.5%, respectively, **P = 0.026**; **Table 4**). In addition, the reported number of live births was higher among HPV positive women compared to HPV negative women [median 1 (IOR 1 – 2); median 2 (IQR 1 – 3) respectively, **P = 0.015**; **Table 4**]. There was no significant difference among HPV positive and HPV negative women in terms of the age of sexual debut, number of lifetime partners, number of sex acts in the past 30 days, condom use, and the presence of inflammatory STIs (gonorrhoea, chlamydia, trichomonas, mycoplasma and HSV-2; **Table 4**).

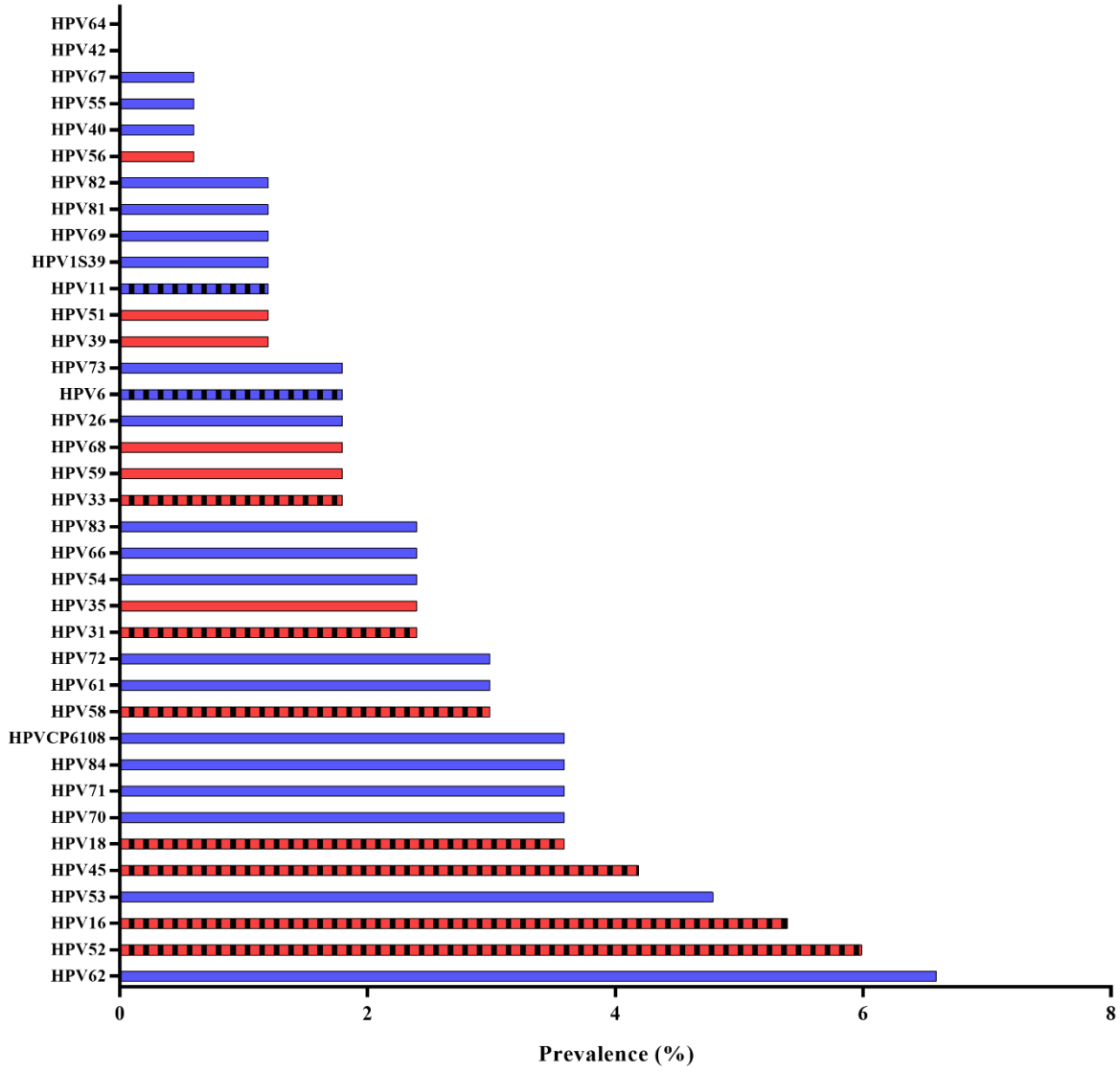

Figure 15. Type-specific HPV prevalence among participants. *The red bars indicate HR HPV genotypes, while the blue bars indicate LR HPV genotypes detected in the population of 167 women at baseline. Vaccine strains are indicated by stripes.*

Table 4. Baseline characteristics by HPV status.

DEMOGRAPHICS		OVERALL (N = 167) % (n)	HPV+ (N = 85) % (n)	HPV- (N = 82) % (n)	P-VALUE
Age mean (SD)		29.6 (5.5)	28.4 (4.9)	30.9 (5.9)	**0.004***
Age (years)	20-25	18.6% (31)	20.0% (17)	17.1% (14)	**0.032**
	25-29	41.3% (69)	49.4% (42)	32.9% (27)	
	>= 30	40.1% (67)	30.6% (26)	50.0% (41)	
Education					
	Less than primary school	39.5% (66)	31.8% (27)	47.6% (39)	0.204
	Primary school complete	2.4% (4)	2.4% (2)	2.4% (2)	
	High school complete	53.3% (89)	60.0% (51)	46.3% (38)	
	Tertiary complete	4.8% (8)	5.9% (5)	3.7% (3)	
Sexual Behaviour median (IQR)					
Age of Sexual Debut		18 (16 - 19)	18 (16 - 19)	18 (16 - 20)	0.323
Age of regular/stable partner		32 (28 - 37)	34 (30 - 37)	32 (28 - 36)	**0.005***
Age of first partner		21 (19 - 24)	20 (19 - 24)	21 (19 - 24)	0.426
Number of live births		1 (1 - 2)	1 (1 - 2)	2 (1 - 3)	**0.015***
Age at first pregnancy		19 (17 - 22)	19 (17 - 21)	19.5 (18 - 23)	0.191
Age when started menstruating		14 (13 - 16)	14 (13 - 15)	14 (13 - 16)	0.701
Number of lifetime partners		2 (2 - 4)	3 (2 - 4)	2 (1 - 4)	0.264
Number of pregnancies		2 (1 - 2)	1 (1 - 2)	2 (1 - 3)	0.094
Number of vaginal sex acts in the last 30 days		4 (2 – 8)	4 (3 – 8)	4 (2 – 6)	0.110
Anal sex act in the last 30 days	Yes	1.2% (2)	0	2.4% (2)	0.240
	No	98.8% (165)	100.0% (85)	97.6% (80)	
Partner HIV Status	No answer	12.6% (21)	16.5% (14)	8.5% (7)	0.272
	Positive	2.4% (4)	3.5% (3)	1.2% (1)	
	Negative	65.9% (110)	64.7% (55)	67.1% (55)	
	Unknown	19.2% (32)	15.3% (13)	23.2% (19)	
Partner Circumcision	No answer	14.4% (24)	20.0% (17)	8.5% (7)	0.093
	Yes	29.3% (49)	30.6% (26)	28.1% (23)	
	No	54.5% (91)	44.0% (40)	62.2% (51)	
	Unknown	1.7% (3)	2.4% (2)	1.2% (1)	
Relationship Status	Married	15.6% (26)	9.4% (8)	22.0% (18)	0.074
	Stable partner	80.8% (135)	87.1% (74)	74.4% (61)	
	Casual Partner	3.6% (6)	3.5% (3)	3.7% (3)	
Living with regular partner	Yes	23.4% (39)	16.5% (14)	30.5% (25)	**0.026***
	No	76.6% (128)	83.5% (71)	69.5% (57)	
How often see regular/stable partner	About every day	27.4% (45/164)	24.1% (20/83)	30.9% (25/81)	0.310
	About every week	43.9% (72/164)	50.6% (42/83)	37.0% (30/81)	
	About every month	26.2% (43/164)	24.1% (20/83)	28.4% (23/81)	
	Less than every month	2.4% (4/164)	1.2% (1/83)	3.7% (3/81)	
Contraceptive type	Depo-provera	57.8% (93/161)	51.9% (42/81)	63.8% (51/80)	0.158
	Oral contraceptive	21.1% (34/161)	19.8% (16/81)	22.5% (18/80)	
	Nur-isterate	15.5% (25/161)	1.0% (17/81)	10.0% (8/80)	
	Other	5.6% (9/161)	7.4% (6/81)	3.8% (3/80)	
Use of Male Condoms	Always	37.7% (63)	34.1% (29)	41.5% (34)	0.638
	Sometimes	48.5% (81)	45.1% (37)	45.1% (37)	
	Never	13.8% (23)	13.4% (11)	13.4% (11)	
STI Testing					
Gonorrhoea	Negative	96.2% (150/156)	95.0% (76/80)	97.4% (74/76)	1.000
	Positive	3.8% (6/156)	5.0% (4/80)	2.6% (2/76)	
Chlamydia	Negative	92.3% (144/156)	93.7% (75/80)	90.8% (69/76)	0.363
	Positive	7.7% (12/156)	6.3% (5/80)	9.2% (7/76)	
Trichomonas	Negative	95.5% (149/156)	95.0% (76/80)	96.1% (76/76)	1.000
	Positive	4.5% (7/156)	5.0% (4/80)	3.9% (3/76)	
Mycoplasma	Negative	95.5% (149/156)	95.0% (76/80)	96.1% (73/76)	0.720
	Positive	4.5% (7/156)	5.0% (4/80)	3.9% (3/76)	
HSV-2 serostatus	Negative	10.2% (17)	10.6% (9)	9.8% (8)	1.000
	Positive	89.8% (150)	89.4% (76)	90.2% (74)	
Any STI [a]	No	82.6% (138)	82.4% (70)	82.9% (68)	1.000
	Yes	17.4% (29)	17.6% (15)	17.1% (14)	
Bacterial Vaginosis score [median (IQR)]		1 (0 - 4)	2 (0 - 4.5)	1 (0 - 3)	0.330

**P<0.05* were considered statistically significant and are listed in bold. [a]Any STI defined as infection with Gonorrhoea, Chlamydia, Trichomonas and/or Mycoplasma. IQR, interquartile range; SD, standard deviation.

Numerous studies have demonstrated that STIs and BV are able to induce a robust immune response and contribute to genital inflammation (Levine *et al.*, 1998; Fichorova *et al.*, 2001a; Reddy *et al.*, 2004; Yudin *et al.*, 2003). STIs increase the production of pro-inflammatory cytokines, which in turn, recruit immune cells and impact epithelial barrier integrity (Anahtar *et al.*, 2015; Arnold *et al.*, 2016; Kaul *et al.*, 2008). These immune response are presumably elicited in order to facilitate pathogen clearance and even defend against reinfection (Kaul *et al.*, 2008), but may render individuals more vulnerable to HIV infection (Laga *et al.*, 1993; van de Wijgert *et al.*, 2009; Van Der Pol *et al.*, 2008; Mlisana *et al.*, 2012; Masson *et al.*, 2014; Taha *et al.*, 1998). However, whether HPV infection has a similar effect on the genital mucosa is yet unclear. Here, the relationship between HPV infection and three biomarkers of inflammation associated with increased risk of HIV acquisition: genital cytokines, epithelial barrier integrity, and immune cell activation was investigated.

6.3. Prevalent HPV and biomarkers of inflammation

6.3.1. Association between prevalent HPV and genital cytokine concentrations

To determine whether HPV infection was associated with a distinct cytokine profile, the concentrations of 48 cytokines involved in chemotaxis, growth, inflammation, adaptive responses, and regulation were measured in baseline CVL specimens (**Table 5**). Cytokine concentrations were similar between the groups, with the exception of the adaptive cytokine IL-5, which trended towards a higher concentration among HPV positive women (**P = 0.078**; **Table 5**).

6.3.2. Association between prevalent HPV and epithelial barrier integrity

An intact epithelium is an important physical barrier protecting against HIV entry. Elevated levels of pro-inflammatory cytokines disrupts tight junctions between epithelial cells, thereby reducing epithelial barrier integrity (Nazli *et al.*, 2010), as measured by MMPs, the enzymes responsible for the degradation and remodelling of the epithelial barrier (Arnold *et al.*, 2016). HPV is an epithelial virus and may facilitate HIV dissemination through disruption of the epithelial barrier (Herfs *et al.*, 2011). Soluble protein biomarkers of epithelial barrier integrity were compared between HPV positive and HPV negative women (**Table 6**). However, concentrations of MMPs did not differ between the groups (**Table 6**), suggesting that prevalent HPV infection may not impact epithelial barrier integrity.

Table 5. Cytokine concentrations among HPV- and HPV+ women.

	OVERALL *(N = 163)*	**HPV- *(N = 81)***	**HPV+ *(N = 82)***	
Cytokine	**Median (IQR) log_{10} pg/ml**	**Median (IQR) log_{10} pg/ml**	**Median (IQR) log_{10} pg/ml**	**P-value**
IL-1α	2,154 (1,806-2,522)	2,074 (1,653-2,616)	2,233 (1,854-2,503)	0.270
IL-1β	1,706 (1,106-2,295)	1,668 (1,086-2,348)	1,721 (1,109-2,290)	0.926
IL-6	0,948 (0,700-1,337)	0,897 (0,633-1,363)	0,984 (0,768-1,309)	0.270
IL-12P40	2,431 (2,317-2,524)	2,429 (2,313-2,515)	2,432 (2,321-2,551)	0.787
IL-12P70	1,761 (1,495-2,050)	1,712 (1,475-1,969)	1,827 (1,531-2,072)	0.208
IL-18	2,351 (1,945-2,720)	2,374 (1,934-2,734)	2,267 (1,977-2,730)	0.901
MIF	3,559 (3,241-3,873)	3,583 (3,237-3,902)	3,534 (3,240-3,860)	0.765
TNF-α	1,071 (0,859-1,413)	1,026 (0,775-1,494)	1,102 (0,914-1,363)	0.418
TNF-β	0,548 (0,405-0,708)	0,544 (0,398-0,652)	0,570 (0,407-0,720)	0.381
TRAIL	1,486 (1,250-1,875)	1,467 (1,240-1,835)	1,495 (1,289-1,879)	0.427
CTACK	1,272 (1,060-1,453)	1,274 (1,047-1,412)	1,264 (1,095-1,486)	0.515
EOTAXIN	0,814 (0,477-1,046)	0,794 (0,434-1,023)	0,833 (0,619-1,062)	0.321
GRO-α	2,882 (2,320-3,494)	2,758 (2,211-3,475)	2,989 (2,402-3,549)	0.331
IL-8	2,722 (2,353-3,170)	2,701 (2,253-3,174)	2,742 (2,363-3,132)	0.690
IL-16	1,603 (1,424-1,869)	1,617 (1,367-1,858)	1,590 (1,448-1,905)	0.664
IP-10	2,325 (1,692-2,913)	2,351 (1,661-2,792)	2,321 (1,782-3,003)	0.455
MCP-1	1,469 (1,368-1,569)	1,477 (1,368-1,568)	1,467 (1,356-1,607)	0.927
MCP-3	1,023 (0,807-1,207)	1,039 (0,786-1,180)	0,990 (0,810-1,233)	0.958
MIG	2,579 (2,061-3,117)	2,589 (1,974-3,052)	2,559 (2,078-3,222)	0.289
MIP-1α	0,064 (-0,187-0,344)	0,061 (-0,201-0,347)	0,064 (-0,163-0,339)	0.952
MIP-1β	1,220 (0,822-1,738)	1,203 (0,792-1,688)	1,245 (0,866-1,773)	0.352
RANTES	0,797 (0,455-1,078)	0,804 (0,372-1,047)	0,793 (0,589-1,190)	0.221
IFN-α2	1,256 (1,143-1,399)	1,246 (1,154-1,386)	1,267 (1,119-1,409)	0.656
β-NGF	-0,051 (-0,509-0,332)	-0,076 (-0,678-0,261)	-0,032 (-0,485-0,405)	0.437
FGF BASIC	1,423 (1,363-1,504)	1,423 (1,360-1,508)	1,422 (1,367-1,499)	0.981
G-CSF	2,330 (1,876-2,859)	2,286 (1,703-2,813)	2,388 (1,957-2,925)	0.141
GM-CSF	1,843 (1,795-1,908)	1,851 (1,788-1,911)	1,839 (1,796-1,902)	0.542
HGF	2,302 (1,924-2,799)	2,247 (1,851-2,797)	2,399 (1,949-2,820)	0.528
IL-3	2,029 (1,949-2,156)	2,029 (1,943-2,138)	2,018 (1,953-2,188)	0.581
IL-7	0,862 (0,681-1,051)	0,795 (0,597-1,060)	0,871 (0,704-1,051)	0.256
IL-9	0,733 (0,566-0,863)	0,708 (0,553-0,864)	0,738 (0,617-0,867)	0.302
LIF	1,113 (0,940-1,291)	1,103 (0,931-1,292)	1,145 (0,963-1,293)	0.668
M-CSF	1,851 (1,601-2,132)	1,793 (1,578-2,132)	1,981 (1,613-2,130)	0.200
PDGF-BB	1,126 (0,895-1,328)	1,103 (0,867-1,311)	1,151 (0,970-1,367)	0.147
SCF	1,016 (0,750-1,440)	0,990 (0,710-1,323)	1,062 (0,787-1,482)	0.227
SCGF-β	2,687 (2,302-3,060)	2,645 (2,137-2,980)	2,746 (2,372-3,076)	0.231
SDF-1α	2,421 (2,133-2,676)	2,378 (2,089-2,666)	2,443 (2,212-2,690)	0.198
VEGF	2,399 (2,064-2,723)	2,336 (1,970-2,699)	2,437 (2,123-2,746)	0.255
IFN-γ	1,369 (1,153-1,561)	1,359 (1,072-1,619)	1,386 (1,228-1,546)	0.476
IL-2	0,446 (0,188-0,668)	0,458 (0,242-0,659)	0,418 (0,174-0,675)	0.486
IL-4	0,013 (-0,119-0,179)	-0,009 (-0,140-0,200)	0,037 (-0,072-0,164)	0.535
IL-5	0,236 (-0,921-0,498)	0,041 (-1,222-0,443)	0,299 (-0,189-0,522)	0.078
IL-13	0,543 (0,373-0,735)	0,537 (0,366-0,730)	0,563 (0,386-0,738)	0.607
IL-15	0,310 (-1,260-0,633)	0,283 (-1,260-0,633)	0,317 (-1,260-0,687)	0.491
IL-17	1,086 (0,957-1,302)	1,081 (0,946-1,304)	1,093 (0,977-1,291)	0.542
IL-2RA	1,318 (1,115-1,498)	1,329 (1,120-1,501)	1,316 (1,094-1,504)	0.620
IL-10	1,629 (1,494-1,784)	1,620 (1,452-1,770)	1,636 (1,521-1,812)	0.289
IL-1RA	4,101 (3,939-4,220)	4,107 (3,946-4,219)	4,096 (3,931-4,227)	0.901

P-values <0.05 were considered significant. Nonparametric comparisons were conducted using the Mann Whitney U test.
***Cytokine/Chemokine functions:** Pro-inflammatory, Chemokines, Growth Factors, Adaptive response, Anti-inflammatory.*

Table 6. MMP concentrations by HPV status.

	OVERALL ***(N = 163)***	**HPV-** ***(N = 81)***	**HPV+** ***(N = 82)***	
	Median (IQR) log_{10} pg/ml	**Median (IQR) log_{10} pg/ml**	**Median (IQR) log_{10} pg/ml**	**P-value**
MMP-1	3,200 (2,780-3,450)	3,210 (2,795-3,480)	3,190 (2,750-3,420)	0.596
MMP-2	3,460 (2,900-3,840)	3,400 (2,860-3,900)	3,560 (3,128-3,803)	0.535
MMP-3	2,950 (2,630-3,230)	2,920 (2,640-3,210)	3,020 (2,618-3,263)	0.660
MMP-7	2,440 (2,000-3,180)	2,420 (1,870-3,135)	2,455 (2,088-3,223)	0.261
MMP-8	5,010 (4,210-6,230)	5,010 (4,275-6,520)	5,010 (4,150-6,093)	0.648
MMP-9	4,720 (3,970-5,540)	4,630 (4,015-5,660)	4,750 (3,895-5,440)	0.670
MMP-10	2,940 (2,390-3,290)	2,960 (2,390-3,275)	2,925 (2,385-3,295)	0.877
MMP-12	2,930 (2,520-3,400)	2,930 (2,540-3,310)	2,965 (2,508-3,435)	0.926
MMP-13	2,270 (1,740-2,650)	2,310 (1,725-2,665)	2,270 (1,785-2,623)	0.857

P-values <0.05 were considered significant. Unpaired nonparametric comparisons were conducted using the Mann Whitney U test.

6.3.3. Association between prevalent HPV and immune cell recruitment

The frequency of activated ($CD38^+$ and/or $HLA\text{-}DR^+$) or proliferating ($Ki67^+$) T cells ($CD3^+$ $CD4^+$ or $CD8^+$), NK cells ($CD56^+CD16^+$) and activated $CCR5^+CD4^+$ T cell targets for HIV replication was compared between HPV positive and HPV negative women (**Table 7**). Cellular frequencies were similar between the groups, with the exception of the $CD4^+$ T cell population, which trended towards a reduced frequency among HPV positive women (**P = 0.056**; **Table 7**).

Table 7. Cellular frequencies by HPV status.

	OVERALL *(N = 163)*	HPV- *(N = 80)*	HPV+ *(N = 83)*	
	Median % (IQR)	**Median % (IQR)**	**Median % (IQR)**	**P-value**
LYMPHOCYTES	2,360 (1,020-5,590)	2,325 (1,008-6,053)	2,610 (1,020-5,410)	0.852
CD3	11,200 (2,330-25,500)	10,350 (1,883-27,980)	12,000 (2,650-23,800)	0.908
CD3CD38DR$^+$CCR5$^+$	5,670 (1,490-14,000)	6,060 (1,395-15,100)	5,225 (1,950-13,850)	0.942
CD3CCR5$^+$CD38$^+$	17,650 (6,800-30,100)	16,800 (6,170-31,600)	19,400 (6,950-28,400)	0.808
CD3CCR5$^+$DR$^+$	11,300 (4,620-28,000)	11,200 (4,515-24,150)	11,700 (4,643-32,700)	0.512
CD3CCR5$^+$KI67$^+$	7,725 (0,000-21,950)	6,320 (0,000-18,230)	9,510 (0,000-24,700)	0.380
CD3CD38$^+$DR$^+$	11,100 (5,058-24,330)	10,700 (4,935-22,250)	11,200 (5,055-25,300)	0.700
CD3CCR5$^+$	43,350 (23,950-64,080)	41,700 (21,200-66,700)	44,900 (24,500-63,300)	0.989
CD3CD38$^+$	39,700 (21,300-58,100)	38,650 (25,000-57,400)	43,500 (18,100-61,500)	0.908
CD3HLA-DR	25,050 (12,480-47,080)	24,900 (11,450-41,950)	25,550 (14,500-51,900)	0.510
CD3KI67	18,700 (0,099-37,100)	14,100 (0,106-36,080)	22,900 (0,098-38,100)	0.433
CD3 -	37,250 (23,450-58,950)	40,700 (24,330-60,730)	33,500 (22,250-58,430)	0.450
NK Cells [2]	4,365 (1,380-14,030)	5,465 (1,324-12,830)	4,020 (1,340-17,000)	0.954
CD3 Activation [1]	75,000 (55,500-91,000)	72,850 (54,450-91,480)	75,600 (59,500-90,100)	0.860
CD4	60,000 (48,100-68,900)	62,250 (49,350-72,000)	57,500 (46,200-66,700)	0.056
CD4CD38DR$^+$CCR5$^+$	4,270 (0,484-12,100)	4,300 (0,371-11,600)	4,125 (0,535-12,130)	0.987
CD4CCR5$^+$CD38$^+$	18,950 (6,033-32,280)	18,300 (4,940-33,600)	20,400 (6,360-32,200)	0.725
CD4CCR5$^+$DR$^+$	8,970 (2,210-25,900)	9,090 (2,500-20,550)	8,940 (2,045-27,750)	0.887
CD4CCR5$^+$KI67$^+$	3,610 (0,000-23,250)	3,390 (0,000-19,730)	3,830 (0,000-27,330)	0.713
CD4CD38$^+$DR$^+$	8,305 (1,813-17,950)	8,535 (1,788-18,080)	8,035 (1,768-17,480)	0.843
CD4CCR5	45,450 (24,900-69,000)	45,300 (25,000-69,800)	45,700 (22,200-66,800)	0.798
CD4CD38	42,600 (21,900-59,300)	40,100 (23,530-59,830)	44,100 (19,100-58,300)	0.861
CD4HLA-DR	18,550 (6,720-34,650)	17,300 (7,673-32,480)	19,000 (4,898-39,680)	0.826
CD4KI67	10,500 (0,000-34,900)	10,400 (0,000-33,680)	10,500 (0,000-35,700)	0.895
CD4 Activation [1]	75,000 (54,700-90,200)	75,800 (52,980-91,580)	75,000 (55,000-89,400)	0.706
CD8	35,700 (25,400-46,400)	35,200 (25,580-48,350)	35,800 (25,000-46,300)	0.978
CD8CD38DR$^+$CCR5$^+$	5,310 (0,000-13,300)	4,170 (0,000-12,350)	6,280 (0,419-14,080)	0.316
CD8CCR5$^+$CD38$^+$	11,500 (3,233-22,500)	10,300 (3,030-22,300)	13,100 (4,260-24,600)	0.445
CD8CCR5$^+$DR$^+$	11,600 (1,800-25,400)	10,900 (0,729-19,050)	13,200 (2,273-27,130)	0.267
CD8CCR5$^+$KI67$^+$	3,920 (0,000-19,150)	2,355 (0,000-14,550)	7,275 (0,000-25,300)	0.280
CD8CD38$^+$DR$^+$	12,500 (2,638-30,750)	11,800 (2,060-25,830)	13,100 (2,783-33,700)	0.491
CD8CCR5	31,750 (16,300-53,280)	30,900 (15,400-52,800)	31,900 (18,200-55,200)	0.802
CD8CD38	37,800 (14,300-56,500)	35,450 (14,080-52,000)	38,700 (15,300-60,400)	0.455
CD8HLA-DR	35,900 (10,630-58,430)	33,600 (9,418-57,150)	36,850 (11,600-62,530)	0.628
CD8KI67	13,700 (0,000-40,000)	14,250 (0,000-38,250)	13,600 (0,000-40,400)	0.771
CD8 Activation [1]	72,800 (53,300-90,500)	72,100 (53,780-91,900)	72,900 (51,200-89,300)	0.970

P-values <0.05 were considered significant. Unpaired nonparametric comparisons were conducted using the Mann Whitney U test. [1]Activation refers to cells expressing CCR5, HLA-DR and/or CD38. [2]NK cells refers to those expressing CD56+CD16+ markers.

Taken together, the detection of any HPV genotype in CVL specimens was not associated with significant increases in cytokine concentrations, markers of immune cell activation or epithelial barrier integrity. To limit the potential for the inflammatory nature of discharge-associated STIs and BV to mask a contribution of HPV to genital immunity, regression models were used to validate the above observations by controlling for the presence of discharge-associated STIs and nugent score (**Figure 16**; **Appendix B**).

Controlling for an impact of STIs and BV on genital cytokine concentrations, HPV infection was associated with increased concentrations of the chemokine SDF-1a [**β = 0.148 pg/ml**, **P = 0.036**; **Figure 16A**]. Although not statistically significant, being HPV positive was associated with trending decreases of MMP-9 concentrations [**β = -0.357 pg/ml**, **P = 0.092**; **Figure 16B**] and reduced frequencies of CD4 cells [**β = -6.315 pg/ml**, **P = 0.054**; **Figure 16C**].

Based on their respective well-established roles in HIV acquisition, a relationship between genital inflammation (Masson et al., 2015) and HPV status was also investigated. Genital inflammation, as defined by having 5 or more of 9 pro-inflammatory cytokines and chemokines (IL-1α, IL-1β, IL-6, TNF-α, IL-8, IP-10, MCP-1, MIP-1α and MIP-1β) above the 75th percentile (Masson *et al.*, 2015), did not distinguish HPV-infected from uninfected women (**P = 0.700**), suggesting that being HPV positive was not associated with this measurement of genital inflammation.

In summary here, *HPV infection predicted increased concentrations of the chemokine SDF-1α, but was not associated with markers of epithelial barrier integrity, immune cell activation, or genital inflammation.* Further investigations were therefore conducted to determine whether other features of HPV infection, also associated with HIV infection, had an impact on genital immunity; namely, the number of infecting strains, the oncogenic potential of the HPV, and coinfection with other STI.

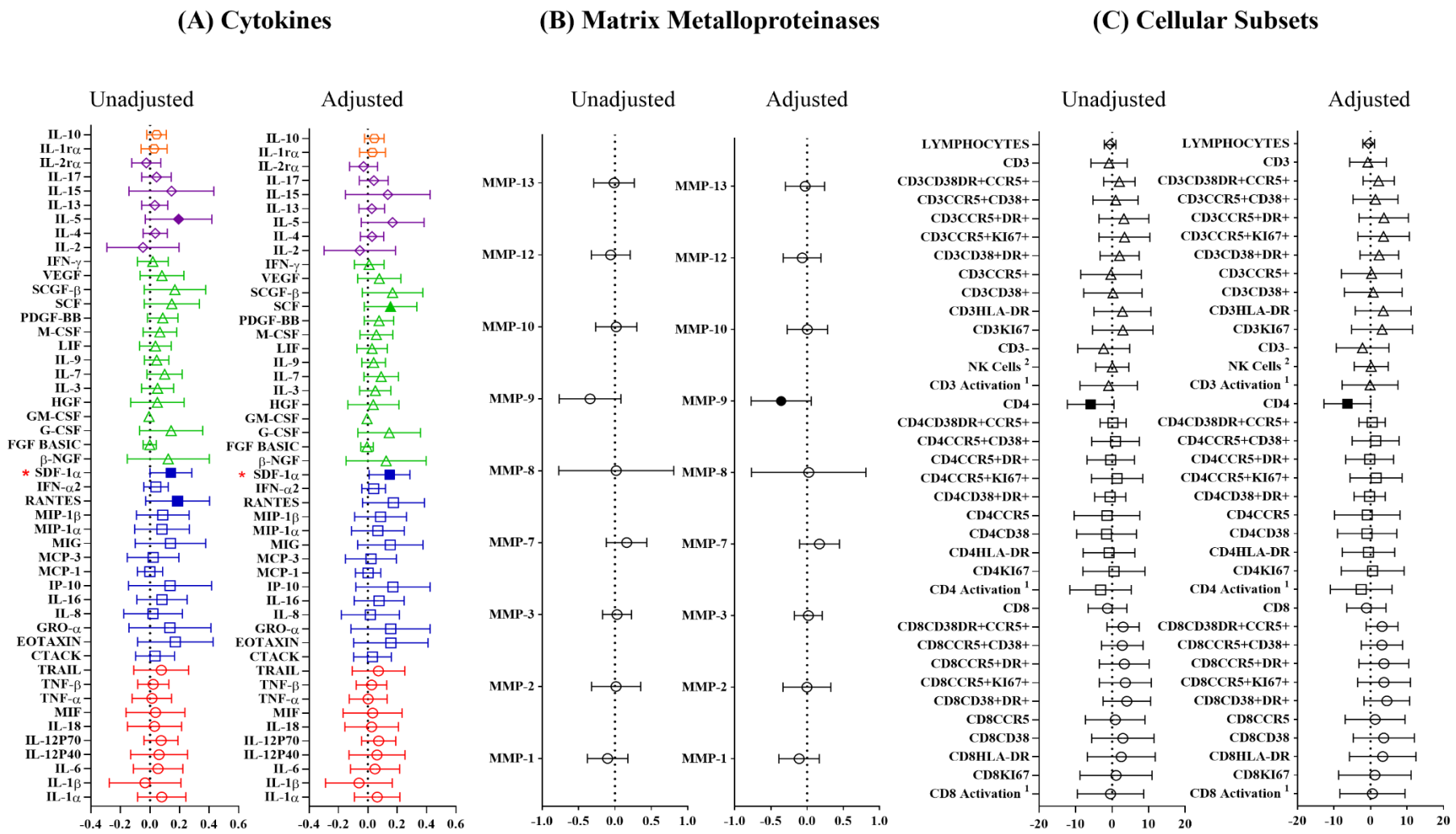

Figure 16. Association between HPV status and biomarkers of genital inflammation. *β-coefficients and corresponding P-values were determined using linear regression models. B-coefficients are indicated by shapes and error bars indicate 95% confidence intervals. Significant P-values (P<0.05) are indicated by (*****) and shading, whilst trends (P<0.1) are indicated by shading only. [1]Activation refers to cells expressing CCR5, HLA-DR and/or CD38. [2]NK cells refers to those expressing $CD56^{+}CD16^{+}$ markers.* ***Cytokines are ordered according to functionality: pro-inflammatory, chemokines, growth factors, adaptive response and anti-inflammatory cytokines****.*

6.4. Association between multiple HPV genotypes and genital immunity

To determine whether having multiple concurrent HPV infections contributes to the genital immune response, HPV positive women were stratified into groups based on the number of infecting HPV types. Women were classified as having "1" or "2+" based on whether they had one or multiple HPV genotypes detected in CVL specimens. Concentrations of cytokines, immune cell activation, and epithelial barrier integrity were compared between these groups (**Figure 17**; **Appendix C**).

In univariate analysis, having multiple infecting HPV strains was inversely associated with concentrations of the growth factor M-CSF [**β = -0.162 pg/ml**, **P = 0.044**; **Figure 17A**]. However, after controlling for STI and BV, this significant association with M-CSF was lost, and an association between multiple genotypes and reduced concentrations of the adaptive cytokine IL-5 was observed [**β = -0.170 pg/ml**; **P = 0.033**; **Figure 17A**]. Having multiple concurrent HPV infections was not significantly associated with MMP concentrations (**Figure 17B**), with immune cell activation (**Figure 17C**), nor with genital inflammation as defined by elevated levels of 5/9 pro-inflammatory cytokines and chemokines (**P = 0.215**).

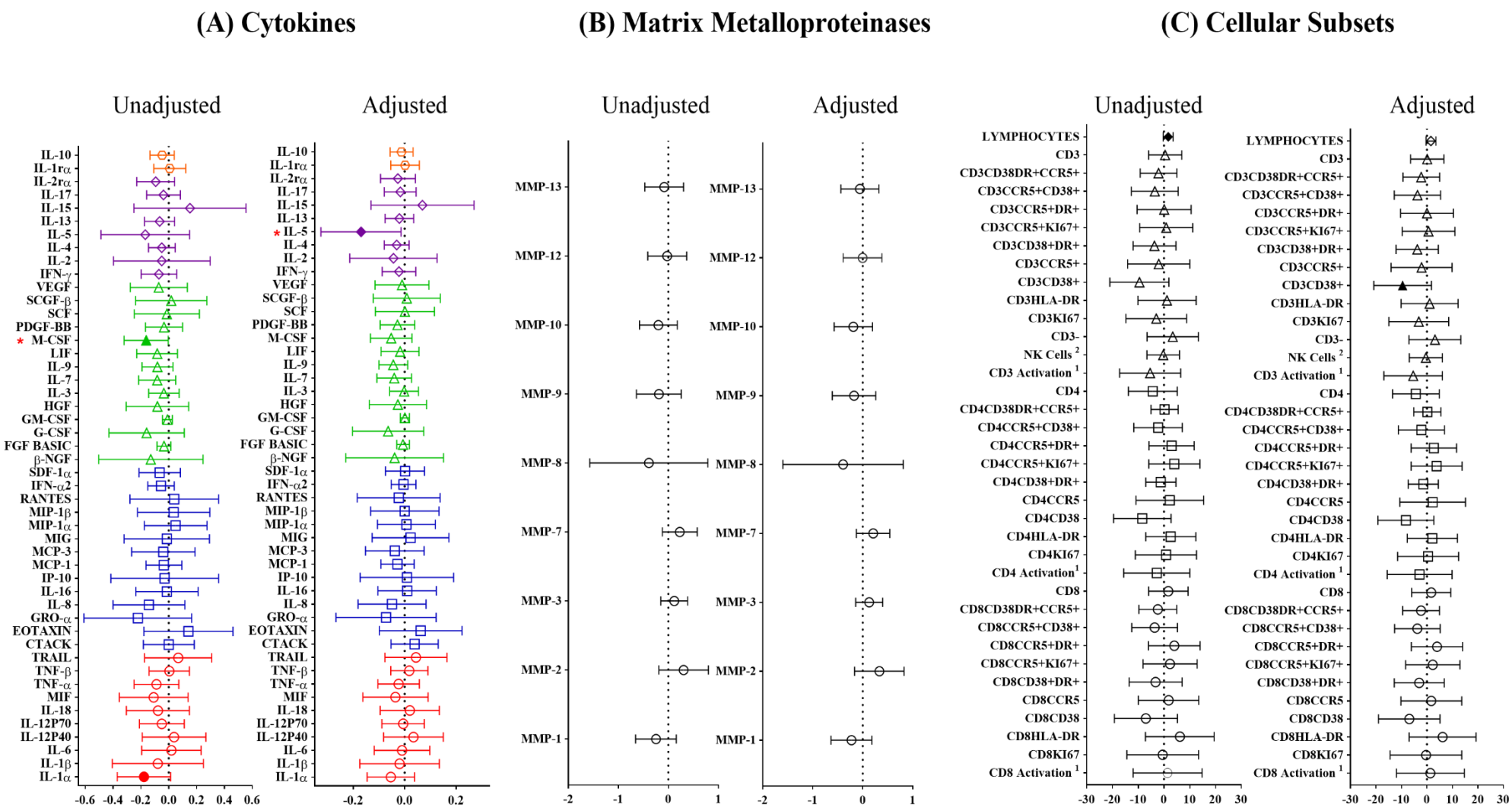

Figure 17. Association between multiple HPV genotypes and biomarkers of genital inflammation. *β-coefficients and corresponding P-values were determined using linear regression models. B-coefficients are indicated by shapes and error bars indicate 95% confidence intervals. Significant P-values (P<0.05) are indicated by (*) and shading, whilst trends (P<0.1) are indicated by shading only.* [1]*Activation refers to cells expressing CCR5, HLA-DR and/or CD38.* [2]*NK cells refers to those expressing $CD56^{+}CD16^{+}$ markers.* ***Cytokines are ordered according to functionality: pro-inflammatory, chemokines, growth factors, adaptive response and anti-inflammatory cytokines.***

6.5. Association between oncogenic HPV and genital immunity

Soluble protein biomarkers of inflammation and immune cell activation was compared between women with at least 1 HR-HPV type (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV68) and women infected with LR-HPV types only (**Figure 18**; **Appendix D**).

In univariate analysis, infection with HR-HPV was associated with the chemokine MCP-1 (**β = 0.124 pg/ml**, **P = 0.052**), the adaptive cytokine IL-13 (**β = 0.119 pg/ml**, **P = 0.028**) and the pro-inflammatory cytokine IL-12p70 [**β = 0.154 pg/ml**, **P = 0.054**; **Figure 18A**]. Controlling for STI and BV in multivariate regression models, infection with HR-HPV was significantly associated with increased concentrations MCP-1 (**β = 0.127 pg/ml**, **P = 0.046**) and IL-13 (**β = 0.117 pg/ml**, **P = 0.031**). In addition, although not statistically significant, women with HR-HPV were more inclined to have increased concentrations of the pro-inflammatory cytokines: IL-1β (**β = 0.268 pg/ml**, **P = 0.078**) and IL-12P70 (**β = 0.150 pg/ml**, **P = 0.064**), chemokines: eotaxin (**β = 0.284 pg/ml**, **P = 0.073**), IL-8 (**β = 0.222 pg/ml**, **P = 0.088**), IP-10 (**β = 0.341 pg/ml**, **P = 0.055**) and MIG (**β = 0.268 pg/ml**, **P = 0.067**), growth factor: VEGF (**β = 0.175 pg/ml**, **P = 0.088)** and the adaptive cytokine IL-2 [**β = 0.307 pg/ml**, **P = 0.066**; **Figure 18A**]. Even though HR-HPV was associated with several cytokines, no association was observed between oncogenicity and the definition of genital inflammation, suggesting that oncogenic HPV infection was not associated with this measurement of genital inflammation.

HR-HPV infection trended towards an association with increased MMP-3 concentrations [**β = 0.239 pg/ml**, **P = 0.075**; **Figure 18B**]. Further, HR-HPV infection was significantly associated with greater frequencies of lymphocytes [**β = 1.987 pg/ml**, **P = 0.042**; **Figure 18C**]. It is important to note that the number of infecting HPV types did not differ significantly between women with LR-HPV and HR-HPV infections [median 0 (IQR 0 – 2); median 1 (IQR 0 – 3) respectively, **P = 0.109**].

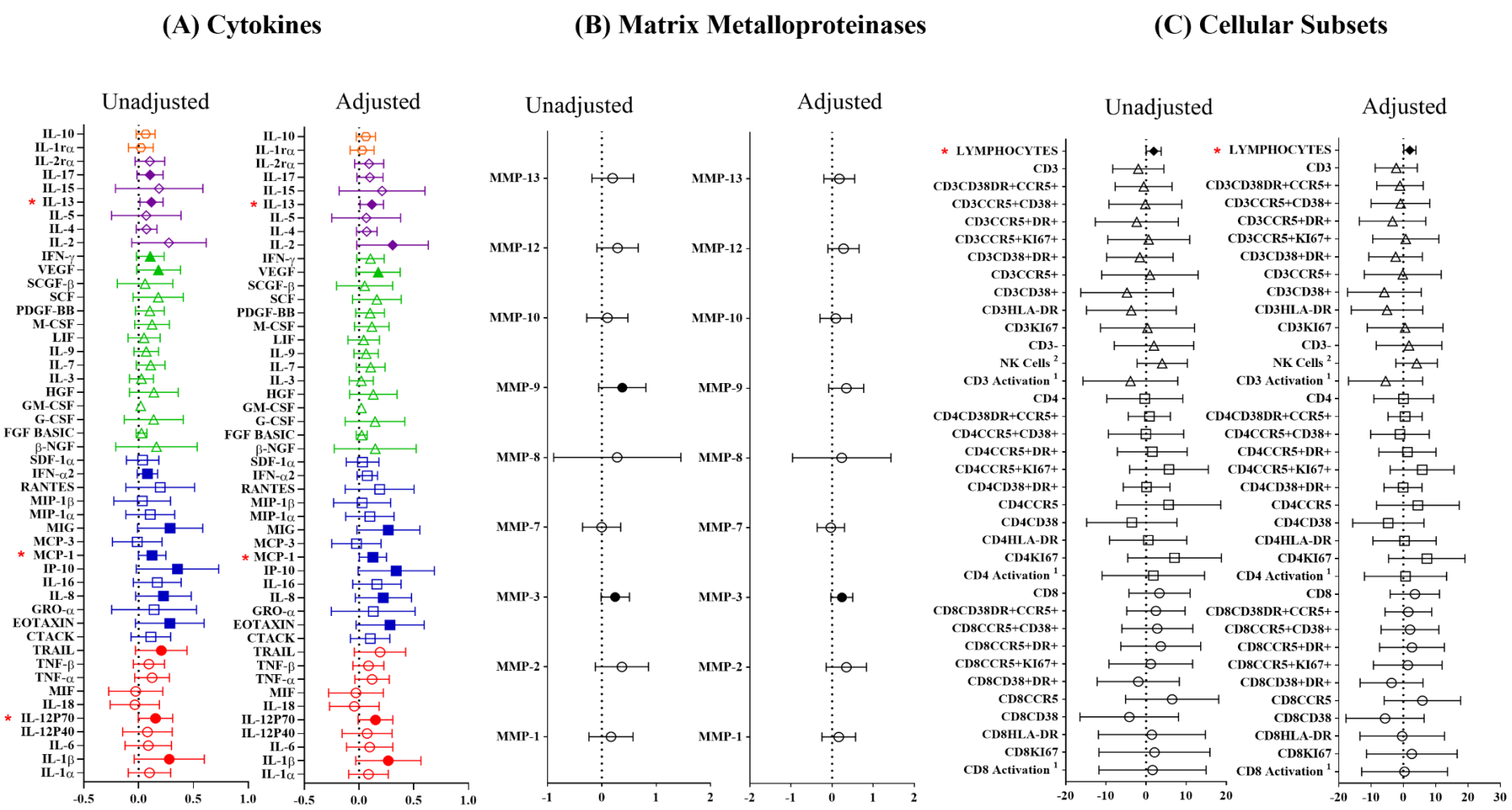

Figure 18. Association between oncogenic HPV and biomarkers of genital inflammation. *β-coefficients and corresponding P-values were determined using linear regression models. B-coefficients are indicated by shapes and error bars indicate 95% confidence intervals. Significant P-values (P<0.05) are indicated by (*) and shading, whilst trends (P<0.1) are indicated by shading only.* [1]*Activation refers to cells expressing CCR5, HLA-DR and/or CD38.* [2]*NK cells refers to those expressing $CD56^{+}CD16^{+}$ markers.* ***Cytokines are ordered according to functionality:*** ***pro-inflammatory****,* ***chemokines****,* ***growth factors****,* ***adaptive response*** *and* ***anti-inflammatory*** *cytokines.*

6.6. HPV, discharge-associated STIs, and biomarkers of inflammation

To investigate a potential compounding effect of co-infecting HPV and inflammatory STI on biomarkers of inflammation, the biomarkers were compared between participants categorised as uninfected (HPV-STI-), having discharge-associated STI only (HPV-STI+), having HPV only (HPV+STI-), and co-infected (HPV+STI+; **Table 8**; **Appendix E**).

6.6.1. Association between HPV, STIs, and genital cytokine concentrations

HPV+STI- women had increased concentrations of the adaptive cytokine IL-5, pro-inflammatory cytokine IL-6, and the growth factor G-CSF compared to uninfected women, indicating that HPV infection is associated with a distinct cytokine signature [**Table 8**; **Appendix E**: **Table 10**]. However, these associations did not withstand adjustment for multiple comparisons. Infection with STI only was associated with elevated levels of several cytokines relative to uninfected women; with concentrations of IL-1β, IL-6, TNF-α, TRAIL, CTACK, IL-16, MIG, MIP-1α, RANTES, β-NGF, FGF, HGF, SCF, SCGF-β and IL-5 remaining significant even after adjustment. Infection with STIs induced a robust pro-inflammatory cytokine response compared to HPV infection [**Table 8**; **Appendix E**: **Table 10**]. Women with an HPV/STI coinfection were associated with a similar cytokine profile as women with STI only, and coinfection was associated with significant increases in additional cytokines: TNF-β, MCP-3, IL-9 and M-CSF relative to uninfected women [**Table 8**; **Appendix E**: **Table 10**]. Additionally, comparisons between the STI only and coinfection groups demonstrated that for some cytokines (IL-6 and IL-1RA) the effect of concomitant HPV infection was a reduction of the cytokine when compared to the concentrations observed in women with STI alone. These data suggested that the inflammatory response observed in co-infected women was solely driven by discharge-associated STIs and HPV infection had limited impact on the cytokine milieu in HPV+STI+ women. As expected, STI was significantly associated with genital inflammation status relative to uninfected women (**P = 0.0054**), suggesting that infection with non-HPV related pathogens induced an inflammatory response at the genital mucosa.

Taken together, these data suggests that *infection with this panel of discharge-associated agents was related to an inflammatory response, more so than HPV infection alone; and that HPV co-infection with STI has a limited impact on cytokine concentrations*.

Table 8. Cytokine concentrations among HPV-STI-, HPV+STI-, HPV-STI+ and HPV+STI+ women.

Cytokine	HPV-STI- *(N = 67)*	HPV ONLY *(N = 66)*	STI ONLY *(N = 14)*	HPV+STI+ *(N = 15)*
	Median (IQR) log_{10} pg/ml	Median (IQR) log_{10} pg/ml	Median (IQR) log_{10} pg/ml	Median (IQR) log_{10} pg/ml
IL-1α	2,032 (1,614-2,473)	2,143 (1,834-2,444) #	2,683 (1,930-3,111)*	2,540 (2,247-2,797)*
IL-1β	1,563 (1,009-2,098)	1,557 (1,044-2,251)	2,395 (1,650-2,826)*	2,118 (1,719-2,335)*
IL-6	0,795 (0,522-1,256)	1,004 (0,787-1,351)*	1,207 (0,972-1,735)* #	0,892 (0,711-1,055)
IL-12P40	2,425 (2,296-2,507)	2,423 (2,320-2,520)	2,551 (2,370-2,731)*	2,512 (2,317-2,594)
IL-12P70	1,709 (1,457-1,939)	1,774 (1,476-2,072)	1,892 (1,481-2,160)	1,943 (1,617-2,054)
IL-18	2,351 (1,855-2,677)	2,293 (1,954-2,684)	2,541 (2,037-2,801)	2,219 (2,113-3,025)
MIF	3,551 (3,244-3,904)	3,549 (3,249-3,834)	3,617 (3,197-3,902)	3,487 (3,085-3,948)
TNF-α	0,983 (0,769-1,388)	1,074 (0,906-1,348)	1,369 (1,085-1,874)*	1,209 (1,031-1,483)
TNF-B	0,542 (0,387-0,633)	0,537 (0,405-0,661) #	0,560 (0,403-1,005)	0,734 (0,524-0,848)*
TRAIL	1,420 (1,092-1,738)	1,472 (1,236-1,842) #	1,861 (1,448-2,354)*	1,866 (1,489-2,004)*
CTACK	1,222 (1,040-1,402)	1,216 (1,084-1,405) #	1,392 (1,284-1,759)*	1,481 (1,195-1,590)*
EOTAXIN	0,732 (0,418-0,996)	0,823 (0,475-1,063)	1,003 (0,460-1,299)	0,900 (0,732-1,064)
GRO-A	2,693 (2,192-3,456)	2,956 (2,352-3,484)	3,075 (2,778-3,754)	3,206 (2,532-3,783)
IL-8	2,601 (2,143-3,038)	2,692 (2,344-3,050)	3,028 (2,589-3,638)*	2,962 (2,669-3,221)
IL-16	1,542 (1,349-1,816)	1,578 (1,437-1,890)	1,876 (1,673-2,332)*	1,828 (1,456-2,137)*
IP-10	2,306 (1,599-2,728)	2,322 (1,704-2,959)	2,636 (2,290-3,472)*	2,280 (1,901-3,523)
MCP-1	1,458 (1,368-1,544)	1,469 (1,356-1,611)	1,540 (1,377-1,800)	1,458 (1,316-1,485)
MCP-3	1,036 (0,751-1,156)	0,975 (0,774-1,185) #	1,047 (0,920-1,457)	1,184 (0,958-1,365)*
MIG	2,422 (1,821-2,889)	2,466 (2,059-3,046)	3,200 (2,447-3,821)*	3,247 (2,299-3,516)*
MIP-1α	0,009 (-0,252-0,316)	0,008 (-0,204-0,313)	0,314 (0,028-0,698)*	0,193 (-0,004-0,483)
MIP-1B	1,043 (0,728-1,663)	1,281 (0,846-1,752)	1,487 (1,135-1,877)*	1,224 (1,015-1,940)
RANTES	0,754 (0,340-0,975)	0,793 (0,525-1,114)	1,161 (0,796-1,527)*	1,035 (0,732-1,251)*
IFN-α2	1,225 (1,143-1,345)	1,242 (1,119-1,369) #	1,379 (1,233-1,648)*	1,437 (1,160-1,518)*
β-NGF	-0,131 (-2,000-0,246)	-0,117 (-0,498-0,302)	0,221 (-0,144-0,754)*	0,366 (-0,377-0,630)*
FGF BASIC	1,409 (1,358-1,493)	1,422 (1,371-1,482)	1,503 (1,426-1,587)*	1,481 (1,353-1,562)
G-CSF	2,199 (1,591-2,701)	2,395 (2,066-2,965)*	2,517 (2,202-3,307)*	2,239 (1,800-2,730)
GM-CSF	1,840 (1,789-1,908)	1,855 (1,802-1,907) #	1,890 (1,775-1,932)	1,799 (1,751-1,861)
HGF	2,167 (1,747-2,748)	2,323 (1,918-2,780)	2,788 (2,227-3,329)*	2,575 (2,299-2,915)*
IL-3	2,024 (1,891-2,114)	1,997 (1,951-2,154)	2,118 (1,996-2,479)*	2,258 (1,964-2,326)*
IL-7	0,781 (0,547-1,049)	0,865 (0,683-1,031)	0,992 (0,741-1,073)	0,903 (0,780-1,086)
IL-9	0,655 (0,515-0,844)	0,733 (0,590-0,848)	0,785 (0,617-1,021)	0,815 (0,717-0,930)*
LIF	1,073 (0,899-1,262)	1,075 (0,936-1,239) #	1,164 (1,100-1,620)*	1,305 (1,216-1,375)*
M-CSF	1,778 (1,565-2,020)	1,926 (1,598-2,121)	1,995 (1,661-2,350)	2,056 (1,729-2,203)*
PDGF-BB	1,078 (0,817-1,300)	1,127 (0,966-1,361)	1,270 (1,040-1,669)*	1,276 (0,971-1,392)*
SCF	0,960 (0,694-1,232)	1,010 (0,765-1,337)	1,443 (0,894-1,810)*	1,477 (0,887-1,706)*
SCGF-β	2,548 (2,090-2,900)	2,734 (2,384-3,037)	3,137 (2,438-3,350)*	2,756 (2,367-3,094)
SDF-1A	2,303 (2,054-2,646)	2,426 (2,188-2,679)	2,511 (2,375-2,734)*	2,511 (2,216-2,736)
VEGF	2,333 (1,943-2,571)	2,405 (2,092-2,710)	2,581 (2,108-3,058)	2,681 (2,275-2,833)
IFN-G	1,312 (1,060-1,536)	1,371 (1,192-1,530)	1,562 (1,222-1,826)*	1,448 (1,335-1,615)
IL-2	0,446 (0,236-0,659)	0,436 (0,187-0,680)	0,546 (0,389-0,886)	0,322 (-0,495-0,520)
IL-4	-0,013 (-0,181-0,158)	0,025 (-0,107-0,133)	0,204 (-0,034-0,399)*	0,093 (-0,066-0,290)
IL-5	-0,051 (-1,222-0,403)	0,243 (-0,253-0,500)*	0,496 (0,266-0,727)*	0,427 (0,255-0,593)*
IL-13	0,535 (0,332-0,728)	0,563 (0,329-0,733)	0,614 (0,392-0,813)	0,543 (0,403-0,780)
IL-15	0,093 (-1,260-0,614)	0,350 (-1,260-0,700)	0,532 (-1,260-0,943)	0,188 (-1,260-0,496)
IL-17	1,069 (0,937-1,266)	1,093 (0,975-1,279)	1,201 (1,034-1,698)*	1,129 (0,977-1,406)
IL-2rα	1,272 (1,115-1,420)	1,241 (1,081-1,420) #	1,476 (1,319-1,957)*	1,562 (1,246-1,673)*
IL-10	1,606 (1,434-1,715)	1,626 (1,513-1,815)	1,694 (1,518-1,873)*	1,640 (1,608-1,773)
IL-1RA	4,107 (3,911-4,211)	4,115 (3,985-4,248) #	4,108 (4,000-4,291) #	3,943 (3,749-4,095)

*Mann-Whitney U tests were used to compare medians between groups. P-values<0.05 were considered significant. * denote significant differences between HPV-STI- and HPV only, or STI only or HPV+STI+; while # represents a statistical difference in medians between HPV+STI+ and HPV only, or STI only. * or # in red print indicate p values significant after multiple comparisons adjustment by Dunn's post-testing.* ***Cytokines are ordered according to functionality: pro-inflammatory, chemokines, growth factors, adaptive response and anti-inflammatory cytokines.***

6.6.2. Association between HPV, STIs, and epithelial barrier integrity

Women with STI only had significantly higher levels of MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-12 and MMP-13 compared to uninfected women [**Table 9**; **Appendix E**: **Table 11**]. Coinfection was associated with increases in similar MMPs [MMP-2, MMP-3 and MMP-12] as the HPV-STI+ group. Additionally, women with HPV only had significantly reduced concentrations of MMP-2, MMP-12 and MMP-13 compared to HPV+STI+ women, suggesting that STI alone contributed to increases of these enzymes observed in co-infected women.

Table 9. *MMP concentrations among HPV-STI-, HPV+STI-, HPV-STI+ and HPV+STI+ women.*

Protein	HPV-STI- *(N = 67)*	HPV ONLY *(N = 66)*	STI ONLY *(N = 14)*	HPV+STI+ *(N = 15)*
	Median (IQR) log_{10} pg/ml	**Median (IQR) log_{10} pg/ml**	**Median (IQR) log_{10} pg/ml**	**Median (IQR) log_{10} pg/ml**
MMP-1	2,690 (3,150-3,440)	2,435 (3,150-3,420)	3,163 (3,550-3,848)*	3,160 (3,350-3,480)
MMP-2	2,700 (3,340-3,770)	2,838 (3,525-3,780) #	3,415 (4,050-4,355)*	3,400 (3,730-4,160)*
MMP-3	2,630 (2,810-3,170)	2,510 (2,930-3,273)	2,813 (3,265-3,603)*	2,810 (3,170-3,240)*
MMP-7	1,810 (2,420-3,110)	2,003 (2,515-3,150)	2,098 (2,760-3,330)	2,210 (2,460-3,610)
MMP-8	4,190 (5,000-5,920)	4,108 (4,950-6,223)	4,905 (6,560-7,623)*	4,930 (5,240-6,050)
MMP-9	3,890 (4,500-5,410)	3,680 (4,635-5,443)	4,340 (5,685-6,335)*	4,510 (5,000-5,440)
MMP-10	2,290 (2,890-3,180)	2,270 (2,960-3,313)	2,793 (3,135-3,538)	2,700 (2,900-3,010)
MMP-12	2,440 (2,830-3,230)	2,268 (2,855-3,408) #	2,895 (3,330-4,013)*	2,840 (3,240-3,700)*
MMP-13	1,710 (2,140-2,630)	1,708 (2,185-2,605) #	2,278 (2,595-2,965)*	2,270 (2,490-2,690)

*Mann-Whitney U tests were used to compare medians between groups. P-values<0.05 were considered significant. * denote significant differences between HPV-STI- and HPV only, or STI only or HPV+STI+; while # represents a statistical difference in medians between HPV+STI+ and HPV only, or STI only. * or # in red print indicate p values significant after multiple comparisons adjustment by Dunn's post-testing.*

6.6.3. Association between HPV, STIs, and immune cell recruitment

The inflammatory nature of STIs extended to the investigation of immune cell subsets in the context of HPV [**Table 10**; **Appendix E**: **Table 12**]. Women with STI only had increased frequencies of activated ($CD3^+CD38^+$), highly activated ($CD3^+CD38^+DR^+$), total activated and activated $CCR5^+CD3^+$ T cell targets ($CD3^+CCR5^+CD38^+$, $CD3^+CD38^+DR^+CCR5^+$) compared to uninfected women. These T cell associations were attributable to the CD8+ population since activated ($CD8^+CD38^+$), highly activated ($CD8^+CD38^+DR^+$) and activated $CCR5^+CD8^+$ T cell targets ($CD8^+CCR5^+CD38^+$, $CD8^+CD38^+DR^+CCR5^+$) were significantly higher among HPV-STI+ women relative to uninfected women [**Table 10**; **Appendix E**: **Table 12**]. However, these associations did not withstand multiple comparisons. These same cell subsets were increased among women with a coinfection; in addition, co-infected women had increased frequencies of activated ($CD3^+DR^+$) CD3 cells, activated $CCR5^+CD4^+$ T cell targets ($CD4^+CCR5^+CD38^+$)

and activated $CD8^+$ cells ($CD8^+DR^+$) compared to uninfected women. Furthermore, women with HPV only had reduced frequencies of multiple cellular subsets [($CD3^+DR^+$, CD3 total activation, $CD4^+CD38^+$, $CD4^+CCR5^+CD38^+$, CD4 total activation, $CD8^+CD38^+$, $CD8^+DR^+$, $CD8^+CD38^+DR^+$ and CD8 total activation), **Table 10**; **Appendix E**: **Table 12**] relative to co-infected women, suggesting that *cellular associations observed in HPV+STI+ women were driven by discharge-associated STIs.*

Table 10. Cellular frequencies among HPV-STI-, HPV+STI-, HPV-STI+ and HPV+STI+ women.

	HPV-STI- ***(N = 66)***	**HPV ONLY** ***(N = 69)***	**STI ONLY** ***(N = 14)***	**HPV+STI+** ***(N = 13)***
Cell Type	**Median % (IQR)**	**Median % (IQR)**	**Median % (IQR)**	**Median % (IQR)**
LYMPHOCYTES	1,083 (2,170-5,350)	0,982 (2,290-5,715)	0,631 (3,520-7,860)	1,050 (2,940-4,365)
CD3	1,178 (10,350-27,730)	2,755 (13,400-23,450)	3,678 (10,190-38,700)	1,290 (11,400-24,350)
$CD3CD38DR^+CCR5^+$	0,829 (4,770-13,530)	1,550 (5,090-13,730)	6,935 (10,300-26,850)*	3,625 (6,450-30,500)
$CD3CCR5^+CD38^+$	4,650 (16,400-25,600)	6,170 (17,900-27,800)	13,250 (28,800-42,600)*	15,600 (21,900-40,950)
$CD3CCR5^+DR^+$	3,403 (8,990-19,980)	3,883 (11,000-28,500)	8,050 (14,600-51,600)	6,885 (25,200-50,200)*
$CD3CCR5^+KI67^+$	0,000 (3,800-13,850)	0,000 (8,765-24,900)	0,074 (17,700-57,350)	0,000 (16,600-28,050)
$CD3CD38^+DR^+$	3,135 (10,200-20,450)	4,680 (11,050-24,550)	13,250 (18,900-28,850)*	6,150 (16,900-47,000)
$CD3CCR5^+$	21,100 (38,700-59,950)	22,250 (44,600-57,850)	34,200 (57,450-73,050)	30,950 (46,700-84,000)
$CD3CD38^+$	21,180 (37,600-52,380)	16,900 (42,400-53,650)	32,700 (54,700-73,550)*	26,350 (61,700-75,600)
CD3HLA-DR	10,550 (23,900-40,900)	13,230 (24,950-49,530) #	21,150 (27,800-61,050)	24,000 (34,900-66,500)*
CD3KI67	0,513 (13,500-31,180)	0,049 (21,400-40,750)	0,072 (21,600-63,280)	3,342 (29,000-38,250)
CD3 -	23,650 (41,500-59,780)	21,850 (35,500-58,850)	24,880 (29,750-67,780)	22,550 (33,200-59,800)
NK Cells [2]	1,580 (5,520-12,350)	1,830 (4,080-17,500)	0,403 (2,730-20,650)	1,283 (3,745-12,530)
CD3 Activation [1]	53,250 (68,200-91,030)	55,150 (74,600-86,850) #	75,250 (86,050-93,630)*	76,500 (90,100-98,850)*
CD4	49,030 (62,250-72,880)	48,350 (58,000-67,050)	53,780 (64,450-70,300)	29,050 (45,900-63,850)
$CD4CD38DR^+CCR5^+$	0,084 (3,715-10,980)	0,608 (4,125-12,180)	4,065 (7,610-18,900)	1,260 (5,910-15,200)
$CD4CCR5^+CD38^+$	3,370 (16,700-30,700)	4,725 (18,600-31,500) #	9,128 (26,950-38,280)	18,300 (27,300-56,000)*
$CD4CCR5^+DR^+$	2,270 (8,740-19,380)	2,090 (8,940-28,300)	5,310 (12,600-37,850)	1,260 (9,460-28,450)
$CD4CCR5^+KI67^+$	0,000 (3,770-19,250)	0,000 (3,400-27,780)	0,000 (0,275-37,950)	0,000 (18,200-26,550)
$CD4CD38_+DR^+$	1,230 (8,160-17,450)	1,890 (8,770-18,230)	7,685 (12,300-21,750)	2,180 (6,920-21,850)
CD4CCR5	24,800 (44,700-69,200)	19,750 (43,300-62,800)	24,380 (56,550-75,830)	30,700 (54,500-95,450)
CD4CD38	21,280 (39,650-58,100)	17,700 (42,900-54,600) #	26,080 (56,150-74,880)	30,800 (63,600-75,400)
CD4HLA-DR	7,140 (16,800-32,750)	5,500 (19,100-41,550)	9,795 (20,300-43,950)	3,050 (10,800-35,950)
CD4KI67	0,000 (12,050-33,430)	0,000 (8,860-36,850)	0,000 (0,240-44,880)	0,000 (25,000-36,050)
CD4 Activation [1]	50,850 (72,750-91,580)	53,000 (72,700-84,400) #	62,350 (83,050-91,980)	74,650 (84,300-98,950)
CD8	24,080 (35,650-49,100)	24,400 (36,600-45,700)	29,600 (32,350-44,430)	20,700 (35,100-54,000)
$CD8CD38DR^+CCR5^+$	0,000 (3,615-9,955)	0,000 (6,280-13,700)	4,735 (11,600-17,100)*	3,165 (8,320-30,550)
$CD8CCR5^+CD38^+$	0,597 (9,300-18,550)	3,585 (12,900-22,850)	9,155 (18,250-46,430)*	6,360 (18,100-36,050)
$CD8CCR5^+DR^+$	0,137 (8,110-19,130)	1,958 (12,150-26,500)	8,830 (14,300-27,950)	4,765 (23,600-51,350)
$CD8CCR5^+KI67^+$	0,000 (2,380-13,900)	0,000 (5,265-26,180)	0,000 (0,171-47,150)	0,000 (9,520-24,900)
$CD8CD38^+DR^+$	0,163 (10,400-21,600)	1,830 (12,700-30,980) #	15,050 (18,800-34,800)*	5,865 (34,900-68,700)*
CD8CCR5	14,250 (28,500-51,250)	17,450 (31,900-50,000)	24,830 (42,500-64,200)	17,150 (32,600-70,750)
CD8CD38	8,730 (33,200-50,200)	13,150 (38,600-54,100) #	32,050 (50,100-75,680)*	23,700 (67,400-81,800)*
CD8HLA-DR	6,210 (29,900-54,050)	10,550 (36,650-57,150) #	29,350 (40,000-68,150)	28,750 (54,300-85,850)*
CD8KI67	0,000 (13,500-35,100)	0,000 (11,700-41,050)	0,000 (20,000-97,680)	0,000 (26,100-39,700)
CD8 Activation [1]	45,230 (67,950-90,580)	50,500 (70,800-88,150) #	73,580 (85,150-94,200)*	71,900 (88,400-99,750)*

*Mann-Whitney U tests were used to compare medians between groups. P-values<0.05 were considered significant. * denote significant differences between HPV-STI- and HPV only, or STI only or HPV+STI+; while # represents a statistical difference in medians between HPV+STI+ and HPV only, or STI only. * or # in red print indicate p values significant after multiple comparisons adjustment by Dunn's post-testing. [1]Activation refers to cells expressing CCR5, HLA-DR and/or CD38. [2]NK cells refers to those expressing $CD56^+CD16^+$ markers.*

7. DISCUSSION

The association between STIs, BV and their contribution to genital inflammation (Levine *et al.*, 1998; Fichorova *et al.*, 2001a; Reddy *et al.*, 2004; Yudin *et al.*, 2003) and increased HIV risk has been well established (Laga *et al.*, 1993; van de Wijgert *et al.*, 2009; Van Der Pol *et al.*, 2008; Mlisana *et al.*, 2012; Masson *et al.*, 2014; Taha *et al.*, 1998). However, even though HPV is one of the most common STIs globally, and is associated epidemiologically with an increased risk of HIV acquisition (Smith-McCune *et al.*, 2010b; Averbach *et al.*, 2010; Auvert *et al.*, 2011; Konopnicki *et al.*, 2013a; Low *et al.*, 2011), the underlying mechanisms of this remain elusive. Therefore, the hypothesis of this study was that the immune responses associated with HPV (whether in the context of multiple concurrent HPV infections, high or low risk HPV infection, and coinfection with other STI) contribute to a genital immune environment conducive to an increased risk of HIV infection. Specifically, associations between HPV and biological factors known to increase HIV risk, namely genital cytokines and inflammation (Nicol *et al.*, 2005; Masson *et al.*, 2015), genital epithelial microabrasions (Herfs *et al.*, 2011; Arnold *et al.*, 2016), and HIV target cells frequencies (Arnold *et al.*, 2016; Kobayashi *et al.*, 2004) were investigated.

The study population included 167 HIV negative, sexually active women aged 18-40 years attending the CAPRISA eThekwini and Vulindela Clinics or their neighbouring public sector family-planning services in KZN. In this study, more than half the population had detectable HPV DNA at baseline. As expected, HPV positive women were significantly younger than their HPV negative counterparts. Younger, sexually active women generally have the highest prevalence of HPV infection and are at greatest risk of acquiring HPV (Burchell *et al.*, 2006; Dunne *et al.*, 2007; Kahn *et al.*, 2007; Manhart *et al.*, 2006). HPV prevalence has often been described as bimodal, with peaks among younger women and those 55 years or older (Herrero *et al.*, 2000; Castle *et al.*, 2005). Younger women are more likely to have a transformation zone remaining on the ectocervix and are at increased risk of developing premalignant lesions of the cervix due to direct exposure to carcinogenic agents (Autier *et al.*, 1996). With increasing age, the transformation zone shifts upward into the endocervix, becoming less susceptible to carcinogenic agents (Autier *et al.*, 1996) such as HPV. Additionally, younger women have a large area of cervical epithelium undergoing squamous metaplasia; and during this period the cervical cells are more vulnerable to HPV infection and persistence (Trottier *et al.*, 2006; Moscicki *et al.*, 1999; Louie *et al.*, 2009; Moscicki *et al.*, 2006; Castle *et al.*, 2006).

HPV positive women were also more likely to have a partner with a higher mean age, and were less likely to be living with a regular partner than HPV negative women. Young women in African countries are known to engage in sexual relationships with older men for financial gain, sexual gratification and recognition from peers, which generally outweigh the concern of acquiring STIs and HIV infection (Longfield *et al.*, 2004). In fact, young women in age-disparate relationships are reportedly more concerned about the risk of becoming pregnant, rather than acquiring STIs and HIV infection (Silberschmidt *et al.*, 2001; Jones, 2006; Nkosana *et al.*, 2007). This age disparate dynamic was further demonstrated in a recent study which showed that young women in KZN acquire HIV infection through sexual relations with older men, who in turn acquired the virus from women of similar age (de Oliveira *et al.*, 2017). Additionally, women in casual relationships are more likely to acquire incident HPV infections. Sexual intercourse with a new partner or multiple partners has also been associated with increased risk of acquiring HPV infection, progression to pre-cancer lesions and invasive cervical cancer (Zitkute *et al.*, 2016; Burk *et al.*, 1996). Programmatic strategies that emphasize the importance of condom use, decreasing peer pressure to pursue such relationships, and improving women's access to alternative sources of income are key in averting age-disparate relationships among young African women (Longfield *et al.*, 2004).

In this cohort of women, parity was also higher among HPV positive women compared to HPV negative women. High parity is known to increase the risk of squamous-cell carcinoma of the cervix among HPV positive women (Boyd *et al.*, 1964; Muñoz *et al.*, 2002; Jensen *et al.*, 2013; Almonte *et al.*, 2011). Although the exact mechanism by which parity influences the risk of cervical cancer is unclear, factors may include: poor nutrition, poorly managed parturition, hormones, the method of delivery and reduced immunity during pregnancy (Muñoz *et al.*, 2002; Autier *et al.*, 1996), all likely to impact the integrity of the genital epithelium, the portal for HPV entry (Stanley, 2012).

Taken together, the baseline epidemiologic findings observed in this study support established demographic, behavioural and clinical associations with HPV infection. Since none of these factors except STIs and nugent score were associated with genital inflammation status at baseline, only these were controlled for in multivariate analyses investigating the impact of HPV infection on biomarkers of inflammation.

In this study, concordant with reports of the inflammatory nature of STIs (Levine *et al.*, 1998; Fichorova *et al.*, 2001a; Reddy *et al.*, 2004; Anahtar *et al.*, 2015; Arnold *et al.*, 2016; Kaul *et al.*, 2008), having a discharge-associated STI alone was significantly associated with several genital cytokines, and genital inflammation status, compared to women uninfected by HPV or discharge-associated STI (HPV-STI- women). These data suggest that infection with these non-HPV pathogens may result in increased HIV risk, since the definition of inflammation used is associated with HIV seroconversion (Masson *et al.*, 2015). Genital inflammation, however, was not associated with prevalent HPV infection, the number of infecting types, or the oncogenic potential of the HPV. These findings were corroborated by other studies using a similar definition of genital inflammation (Kriek *et al.*, 2016; Shannon *et al.*, 2017). Although HPV infection is not associated with this definition of genital inflammation [which was originally generated in association with other discharge and ulcer-related STI (Masson *et al.*, 2014), HPV infection was indeed associated with a more subtle immune response, and further investigation to further characterise this response is warranted.

Prevalent HPV infection was significantly associated with increases in the chemokine SDF-1α at baseline, even after controlling for inflammatory STI and BV. SDF-1α is involved in cell growth and apoptosis, and is associated with HPV infection (Jaafar *et al.*, 2009) and invasion and metastasis of various cancer types (Feig *et al.*, 2013; Teng *et al.*, 2016; Chen *et al.*, 2015). A recent study by Song *et al*. suggested that the relationship between SDF-1α and cervical cancer may be mediated by SDF-1α-associated activation of the NF-κB pathway via interactions with its ligand, CXCR4 (Song *et al.*, 2017). This pathway participates in the regulation of cell proliferation, apoptosis and angiogenesis in cervical cancer (Song *et al.*, 2017). Elevated levels of this chemokine among HPV positive women may indicate increased risk of progression to cervical cancer if infection persists. Moreover, CXCR4 is a co-receptor for HIV-1, and the binding of SDF-1α to it inhibits viral entry (Maréchal *et al.*, 1999). In addition to SDF-1α inhibiting the entry of CXCR4-tropic HIV, it is known to increase the infectivity of the CCR5-tropic HIV-1 envelope (Maréchal *et al.*, 1999). An understanding of whether the association observed between SDF-1α and HPV infection contributes to protection from CXCR4-tropic HIV, or promotion of CCR5-tropic infectivity in HPV-infected women would require assessments outside the scope of this study e.g. the additional measurement of $CXCR4^{+}$ T cell populations, a longitudinal analysis of HIV outcome, etc. However, the

association between SDF-1α and HPV infection suggests a potential impact on HIV risk, as well as on progression to cervical cancer.

Prevalent HPV infection was not associated with genital inflammation, immune cell activation, or markers of epithelial barrier integrity. However, although not significant, a trend towards a relationship between prevalent HPV infection and reduced frequencies of CD4 cells was observed, implying that a compromised immune response may be associated with an increased risk HPV acquisition. While this investigation took place in an HIV negative population, this observation of reduced CD4 cells in HPV-infected women is broadly in keeping with numerous studies demonstrating that reduced CD4 count is associated with increased prevalence of HPV infection in HIV positive women (Ortiz *et al.*, 2017; Piper *et al.*, 1999; Denny *et al.*, 2012; da Silva *et al.*, 2015). Among HIV positive women, higher CD4 counts were shown to reduce the risk of persistent infection with HR-HPV types (Konopnicki *et al.*, 2013b; Kang *et al.*, 2012). Some studies even report that CD4 counts are a stronger predictor of HPV infections than HIV viral load (Denny *et al.*, 2012; da Silva *et al.*, 2015; Konopnicki *et al.*, 2013b). Although prevalent HPV infection was not associated with the upregulation of pro-inflammatory cytokines nor immune cell recruitment, further investigations were conducted to determine whether other features of HPV infection, also associated with HIV infection, had an impact on genital immunity.

This study examined the contribution of multiple concurrent HPV infections as the epidemiologic link between HPV infection and increased HIV risk. Although several studies have demonstrated that having multiple infections is associated with a significantly increased risk of HIV infection (Konopnicki *et al.*, 2013a; Averbach *et al.*, 2010), there was no association between infection with multiple HPV types and pro-inflammatory cytokines, immune cell recruitment or epithelial barrier disruption in this study. However, multiple concurrent infections were associated with reduced concentrations of the adaptive cytokine IL-5. IL-5, a Th2-affiliated cytokine, is generally associated with non-specific innate immunity to environmental allergens (Marks *et al.*, 2011b), and is known to regulate the expression of genes involved in the proliferation, maturation and effector functions of B cells and eosinophils. It is possible that this limited immune response among women with multiple infections may be a mechanism whereby HPV evades host immune responses. While we and others (Marks *et al.*, 2011b) observed a signature of elevated IL-5 concentrations in HPV infected women relative

to uninfected women, perhaps indicative of an immune response to current infection, it is interesting that having more than one HPV genotype detectable at the genital epithelium corresponds to reduced levels of this cytokine. This may allude to potential immune evasion strategies that are activated by HPV upon infection with multiple types in an effort to remain undetectable by the host immune responses. Having multiple concurrent HPV infection was not associated with an upregulated immune response at the genital tract as originally postulated. Although other studies corroborate these findings that infection with multiple HPV does not affect the concentrations of pro-inflammatory cytokines genital tract (Kriek *et al.*, 2016), it was however, associated with a reduction in the concentrations of the adaptive response cytokine IL-5. It is important to note that there are more than 120 HPV types in existence and only 37 types were assessed in this study. Therefore the number of concurrently infecting HPV types may be under-represented and other HPV types may also contribute to an immune response at the genital mucosa.

Since the number of infecting HPV types was not associated with markers of a traditional inflammatory response, it was further investigated whether the type of infecting HPV was associated with biomarkers of inflammation instead. Having a HR-HPV infection predicted significant increases in the chemokine MCP-1 and the adaptive cytokine IL-13. MCP-1 recruits monocytes, memory T cells and DCs to sites of infection (Carr *et al.*, 1994; Xu *et al.*, 1996) and is included in the definition of genital inflammation used here (Masson *et al.*, 2015). MCP-1 has been linked to chronic inflammatory diseases (Baggiolini *et al.*, 1994; Ransohoff *et al.*, 1996), antitumor immunity (Huang *et al.*, 1994; Rollins *et al.*, 1991), atherosclerosis (Charo *et al.*, 2004) and cervical cancer (Kleine-Lowinski *et al.*, 1999; Kleine-Lowinski *et al.*, 2003; Rösl *et al.*, 1994). Several studies have shown that MCP-1 expression and infiltrating cells of the monocyte/macrophage lineage were only detectable in premalignant precursor cells and absent in high-grade lesions of cervical cancer patients (Kleine-Lowinski *et al.*, 1999; Riethdorf *et al.*, 1996; Spinillo *et al.*, 1993). Furthermore, HIV-infected women were found to have elevated concentrations of this chemokine compared to HIV negative women (Kriek *et al.*, 2016), suggesting a potential link between increased concentrations of MCP-1 among women with HR-HPV infection and increased risk of HIV acquisition.

The role of IL-13 in genital inflammation and HIV acquisition is unclear. Older women with HR-HPV have been shown to have greater concentrations of IL-13, also a Th2-associated cytokine linked to non-specific innate immunity and response to environmental allergens (Marks *et al.*, 2011b). IL-13, which is responsible for the activation and control of eosinophils (Pope *et al.*, 2001; Rothenberg *et al.*, 1999; Zimmermann *et al.*, 2003), were elevated among women with HR-HPV (Marks *et al.*, 2011a). Eosinophils have been investigated as mediators of tumour immunity, and eosinophilia as a potential prognostic marker in oral cancers (Martinelli-Klay *et al.*, 2009). These data may indicate that increased concentrations of IL-13 are associated with a non-specific immune response toward HR-HPV infection, similar to the host immune response elicited toward environmental allergens.

Although not statistically significant, there was a trend toward increased concentrations of the pro-inflammatory cytokines IL-1β and IL-12p70; chemokines eotaxin, IL-8, IP-10 and MIG; the growth factor, VEGF; and the adaptive cytokine IL-2 among women with HR-HPV infection. Both IL-1β and IL-12p70 are pro-inflammatory and elevated concentrations of these cytokines is associated with active STI infection (Masson *et al.*, 2014). In support of these findings, a recent study found that HPV positive participants had upregulated genital concentrations of IP-10 and MIG (Shannon *et al.*, 2017), both of which are IFN-γ-induced, bind CXCR3 (Hsieh *et al.*, 2006), and may indicate immune activation. Genital levels of both chemokines are reduced among women who remained HIV negative despite frequent HIV exposure (Lajoie *et al.*, 2012). However, elevated levels of IL-8 and IP-10 are significantly associated with HIV seroconversion among South African women (Masson *et al.*, 2015) and form part of the definition of inflammation used in this study. Although the exact mechanisms by which IP-10 and MIG enhance HIV susceptibility is unknown, Shannon *et al*. found both chemokines to be moderately correlated with increases in $CCR5^{+}CD4^{+}$ T cells (Shannon *et al.*, 2017). It has been suggested that the innate immune response may be critical for HPV clearance (Daud *et al.*, 2011; Scott *et al.*, 2013). In keeping with this, treatment of HR-HPV infections with Imiquimod, a TLR-7 agonist, induces IFN-α, IL-1, IL-6, IL-8, IL-10 and IL-12, promotes migration of Langerhans cells and enhances antigen presentation, resulting in the clearance of infection (Stanley, 2002; Schon *et al.*, 2004). Although, the cross-sectional design of this study prevents conclusions to be made regarding HPV clearance, there was a trend towards infection with oncogenic HPV and increased concentrations of IL-1, IL-8 and IL-12, all of which are associated with the clearance of HPV infection (Stanley, 2002; Schon *et al.*, 2004). Moreover,

women with an oncogenic HPV infection were more inclined to have increased concentrations of the adaptive cytokine IL-2. Clearance of HPV infection is mediated by a robust $CD8^+$ cytotoxic T lymphocyte response which is characterized by elevated concentrations of IL-2 and IFN-γ (Stanley, 2006). Thus increased levels of IL-2 may be indicative of an attempt to clear HPV infection.

A trend towards increases in eotaxin was also observed among women with HR-HPV. Eotaxin is another Th2-induced chemokine that is primarily responsible for the recruitment and regulation of eosinophils and basophils (Marks *et al.*, 2011b), again suggesting that a non-specific innate immune response may be elicited toward infection with HR-HPV. Even though this study did not assess CIN nor progression to cervical cancer, the trend toward increased concentrations of VEGF among women HR-HPV infection may indicate progression to cervical cancer, since VEGF has been implicated as an early marker of cervical carcinogenesis (Branca *et al.*, 2006).

Although there were no significant associations between oncogenic HPV and markers of epithelial barrier integrity, women with HR-HPV had a trend toward increased concentrations of MMP-3. MMPs play a pivotal role in the degradation of the extracellular matrix and basement membrane and hence, may play a key role in cancer development (Stott-Miller *et al.*, 2011). MMP-3 concentrations are known to be upregulated in tumours and immunohistochemistry has revealed over-expression of MMP-3 in cancers compared to normal cervical epithelium (Rajkumar *et al.*, 2011). Additionally, HPV may facilitate HIV entry and dissemination through disruption of the epithelial barrier (Herfs *et al.*, 2011). However, this study was not designed to address this, and further investigation is warranted.

Additionally, this study demonstrated a significant association between HR-HPV and increased frequencies of lymphocytes. Increased lymphocyte frequencies among women with HR-HPV may indicate immune cell recruitment to the site of infection in an attempt to clear infection. Histological examination of regressing genital warts have shown a large infiltrate into the wart stroma and epithelium of T cells ($CD4^+$ and $CD8^+$) and macrophages (Coleman *et al.*, 1994). The host immune response to HPV is mediated by T-lymphocytes, which are also primary target cells for HIV (Houlihan *et al.*, 2012; Stanley, 2001). Recruitment of HIV target cells ($CD4^+CCR5^+$) to the female genital tract to clear HPV infection may also provide an immune environment conducive to HIV acquisition. Although some of these associations were not

statistically significant, elevated levels of these cytokines, MMPs, and immune cell recruitment among women with HR-HPV may provide a possible mechanistic link between HR-HPV and a contribution toward genital inflammation which is associated with HIV acquisition.

Lastly, investigations were conducted to determine the contribution of HPV infection to genital inflammation in the context of other STI (*C. trachomatis*, *T. vaginalis*, *M. genitalium* and/or *N. gonorrhoeae)*. STIs are a well-established contributor to genital inflammation and increased HIV risk (Levine *et al.*, 1998; Fichorova *et al.*, 2001a; Reddy *et al.*, 2004; Anahtar *et al.*, 2015; Arnold *et al.*, 2016; Kaul *et al.*, 2008). The immune responses that are elicited by the host in order to facilitate pathogen clearance and defend against reinfection also may render individuals more vulnerable to HIV infection (Laga *et al.*, 1993; van de Wijgert *et al.*, 2009; Van Der Pol *et al.*, 2008; Mlisana *et al.*, 2012; Masson *et al.*, 2014; Taha *et al.*, 1998). Infection by sexually transmitted pathogens have been shown to influence the persistence and progression or clearance on concurrent HPV infection (Castle *et al.*, 2003). Multiple STIs were found to be risk factors for cervical cancer, suggesting that non-HPV STIs may act as HPV cofactors (Castle *et al.*, 2003). Among women with HR-HPV infection, antibodies to *C. trachomatis* was associated with an increased risk of cervical cancer (Smith *et al.*, 2002). A study by Silins *et al*. demonstrated that history of previous *C. trachomatis* infection was associated with the persistence of HPV infection (Silins *et al.*, 2005). *T. vaginalis* has been associated with increased risk of squamous intraepithelial lesions, CIN based on biopsy results and HR-HPV infection (Verteramo *et al.*, 2009; Watts *et al.*, 2005; Noel *et al.*, 2010; Yap *et al.*, 1995). *T. vaginalis* infection produces microabrasions in the cervical epithelium, thereby facilitating HPV entry (Thurman *et al.*, 2011). Moreover, *Neisseria gonorrhoea* and *Mycoplasma genitalium* have been linked to persistence of HR-HPV infection (de Abreu *et al.*, 2016; Biernat-Sudolska *et al.*, 2011).

In this study women with HPV only (HPV+STI-) had greater levels of IL-5, IL-6 and G-CSF compared to uninfected women. However, HPV-STI+ women had elevated levels of these and several other cytokines, MMP concentrations and greater immune cell recruitment compared to HPV-STI- women, indicative of the inflammatory nature of the discharge-associated STIs assessed. A study by *Fichorova et al.* showed that human endocervical, ectocervical and vaginal cell lines that were actively invaded by *N. gonorrhoeae* had elevated concentrations of IL-8 and IL-6 (Fichorova *et al.*, 2001a). Cervical secretions of Chlamydia positive women revealed upregulated recruitment of CD8 lymphocytes to the genital tract and increased

concentrations of IFN-γ, TNF-α, IL-10 and IL-12 (Reddy *et al.*, 2004), all of which have been broadly substantiated in this study. Moreover, persistent infection with *M. genitalium* has been associated with elevated concentrations of IL-8, MCP-1 and MIP-1β (McGowin *et al.*, 2012). Additionally, discharge-related STIs were significantly associated with the definition of inflammation validating the findings of Masson *et al*. which additionally demonstrated that chlamydia and gonorrhoea were highly inflammatory relative to the other STIs measured (Masson *et al.*, 2014). Data from these studies are broadly in keeping with the findings here, which demonstrated distinct pro-inflammatory host responses associated with these discharged-related STIs. These immune response associated with STIs in this study, may be associated with increased risk of HIV infection.

On the contrary, women with HPV only also had reduced levels of multiple cytokines, MMPs and several immune cell subsets, (most notably activated $CCR5^{+}CD4$ HIV target cells), relative to HPV+STI+ women. These data indicate that the inflammatory response associated with coinfection was driven by infection with the panel of discharge-associated agents assessed. Furthermore, women with an HPV/STI coinfection had lower concentrations of the pro-inflammatory cytokine IL-6 and anti-inflammatory cytokine IL-1RA compared to women with STI only, suggesting that the effect of concomitant HPV infection is a reduction of cytokine production. Anti-inflammatory cytokines function in the downregulation of pro-inflammatory cytokines in order to maintain homeostasis (Gérard *et al.*, 1993; Howard *et al.*, 1993), however, an excessive anti-inflammatory response may lead to immune suppression (Davidoff, 1996; Fisher *et al.*, 1996). A reduction of these cytokines in HPV+STI+ women may be attributed to the ability of HPV to evade host immune responses. Evasion of the host immunity has been considered vital for HPV to persist and cause HPV-related cancers (Senba *et al.*, 2012).

This study had several limitations, most notably, the cross-sectional design prevented the classification of HPV infections as persistent or transient. It is likely that different immune signatures may have been associated with the persistence or clearance of HPV infection, or even impending acquisition. Additionally, upon categorization of women according to multiple genotypes and oncogenicity, the sample size was considerably reduced and perhaps a larger cohort would demonstrate significant associations rather than several trends as seen in women with HR-HPV. The use of CVL specimens for the measurement of cytokine concentrations may be limiting since CVLs are diluted and may prevent measurement of cytokines present at

lower levels. Perhaps the use of Softcup specimens may be a more suitable method of measuring cytokine concentrations in genital specimens since these samples are concentrated. Several other factors that may have contributed to genital inflammation were not assessed in this study, including: age, oral contraceptive use, menstrual cycles, microbiome composition, HPV viral load, and the use of vaginal insertive products. It is important to note that tight junction proteins and other adhesion molecules may have been better measures of epithelial barrier integrity as a reduction in these would provide HPV with direct access to basal epithelial cells. Finally, disease outcomes such as progression to cervical cancer or HIV seroconversion were not measured in this study, therefore conclusive statements regarding HPV, STIs and disease cannot be made.

It was originally hypothesized that the immune responses associated with prevalent HPV, with having multiple concurrent HPV infections, with oncogenic HPV and an HPV/STI coinfection were associated with a genital immune response conducive to HIV acquisition. When investigating the compounding effect of co-infecting HPV and inflammatory STI on biomarkers of inflammation, several cytokines, MMPs and immune cell subsets were elevated among women with STI only (HPV-STI+) compared to uninfected women (HPV-STI-); suggesting, as expected, that the panel of discharge-associated STIs assessed in this study, are highly inflammatory and significantly associated with genital inflammation. Interestingly, in this analysis, HPV infection in the absence of other STI was associated with reductions in selected cytokine and MMP concentrations, and with reduced frequencies of immune cells relative to women with coinfection, indicative of a modulatory effect on the STI-associated immune response. In multivariate models controlling for STI and BV, HPV infection (not taking into account the number or type of infecting HPV) predicted increased concentrations of the chemokine SDF-1α, but was not associated with markers of epithelial barrier integrity, or immune cell activation. Further investigations were conducted to determine whether multiple HPV infections, oncogenicity and coinfection with other STI had an impact on genital immunity. Having multiple HPV infections was associated with reduced concentrations of IL-5, but was not, however, significantly associated with MMP concentrations nor with T cell activation. Further, infection with HR-HPV was associated with increased concentrations of MCP-1 and IL-13, and was also significantly associated with greater frequencies of lymphocytes at the genital tract, but not with markers of epithelial barrier integrity. This study demonstrates a distinct immune response associated with HPV infection, and more specifically

oncogenic HPV strains, which may contribute to an environment conducive to HIV infection by virtue of its relationship with cytokines and the recruitment of immune cells. HPV infection is not associated with the traditional definition of genital inflammation and may be associated more with a Th-2 immune response. This study is one of few that investigated immune responses associated with the risk of acquiring HIV infection among HPV positive women in KZN, and one of the first to investigate the contribution of an HPV/STI coinfection on biomarkers of genital inflammation. The HPV prevalence among this population of women may have important public health implication related to the role-out of HPV vaccinations. Furthermore, these findings among women with HR-HPV infection, if validated, has potential to be used in future cancer control strategies.

8. CONCLUSION

In conclusion, this study demonstrated that while discharge-related STIs are highly inflammatory, a more subtle immune profile was associated with HPV infection, whether in terms of viral oncogenicity, having multiple HPV infections, and even on HPV/STI coinfection. In this study, HPV was unlike discharge-associated STIs in terms of the cytokine profile, the impact on epithelial barrier integrity, and immune activation, and implied a more modest associated immune response that did not overtly relate to an increased potential for HIV risk. However, the observed association between HR-HPV and the genital cytokine and immune cell profiles suggests the need for longitudinal investigation to conclusively assess whether genital inflammation associated with oncogenic persistent HPV infection does indeed translate to increased risk of HIV acquisition.

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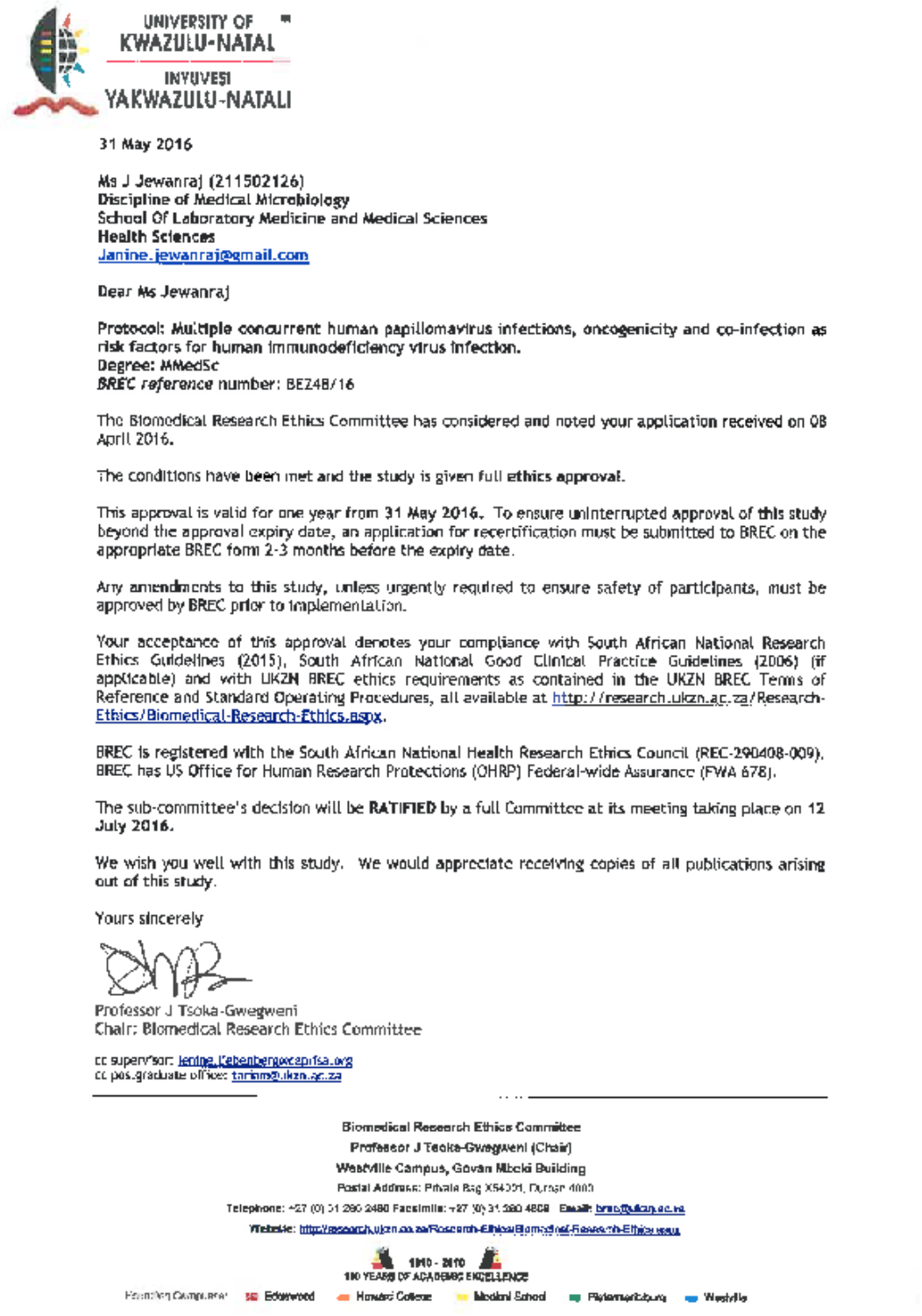
10. APPENDIX

APPENDIX A



UNIVERSITY OF KWAZULU-NATAL
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31 May 2016

Ms J Jewanraj (211502126)
Discipline of Medical Microbiology
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Health Sciences
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Dear Ms Jewanraj

Protocol: Multiple concurrent human papillomavirus infections, oncogenicity and co-infection as risk factors for human immunodeficiency virus infection.
Degree: MMedSc
BREC reference number: BE248/16

The Biomedical Research Ethics Committee has considered and noted your application received on 08 April 2016.

The conditions have been met and the study is given full ethics approval.

This approval is valid for one year from 31 May 2016. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

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

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

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

The sub-committee's decision will be **RATIFIED** by a full Committee at its meeting taking place on 12 July 2016.

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor J Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

cc supervisor: [illegible]
cc pos.graduate office: [illegible]

Biomedical Research Ethics Committee
Professor J Tsoka-Gwegweni (Chair)
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Figure 1. *BREC Approval Letter.*

UNIVERSITY OF
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INYUVESI
YAKWAZULU-NATALI

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Website: http://research.ukzn.ac.za/ResearchEthics/BiomedicalResearchEthics.aspx

28 March 2017

Ms J Jewanraj (211502126)
Discipline of Medical Microbiology
School Of Laboratory Medicine and Medical Sciences
Health Sciences
Janine.jewanraj@gmail.com

Dear Ms Jewanraj

Protocol: Multiple concurrent human papillomavirus infections, oncogenicity and co-infection as risk factors for human immunodeficiency virus infection, Degree: MMedSc.
***BREC reference* number: BE248/16**

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	31 May 2017
Expiration of Ethical Approval:	30 May 2018

I wish to advise you that your application for Recertification received on 20 March 2017 for the above protocol has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

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

This approval will be ratified by a full Committee at its meeting taking place on 11 April 2017.

Yours sincerely

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

Figure 2. *BREC Recertification Approval Letter.*

APPENDIX B

Association between prevalent HPV and soluble protein biomarkers of inflammation

Table 1. Cytokine concentrations among HPV- (n = 81) and HPV+ (n = 82) women.

	Unadjusted			Adjusted		
Cytokine	**β-Coefficient**	**95% CI**	**P-Value**	**β-Coefficient**	**95% CI**	**P-Value**
IL-1α	0,080	(-0,085-0,244)	0,341	0,064	(-0,091-0,219)	0,419
IL-1β	-0,033	(-0,276-0,209)	0,787	-0,060	(-0,287-0,166)	0,599
IL-6	0,055	(-0,113-0,223)	0,520	0,049	(-0,118-0,216)	0,563
IL-12P40	0,062	(-0,131-0,254)	0,527	0,063	(-0,128-0,254)	0,518
IL-12P70	0,075	(-0,041-0,190)	0,204	0,074	(-0,042-0,189)	0,208
IL-18	0,031	(-0,153-0,214)	0,742	0,026	(-0,156-0,209)	0,775
MIF	0,037	(-0,162-0,236)	0,714	0,033	(-0,167-0,233)	0,745
TNF-α	0,013	(-0,121-0,146)	0,852	0,002	(-0,127-0,131)	0,974
TNF-β	0,022	(-0,084-0,128)	0,679	0,025	(-0,078-0,129)	0,631
TRAIL	0,076	(-0,110-0,262)	0,420	0,073	(-0,106-0,252)	0,422
CTACK	0,034	(-0,097-0,166)	0,606	0,032	(-0,096-0,160)	0,623
EOTAXIN	0,171	(-0,084-0,427)	0,187	0,157	(-0,096-0,410)	0,223
GRO-α	0,135	(-0,142-0,412)	0,337	0,156	(-0,114-0,425)	0,256
IL-8	0,021	(-0,178-0,220)	0,837	0,017	(-0,181-0,215)	0,865
IL-16	0,082	(-0,089-0,252)	0,346	0,077	(-0,093-0,247)	0,371
IP-10	0,136	(-0,145-0,416)	0,341	0,171	(-0,082-0,424)	0,183
MCP-1	0,000	(-0,086-0,085)	0,992	0,002	(-0,084-0,087)	0,972
MCP-3	0,021	(-0,153-0,196)	0,812	0,022	(-0,151-0,195)	0,805
MIG	0,139	(-0,099-0,377)	0,251	0,153	(-0,069-0,376)	0,176
MIP-1α	0,081	(-0,104-0,266)	0,391	0,069	(-0,112-0,249)	0,453
MIP-1β	0,087	(-0,091-0,265)	0,338	0,086	(-0,090-0,262)	0,336
RANTES	0,187	(-0,030-0,403)	**0,090**	0,174	(-0,037-0,386)	0,105
IFN-α2	0,041	(-0,042-0,123)	0,331	0,040	(-0,040-0,120)	0,325
SDF-1α	0,142	(0,001-0,284)	**0,049***	0,148	(0,010-0,287)	**0,036***
β-NGF	0,124	(-0,154-0,402)	0,380	0,125	(-0,147-0,397)	0,366
FGF BASIC	-0,002	(-0,046-0,043)	0,944	-0,005	(-0,048-0,038)	0,813
G-CSF	0,143	(-0,071-0,357)	0,188	0,145	(-0,068-0,359)	0,180
GM-CSF	-0,007	(-0,034-0,021)	0,632	-0,006	(-0,033-0,021)	0,670
HGF	0,050	(-0,131-0,231)	0,587	0,037	(-0,137-0,212)	0,672
IL-3	0,051	(-0,057-0,160)	0,351	0,051	(-0,056-0,158)	0,347
IL-7	0,099	(-0,020-0,218)	0,103	0,091	(-0,027-0,208)	0,128
IL-9	0,045	(-0,038-0,128)	0,282	0,040	(-0,041-0,120)	0,332
LIF	0,037	(-0,070-0,144)	0,500	0,029	(-0,074-0,133)	0,577
M-CSF	0,067	(-0,047-0,182)	0,245	0,058	(-0,053-0,170)	0,300
PDGF-BB	0,087	(-0,017-0,190)	0,101	0,077	(-0,023-0,177)	0,130
SCF	0,148	(-0,039-0,335)	0,120	0,155	(-0,024-0,333)	**0,089**
SCGF-β	0,169	(-0,040-0,378)	0,112	0,169	(-0,038-0,375)	0,109
VEGF	0,081	(-0,068-0,229)	0,284	0,078	(-0,069-0,225)	0,298
IFN-γ	0,019	(-0,086-0,124)	0,725	0,009	(-0,092-0,111)	0,855
IL-2	-0,048	(-0,291-0,196)	0,700	-0,055	(-0,298-0,188)	0,656
IL-4	0,036	(-0,046-0,118)	0,383	0,029	(-0,050-0,108)	0,473
IL-5	0,193	(-0,032-0,419)	**0,092**	0,169	(-0,045-0,384)	0,122
IL-13	0,033	(-0,056-0,121)	0,466	0,027	(-0,060-0,115)	0,536
IL-15	0,145	(-0,142-0,433)	0,320	0,136	(-0,153-0,425)	0,354
IL-17	0,045	(-0,056-0,145)	0,381	0,041	(-0,057-0,139)	0,411
IL-2rα	-0,025	(-0,124-0,074)	0,623	-0,029	(-0,124-0,067)	0,553
IL-1rα	0,028	(-0,061-0,116)	0,537	0,032	(-0,056-0,120)	0,472
IL-10	0,044	(-0,022-0,110)	0,193	0,044	(-0,022-0,110)	0,192

P-values were determined using linear regression models. P<0.05 indicated by (*) and trending p-values in ***bold****. Cytokine/Chemokine functions: Pro-inflammatory, Chemokines, Growth Factors, Adaptive response, Anti-inflammatory.*

Table 2. MMP concentrations among HPV- (n = 81) and HPV+ (n = 82) women.

	Unadjusted			Adjusted		
Protein	**β-Coefficient**	**95% CI**	**P-Value**	**β-Coefficient**	**95% CI**	**P-Value**
MMP-1	-0,099	(-0,377-0,179)	0,483	-0,110	(-0,387-0,167)	0,434
MMP-2	0,017	(-0,321-0,354)	0,922	-0,002	(-0,331-0,328)	0,434
MMP-3	0,029	(-0,168-0,226)	0,769	0,018	(-0,175-0,210)	0,857
MMP-7	0,161	(-0,116-0,438)	0,253	0,172	(-0,104-0,448)	0,221
MMP-8	0,019	(-0,771-0,808)	0,962	0,023	(-0,767-0,813)	0,955
MMP-9	-0,341	(-0,764-0,082)	0,114	-0,357	(-0,772-0,059)	**0,092**
MMP-10	0,018	(-0,265-0,300)	0,901	0,004	(-0,277-0,285)	0,977
MMP-12	-0,057	(-0,325-0,210)	0,674	-0,067	(-0,329-0,195)	0,615
MMP-13	-0,011	(-0,289-0,266)	0,937	-0,028	(-0,299-0,243)	0,838

*P-values were determined using linear regression models. Trending values indicated in **bold**.*

Table 3. Cellular frequency among HPV- (n = 80) and HPV+ (n = 83) women.

	Unadjusted			Adjusted		
Cell Type	**β-Coefficient**	**95% CI**	**P-Value**	**β-Coefficient**	**95% CI**	**P-Value**
LYMPHOCYTES	-0,559	(-2,140-1,021)	0,486	-0,479	(-2,067-1,108)	0,552
CD3	-0,819	(-5,771-4,132)	0,744	-0,692	(-5,701-4,318)	0,785
CD3CD38DR$^+$CCR5$^+$	1,999	(-2,286-6,283)	0,358	2,235	(-2,028-6,498)	0,302
CD3CCR5$^+$CD38$^+$	0,979	(-5,206-7,165)	0,755	1,367	(-4,790-7,525)	0,662
CD3CCR5$^+$DR$^+$	3,249	(-3,587-10,085)	0,349	3,688	(-3,061-10,437)	0,282
CD3CCR5$^+$KI67$^+$	3,393	(-3,604-10,390)	0,340	3,608	(-3,417-10,632)	0,312
CD3CD38$^+$DR$^+$	2,066	(-3,277-7,408)	0,446	2,426	(-2,853-7,706)	0,365
CD3CCR5$^+$	-0,283	(-8,567-8,002)	0,946	0,295	(-7,938-8,528)	0,944
CD3CD38$^+$	0,228	(-7,769-8,225)	0,955	0,790	(-7,109-8,689)	0,844
CD3HLA-DR	2,860	(-4,920-10,639)	0,469	3,516	(-4,144-11,175)	0,366
CD3KI67	2,939	(-5,332-11,210)	0,484	3,191	(-5,145-11,527)	0,451
CD3 -	-2,286	(-9,404-4,831)	0,527	-2,161	(-9,370-5,048)	0,555
NK Cells [2]	0,067	(-4,472-4,606)	0,977	0,218	(-4,424-4,859)	0,926
CD3 Activation [1]	-0,924	(-8,772-6,924)	0,816	-0,105	(-7,747-7,538)	0,978
CD4	-5,858	(-12,251-0,535)	**0,072**	-6,315	(-12,728-0,098)	**0,054**
CD4CD38DR$^+$CCR5$^+$	0,273	(-3,324-3,870)	0,881	0,463	(-3,140-4,065)	0,800
CD4CCR5$^+$CD38$^+$	0,955	(-5,552-7,462)	0,772	1,463	(-4,982-7,908)	0,655
CD4CCR5$^+$DR$^+$	-0,389	(-6,881-6,103)	0,906	-0,206	(-6,760-6,348)	0,951
CD4CCR5$^+$KI67$^+$	1,383	(-5,638-8,405)	0,698	1,556	(-5,540-8,652)	0,666
CD4CD38$^+$DR$^+$	-0,520	(-4,778-3,738)	0,810	-0,246	(-4,515-4,024)	0,910
CD4CCR5	-1,393	(-10,376-7,590)	0,760	-0,871	(-9,878-8,135)	0,849
CD4CD38	-1,495	(-9,675-6,684)	0,719	-0,923	(-9,039-7,193)	0,823
CD4HLA-DR	-0,837	(-7,923-6,249)	0,816	-0,547	(-7,711-6,618)	0,880
CD4KI67	0,514	(-7,978-9,007)	0,905	0,601	(-8,006-9,209)	0,890
CD4 Activation [1]	-3,120	(-11,541-5,301)	0,465	-2,539	(-10,966-5,887)	0,553
CD8	-1,271	(-6,601-4,059)	0,638	-1,106	(-6,488-4,275)	0,685
CD8CD38DR$^+$CCR5$^+$	3,035	(-1,387-7,457)	0,177	3,194	(-1,157-7,546)	0,149
CD8CCR5$^+$CD38$^+$	2,805	(-2,900-8,511)	0,333	3,145	(-2,506-8,795)	0,273
CD8CCR5$^+$DR$^+$	3,340	(-3,470-10,149)	0,334	3,676	(-3,121-10,473)	0,287
CD8CCR5$^+$KI67$^+$	3,661	(-3,457-10,779)	0,311	3,722	(-3,463-10,907)	0,308
CD8CD38$^+$DR$^+$	4,023	(-2,478-10,524)	0,223	4,493	(-1,794-10,780)	0,160
CD8CCR5	0,857	(-7,291-9,006)	0,836	1,267	(-6,915-9,449)	0,760
CD8CD38	2,951	(-5,593-11,495)	0,496	3,642	(-4,742-12,025)	0,392
CD8HLA-DR	2,519	(-6,778-11,817)	0,593	3,343	(-5,747-12,433)	0,469
CD8KI67	1,089	(-8,781-10,960)	0,828	1,219	(-8,746-11,185)	0,809
CD8 Activation [1]	-0,416	(-9,523-8,691)	0,928	0,532	(-8,382-9,446)	0,906

*P-values were determined using linear regression models. Trending p- values in **bold.** [1]Activation refers to cells expressing CCR5, HLA-DR and/or CD38. [2]NK cells refers to those expressing CD56$^+$CD16$^+$ markers.*

APPENDIX C

Association between multiple concurrent HPV infections and soluble protein biomarkers of inflammation

Table 4. Cytokine concentrations among women 1 (n = 48) vs 2+ (n = 37) HPV genotypes.

	Unadjusted			Adjusted		
Cytokine	**β-Coefficient**	**95% CI**	**P-Value**	**β-Coefficient**	**95% CI**	**P-Value**
IL-1α	-0,178	(-0,371-0,014)	**0,069**	-0,054	(-0,146-0,038)	0,248
IL-1β	-0,078	(-0,405-0,249)	0,635	-0,020	(-0,175-0,135)	0,798
IL-6	0,019	(-0,195-0,233)	0,858	-0,010	(-0,118-0,098)	0,851
IL-12P40	0,039	(-0,190-0,268)	0,737	0,034	(-0,082-0,150)	0,560
IL-12P70	-0,050	(-0,211-0,112)	0,544	-0,006	(-0,089-0,077)	0,881
IL-18	-0,078	(-0,304-0,148)	0,495	0,020	(-0,095-0,135)	0,733
MIF	-0,108	(-0,355-0,139)	0,386	-0,036	(-0,163-0,091)	0,573
TNF-α	-0,089	(-0,250-0,072)	0,277	-0,023	(-0,104-0,057)	0,564
TNF-β	0,004	(-0,141-0,149)	0,958	0,018	(-0,054-0,090)	0,622
TRAIL	0,068	(-0,174-0,309)	0,578	0,044	(-0,077-0,164)	0,473
CTACK	0,001	(-0,183-0,184)	0,994	0,038	(-0,053-0,130)	0,408
EOTAXIN	0,142	(-0,178-0,462)	0,380	0,062	(-0,099-0,223)	0,444
GRO-α	-0,222	(-0,610-0,165)	0,257	-0,072	(-0,267-0,122)	0,462
IL-8	-0,142	(-0,401-0,116)	0,276	-0,050	(-0,182-0,083)	0,458
IL-16	-0,013	(-0,237-0,211)	0,910	0,009	(-0,104-0,123)	0,871
IP-10	-0,029	(-0,416-0,358)	0,881	0,009	(-0,173-0,190)	0,926
MCP-1	-0,035	(-0,163-0,094)	0,593	-0,028	(-0,093-0,037)	0,391
MCP-3	-0,039	(-0,267-0,189)	0,736	-0,039	(-0,153-0,075)	0,501
MIG	-0,014	(-0,321-0,293)	0,928	0,023	(-0,127-0,172)	0,764
MIP-1α	0,050	(-0,176-0,275)	0,662	0,007	(-0,106-0,119)	0,906
MIP-1β	0,035	(-0,225-0,295)	0,788	0,000	(-0,133-0,133)	0,997
RANTES	0,040	(-0,279-0,359)	0,805	-0,023	(-0,184-0,138)	0,776
IFN-α2	-0,055	(-0,151-0,040)	0,253	-0,004	(-0,053-0,044)	0,853
SDF-1α	-0,065	(-0,213-0,084)	0,387	0,001	(-0,075-0,077)	0,976
β-NGF	-0,129	(-0,504-0,246)	0,497	-0,039	(-0,230-0,151)	0,684
FGF BASIC	-0,034	(-0,084-0,015)	0,175	-0,006	(-0,031-0,019)	0,617
G-CSF	-0,159	(-0,430-0,111)	0,245	-0,064	(-0,203-0,074)	0,357
GM-CSF	-0,010	(-0,045-0,025)	0,583	0,001	(-0,017-0,018)	0,936
HGF	-0,082	(-0,307-0,144)	0,473	-0,026	(-0,138-0,085)	0,639
IL-3	-0,035	(-0,145-0,075)	0,531	-0,003	(-0,058-0,053)	0,929
IL-7	-0,083	(-0,217-0,050)	0,217	-0,041	(-0,108-0,027)	0,234
IL-9	-0,081	(-0,192-0,030)	0,152	-0,044	(-0,100-0,012)	0,120
LIF	-0,083	(-0,230-0,063)	0,261	-0,018	(-0,092-0,056)	0,628
M-CSF	-0,162	(-0,321--0,004)	**0,044**	-0,053	(-0,133-0,028)	0,197
PDGF-BB	-0,034	(-0,168-0,100)	0,614	-0,028	(-0,096-0,040)	0,411
SCF	-0,013	(-0,248-0,221)	0,910	0,001	(-0,114-0,115)	0,993
SCGF-β	0,018	(-0,238-0,274)	0,889	0,008	(-0,122-0,139)	0,898
VEGF	-0,072	(-0,276-0,133)	0,489	-0,011	(-0,115-0,094)	0,839
IFN-γ	-0,071	(-0,199-0,057)	0,272	-0,022	(-0,087-0,043)	0,497
IL-2	-0,050	(-0,398-0,298)	0,776	-0,044	(-0,214-0,126)	0,609
IL-4	-0,050	(-0,146-0,046)	0,305	-0,031	(-0,079-0,017)	0,203
IL-5	-0,169	(-0,487-0,150)	0,295	-0,170	(-0,326--0,014)	**0,033***
IL-13	-0,065	(-0,173-0,043)	0,234	-0,020	(-0,076-0,035)	0,466
IL-15	0,153	(-0,250-0,556)	0,452	0,069	(-0,131-0,270)	0,494
IL-17	-0,038	(-0,160-0,084)	0,536	-0,017	(-0,079-0,045)	0,582
IL-2rα	-0,094	(-0,230-0,043)	0,177	-0,027	(-0,094-0,041)	0,438
IL-1rα	0,008	(-0,107-0,122)	0,895	0,002	(-0,054-0,057)	0,955
IL-10	-0,048	(-0,135-0,040)	0,279	-0,012	(-0,057-0,032)	0,583

*P-values were determined using linear regression models. P<0.05 indicated by (*) and trending p-values in **bold**. Cytokine/Chemokine functions: Pro-inflammatory, Chemokines, Growth Factors, Adaptive response, Anti-inflammatory.*

Table 5. MMP concentrations among women 1 (n = 48) vs 2+ (n = 37) HPV genotypes.

	Unadjusted			Adjusted		
Protein	**β-Coefficient**	**95% CI**	**P-Value**	**β-Coefficient**	**95% CI**	**P-Value**
MMP-1	-0,247	(-0,653-0,159)	0,229	-0,225	(-0,634-0,184)	0,277
MMP-2	0,306	(-0,190-0,802)	0,223	0,334	(-0,161-0,828)	0,183
MMP-3	0,117	(-0,151-0,385)	0,388	0,129	(-0,140-0,398)	0,343
MMP-7	0,230	(-0,119-0,578)	0,194	0,208	(-0,125-0,541)	0,217
MMP-8	-0,390	(-1,570-0,790)	0,512	-0,392	(-1,597-0,812)	0,519
MMP-9	-0,190	(-0,635-0,256)	0,400	-0,173	(-0,606-0,261)	0,430
MMP-10	-0,197	(-0,576-0,182)	0,304	-0,188	(-0,572-0,196)	0,333
MMP-12	-0,023	(-0,412-0,365)	0,905	-0,002	(-0,391-0,386)	0,990
MMP-13	-0,081	(-0,468-0,305)	0,676	-0,057	(-0,436-0,321)	0,764

P-values were calculated using linear regression models.

Table 6. Cell frequencies among women 1 (n = 47) vs 2+ (n = 36) HPV genotypes.

	Unadjusted			Adjusted		
Cell Type	**β-Coefficient**	**95% CI**	**P-Value**	**β-Coefficient**	**95% CI**	**P-Value**
LYMPHOCYTES	1,655	(-0,256-3,566)	**0,089**	1,603	(-0,333-3,538)	0,103
CD3	0,462	(-5,967-6,892)	0,887	0,133	(-6,418-6,685)	0,968
CD3CD38DR$^+$CCR5$^+$	-2,079	(-9,212-5,054)	0,563	-2,193	(-9,413-5,027)	0,547
CD3CCR5$^+$CD38$^+$	-3,556	(-12,644-5,532)	0,439	-3,718	(-12,812-5,375)	0,418
CD3CCR5$^+$DR$^+$	0,064	(-10,385-10,514)	0,990	0,021	(-10,349-10,392)	0,997
CD3CCR5$^+$KI67$^+$	0,863	(-9,447-11,173)	0,868	0,652	(-9,677-10,981)	0,900
CD3CD38$^+$DR$^+$	-3,691	(-12,021-4,639)	0,380	-3,784	(-12,086-4,518)	0,367
CD3CCR5$^+$	-2,020	(-14,101-10,061)	0,740	-2,062	(-14,021-9,897)	0,732
CD3CD38$^+$	-9,549	(-20,989-1,891)	0,101	-9,553	(-20,873-1,768)	**0,097**
CD3HLA-DR	1,188	(-10,114-12,490)	0,835	1,031	(-10,133-12,194)	0,855
CD3KI67	-2,980	(-14,762-8,802)	0,616	-3,169	(-14,909-8,570)	0,592
CD3-	3,421	(-6,522-13,364)	0,495	3,141	(-7,076-13,358)	0,542
NK Cells [2]	-0,282	(-6,622-6,058)	0,929	-0,401	(-6,894-6,091)	0,542
CD3 Activation [1]	-5,386	(-17,224-6,451)	0,368	-5,395	(-16,878-6,088)	0,352
CD4	-4,353	(-13,838-5,131)	0,364	-4,325	(-13,502-4,852)	0,351
CD4CD38DR$^+$CCR5$^+$	0,244	(-5,054-5,541)	0,927	0,160	(-5,172-5,491)	0,953
CD4CCR5$^+$CD38$^+$	-2,263	(-11,675-7,150)	0,634	-2,170	(-11,229-6,888)	0,635
CD4CCR5$^+$DR$^+$	2,972	(-5,782-11,726)	0,501	2,735	(-6,149-11,619)	0,542
CD4CCR5$^+$KI67$^+$	4,021	(-5,903-13,944)	0,422	3,813	(-6,175-13,801)	0,449
CD4CD38$^+$DR$^+$	-1,223	(-7,086-4,641)	0,679	-1,425	(-7,318-4,469)	0,632
CD4CCR5	2,288	(-10,836-15,413)	0,730	2,280	(-10,618-15,178)	0,726
CD4CD38	-8,401	(-19,589-2,787)	0,139	-8,244	(-19,198-2,711)	0,138
CD4HLA-DR	2,652	(-7,039-12,342)	0,588	2,161	(-7,678-12,000)	0,663
CD4KI67	0,772	(-11,092-12,635)	0,897	0,477	(-11,521-12,475)	0,937
CD4 Activation [1]	-2,781	(-15,615-10,054)	0,668	-2,860	(-15,598-9,877)	0,656
CD8	1,695	(-6,000-9,390)	0,662	1,708	(-5,985-9,402)	0,660
CD8CD38DR$^+$CCR5$^+$	-2,386	(-9,710-4,937)	0,519	-2,305	(-9,546-4,936)	0,528
CD8CCR5$^+$CD38$^+$	-3,662	(-12,544-5,219)	0,414	-3,736	(-12,686-5,215)	0,409
CD8CCR5$^+$DR$^+$	3,981	(-6,049-14,011)	0,432	3,963	(-6,104-14,030)	0,409
CD8CCR5$^+$KI67$^+$	2,354	(-8,159-12,867)	0,657	2,307	(-8,346-12,960)	0,668
CD8CD38$^+$DR$^+$	-3,237	(-13,534-7,059)	0,533	-2,995	(-12,814-6,824)	0,545
CD8CCR5	1,811	(-9,939-13,562)	0,760	1,748	(-10,175-13,671)	0,771
CD8CD38	-7,023	(-19,305-5,259)	0,259	-6,916	(-19,013-5,182)	0,259
CD8HLA-DR	6,162	(-7,186-19,511)	0,361	6,168	(-6,954-19,289)	0,352
CD8KI67	-0,479	(-14,383-13,424)	0,945	-0,351	(-14,416-13,713)	0,960
CD8 Activation [1]	1,396	(-12,025-14,816)	0,837	1,373	(-11,963-14,710)	0,838

*P-values were calculated using linear regression models. Trending values indicated in **bold**. [1]Activation refers to cells expressing CCR5, HLA-DR and/or CD38. [2]NK cells refers to those expressing CD56$^+$CD16$^+$ markers.*

APPENDIX D

Association between oncogenic HPV infections and soluble protein biomarkers of inflammation

Table 7. Cytokine concentrations among women with LR (n = 39) and HR (n = 43) HPV.

	Unadjusted			Adjusted		
Cytokine	**β-Coefficient**	**95% CI**	**P-Value**	**β-Coefficient**	**95% CI**	**P-Value**
IL-1α	0,099	(-0,095-0,293)	0,312	0,087	(-0,095-0,268)	0,344
IL-1β	0,280	(-0,039-0,599)	**0,084**	0,268	(-0,031-0,567)	**0,078**
IL-6	0,088	(-0,123-0,300)	0,407	0,098	(-0,114-0,309)	0,359
IL-12P40	0,082	(-0,145-0,308)	0,475	0,074	(-0,154-0,303)	0,518
IL-12P70	0,154	(-0,003-0,311)	**0,054***	0,150	(-0,009-0,309)	**0,064**
IL-18	-0,035	(-0,260-0,191)	0,761	-0,044	(-0,270-0,183)	0,702
MIF	-0,027	(-0,273-0,219)	0,828	-0,028	(-0,278-0,222)	0,821
TNF-α	0,124	(-0,035-0,282)	0,125	0,120	(-0,037-0,277)	0,132
TNF-β	0,094	(-0,048-0,237)	0,192	0,086	(-0,055-0,227)	0,228
TRAIL	0,207	(-0,029-0,443)	**0,085**	0,192	(-0,042-0,425)	0,107
CTACK	0,113	(-0,068-0,293)	0,217	0,103	(-0,077-0,282)	0,259
EOTAXIN	0,285	(-0,028-0,598)	**0,074**	0,284	(-0,027-0,595)	**0,073**
GRO-α	0,142	(-0,245-0,528)	0,468	0,131	(-0,252-0,515)	0,497
IL-8	0,228	(-0,026-0,481)	**0,077**	0,222	(-0,034-0,479)	**0,088**
IL-16	0,171	(-0,049-0,390)	0,125	0,163	(-0,058-0,383)	0,146
IP-10	0,355	(-0,022-0,731)	**0,064**	0,341	(-0,008-0,690)	**0,055**
MCP-1	0,124	(-0,001-0,249)	**0,052***	0,127	(0,002-0,252)	**0,046***
MCP-3	-0,012	(-0,238-0,215)	0,918	-0,024	(-0,249-0,201)	0,834
MIG	0,287	(-0,010-0,585)	**0,058**	0,268	(-0,020-0,556)	**0,067**
MIP-1α	0,107	(-0,116-0,329)	0,344	0,099	(-0,121-0,319)	0,372
MIP-1β	0,034	(-0,225-0,292)	0,796	0,028	(-0,233-0,290)	0,829
RANTES	0,198	(-0,115-0,512)	0,212	0,188	(-0,126-0,502)	0,237
IFN-α2	0,081	(-0,013-0,175)	**0,088**	0,075	(-0,018-0,169)	0,111
SDF-1α	0,037	(-0,111-0,184)	0,624	0,033	(-0,116-0,182)	0,663
β-NGF	0,163	(-0,208-0,535)	0,384	0,149	(-0,225-0,522)	0,430
FGF BASIC	0,027	(-0,023-0,076)	0,284	0,024	(-0,025-0,073)	0,332
G-CSF	0,139	(-0,131-0,408)	0,309	0,146	(-0,126-0,418)	0,289
GM-CSF	0,019	(-0,016-0,054)	0,278	0,021	(-0,013-0,056)	0,219
HGF	0,139	(-0,083-0,361)	0,216	0,130	(-0,087-0,348)	0,237
IL-3	0,027	(-0,082-0,136)	0,626	0,022	(-0,088-0,131)	0,695
IL-7	0,110	(-0,022-0,241)	0,101	0,106	(-0,026-0,238)	0,113
IL-9	0,070	(-0,041-0,181)	0,214	0,065	(-0,046-0,175)	0,248
LIF	0,049	(-0,097-0,195)	0,503	0,043	(-0,102-0,188)	0,556
M-CSF	0,123	(-0,036-0,281)	0,128	0,117	(-0,041-0,275)	0,145
PDGF-BB	0,103	(-0,027-0,234)	0,120	0,101	(-0,030-0,233)	0,130
SCF	0,179	(-0,050-0,408)	0,124	0,163	(-0,059-0,386)	0,149
SCGF-β	0,059	(-0,194-0,313)	0,642	0,052	(-0,204-0,308)	0,689
VEGF	0,182	(-0,018-0,382)	**0,074**	0,175	(-0,027-0,377)	**0,088**
IFN-γ	0,106	(-0,019-0,232)	**0,096**	0,105	(-0,021-0,230)	0,102
IL-2	0,277	(-0,063-0,617)	0,109	0,307	(-0,021-0,634)	**0,066**
IL-4	0,073	(-0,022-0,167)	0,130	0,071	(-0,023-0,165)	0,137
IL-5	0,070	(-0,248-0,388)	0,662	0,066	(-0,250-0,381)	0,679
IL-13	0,119	(0,013-0,224)	**0,028***	0,117	(0,011-0,224)	**0,031***
IL-15	0,188	(-0,211-0,587)	0,352	0,211	(-0,182-0,603)	0,288
IL-17	0,104	(-0,015-0,223)	**0,085**	0,100	(-0,020-0,219)	0,101
IL-2rα	0,103	(-0,032-0,238)	0,134	0,093	(-0,040-0,225)	0,167
IL-10	0,064	(-0,022-0,150)	0,144	0,063	(-0,024-0,150)	0,152
IL-1rα	0,021	(-0,093-0,134)	0,717	0,028	(-0,082-0,137)	0,618

P-values were determined using linear regression models. P<0.05 indicated with (*) and trends in ***bold****. Cytokine/Chemokine functions: Pro-inflammatory, Chemokines, Growth Factors, Adaptive response, Anti-inflammatory.*

Table 8. MMP concentrations among women with LR (n = 39) and HR (n = 43) HPV.

	Unadjusted			Adjusted		
Protein	**β-Coefficient**	**95% CI**	**P-Value**	**β-Coefficient**	**95% CI**	**P-Value**
MMP-1	0,169	(-0,235-0,573)	0,408	0,163	(-0,245-0,571)	0,429
MMP-2	0,371	(-0,118-0,860)	0,135	0,349	(-0,142-0,840)	0,161
MMP-3	0,247	(-0,014-0,508)	**0,063**	0,239	(-0,025-0,503)	**0,075**
MMP-7	-0,002	(-0,351-0,347)	0,991	-0,031	(-0,365-0,303)	0,854
MMP-8	0,285	(-0,885-1,455)	0,630	0,236	(-0,962-1,434)	0,696
MMP-9	0,378	(-0,057-0,814)	**0,088**	0,347	(-0,078-0,772)	0,108
MMP-10	0,103	(-0,275-0,480)	0,590	0,090	(-0,294-0,473)	0,643
MMP-12	0,289	(-0,090-0,669)	0,133	0,280	(-0,100-0,661)	0,147
MMP-13	0,200	(-0,181-0,581)	0,299	0,177	(-0,197-0,552)	0,349

*P-values were calculated using linear regression model. Trending values indicated in **bold**.*

Table 9. Cellular frequencies among women with LR (n = 39) and HR (n = 44) HPV.

	Unadjusted			Adjusted		
Cell Type	**β-Coefficient**	**95% CI**	**P-Value**	**β-Coefficient**	**95% CI**	**P-Value**
LYMPHOCYTES	1,910	(0,024-3,795)	**0,047***	1,987	(0,077-3,898)	**0,042***
CD3	-1,913	(-8,284-4,458)	0,552	-2,214	(-8,725-4,296)	0,500
CD3CD38DR+CCR5+	-0,567	(-7,645-6,512)	0,874	-1,055	(-8,252-6,142)	0,771
CD3CCR5+CD38+	-0,154	(-9,212-8,904)	0,973	-0,886	(-9,986-8,214)	0,847
CD3CCR5+DR+	-2,303	(-12,639-8,034)	0,659	-3,346	(-13,637-6,945)	0,519
CD3CCR5+KI67+	0,680	(-9,532-10,892)	0,895	0,741	(-9,536-11,018)	0,886
CD3CD38+DR+	-1,533	(-9,816-6,750)	0,714	-2,409	(-10,695-5,877)	0,564
CD3CCR5+	0,982	(-11,021-12,984)	0,871	-0,198	(-12,126-11,731)	0,974
CD3CD38+	-4,731	(-16,235-6,773)	0,416	-5,913	(-17,321-5,495)	0,305
CD3HLA-DR	-3,654	(-14,821-7,514)	0,517	-5,074	(-16,124-5,976)	0,363
CD3KI67	0,384	(-11,333-12,101)	0,948	0,523	(-11,199-12,245)	0,929
CD3-	1,964	(-7,914-11,842)	0,693	1,757	(-8,398-11,913)	0,731
NK Cells [2]	4,036	(-2,205-10,276)	0,201	4,096	(-2,336-10,527)	0,207
CD3 Activation [1]	-3,892	(-15,675-7,891)	0,513	-5,519	(-16,961-5,922)	0,340
CD4	-0,306	(-9,772-9,160)	0,949	0,060	(-9,139-9,258)	0,990
CD4CD38DR+CCR5+	0,829	(-4,415-6,073)	0,754	0,535	(-4,769-5,839)	0,841
CD4CCR5+CD38+	-0,005	(-9,365-9,355)	0,999	-1,057	(-10,096-7,982)	0,817
CD4CCR5+DR+	1,558	(-7,130-10,246)	0,722	1,239	(-7,618-10,095)	0,781
CD4CCR5+KI67+	5,725	(-4,061-15,511)	0,248	5,802	(-4,086-15,690)	0,246
CD4CD38+DR+	0,144	(-5,670-5,958)	0,961	-0,104	(-5,976-5,768)	0,972
CD4CCR5	5,641	(-7,341-18,624)	0,390	4,508	(-8,318-17,333)	0,486
CD4CD38	-3,517	(-14,752-7,718)	0,535	-4,684	(-15,708-6,340)	0,400
CD4HLA-DR	0,537	(-9,078-10,152)	0,912	0,347	(-9,455-10,148)	0,944
CD4KI67	7,105	(-4,572-18,781)	0,230	7,251	(-4,596-19,098)	0,227
CD4 Activation [1]	1,815	(-10,938-14,568)	0,778	0,638	(-12,073-13,348)	0,921
CD8	3,336	(-4,279-10,950)	0,386	3,554	(-4,082-11,190)	0,357
CD8CD38DR+CCR5+	2,441	(-4,811-9,693)	0,505	1,524	(-5,691-8,740)	0,675
CD8CCR5+CD38+	2,821	(-6,013-11,655)	0,527	2,035	(-6,914-10,985)	0,652
CD8CCR5+DR+	3,662	(-6,277-13,601)	0,466	2,632	(-7,407-12,671)	0,603
CD8CCR5+KI67+	1,193	(-9,229-11,615)	0,820	1,373	(-9,235-11,981)	0,797
CD8CD38+DR+	-1,936	(-12,150-8,278)	0,707	-3,665	(-13,422-6,093)	0,457
CD8CCR5	6,483	(-5,104-18,070)	0,269	5,905	(-5,911-17,720)	0,323
CD8CD38	-4,179	(-16,437-8,080)	0,500	-5,675	(-17,765-6,414)	0,353
CD8HLA-DR	1,459	(-11,828-14,745)	0,828	-0,360	(-13,490-12,769)	0,957
CD8KI67	2,094	(-11,704-15,892)	0,763	2,617	(-11,390-16,623)	0,711
CD8 Activation [1]	1,631	(-11,694-14,955)	0,808	0,356	(-12,940-13,652)	0,958

P-values were calculated using linear regression models. P<0.05 are indicated by (*). [1]Activation refers to cells expressing CCR5, HLA-DR and/or CD38. [2]NK cells refers to those expressing $CD56^{+}CD16^{+}$ markers.

APPENDIX E

Association between HPV, STI and soluble protein biomarkers of inflammation

Table 10. Cytokine concentrations among HPV-STI-, HPV+STI-, HPV-STI+ and HPV+STI+ women.

Cytokine	HPV-STI- *(N = 67)*	HPV ONLY *(N = 66)*	STI ONLY *(N = 14)*	HPV+STI+ *(N = 15)*	
	Median (IQR) log_{10} pg/ml	Median (IQR) log_{10} pg/ml	Median (IQR) log_{10} pg/ml	Median (IQR) log_{10} pg/ml	P-Value
IL-1α	2,032 (1,614-2,473)	2,143 (1,834-2,444) #	2,683 (1,930-3,111)*	2,540 (2,247-2,797)*	**0.006**[a]
IL-1β	1,563 (1,009-2,098)	1,557 (1,044-2,251)	2,395 (1,650-2,826)*	2,118 (1,719-2,335)*	**0.005**[a]
IL-6	0,795 (0,522-1,256)	1,004 (0,787-1,351)*	1,207 (0,972-1,735)* #	0,892 (0,711-1,055)	**0.004**[a]
IL-12P40	2,425 (2,296-2,507)	2,423 (2,320-2,520)	2,551 (2,370-2,731)*	2,512 (2,317-2,594)	0.065
IL-12P70	1,709 (1,457-1,939)	1,774 (1,476-2,072)	1,892 (1,481-2,160)	1,943 (1,617-2,054)	0.230
IL-18	2,351 (1,855-2,677)	2,293 (1,954-2,684)	2,541 (2,037-2,801)	2,219 (2,113-3,025)	0.352
MIF	3,551 (3,244-3,904)	3,549 (3,249-3,834)	3,617 (3,197-3,902)	3,487 (3,085-3,948)	0.960
TNF-α	0,983 (0,769-1,388)	1,074 (0,906-1,348)	1,369 (1,085-1,874)*	1,209 (1,031-1,483)	**0.029**[a]
TNF-B	0,542 (0,387-0,633)	0,537 (0,405-0,661) #	0,560 (0,403-1,005)	0,734 (0,524-0,848)*	0.072
TRAIL	1,420 (1,092-1,738)	1,472 (1,236-1,842) #	1,861 (1,448-2,354)*	1,866 (1,489-2,004)*	**0.001**[a]
CTACK	1,222 (1,040-1,402)	1,216 (1,084-1,405) #	1,392 (1,284-1,759)*	1,481 (1,195-1,590)*	**0.004**[a]
EOTAXIN	0,732 (0,418-0,996)	0,823 (0,475-1,063)	1,003 (0,460-1,299)	0,900 (0,732-1,064)	0.117
GRO-A	2,693 (2,192-3,456)	2,956 (2,352-3,484)	3,075 (2,778-3,754)	3,206 (2,532-3,783)	0.311
IL-8	2,601 (2,143-3,038)	2,692 (2,344-3,050)	3,028 (2,589-3,638)*	2,962 (2,669-3,221)	0.108
IL-16	1,542 (1,349-1,816)	1,578 (1,437-1,890)	1,876 (1,673-2,332)*	1,828 (1,456-2,137)*	**0.022**[a]
IP-10	2,306 (1,599-2,728)	2,322 (1,704-2,959)	2,636 (2,290-3,472)*	2,280 (1,901-3,523)	0.153
MCP-1	1,458 (1,368-1,544)	1,469 (1,356-1,611)	1,540 (1,377-1,800)	1,458 (1,316-1,485)	0.465
MCP-3	1,036 (0,751-1,156)	0,975 (0,774-1,185) #	1,047 (0,920-1,457)	1,184 (0,958-1,365)*	0.091
MIG	2,422 (1,821-2,889)	2,466 (2,059-3,046)	3,200 (2,447-3,821)*	3,247 (2,299-3,516)*	**0.004**[a]
MIP-1α	0,009 (-0,252-0,316)	0,008 (-0,204-0,313)	0,314 (0,028-0,698)*	0,193 (-0,004-0,483)	**0.026**
MIP-1B	1,043 (0,728-1,663)	1,281 (0,846-1,752)	1,487 (1,135-1,877)*	1,224 (1,015-1,940)	0.070
RANTES	0,754 (0,340-0,975)	0,793 (0,525-1,114)	1,161 (0,796-1,527)*	1,035 (0,732-1,251)*	**0.005**[a]
IFN-α2	1,225 (1,143-1,345)	1,242 (1,119-1,369) #	1,379 (1,233-1,648)*	1,437 (1,160-1,518)*	**0.011**
β-NGF	-0,131 (-2,000-0,246)	-0,117 (-0,498-0,302)	0,221 (-0,144-0,754)*	0,366 (-0,377-0,630)*	**0.016**[a]
FGF BASIC	1,409 (1,358-1,493)	1,422 (1,371-1,482)	1,503 (1,426-1,587)*	1,481 (1,353-1,562)	**0.043**[a]
G-CSF	2,199 (1,591-2,701)	2,395 (2,066-2,965)*	2,517 (2,202-3,307)*	2,239 (1,800-2,730)	**0.053**
GM-CSF	1,840 (1,789-1,908)	1,855 (1,802-1,907) #	1,890 (1,775-1,932)	1,799 (1,751-1,861)	0.165
HGF	2,167 (1,747-2,748)	2,323 (1,918-2,780)	2,788 (2,227-3,329)*	2,575 (2,299-2,915)*	**0.005**[a]
IL-3	2,024 (1,891-2,114)	1,997 (1,951-2,154)	2,118 (1,996-2,479)*	2,258 (1,964-2,326)*	**0.030**
IL-7	0,781 (0,547-1,049)	0,865 (0,683-1,031)	0,992 (0,741-1,073)	0,903 (0,780-1,086)	0.166
IL-9	0,655 (0,515-0,844)	0,733 (0,590-0,848)	0,785 (0,617-1,021)	0,815 (0,717-0,930)*	0.068
LIF	1,073 (0,899-1,262)	1,075 (0,936-1,239) #	1,164 (1,100-1,620)*	1,305 (1,216-1,375)*	**0.006**[a]
M-CSF	1,778 (1,565-2,020)	1,926 (1,598-2,121)	1,995 (1,661-2,350)	2,056 (1,729-2,203)*	0.065
PDGF-BB	1,078 (0,817-1,300)	1,127 (0,966-1,361)	1,270 (1,040-1,669)*	1,276 (0,971-1,392)*	**0.040**
SCF	0,960 (0,694-1,232)	1,010 (0,765-1,337)	1,443 (0,894-1,810)*	1,477 (0,887-1,706)*	**0.012**[a]
SCGF-β	2,548 (2,090-2,900)	2,734 (2,384-3,037)	3,137 (2,438-3,350)*	2,756 (2,367-3,094)	**0.036**[a]
SDF-1A	2,303 (2,054-2,646)	2,426 (2,188-2,679)	2,511 (2,375-2,734)*	2,511 (2,216-2,736)	0.109
VEGF	2,333 (1,943-2,571)	2,405 (2,092-2,710)	2,581 (2,108-3,058)	2,681 (2,275-2,833)	0.164
IFN-G	1,312 (1,060-1,536)	1,371 (1,192-1,530)	1,562 (1,222-1,826)*	1,448 (1,335-1,615)	0.087
IL-2	0,446 (0,236-0,659)	0,436 (0,187-0,680)	0,546 (0,389-0,886)	0,322 (-0,495-0,520)	0.264
IL-4	-0,013 (-0,181-0,158)	0,025 (-0,107-0,133)	0,204 (-0,034-0,399)*	0,093 (-0,066-0,290)	**0.046**
IL-5	-0,051 (-1,222-0,403)	0,243 (-0,253-0,500)*	0,496 (0,266-0,727)*	0,427 (0,255-0,593)*	**0.001**[a]
IL-13	0,535 (0,332-0,728)	0,563 (0,329-0,733)	0,614 (0,392-0,813)	0,543 (0,403-0,780)	0.657
IL-15	0,093 (-1,260-0,614)	0,350 (-1,260-0,700)	0,532 (-1,260-0,943)	0,188 (-1,260-0,496)	0.171
IL-17	1,069 (0,937-1,266)	1,093 (0,975-1,279)	1,201 (1,034-1,698)*	1,129 (0,977-1,406)	**0.054**
IL-2rα	1,272 (1,115-1,420)	1,241 (1,081-1,420) #	1,476 (1,319-1,957)*	1,562 (1,246-1,673)*	**0.002**[a]
IL-10	1,606 (1,434-1,715)	1,626 (1,513-1,815)	1,694 (1,518-1,873)*	1,640 (1,608-1,773)	0.150
IL-1RA	4,107 (3,911-4,211)	4,115 (3,985-4,248) #	4,108 (4,000-4,291) #	3,943 (3,749-4,095)	0.113

*Mann-Whitney U tests were used to compare medians between groups. P-values<0.05 were considered significant. * denote significant differences between HPV-STI- and HPV only, or STI only or HPV+STI+; while # represents a statistical difference in medians between HPV+STI+ and HPV only, or STI only. * or # in red print indicate p values significant after multiple comparisons adjustment by Dunn's post-testing.* ***Cytokines are ordered according to functionality: pro-inflammatory, chemokines, growth factors, adaptive response and anti-inflammatory cytokines.***

Table 11. MMP concentrations among HPV-STI-, HPV+STI-, HPV-STI+ and HPV+STI+ women.

Protein	HPV-STI- *(N = 67)*	HPV ONLY *(N = 66)*	STI ONLY *(N = 14)*	HPV+STI+ *(N = 15)*	
	Median (IQR) log_{10} pg/ml	**Median (IQR) log_{10} pg/ml**	**Median (IQR) log_{10} pg/ml**	**Median (IQR) log_{10} pg/ml**	**P-Value**
MMP-1	2,690 (3,150-3,440)	2,435 (3,150-3,420)	3,163 (3,550-3,848)*	3,160 (3,350-3,480)	**0.018**
MMP-2	2,700 (3,340-3,770)	2,838 (3,525-3,780) #	3,415 (4,050-4,355)*	3,400 (3,730-4,160)*	**0.002**[a]
MMP-3	2,630 (2,810-3,170)	2,510 (2,930-3,273)	2,813 (3,265-3,603)*	2,810 (3,170-3,240)*	**0.027**
MMP-7	1,810 (2,420-3,110)	2,003 (2,515-3,150)	2,098 (2,760-3,330)	2,210 (2,460-3,610)	0.199
MMP-8	4,190 (5,000-5,920)	4,108 (4,950-6,223)	4,905 (6,560-7,623)*	4,930 (5,240-6,050)	**0.020**[a]
MMP-9	3,890 (4,500-5,410)	3,680 (4,635-5,443)	4,340 (5,685-6,335)*	4,510 (5,000-5,440)	**0.017**[a]
MMP-10	2,290 (2,890-3,180)	2,270 (2,960-3,313)	2,793 (3,135-3,538)	2,700 (2,900-3,010)	0.310
MMP-12	2,440 (2,830-3,230)	2,268 (2,855-3,408) #	2,895 (3,330-4,013)*	2,840 (3,240-3,700)*	**0.013**
MMP-13	1,710 (2,140-2,630)	1,708 (2,185-2,605) #	2,278 (2,595-2,965)*	2,270 (2,490-2,690)	**0.027**

*Mann-Whitney U tests were used to compare medians between groups. P-values<0.05 were considered significant. * denote significant differences between HPV-STI- and HPV only, or STI only or HPV+STI+; while # represents a statistical difference in medians between HPV+STI+ and HPV only, or STI only. * or # in red print indicate p values significant after multiple comparisons adjustment by Dunn's post-testing.*

Table 12. Cellular frequencies among HPV-STI-, HPV+STI-, HPV-STI+ and HPV+STI+ women.

	HPV-STI- *(N = 66)*	HPV ONLY *(N = 69)*	STI ONLY *(N = 14)*	HPV+STI+ *(N = 13)*	
Cell Type	**Median % (IQR)**	**Median % (IQR)**	**Median % (IQR)**	**Median % (IQR)**	**P-Value**
LYMPHOCYTES	1,083 (2,170-5,350)	0,982 (2,290-5,715)	0,631 (3,520-7,860)	1,050 (2,940-4,365)	0.971
CD3	1,178 (10,350-27,730)	2,755 (13,400-23,450)	3,678 (10,190-38,700)	1,290 (11,400-24,350)	0.773
CD3CD38DR+CCR5+	0,829 (4,770-13,530)	1,550 (5,090-13,730)	6,935 (10,300-26,850)*	3,625 (6,450-30,500)	0.060
CD3CCR5+CD38+	4,650 (16,400-25,600)	6,170 (17,900-27,800)	13,250 (28,800-42,600)*	15,600 (21,900-40,950)	0.056
CD3CCR5+DR+	3,403 (8,990-19,980)	3,883 (11,000-28,500)	8,050 (14,600-51,600)	6,885 (25,200-50,200)*	0.081
CD3CCR5+KI67+	0,000 (3,800-13,850)	0,000 (8,765-24,900)	0,074 (17,700-57,350)	0,000 (16,600-28,050)	0.353
CD3CD38+DR+	3,135 (10,200-20,450)	4,680 (11,050-24,550)	13,250 (18,900-28,850)*	6,150 (16,900-47,000)	**0.037**
CD3CCR5+	21,100 (38,700-59,950)	22,250 (44,600-57,850)	34,200 (57,450-73,050)	30,950 (46,700-84,000)	0.163
CD3CD38+	21,180 (37,600-52,380)	16,900 (42,400-53,650)	32,700 (54,700-73,550)*	26,350 (61,700-75,600)	**0.045**
CD3HLA-DR	10,550 (23,900-40,900)	13,230 (24,950-49,530) #	21,150 (27,800-61,050)	24,000 (34,900-66,500)*	0.111
CD3KI67	0,513 (13,500-31,180)	0,049 (21,400-40,750)	0,072 (21,600-63,280)	3,342 (29,000-38,250)	0.656
CD3-	23,650 (41,500-59,780)	21,850 (35,500-58,850)	24,880 (29,750-67,780)	22,550 (33,200-59,800)	0.909
NK Cells [2]	1,580 (5,520-12,350)	1,830 (4,080-17,500)	0,403 (2,730-20,650)	1,283 (3,745-12,530)	0.972
CD3 Activation [1]	53,250 (68,200-91,030)	55,150 (74,600-86,850) #	75,250 (86,050-93,630)*	76,500 (90,100-98,850)*	**0.007[a]**
CD4	49,030 (62,250-72,880)	48,350 (58,000-67,050)	53,780 (64,450-70,300)	29,050 (45,900-63,850)	0.135
CD4CD38DR+CCR5+	0,084 (3,715-10,980)	0,608 (4,125-12,180)	4,065 (7,610-18,900)	1,260 (5,910-15,200)	0.375
CD4CCR5+CD38+	3,370 (16,700-30,700)	4,725 (18,600-31,500) #	9,128 (26,950-38,280)	18,300 (27,300-56,000)*	0.098
CD4CCR5+DR+	2,270 (8,740-19,380)	2,090 (8,940-28,300)	5,310 (12,600-37,850)	1,260 (9,460-28,450)	0.843
CD4CCR5+KI67+	0,000 (3,770-19,250)	0,000 (3,400-27,780)	0,000 (0,275-37,950)	0,000 (18,200-26,550)	0.959
CD4CD38+DR+	1,230 (8,160-17,450)	1,890 (8,770-18,230)	7,685 (12,300-21,750)	2,180 (6,920-21,850)	0.409
CD4CCR5	24,800 (44,700-69,200)	19,750 (43,300-62,800)	24,380 (56,550-75,830)	30,700 (54,500-95,450)	0.337
CD4CD38	21,280 (39,650-58,100)	17,700 (42,900-54,600) #	26,080 (56,150-74,880)	30,800 (63,600-75,400)	0.074
CD4HLA-DR	7,140 (16,800-32,750)	5,500 (19,100-41,550)	9,795 (20,300-43,950)	3,050 (10,800-35,950)	0.894
CD4KI67	0,000 (12,050-33,430)	0,000 (8,860-36,850)	0,000 (0,240-44,880)	0,000 (25,000-36,050)	0.978
CD4 Activation [1]	50,850 (72,750-91,580)	53,000 (72,700-84,400) #	62,350 (83,050-91,980)	74,650 (84,300-98,950)	0.092
CD8	24,080 (35,650-49,100)	24,400 (36,600-45,700)	29,600 (32,350-44,430)	20,700 (35,100-54,000)	0.995
CD8CD38DR+CCR5+	0,000 (3,615-9,955)	0,000 (6,280-13,700)	4,735 (11,600-17,100)*	3,165 (8,320-30,550)	0.067
CD8CCR5+CD38+	0,597 (9,300-18,550)	3,585 (12,900-22,850)	9,155 (18,250-46,430)*	6,360 (18,100-36,050)	0.084
CD8CCR5+DR+	0,137 (8,110-19,130)	1,958 (12,150-26,500)	8,830 (14,300-27,950)	4,765 (23,600-51,350)	0.157
CD8CCR5+KI67+	0,000 (2,380-13,900)	0,000 (5,265-26,180)	0,000 (0,171-47,150)	0,000 (9,520-24,900)	0.694
CD8CD38+DR+	0,163 (10,400-21,600)	1,830 (12,700-30,980) #	15,050 (18,800-34,800)*	5,865 (34,900-68,700)*	**0.030**
CD8CCR5	14,250 (28,500-51,250)	17,450 (31,900-50,000)	24,830 (42,500-64,200)	17,150 (32,600-70,750)	0.470
CD8CD38	8,730 (33,200-50,200)	13,150 (38,600-54,100) #	32,050 (50,100-75,680)*	23,700 (67,400-81,800)*	**0.033**
CD8HLA-DR	6,210 (29,900-54,050)	10,550 (36,650-57,150) #	29,350 (40,000-68,150)	28,750 (54,300-85,850)*	0.072
CD8KI67	0,000 (13,500-35,100)	0,000 (11,700-41,050)	0,000 (20,000-97,680)	0,000 (26,100-39,700)	0.902
CD8 Activation [1]	45,230 (67,950-90,580)	50,500 (70,800-88,150) #	73,580 (85,150-94,200)*	71,900 (88,400-99,750)*	**0.014**

*Mann-Whitney U tests were used to compare medians between groups. P-values<0.05 were considered significant. * denote significant differences between HPV-STI- and HPV only, or STI only or HPV+STI+; while # represents a statistical difference in medians between HPV+STI+ and HPV only, or STI only. * or # in red print indicate p values significant after multiple comparisons adjustment by Dunn's post-testing.[1]Activation refers to cells expressing CCR5, HLA-DR and/or CD38. [2]NK cells refers to those expressing $CD56^{+}CD16^{+}$ markers.*