# Investigation of intravaginal practices as a factor associated with a high prevalence of genital human papillomavirus infection in adolescent girls.

By

Mntambo Ntombenhle

215006731

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Of

Master of Science

Department of Biochemistry,

CAPRISA Mucosal Immunology laboratory,

School of Life Sciences,

College of Agriculture, Engineering and Science,

University of KwaZulu-Natal,

Pietermaritzburg

Supervisor: Dr. Pamela Gumbi

Co-supervisor: Dr. Sinaye Ngcapu

# PREFACE

All of the experimental work described in this thesis was carried out at the Centre for the AIDS Programme of Research in South Africa, Nelson R. Mandela School of Medicine, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa, under the supervision of Dr. Pamela Gumbi and co-supervision of Dr. Ngcapu. The research was financially supported by Poliomyelitis Research Foundation (PRF).

This research work has never been submitted to any other tertiary institution, where the work of others has been used, it is acknowledged in the text. All of the results reported are due to investigations by the candidate.

Ntombenhle Mntambo Signed: [redacted]

Dr. Pamela Gumbi Signed: [redacted]…

As the candidate's supervisor, I agree with the submission of this thesis.

Date: 29 November 2021

# DECLARATION

I Ntombenhle Mntambo declare that:

The research reported in this thesis, except where otherwise indicated or acknowledged, is my original work. This work has not been submitted for any degree or examination at any other university. It does not contain other persons' data, pictures, graphs, or another person's information unless specifically acknowledged as being sourced from another person. No other person's writing is contained in this thesis unless specifically acknowledged as being sourced from other researchers. Where written sources have been quoted and where the precise words have been used, the writings are placed within quotes and referenced.

# DEDICATION

I dedicate this work to my family.

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# ETHICS DECLARATION

The study protocol was approved by the BREC of the University of KwaZulu-Natal, ref no: BREC/00001015/2020. GCP and HSP were followed and written informed consent was obtained from all participants before enrolment. The identity of participants was kept confidential.

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# ABBREVIATIONS

| | |
|---|---|
| % | Percentage |
| + | Positive |
| - | Negative |
| µl | Microlitre |
| AMPs | Antimicrobial peptides |
| APC | Alkaline Phosphatase Conjugate |
| APC-H7 | Allophycocyanin-H7 |
| BD | Becton Dickson |
| BV | Bacterial Vaginosis |
| BV711 | Brilliant Violet 711 |
| CAPRISA | Centre for the AIDS Programme of Research in South Africa |
| CCR5 | C-C chemokine receptor type 5 |
| CT | Chlamydia trachomatis |
| DNA | deoxyribonucleic acid |
| ELISA | Enzyme-linked Immunosorbent Assay |
| FACS | Fluorescence-activated cell sorting |
| FBS | Fetal Bovine Serum |
| FDA | Food and Drug Administration |
| FMO | Fluorescent Minus One |
| FRT | Female reproductive tract |
| HBD | human β-defensin |

| | |
|---|---|
| HIV | Human immunodeficiency virus |
| HLA-DR | Human leukocyte antigen |
| HPV | Human Papilloma Virus |
| IFN | Interferon |
| KZN | KwaZulu-Natal |
| L. crispatus | Lactobacillus crispatus |
| ml | Milliliter |
| $^{o}$C | Degrees Celsius |
| OR | odds ratio |
| PBS | Phosphate buffer saline |
| PCR | Polymerase Chain Reaction |
| RPMI | Roswell Park Memorial Institute |
| RPRM | Robust Poisson regression models |
| STI | Sexually transmitted infection |
| TLR | Toll-like receptor |
| VIPs | Vaginal Inserted Products |
| Vivid | Violet-fluorescent reactive dye |
| VLPs | virus-like particles |

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# ABSTRACT

**Background:** Human papillomavirus (HPV) is a common sexually transmitted infection (STI) in women which mainly infects the mucosal areas. Young women are disproportionately infected by HPV, and factors that may render adolescents or younger women more vulnerable to HPV acquisition than older women have not been fully elucidated. This study aimed to investigate the associations between the prevalence of HPV, the use of intravaginal products, the immune activation status of cervical T cells, and alterations of the composition and concentrations of antimicrobial peptides (AMPs) in vaginal fluid among adolescent females close to their sexual debut and older women in KwaZulu-Natal.

**Methodology:** Genital specimens (cervical cytobrush and cervicovaginal swabs) were collected from 154 female participants aged 14-19 and 25-35 years. From cervicovaginal swabs, HPV genotyping was done using a deoxyribonucleic acid (DNA) Flow hybridization system. Flow cytometry was conducted from cervical cytobrush specimens (evaluating CD38+, HLA-DR+, and CCR5+ expressions) to assess T-cell immune activation status. Enzyme-linked Immunosorbent Assay (ELISA) kits were used to measure genital concentrations of human β-defensin (HBD-1, HBD-2), and psoriasin from cervicovaginal swabs. Statistical tests conducted were Robust Poisson regression models (RPRM), the Tukey multiple comparison adjustment, t-test, and Mann Whitney U test. *P* values of <0.05 were statistically significant.

**Results:** HPV prevalence was 85%, with high-risk genotypes being the most prevalent. The majority of the cohort was infected with multiple genotypes (76.62%). Genotypes associated with cancer and current Gardasil®9 HPV vaccine targets were more common (53.9%) than those associated with genital warts (14.9%). The risk of HPV in adolescent females was 15.9% higher than in adult females. The use of vaginal inserted products (VIPs) was associated with a 40% higher risk of contracting genotypes related to cancer (p=0.0503) compared to non-users. The risk of HPV infection for adolescents using VIPs was 23% higher than that of adults using VIPs. However, these differences were not statistically significant at a 5% level of significance. Sexual debut after 18 years significantly reduced the risk of overall HPV infection (p=0.0040) and infection with genotypes associated with cancer (p=0.0024). When comparing HPV infected adolescents and adults, the proportion of activated CD4+ T cells was significantly higher in adolescents, particularly in CD4+ HLA-DR+ cells (p=0.0008). CD8+ T cells showed no difference. A significantly higher concentration of HBD-2 was observed in HPV+ adults compared to HPV- adults (p=0.0215) and HPV+ adolescents (p=0.0189).

**Conclusion:** The overall HPV prevalence is higher than the previously reported prevalence in KwaZulu-Natal province. In addition, we demonstrate that the use of VIPs may be associated with some HPV infection risk, particularly in adolescent females. This finding suggests that young women should be warned about the potential risk of using VIPs. We also confirm that the age-phase, delay in sexual debut, and the number of sexual life partners have significant associations with HPV genotypes linked to cancer, highlighting the importance and the urgency of vaccinating young girls with Gardasil®9 HPV vaccine. Adolescents with HPV have significantly higher levels of activated CD4+ T cells in their cervical mucosa, suggesting the presence of reactive activated cells that lack efficiency in the clearing of HPV infection in this age group. HPV infection upregulates HBD-2 levels during HPV infection, notably significantly higher in adult females than in adolescents. This investigation has generated new insights into the risk factors for HPV acquisition in young women. These findings need to be confirmed further by larger cohort size studies, essential for HPV prevention.

# CHAPTER 1: INTRODUCTION

## 1.1 Background of the study

Human papillomavirus (HPV) is one of the most common sexually transmitted infections (STIs) in women, which infects the mucosal areas of the vagina, vulva, cervix, and anus (Burchell et al., 2006). The risk is skewed towards young women who engage in sexual acts early in their reproductive years (Newton-Levinson et al., 2016, Mbulawa et al., 2018) and the highest incidence rates are seen in women who sexually debuted before 16 years of age (Schiffman et al., 2005). A considerable proportion (80%) of sexually active individuals contract genital HPV during their lifetime (Einstein et al., 2009) and those in whom infection persists have a significantly higher risk of developing cervical cancer (Bahadoor-Yetman et al., 2016). Several factors have been associated with HPV natural history. These include early sexual debut, infection with other STIs including, human immunodeficiency virus (HIV), increased number of lifetime and current sexual partners (Insinga et al., 2010). However, factors that may render adolescents or younger women more vulnerable to HPV acquisition than older women are not well understood. Adolescence is an essential phase of physiological growth and development of the immune system (Simon et al., 2015).

Critical to developmental changes in the reproductive system is the ability of the young adolescent immune system to mount a response against pathogens quickly. The immune system clears most of the HPV infection, however, the precise mechanisms are still not completely understood (Barros et al., 2018). The innate and adaptive immunity have both been shown to be associated with HPV elimination in several studies, although they do not clearly show how this is established (Frazer, 2009). There is a possibility that HPV infection could affect the differentiation of cervical immune T cells uniquely. Changes in adaptive immunity can result in quicker progression from high-risk HPV infection to cervical cancer (Song et al., 2015). The changes and modifications induced by high-risk HPV infection could result in the adjustment of the immune system, creating an environment suitable for persistent infection (Song et al., 2015). T cell activation is critical in HPV infection. The increased prevalence of HPV infections in immunocompromised individuals, including immunosuppression because of HIV infection, displays the focal significance of the CD4 T cell population in the control of established HPV infections. A study by Stanley and Sterling (2014) showed that compromised

CD4+ T cells promoted the occurrence of HPV-associated cancer in patients with HIV infection. In addition, the various cellular components involved during adaptive epithelial immune responses have been demonstrated in both cutaneous and mucosal HPV infected tissues (Scott et al., 2001). Evans et al. (1997) identified cytotoxic mediated immunity against HPV in lymph nodes and tumors of cervical cancer patients (Evans et al., 1997).

The Innate immune system also plays a significant role in defense against HPV infections. Antimicrobial peptides are small peptides, most of which are known to be cationic. These molecules play multiple roles as host defense peptides, which form part of innate immunity (Lei et al., 2019). The precise mechanism by which AMPs carry out their antimicrobial action is not entirely understood, however, they are suspected to bind and interact with target cell membranes, creating pores for antimicrobial activity (Ganz, 2003). Many of these peptides have been discovered in epithelial layers, phagocytic cells, and body fluids, and they belong to three main families: defensins, cathelicidins, and S100 proteins. Defensin peptides are cysteine-rich residues divided into three subfamilies (alpha, beta, and theta). The β-defensins play a vital role in the innate immunity to infections, including HPV. An upregulated expression of epithelial human β-defensins (HBD-1, -2, -3) was identified in HPV-associated lesions and this study demonstrated epithelial defensin expression in 100% patient-derived papilloma tissue samples (Chong et al., 2006). The cathelicidin, LL37 has been shown to have direct antimicrobial activity against many different infection-causing micro-organisms and can also modulate immune response (Harten et al., 2018). Although in the context of HPV not much has been reported, it is expressed in HPV-induced common skin warts (Conner et al., 2002) and genital tissues (Valore et al., 2002). Psoriasin also called S100A7 belongs to the S100 family of calcium-binding proteins and is the most abundant antimicrobial peptide in healthy skin. One study showed downregulation of psoriasin in patients with premalignant HPV-induced high grade squamous intraepithelial lesions before surgical treatment (Alvendal et al., 2019). However, this negative association between psoriasin and HPV lesions was not observed in patients with other tumours which were not associated with HPV.

In addition to the developing immune system, the skewing of HPV risk towards adolescents and young women may be attributed to different behavioural factors. Transmission risk is largely attributed to engaging in risky sexual behaviours especially those that are unique to the adolescent phase (Wigfall et al., 2012). These may include having unprotected sexual intercourse, engaging in riskier sexual activities, the pressure of retaining partners, and

intravaginal practices, which will be the main focus of this study. Intravaginal practices refer to the behaviour in which women use various substances to alter the structure and environment of the vagina for the management of vaginal health and sexual life. These practices include (i) intravaginal cleaning and wiping for hygiene purposes (e.g, douching or washing with liquids, or wiping with cloth or newspaper) or (ii) intravaginal insertion of substances to dry or tighten the vagina for sexual pleasure (Low et al., 2011). This is a common habit among African women across sub-Saharan Africa. They report the use of various intravaginal products/vaginal inserted products (VIPs) to create a warm and tight vaginal environment to please male partners (Alcaide et al., 2017).

VIPs have been associated with adverse health outcomes, including sexually transmitted bacterial infections and cervical cancer (Cottrell, 2010). Studies have associated Intravaginal insertion of commercial products with *bacterial vaginosis* (BV) (Hassan et al., 2007) and subsequently, a greater risk of STI acquisition, including HIV and HPV. Although very few studies have looked at the association between vaginal practices and HPV infection, there is contradictory data from those that have looked at this association (Bui et al., 2016). However, it has been hypothesised that increased risk to viral infections like HPV is brought about by the use of vaginal products. These products may cause physical abrasions and disrupt the vaginal stratified squamous epithelium by removing of natural mucus secretions and disturbing local innate immunity, creating a compromised environment, easily accessible to pathogens including HPV (Bui et al., 2018). Conversely, the use of VIPs for cleansing after sexual activity may be beneficial for clearing transmitted HPV and reducing infection risk (Bui et al., 2018). These findings highlight the need to examine the further association of VIPs with HPV infection, particularly in regions where VIPs are quite common and HIV incidence is high. A high HPV prevalence in South African women below 25 years of age has been previously reported in the KwaZulu- Natal province (76%) (Ebrahim et al., 2016) and (74%) (Liebenberg et al., 2019), in this same region, high HIV burden (Abdool Karim et al., 2017) and VIPs use among adolescent girls and young women are common (Humphries et al., 2019).

## 1.2 Aim and Objectives of the study

This study aimed to investigate the associations between prevalence of HPV, the use of intravaginal products, the immune activation status of cervical T cells, and alterations of the composition and concentrations of AMPs in vaginal fluid among adolescent females close to their sexual debut and older women in KwaZulu-Natal (KZN).

**To achieve this aim, the objectives were:**

1. To determine the distribution of high-risk and low-risk HPV genotypes in adolescent girls and adult women.
2. To evaluate the relationship between the vaginal inserted product use and HPV genotypes.
3. To examine whether the immune activation status of CD4 and CD8T cells is associated with HPV infection.
4. To determine whether AMPs, HBD- 1, -2, and psoriasin concentrations in the genital tract are related to HPV status and use of VIPs.

**Hypothesis:**

The lower reproductive tract of adolescents (14-19 years), soon after sexual debut, represents a naive reactive state characterised by a deficiency of antimicrobial polypeptides with delayed response to pathogens, including HPV. We hypothesize that in this naïve age group of young women, the use of traditional and commercial vaginal inserted products (including sex enhancers) is common compared to their adult counterparts. Vaginal inserted products may compound the risk of HPV infection and persistence, which may lead to a higher risk of cervical cancer, compromised adaptive and innate immunity.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 Human Papilloma virus.

HPV is a common STI known to affect both males and females. About 70% to 90% of the HPV infection is cleared by the immune system in less than two years (Best et al., 2012). However, the infection that persists can change the cervix cells into pre-cancer cells which show no symptoms. The cells with early pre-cancer development can go back to normal on their own or get treated, however, like the HPV infection the persistence of pre-cancer cells can lead to cervical cancer (Schiffman and Wentzensen, 2013). Cervical cancer is the fourth most frequent cancer among women globally, with 570 000 new cases diagnosed in 2018 (Bray et al., 2018). The burden of cervical cancer in South Africa for 2018 was estimated as 12 983 annual number of cases with 5 595 annual deaths (Bruni et al., 2018, Bruni et al., 2019), Bruni et al., 2019). Most HPV strains do not cause any symptoms, but certain strains can cause genital warts, while other types are the principal cause of invasive cervical cancer (Schiffman et al., 1993).

There are more than 200 HPV genotypes (Kocjan et al., 2015), these are categorised as high and low-risk genotypes, and these will be described in more detail in Section 2.1.2. High-risk types are the critical factor for the pathogenesis of various cancers (Park et al., 2019). HPV 16 and 18 are the most common high-risk types, accounting for approximately 70% of cervical cancer around the world (Bansal et al., 2016). In addition, HPV infection is also associated with cancers of the vulva, penis, anus, vagina as well as head and neck cancers. Low-risk HPV infections cause warts on the skin and genitals. Warts can appear from a few weeks to a few months and even years after exposure to HPV (Yanofsky et al., 2012).

### 2.1.1 HPV structure and genome

HPV has a non-enveloped circular double-stranded DNA genome made up of approximately 7900 nucleotides (Taberna et al., 2017). Figure 2.1 displays a typical genomic organisation of HPV divided into three main regions (Shanmugasundaram and You, 2017). The first region is an upstream regulatory region (URR), also known as the long central region, this is a non-coding region that takes up 10% of the viral genome. This is where replication begins and where there are multiple transcription factor binding sites necessary for the regulation of viral

transcription (de Villiers, 2013).

The second region occupies over 50% of the viral genome with open reading frames which encode E1, E2, E4, E5, E6, and E7 regulatory proteins, each holding important functions in the genome. E1 has DNA helicase activity, once it attaches to the viral origin of replication it can drive the viral DNA replication process. E2 regulates cellular gene expression, it recruits E1 to the viral origin for replication and plays an important role in the transfer of the viral genome to daughter cells when the host cell divides. E4 takes part in the assembly of the virion. E5 controls cell growth and differentiation. E6 is an oncoprotein (Hoppe-Seyler et al., 2018), it works with E7 to maintain a suitable environment for viral replication and is known to target and bind p53 to tamper with its tumor-suppressive function and direct it to degradation (Scheffner et al., 1993). E7 also controls the cell cycle (Taghizadeh et al., 2019).

The third region is the late region, which covers almost 40% of the genome and is downstream of the early region, encoding the L1 and L2 proteins of the viral capsid. These are known as major and minor proteins, respectively, and are known to promote the formation of complete virions. L1 is a 55 kDa protein that self-assembles into virus-like particles (VLPs) considered as strong immunogens and used as a target for HPV vaccine development (Buck et al., 2013). The capsid protein L2 cannot form VLPs but co-assembles with L1 into VLPs to enhance their assembly (Wang and Roden, 2013).

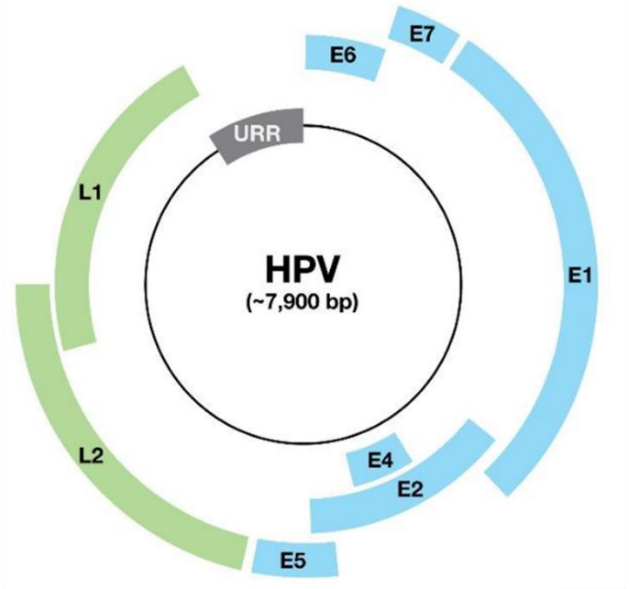


**Figure 2.1: Genomic organisation of HPV.** Composed of approximately 7900 base pairs. Consists of the upstream regulator region (URR), six early genes (E, in blue), and the late region (in green) which codes for L1 and L2, the major and minor viral capsid proteins, respectively. Adapted from Shanmugasundaram and You (2017).

### 2.1.2 HPV classification

HPVs are small viruses with circular double-stranded DNA. They belong to a family of non-enveloped DNA viruses called papillomaviridae which is a large group of different viruses and many HPV types classified into different genera. Five main HPV genera were discovered and are currently known: $\alpha$-papillomavirus, $\beta$-papillomavirus, $\gamma$-papillomavirus, mu-papillomavirus, and nu-papillomavirus (Crow, 2012) (Figure 2.2). HPV genotypes from each genus are also grouped into different species and further separated as high-risk and low-risk genotypes according to their oncogenic properties (Sehnal et al., 2019). The classification was made based on the nucleotide sequence of the open reading frame which encodes the capsid L1 protein (Murahwa et al., 2019). HPV is among the causes of cancer (zur Hausen, 1996), it is the second leading cause of cervix cancer (Walboomers et al., 1999) and the mucosal HPV types commonly associated with cervical cancer, anal, vaginal, penile, and oral cancer are in the $\alpha$-papillomavirus genus (Olivero et al., 2018). Out of all the HPV genotypes, hpv16 is considered the most oncogenic, with the highest level of danger and amounts to most cervical cancer cases (54.4 %) and other cancers in the world (Song et al., 2019). All the common subtypes linked to cancer and the subtypes responsible for over 90% of the genital warts are found under the $\alpha$ -papillomavirus genus.

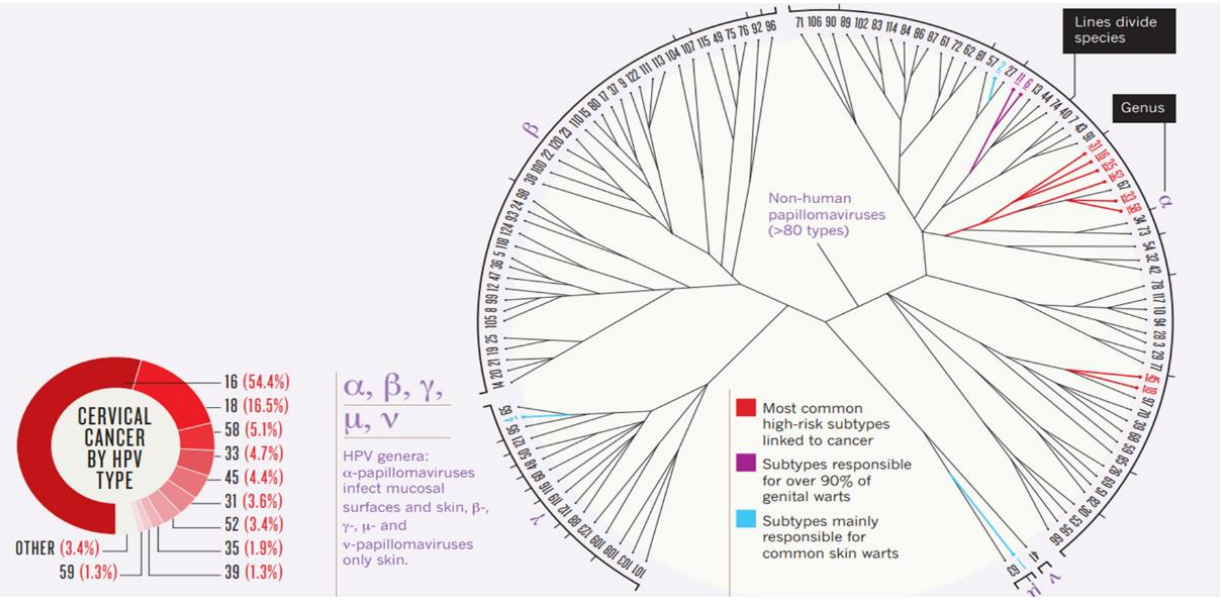


**Figure 2.2: Classification of the human papillomavirus.** A subdivision of HPV is displayed with 5 different genera, $\alpha$-papillomavirus, $\beta$-papillomavirus, $\gamma$-papillomavirus, μ-papillomavirus, and ν-papillomavirus named in the order from the genus with more genotypes to the least. Under these are all known HPV genotypes classified into different species separated by lines in the diagram. α-papillomaviruses infect mucosal surfaces and skin while β-, γ-, μ- and ν-papillomaviruses only infect the skin. The most common high-risk subtypes that cause cancer are shown in blue colour and their link to cervical cancer is given in percentage (%). Adapted from Crow, (2012).

## 2.2 Burden of HPV and HPV-related cancer.

### 2.2.1 Global burden of HPV

According to the HPV information centre, the global prevalence of HPV infection is around 12%, with the overall HPV prevalence the highest in sub-Saharan Africa (24%) followed by Eastern Europe (21%) and the lowest in Western Asia (1.7%) (Bruni et al., 2019). These data were obtained from women with normal cervical cytology and corresponded to the results from a study by Bruni et al., (2010). HPV is considered to be one of the leading causes of infection-related cancer in mankind (Serrano et al., 2018). The incidence rate of cervical cancer caused by HPV per 100 000 women per year in the world was observed to be 13.1, with a mortality rate of 6.9 in 100 000 women (Ferlay et al., 2019). In Africa, the incidence rate was estimated to be 27.6 in 100 000, with the mortality rate being 20 in 100 000 and the highest compared to other continents. The continent following Africa is Asia, with the cervical cancer incidence rate and mortality rate of 11.9 in 100 000 and 6.2 in 100 000, respectively (Ferlay et al., 2019).

Globally, the prevalence of HPV is higher in females than in males, with the highest incidence predominantly observed in females under the age of 25, with a decline in older females (Aro et al., 2019). In this age group alone, it is 19.2% worldwide and 43.9% in Africa (Bruni et al., 2010). The study also investigated the age-specific prevalence of HPV worldwide and in four different continents, and showed that young women under the age of 25 were disproportionately affected by HPV (Serrano et al., 2018) (Figure 2.3). Africa was observed to have a very high percentage of HPV incidents in all age groups compared to the other continents in the study. Asia had the lowest prevalence among the 25 years and younger age groups (Bruni et al., 2010).

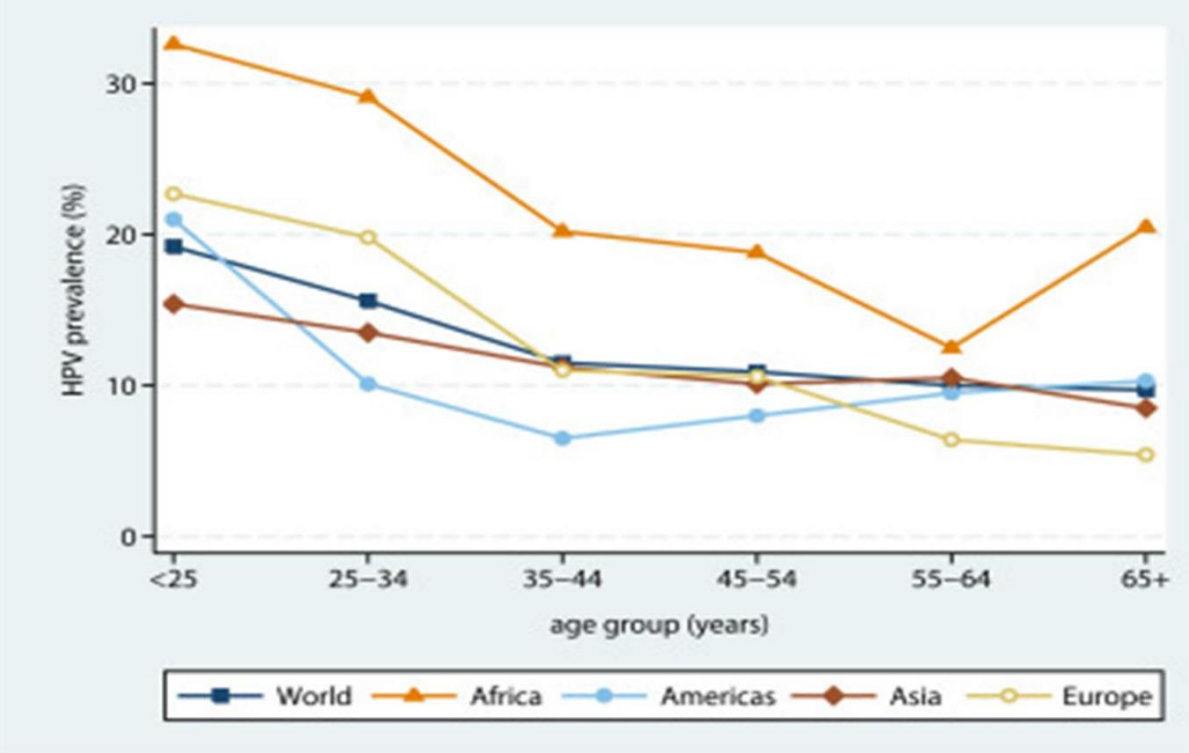


**Figure 2.3: Age-specific prevalence of HPV (%) in the world and four regions.** Women with normal cervical cytology in the world and four selected regions (Africa, Americas, Asia, and Europe) were assessed for HPV prevalence. In all regions, a peak in HPV infection was observed at younger ages (< 25 years). HPV prevalence in Africa is the highest in all age groups. Taken from Serrano et al., (2018).

### 2.2.2 HPV burden in South Africa

In KwaZulu-Natal, South Africa, the prevalence of HPV in sexually active females was found to be 76.3%. The total proportion of women with high-risk genotypes was 54.5%, and the prevalence of high-risk genotypes was 71.3% among women infected with HPV (Ebrahim et al., 2016). In Gauteng, a high HPV infection rate of 85% was reported in women under the age of 25 years, while in the Western Cape it was estimated to be around 44% -71% (Mbulawa et al., 2018, Giuliano et al., 2015). The incidence rate of cervical cancer cases attributable to HPV in South Africa was estimated to be 43.5 in 2018 (Bruni et al., 2019). The rate of anogenital cancers other than the cervix (vulvar, vaginal, anal, and penile) was estimated to be 1.7% (Bruni et al., 2019).

## 2.3 HPV infection in young women and adolescent girls.

Women are the most susceptible to HPV infection compared to males (Bruni et al., 2010). In general, women are biologically more vulnerable to STIs like HPV, especially during sexual intercourse. This is because the vaginal surface is larger and more prone to sexual secretions compared to the primarily skin-covered penis (Chopra et al., 2015). Furthermore, the quantity of potentially infected male ejaculate deposited in a woman's vaginal cavity during intercourse is greater than the volume of potentially infected cervical and vaginal fluids to which males are exposed. According to Bruni et al. (2010), HPV peaks in younger women around the age of sexual debut and declines in the late 20s and 30s. This places emphasis on the burden of HPV in females at a very young age as well as exposure to potential cancers associated with this virus.

### 2.3.1. Risk factors associated with HPV infection

Most studies have reported young women and adolescent girls as the most infected with HPV (Giuliano et al., 2002). However, the behavioural, psychological, and immunological factors contributing to such findings have not been fully elucidated. Some of the known HPV risk factors are listed below:

**i. Number of sex partners**

The number of sexual partners has long influenced the prevalence of HPV (Mbulawa et al., 2018), having multiple partners increases chances of getting HPV and a higher risk of HPV is more strongly associated with having sex with a new partner than

with an existing partner (Johnson et al., 2012). The lifetime number of sexual partners was found to be an important risk factor for acquiring HPV, even after they had controlled for sexual behaviours around the time of HPV infection, with 21% of HPV infections detected, which were attributable to the number of sex partners (González et al., 2010).

### ii. Long-term hormonal contraceptive use

In some case-control studies, long-term use of combined oral contraceptives has been associated with the diagnosis of cervical cancer in HPV-positive women (Moreno et al., 2002, Gadducci et al., 2020), although this association is unclear. Another study found that long-term use of combined oral contraceptives over a 6-year period was associated with an increased risk of the common HPV infection in a cohort of long-term users of hormonal and non-hormonal contraceptives (Marks et al., 2011). In contrast, no association was observed among depot-medroxyprogesterone acetate users in Thailand (Marks et al., 2011).

### iii. Tobacco smoking

Smoking and environmental tobacco smoke exposure has been shown by a few studies in the literature to be risk factors for HPV infection. A US study examining tobacco smoke exposure as a risk factor for HPV infection in women aged 18-26 years found a strong association between tobacco smoke exposure and HPV in adolescents. This study showed a strong dose-response relationship between tobacco smoke exposure and HPV infection, the highest risk being among active smokers (Kum-Nji et al., 2019).

### iv. Condom usage

The use of condoms has been shown to have a protective effect against many STIs including HPV, when used correctly and consistently (Lam et al., 2014). Those who report little, or no condom use were more likely to have HPV DNA detected than those who reported using condoms most of the time or always (Lam et al., 2014). In a study where they compared women who reported no condom use to those who reported using them every time, it was found that those who always used condoms were slightly less likely to become HPV positive (Ho et al., 1998). However, some

studies have shown that condom use may prevent progression to lesions but not the actual infection by HPV (Manhart and Koutsky, 2002).

### v. HIV infections and other STIs

Many studies have recognised an association between STIs and HPV infection, different other STIs are frequently present in HPV-infected women. *Trichomonas vaginalis* was positively correlated with the presence of HPV, which indicated that this infection increases the risk of HPV infection in women (Paula et al., 2020). Nitrosamines produced by *Trichomonas vaginalis* have been suggested to promote cervical inflammation and result in injury which allows HPV to invade the basal layer of the cervical epithelium (Mercer and Johnson, 2018). HPV incidence was found to be significantly higher among women with *Chlamydia trachomatis* co-infection as compared to those without, however, they could not confirm whether this association differed among HPV genotypes (Vriend et al., 2015).

### vi. Early sexual debut

Sexual debut refers to the time one had their first sexual experience, and an early sexual debut is defined as having had sexual intercourse at or before 14 years of age (Girmay et al., 2019). Several studies have suggested that initiating sexual intercourse at an early age increases young people's risk of infection with HIV and STIs (Durowade et al., 2017). This is partly because they are more likely to engage in riskier sex or entertain multiple sex partners with less likelihood of condom use (Mazengia and Worku, 2009). Early age at first sexual intercourse has been associated with an increased risk of high-risk HPV (Louie et al., 2009).

### vii. Weakened immune system

Twelve percent of HPV infections have been shown to be due to weakened immune systems, suggesting an immune response as an important determinant of HPV infections (González et al., 2010).

### viii. Multiparity

The number of pregnancies has been associated with an increased risk of cervical cancer in HPV-positive women (Hinkula et al., 2004). The HPV prevalence in pregnant women was also found to increase with gestational age, increasing from 8.0% in the first trimester to 16.7% in the second trimester, and 23.1% in the third trimester (Smith et al., 1991).

## 2.4 Intravaginal practices in young women and adolescent girls.

Vaginal practices are associated with adverse health effects such as sexually transmitted diseases and cervical cancer (Cottrell, 2010). Few studies have investigated the relationship between vaginal practices and HPV infection, and those that have examined this association have found contrary results (Bui et al., 2018, Lee et al., 2014). Intravaginal practices are a variety of behaviours by women, done to manage their vaginal health, hygiene, and sexual life, these include both intravaginal cleansing and intravaginal insertion of products (Braunstein and van de Wijgert, 2005). These practices are reportedly higher in the region of Sub-Saharan Africa and are a common habit among women, particularly black African women, irrespective of age (Esber et al., 2016). Various vaginal inserted products are commonly used by women, and these may be used in the form of creams, cleansers, and lubricants. The products which were previously found to be common among adolescents and young women in rural KZN and a cohort of African American women are listed in (Table 2.1). Some of the products were found to be common among younger women (Brown et al., 2016). Younger women are also the most to report the influence of their partner in washing intravaginally compared to older women (Brown et al., 2016). These practices are believed to provide increased pleasure for men, however, for women, these may cause friction and possibly damage the delicate vaginal mucosal lining (Ramjee and Daniels, 2013).

**Table 2.1: Common products and reasons for use on the vagina.**

| Common products | Obtained | Ingredients & Application | Motivation for use |
|---|---|---|---|
| **Kuber (Humphries et al., 2019)** | Commercially | Tobacco leaves, slaked lime paste, and Delta-9-tetrahydrocannabinol, inserted into the vagina or placed under the tongue. | Drying agent, stimulate sexual performance, mood elevation. |
| **Snuff (Humphries et al., 2019)** | Commercially | Tobacco leaves, inserted into the vagina. | Sexual stimulant tightens the vagina. |
| **Oils or sexual lubricants (Brown et al., 2016)** | Commercially | Glycerine, coconut, olives. Applied on the vagina. | Lubrication, sexual pleasure, and reduce dryness. |
| **Petroleum jelly (Brown et al., 2016)** | Commercially | a mixture of hydrocarbons, natural waxes, and mineral oils. Applied into the vagina. | Reduce dryness and discomfort. |
| **Traditional herbs (Humphries et al., 2019)** | Traditional healers | A mixture of herbs, boiled and used to steam the vagina. | Tightening |
| **Wet wipes** | Commercially | Cotton or polyester and polypropylene saturated with a cleaning solution. Wipe on the vagina. | Hygiene |
| **Bluestone (Humphries et al., 2019)** | Traditionally | Copper Sulphate, put in cold water to wash the vulva. | Tightening |
| **Alum (Gafos et al., 2010)** | Commercially | Combination of aluminium sulphate and potassium sulphate. Inserted onto the vagina or diluted in water. | Dries and tightens the vagina, also used for vaginal cleansing. |

## 2.5 Effects of intravaginal practices and the risk of HPV acquisition.

The female reproductive tract (FRT) is divided into two immunological regions, one is the upper FRT made up of the endocervix, uterus, and oviduct, the other region is the lower FRT which includes the vagina and ectocervix (Lee et al., 2015). The vagina is the muscular part of the genital tract that connects the vulva to the cervix. The proximal end of the cervix is called the internal os and leads to the uterus, while the distal end is called the external os and leads to the vagina (Jain and Limaiem, 2021). The boundary between the two cervical regions (endocervix and ectocervix) is referred to as cervical transformation zone, which is an area of cellular changes, composed mainly of cervical epithelial cells (De Tomasi et al., 2019) (Figure 2.4). The cervical tissue consists of stromal and epithelial cells separated by a basement membrane (De Tomasi et al., 2019). The lower part of the female reproductive tract is susceptible to various microorganisms when in contact with the external environment. For this reason, the reproductive tract of a normal and healthy female is composed of protective proteins, cells, and layers / linings (Reis Machado et al., 2014). The vaginal mucosa is lined with the mucosal surface that acts as a physical immunological barrier, keeping potential pathogens away from vaginal epithelial cells. It contains glycosylated mucosal proteins, such as mucins, which form a dense, lubricated physical barrier that prevents pathogen access to epithelial cells (Amabebe and Anumba, 2018). The mucosa is rich in nutrients such as glucose or amino acids, which allow bacteria to colonize and form the vaginal microbiota. A healthy microbiota is a balance of healthy microbes predominated by the *Lactobacilli species* with the main strains being: *Lactobacillus iners*, *Lactobacillus crispatus* (*L. crispatus)* , *Lactobacillus gasseri*, and *Lactobacillus jensenii* (Fettweis et al., 2014). The presence of these microorganisms protects the vaginal mucosa from potential pathogens by competing for the nutrients, blocking their adhesion (Kalia et al., 2020). Glycogen and other carbohydrates produced by the stratified squamous epithelium of the vagina promote the production of antibacterial compounds and lactic acid by Lactobacillus species, creating an acidic vaginal environment (pH 3.5-4.5) that inhibits pathogen growth (Graver and Wade, 2011).

The vagina is a delicate self-cleaning organ and does not require any special cleaning products. Despite this, a number of women have reported the use of vaginal products for hygiene purposes while some have reported inserting products on the vagina for sexual enhancement. Insertion of these products may cause chemical damage and physical abrasions, which may be exacerbated during sexual intercourse (Hilber et al., 2010). In addition, these practices may result in the intravaginal mucosal integrity being affected in various ways, some contributing

to the growth of dangerous organisms. Consequently, this may increase susceptibility to BV, placing women at an even greater risk of STI acquisition, including HIV and HPV (Cohen et al., 2012). A study that investigated the effects of feminine hygiene products on the vaginal mucosal biome produced results that provided an experimental warning on the use of products such as nonoxynol-9, vagisil, and lubricants as they may weaken the vaginal barrier by destroying *L. crispatus* and possibly other normal microflora species not assessed on the study (Fashemi et al., 2013). Not much is known about the relationship between the use of vaginal inserted products and the HPV infection. The studies that have looked at this association have found varying results. It has been suggested that susceptibility to HPV may be as a result of a ruptured stratified vaginal squamous epithelium, caused by material some use for douching, wiping (e.g., newspaper), and other intravaginal products and their harmful components (Bui et al., 2018). Douching has been shown to be associated with an increased risk of any HPV genotype, HPV infection with multiple genotypes, or the re-detection of HPV 16 in follow-up tests (Moscicki et al., 2013). Contrary, others found that douching lowers the likelihood of genital warts (HPV 6 or 11) (Low et al., 2010) and significantly decreased the prevalence of HPV infection (Lee et al., 2014). Intravaginal washing with some chemicals may disturb the vaginal microbiota and increase the vaginal pH, making it easy for unhealthy microorganisms to proliferate in the vagina, thus facilitating the entry of viral infections. Inversely, cleaning the vagina, particularly after sexual intercourse, can help eliminate the spread of HPV and reduce the risk of infection (Bui et al., 2018). This showcases the need for more research and evidence on the association of HPV infection and intravaginal inserted products (VIPs), particularly in African countries where the use of VIPs is very high.

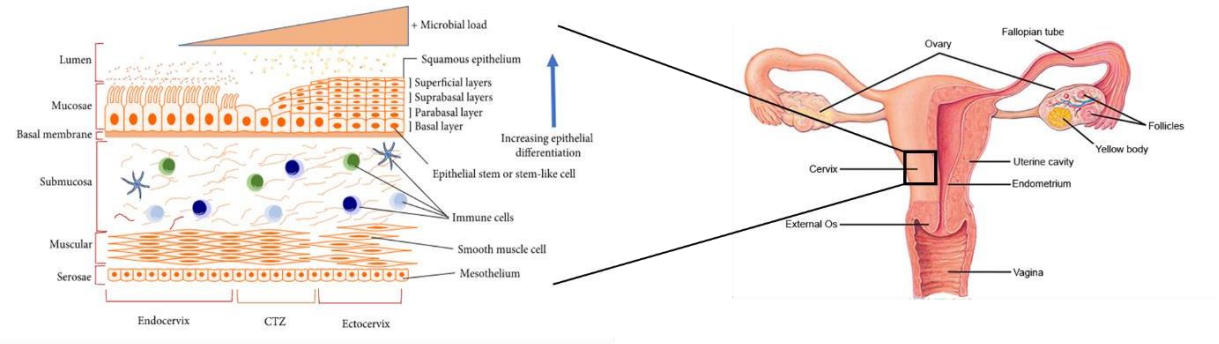


**Figure 2.4: Illustration of the normal, healthy female genital tract concentrating on the cervix.** The cervix is divided into the two main compartments, the ectocervix and endocervix, which are bordered by the cervical transformation zone (CTZ) and their associated immune cells. The cervical tissue consists of stromal and epithelial cells separated by a basement membrane. The ectocervix and vagina have squamous stratified epithelia, while the endocervix and uterus, have simple columnar epithelia. Adapted from Tomasi et al., (2019).

## 2.6 HPV vaccination

The high burden of HPV especially among adolescents requires the development of effective vaccination therapies. The first vaccine against HPV was approved by the Food and Drug Administration (FDA) in 2006 (Tomljenovic and Shaw, 2012). Three vaccines are known and commercially available (Lee et al., 2016), the first one is a bivalent HPV2 vaccine which is directed against the two commonly known high-risk oncogenic types, HPV 16 and HPV 18 (Control and Prevention, 2010a). The second vaccine is the quadrivalent HPV4, this vaccine acts against both cancer-causing types (HPV 16 and 18) as well as the low-risk types (HPV 6 and 11) known for causing genital warts (Control and Prevention, 2010b). The third vaccine is a 9-valent vaccine which was FDA approved in 2014, this vaccine is directed against 9 strains, some of which are targets of the previously mentioned genotypes (HPV 16, 18, 6, and 11) and 5 additional oncogenic strains (HPV 31, 33, 45, 52, 58) associated with cervical cancer (Petrosky et al., 2015). These vaccines were proven effective specifically against the two well-known carcinogenic HPV types (HPV 16 and 18) and promising in the prevention and reduction of cervical cancer (Joura and Pils, 2016, St Laurent et al., 2018).

HPV vaccination is given to adolescents from the age of 9 to 12 years (Rodriguez et al., 2019), as it was observed most effective when one had received it before their first sexual activity (Kudo et al., 2019, Wright et al., 2008). A school-based HPV vaccination program was launched in South Africa in 2014, with 87% of 4th grade girls receiving a single dose of the

bivalent vaccine, only 10% had mild side effects. This demonstration project was a success, partly because parents were familiar with vaccination (Delany-Moretlwe et al., 2018). Scientists are working on developing or improving the existing vaccines, while also facing the main struggle of getting people to make use of these vaccines for their well-being. This showcases the need for more education and outreach programs on cervical cancer, HPV, and vaccination, particularly in the less developed regions.

## 2.7 HPV immunity

Studies have shown that at least half of young women are infected with HPV after their first sexual encounter, and more than 80% of all women are infected at least once in their lives. It has also been observed that about 90% of HPV infections resolve within 2 years and 10% of infections continue until they eventually develop into cancer (Okunade, 2020). Immune responses clear the HPV infection in most healthy individuals (Malejczyk et al., 1997, Beachler et al., 2016). These responses are by the immune system which consists of innate immunity known as the first line of defence and exerts a nonspecific response on the infection (Iida et al., 2018), and adaptive immunity, the second line of defense that occurs in two ways: cell-mediated immune response by T cells and humoral immune response controlled by activated B cells and antibodies (Warrington et al., 2011). The clearance of HPV is said to be spontaneous but there is not a specific and clear mechanism that has been explained following HPV clearance by the immune system. Since a large percentage of this infection gets cleared, this indicates that the host defence mechanisms can effectively clear HPV infection.

### 2.7.2 Innate immune responses

The innate immune system as the first line of defence senses a threat through signals from molecules that are foreign and therefore would not normally be found in the human body. These foreign substances are recognised by pattern recognition receptors such as Toll-like receptors (TLR) (Takeda and Akira, 2005) and this triggers the sentinel cells to bring together the innate immune effectors and activate adaptive immunity (Iwasaki and Medzhitov, 2015). High expression of TLR -7, 8, and 9 have been shown in previous studies to be associated with the elimination of HPV (Daud et al., 2011).

Some of the innate immune defences include physical barriers like the mucous membrane which secretes protective fluids and antimicrobial peptides. AMPs are small host defense

peptides that are cationic and play key antimicrobial roles (Lei et al., 2019). These peptides can stop the invasion of foreign microorganisms, although the precise mechanism by which AMPs carry out their antimicrobial action is not completely understood, it has been established that they bind and interact with target cell membranes and induce cell damage (Ganz, 2003). The known group of AMPs includes alpha and β-defensins, cathelicidin, psoriasin, and RNase7. Human defensins are produced by neutrophils and epithelial cells, grouped into alpha and β-defensins all known to protect the surface of the human body against invasion of several pathogens (Bevins et al., 1999). There is only one cathelicidin gene identified in humans and it encodes the peptide LL-37, this peptide is expressed in circulating neutrophils, myeloid bone marrow cells, and epithelial cells of the skin. LL-37 has direct and indirect antimicrobial factors, acts to promote wound healing, and can also modulate adaptive immunity (Bowdish et al., 2005). Psoriasin is an AMP found mostly in healthy skin and belongs to a group of calcium-binding proteins known as the S100 proteins.

Few studies have investigated the association between AMPs and HPV. Wiens et al (2015), showed that alpha defensin 5 (HD5) can prevent furin from accessing L2 by binding directly to the viral capsid and inhibiting the cleavage of L2, important for HPV internalization in human hosts (Wiens and Smith, 2015). High levels of HBD-3 were observed in the cytoplasm of cancer cells co-expressing the HPV 16 E6 protein in their nucleus through immunofluorescence imaging, this suggested that cancer cells of HPV associated head and neck squamous cell carcinomas over-express HBD-3 (DasGupta et al., 2016). Another study showed downregulation of psoriasin in patients with HPV-induced high-grade squamous intraepithelial lesions, this differed from what others had seen with other tumours which are not HPV associated (Alvendal et al., 2019). In most cancers, psoriasin is over-expressed and considered to promote the progression of tumours (Moubayed et al., 2007). This raised a question of whether this could be one of the ways in which HPV can persist by reducing the immune response.

### 2.7.3 Adaptive immune responses

This immunity is triggered through the actions of the innate immune system, it is very important as it is recruited or activated when innate immunity fails to eliminate pathogens. There are two types of adaptive responses: cell-mediated immune responses controlled by T cells and humoral immune responses mediated by the production of B-cell antibodies (Caminero et al., 2021). Cell-mediated immunity defends an organism by activating antigen-specific cytotoxic T

cells, which cause apoptosis of cells that display foreign antigens, such as virus-infected cells (Marshall et al., 2018). The humoral immune response works by the activation of B cells, which when activated secrete antibodies known as immunoglobulins. These circulate in the bloodstream and permeate other body fluids, where they bind selectively to the foreign antigen that triggered their secretion. Antibody binding renders viruses and microbial toxins inactive by preventing them from binding to receptors on host cells, making them more susceptible to phagocytosis instead (Marshall et al., 2018).

Adaptive immune responses have been implicated in the control of early HPV infections (Westrich et al., 2017). CD4 T cell responses are very important in launching an efficient immune response against the E6, E7, and possibly the E2 protein of high-risk HPV (de Jong et al., 2002, Stanley, 2009), this was further supported by a study that displayed an E6 specific memory T helper cell response against HPV16 (Welters et al., 2003). Cytotoxic effector cells induce cytotoxicity which is also among the important approaches in controlling and clearing viral infections including HPV. Cytotoxic T cell responses against the E6 protein were detected in a study that investigated cytotoxic responses against E6 and E7 HPV-16 proteins in subjects without cervical abnormalities and those with cervical intraepithelial neoplasm.

### 2.7.4 Evasion of the immune system by HPV

With all these possible countermeasures which have proven to succeed in clearing most of the HPV infections, some of the infections, however, still manage to stay in the system unnoticed for quite a long time. This is possible because HPV can evade the immune system through several mechanisms (Figure 2.5) (Grabowska and Riemer, 2012, Westrich et al., 2017). HPV infection invades the human host through the basal keratinocytes of the mucosal epithelium (Münger et al., 1989). The virus infects only epithelial cells, and the entire reproductive cycle occurs away from immune effector cells. HPV uses the keratinocyte differentiation pathway for its replication and assembly. It has no cytolytic effects on the host cells as it uses cells already destined to die by anoikis and there is no viremic phase of the life cycle, therefore no inflammation occurs during the HPV infection cycle (Stanley, 2012). The immune effector cells are not alerted about the abnormal activity in the host, and the virus is barely recognized, so no immune response is triggered. HPV further weakens the immune system by destroying adaptive immunity. It interferes with type 1 interferons (IFN), IFN-α, and IFN-β through its E6 and E7 oncoproteins (Beglin et al., 2009), thereby preventing the initiation of antiviral activity. The infected keratinocytes are compromised in a way that does not allow the release of

inflammatory cytokines leading to no activation of skin Langerhans cells required to induce adaptive immunity (Morris et al., 1983). HPV also downregulates HLA class 1 by trapping peptide-loaded HLA class 1 receptors on the Golgi body through E5 proteins thus avoiding cytotoxic detection (Ashrafi et al., 2006, Zhou et al., 2019). Although this activates natural killer cells, very few are resident or move to this cell layer.

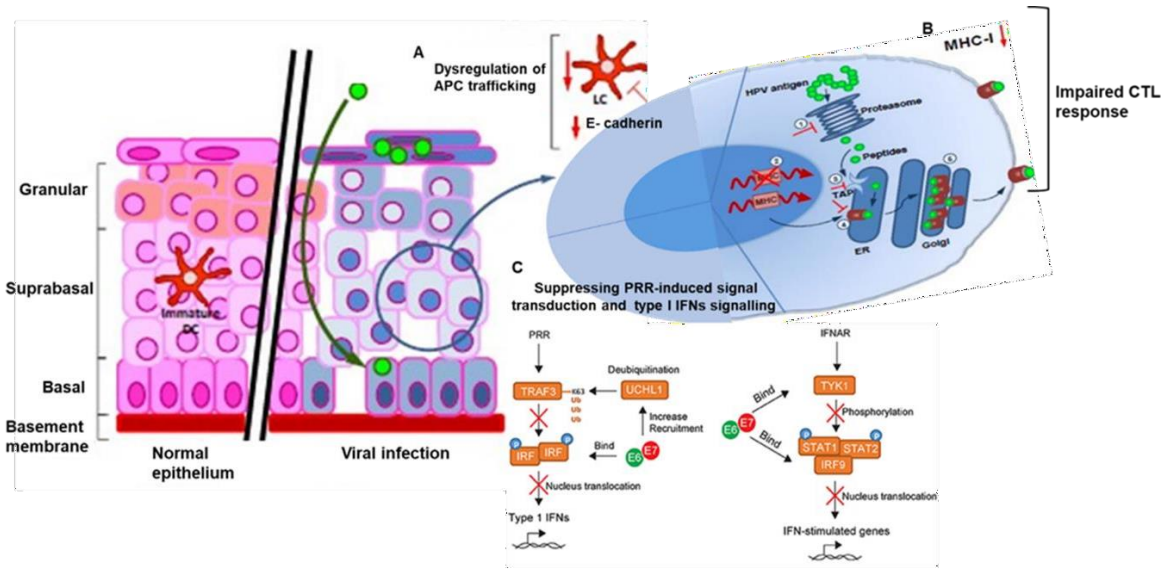


**Figure 2.5: Immune evasion mechanisms used by HPV oncoproteins.** HPV can avoid the immune system and stay unnoticed for a long time through (A) the E6-mediated reduced expression of the epithelial adhesion molecule E-cadherin on HPV-infected keratinocytes, a molecule used by Langerhans cells (LCs) for adhesion to keratinocytes and by modulating APC trafficking. (B) inhibiting the expression of proteasome subunits, MHC class I, TAP and reducing MHC-I trafficking by directly interacting with the MHC-I heavy chain and trapping MHC-I molecules in the Golgi apparatus. (C) High-risk HPV E6 and E7 inhibit the activation of TNF receptor-related factor 3 (TRAF3) by blocking pathogen recognition receptors (PRR) signal transduction cascades through upregulating ubiquitin C-terminal hydrolase L1 (UCHL1) and by binding to the transcription factor regulated by interferon (IRF), preventing its transcriptional activity in the nucleus. These oncoproteins also bind to tyrosine kinase 2 (TYK2) to hinder the phosphorylation of STAT1 and STAT2 and interact with IRF9 to prevent it from binding to phosphorylated STAT1 and STAT2 to activate IFN-stimulating genes, thereby interfering with the IFN-α / β receptor (IFNAR) signalling pathway. Adapted from Grabowska and Riemer (2012) and Zhou et al., (2019).

## 2.8 Conclusion

While great progress has been made in understanding how HPV evades the immune system, knowledge gaps exist. The pathogenesis of the HPV infection is not entirely understood, especially in terms of the risk factors involved and how it affects or is affected by the immune system. The goal of this study was to research the use of intravaginal inserted products by adolescent females and adult women as a risk factor for HPV infection, in addition, it also focuses on how some of the immune components impact on HPV clearance.

# CHAPTER 3: MATERIALS AND METHODS

## 3.1. General reagents used

Phosphate Buffered Saline (PBS, Lonza, Walkersville, MD USA), R10 medium (made of Roswell Park Memorial Institute Medium (RPMI) 1640, 10% heat-inactivated Fetal Bovine Serum (FBS), 6% penicillin/streptomycin) from Lonza, Walkersville, MD USA and Cellfix (Becton Dickinson Benelux N.V., Belgium).

## 3.2. Study setting and cohort

This study was nested within the parent study – called MIST (Mucosal Injury from Sexual Trauma) which is a longitudinal cohort study currently ongoing at the CAPRISA (Centre for the AIDS Programme of Research in South Africa) Clinical Trials Unit at Vulindlela, KwaZulu-Natal, Biomedical Research Ethics Committee (BREC) reference number-BF504/17. The MIST study was aimed at investigating unique socio-behavioural, reproductive tract anatomical and biological characteristics around sexual debut in adolescent females (14-19 years) in response to early sexual exposure, male semen products, mucosal trauma and wound healing compared to a group of older women (25-35 years). Participants came for study visits at baseline (no use of vaginal products and no sexual intercourse for at least 2 weeks) and two reported sexual acts.

For this study, specimens collected at baseline from 154 sexually active adolescent girls and adult women aged 14-19 and 25-35 years, respectively, were utilized. Of the 154 female participants, 96 were adolescent girls and 58 were adult women. Participants who were HIV positive, pregnant, had a history of cervical disease, and had taken any form of antibiotic in the past month prior to enrolment were excluded from the study. The HPV vaccination status for all the study participants was unknown. Written informed consent was obtained from all participants before enrolment and for minors between the ages of 14-17 years, both assent and parental informed consent were sought. This study protocol was approved by the BREC of the University of KwaZulu-Natal, ref no: BREC/00001015/2020.

## 3.3. Assessment of socio-behavioural factors

A questionnaire with questions relating to multiple levels affecting an individual's specific socio-behavioural risk profile was administered. Specifically, a detailed questionnaire was administered at the CAPRISA clinic assessing behaviour, sexual practices, vaginal practices, menstrual and health history. In addition to the questionnaires, all study participants were given diaries to record sexual behaviour, coital frequency, and vaginal product use that was reviewed at scheduled visits to assist with recall and accurate capturing of sexual behaviour.

## 3.4. Specimens collection and STI testing

From the parent study, a pregnancy test was performed at each visit using a urine dipstick pregnancy test and must have been negative prior to any genital specimen collection. The remainder of the urine specimens collected for pregnancy testing were then used to test *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) infections by the Cepheid Xpert CT/NG assay, performed on the GeneXpert® Instrument Systems at the Vulindlela site laboratory. From each participant and at each visit, vaginal samples were collected under speculum examination by clinicians. A lateral vaginal wall/posterior fornix swab was used to test BV by Nugent score and to detect *Trichomonas vaginalis*. These two tests were completed at Neuberg Global Laboratories, Amanzimtoti, South Africa. Vaginal pH was measured by the clinical staff by placing the vaginal swab on a piece of pH test paper and comparing change against a reference chart. Other specimens that were collected from the parent study include, the endocervical cytobrush, cervicovaginal swabs, soft cups, and vulvovaginal swabs, for this project, endocervical swabs collected at baseline visit were used for the measurement of AMP concentrations (Section 3.5) and HPV genotyping (Section 3.6). These swabs were placed in 1 milliliter (ml) tubes and kept at -80°C until use. In addition, cervical cytobrush specimens collected at baseline were used to measure cervical T cell activation, directly *ex vivo* (Section 3.7).

## 3.5. Measurement of Antimicrobial peptides concentrations by Enzyme-linked Immunosorbent Assay.

The concentrations of HBD-1 and HBD-2 and Psoriasin (S100A7) were measured in vaginal secretions using sandwich ELISA HBD-1 and HBD-2 ELISA kits (Novus Biologicals, Inc., Centennial, Colorado, USA, Catalog# NBP2-67933, NBP2-77363, respectively) and S100A7 ELISA kit (Elabscience Biotechnology, Inc., Texas, USA, Catalog# E-EL-H1296) according to the manufacturer's instructions. Before the ELISA experiment, PBS (650 µl) was added to each endocervical swab, and the samples were left to thaw at 4°C overnight. A portion of each sample (150 µl) was centrifuged (Eppendorf Centrifuge 5430 R, Eppendorf AG, Hamburg, Germany) at 425×g for 10 minutes at 4°C, and the supernatant was stored at -80°C until the time of the assay. A standard working solution (100 µl per well) was added to the first two columns of the plate and thawed samples were added to other wells (100 µl per well), the plate was covered and incubated at 37°C for 90 minutes. After the incubation period, the liquid was removed from each well and a biotinylated detection antibody (100 µl) was immediately added to each well, mixed gently, and incubated at 37°C for 1 hour. The solution was decanted, and wells were washed 3 times for 2 minutes with 350 µl of wash buffer which was prepared before the experiment. Avidin-Horseradish Peroxidase conjugate working solution (100 µl) was then added to each well and the plate was incubated at 37°C for 30 minutes. After removing the solution from each well, the wash step was repeated 5 times. Thereafter, a substrate reagent was added to each well for incubation of 15 to 25 minutes at 37°C according to the actual colour change, the plate was covered the whole time for protection against light. The reaction was then terminated by the addition of a stop solution (50 µl) in the same order as when the substrate solution was added. The optical density in each well was measured at once with a VersaMax microplate reader (Molecular devices, Sunnyvale, California, USA) set at a wavelength of 450 nm.

## 3.6. Human papillomavirus (HPV) genotyping

### 3.6.1. Polymerase Chain Reaction (PCR)

Stored endocervical swabs were in 400 µl of PBS, these were vortexed and a volume of 100 µl of the samples was removed and pipetted into a 96 well plate. The plate was centrifuged (Eppendorf Centrifuge 5810, Eppendorf AG, Hamburg, Germany) at a maximum speed of

2250×g for 30 minutes. The supernatant was decanted, and the cell pellet was re-suspended in 300 µl DNase/RNase-free double distilled water, the sample was mixed by vortexing at low-medium speed. A volume of 30 µl of the suspension was used as a DNA template for PCR.

A volume of 30 µl of the suspension was added into the PCR tubes/strips containing the lyophilised PCR mix per sample to be analysed. The mix was homogenized by pipetting or centrifuging for a few seconds. The PCR tubes were placed in a thermal cycler (Applied Biosystems™ SimpliAmp™ Thermal Cycler, Thermo Fisher Scientific Inc) under the following amplification conditions:

**Table 3. 1: PCR conditions.**

| No. of Cycles | Temperature | Time |
|---|---|---|
| 1 Cycle | 25°C | 10 min |
| 1 Cycle | 94°C | 3 min |
| 15 Cycles | 94°C | 30 s |
| | 47°C | 30 s |
| | 72°C | 30 s |
| 35 Cycles | 94°C | 30 s |
| | 65°C | 30 s |
| | 72°C | 30 s |
| 1 Cycle | 72°C | 5 min |
| | 8°C | ∞ |

The PCR samples were denatured before the hybridization procedure at 95°C for 10 minutes in a thermocycler and cooled on ice for at least 2 minutes. Reagent A (Hybridization solution) was pre-warmed at 41°C.

### 3.6.2. Hybridization process

To begin the hybridization process, the hybriSpot machine (HS12, Vitro, S.A., Sevilla, Spain) was set to wizard mode for automatic display of instructions, HPV test was selected, and each HPV Chip was placed in the indicated position in the HS12 device chamber. The temperature of the chamber was automatically set to 41°C. A volume of 300 µl of pre-heated Reagent A (Hybridization solution) was dispensed into each Chip and incubated at 41°C for at least 2 minutes, when the time was up the reagent was automatically removed by vacuum. A volume

of 270 µl of preheated Reagent A (41°C) and 30 µl of each denatured PCR product was dispensed into the HPV Chip and incubated at 41°C for 8 minutes. The reagent was automatically removed by vacuum and 3 washes were performed with 300 µl of pre-heated Reagent A (41°C). The temperature of the chamber was then set automatically to 29°C. A volume of 300 µl of Reagent B (Blocking solution) was dispensed into each Chip and incubated for 5 minutes. Thereafter, the reagent was automatically removed by vacuum. A volume of 300 µl of Reagent C (Streptavidin-Alkaline Phosphatase) was dispensed into each Chip and Incubated for 5 minutes at 29°C. The reagent was automatically removed by vacuum and the chamber temperature was set to 36°C. Four washes were performed with 300 µl of Reagent D (Washing buffer I), then 300 µl of Reagent E (substrate and chromogen) was dispensed into each Chip and Incubated at 36°C for 10 minutes. The reagent was automatically removed by vacuum and 2 washes were performed by dispensing 300 µl of Reagent F (Washing buffer II) into each Chip. The reagent was then automatically removed by vacuum and Chips were picked up for image capture and analysis. The sample management, image capture, analysis, report, and LIS connection were supported by the hybriSoft software, version 2.0 (Vitro, S.A.).

## 3.7. Immune activation status of CD4 T cells and CD8 T cells.

### 3.7.1. Cervical mucosal sample collection and processing.

A Digene cervical cytobrush specimen was collected under speculum examination by inserting the brush into the endocervical os and rotating it for one 360° turn to obtain cervical cells, as previously described (Gumbi et al., 2008), maintained at 4°C in a benchtop cooler in 3 ml transport medium (RPMI containing 10% FBS and Pen/Strep/Amphotericin B) and transported to the laboratory within four hours of collection. In the laboratory, the cervical cytobrush was rotated against the side of the centrifuge tube to dislodge the cells from the cytobrush, then flushed with the 3 ml of transport medium it comes with and flushed for the second time with another 3ml of fresh medium. The cell suspension was centrifuged at 300×g with brakes for 7 minutes at 4°C to pellet the cervical cells. Supernatants were decanted into small vials and stored at - 80°C for later use and the cell pellet was retained and used for evaluation of T cell immune activation status by Fluorescence- activated cell sorting (FACS).

### 3.7.2 Flow cytometry panel

The panel was pre-optimized for use on the BD LSR Fortessa$^{TM}$ flow cytometer (Becton Dickinson Immunocytometry Systems). This flow cytometer can be configured with up to 7

lasers (blue, red, violet, UV, and yellow-green) and can accommodate the detection of up to 18 colors simultaneously. The viability marker fluoresces in the Pacific blue channel. Therefore, CD14, CD19, and Dead cells were placed on the same channel (Pacific blue channel, also called a dump channel), as these were to be excluded. CD3 was placed in the Allophycocyanin-H7 (APC-H7) channel, CD4 was placed in the Phycoerythrin cyanine 5.5 (PECy5.5) channel, CD8 was placed in the Brilliant Violet 711 (BV711) channel, Human leukocyte antigen (HLA-DR) was placed in the Alexa-fluor-700 channel, CD38 was placed in the Phycoerythrin cyanine 7 (PE-Cy7) channel, and C-C chemokine receptor type 5 (CCR5) was placed in the Alkaline Phosphatase Conjugate (APC) channel.

### 3.7.3. Staining of non-viable cells using live/dead fixable dead cell stain (Vivid)

The stock concentration of Vivid was prepared by reconstituting with 50 µl Dimethyl Sulfoxide. The reconstituted Vivid was diluted in a 1:40 ratio with PBS in a labelled Eppendorf. The Vivid working solution was always made fresh and used to make the master mix according to the number of reactions.

For sample staining, the cell pellet that was retained during cytobrush processing was re-suspended in fresh R10 medium (RPMI 1640, 10% FBS, 6% penicillin/streptomycin) and the tubes were centrifuged at 300×g for 7 minutes at 4°C, the supernatant was poured out and the pellet re-suspended in 5 ml of fresh medium. A volume of 2 ml of the cell suspension was pipetted into a falcon tube (Becton Dickson) and the remaining volume of the tube was filled with PBS centrifuged (HERMLE Z 446 centrifuge) at 170×g for 5 minutes to wash the sample. After centrifugation, the supernatant was poured out and the pellet re-suspended in 50 µl of pre-titrated Vivid (live/dead cell marker), vortexed, and incubated in the dark, at room temperature for 20 minutes. The cells were then washed with 2% FBS, centrifuged at 170×g for 5 minutes.

### 3.7.4. Staining of cells for chemokine receptor CCR5 and surface makers

The cells were stained for chemokine receptor CCR5 by adding 50 µl master mix of APC labelled anti-CCR5 prepared according to the number of reactions or samples (Table 3.2). The samples were incubated in the incubator set at 37°C for 20 minutes. After incubation, the samples were washed with 2% FBS at 170×g for 5 minutes. The cells were then stained for surface markers (APC-H7-labelled anti-CD3, PECy5.5-labelled anti-CD4, BV711-labelled

anti-CD8, PE-Cy7-labeled anti-CD38, Alexa flour 700-labelled anti-HLA-DR, Pacific blue-labelled anti-CD19, and anti-CD14) by adding 50 µl of the master mix per one reaction (Table 3.2), vortexed and incubated at room temperature for 20 minutes. The cells were washed with 2% FBS, centrifuged at 170×g for 5 minutes. The cell pellet was re-suspended in 150 µl Cellfix. Cells were stored at 4°C and acquired using a BD LSRFortessa$^{TM}$, within 48 hours of staining. All fluorescently labelled antibodies were obtained from Becton, Dickinson, and Company (San Jose, CA, USA).

**Table 3. 2: Antibodies (chemokines and surface markers) with the pre-titrated volumes for a master mix preparation of one reaction.**

| **Chemokine master mix stained at 37 degrees Celsius (ºC)** | |
|---|---|
| CCR5 APC | 10 µl |
| 2% FBS | 40 µl |
| **Surface markers master mix stained at room temperature** | |
| CD3 APC-H7 | 1.5 µl |
| CD4 PE-Cy5.5 | 0.5 µl |
| CD8 BV711 | 0.8 µl |
| CD14 Pac blue | 1 µl |
| CD19 Pac blue | 2 µl |
| CD38 PE-Cy7 | 1 µl |
| HLA-DR Alexa flour 700 | 2 µl |
| 2% FBS | 41.2 µl |

### 3.7.5. Compensation controls

These are single-stained controls used to calculate fluorescent spillover between channels. The compensation beads (BD Bioscience) were stained with individual fluorochrome-conjugated antibodies for use as single colour compensation controls. These were prepared by first labelling 8 FACS tubes according to each fluorochrome. For Pacific blue, either CD14 or CD19 was used. The negative control which only had beads was also included. The compensation beads were first vortexed, then one drop was placed in each labelled FACS tube followed by the addition of antibodies to the corresponding tube at the optimal titre, leaving out the negative control tube. All the antibodies used anti-mouse compensation beads. The tubes were centrifuged at 800×g for 4 minutes at 4°C. The tubes were vortexed for resuspension and incubated at room temperature in the dark for 20 minutes. A volume of 1 ml PBS was added to

each tube and centrifuged at 800×g for 3 minutes at 4°C. The supernatant was removed and 150 µl of 1× cellfix was added to each tube and the tubes were vortexed briefly for resuspension before acquisition.

### 3.7.6. Preparation of Rainbow beads

The detected fluorescence intensity on a flow cytometer is influenced by different parameters such as the laser power, alignment, temperature, and optimal efficiency. When setting up an experiment, the sensitivity of the detectors is modified to optimize instrument performance for the run. As these voltages may not be optimal on the next run, acquiring rainbow beads and adjusting for target values before every experiment is important (Mizrahi et al., 2018). These were prepared by diluting one drop of well-mixed rainbow beads in 300 µl of flow buffer.

### 3.7.8 Fluorescence minus one (FMO) control

These controls were prepared by first labelling FACS tubes with each fluorochrome (APC-H7 labelled anti-CD3, PE-Cy5.5–labelled anti-CD4, BV711–labelled anti-CD8, PE-Cy7-labeled anti-CD38, Alexa flour 700-labelled anti-HLA-DR, Pacific blue-labelled - Live/dead). Approximately $1\times10^6$ of previously isolated peripheral blood mononuclear cell (PBMC) cell suspension was added into each tube. PBMC cells were spun at 800×g for 10 minutes, the supernatant was decanted, and the tubes flicked to re-suspend the cells. An appropriate volume of each antibody (according to the antibody titrations) was added minus the antibody being tested, the one labelled in the tube, and centrifuged at 800×g for 3 minutes at 4ºC. The tubes were vortexed and re-suspended, incubated for 20 minutes in the dark, at room temperature. PBS (1ml) was added to each tube and centrifuged at 800×g for 3 minutes at 4ºC. The supernatants were decanted, and 150µl of 1× cell fix (1ml of 10× cell fix added to 9ml distilled water) was added to each tube and vortexed briefly.

### 3.7.9. Data acquisition

Stained cells were acquired on the BD LSRFortessa™ flow cytometer with FacsDiva software. The number of events collected was 500000-1000000. Data were analyzed using FlowJo version 10.7.1 (Treestar; Ashland, OR). FMO controls were used to set gates.

### 3.7.10. Gating strategy

The gating strategy is done to ensure that the gates are placed upon the cell populations of interest to investigate and quantify these populations. The first step in the gating strategy used in this study included the time plot, which is where a gate is placed in parts where there are no interferences, this removes any inconsistencies that might have occurred during the sample acquisition process. The second plot was the forward scatter- height against forward scatter- area, which ensures that the fluorescence detected is from single cells by excluding the duplets or any clumped cells. When distinguishing cell populations, the first step is based on forward and side scatter properties which give an estimation of the size, and the granularity of the cells, to help distinguish between cell types. The cell type of interest were cervical lymphocytes. Plotting the dump channel (pacific blue) against CD3 APC-H7 ensured the exclusion of cell debris, dead cells, and monocytes by gating on the positive CD3 lymphocyte population to further examine T cells. The CD3+ T cells were further identified and gated by the expression of CD4+ and CD8+ T cells subsets, the relative expression of CD38+, HLA-DR+ activation markers, and the C-C chemokine receptor, CCR5+ (Figure 3.1).

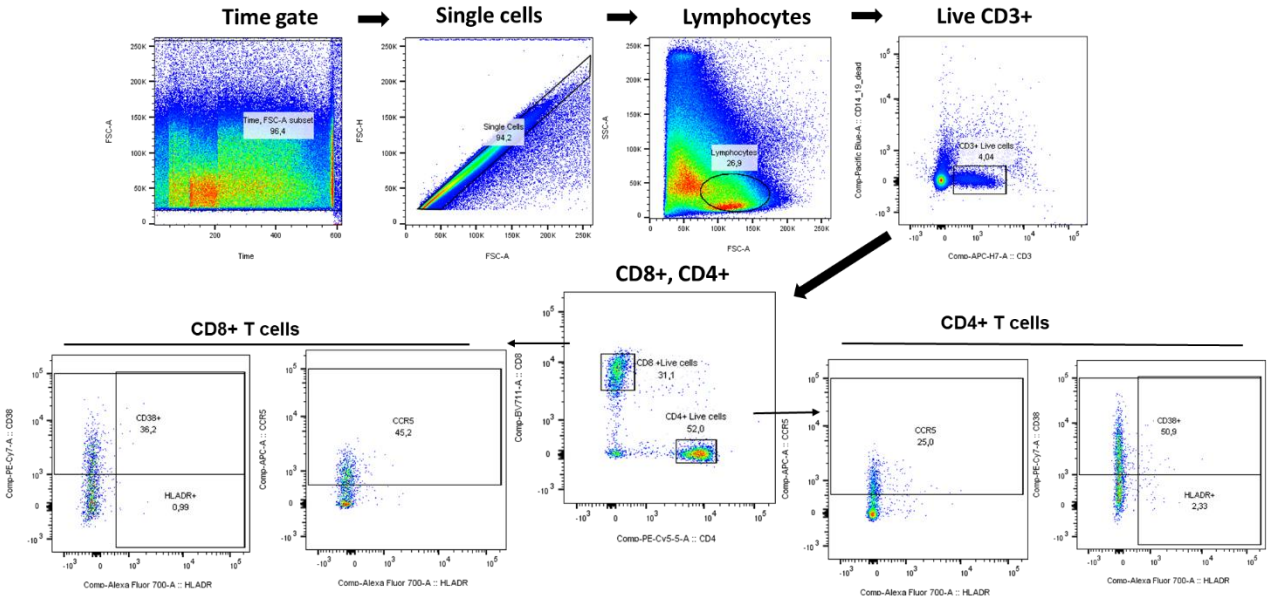


**Figure 3.1: The sequential gating strategy applied for the flow cytometry analysis.** The first flow plot shows the measurement of forward scatter height and time, the second plot shows the measurement of forward scatter height and forward scatter area to determine the single cells inside a diagonal gate. From the singlets, the analyses were narrowed by plotting side scatter area, and forward area scatter to identify cervical lymphocytes (inside an oval gate) while excluding B cells, monocytes, granulocytes, and dead cells. From the lymphocytes gate, a viability gate is set up by plotting dump channel using Pacific blue (dead cells, CD14 and CD19) with CD3+ T cells and gating on live CD3+ T cells. Live CD3+ T cells were further subdivided into CD4+, and CD8+ T cells and these were subsequently analysed for CCR5 and activation markers of interest HLA-DR+ and CD38+.

## 3.8. Statistical analysis

The raw data analysis and statistical tests were performed with Graph-Pad Prism version 7 (GraphPad Software, San Diego, CA). Robust Poisson regression models (RPRM) were fitted to investigate the prevalence of HPV in women. The prevalence obtained did not include the pre- known prevalence, meaning it was higher than normal. Considering the cut-off value (<10%) of a rare outcome (Ranganathan et al., 2015) and the cut-off risks (<20%) of two groups to be compared, we considered the outcome for this study to be frequent. As suggested by Davies et al. (1998), the cut-off risks (<20%) of a group category to be compared confirm if the outcome is frequent (Davies et al., 1998). In our study, the two population groups of interest were age-phase and the VIPs use. The authors indicated that when the outcome is common, odds ratio (OR) turns to exaggerate the effect size (Chen et al., 2018). Based on these observations the reasonable choice of the measure for the effect size was the relative risk as opposed to OR. To compare the differences between tested groups, the Tukey multiple comparison adjustment was used. For T cells and AMP data, comparisons were done using the Kruskal-Wallis Test and the Mann Whitney U test. $p$ values of $<0.05$ were statistically significant.

# CHAPTER 4: RESULTS

## 4.1. The distribution of high-risk and low-risk HPV genotypes in adolescent girls and adult women.

The distribution of HPV genotypes in adolescent girls and adult women was determined. The HPV prevalence in the total study population was found to be 85% (95% CI 78.44-90.29%). Among these, 19% had low-risk genotype(s) only, 21% had high-risk genotype(s) only and 45% had both low-risk and high-risk genotypes. In adolescents, the prevalence was 90%, among these, 18% had low-risk genotype(s) only, 16% had high-risk genotype(s) only and 56% had both low-risk and high-risk genotypes (Figure 4.1A). The prevalence of HPV in adults was slightly lower (78%) compared to adolescents, among the infected adults, 21% had low-risk HPV genotype(s) only, 31% had high-risk genotype(s) only and 26% had both low-risk and high- risk genotypes (Figure 4.1A).

There were more HPV genotypes detected in adolescents compared to adults. The most prevalent low-risk genotypes in adolescents were HPV62/81 (32%), HPV6 (17%), HPV67 (16%), HPV44/55 (15%) and HPV42, 54 (10%) while the common low-risk genotypes in adults were HPV62/81 (21%), HPV44/55 (17%) and HPV71 (10%). The common high-risk genotypes in adolescents were HPV52 (21%), HPV39, 59 (16%), HPV35 (14%), HPV45 (13%), HPV31, 51 (11%) and HPV16, 18, 56, 58 (10%) and in adults, it was HPV35 (19%) and HPV16 (10%). HPV genotypes that were common in both adolescents and adults were HPV62/81, HPV44/55, HPV16,45 (10%), HPV39, 59 (11%) and HPV35, 52 (16%) (Figure 4.1B).

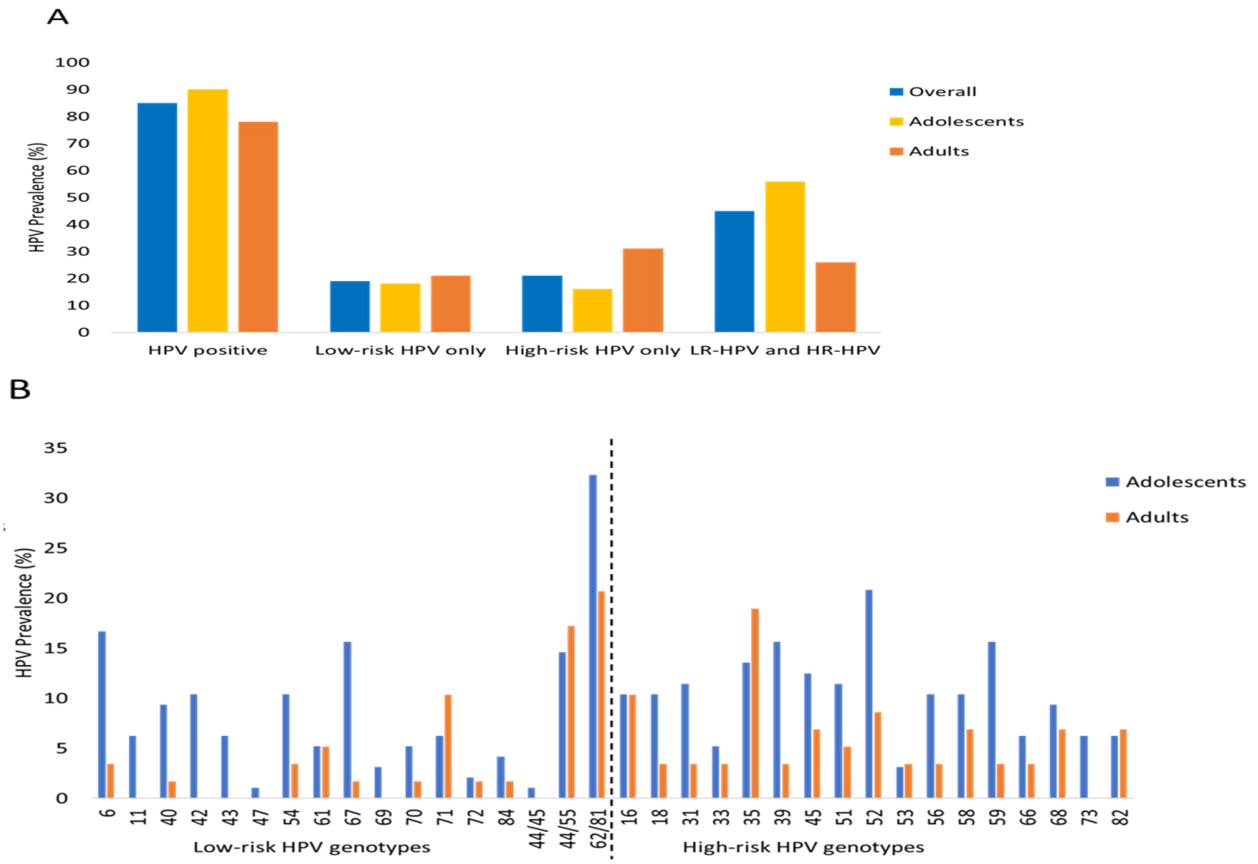


**Figure 4.1: HPV prevalence (%) in sexually active adolescents and adults**. The bar graphs show (A) the prevalence of overall HPV, low-risk HPV, high-risk HPV, and the prevalence of both low and high-risk HPV in overall females, adolescents, and adults. Low-risk HPV genotypes are defined as HPV type 6, 11, 40, 42, 43, 47, 54, 61, 67, 69, 70, 71, 72, 84, 44/45, 44/55 and 62/81 while High-risk HPV types are defined as HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82, according to those detected in this study. (B) The prevalence of each low-risk and high-risk genotype was also analysed and is represented in a bar graph, a dotted line is used as a form of separation between low-risk genotypes and high-risk genotypes. Statistical comparisons of HPV prevalence between adolescents and adults were done by conducting a t-test (statistical values not shown as they were not significant).

## 4.2. The distribution of high-risk and low-risk HPV genotypes in users and non-users of VIPs.

Twenty-six percent of the participants enrolled in this study were using VIPs. A variety of products were reported, and these products were used for different purposes (Table 4.1). Depending on the reason for use, both the frequency of use and the timing were diverse. In adolescent girls, the use of alum powder (potassium aluminium sulfate) was common (52%) and

this was followed by ibhodwe labafazi (scented petroleum jelly) at 17%. Alum and ibhodwe labafazi were mainly used for sexual pleasure (i.e., vaginal tightening and drying) and sexual stimulation, respectively. In adult women, snuff (tobacco leaves) and ibhodwe labafazi were the most commonly used products, 17% and 44%, respectively. Snuff was mainly used for sexual stimulation and vaginal tightening (Table 4.1).

The impact of VIPs use on HPV risk was investigated. Figure 4.2 displays the prevalence of HPV as well as the distribution of HPV genotypes in participants who use VIPs compared to those who do not. Overall, VIPs users had a slightly higher HPV prevalence compared to non-users, although this was not statistically significant when a t-test was used (Figure 4.2A). VIPs users were more likely to be infected with both high-risk and low-risk genotypes, whereas the non-users had a higher prevalence of high-risk genotypes only. The most common low-risk genotypes detected in the study cohort amongst the VIPs users were HPV62/81 (24%), HPV6 (17%), HPV71 (15%), HPV67 (12%), and HPV54, 61, 44/55 were detected in 10% of participants. In the non-users, the common low-risk genotypes were HPV62/81 (25%), HPV44/55 (18%) and HPV67 (10%). The most common high-risk genotypes amongst the VIPs users were HPV52 (24%), HPV16 (15%), HPV39 (12%), and HPV18, 33, 51, 53, 56, 59 were detected in 10% of infected individuals. High-risk genotypes which were mostly detected in those who did not use VIPs were HPV35 (19%), HPV52 (13%), HPV45, 59 (12%), HPV39 (11%), HPV31, 58, 68 (10%) (Figure 4.2B).

When adolescents were analysed separately from adults, the most prevalent low-risk genotypes in the VIPs users were HPV62/81 (39%), HPV6 (26%), HPV67 (22%), HPV54 (17%), HPV40, 61, 71 (13%), while in non-users the most prevalent low-risk genotypes were HPV62/81 (30%), HPV44/55 (18%), HPV6, 67 (14%) and HPV42 (11%). The common high-risk genotypes in adolescent VIPs users were HPV52 (35%), HPV16, 39 (22%), HPV59 (17%) and HPV73 (13%), while the most common high-risk genotypes in non-users were HPV35, 52 (16%), HPV59 (15%), HPV31, 39, 45 (14%), HPV51, 58 (12%), HPV18, 56, 68 (11%) (Figure 4.2C). In adult VIPs users, the common low-risk genotypes were HPV62/81 (33%) and HPV44/55, 71 (17%), and in non-users, HPV62/81 (15%) and HPV44/55 (18%) were the most common low-risk genotypes. The most prevalent high-risk genotypes in adult users were HPV18, 33, 51, 52, 53, 56, 58 and 73 (10%) while in non-users, HPV35 (25%) and HPV16 (13%) were the common high-risk genotypes (Figure 4.2D).

**Table 4.1: Vaginal inserted products used by adolescents and adults, description and reason for use.**

| Name of product | Ingredients/description | Reason for use | No. of participants who used the product, no. (%) | |
|---|---|---|---|---|
| | | | Adolescents (n=23) | Adults (n=18) |
| Alum | Combination of aluminium sulphate and potassium sulphate | Dries and tightens the vagina | 12(52) | 1(6) |
| Snuff | Tobacco leaves | Sexual stimulation, tightens the vagina | 1(4) | 3(17) |
| Powder substance | Unknown | Tightens and heats the vagina | 0 | 1(6) |
| Vinegar | Fermented ethanol or sugars by acetic acid bacteria | Detoxification, warms up the vagina | 0 | 1(6) |
| Herbal vaseline | Paraffin oil, paraffin wax, petroleum, fragrance, vitamin E | Lubrication | 0 | 1(6) |
| Tsitsi powder | Unknown | Dries, tightens the vagina | 0 | 1(6) |
| Halls sweet | Menthol, Eucalyptus oil, glucose Syrup, Sucrose | Tightens and warms up the vagina | 1(4) | 0 |
| Idliso labafazi | Unknown | Sexual stimulation, tightens the vagina | 0 | 1(6) |
| Blue stone | Copper Sulphate | Tightens the vagina | 0 | 1(6) |
| Ugogotshitshi | Unknown | Tightens the vagina | 1(4) | 0 |
| Traditional herbs | A variety of herbs | Detoxification, tighten and warm up the vagina | 1(4) | 0 |
| Ice cubes | Water | Tighten the vagina | 1(4) | 0 |
| Holy ash | Burnt dried wood, burnt cow dung | Sexual stimulation, dries and warms the vagina | 1(4) | 0 |
| Tartaric | potassium hydrogen tartrate, calcium tartrate | Dries, tightens the vagina | 1(4) | 0 |
| Ibhodwe labafazi (women's pot) | Pink vaseline/cream | Detoxification/freshening, sexual stimulation, dries, lubricates, tightens and warms up the vagina | 4(17) | 8(44) |
| Newspaper | Any plain print newspaper | Dries the vagina | 1(4) | 0 |


**Figure 4.2: Distribution and Prevalence of low-risk and high-risk HPV genotypes in users and non-users of virginal inserted products (VIPs)**. These bar graphs show (A) the prevalence of overall HPV, low-risk HPV, high-risk HPV and of both low-risk and high-risk HPV. The distribution of each HPV genotype in overall users and non-users (B), adolescent users and non-users (C) as well as in adult users and non-users (D). Low-risk HPV genotypes are defined as HPV type 6, 11, 40, 42, 43, 47, 54, 61, 67, 69, 70, 71, 72, 84, 44/45, 44/55 and 62/81 while High-risk HPV types are defined as HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82. A dotted line is used as a form of separation between low-risk genotypes and high-risk genotypes. Statistical comparisons of HPV prevalence between users and non-users were done by conducting a t-test (statistical values not shown as they were not significant).

## 4.3. Factors associated with HPV acquisition risk in adolescent girls and adult women and the impact of VIPs use.

Several factors which have been previously associated with HPV natural history were described in the sample of women in this study (Table 4.2). The two population groups of interest were age-phase and VIPs use. From Table 4.2, the adolescent group dominated the study population, contributing 62.75% which is more than the adult group by 25.50%. The VIPs users contributed only 26.6% of the women in the study. Some of the factors previously associated with HPV natural history were similar between adolescent girls and adult women. These factors included sexual debut, number of sexual partners, and vaginal pH. The median age at first sex for adolescents was 16 years and ranged from 15 to 17 years, whereas for adults it was 17.5 years with a range of 16 to 18 years. Adolescents reported an average of 2 sexual lifetime partners compared to adults who reported an average of 3 sexual lifetime partners. The median vaginal pH was 4.7 for both adolescent girls and adult women. More than 50% of both adolescent girls and adult women had BV. On the other hand, the use of condoms was less common in adolescent girls (10.42%) compared to adult women (22.41%). Furthermore, STIs such as *Neisseria Gonorrhoeae* and *chlamydia* were more common in adolescent girls compared to adult women. The prevalence of *Neisseria Gonorrhoeae* was 8.33% in adolescent girls compared to 3.45% in adult women, and the prevalence of *chlamydia* was 35.42% in adolescent girls compared to 8.62% in adult women. Adults (53.45%) were more likely to use contraceptives compared to adolescent girls (37.50%).

When VIPs users were compared to non-users (irrespective of age), it was observed that most of the factors previously associated with HPV natural history were similar between these two groups (Table 4.2). VIPs non-users were more likely to use condoms compared to VIPs users (16.81% compared to 9.76%), even though *Neisseria Gonorrhoeae* was slightly more common in non-users (7.96%) compared to users (2.44%).

**Table 4.2: Population group characteristics within the age-phase group and the vaginal inserted products (VIPs) non-users and users.**

| | Age-Phase | | VIPs | | |
|---|---|---|---|---|---|
| | Adolescent | Adult | Non-user | User | **Total** |
| **population group** | 96 (62.34) | 58 (37.66) | 113 (73.38) | 41 (26.62) | 154 (100.00) |
| **population group characteristics** | | | | | |
| Age at first sex | 16 [15-17] | 17.5 [16-18] | 16 [15-18] | 16 [15-17] | 16 [15-17] |
| Sex lifetime partners | 2 [1-3] | 3 [2-5] | 2 [1-3] | 2 [1-5] | 2 [1-3] |
| Condom use | 10 (10.42) | 13 (22.41) | 19 (16.81) | 4 (9.76) | 23 (14.94) |
| Using Contraceptives | 36 (37.50) | 31 (53.45) | 49 (43.36) | 18 (43.90) | 67 (43.51) |
| *Oral* | 5 (5.21) | 1 (1.72) | 5 (4.42) | 1 (2.44) | 6 (3.90) |
| *Injectable* | 31 (32.29) | 30 (51.72) | 44 (38.94) | 17 (41.46) | 61 (39.61) |
| Vaginal PH | 4.7 [4.7-5.3] | 4.7 [4.7-5.3] | 4.7 [4.7-5.3] | 4.7 [4.7-5.3] | 4.7 [4.7-5.3] |
| Bacterial Vaginosis | 61 (63.54) | 34 (58.62) | 70 (61.95) | 25 (60.98) | 95 (61.69) |
| At least one of sexually transmitted disease | 41 (42.71) | 9 (15.52) | 37 (32.74) | 13 (31.71) | 50 (32.47) |
| *Neisseria Gonorrhoeae* | 8 (8.33) | 2 (3.45) | 9 (7.96) | 1 (2.44) | 10 (6.49) |
| *Chlamydia* | 34 (35.42) | 5 (8.62) | 30 (26.55) | 9 (21.95) | 39 (25.32) |
| *Trichomonas* | 6 (6.25) | 3 (5.17) | 6 (5.31) | 3 (7.32) | 9 (5.84) |

Parenthesis () represents the percentage of the number of women; Square brackets [], represent the interquartile range of the median.

The HPV risk factors were investigated according to 3 endpoints, factors associated with (i) the overall prevalence of HPV, (ii) the prevalence of HPV genotypes associated with genital warts (HPV6 and HPV11), and (iii) the HPV genotypes associated with cancer (16, 18, 31, 33, 45, 52, 58), particularly, those targeted by the available vaccines (Table 4.3). The proportions of overall positive HPV, genotypes associated with genital warts, and genotypes associated with cancer by each group of the age-phase, VIPs use, and other characteristics of the study participants were calculated. As mentioned above, the overall prevalence of HPV was 85.1%, while the prevalence of the genotypes associated with genital warts was 14.9% and the prevalence of the genotypes associated with cancer was found to be 53.9%. The genotypes associated with genital warts were more prevalent in adolescents (21.9%) compared to adults (3.4%), while the genotypes associated with cancer were prevalent more in adults (63.8%) than in adolescents (47.9%). The proportions of the 3 endpoints were further calculated within each group of VIPs use and other population characteristics. Individuals who used VIPs had a slightly higher prevalence of genotypes associated with genital warts (17.1%), but a lower prevalence of the genotypes associated with cancer (41.5%) compared to non-users (58.4%). On the other hand, most participants did not use condoms or contraceptives. As such, the impact of these factors on HPV prevalence cannot be concluded. In addition, all women who had HPV genotypes associate

with genital warts had STIs, indicating a zero prevalence of HPV genotypes associated with genital warts in women who had no STIs.

**Table 4.3: HPV prevalence by age phase, VIPs use, and clinical characteristics.**

| | | | Tested HPV Positive | | HPV Genotype related to Genital warts | | Cancer | |
|---|---|---|---|---|---|---|---|---|
| | ***Category*** | **Women** | No | *Yes* | No | *Yes* | No | *Yes* |
| Population | *Women* | 154 (100.0) | 23 (14.9) | 131 (85.1) | 131 (85.1) | 23 (14.9) | 71 (46.1) | 83 (53.9) |
| Age-Phase | *Adolescent* | 96 (62.3) | 10 (10.4) | 86 (89.6) | 75 (78.1) | 21 (21.9) | 50 (52.1) | 46 (47.9) |
| | *Adult* | 58 (37.7) | 13 (22.4) | 45 (77.6) | 56 (96.6) | 2 (3.4) | 21 (36.2) | 37 (63.8) |
| VIP use | *User* | 41 (26.6) | 5 (12.2) | 36 (87.8) | 34 (82.9) | 7 (17.1) | 24 (58.5) | 17 (41.5) |
| | *Non-user* | 113 (73.4) | 18 (15.9) | 95 (84.1) | 97 (85.8) | 16 (14.2) | 47 (41.6) | 66 (58.4) |
| Condom use | *Yes* | 23 (14.9) | 4 (17.4) | 19 (82.6) | 20 (87.0) | 3 (13.0) | 9 (39.1) | 14 (60.9) |
| | *No* | 131 (85.1) | 19 (14.5) | 112 (85.5) | 111 (84.7) | 20 (15.3) | 62 (47.3) | 69 (52.7) |
| Bacterial Vaginosis | *Present* | 95 (61.7) | 14 (14.7) | 81 (85.3) | 81 (85.3) | 14 (14.7) | 42 (44.2) | 53 (55.8) |
| | *Absent* | 59 (38.3) | 9 (15.3) | 50 (84.7) | 50 (84.7) | 9 (15.3) | 29 (49.2) | 30 (50.8) |
| STI | *Present* | 149 (96.8) | 22 (14.8) | 127 (85.2) | 126 (84.6) | 23 (15.4) | 69 (46.3) | 80 (53.7) |
| | *Absent* | 5 (3.2) | 1 (20.0) | 4 (80.0) | 5 (100.0) | 0 | 2 (40.0) | 3 (60.0) |
| Contraceptives | *Yes* | 6 (3.9) | 1 (16.7) | 5 (83.3) | 5 (83.3) | 1 (16.7) | 4 (66.7) | 2 (33.3) |
| | *No* | 148 (96.1) | 22 (14.9) | 126 (85.1) | 126 (85.1) | 22 (14.9) | 67 (45.3) | 81 (54.7) |

Parenthesis () represent the percentage of the number of women. HPV genotypes associated with genital warts (HPV6 and HPV11), genotypes associated with cancer (HPV 16, 18, 31, 33, 45, 52, 58).

To visualize whether the proportions of HPV prevalence with VIPs differs per age-phase, the logit of the proportions of HPV, HPV genotypes associated with genital warts, and HPV genotype associated with cancer were calculated then an interaction plot was plotted in Figures 4.3, 4.4, and 4.5, respectively. Figures 4.3A, 4.4A and 4.5A showed that the adolescent group seems to be more prevalent to the risk of HPV than the adult group. This observation is reflected even in the categories of the use of VIPs. There seem to be an interaction effect between age-phase and VIPs, suggesting that the prevalence due to age-phase differs by whether the woman is using VIPs or not.

From Figure 4.3.B, there seemed to be a slightly reducing slope that had a linear functional form between the logit of the proportion of HPV positives and the number of sex lifetime partners. This was slightly different for HPV genotypes associated with genital warts as there was a change from a linear relationship to a decline as the number of sex lifetime partners increased (Figure 4.4B). Figure 4.5B showed a slight increase in the prevalence of HPV genotypes

associated with cancer with increasing number of sexual partners. There was an increasing risk of overall HPV and HPV genotypes associated with cancer as age at first sex increased until 17 years which was indicated by a positive slope and a sudden reduction for those who had their first sex at 18 years and above (Figure 4.3C and 4.5C). On the other hand, Figure 4.4C showed a similar prevalence of HPV genotypes associated with genital warts for those who started sex at 15 years and a decline after the age of 17 years.

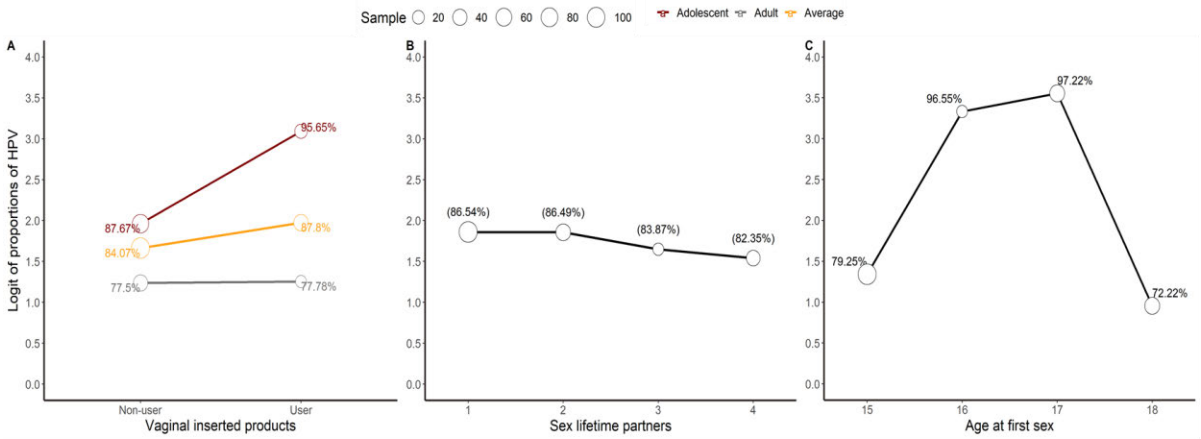


**Figure 4.3: Relationship between HPV prevalence and age-phase interaction with VIPs, number of sex lifetime partners and age at first sex.** The figure shows the interaction plot with logit of the proportions of overall HPV prevalence with vaginal inserted products plotted in (A) and the logit of the HPV prevalence plotted with the number of sex lifetime partners (B) and age at first sex (C).

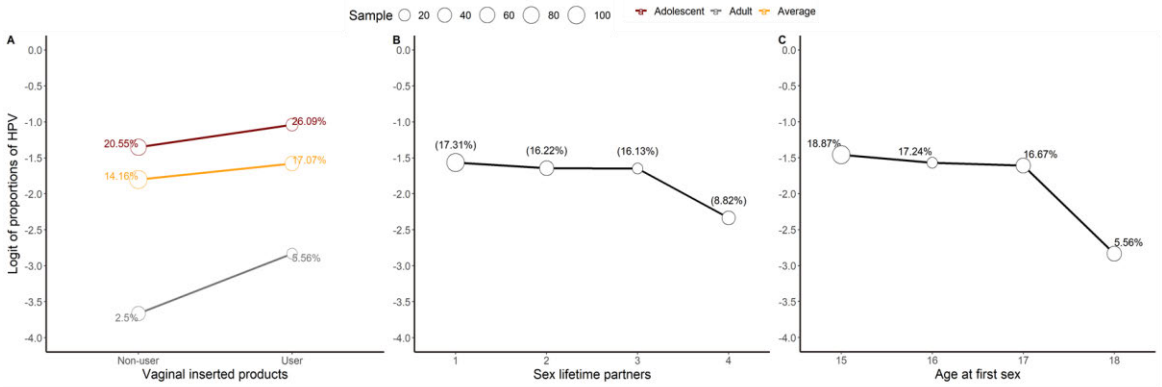


**Figure 4.4: Relationship between the prevalence of HPV genotypes associated with genital warts and age-phase interaction with VIPs, number of sex lifetime partners and age at first sex.** The figure shows the interaction plot with logit of the proportions of HPV prevalence with vaginal inserted products plotted in (A) and the logit of the HPV prevalence plotted with the number of sex lifetime partners (B) and age at first sex (C).

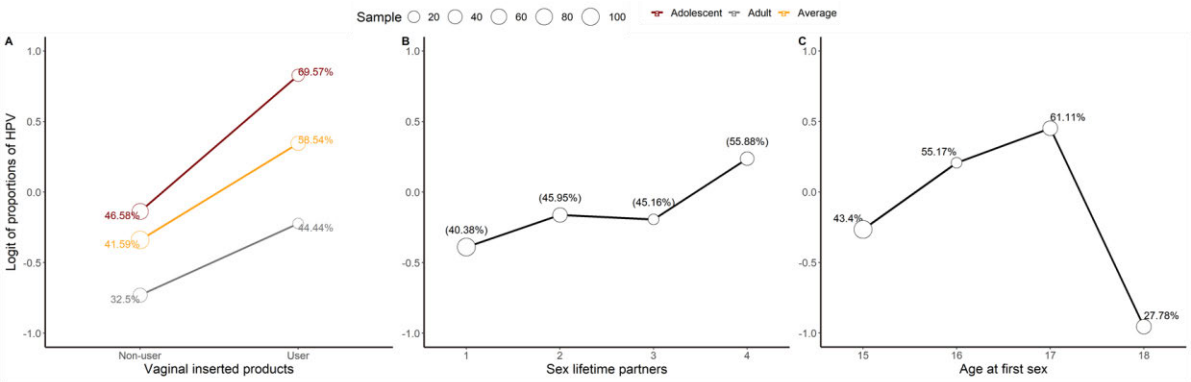


**Figure 4.5: Relationship between the prevalence of HPV genotypes associated with cancer and age-phase interaction with VIPs, number of sex lifetime partners and age at first sex.** The figure shows the interaction plot with logit of the proportions of HPV prevalence with vaginal inserted products plotted in (A) and the logit of the HPV prevalence plotted with the number of sex lifetime partners (B) and age at first sex (C).

Further analysis was done by determining the 95% confidence interval of the risk of HPV in this study population. Since the outcome (HPV risk) was considered to be frequent, the choice of the measure for the effect size was the relative risk as opposed to the odds ratio. The relative risk for the age-phase was 1.1546 at 95% CI (0.9896-1.3472) and 1.0444 at 95% CI (0.9084-1.2007) for the VIPs use, meaning that the risk of HPV for adolescents was 15.46% higher compared to the adults and for women using VIPs, it was 4.44% higher than those who were not using VIPs, respectively. To fit a binary outcome and to adjust for several covariates, a RPRM was shown to be stable and suitable for the analysis of this study. Three RPRMs were fitted to investigate the prevalence of overall HPV, HPV genotypes associated with genital warts, and HPV genotypes associated with cancer in the study population. These series of models were utilised to further interrogate the following:

• Are adolescent females more likely than adults to be at risk of HPV, HPV genotypes associated with genital warts, and HPV genotypes associated with cancer thus, are these HPV outcomes independent of age-phase?

• Are females using VIPs more likely than non-users to be at risk of HPV, HPV genotypes associated with genital warts, and HPV genotypes associated with cancer thus, is the HPV risk independent of VIPs use?

• Do these (age-phase, VIPs or interaction of age-phase and VIPs) effects change when the characteristics of women are adjusted for?

The resulting relative risk estimates of HPV together with the 95% confident intervals are presented in Table 4.4. From the un-adjusted model with the interaction of age-phase and VIPs,

the results indicated that the risk of HPV for adolescents using VIPs was 23% higher than that of adults using VIPs. However, this risk ratio was not significant at a 5% level of significance since the 95% CI of (0.946-1.598) included 1, hence the interaction term was not included further. In the un-adjusted model, the estimated HPV prevalence for adolescents was about 90.9% while for VIPs users, it was 86.8% with 95% CI of (84.9%-97.3%) and (77.3%-97.4%), respectively. This prevalence had an absolute difference of 12.5% with adolescents being at higher risk than adults and 4.7% with VIPs users being at higher risk than VIPs non-users. The ratio of the two-prevalence resulted to an HPV risk ratio of 1.159 for age-phase and 1.057 for VIPs which were not significant at 5% level of significance. This meant that the risk of HPV in adolescent females was 15.9% higher than adult females while for VIPs users, it was 5.7% higher than non-users. However, these differences were not statistically significant at 5% level of significance. After adjusting for other characteristics, the HPV risk due to age-phase reduced by 4.3% and by only 0.1% for VIPs. The adjusted model indicated that there was still no significant difference in the risk of HPV between age-phase or VIPs at 5% level of significance.

**Table 4.4: HPV prevalence and risk ratio differences between age-phase and vaginal inserted products.**

| | | Model | | |
|---|---|---|---|---|
| **Effect** | **Class** | Interaction Un-adjusted | No Interaction Un-Adjusted | No Interaction Adjusted |
| **Prevalence** | | | | |
| Phase | *Adolescent* | 0.916 [0.861,0.974] | 0.909 [0.849,0.973] | 0.865 [0.698,1.000] |
| | *Adult* | 0.776 [0.669,0.901] | 0.784 [0.681,0.902] | 0.775 [0.616,0.975] |
| VIP | *User* | 0.863 [0.757,0.983] | 0.868 [0.773,0.974] | 0.838 [0.672,1.000] |
| | *Non-user* | 0.824 [0.750,0.905] | 0.821 [0.750,0.898] | 0.800 [0.647,0.989] |
| Phase*VIP | *Adolescent (User)* | 0.957 [0.877,1.000] | | |
| | *Adolescent (Non-user)* | 0.877 [0.804,0.955] | | |
| | *Adult (User)* | 0.778 [0.608,0.996] | | |
| | *Adult (Non-user)* | 0.775 [0.656,0.916] | | |
| **Risk ratio** | | | | |
| Phase | *Adolescent-Adult* | 1.179 [1.004,1.386] | 1.159 [0.994,1.352] | 1.116 [0.942,1.322] |
| VIP | *User-Non-user* | 1.046 [0.891,1.229] | 1.057 [0.924,1.210] | 1.047 [0.912,1.203] |
| Phase*VIP | *User: Adolescent-Adult* | 1.230 [0.946,1.598] | | |
| | *Non-user: Adolescent-Adult* | 1.131 [0.938,1.365] | | |

Square brackets [], represent confidence intervals (CI) at 95%

A similar analysis was done for the risk of HPV genotypes linked to genital warts and cervical cancer. The unadjusted and adjusted models indicated that there was no significant difference in the risk of these HPV genotypes between age-phases or VIPs at 5% level of significance (data not shown).

Overall, the factor associated with HPV status in the HPV prevalence was sexual debut with a p-value of 0.004 (≤15 years vs 16 years, p-value = 0.048 and, 15 years vs 17 years, p-value = 0.034; Tukey multiple comparison adjustment). Women who had their first sex at 16 years had a 24.07% risk of HPV higher than those who had theirs at 15 years or less (95% CI of 1.0013, 1.5373) and the relative risk of those who started sex at 17 years compared to those who started at 15 years was 1.2455 with 95% CI of (1.0116, 1.5334) (Table 4.5). For those with genotypes associated with genital warts, a significant difference in HPV prevalence between adolescents and adults (p-value = 0.0075) was observed. The adolescent group was 7 times more at risk of having genotypes associated with genital warts compared to adults, this difference had the relative risk of 7.2427 with 95% CI of (1.2720, 41.2400) (Table 4.5). For the group with genotypes associated with cancer, three significant associations were observed. There was a significant effect of age-phase in HPV prevalence (p-value = 0.013), the relative risk for age-phase was 1.7019 with a 95% CI of (1.0994 2.6345), which meant that there was a 70% higher risk of having genotypes associated with cancer in adolescents than in adults. Having genotypes associated with cancer was also linked to sexual debut (p-value = 0.045) and the number of sex lifetime partners (p-value = 0.0024). There was a positive slope for the number of sex lifetime partners that had a relative risk of 1.2752 with a 95% CI of (1.0955, 1.4843), which meant that for every additional partner the risk of contracting HPV genotypes associated with cancer increased by 27%. Also, VIPs use was at the boundary of 0.05 with a p-value of 0.0503 and a relative risk of 1.4016 with 95% CI of (1.0210, 1.9241) which meant that VIPs users were at 40% higher risk of HPV genotypes associated with cancer than VIPs non-users.

**Table 4.5: Score statistics for factors associated with HPV.**

| | Tested | | HPV Genotype related to | | | |
|---|---|---|---|---|---|---|
| | HPV Positive | | Genital warts | | Cancer | |
| **Variable** | $\chi^2_{df}$ | $p$-value | $\chi^2_{df}$ | $p$-value | $\chi^2_{df}$ | $p$-value |
| Age-Phase | $1.63_{(1)}$ | 0.2019 | $7.14_{(1)}$ | **0.0075** | $6.16_{(1)}$ | **0.0130** |
| VIP use | $0.43_{(1)}$ | 0.5145 | $0.47_{(1)}$ | 0.4930 | $3.83_{(1)}$ | 0.0503 |
| Bacterial Vaginosis | $0.01_{(1)}$ | 0.9244 | $0.08_{(1)}$ | 0.7787 | $0.51_{(1)}$ | 0.4771 |
| Condom use | $0.03_{(1)}$ | 0.8645 | $0.10_{(1)}$ | 0.7518 | $0.01_{(1)}$ | 0.9253 |
| Age at first sex | $13.31_{(3)}$ | **0.0040** | $0.06_{(1)}$ | 0.8110 | $8.05_{(3)}$ | **0.0450** |
| Sex lifetime partners | $0.87_{(1)}$ | 0.3522 | $0.32_{(1)}$ | 0.5734 | $9.23_{(1)}$ | **0.0024** |
| STI | $0.27_{(1)}$ | 0.6044 | | | $0.69_{(1)}$ | 0.4075 |
| Contraceptives | | | $0.01_{(1)}$ | 0.9419 | | |

Parenthesis (), represent the degrees of freedom (df)

## 4.4. The immune activation status of CD4+ and CD8+ cervical T cells and HPV status.

### 4.4.1. T cell activation in adolescents and adults who are HPV- and HPV+.

To examine whether the immune activation status of CD4+ and CD8+ T cells is associated with HPV clearance, the activation levels of CD4+ T cells (overall CD4+ cells, CD4+ CCR5+, CD4+ CD38+, CD4+ HLA-DR+) and CD8+ T cells (overall CD8+ cells, CD8+ CCR5+, CD8+ CD38+, CD8+ HLA-DR+) was measured in HPV negative and in HPV positive participants. There was no significant difference observed in the activation level of both CD4+ T cells and CD8+ T cells amongst adolescent and adult women who were HPV- and HPV +. Only CD4+ HLA-DR+ T cells were significantly higher in HPV+ adolescents compared to HPV+ adult women (p-value = 0.0008) (Figure 4.6), indicating significantly more levels of activated CD4+ T cells in the genital tract of adolescents infected with HPV compared to adults infected with HPV.

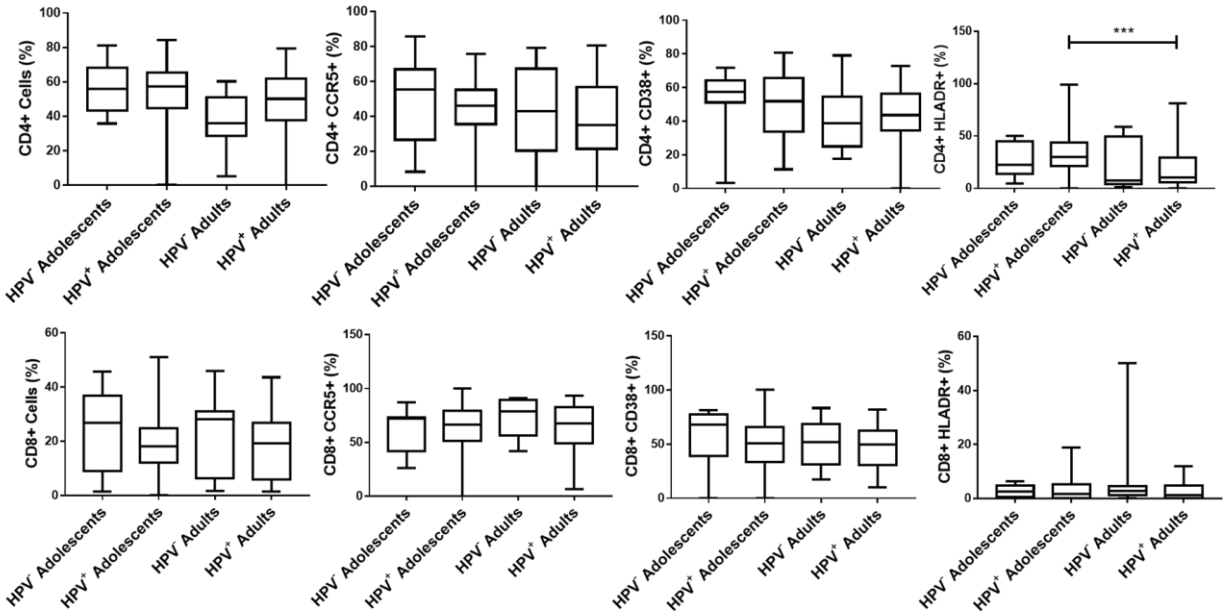


**Figure 4.6: The analysis of CD4+ and CD8+ T cell activation in adolescents and adults who are HPV- and HPV+.** The box and whisker plots show the percentage of CD4+ T cells, CD4+ CCR5+, CD4+ CD38+ cells and CD4+ HLADR+ cells as well as the percentage of CD8+ T cells, CD8+ CCR5+, CD8+ CD38+ cells and CD8+ HLADR+ cells. Participants were separated according to age and HPV status. The lines extending from the box are whiskers, indicate variability outside the upper and lower quartiles. Statistical comparisons were performed using the Mann-Whitney test. Significant differences are shown by an asterisk (*) system ($P \leq 0.001$***).

There was no difference observed in the activation status of both CD4+ T cells and CD8+ T cells when participants were grouped according to HPV status, irrespective of age group (Figure 4.7).

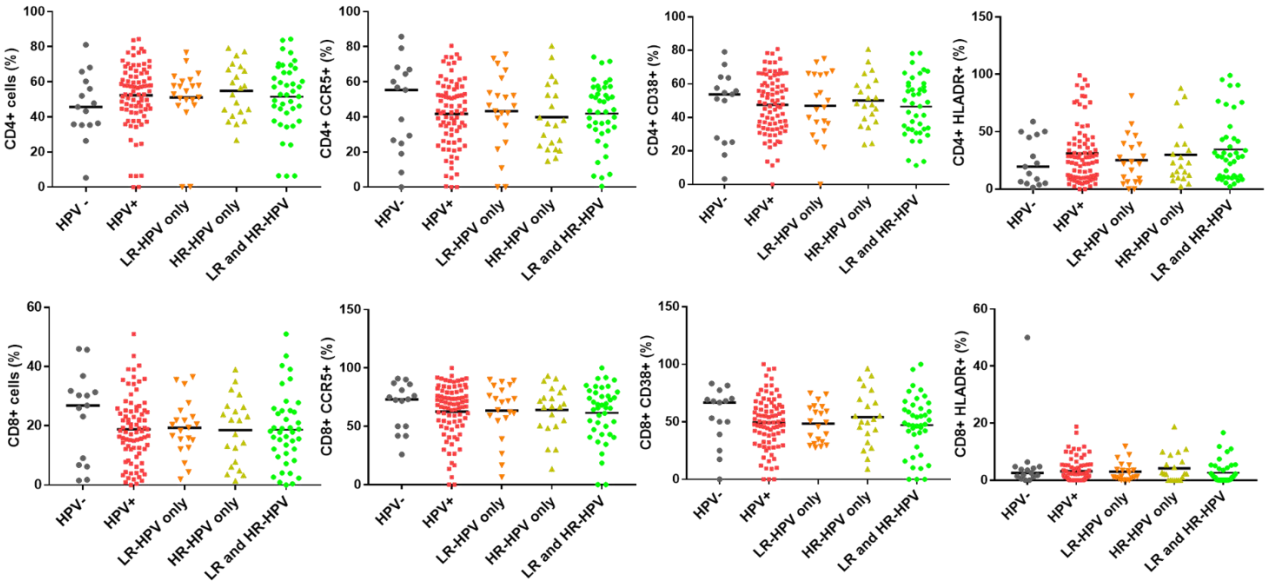


**Figure 4.7: T cell activation in HPV negative and HPV positive individuals (Overall HPV, low-risk (LR) HPV only, high-risk (HR) HPV only and LR and HR HPV).** The dot plots show the percentages of CD4+ T cells, CD4+ CCR5+, CD4+ CD38+ cells and CD4+ HLADR+ cells as well as the percentage of CD8+ T cells, CD8+ CCR5+, CD8+ CD38+ cells and CD8+ HLADR+ cells. Each dot represents one participant. The median is indicated by a horizontal line within the data points. Statistical tests were performed by the Kruskal-Wallis test (values not shown as they were not significant).

### 4.4.2. The impact of vaginal product use on the immune activation status of CD4 and CD8T cells and HPV status.

CD4+ and CD8+ T cell activation in participants stratified according to HPV status and use of VIPs was investigated. There was no significant difference in the expression of activated T cells in VIPs users compared to non-users. Furthermore, HPV status did not impact on the activation status in VIPs users compared to non-users (Figure 4.8).

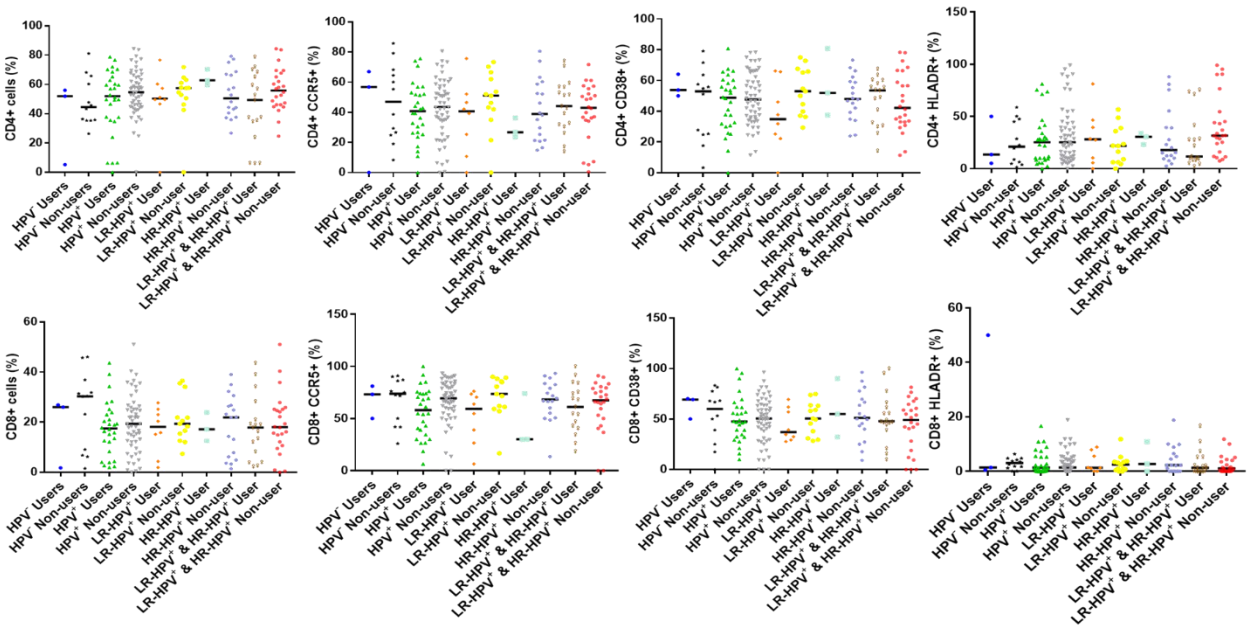


**Figure 4.8: Immune activation status of CD4+ and CD8+ T cells in HPV- and HPV+ women who are users or non-users of vaginal inserted products (VIPs).** The dot plots represent the proportions of activated T cells in women characterised by HPV status; HPV-, HPV+ (overall), LR-HPV+ (low-risk HPV), HR-HPV+ (high-risk HPV), LR-HPV and HR-HPV (positive for both low-risk and high-risk genotypes) and VIPs. The median is indicated by a horizontal line within the data points. Statistical tests were performed by the Kruskal-Wallis test (values not shown as they were not significant).

## 4.5 The influence of human antimicrobial peptides, human β-defensins (HBD-1, -2), and psoriasin concentrations on HPV clearance.

### 4.5.1. Impact of the HPV status on the concentration of AMPs.

There was no difference observed in concentration levels of HBD-1 between HPV- and HPV+ adolescents. Similarly, there was no difference in HBD-1 concentrations between HPV- and HPV+ adults. No difference was observed in the concentration of HBD-2 between HPV- and HPV+ adolescents, while a significant difference was observed between HPV- adults compared to HPV+ adults (p-value = 0.0215) with the HPV- individuals showing a significantly lower concentration of HBD-2. Furthermore, there was also a significantly higher concentration of HBD-2 in HPV+ adults compared to HPV+ adolescents (p-value = 0.0189). Similar to HBD-1, no differences were observed in the concentration of psoriasin between HPV- and HPV+ adolescents. The adults also did not show any significant differences in the concentrations of psoriasin (Figure 4.9A). Irrespective of age, the concentration of psoriasin was significantly

higher in HPV- individuals (p-value = 0.0012) compared to the low-risk HPV group. The group with low-risk HPV only also had significantly lower levels of psoriasin (p-value = 0.0478) compared to the group with both low-risk and high-risk HPV (Figure 4.9B).

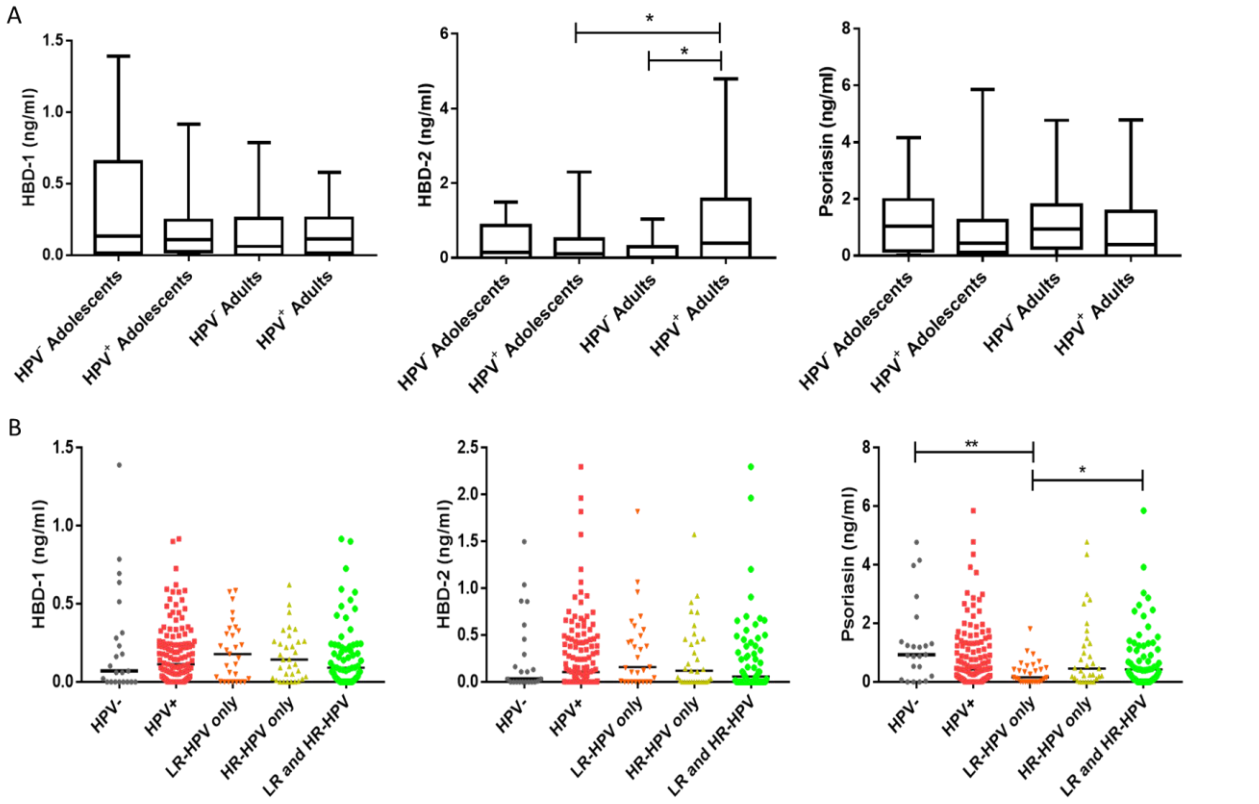


**Figure 4.9: Concentrations of AMPs (HBD-1, HBD-2 and Psoriasin) in the cervicovaginal fluid.** (A) Shown in box and whisker plots are the concentrations of antimicrobial peptides (AMPs) in HPV- and HPV+ adolescents and adults. (B) Concentrations of AMPs in participants who are HPV-, HPV+, with low-risk HPV only, high-risk HPV only and positive for both low-risk and high-risk HPV are represented in dot plots. The median is indicated by a horizontal line within the data points. Significant differences are shown by an asterisk (*) system ($P > 0.05$ ns, $P \leq 0.05$ *, $P \leq 0.01$ ** and $P \leq 0.001$***). Statistical tests were performed by the Kruskal-Wallis test and the Mann Whitney U test.

### 4.5.2. The impact of VIPs use on the concentration of AMPs.

No difference was observed in the concentration of all three peptides (HBD-1, HBD-2 and Psoriasin) between those who reported VIPs use and those who did not (Figure 4.10A). The concentration of these AMPs in individuals separated according to both HPV status and VIPs use was similar with no statistically significant differences observed (Figure 4.10B).

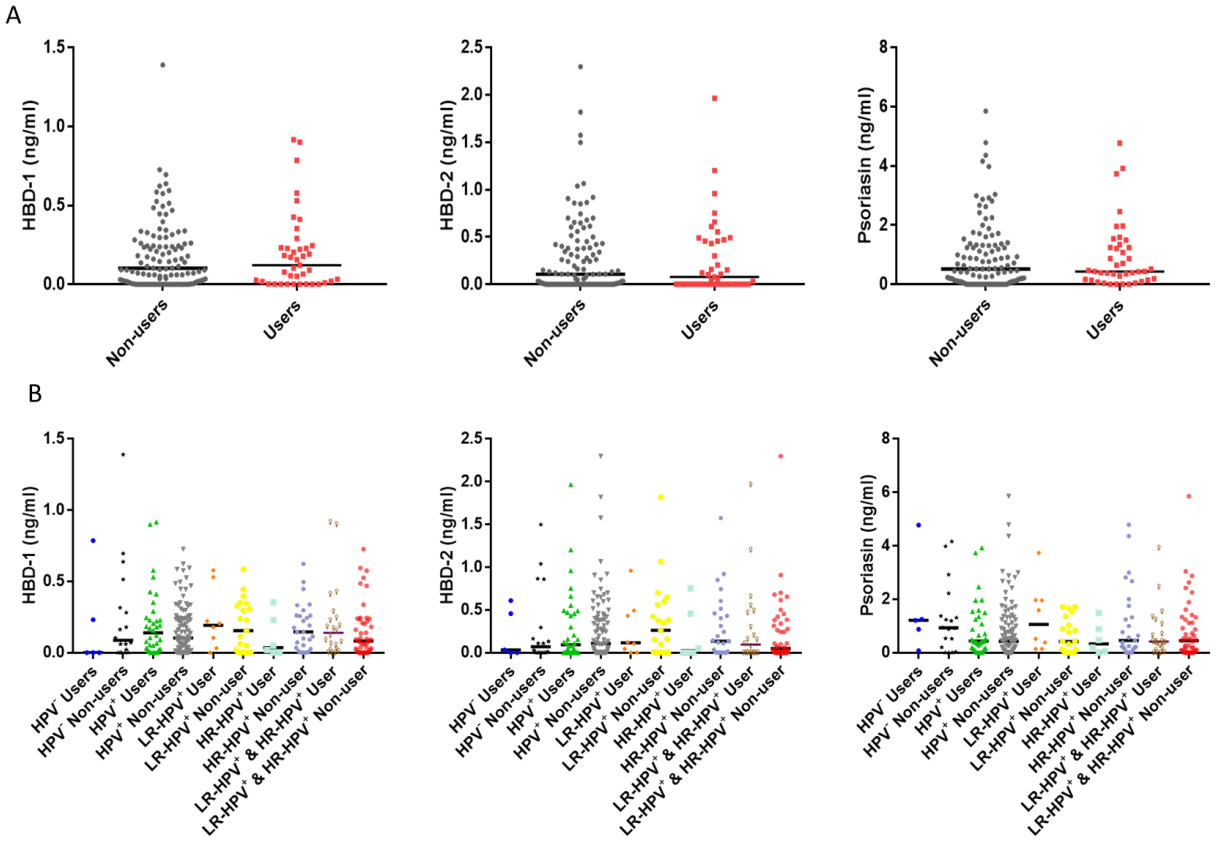


**Figure 4.10: Concentrations of AMPs (HBD-1, HBD-2 and Psoriasin) in the cervicovaginal fluids.** (A) Displayed are concentrations of antimicrobial peptides (AMPs); human beta-defensin (HBD)-1, -2 and Psoriasin in users and non-users of VIPs, as well as in (B) participants stratified according to the HPV status and the use of VIPs. The median is indicated by a horizontal line within the data points. Statistical tests were performed by the Kruskal-Wallis test.

# CHAPTER 5: DISCUSSION

It is well known that HPV is the leading cause of cervical cancer and is the most common STI, especially in young women. However, gaps exist in understanding the risk factors leading to higher susceptibility to HPV infection in young females. In addition, few studies have investigated the associations between VIPs and genital HPV infection. The high incidence of HPV in adolescents has been mostly correlated to risky sexual behaviour. This study tested the hypothesis that in the naïve age group, the use of VIPs is common compared to adult women. These products may compound the risk of the HPV infection, particularly in the lower reproductive tract of adolescents, which soon after sexual debut, represents a naive reactive state characterised by a deficiency of antimicrobial polypeptides with a delayed response to pathogens, including HPV resulting in a compromised innate and adaptive immunity. This study confirmed the higher risk of HPV in adolescent females than adult females and some slight risk in VIPs users compared to non-users.

The overall HPV prevalence was 85% and was greater than the previous studies looking at a similar population of young females in KZN. The most recent report of HPV prevalence in a cohort of sexually active young females in Kwazulu-Natal was 74% (Liebenberg et al., 2019). Ebrahim et al. (2016), also studied HPV burden in young women in KZN and found an overall HPV prevalence of 76.3%. These differences might be due to HPV tests used. This study cohort was dominated by individuals in whom multiple HPV genotypes were detected (2 or more, 76.62%). The high prevalence of infection with multiple HPV genotypes is a cause for concern. Moreover, Liebenberg et al. (2019) found that approximately 75% of the KZN young females had multiple HPV genotypes. The prevalence of the genotypes associated with genital warts and the genotypes associated with cancer (those targeted by the current vaccine) was 14,9% and 53,9%, respectively. Therefore, more than half of this study cohort had genotypes associated with cancer, highlighting the importance and the urgency of vaccinating young girls with Gardasil®9 HPV vaccine. Several other studies have reported the high prevalence of genotypes targeted by the current vaccine (Mboumba Bouassa et al., 2019), this has been observed mostly by studies in women with intraepithelial lesions (Taku et al., 2021, Paz-Zulueta et al., 2018). Among the cancer-related genotypes targeted by the current vaccines, HPV52 was the most prevalent genotype (16%), particularly in adolescents (21%), followed by HPV16 and HPV 45,

both with a prevalence of 10%. In the overall cohort, there was also a high prevalence of high-risk HPV35 (16%), a genotype not targeted by any of the HPV vaccines. Other studies have also noted with concern a high prevalence of this genotype (Mbulawa et al., 2021, Taku et al., 2021). A study that investigated an association of HPV35 with cervical carcinogenesis in African women revealed this genotype to have been found in 2% of invasive cervical cancers worldwide and up to 10% in Sub-Saharan Africa (Pinheiro et al., 2020). The high burden of HPV35 infection may be worth looking into further, especially since Africa is already leading with cervical cancer cases and mortality rate, with HPV being the leading cause (Bruni et al., 2019).

Only 26,62% of this study cohort reported using VIPs, and the adolescent group occupied the larger part of this percentage. Sexual pleasure was the primary motivation behind the use of the majority of the products. Contrary, the reason behind the use of VIPs in the two studies conducted in the Western Cape and Gauteng provinces was mainly for hygiene purposes (Myer et al., 2004, Lazarus et al., 2019). Alum powder (potassium aluminium sulfate) was the most popular product shared between adolescents and adult females. The motivation for the use of this product is vaginal tightening and the dry sex effect. Dry sex may compromise the integrity of the vaginal epithelium tissue and put women at a heightened risk of acquiring STIs (Levin, 2005). Most women in this cohort reported just one product use, whereas other studies have found that some women use multiple products, and this varies from person to person (Turner et al., 2010, Crann et al., 2018). Indeed, this study showed some interaction between age-phase and VIPs, suggesting that the prevalence due to age-phase differs by whether the woman is using VIPs or not. Showing this interaction might require a large sample size than the one in this study. Other studies have found douching to be associated with an increased risk of HPV (Moscicki et al., 2013). In contrast, others have observed douching to significantly decrease the prevalence of HPV infection (Lee et al., 2014).

To better understand the risk factors for HPV and the impact of VIPs use, three study endpoints (prevalence of HPV positive, genital warts related HPV genotype, and cancer-related HPV genotype) were correlated with age-phase and with the proportions of positive HPV by each group of the age-phase, VIPs use, and other characteristics. As expected, and proven by other studies, sexual debut significantly impacted HPV prevalence (Houlihan et al., 2016). The results revealed an increase in HPV prevalence in individuals who started sex at 16 years, the

prevalence peaked at 17 years, and there was a sudden decline at 18 years and above. Also, individuals who sexually debuted at 16 and 17 years were at higher risk of HPV than those who started at 15 years. The sexual debut was associated with HPV even when looking only at genotypes associated with cancer. However, Mbulawa et al. (2021) found no association between age and sexual debut with the HPV infection. The prevalence of the genotypes related to cancer was associated with the number of sex lifetime partners and age-phase, the risk being higher for the adolescent group than adults. Interestingly, the use of VIPs was at the boundary of the alpha value (0.05), with a relative risk of the VIPs users being at a higher risk of being infected with genotypes associated with cancer than non-users.

The prevalence of HPV genotypes associated with genital warts was significantly associated with age-phase. Adolescents were the ones at a higher risk of having these genotypes compared to adults. As opposed to the protective effect of condoms observed by other studies against HPV (Lam et al., 2014), This study did not observe the effectiveness of condom use in reducing HPV transmission. This surprising finding is possibly due to a small number of individuals reporting condom use. The few who used condoms were not assessed for how often, which is essential for determining the protective effect. There was also no significant impact of contraceptives, BV, and STIs on the overall HPV prevalence, the prevalence of the genotypes associated with genital warts, and genotypes associated with cancer.

The association between T cell immune activation and HPV is not entirely understood. It has, however, been established that the immune cells in the cervical mucosa are essential for the control of HPV infection (Mbuya et al., 2020). To determine whether the T cell immune activation status is associated with the HPV status, this study characterised the expression of the HIV co-receptor CCR5 and the activation markers (CD38 and HLA-DR) on cervical T cells according to the HPV status and the use of VIPs. The results showed no significant difference in the expression of the co-receptor CCR5 on CD4+ T cells between HPV- and HPV+ groups, suggesting that the HPV infection does not induce much change in the proportion of CD4+ T cells expressing CCR5. Similar findings of Mbuya et al. reported that HPV was not associated with a significant difference in the frequency of CCR5+ CD4 cervical T cells and, therefore, does not alter CCR5 expression (Mbuya et al., 2020). The frequency of this receptor on CD4+ T cells was still not significantly different even when data was further stratified into those who use VIPs and those who do not. Neither the use of VIPs nor HPV infection was associated with

a significant change in the expression of CCR5 on CD4+ and CD8+ cells. A significant increase in the number of activated cervical CD4+T cells expressing HLA-DR in adolescents compared to adults with HPV is interesting. This finding may suggest elevated levels of antigen presentation by CD4+ T cells in adolescents with HPV infection. However, how and why antigen presentation by CD4+ T cells gets elevated in the genital tracts of adolescents is not fully understood here and may require further investigation. Mbuya et al. (2020) also found significantly higher frequencies of HLA-DR+ CD4+ T cells in HPV+ females. However, their study mainly focused on adults. Other studies found no associations between HPV infection and cervical T cell activation status (Shannon et al., 2017). Stratification by VIPs use did not significantly affect the proportion of activated CD4+ and CD8+ cells, implying that VIPs use does not seem to impact T cell activation status in the cervical mucosa.

Antimicrobial peptides form an essential part of the innate system and have been shown to play an important role in the defense against genital HPV infections. The concentrations of three antimicrobial peptides, HBD-1, HBD-2, and psoriasin were analysed and compared in HPV+ and HPV- females. There were no significant differences in HBD-1 concentrations between all the groups investigated. Contrary, other studies have reported a significant upregulation of this peptide in HPV-induced lesions compared to normal controls (Chong et al., 2006, Erhart et al., 2011). Erhart et al. found expressions of HBD-2 and Psoriasin significantly high in patients with condyloma acuminatum (genital warts caused by HPV). The results of this study were similar for HBD-2; there were significantly elevated levels of HBD-2 in HPV+ adult women compared to HPV- adults and HPV+ adolescents. This finding suggests that adolescents infected with HPV have significantly less upregulation of HBD-2 compared to infected adults. A higher concentration of HBD-2 in low-risk HPV infection than high-risk HPV was reported previously (Szukiewicz et al., 2016). For psoriasin, the opposite was observed. Irrespective of age, the concentration of psoriasin was significantly higher in HPV- individuals than those who tested positive for low-risk HPV genotypes. The low-risk HPV+ group showed a significantly lower concentration of psoriasin than those who tested positive for two or more subtypes comprised of both low-risk and high-risk HPV. Alvendal et al. found downregulated expression of psoriasin in individuals with HPV-induced mucosal lesions, which, after the infection had cleared, were the same as in healthy controls (Alvendal et al., 2019). Stratification by the use of VIPs did not reveal a significant effect on the concentrations of all three peptides, which may be due to the small sample size.

One of the study limitations was that the VIPs users were a small sample size with a wide range of products, possibly used differently depending on the individual. In addition, these products are likely to have different effects on the genital tract. The cross-sectional nature of this study limits the focus to be on HPV prevalence with the absence of other observations such as clearance of the HPV infection.

## Conclusion

In summary, the risk of cervical cancer remains a threat in younger women. This study confirms that age-phase, age at first sex, and the number of sex lifetime partners are the main factors associated with cancer-related HPV genotypes. In addition, the adolescent group was seven times more at risk of HPV genotype related to genital warts than adults. These findings highlight the urgency of expanding HPV vaccination programs to vaccinate more young girls before their sexual debut. VIPs users were 40% higher at risk of cancer-related HPV genotypes than VIPs non-users, even though the sample size was small, suggesting that young women should be warned about the potential dangers of using VIPs. When this age group is infected with HPV, cervical CD4+ T cells expressing HLA-DR, associated with activation and antigen presentation are elevated. This finding warrants further investigation. This study also revealed that an upregulation of HBD-2 associated with HPV infection is significantly less in adolescents than adults, indicating limited concentrations in this naïve age group.

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