Cervical neoplasia in women with and without HIV-related immune depletion: Epidemiology and pathogenesis related to HPV types.

by

Matthys Cornelis van Aardt

submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy

in the Faculty of Health Sciences

in the subject

Obstetrics and Gynaecology (Gynaecologic Oncology)

at the

University of Pretoria

Supervisor: Prof G. Dreyer

April 2016

ABSTRACT

Objectives: Despite current comprehension of HPV epidemiology, the question of how HPV type distribution in the South African population differs from that in the rest of the world remains largely unanswered for both prevalence and oncogenic potential. The primary objective of this study was to investigate the causal relationship between oncogenic or high-risk HPV (HrHPV) types and disease of the cervix, ranging from healthy women to pre-neoplastic and malignant disease. The secondary objective was to investigate the potential differences in the importance of oncogenic HPV types between HIV-infected and HIV-non-infected women. This information is crucial to design country-specific primary and secondary prevention programmes.

Methods and materials: This study consists of five smaller studies to address the research problem. Firstly, we investigated the prevalence and distribution of HPV types in sample South African women representative of the general population and subset women with AIDS, both with normal cytology. Secondly, we assessed HPV types present in patients with biopsy-confirmed CIN II/III and to compare HPV type distribution between HIV-infected- and -non-infected women. DNA typing was done on the surface as well as in tissue samples of the dysplastic lesion, in an attempt to identify the lesion-causing virus. Immunohistochemical markers was utilised to insure accurate histological diagnosis and reduce inter- and intra-observer variability. Lastly, we investigated type-specific prevalence in women with invasive cervical cancer (ICC) with and without HIV co-infection. All the above data was then collated to determine the importance of HPV types in cervical oncogenesis in South African women with and without HIV.

Results: High-risk HPV DNA was detected from 45% of women with normal cytology, 93% of CIN II/III and 88% of ICC. The four most prevalent HrHPV types found in women without cytological abnormalities were HPV 16, 51, 58 and 45; among women with CIN II/III HPV 16, 52, 35 and 18 were the most common single types; and in ICC samples HPV 16, 18, 45 and 35 were most common. HPV 16, 18, 31, 33, 35, 51, 52 and 56 were all found to be important causes of cervical dysplasia. HPV 16, 18 and 35 were more common in ICC than in women with normal cytology, while HPV 16, 18 and 45 were more common in ICC than pre-invasive disease.

Infection with HPV and with multiple HPV types was more common among HIV-positive women in all disease groups of the study. Among HIV-positive women HPV 18, 35, 45 and 56 seem to be more important in CIN II/III, while HPV 18, 33, 45 and 58 may be more important causes of ICC. Only HPV 45 was statistically significantly more common among HIV-positive women.

Conclusion: The studied population of South African women differs significantly from published data. We also described potential differences in the oncogenic importance of specific HPV types among immune depleted women never discussed before. It is recommended that efforts for both vaccination and screening should be focused only on HPV alpha-9 and alpha-7 groups and firstly only on HPV 16, 18, 45 and 35.

Keywords: Human papillomavirus (HPV), human immunodeficiency virus (HIV), cervical intraepithelial neoplasia (CIN), cervical screening, normal cytology, cervical cancer.

CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
Cdk2	Cyclin-dependent kinase 2
C-HPV	Consensus human papillomavirus
CIN	Cervical intraepithelial neoplasia
CT	Collection tube
DNA	Deoxyribonucleic acid
FFPE	Formalin-fixed paraffin-embedded
FT	Filter tube
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
Hr	High-risk
HrHPV	High-risk human papillomavirus
Hrr	High-risk rest
HSIL	High-grade squamous intraepithelial lesions
ICC	Invasive cervical cancer
IHC	Immunohistochemical
LA	Linear array
LBA	Line blot assay
LBC	Liquid-based cytology
LCM	Laser capture micro-dissection
LiPA	Line probe assay
LLETZ	Large loop excision of the transformation zone
LrHPV	Low-risk human papillomavirus
LSIL	Low-grade squamous intraepithelial lesions
mRNA	Messenger ribonucleic acid
NHLS	National Health Laboratory Service
NPV	Negative predictive value

ORF	Open reading frames
p16	$p16^{INK4a}$
PCR	Polymerase chain reaction
pRb	Retinoblastoma tumour suppressor protein
PrHrHPV	Probable high-risk human papillomavirus
RNA	Ribonucleic acid
S-HPV	Surface human papillomavirus
SIL	Squamous intraepithelial lesions
T-HPV	Targeted-tissue-based human papillomavirus
vHr	Very high-risk
vvHr	Very very high-risk

INTRODUCTION TO THE STUDY

The introduction focuses on the background to the study and the reason for the research. The chapter also aims to explain the research problem and the objective of this thesis. The thesis consists of five separate studies and a concluding chapter at the end.

BACKGROUND

Human papillomavirus (HPV) infections are globally the most frequent transmitted sexual infection. In South Africa, it is estimated that no less than 21% of females in the general population are currently infected with HPV of the uterine cervix and genital tract. Epidemiological data indicates that there has been a substantial increase in HPV infections over the last 10 years in Southern Africa. This increase is especially related to the immune depletion associated with the human immunodeficiency virus (HIV). Up to 85% of HIV-infected women may be co-infected with HPV.[1]

Human papillomavirus infection plays a fundamental role in the development of disease, ranging from benign anogenital warts and papillomatosis of the respiratory tract to cervical, anogenital and oropharyngeal cancer.[2] The uterine cervix in relation to morbidity and mortality remains extremely common and of importance.[3]

Although genital HPV infections are primarily sexually transmitted, other means of transmission have also been documented. Nonsexual transmission of genital HPV types include vertical and perinatal, transplacental and horizontal transmission.[4]

As early as 1842, Rigoni-Stern noticed that cervical cancer almost always occurred in married women and rarely in celibate women. It was, however, not until 1974 that cervical cancer was attributed to an environmental factor, likely a virus, causing atypical metaplasia of the columnar epithelium that might

progress to cancer. In 1976 and 1977 numerous groups detected HPV within the nuclei of abnormal epithelial cells and it was hypothesised that HPV was significant in the pathophysiology of cervical cancer. Following expansion in HPV research in the years after 1977, it is known today that 99.7% of cervical cancers are caused by HPV.[5]

Human papillomavirus is a member of the Papillomaviridae family. It is a small circular double-stranded deoxyribonucleic acid (DNA) virus with close to 8 000 nucleotide base pairs and, depending on the genotype, has between seven and nine open reading frames (ORFs). Papillomaviruses are categorised into 16 different genera, with the majority of HPVs classified as either alpha or beta genus. Alpha genus consists mainly of genital or mucosal HPV, whereas HPV from the beta genus is primarily responsible for inconsistent skin infections.[6]

Up to 2015, more than 150 genotypes have been identified with 40 of these known to infect the intraepithelial layer of the anogenital tract. Cervical cancer is strongly associated with 15 of these oncogenic- or high-risk HPV (HrHPV) types.[1,7] HPV types associated with infection of the lower genital tract stem form five species: alpha 5, 6, 7, 9 and 10. Alpha 7 (HPV types 18, 39, 45, 59, 68, 70) and alpha 9 (HPV types 16, 31, 33, 35, 52, 58, 67) predominate.[7]

The virus's life cycle begins when the virus gains access to the basement membrane through minute abrasions of the skin, cervical and/or anal transformation zones. Through endocytosis the virus enters the basal cells followed by breakdown of the envelope proteins. The viral genome is arranged separate from the host DNA as episomes. If the host immune system is intact, around 80% of genital infections will be cleared in less than two years. Persistent HPV infections occur when the viral DNA is integrated into that of the host cell.[1,7]

Women infected with HIV are known to have a higher risk of HPV infections and associated anogenital neoplasia than HIV-non-infected women. Determining the interaction between HPV and HIV remains a challenge, mainly because the mechanism by which immune depletion adds to the higher chance of HPV-

associated disease is poorly understood.[8] In one study, HIV-infected women were almost twice and three times more likely to be infected with high- and low-risk HPV types as the HIV-negative control.[9]

It is, however, known that women infected with HIV have an increased prevalence of newly acquired HPV infections, with persistent infections more likely to occur. CD4 cell counts and HIV ribonucleic acid (RNA) viral load are used to monitor HIV disease progression. Deterioration in immune function is associated with an increased risk of HPV infection and -related disease. The duration of HIV infection is important in the explaining of and understanding the interaction between HIV and HPV infection.[10]

Evidence indicates that HPV infection increases significantly shortly after newly acquired HIV infection, suggesting that HIV causes immune suppression of the genital mucosa.[10-12] This dysfunction in local immunity might be a result of reduced CD4+ T-cells concentrated in the mucosa.[10,11] Reactivation of latent HPV infections might also be mediated by these changes.[10]

Highly active antiretroviral therapy (HAART) can, to some extent, restore immunity in HIV-infected women. By increasing life expectancy, women on HAART may have a higher risk of oncogenic HPV exposure. An inability to clear these infections could result in higher rates of cervical cancer and its precursors. However, an American study concluded that effective and compliant use of HAART considerably lowered the load of HPV infection. This conclusion might help to clarify why cervical cancer does not appear to be higher in the HAART era in spite of women living longer.[13]

Highly active antiretroviral therapy regimes are constantly changing and it is crucial to continuously evaluate the effect that evolutions and advances in drugs and drug combinations have on HPV infection and HPV-related disease.[8]

Despite recent advances in cervical cancer treatment, prevention remains the most important intervention to reduce the burden of cervical cancer in women,

regardless of their immune status.[14] The importance of utilising HPV DNA testing as part of the screening for cervical disease was highlighted in the POBASCAM trial.[15] This randomised controlled trial showed protection against cervical cancer through the identification and appropriate treatment of cervical abnormalities (cervical intraepithelial neoplasia (CIN) II or worse) sooner.[15]

On the basis of guidelines in use, women with HIV infection should have two cervical cytology screenings in the 12 months following diagnosis. Yearly screening should follow if no abnormalities are detected.[16]

Identifying E6 and E7 messenger-RNA (mRNA), indicative of the oncogenic ability of HPV infection, is the rationale behind HPV RNA testing. The FASE study compared RNA and DNA testing with cytology and found both RNA (AHPV) and DNA (HC2) to be very sensitive for high-grade lesions (CIN II and III) – much higher than liquid-based cytology (LBC). The specificity of HC2 (DNA) was lower compared to AHPV (RNA) and LBC, which had comparable specificity. Adding LBC to HPV testing, RNA or DNA, improved sensitivity but worsened specificity. The authors suggested that RNA testing (AHPV) might be an alternative for screening for premalignant cervical lesions in women 20 years and older.[17]

This evidence was supported by another study that concluded that HPV RNA compared to HPV DNA testing had similar sensitivity but that HPV was more specific and had a higher positive predictive value, thus bettering screening for cervical cancer.[18] A systemic review suggested that mRNA tests have a role in diagnosing cervical disease but that further studies, including financial assessment, are required to ascertain clinical use of HPV mRNA testing.[19]

Immune system integrity appears to be a less important factor in progression of disease from CIN II or worse to cancer than progression of low-grade lesions (CIN I) to high-grade lesions (CIN II). Other factors, most likely genetic changes, are responsible for delayed development of invasive malignancy.[20]

As mentioned, knowledge of the interaction of HAART and HPV infection and its effect on cervical neoplasia in HIV-positive women is limited. Women of advanced age have higher incidences of cervical cancer, and the disease is likely to increase in women living with HIV because of HAART-associated increase in longevity.[13]

MOTIVATION FOR THE STUDY

Infection with one or more oncogenic HPV types is very common. However, invasive cervical cancer develops in only a small percentage of theses women. The discrepancy between the incidence of infection and cancer progression and the long period between HPV infection and detecting invasive cancer highlights the importance of complex interactions between viral, environmental and host-associated factors.[21]

On the basis of their association with malignancy, HPV types can be categorised as either high- or low risk. Present in 99.7% of patients with cervical cancer, the five most commonly found HPV types are HPV 16, 18, 33, 45 and 31, with HPV 16 and 18 responsible for more than 70%. Despite current comprehension of HPV epidemiology, the question of how the type distribution of HPV in the South African population differs from that in the rest of the world remains largely unanswered.

RESEARCH PROBLEM

The primary intention of this study was to investigate the causal relationship between high-risk or oncogenic HPV and disease of the cervix, ranging from pre-neoplastic to malignant disease. The secondary intention was to investigate the difference in the prevalence of oncogenic HPV types between HIV-infected patients and HIV-non-infected patients, in relation to disease of the cervix. If we can show which serotypes are more prevalent in groups with different levels of immunity, future vaccine design strategies and cervical screening programmes might improve. Information on type-specific distribution of HPV infections is also important to monitor patients in the post-vaccination era.

This thesis consists of five studies for addressing the research problem:

- HPV type distribution in women in the general population without cytological abnormalities (Chapter 1)
- Oncogenic and incidental HPV types associated with cervical pre-neoplasia in HIV-positive- and HIV-negative women (Chapter 2)
- HPV type or types demonstrated within tissue samples of histologically confirmed cervical intraepithelial lesions in women with and without HIV-related immune depletion (Chapter 3)
- Targeted tissue-based HPV types detected within CIN lesions in HIV-positive and -negative women: reducing inter- and intra-observer variability with $p16^{ink4a}$ and Ki-67 (Chapter 4)
- HPV type distribution in women with invasive cervical cancer and the effect of HIV-induced immune dysfunction (Chapter 5)

GENERAL OBJECTIVES

The general objective of this doctoral thesis was to determine if HPV type-related development of cervical neoplasia and HPV types associated with disease progression from pre-malignant- to malignant lesions differs in relation to immune system integrity. This question is crucial for cervical-cancer-screening test selection and lies at the basis of cancer-prevention policies and programmes.

In Chapter 1, we aimed to investigate the distribution of HPV types in a sample of women representative of the South African general population, with normal cytological screening tests. In a smaller population of South African women with acquired immunodeficiency syndrome (AIDS) and normal cervical cytology we also aimed to determine HPV type distribution

In Chapter 2, we aimed to assess HPV types present in patients with biopsy-confirmed cervical intraepithelial lesions and to compare the HPV type distribution between HIV-infected- and HIV non-infected women. We also aimed to describe the prevalence of oncogenic or high-risk HPV types as immune

function deteriorates, as indicated by CD4 cell count and duration on antiretroviral therapy.

In Chapter 3, we aimed to study which HPV types identified with DNA typing on the surface were also found within the dysplastic lesion, in an attempt to identify the specific virus causing the lesion. We also aimed to investigate the effect of the immune system integrity on tissue HPV genotyping.

In Chapter 4, we aimed to utilise immunohistochemical markers to reduce inter- and intra-observer variability in diagnosing true pre-malignant lesions and to study the HPV type/s present in these lesions.

In Chapter 5, we aimed to study which HPV types are prevalent in women with cervical cancer in our population and to compare the cancer-associated HPV types with HIV status

GENERAL HYPOTHESIS

The general hypothesis of this doctoral thesis was that HPV type-related development of cervical neoplasia and HPV types associated with disease progression from pre-malignant to malignant lesions differ in relation to immune system integrity. The hypotheses of the individual studies are addressed in the mentioned chapters.

GENERAL METHODS

The oncology unit of the Department of Obstetrics and Gynaecology at the University of Pretoria has done various studies during the past years, staring in 2003, on HPV prevalence. These studies included HPV prevalence in the general population, as well as the distribution in women with cervical abnormalities, ranging from pre-neoplastic lesions to invasive cancers. Data from these datasets was combined and study groups expanded, where applicable, as well as utilising new research methods to help provide answers to research questions set out in this thesis.

REFERENCES

1. Richter KL. Understanding and incorporating human papillomavirus testing in cervical cancer screening: a South African perspective. South Afr J Gynaecol Oncol. 2011;3(1):9-14.
2. Steben M, Duarte-Franco E. Human papillomavirus infection: Epidemiology and pathophysiology. Gynecol Oncol. 2007;107(Suppl):S2-5.
3. Palefsky J. Human papillomavirus infection among HIV-infected individuals. Implications for development of malignant tumors. Hematol Oncol Clin North Am. 1991;5(2):357-370.
4. Bosch FX, Qiao Y, Castellsagué X. The epidemiology of human papillomavirus infection and its association with cervical cancer. Int J Gynaecol Obstet. 2006;94(Suppl 1):S8-21
5. Gurumurthy M, Peevor R, Fiander A. Human papillomavirus update (including vaccination). Obstet Gynaecol Reprod Med. 2013;23(8):231-237
6. Lazarczyk M, Cassonnet P, Pons C, Jacob Y, Favre M. The EVER Proteins as a natural barrier against papillomaviruses: A new insight into the pathogenesis of human papillomavirus infections. Microbiol Mol Biol Rev. 2009;73(2):348-370.
7. Carter JR, Ding Z, Rose BR. HPV infection and cervical disease: a review. Aust N Z J Obstet Gynaecol. 2011;51(2):103-108.
8. Palefsky JM, Holly EA. Chapter 6: Immunosuppression and Co-infection with HIV. J Natl Cancer Inst Monogr. 2003;31:41-6.
9. Ahdieh L, Klein RS, Burk R, Cu-Uvin S, Schuman P, Duerr A, et al. Prevalence, incidence, and type-specific persistence of human papillomavirus in human immunodeficiency virus (HIV)-positive and HIV-negative women. J Infect Dis. 2001;184(6):682-690.
10. Wang C, Wright TC, Denny L, Kuhn L. Rapid rise in detection of human papillomavirus (HPV) infection soon after incident HIV infection among South African women. J Infect Dis. 2011;203(4):479-486.

11. Nowak RG, Gravitt PE, Morrison CS, Gange SJ, Kwok C, Oliver AE, et al. Increases in human papillomavirus detection during early HIV infection among women in Zimbabwe. J Infect Dis. 2011;203(8):1182-1191.
12. Auvert B, Marais D, Lissouba P, Zarca K, Ramjee G, Williamson AL. High-risk human papillomavirus is associated with HIV acquisition among South African female sex workers. Infect Dis Obstet Gynecol. 2011;2011:692012. Epub 2011 Jul 3.
13. Minkoff H, Zhong Y, Burk RD, Palefsky JM, Xue X, Watts DH, et al. Influence of adherent and effective antiretroviral therapy use on human papillomavirus infection and squamous intraepithelial lesions in human immunodeficiency virus-positive women. J Infect Dis. 2010;201(5):681-690.
14. Einstein MH, Phaeton R. Issues in cervical cancer incidence and treatment in HIV. Curr Opin Oncol. 2010;22:449-455.
15. Rijkaart DC, Berkhof J, Rozendaal L, van Kemenade FJ, Bulkmans NW, Heideman DA, et al. Human papillomavirus testing for the detection of high-grade cervical intraepithelial neoplasia and cancer: final results of the POBASCAM randomised controlled trial. Lancet Oncol. 2012;13(1):78-88.
16. Gravitt PE, Kirk GD. Progress and pitfalls in defining the influence of highly active antiretroviral therapy on human papillomavirus-associated cervical disease. J Infect Dis. 2010;201(5):650-652.
17. Monsonego J, Hudgens MG, Zerat L, Zerat JC, Syrjänen K, Halfon P, et al. Evaluation of oncogenic human papillomavirus RNA and DNA tests with liquid-based cytology in primary cervical cancer screening: the FASE study. Int J Cancer. 2011;129(3):691-701.
18. Coquillard G, Palao B, Patterson B. Quantification of intracellular HPV E6/E7 mRNA expression increases the specificity and positive predictive value of cervical cancer screening compared to HPV DNA. Gynecol Oncol. 2011; 120:89-93.
19. Burger EA, Kornør H, Klemp M, Lauvrak V, Kristiansen IS. HPV mRNA tests for the detection of cervical intraepithelial neoplasia: A systematic review. Gynecol Oncol. 2011;120(3):430-438.

20. Palefsky JM. Human papillomavirus infection in HIV-infected persons. Top HIV Med. 2007;15(4):130-133.

21. Kostopoulou EA, Koukoulis G. Immunohistochemistry in the diagnosis of squamous intraepithelial lesions of the uterine cervix. In: Van den Broeck D, editor. Human papillomavirus and related diseases - from bench to bedside - a clinical perspective. InTech; 2012. p. 41-64. Available from: http://www.intechopen.com/books/human-papillomavirus-and-related-diseases-from-bench-to-bedside-a-clinical-perspective/immunohistochemistry-in-the-diagnosis-of-squamous-intraepithelial-lesions-of-the-uterine-cervix

CHAPTER 1

HUMAN PAPILLOMAVIRUS (HPV) TYPE DISTRIBUTION IN WOMEN IN THE GENERAL POPULATION WITHOUT CYTOLOGICAL ABNORMALITIES

1.1. INTRODUCTION

1.1.1. Burden of disease

Cervical cancer is the third most common malignancy diagnosed worldwide. It is estimated that in 2008, new cases of cervical cancer totalled 530 000. In the same year, 275 000 deaths from cervical cancer occurred. Low-resource countries carry the burden, as over 85% of cervical cancer occurs in these nations.[1]

1.1.2. Human papillomavirus (HPV) infections

Persistent infections with specific HPV genotypes strongly predict malignancies that relate to HPV, but HPV type distribution in the general population is not a true reflection of high-risk types found in cancer that is attributed to HPV.[2]

Precursor lesions to cervical cancer do not develop in all women with persistent high-risk HPV (HrHPV) infections.[3] Approximately 70% of new HPV infections are cleared by women's immune systems within one year, or become undetectable.[4] However, cervical cancer is almost always caused by persistent HPV infections.[1]

1.1.3. Mechanism of HPV-induced oncogenesis

Malignant transformation is dependent on the production of oncoproteins E6 and E7 by the matching E6 and E7 genes. E6 and E7 bind and facilitate degradation of p53 and retinoblastoma protein (pRb) respectively. Both p53 protein and pRb are important for cell-replication control, programmed cell death (apoptosis) and genomic stability.[5,6,7]

E6 inactivates the p53 gene through gene mutation, which indirectly neutralises the p53 protein, leading to uncontrolled replication of cells and dysregulation of the host cell genome. The pRb pathway, essential for cell regulation, is disrupted

because E7 protein degrades pRb and causes loss of inhibitory cellular proteins.[5,6,7]

1.1.4. HPV prevalence in women without cytological abnormalities

The adjusted prevalence, in 2010, of HPV infections in women with no cytological abnormalities was estimated to be 11.7% worldwide.[8] The prevalence of HPV infections in women without cytological abnormalities differs considerably between countries and regions, as well as within regions – ranging from 1.6% to 41.9%.[9] On average, HPV prevalence in regions in Europe, North America and Asia is lower than that in regions in Africa and Latin America, especially in human immunodeficiency virus (HIV)-infected groups.[1,9,10]

A prospective study performed on university students in the USA showed that 30% were HPV-infected within one year after initiating sexual intercourse and 54% within four years.[11] It appears as if HrHPV infections even out in American women in the general population who are older than 45 years of age, and that the percentage of women who are positive for HPV DNA drops below 5%.[12]

Among women with normal cytology, a meta-analysis, published in 2010, identified the highest prevalence of HPV to be women less than 25 years of age, with reported rates of 23.2%. The prevalence rate decreased to 8.7% in women between 25 and 34 years and less than 5% in women above 35 years.[9]

1.1.5. HPV prevalence in women with invasive cervical cancer

The five most commonly found HPV types in 99.7% of patients with cervical cancer are HPV 16, 18, 33, 45 and 31. HPV 16 and 18 are responsible for more than 70% of cervical cancer.[5,13] Other factors associated with cervical carcinogenesis include smoking, nutritional deficiencies, genetic factors, HIV co-infection and infections of the genital tract.[14]

Internationally HPV 16 is the most frequent infection and estimated to be associated with around 56% of all cervical cancers.[15] Testing women for HPV

DNA identifies women with cervical neoplasia and also the ones at risk for future disease.[16]

1.1.6. HIV and HPV

Women infected with HIV are known to have high HPV infection rates.[17] Following the extensive growth of the HIV pandemic in Sub-Saharan Africa, HPV infections are on an upward trend.[10]

Women with one HPV type infection have a higher chance to simultaneously be infected by other HPV subtypes. In theory, currently available vaccines against HPV 16 and 18 could potentially cause a rise or fall in other HPV types not covered by the currently available vaccines.[18]

1.1.7. Motivation for the study

Utilising HPV testing as a screening method in many countries has become an acceptable alternative to cytology. Current recommendations are that screening is extended to three years if both HPV DNA and cytology are negative. The treatment for women with HrHPV infections and cytological abnormalities is clear. Currently, there are no universally accepted guidelines for women testing positive for HrHPV without cytological abnormalities. These women have a higher risk of future high-grade cervical intraepithelial lesions (CIN II and III).[19]

Epidemiological data on HPV distribution in the general population is crucial in the light of new broad-spectrum HPV vaccines that are currently under development. It is also important to have this data before introducing HPV-based screening tests in a population for use in cost-benefit modelling studies, and to evaluate the impact on healthcare infrastructure. The data would also provide a baseline against which the impact of current HPV vaccines could be monitored in the future.[9,16]

1.2. OBJECTIVES AND HYPOTHESES

1.2.1. Objectives

The objectives of this study were to:

- Investigate the distribution of HPV types in a sample of women representative of the general population, in South Africa, with normal cytological screening tests;
- Determine HPV type distribution in a smaller population of South African women with acquired immunodeficiency syndrome (AIDS) and normal cervical cytology;
- Compare the two study populations with normal cytology with each other in relation to HPV prevalence and type-specific distribution; and
- Compare findings from our population with data from Africa and other continents to determine possible similarities and differences.

1.2.2 Hypotheses

The hypotheses of this study were that:

- HPV type distribution in the respective study populations with normal cytological screening would be different to the distribution found in the international literature
- HPV type distribution would be different among the HIV-infected subgroup of women with normal cervical cytology when compared to the subgroup representing the general population with normal cytological screening tests

1.3. MATERIAL AND METHODS

1.3.1 Study design

This was a retrospective descriptive study, based on data from a dataset that was created from 2008 to 2010. The study comprised two study populations and the main inclusion criterion was a normal cervical cytological screening test. Study population A (Pop A) was representative of the general population and study population B (Pop B) of women with AIDS.

1.3.2. Consent process and ethical considerations

Patients received counselling and an informed consent document that explained the method and voluntary nature of the study. In Pop A, counselling and informed consent obtained by trained medical personnel motivated patients to undergo HIV testing, but it was explained clearly that testing was voluntary. The HIV results of patients in Pop A were not included in this study. Written informed consent and standard management protocols were the same for both study populations. Patients with abnormal cytology were managed according to standard treatment protocols.

The different protocols for this study were approved by the Ethics Committee of the Faculty of Health Sciences of the University of Pretoria (131/2005, 210/2008, 189/2012).

1.3.3. Patient recruitment

Patients in this study used public health care and the vast majority were black South African women.

Pop A was representative of women attending public healthcare facilities, and included women attending five primary health clinics in the Tshwane region. The women were invited to screen for cervical abnormalities with the use of both conventional cytology (a Papanicoloau smear) and HPV DNA genotyping. One-

thousand-two-hundred-and-sixty patients had no cytological abnormalities and received HPV DNA genotyping. Complete demographic data was available for 1 238 patients.

Pop B was a subset of women infected with HIV who presented at the immunology outpatient department for the initiation and review of their highly active antiretroviral therapy (HAART). These women were also invited to screen using conventional cytology and HPV DNA genotyping. CD_4 cell count, used as one of the indicators to initiate HAART therapy and to monitor treatment, was also recorded. In this study, a CD_4 cell count less than 350/µL was indicative of immune depletion. A total of 83 women who had no cytological abnormalities detected on their cytological testing were recruited from the immunology outpatient department. HPV DNA genotyping was performed on 65 of the 83 women.

1.3.4. Sample collection and transport

Sample collections and transport of these were the same for both study populations. The samples included dry cervical swabs collected by healthcare workers and tampon samples self-collected by the patients. Conventional cytology was performed by professionally trained nurses and qualified doctors working at the respective clinics.

The collected slides were sent to the cytology laboratories at the National Health Laboratory Service (NHLS) and interpreted by qualified cytologists. The cytology laboratory, to assure the quality of the conventional cytology, used standard NHLS protocols. Cervical biopsies to compare the cytological findings with histology were not possible because the primary clinics did not have biopsy forceps and it was not part of the original study design.

1.3.5. HPV DNA testing

Immediately after the swabs and tampon specimens had been collected, they were placed in phosphate-buffered saline and a 10% methanol solution. The

collected samples were sent to the Department of Medical Virology at the University of Pretoria. The swab specimens were transported and stored dry until testing. DNA was extracted by means of the DNA® Isolation Kit (Roche Molecular Systems, Branchburg, USA) on the MagNA Pure Automated Extraction System®. HPV typing was determined by the HPV Linear Array® Genotyping Test. Tests were run for 15 high-risk genotypes: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82; three probable high-risk types: HPV 26, 53 and 66; and 19 low- or undetermined risk types: HPV 6, 11, 40, 42, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, IS39 and CP6108.[20]

1.3.6. Data capturing and analysis

Data were captured on Microsoft® Excel® datasheets, and analysis performed using Stata® statistical software (StataCorp, College Station, USA). Descriptive statistical methods were employed to describe this population. The data analysis consisted of descriptive statistics, mean and standard deviations for continuous data, and frequencies and percentages for categorical data.

The HrHPV types were grouped together on the basis of their prevalence in cervical cancer. The 15 high-risk HPV types were divided into four groups: HPV 16 and/or 18, grouped together and named as “very very high-risk” (vvHr); HPV 35, 45 and/or 52, grouped together and named as “very high-risk” (vHr); HPV 31, 33 and/or 58, grouped together as “high-risk” (Hr); and the other HrHPV types, HPV 39, 51, 56, 59, 68, 73 and/or 82, grouped together as “high-risk rest” (Hrr).

1.4. RESULTS

1.4.1. Socio-demographic characteristics

Pop A

In the period from March 2009 to December 2011, 1 260 patients were recruited who showed no cervical abnormalities on a cytological test. Complete demographic data, including age, were available for 1 238 patients. The age distribution of the study population is illustrated in Figure 1.1. The mean age was 40.94 years (standard deviation 11.99).

Patients were divided according to age into five-year intervals for women aged 20 to 59, and into 10-year intervals for women aged 60 and older. Two-hundred-and-forty (19.39%) women were younger than 30 years of age. Twelve (0.97%) women were younger than 20 years of age, and 19 (1.53%) women were 70 and older.

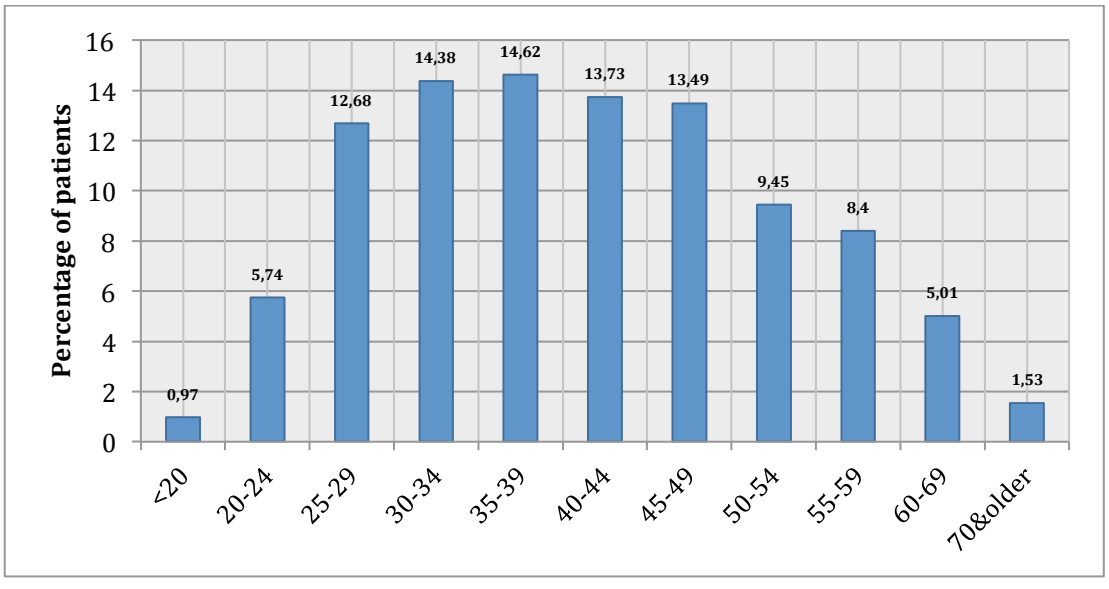


Figure 1.1: Age distribution (percentage) of Pop A

Pop B

The mean age of the 65 patients included was 34.91 years and ranged between 21 and 56 years. Almost half of the patients (32 patients; 49.22%) were between 30 and 39 years of age. Seventeen (26.16%) women were younger than 30 years and 16 (24.62%) women 40 years and older. See Figure 1.2.

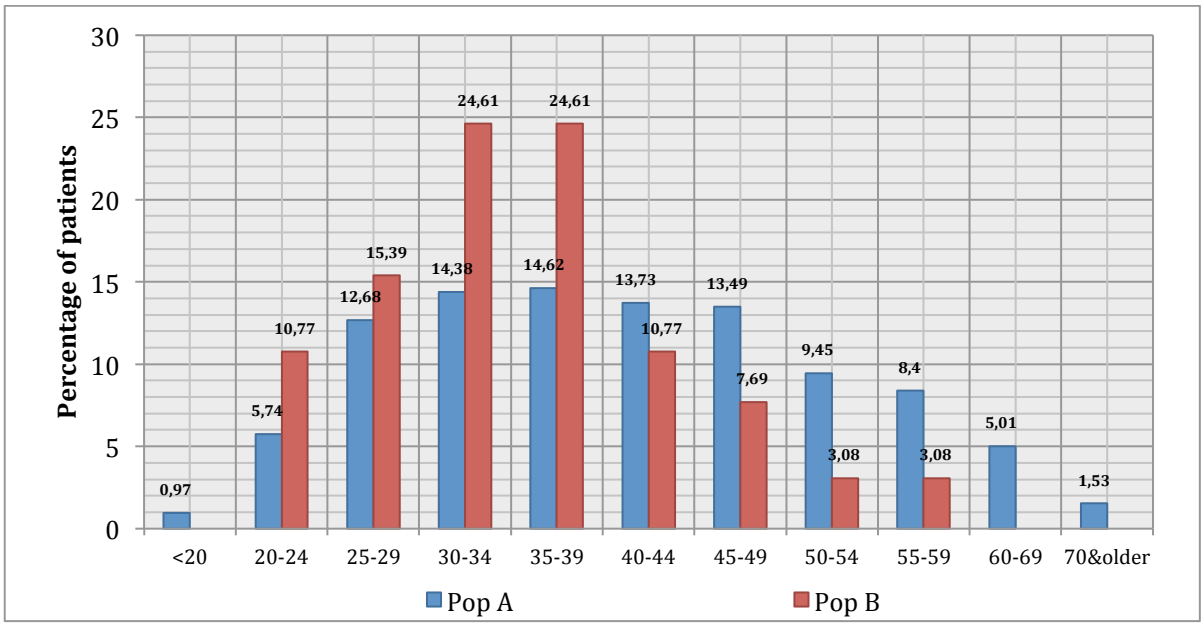


Figure 1.2: Age distribution (percentage) of Pop A an Pop B

Three of the 57 patients with known CD4 cell counts were regarded as immune competent (CD4 cell count > 350/µL). These three patients had been on HAART for more than 12 months. The average CD_4 cell count was 158/µL, with the lowest recorded as 18 and the highest as 408/µL. Four patients had been on HAART for more than 12 months, two patients for less than 12 months and an unknown time for four. The remaining 55 patients were in the process of initiating HAART.

1.4.2. Prevalence of all HPV and low-risk HPV (LrHPV) type infections

Pop A

The prevalence of HPV infections was 67.06% for one or more high- and/or LrHPV types. One-thousand-and-seventy high-risk, 220 probable high-risk, and 1 388 low-risk HPV types were found in 1 260 patients. Four-hundred-and-fifteen (32.94%) of these patients had no viruses identified on HPV DNA typing.

Of the 845 patients with HPV infections, the average number of HPV type infections was just more than three multiple HPV type infections. The distribution of specific HPV types in the general population is illustrated in Figure 1.3.

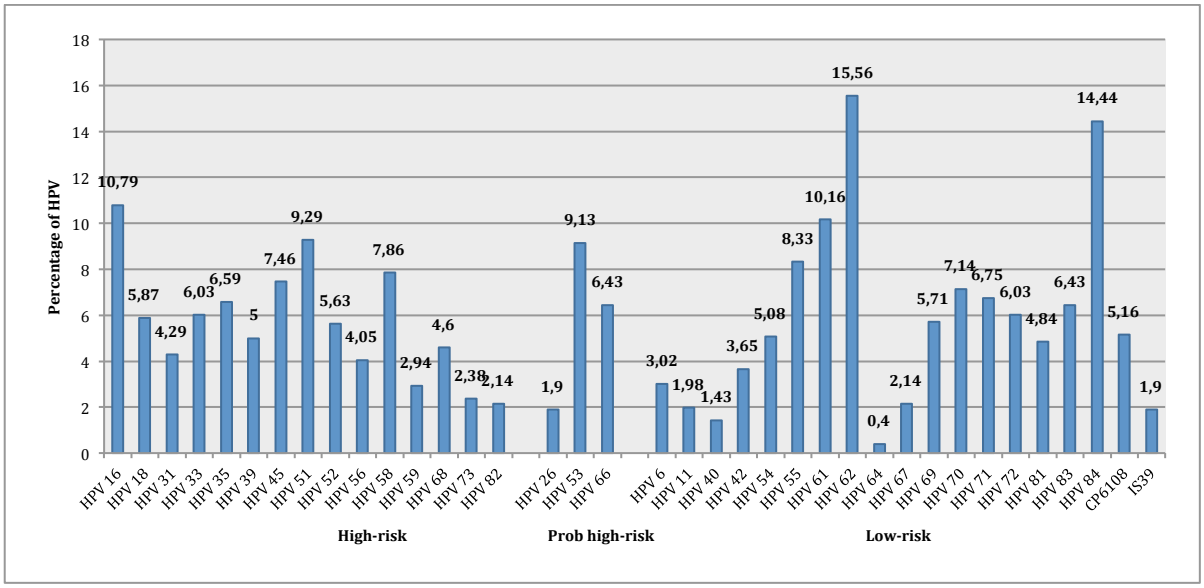


Figure 1.3: Distribution of human papillomavirus types detected in Pop A

Of the the LrHPV types, HPV 62 (15.56%) and HPV 84 (14.4%) were present most frequently. HPV 6 and 11 were uncommon, and were present in 38 (3.02%) and 25 (1.98%) patients, respectively.

Pop B

The prevalence of all HPV types in the study population with AIDS was 87.69%. Eight (12.31%) patients had no HPV infection detected. One-hundred-and-twelve HrHPV infections, 28 probable HrHPV infections (prob HrHPV) and 115 LrHPV infections were observed among the 65 patients. On average, women were infected with around two high-risk types (between zero and ten) and two low-risk types (between zero and seven).

The most prevalent LrHPV type was identical to that found in Pop A. However, HPV 62 (18 patients; 27.69%) was followed by HPV 71 (12 patients; 18.46%). HPV 6 infections were observed in five (7.69%) patients and HPV 11 infections in four (6.15%) patients.

1.4.3. The prevalence of HrHPV types

Pop A

The prevalence of HrHPV infections was 44.92%. A single HrHPV type, illustrated in Figure 1.4, was found in 297 (23.57%) patients. Two-hundred-and-sixty-nine (21.35%) patients had multiple HrHPV-type infections.

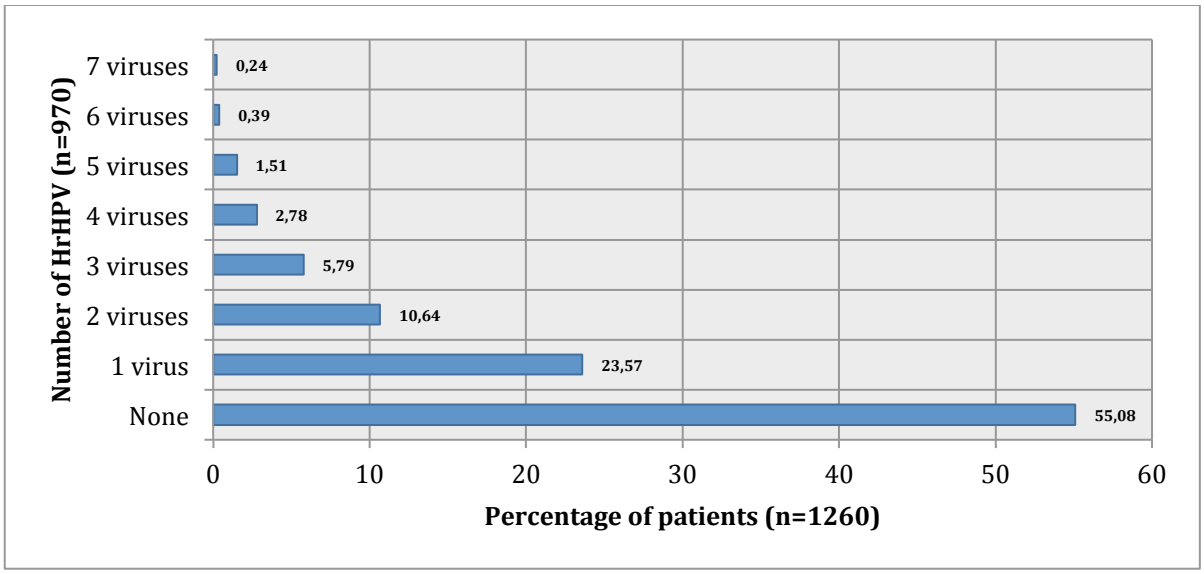


Figure 1.4: Total number of HrHPV types per patient in Pop A

The most common high-risk virus observed was HPV 16, which was present in 136 (10.79%) of the study population, followed by HPV 51 and HPV 58. HPV 18 was observed in 5.87% of the study population and HPV 45 in 7.46%. Figure 1.5 illustrates the 10 most prevalent HrHPV types. One-hundred-and-ninety-seven (15.63%) patients had HPV 16 and/or HPV 18 infections.

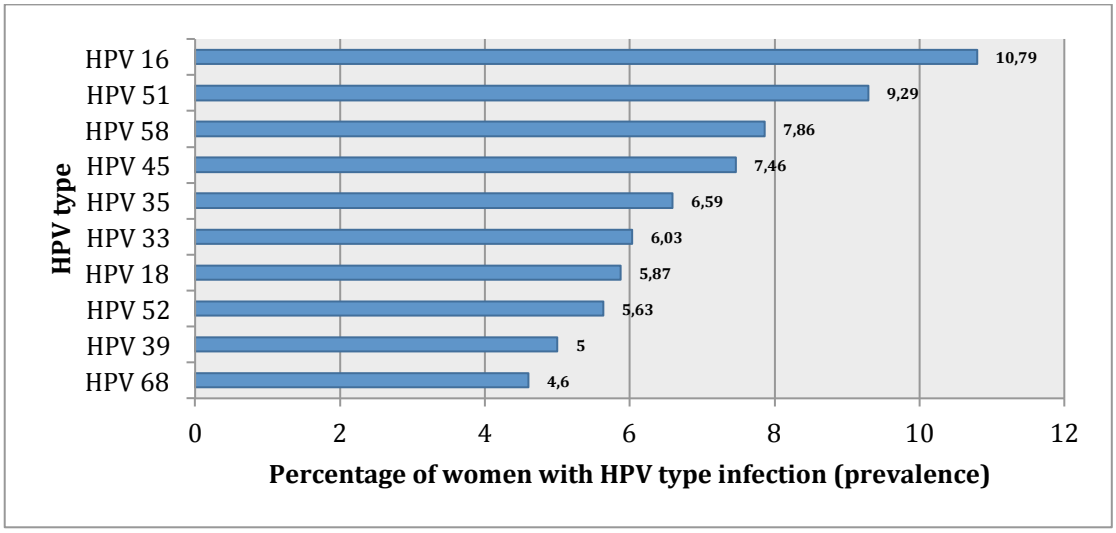


Figure 1.5: The 10 most prevalent HrHPV types found in Pop A

In the different age groups, the highest prevalence of HrHPV infections was seen in women between 20 to 24 years (42 patients; 59.15%), followed by women aged 25 to 29 years (87 patients; 55.41%). The prevalence in women between 35 and 39 years of age was slightly higher than for women between 30 and 34 years (50.28% versus 49.22%). A third peak was seen in women between 60 and 69 years (29 patients; 46.77%). See figures 1.6 and 1.7 for the prevalence of HrHPV and other HPV type categories in different age groups. The percentages are calculated by the number of patients in each age group testing positive for one or more HrHPV type/s.

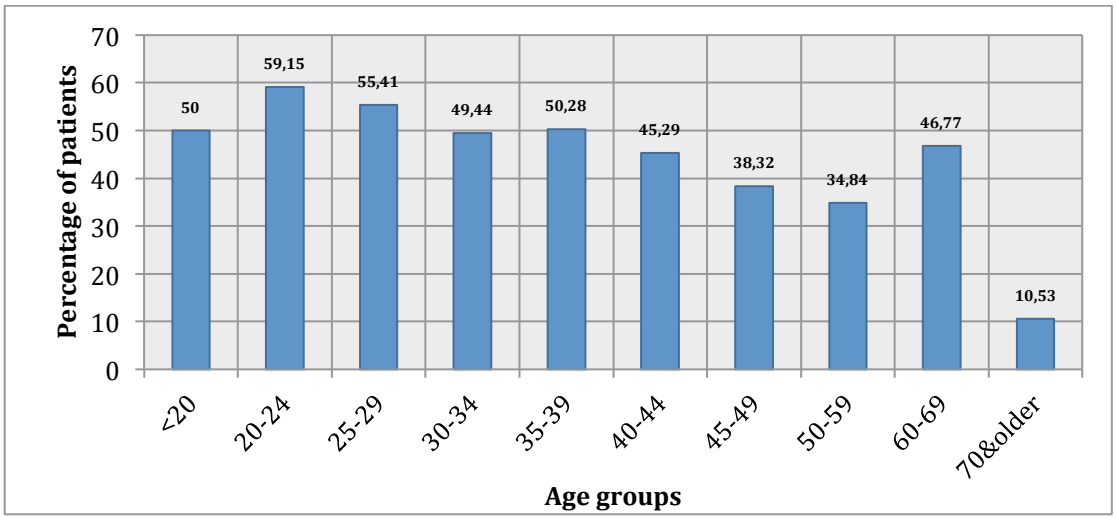


Figure 1.6: Prevalence of HrHPV in different age groups in Pop A

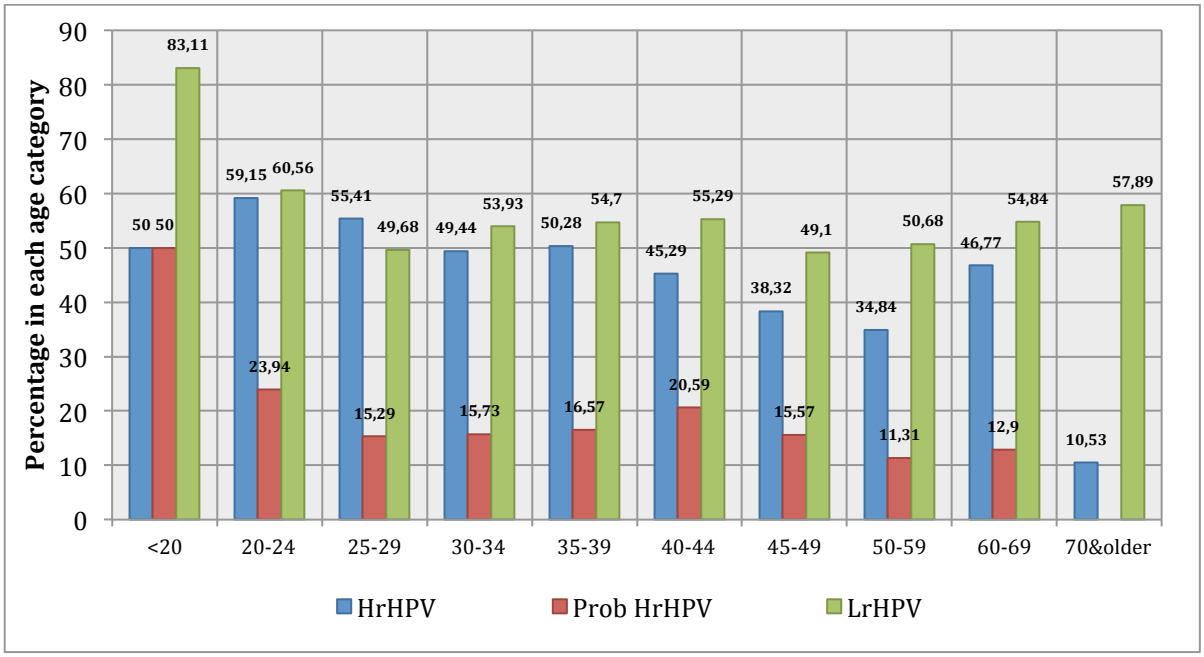


Figure 1.7: Prevalence of HPV categories in different age groups in Pop A

Pop B

Figure 1.8 illustrates the distribution of number of HrHPVs detected per patient and Figure 1.9 illustrates the HrHPV type distribution. The high-risk distribution was different in Pop B from Pop A. HPV 51 infection was most prevalent (14 patients; 21.54%).

The prevalence of HrHPV infections was 78.46% and the prevalence of HPV 16 and/or 18 was high (16 patients; 24.62%). HPV 18 was more prevalent than HPV 16 (10 patients versus 6 patients). See figures 1.9, 1.10 and 1.11.

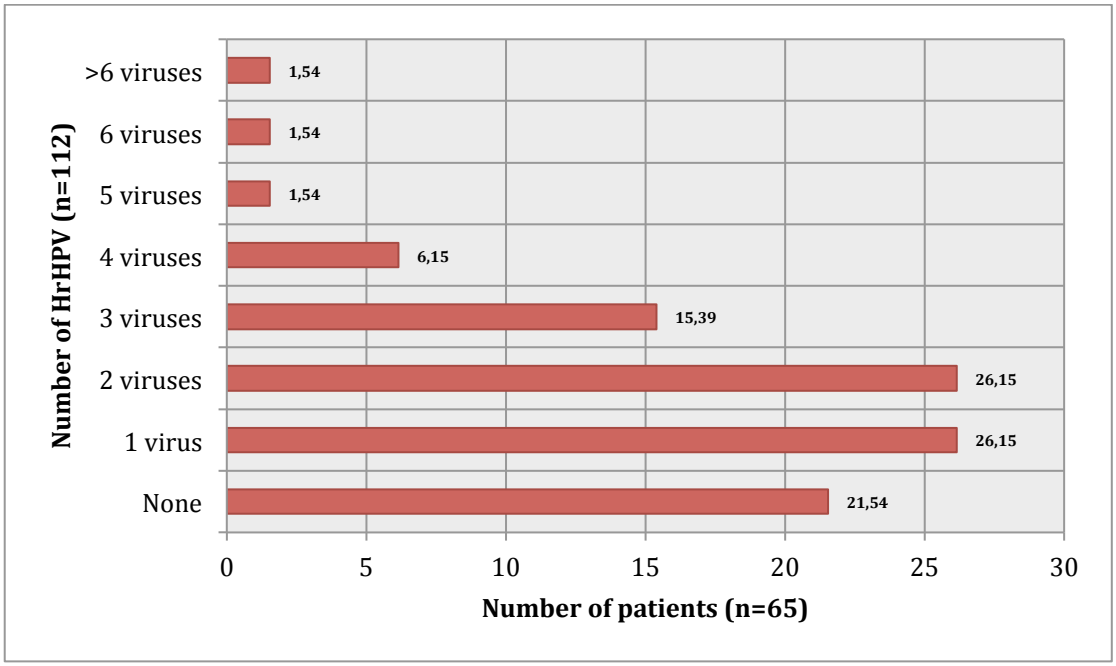


Figure 1.8: Total number of HrHPV types per patient in Pop B

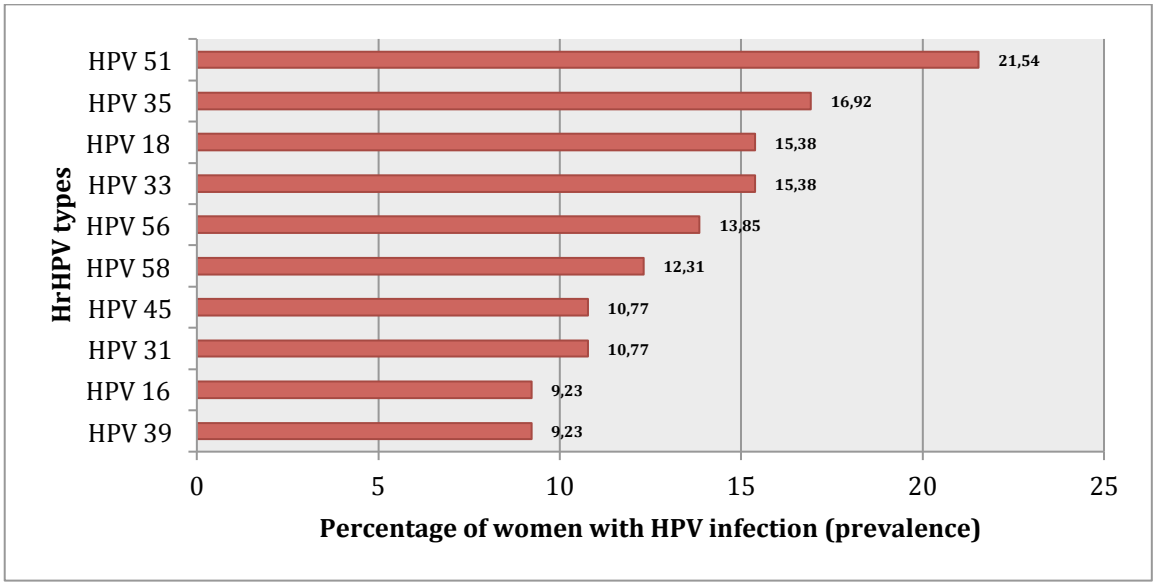


Figure 1.9: Ten most prevalent HrHPV types in Pop B

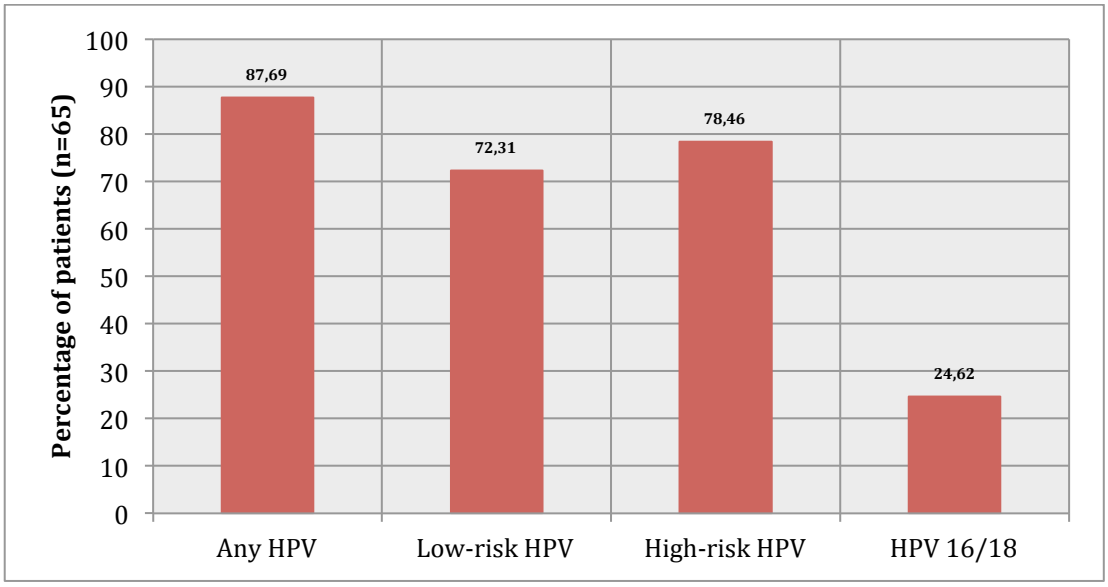


Figure 1.10: Distribution (percentage) of HPV infection in Pop B

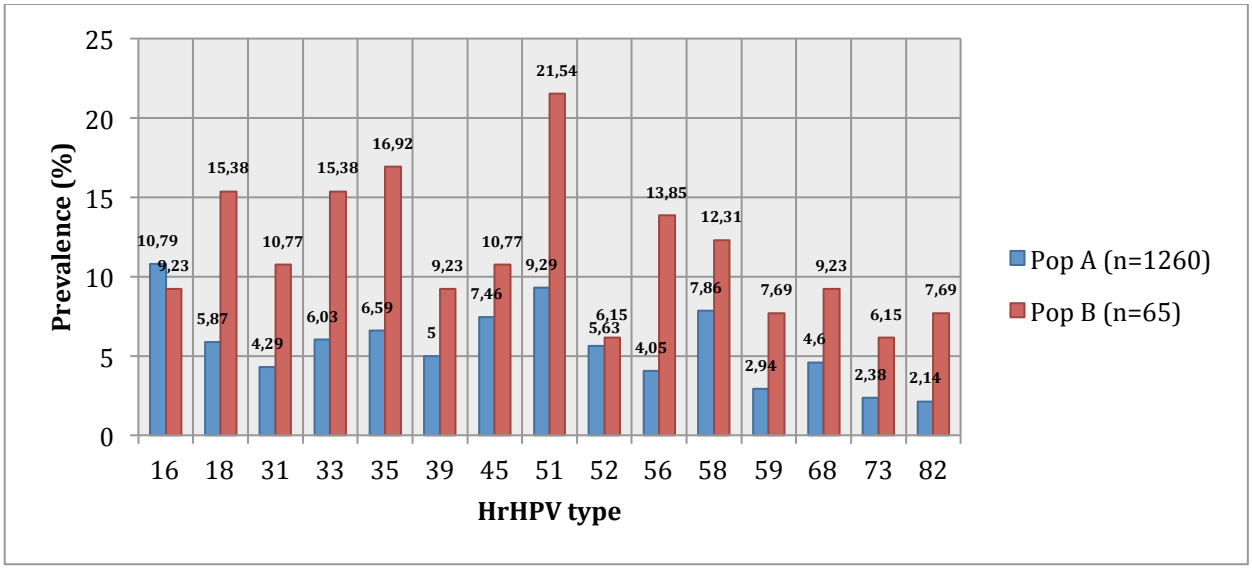


Figure 1.11: All HrHPV type/s detected in Pop A and Pop B

1.4.4. Most prevalent HPV types detected

Pop A

The ten most frequently observed HPV types among the general population with normal cytology are illustrated in Figure 1.12. Half of the viruses ranking under the top 10 were LrHPV types (indicated in blue) with HPV 62 and HPV 84 most

frequent. The four oncogenic HPV types and one probable oncogenic HPV type (indicated in red) were HPV 16, 51, 58, 45 and HPV 53.

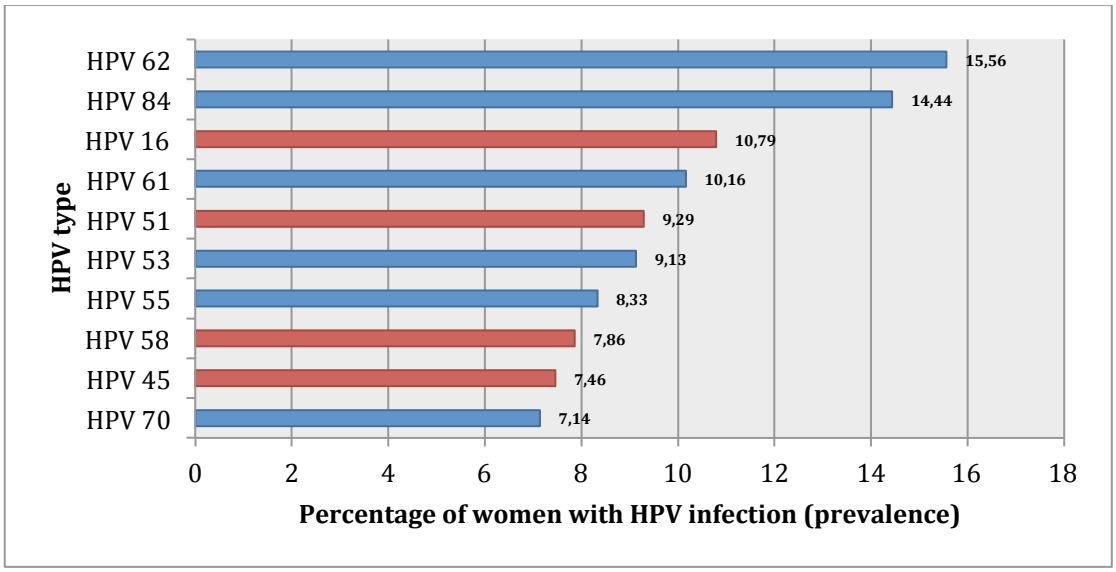


Figure 1.12: Ten most prevalent HPV types among women in Pop A (Red: HrHPV and prob HrHPV types)

Pop B

The ten most prevalent HPV types (see Figure 1.13) were HPV 62, followed in decending order by HPV 51, 66, 71, 35, 70, 84, 18, 33 and 56. Neither HPV 16 nor HPV 45 were among the top ten in Pop B, as was not the case in Pop A.

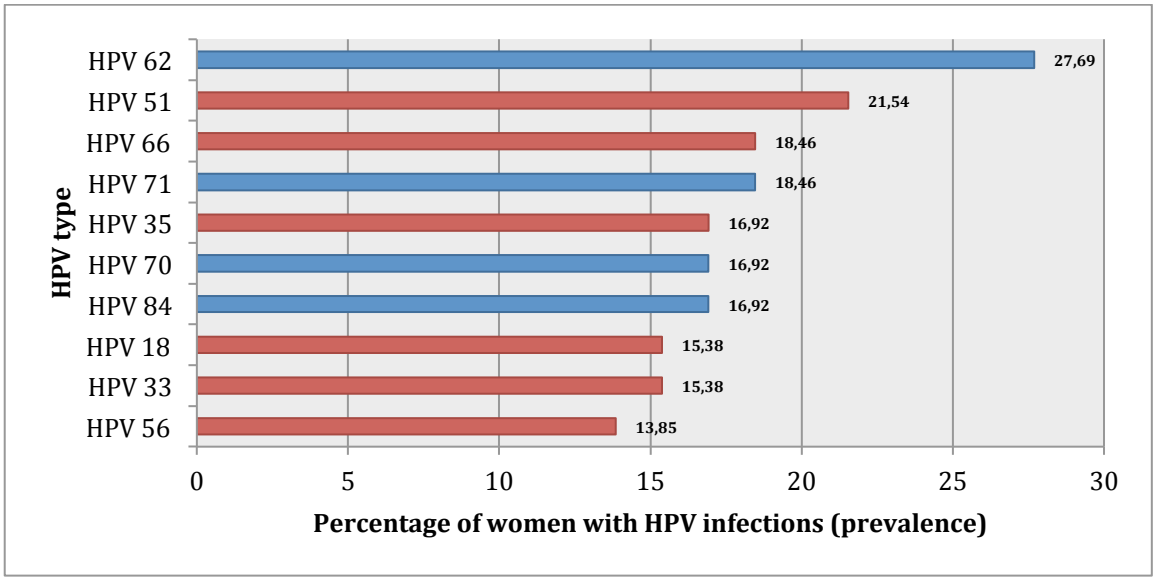


Figure 1.13: Ten most prevalent HPV type infections in Pop B (Red: High-risk and probable HrHPV types)

1.4.5. The eight most oncogenic HPV types in Pop A

As described under methods, the HrHPV types tested were divided in four groups. These were: HPV 16 and/or 18, HPV 35, 45 and/or 52, HPV 31, 33 and/or 58 and all the other HrHPV types grouped together. Twelve (0.95%) patients had one or more viruses present from all four groups. In 67 (5.3%) patients HPV 16 and/or 18 were the only high-risk viruses present and a further 130 (10.31%) patients had HPV 16 and/or 18 present together with different combinations from the other three high-risk groups. A total of 369 (29.29%) patients had HrHPV other than HPV 16 and/or 18. See Table 1.1.

Table 1.1: Top eight oncogenic HPV types

Number of Patients	HPV 16 and/or 18	HPV 35,45 and/or 52	HPV 31,33 and/or 58	Non Top 8 hr HPVs
12	+	+	+	+
14	+	+	+	-
21	+	+	-	+
24	+	+	-	-
16	+	-	+	+
14	+	-	+	-
29	+	-	-	+
67	+	-	-	-
10	-	+	+	+
12	-	+	+	-
36	-	+	-	+
103	-	+	-	-
35	-	-	+	+
64	-	-	+	-
109	-	-	-	+
694	-	-	-	-
1260	**197**	**232**	**177**	**268**

1.4.6. HPV distribution according to age categories in Pop A

In the 1238 patients where age was known, 563 (45.48%) patients tested positive for HrHPV DNA. In the group of women between 20 and 29, more women were infected with HrHPV than not (10.42% versus 7.99%). See Figure 1.14. Women older than 30 years composed 80.61% of the study population and 42.92% of them tested HrHPV DNA positive.

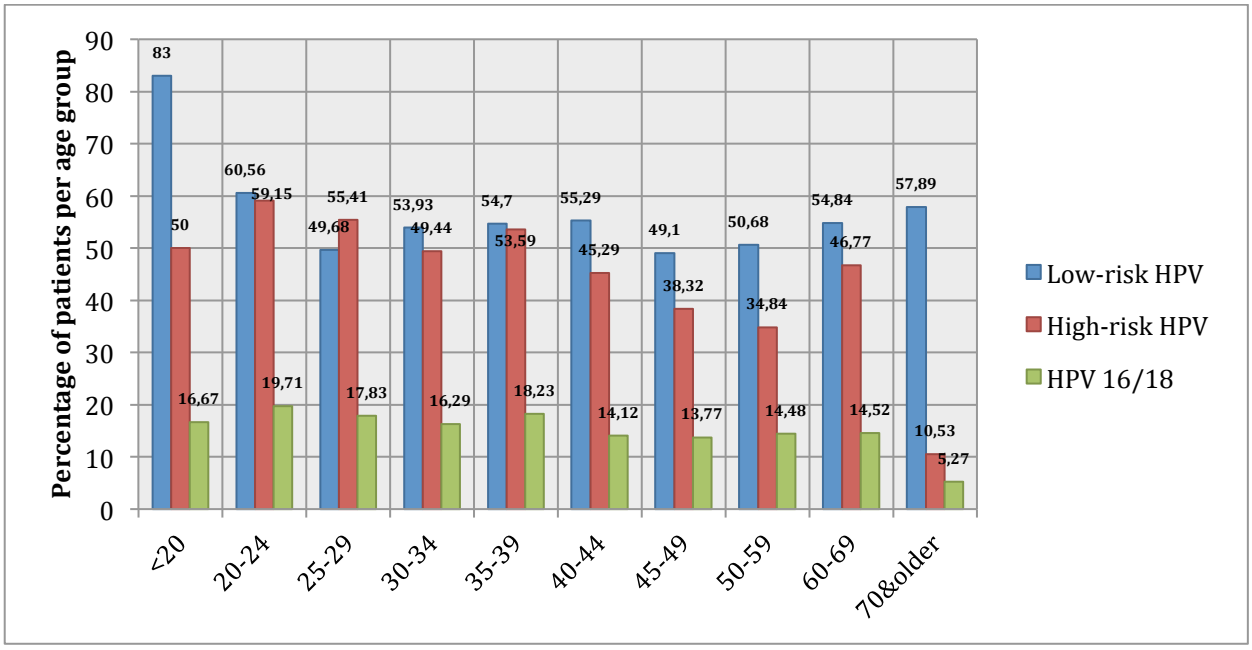


Figure 1.14: HPV prevalence of HPV 16/18, high- and LrHPV types in different age categories in Pop A

One-hundred-and-ninety-five (15.75%) women were infected with HPV 16 and/or 18. The prevalence in women younger than 30 years was 18.33% and in women 30 years and older 15,13%. Two (16.67%) patients younger than 20 years were infected with HPV 16 and/or 18.

The age group with the highest prevalence was women between 20 and 24 years (14 patients; 19.71%), followed by women between 35 and 39 years (33 patients; 18.23%) and between 25 and 29 years (28 patients; 17.83%). Apart from women 70 years and older (1 patient; 5.27%) the group with the lowest prevalence was between the ages of 45 and 49 years (23 patients; 13.77%). See Table 1.2.

Table 1.2: Distribution of HrHPV types in different age groups in relation to four groups

	<20	20-24	25-29	30-34	35-39	40-44	45-49	50-59	60-69	>70	Total
vvHr,vHr,Hr,Hrr	0	2	1	4	1	2	0	2	0	0	12
	0.00	2.82	0.64	2.25	0.55	1.18	0.00	0.90	0.00	0.00	0.97
vvHr,vHr,Hr	0	1	2	2	5	2	1	1	0	0	14
	0.00	1.41	1.27	1.12	2.76	1.18	0.60	0.45	0.00	0.00	1.13
vvHr,vHr,Hrr	1	2	7	3	4	1	2	1	0	0	21
	8.33	2.82	4.46	1.69	2.21	0.59	1.20	0.45	0.00	0.00	1.70
vvHr,vHr	0	1	9	2	3	2	3	3	0	0	23
	0.00	1.41	5.73	1.12	1.66	1.18	1.80	1.36	0.00	0.00	1.86
vvHr,Hr,Hrr	0	1	3	4	3	1	0	3	1	0	16
	0.00	1.41	1.91	2.25	1.66	0.59	0.00	1.36	1.61	0.00	1.29
vvHr,Hr	0	1	2	3	3	1	2	2	0	0	14
	0.00	1.41	1.27	1.69	1.66	0.59	1.20	0.90	0.00	0.00	1.13
vvHr,Hrr	1	0	1	5	8	5	4	4	0	0	28
	8.33	0.00	0.64	2.81	4.42	2.94	2.40	1.81	0.00	0.00	2.26
vvHr	0	6	3	6	6	10	11	16	8	1	67
	0.00	8.45	1.91	3.37	3.31	5.88	6.59	7.24	12.90	5.26	5.41
vHr,Hr,Hrr	0	1	1	1	4	2	1	0	0	0	10
	0.00	1.41	0.64	0.56	2.21	1.18	0.60	0.00	0.00	0.00	0.81
vHr,Hr	0	1	4	1	1	1	1	3	0	0	12
	0.00	1.41	2.55	0.56	0.55	0.59	0.60	1.36	0.00	0.00	0.97
vHr,Hrr	0	4	6	8	5	3	4	4	2	0	36
	0.00	5.63	3.82	4.49	2.76	1.76	2.40	1.81	3.23	0.00	2.91
vHr	1	2	19	19	15	18	10	10	9	0	103
	8.33	2.82	12.1	10.67	8.29	10.59	5.99	4.52	14.52	0.00	8.32
Hr,Hrr	1	3	6	3	9	5	1	4	2	0	34
	8.33	4.23	3.82	1.69	4.97	2.94	0.60	1.81	3.23	0.00	2.75
Hr	0	6	6	8	11	8	11	11	3	0	64
	0.00	8.45	3.82	4.49	6.08	4.71	6.59	4.98	4.84	0.00	5.17
Hrr	2	11	17	19	13	16	13	13	4	1	109
	16.67	15.49	10.83	10.67	7.18	9.41	7.78	5.88	6.45	5.26	8.80
None	6	29	70	90	90	93	103	144	33	17	675
	50.0	40.85	44.59	50.56	49.72	54.71	61.68	65.16	53.23	89.47	54.52
Total	12	71	157	178	181	170	167	221	62	19	1238
	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

vvHr – HPV 16 and/or 18
vHr – HPV 35,45 and/or 52
Hr – HPV 31,33 and/or 58
Hrr – HPV 39,51,56,59,68,73 and/or 82

1.5. DISCUSSION

1.5.1. Age distribution

Prevalent HPV infections are less likely to persist in younger women than in older women. Over a 36-month period, four of five immunocompetent women cleared the infection spontaneously.[12] In this study, the age distribution showed a bell-shaped curve, with a peak between 35 and 39 years of age. The highest prevalence of HrHPV infections was observed in young women. This finding is similar to the findings in another South African study.[21] In the current study, 56% of women younger than 30 years of age were infected with HrHPV.

Results from the current study were comparable to the findings of a Kenyan study, which showed the highest prevalence of HPV infection to be in women between the ages of 25 and 29 years. Fifty-eight per cent of these Kenyan patients tested positive for HPV DNA.[22] However, the findings from the Kenyan study included all women tested, regardless of cervical cytology. The peak in the prevalence of Harp infections in women between 20 and 29 years of age was also noted in a Korean study that was representative of the general population. In the current study, the infection rate was more than double the 23% prevalence found in the Korean population.[16]

In contrast to other African and international studies that showed a decline in HPV prevalence in the general population with increasing age, there was a peak in the prevalence of HrHPV infections in the women in this population between 60 and 69 years of age without cytological abnormalities.[10,19,23] However, a large meta-analysis of women with normal cervical cytology revealed a similar second peak in older women from southern Africa, southern Europe and southern Asia.[9]

The reasons for the peak in this age group were not clear. They might relate to sexual behaviour and/or unscreened and untreated patients. Other reasons might be poor cervical cancer screening implementation, and the highly sensitive method used to test for HPV DNA.[10] Reactivation of latent infections might also

be associated with a decline in immune function associated with ageing.[12] Gravitt et al. suggest that an increased risk of latent HPV infection at 50 years of age might be responsible for the higher prevalence of HPV infection in older women.[24]

1.5.2. Prevalence of any HPV infection and HrHPV types

The prevalence of HPV infection was high. 67.06% of patients were infected with one or more HPV type, and 44.92% of patients were infected with one or more oncogenic HPV type. In comparison to other South African data, the prevalence was slightly lower in this cohort of women with normal cytology than that of 74.6% and 54.3% for all HPV and HrHPV infections, respectively, as reported by Richter et al., for women with and without cytological abnormalities.[10]

The prevalence of HrHPV infections in women with cytological results within normal limits was 20.7% from Cape Town data, but this study was limited to HIV-noninfected women.[19] A global review of the age-specific prevalence of HPV infections reported the prevalence of HPV infections to be between 7% and 60% for Africa, irrespective of cytological findings.[19] Bruni et al. reported the HPV prevalence in women with normal cytology in sub-Saharan Africa to be 24%.[9] The crude prevalence of HPV infections in South Africa, regardless of cervical cytological findings, was reported as 42.2% by Vinodhini et al. These authors also illustrated a marked difference between developed and less developed countries.[1]

The burden of HPV infections in the extended Middle East and North Africa were reported as ranging between 0% and 25% in women with normal cytology.[8] The most recently available prevalence of HPV infections in South Africa, obtained from the World Health Organization/Institut Català d'Oncologica (ICO) HPV information centre, was 21% in women without cytological abnormalities.[25]

The prevalence of HrHPV infections in this study was lower than that reported by Richter et al.,[10] but higher than that reported in other studies carried out in

sub-Saharan Africa.[21] This finding is expected because the current study comprised only women with normal cytology. No differentiation was made between HIV- infected and HIV-noninfected women in this study, which might explain the higher prevalence in this study. The prevalence of any HPV infection in women with and without cervical disease from the USA was reported to be 42.5%, and of any high-risk type 29%.[2] A community-based cohort study in Korea reported the prevalence of HrHPV infections to be 12.6% for study participants.[16]

The prevalence of HrHPV infections in older women in the current study population was almost eight times higher in women aged 60-70 years than that in women who were older than 57 years in the USA.[26]

1.5.3. Specific LrHPV and HrHPV types

The most common HPV type in Pop A was HPV 62, followed by HPV 84 and HPV 16. These findings correlate with data from the USA where HPV 62 and HPV 84 were the most frequent LrHPV infections.[2] Previous published data from southern Africa reported HPV 83 and HPV 53 to be the most prevalent types.[25]

In a more recent publication from Cape Town, HPV 35, 16 and 58 were the most commonly seen infections in women with normal cytology.[21] HPV 56 was the most prevalent in Korea, followed by HPV 18 and HPV 52 in women with and without cervical disease, and HPV 6, 11, 16 and 18 in women with no cytological abnormalities in the extended Middle East and North Africa.[4,16]

When a comparison was made of women with normal cytological findings from different regions globally, HPV 16 was the most common type, except in western Africa, where HPV 31 was the most prevalent. HPV 6 and 11 were uncommon in this population, but slightly more prevalent than the 0.8% reported prevalence for Africa.[9]

In Pop A, HPV 16 was the most common HrHPV type, but in contrast to worldwide reported data, it was followed by HPV 51 and 58 instead of HPV 18.[9] HPV 18 was ranked seventh of all the oncogenic HPV types.

On the basis of findings from Denny et al.,[27] the most frequently identified HPV types in women with invasive cervical cancer in sub-Saharan Africa were HPV 16, 18, 45, 35 and 52. This finding highlights that HPV 45 significantly contributed to cervical cancer.[27] Of the five most frequent HPV type infections in Pop A, three HrHPV types were present, namely HPV 16, 45 and 35. The prevalence of HPV 45 was 7.46%, much more common than the reported prevalence of 0.5% in other women with normal cytology, as reported by Bruni et al.[9]

1.5.4. HPV 16 and/or 18

The prevalence of HPV 16 and/or HPV 18 in Pop A was 15.63%, considerably higher than reported in other world regions in women with and without cervical disease.[1,9] McDonald et al. found HPV 35 and 16 infections to be the most common types in women without cytological abnormalities. HPV 35 was as prevalent as, or more prevalent than, HPV 16.[21] The prevalence (reported by McDonald et al.) of high-risk types HPV 16 and/or 18 in women testing HrHPV positive without cytological abnormalities was 25.39%, compared to 34.64% in the current study.[21]

Approximately one in five women aged 25 years and younger are already exposed to HPV 16 and/or 18, so prophylactic vaccines should be administered before sexual debut in order to prevent HPV 16 and/or 18 infections.[10] In Pop A, the prevalence of HPV 16 and/or 18 was 15.66% in women younger than 25 years of age in the study population. In comparison to other types, Bruni et al. showed that HPV 16 was the most prevalent type and had the highest relative contribution.[9]

In Africa and other areas where HPV is particularly common, the higher prevalence of other HPV types could explain the inverse correlation between the overall HPV prevalence and the contribution of HPV 16.[9] The prevalence of HPV 16 and/or 18 was more than four times that reported by the HPV information centre for South Africa in women with normal cytology, almost twice (15.63% vs. 8.7%) that for Korean women, and almost triple that in females in the USA (15.63% vs. 6.6%) with and without cytological abnormalities.[2,16,25] Smith et al. reported the prevalence of HPV 16 and/or 18, stratified by age, to be less than 8%.[19]

1.5.5. Infection with multiple HPV types

Infections with numerous HPV types are frequently found in women who are sexually active, and especially in HIV-infected women.[28,29] There has been a rise from 4% to 15.7% in multiple HPV infections found in cervical cancer in the past 20 years.[28] However, from the results of one study, it did not seem as if multiple HPV infections had an influence on the natural history of one another.[30]

In Pop A, almost as many women had a single HrHPV type infection as those with multiple HrHPV infections (23.57% vs. 21.35%). It was rare to find multiple HPV infections simultaneously in the earliest studies on HPV, probably because of diagnostic test limitations. Today, it is clear that a woman can harbour multiple HPV infections with different oncogenic types.[28,29]

1.5.6. HPV and HIV

Among the small subgroup of patients with AIDS (Pop B), the prevalence of HPV was extremely high (87.69%), higher than a previously reported prevalence in Africa (75%) in HIV-infected women.[19] A study among high-risk groups confirmed a high prevalence of HPV infections greater than 50%.[19]

A number of studies have indicated that an independent link exists between HIV and acquirement of HPV infections, as well as diseases arising from the infections. HIV-infected and other immune-depleted women have increased

rates of HPV infections and invasive cervical cancer, as well as treatment resistance of HPV-associated disease.[31] Persistent HPV infections are more likely in HIV-infected women together with a higher incidence of cervical dysplasia.[31]

HIV-infected women in comparison to HIV-non-infected women, globally, appear to be infected with HrHPV types other than HPV 16, regardless of whether cytological abnormalities are present or not.[32] Similar trends were seen in this population, with 78.46% of women infected with one or more high-risk types and the prevalence of HPV 16 and/or HPV 18 was 24.62%. HPV 16 was ninth most prevalent and HPV 18 third. The efficacy of the currently available bivalent and quadrivalent vaccines in preventing cervical cancer in HIV-infected women could be questioned because of the underrepresentation of HPV 16 in this population of women.[31,33]

Other southern African studies among HIV-infected women showed a large variety in HPV types, with between 95% to 98% prevalence of any HPV infection and around 85% infection rates of HrHPV types.[34,35] Despite high HIV and HPV infection rates among women living in developing countries, these countries lack proper cervical cancer screening programmes. These women might benefit most from HPV vaccination programmes.[33]

Looking at the effect of HIV on HPV in sexually active partners, Mbulawa et al. found the prevalence of both high- and LrHPV decreasing with increasing age. HIV-infected women were more likely to be infected with any HPV, including HrHPV than HIV-non-infected women. This risk increased as CD_4 cell count decreased.[36] A CD_4 cell count of 200 cells/µL and less was found to be the most significant independent predictor of HrHPV infection and genital warts.[33]

The average CD_4 cell count in Pop B was 158 cells/µL. In South Africa the initiation of HAART is late, with an average CD_4 cell count of 87 cells/µL at the time of initiation. Less than 60% of patients qualifying for HAART received treatment in South Africa in 2010.[10]

Firnhaber et al. demonstrated high incidence and progression rates of cervical lesions among South African women infected with HIV. The use of HAART was associated with a reduction in the incidence and the chance of progression of cervical lesions.[37]

1.6. CONCLUSION

HPV infections were highly prevalent in this study of women without cervical cytological abnormalities and extremely high in women with AIDS. The most prevalent HPV types in Pop A were HPV 62, 84 and 16. The prevalence of HPV 16 and/or 18 was higher than that reported in other world regions, and occurred at a young age. The high prevalence of high-risk HPV types in women with normal cytology is important to consider before implementing HPV-based screening tests in this population, and needs to be addressed with regard to a cost-benefit analysis and the potential impact that it might have on health care in South Africa. These findings are also important to guide future vaccine development and to support the need for early vaccination in this population.

1.7. REFERENCES

1. Vinodhini K, Shanmughapriya S, Das BC, Natarajaseenivasan K. Prevalence and risk factors of HPV infection among women from various provinces of the world. Arch Gynecol Obstet. 2012;285(3):771-777.
2. Hariri S, Unger ER, Sternberg M, Dunne EF, Swan D, Patel S, et al. Prevalence of genital human papillomavirus among females in the United States, the National Health And Nutrition Examination Survey, 2003-2006. J Infect Dis. 2011;204(4):566-573.
3. Castle PE, Rodríguez AC, Burk RD, Herrero R, Wacholder S, Hildesheim A, et al. Long-term persistence of prevalently detected human papillomavirus infections in the absence of detectable cervical precancer and cancer. J Infect Dis. 2011;203(6):814-822.
4. Oakeshott P, Aghaizu A, Reid F. Howell-Jones R, Hay PE, Sadiq ST, et al. Frequency and risk factors for prevalent, incident, and persistent genital carcinogenic human papillomavirus infection in sexually active women: community based cohort study. BMJ. 2012;344:e4168.
5. Richter KL. Understanding and incorporating human papillomavirus testing in cervical cancer screening: a South African perspective. South Afr J Gynaecol Oncol. 2011;3(1):9-14.
6. Carter JR, Ding Z, Rose BR. HPV infection and cervical disease: a review. Aust N Z J Obstet Gynaecol. 2011;51(2):103-108.
7. Van Bogaert LJ. High-risk human papillomavirus screening and testing with immunohistochemical surrogate biomarkers: an alternative to polymerase chain reaction. South Afr J Gynaecol Oncol. 2012;4(1):30-33.
8. Seoud M. Burden of human papillomavirus-related cervical disease in the extended middle East and north Africa - a comprehensive literature review. J Low Genit Tract Dis. 2012;16(2):106-120.
9. Bruni L, Diaz M, Castellsagué X, Ferrer E, Bosch FX, de Sanjosé S. Cervical human papillomavirus prevalence in 5 continents: meta-analysis of 1 million women with normal cytological findings. J Infect Dis. 2010;202(12):1789-1799.

10. Richter K, Becker P, Horton A, Dreyer G. Age-specific prevalence of cervical human papillomavirus infection and cytological abnormalities in women in Gauteng Province, South Africa. S Afr Med J. 2013;103(5):313-317.
11. Winer RL, Lee SK, Hughes JP, Adam DE, Kiviat NB, Koutsky LA. Genital human papillomavirus infection: incidence and risk factors in a cohort of female university students. Am J Epidemiol. 2003;157(3):218-226.
12. Wright TC Jr. Natural history of HPV infections. J Fam Pract. 2009;58(9 Suppl HPV):S3-7.
13. South African Human Papillomavirus Advisory Board. Prophylactic human papillomavirus vaccination against cervical cancer: a summarised resource for clinicians. South Afr J Gynaecol Oncol. 2011;3(1):39-42.
14. South African Human Papillomavirus Advisory Board. Cervical cancer and human papillomavirus: South African guidelines for screening and testing. South Afr J Gynaecol Oncol. 2010;2(1):23-26.
15. Hwang LY, Ma Y, Shiboski SC, Farhat S, Jonte J, Moscicki AB. Active squamous metaplasia of the cervical epithelium is associated with subsequent acquisition of human papillomavirus 16 infection among healthy young women. J Infect Dis. 2012;206(4):504-511.
16. Kim MA, Oh JK, Chay DB, Park DC, Kim SM, Kang ES, et al. Prevalence and seroprevalence of high-risk human papillomavirus infection. Obstet Gynecol. 2010;116(4):932-940.
17. Massad LS, Evans CT, Weber KM, Goderre JL, Hessol NA, Henry D, et al. Changes in knowledge of cervical cancer prevention and human papillomavirus among women with human immunodeficiency virus. Obstet Gynecol. 2010;116(4):941-947.
18. Chaturvedi AK, Katki HA, Hildesheim A, Rodríguez AC, Quint W, Schiffman M, et al. Human papillomavirus infection with multiple types: pattern of coinfection and risk of cervical disease. J Infect Dis. 2011;203(7):910-920.
19. Smith JS, Melendy A, Rana RK, Pimenta JM. Age-specific prevalence of infection with human papillomavirus in females: a global review. J Adolesc Health. 2008;43(4 Suppl):S5-25.

20. Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med. 2003;348(6):518-527.
21. McDonald AC, Denny L, Wang C, Tsai WY, Wright TC Jr, Kuhn L. Distribution of high-risk human papillomavirus genotypes among HIV-negative women with and without cervical intraepithelial neoplasia in South Africa. PLoS One. 2012;7(9):e44332.
22. De Vuyst H, Steyaert S, Van Renterghem L, Claeys P, Muchiri L, Sitati S, et al. Distribution of human papillomavirus in a family planning population in Nairobi, Kenya. Sex Transm Dis. 2003;30(2):137-142.
23. Franceschi S, Herrero R, Clifford GM, Snijders PJ, Arslan A, Anh PT, et al. Variations in the age-specific curves of human papillomavirus prevalence in women worldwide. Int J Cancer. 2006;119(11):2677-2684.
24. Gravitt PE, Rositch AF, Silver MI, Marks MA, Chang K, Burke AE, et al. A cohort effect of the sexual revolution may be masking an increase in human papillomavirus detection at menopause in the United States. J Infect Dis. 2013;207(2):272-280.
25. WHO/ICO Information Centre on Human Papilloma Virus (HPV) and Cervical Cancer. Human papillomavirus and related cancers in South Africa. Summary Report 2010. Available from: www.hpvcentre.net/statistics/reports/ZAF.pdf (accessed 3 February 2013).
26. Lindau ST, Drum ML, Gaumer E, Surawska H, Jordan JA. Prevalence of high-risk human papillomavirus among older women. Obstet Gynecol. 2008;112(5):979-989.
27. Denny L, Adewole I, Anorlu R, Dreyer G, Moodley M, Smith T, et al. Human papillomavirus prevalence and distribution in invasive cervical cancer in sub-Saharan Africa. Int J Cancer. 2014;134(6):1389-1398.
28. Plummer M, Vaccarella S, Franceschi S. Multiple human papillomavirus infections: the exception or the rule? J Infect Dis. 2011;203(7):891-893.
29. Marais DJ, Passmore JA, Denny L, Sampson C, Allan BR, Williamson AL. Cervical and oral human papillomavirus types in HIV-1 positive and

negative women with cervical disease in South Africa. J Med Virol. 2008;80(6):953-959.

30. Campos NG, Rodriguez AC, Castle PE, Herrero R, Hildesheim A, Katki H, et al. Persistence of concurrent infections with multiple human papillomavirus types: a population-based cohort study. J Infect Dis. 2011;203(6):823-827.
31. Denny LA, Franceschi S, de Sanjosé S, Heard I, Moscicki AB, Palefsky J. Human papillomavirus, human immunodeficiency virus and immunosuppression. Vaccine. 2012;30(Suppl 5):168-174.
32. Clifford GM, Gonçalves MA, Franceschi S, HPV, HIV study group. Human papillomavirus types among women infected with HIV: a meta-analysis. AIDS. 2006;20(18):2337-2344.
33. Firnhaber C, Wilkin T. Human papillomavirus vaccines: where do they fit in HIV-infected individuals? Curr HIV/AIDS Rep. 2012;9(3):278-286.
34. Firnhaber C, Zungu K, Levin S, Michelow P, Montaner LJ, McPhail P, et al. Diverse and high prevalence of human papillomavirus associated with a significant high rate of cervical dysplasia in human immunodeficiency virus-infected women in Johannesburg, South Africa. Acta Cytol. 2009;53(1):10-17.
35. Sahasrabuddhe VV, Mwanahamuntu MH, Vermund SH, Huh WK, Lyon MD, Stringer JS, et al. Prevalence and distribution of HPV genotypes among HIV-infected women in Zambia. Br J Cancer. 2007;96(9):1480-1483.
36. Mbulawa ZZ, Marais DJ, Johnson LF, Boulle A, Coetzee D, Williamson AL. Influence of human immunodeficiency virus and CD4 count on the prevalence of human papillomavirus in heterosexual couples. J Gen Virol. 2010;91(Pt 12):3023-3031.
37. Firnhaber C, Westreich D, Schulze D, Williams S, Siminya M, Michelow P, et al. Highly active antiretroviral therapy and cervical dysplasia in HIV-positive women in South Africa. J Int AIDS Soc. 2012;15(2):17382.

CHAPTER 2

ONCOGENIC AND INCIDENTAL HPV TYPES ASSOCIATED WITH CERVICAL PRE-NEOPLASIA IN HIV-POSITIVE- AND HIV-NEGATIVE WOMEN

2.1. INTRODUCTION

2.1.1. Cervical pre-malignant lesions

Cervical cytology, commonly known as a "Papanicolaou" smear, is the most widespread screening method used to detect early changes that, if left untreated, could progress to invasive cervical cancer. Exfoliating cells are collected from the ecto- and endocervical junction, known as the transformation zone, from where cervical dysplasia and cancer originate.[1]

Microscopic changes associated with premalignant and malignant lesions allow grading of abnormal specimens. On the the Bethesda classification system, depending on the number of cellular changes, cervical cytology can be graded as low-grade squamous intraepithelial lesions (LSIL) or high-grade squamous intraepithelial lesions (HSIL).[1]

Cervical cytology is limited to inspection of cells and the diagnosis of cervical intraepithelial neoplasia (CIN) requires the examination of cervical tissue to make a histological diagnosis.[1] "CIN" is a term describing the pre-invasive histological changes that precede invasive cancer of the uterine cervix.[2]

Classifying CIN in three grades is based on the extent of atypical cellular changes. CIN grade I (CIN I) refers to atypical cellular changes limited to the lower third of the epithelium and is considered a low-grade lesion. CIN grade II (CIN II) and III (CIN III), both considered high-grade lesions, refer to more extensive cellular atypia involving less than the basal two-thirds and more than two-thirds of the epithelium respectively.[3]

2.1.2. Risk factors

One of the known predisposing causes for these preneoplastic changes is a persistent infection of one or more of the human papillomavirus (HPV) types. The immune system's inability to resist changes plays a vital role in the

development of cervical carcinoma.[2] Immunocompromised individuals have an increased risk of cervical neoplasia.[4,5]

This increased risk is not only limited to immune dysfunction resulting from human immunodeficiency virus (HIV), but also as a result of other factors like immunosuppressive therapy. In patients on immunosuppressive therapy after renal transplant, one study reported a two to six times higher risk for CIN and a three times higher risk for cervical cancer.[6] Apart from HPV infections and immune suppression, other important cofactors in cervical carcinogenesis are smoking cigarettes and long-term oral contraceptive users.[5]

2.1.3. Progression of CIN

In general, if no treatment is provided, the greater part of low-grade cervical lesions (CIN I) regress, while a substantial number of patients with high-grade lesions (CIN II/III) will progress to invasive cervical cancer. CIN associated with high-risk HPV (HrHPV) types have a higher chance of progressing to a more advanced stage of disease.[7]

High-grade lesions do not inevitably follow low-grade lesions, contradicting the traditional belief regarding an orderly progression of cervical neoplasia from CIN I to invasive cervical cancer. Calculated roughly, it takes 7 to 10 years for an untreated high-grade CIN to progress to cancer, which is longer than the time it takes from initial infection to the development of high-grade CIN.[7] The risk of cervical cancer is greatest for women with CIN III.[1]

2.1.4. Implications of HIV

Globally, with an estimated 33 million people infected, the HIV/AIDS pandemic is placing a huge burden on health care systems and has an enormous impact on women of all ages.[8,9] Around 75% of women infected with HIV are living in sub-Saharan Africa and South Africa has more HIV-infected women than any other country.[10,11]

It is well established that HIV-infected women have a higher susceptibility to HPV infections and HPV-associated lesions, which include CIN II/III and cervical cancer.[11-16] HrHPV types and CIN are up to four times more common in HIV-positive women.[17]

The prevalence of HPV infections and associated cervical intraepithelial neoplasia (CIN), the precursor of cervical cancer, is high in HIV-infected women.[16] Other studies have shown that up to one in five women co-infected with HIV and HPV will develop squamous intraepithelial lesion (SIL) within three years following the diagnosis of HIV. The rate at which pre-malignant lesions progress to cervical cancer appears to be quicker among immunedepleted women and linked to an increase in morbidity and mortality.[18]

2.1.5. HPV prevalence and HIV

Despite a higher prevalence of all HPV type infections among HIV-infected women, there is also an increased prevalence of oncogenic HPV types and multiple-HPV type infections.[10] HPV infections are also more likely to persist when patients are co-infected with HIV.[19]

Although HPV 16, 18, 31, 33, 45, 52 and 58 are regarded as the HPV types with the highest carcinogenic potential, other HPV types (HPV 35, 39, 51, 56, 59, 68, 73 and 82) are also associated with CIN development.[18] Even though HPV 16 and 18 are responsible for up to 70% of cervical cancers, high-grade cervical lesions are more likely to be associated with non-HPV 16/18 types, especially among HIV-infected women.[18,20]

2.1.6. HPV, CIN and immunedepletion

In women with HIV co-infection, studies have shown that the chance of finding cervical HPV DNA together with abnormal cervical cytology increases as CD4 cell count decreases.[21] A decline in CD4 cell count and rising HIV viral load are both risk factors for invasive cervical lesions.[9] However, some aspects of the

relationship between CIN, immune depletion and the effect of highly active antiretroviral therapy (HAART) are not yet clear.[8]

A large cross-sectional analysis conducted in the USA showed a high prevalence of cervical cytological abnormalities among HIV-infected women compared to HIV-non-infected women with similar drug and sexual histories. Only HPV infections, especially types with greater oncogenic potential, and HIV associated immune compromise, in particular a CD4 cell count of less than 200/µl, were found to be associated with the presence of squamous intraepithelial lesions (SIL) in multivariate analysis.[22]

A study done in Brazil showed a strong correlation between immune suppression and the prevalence of SIL. Using CD4 cell count as marker for suppression, patients with a CD4 less than 100/µl showed almost a 3-fold higher prevalence for cytological alterations compared to patients with a CD4 of more than 400/µl.[23]

In another study, HIV-1 RNA plasma levels were shown to be the single most useful way to predict disease progression. Greater rates of coincidental infections by HrHPV types were associated with HIV-1 plasma levels of more than 10 000 copies/ml.[24]

Some data suggests that despite initiation of HAART and associated CD4 cell count increase, most women will not have regression of high-grade (CIN II/III) lesions. It appears as if immune status has a minimal role on either regression of high-grade lesions or cervical cancer advancing from these lesions. Although immune depletion results in a higher chance of premalignant cervical disease, it seems as if other factors contribute to the development of cervical cancer from high-grade cervical lesions.[25,26]

In order to prevent cervical cancer from developing from high-grade lesions, women with CIN II and CIN III are treated. This makes studies on the influence of immune suppression on the likelihood of invasive cancer difficult and unethical.

An indirect method to establish the correlation would be to study the immune status and immune markers in patients who develop cancer.[25]

2.1.7. Motivation for the study

Local data from Africa on the relationship between oncogenic HPV types, immune status and cervical pre-invasive lesions is incomplete.[27,28] The majority of studies in the literature use cytology results synonymously with cervical premalignant changes.[9,29] This highlights the need to compare the prevalence of oncogenic HPV types with histopathologically confirmed CIN in women with different levels of immune competence.

2.2. OBJECTIVE AND HYPOTHESES

2.2.1. Objectives

The objectives of this study were:

- Assessing HPV types present in patients with biopsy-confirmed cervical intraepithelial lesions and to compare HIV-infected- and HIV non-infected women
- Describing the prevalence of HrHPV types as immune function deteriorates, as indicated by CD4 cell count and duration on antiretroviral therapy
- Comparing findings from the study population with data from Africa and other continents

2.2.2. Hypotheses

The hypotheses of this study were that:

- HPV-associated pre-malignant cervical disease (CIN II/III) would vary among women with different levels of immunity
- Oncogenic HPV types would differ in prevalence when immunity deteriorates

- The prevalence of HPV types in the study population would be different from the prevalence of these types in the rest of the world.

2.3. Material and methods

2.3.1. Study design

This is a descriptive study performed at the gynaecologic oncology unit, University of Pretoria. It consists of data obtained from 1 July 2010 to 30 August 2013. Patients included in the study were women aged 18 years and older, referred for treatment of high-grade squamous intraepithelial lesions (HSIL) on conventional cervical cytology (Papanicolaou smears) as part of the national screening programme.

2.3.2. Consent process and ethical considerations

Patients received counselling and an information document that explained the method and voluntary nature of the study. During counselling by trained nursing personnel, patients were motivated to undergo HIV testing as per standard departmental management protocols. It was clearly explained to them that testing was voluntary and not a prerequisite for treatment. Planned treatment was also explained: large loop excision of the transformation zone (LLETZ) or directed biopsies if malignancy was suspected. All patients were informed of their HIV results, if applicable. All patients tested for HIV received post-test counselling and were offered a CD4 cell count. These patients were referred to the appropriate antiretroviral therapy clinic for further management. This study was approved by the Research Ethics Committee of the Faculty of Health Sciences of the University of Pretoria (26/2010, 189/2012).

2.3.3. Patient recruitment

Three-hundred-and-thirty-four (334) consecutive patients referred with HSIL on cervical cytology were invited to participate in the study. Histology results were available for all patients. Only the 270 patients with confirmed CIN II or CIN III

were included in the final study analysis. Of the 64 patients excluded, ten patients had cervical cancer; 25 had CIN I; 12 had cervicitis and eight had no histological abnormalities. The histology was inadequate in nine cases. CD4 cell count was recorded, if applicable, as well as treatment with HAART. On the basis of the 2010 South African guidelines[30], HAART was divided into different groups: patients not yet qualifying for HAART treatment (CD4 cell count > 350/µl); patients in the process of initiating HAART (CD4 cell count ≤ 350/µl); patients treated with HAART for less than six months; six to 12 months; and and patients on treatment for more than 12 months.

2.3.4. Sample collection and transport

Patients underwent colposcopic evaluation and a LLETZ procedure or biopsy as indicated, which were placed in buffered formalin. The samples were transported to the department of Anatomical Pathology at the University of Pretoria, where histological examination was performed.

HPV testing, using a dry swab, was performed on all participating patients. After collection, the swabs were transported in phosphate-buffered saline and 10% methanol solution to the Department of Medical Virology at the University of Pretoria, where HPV DNA testing was performed.

2.3.5. HPV DNA testing

DNA extraction was accomplished by means of the DNA Isolation Kit (Roche Molecular Systems®, Branchburg, NJ) on the MagNa Pure automated extraction system. HPV linear array (LA) genotyping kit (Roche Molecular Systems®, Branchburg, NJ) was used to determine the HPV type. Fifteen high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82), three probable high-risk types (HPV 26, 53 and 66) and 19 low/undetermined risk types (HPV 6, 11, 40, 42, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, IS39 and CP6108) were tested for.[31]

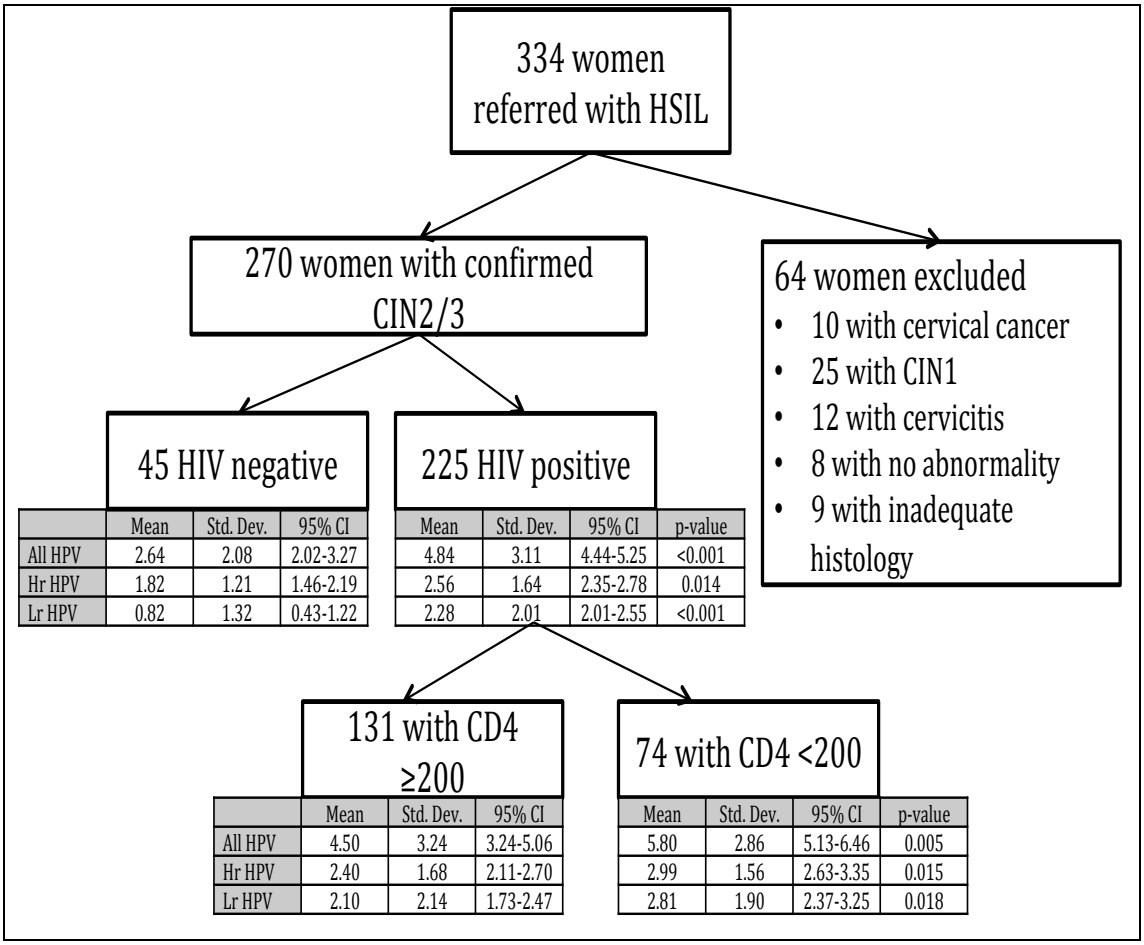
2.3.6. Data capturing and analysis

Data was captured on Microsoft® Excel® datasheets, and analysis performed using Stata® statistical software (StataCorp, College Station, USA). Discrete data was mainly binary in nature and summary statistics were frequency, percentage, 95% confidence intervals, cross-tables and bar charts. Continuous data was summarised with the use of descriptive statistics, mean and standard deviation along with 95% confidence intervals. Comparison between groups was done with Fisher's exact test for discrete outcomes and student's two-sample t-test or Wilcoxon rank sum test for continuous outcomes. Testing was done at the 0.05 level of significance.

2.4. RESULTS

2.4.1. Study population

The ages of patients ranged between 21 and 66. HIV results were available for all women. Of the 270 women, 225 (83.33%) were HIV infected and 45 (16.67%) were not infected. No significant difference existed between the two groups with regard to age of diagnosis (p=0.186). See Figure 2.1.



	Mean	Std. Dev.	95% CI
All HPV	2.64	2.08	2.02-3.27
Hr HPV	1.82	1.21	1.46-2.19
Lr HPV	0.82	1.32	0.43-1.22

	Mean	Std. Dev.	95% CI	p-value
All HPV	4.84	3.11	4.44-5.25	<0.001
Hr HPV	2.56	1.64	2.35-2.78	0.014
Lr HPV	2.28	2.01	2.01-2.55	<0.001

	Mean	Std. Dev.	95% CI
All HPV	4.50	3.24	3.24-5.06
Hr HPV	2.40	1.68	2.11-2.70
Lr HPV	2.10	2.14	1.73-2.47

Mean	Std. Dev.	95% CI	p-value
5.80	2.86	5.13-6.46	0.005
2.99	1.56	2.63-3.35	0.015
2.81	1.90	2.37-3.25	0.018

Figure 2.1: Descriptive statistics at baseline of study population and average number of HPV infections per patient

CD4 cell count was available for 205 (91.1%) patients and HAART treatment and duration were available for all patients. Seventy-four (36.10%) patients had a CD4 cell count less than 200/µl and 131 (63.90%) patients had a CD4 cell count of 200/µl or more.

On the basis of use and duration of highly active antiretroviral therapy (HAART), HIV-infected patients were divided into subgroups. Of the 225 patients, 35 (15.56%) patients did not yet qualify for HAART treatment (CD4 cell count > 350/µl), and 54 (24.00%) patients were in the process of initiating HAART (CD4 ≤ 350/µl). Fifty-six (24.89%), 37 (16.44%) and 43 (19.11%) patients had been treated with HAART for less than six months, for six to 12 months, and for more than 12 months respectively. See Figure 2.2.

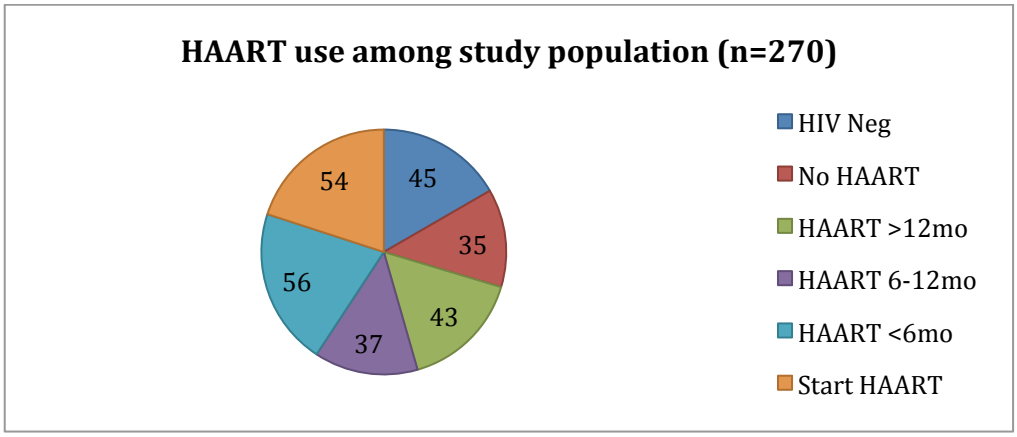


Figure 2.2: HAART use among study population (n=270)

2.4.2. HPV prevalence

The prevalence of any HPV type in patients with CIN II/III was 96.7%; among HIV-negative patients 97.8%, and 96.4% among HIV-positive patients. The prevalence of one or more HrHPV type/s was 93.0% among the entire population – 93.3% and 92.9% in HIV-negative and -positive patients respectively. Twenty-seven (60.0%) HIV-negative patients and 42 (18.7%) HIV-positive patients did not have any low-risk HPV (LrHPV) DNA detected.

2.4.3. Single and multiple HrHPV-type infections

Among the 45 HIV-non-infected patients, 119 HPVs were present, of which 82 were HrHPV. The total number of HPV types detected among the 225 HIV-infected women was 1090, with 577 of these categorised as HrHPV types. The number of HPV types detected per patient was significantly greater among HIV-infected than among –non-infected patients for all HPV types ($p<0.001$), as well as HrHPV ($p=0.014$) types. See Figure 2.1.

A third (33.3%) of HIV-negative patients had a single HPV type infection compared to only 18 (8.0%) HIV-positive patients. Multiple HPV types were present in 29 (64.4%) HIV-negative patients and 183 (81.3%) HIV-positive patients. Three (6.7%) HIV-negative patients had no HrHPV infections, 20 (44.4%) patients had a single HrHPV type infections and the remaining patients

(48.9%) had two or more HrHPV type infections. Forty-four (19.6%) patients co-infected with HIV had a single HrHPV type detected and 165 patients (73.3%) had multiple HrHPV type infections.

2.4.4. HPV type distribution

Among the total group, the most prevalent HrHPV types in descending order of frequency were HPV 16, 58, 35, 52, 51 and 45. The most prevalent HrHPV type in the HIV-negative group, illustrated in Figure 2.3 and Figure 2.4, was HPV 16, followed by HPV 52, 31, 35, 58, 18, 33 and 45. HPV 84 was the most prevalent non-HrHPV type. In the HIV-infected group, illustrated in figures 2.3 and 2.5, HPV 16 was also the most prevalent HrHPV type, followed by HPV 58, 35, 51, 52, 45, 18 and 31. The most prevalent LrHPV type was HPV 62 (See Figure 2.6). There was no statistically significant difference that correlated with the prevalence of specific HrHPV types with HIV status.

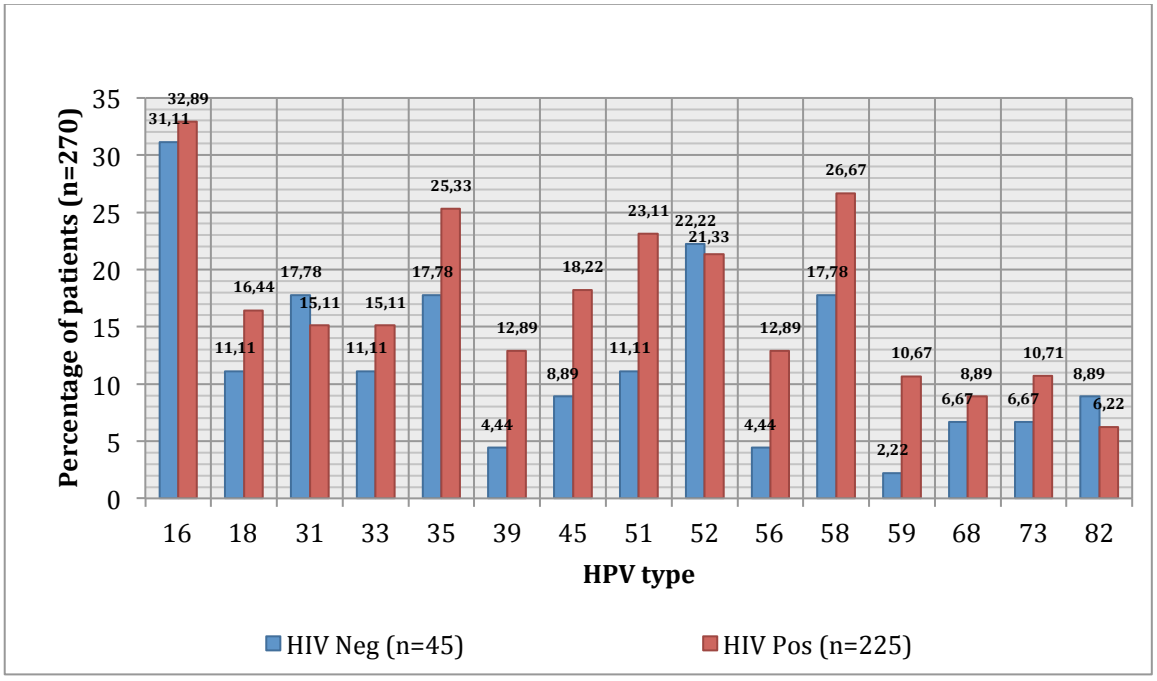


Figure 2.3: Distribution of HrHPV types among HIV-negative and –positive patients with CIN II/III

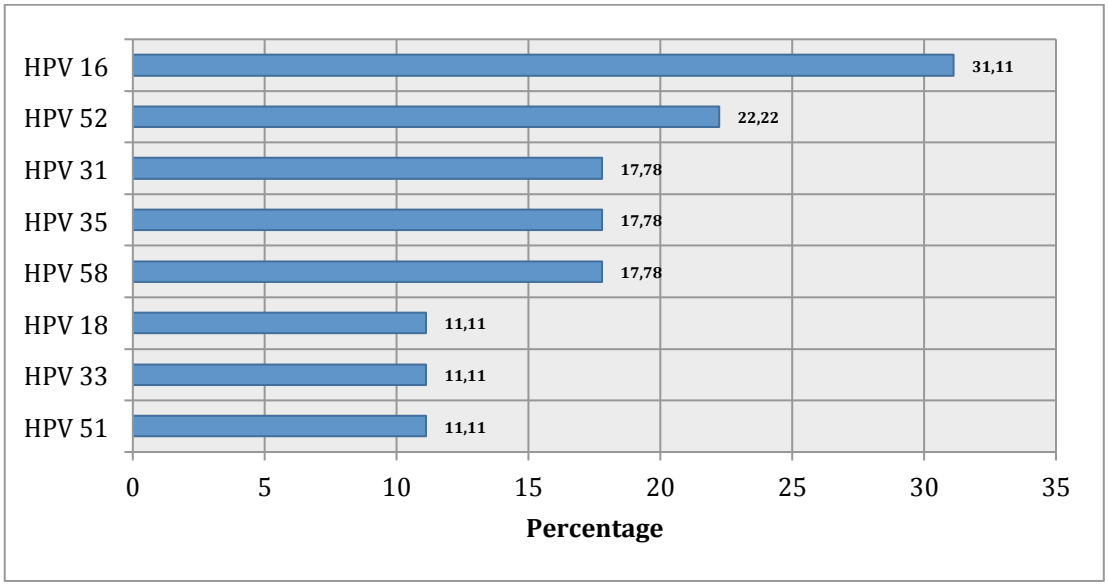


Figure 2.4: Eight most prevalent HrHPV types among HIV-negative patients

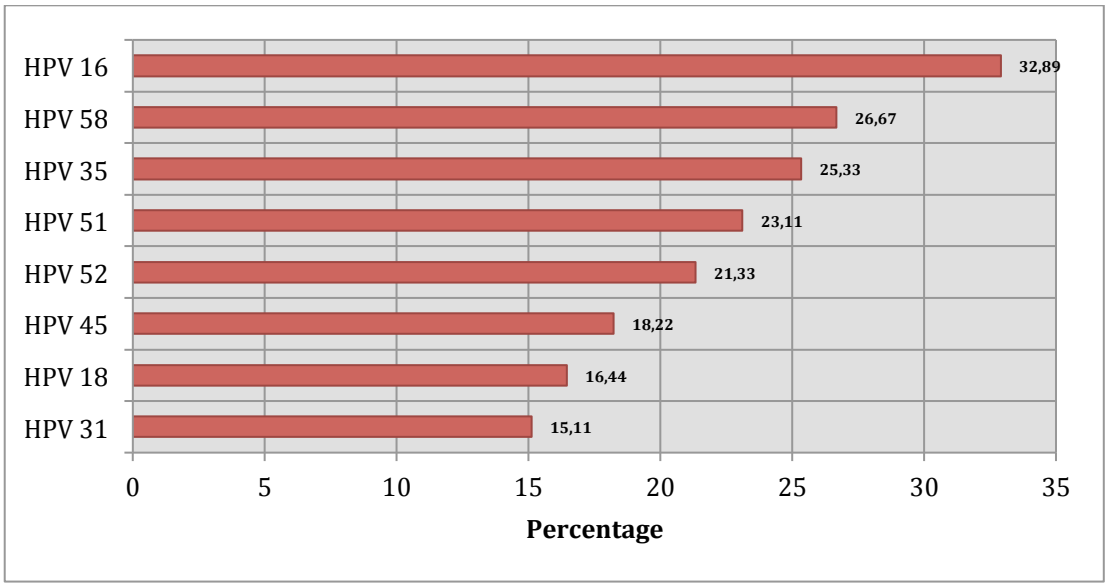


Figure 2.5: Eight most prevalent HrHPV types among HIV-positive patients

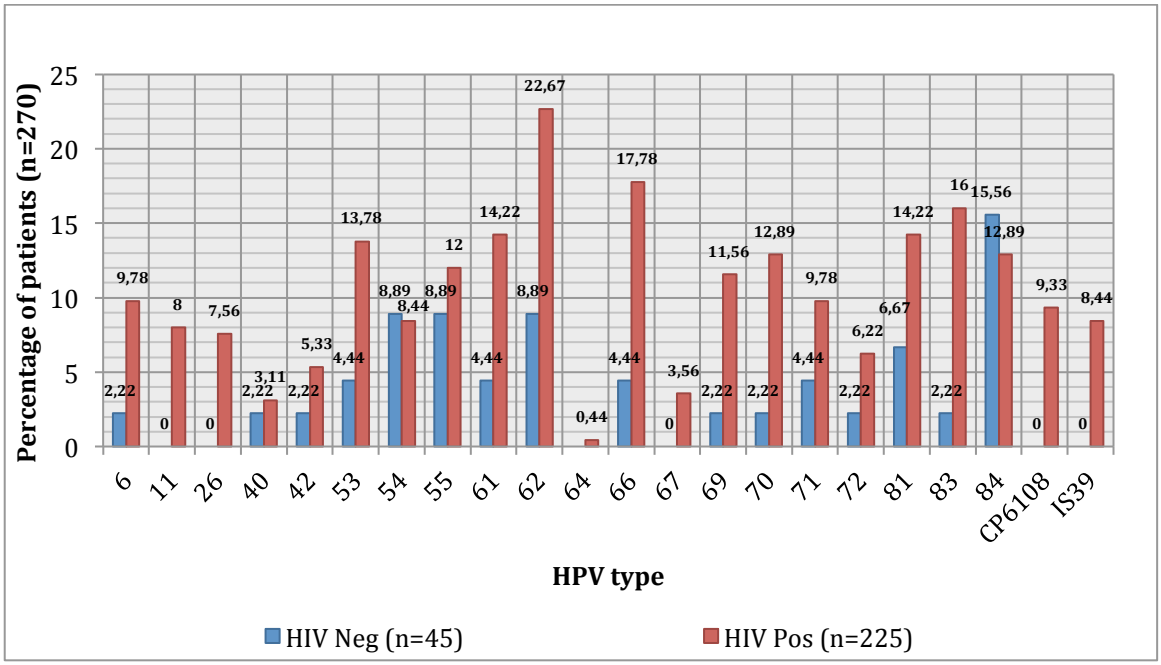


Figure 2.6: Distribution of non-HrHPV types in patients with CIN II/III

2.4.5. Vaccine preventable infections

HrHPV type distribution in relation to high-risk types covered by a HPV 16/18 vaccine (bivalent or quardivalent vaccine), and nine-valent, covering seven HrHPV types: HPV 16, 18, 31, 33, 45, 52, 58, are illustrated in Figure 2.7. HPV 16 and/or 18 were present in 124 patients (45.93%) and 214 patients (79.26%) were infected with HPV 16, 18, 31, 33, 45, 52 and/or 58. Only 37 patients (7.13%) were infected with HrHPV types not included in the 9-valent vaccine. The distribution between HIV-infected and -non-infected patients was very similar, as shown in Figure 2.7.

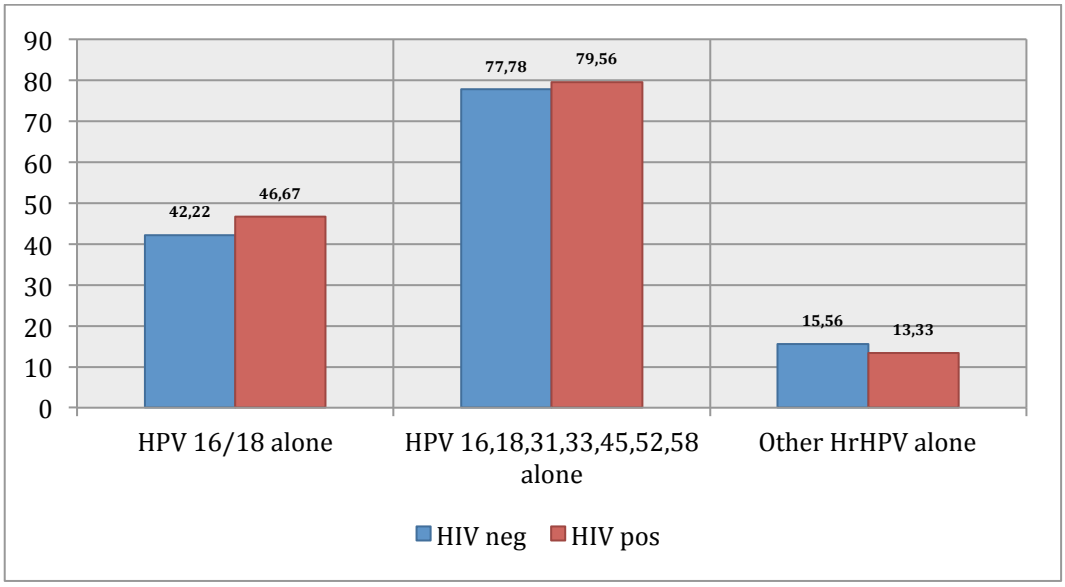


Figure 2.7: HrHPV type distribution of vaccine preventable infections

2.4.6. HPV-type distribution in relation to CD4 cell count

Among the 205 HIV-positive patients with a known CD4 cell count, patients with a CD4 below 200/µl had, on average, significantly more HPV-type infections, including HrHPV and LrHPV types, as illustrated in Figure 2.1. The prevalence of HrHPV types was 90.1% in the CD4 ≥ 200 group, of whom 20.6% had a single HrHPV type and 69.5% were infected with multiple HrHPV types. In the CD4 < 200 group, the prevalence of HrHPV types was 97.3%, of whom 10.8% and 86.5% had single and multiple HrHPV type infections respectively.

The distribution of HrHPV types in relation to CD4 cell count is illustrated in Figure 2.8 and Figure 2.9. The most prevalent HrHPV type in both groups was HPV 16. The eight most prevalent HrHPV types differed as illustrated in Figure 2.10 and 2.11. Compared to women with a CD4 cell count ≥ 200/µl, women with a CD4 cell count < 200/µl had a significant higher prevalence of HPV 51 (p=0.013), HPV 56 (p=0.031) and HPV 73 (p=0.001).

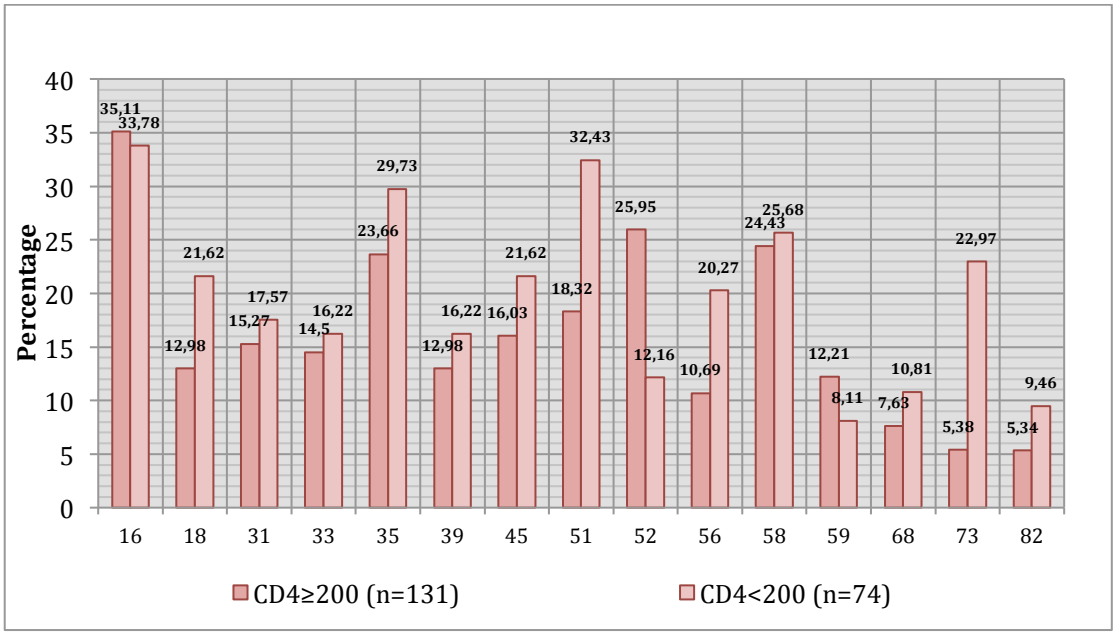


Figure 2.8: Distribution of HrHPV types in HIV-positive patients with CIN II/III in relation to CD4 cell count

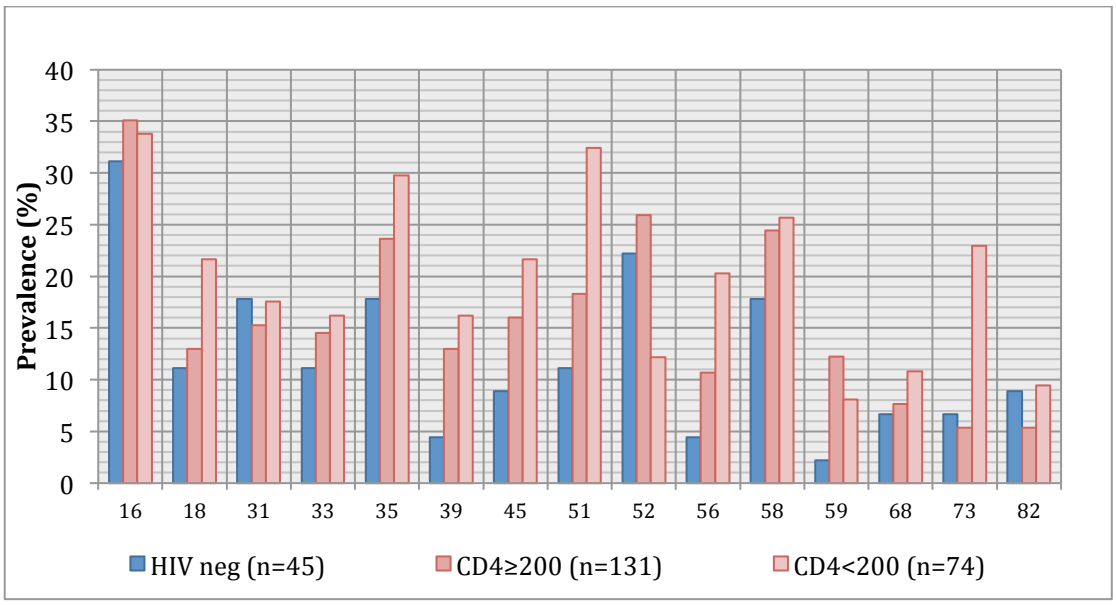


Figure 2.9: Distribution of HrHPV types in patients with CIN II/III in relation to immune competence

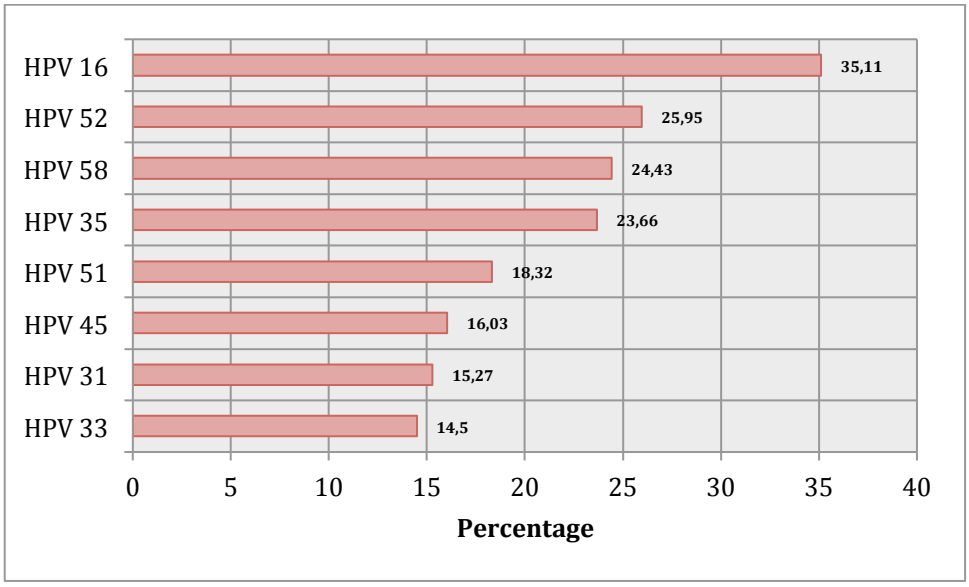


Figure 2.10: Most prevalent HrHPV types in HIV-positive women with a CD4 ≥200

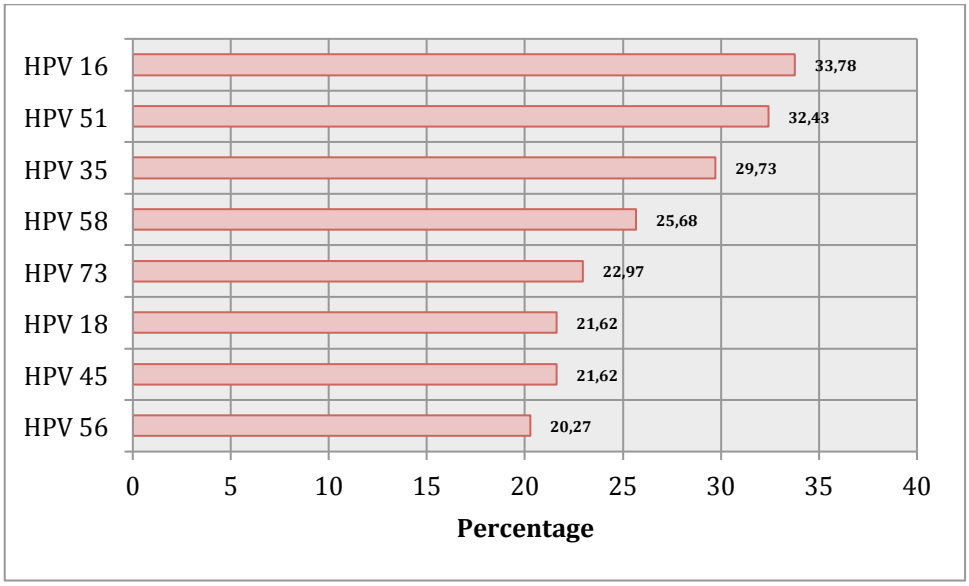


Figure 2.11: Most prevalent HrHPV types in HIV-positive women with a CD4 <200

2.4.7. HPV type distribution in relation to HAART use

The prevalence of HrHPV infections was highest among patients who had been on HAART for between six and 12 months (97.3%) and lowest among patients who had been on HAART for longer than 12 months (88.4%), followed by

patients on HAART for less than six months (91.07%). Except for patients on HAART between six and 12 months, HPV 16 was the most prevalent HrHPV type among all the different subgroups. Table 2.1 illustrates the distribution of different HrHPV types in relation to HAART use and Table 2.2 demonstrates the eight most prevalent HrHPV types in the different groups.

Table 2.1: HrHPV type distribution in relation to HAART therapy

HPV	N %	HIV neg (n=45)	No HAART (n=35)	HAART >12mo (n=43)	HAART 6-12mo (n=37)	HAART <6mo (n=56)	Start HAART (n=54)	Total (n=270)	p-value
16	N	14	15	11	7	22	19	88	0.206
	%	31.11	42.86	25.58	18.92	39.29	35.19	32.59	
18	N	5	5	7	3	11	11	42	0.570
	%	11.11	14.29	16.28	8.11	19.64	20.37	15.56	
31	N	8	5	3	7	11	8	42	0.562
	%	17.78	14.29	6.98	18.92	19.64	14.81	15.56	
33	N	5	4	10	7	6	7	39	0.494
	%	11.11	11.43	23.26	18.92	10.71	12.96	14.44	
35	N	8	5	10	12	15	15	65	0.434
	%	17.78	14.29	23.26	32.43	26.79	27.78	24.07	
39	N	2	3	4	7	8	7	31	0.384
	%	4.44	8.57	9.30	18.92	14.29	12.96	11.48	
45	N	4	5	9	6	11	10	45	0.659
	%	8.89	14.29	20.93	16.22	19.64	18.52	16.67	
51	N	5	7	8	8	15	14	57	0.433
	%	11.11	20.00	18.60	21.62	26.79	25.93	21.11	
52	N	10	9	9	11	12	7	58	0.492
	%	22.22	25.71	20.93	29.73	21.43	12.96	21.48	
56	N	2	2	6	5	8	8	31	0.412
	%	4.44	5.71	13.95	13.51	14.29	14.81	11.48	
58	N	8	8	8	16	15	13	68	0.141
	%	17.78	22.86	18.60	43.24	26.79	24.07	25.19	
59	N	1	5	2	7	4	6	25	0.092
	%	2.22	14.29	4.65	18.92	7.14	11.11	9.26	
68	N	3	2	3	4	4	7	23	0.826
	%	6.67	5.71	6.98	10.81	7.14	12.96	8.52	
73	N	3	1	2	4	11	6	27	0.111
	%	6.67	2.86	4.65	10.81	19.64	11.32	10.04	
82	N	4	2	5	2	1	4	18	0.451
	%	8.89	5.71	11.63	5.41	1.79	7.41	6.67	

Table 2.2: Eight most prevalent HrHPV types in relation to HAART therapy and duration of use (if applicable)

HIV neg (n=45)	No HAART (n=35)	HAART >12mo (n=43)	HAART 6-12mo (n=37)	HAART <6mo (n=56)	Start HAART (n=54)	Total (n=270)
HPV 16	HPV 16	HPV 16	HPV 58	HPV 16	HPV 16	HPV 16
HPV 52	HPV 52	HPV 33	HPV 35	HPV 35	HPV 35	HPV 58
HPV 31	HPV 58	HPV 35	HPV 52	HPV 51	HPV 51	HPV 35
HPV 35	HPV 51	HPV 45	HPV 51	HPV 58	HPV 58	HPV 52
HPV 58	HPV 18	HPV 52	HPV 16	HPV 52	HPV 18	HPV 51
HPV 18	HPV 31	HPV 51	HPV 31	HPV 18	HPV 45	HPV 45
HPV 33	HPV 35	HPV 58	HPV 33	HPV 31	HPV 31	HPV 18
HPV 51	HPV 45	HPV 18	HPV 39	HPV 45	HPV 56	HPV 31

Patients were divided into two groups. Firstly, patients not yet qualifying for HAART (CD4 > 350/µl) and on HAART for 12 months or more were grouped together. This group was compared to a the second group containing patients in the process of initiating HAART (CD4 ≤ 350/µl) and patients on HAART for less than 12 months. Illustrated in Table 2.3, adjusted for age and CD4 cell count on logarithmic scale, the odds were lower to be infected with HPV 18, 33, 45, 51, 52, 59 and 82. There was a significant difference seen for HPV 33 (p=0.029), 59 (p=0.009) and 82 (p=0.034) infections. The odds of an HPV 73 (p=0.004) infection were significantly higher in patients on HAART for less than 12 months or in the process of initiating HAART.

Table 2.3: Association of high-risk HPV types with HAART use adjusted for age and CD4 cell count for patients not requiring HAART or on HAART for 12 months or more

HPV type	Crude OR	Adjusted OR§	95% Confidence interval	P-value
16	1.02	1.17	0.58-2.37	0.120
18	1.23	0.84	0.34-2.09	0.282
31	2.15	2.08	0.77-5.59	0.169
33	0.67	0.54	0.21-1.36	0.029*
35	1.44	1.23	0.57-2.65	0.551
39	1.75	1.87	0.68-5.12	0.639
45	0.95	0.78	0.34-1.83	0.685
51	1.35	0.93	0.42-2.05	0.164
52	0.66	0.98	0.43-2.22	0.132
56	1.42	1.24	0.47-3.28	0.739
58	1.51	1.43	0.65-3.17	0.146
59	1.13	0.93	0.30-2.84	0.009*
68	1.39	1.50	0.45-5.01	0.904
73	4.15	2.39	0.64-8.96	0.004*
82	0.49	0.23	0.06-0.86	0.034*

§Adjusted for age and CD4 count on a logarithmic scale

*Statistically significant

2.4.8. Most probable single oncogenic HPV type identified

The eight most common types of HPV identified in 89% of cervical cancer cases globally, in descending order of frequency, are HPV 16, 18, 45, 31, 33, 52, 58 and 35.[32] If a patient had multiple high-risk HPV types, for example HPV 16, 31, 52 and 82, only the presumed most oncogenic virus, for example HPV 16 was recorded. In both groups HPV 16 was most prevalent.

The HPV distribution curve was more similar to cervical cancer patients in the HIV-positive group than the HIV-negative group. In the HIV-positive group, HPV 52 was more prevalent than HPV 33 and 31, and HPV 35 more prevalent than HPV 58.

Among HIV-negative patients, in descending order of frequency, the single most oncogenic types were HPV 16, 52, 18, 31, 45 and 35. See Figure 2.12.

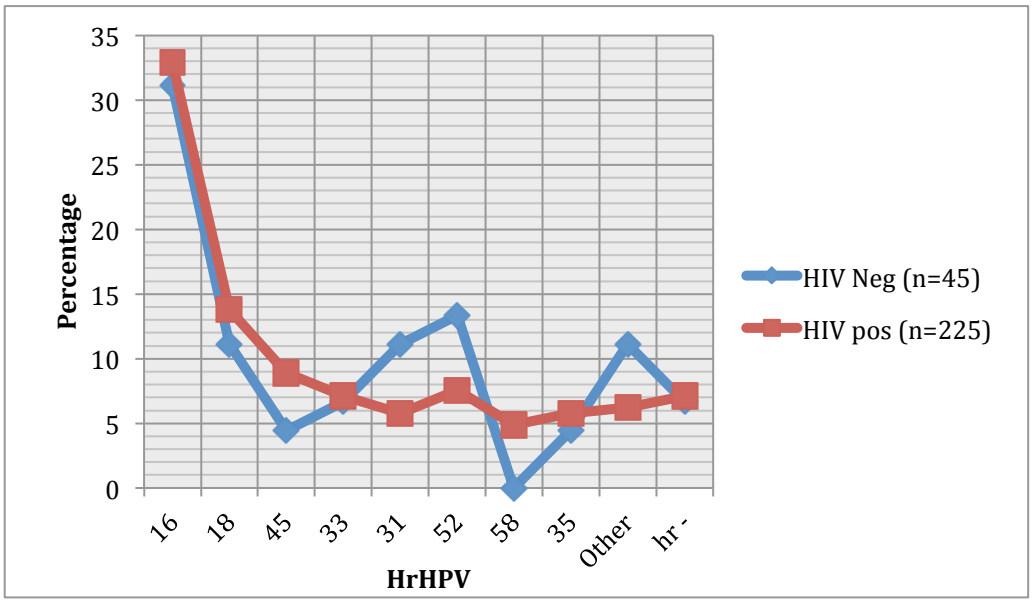


Figure 2.12: Distribution of eight most oncogenic HPV types

2.4.9. Phylogenetic distribution of HrHPV type infections

Dividing all the HrHPV types detected into the different phylogenetic subgroups, a larger percentage of alpha-9 viral type infections were present in HIV-negative group (53/82, 64.63%) than in the HIV-positive group (307/577, 53.21%). In patients co-infected with HIV, a larger percentage of viruses from alpha-7 and alpha-5/6 phylogenetic subgroups were present, as demonstrated in Figure 2.13.

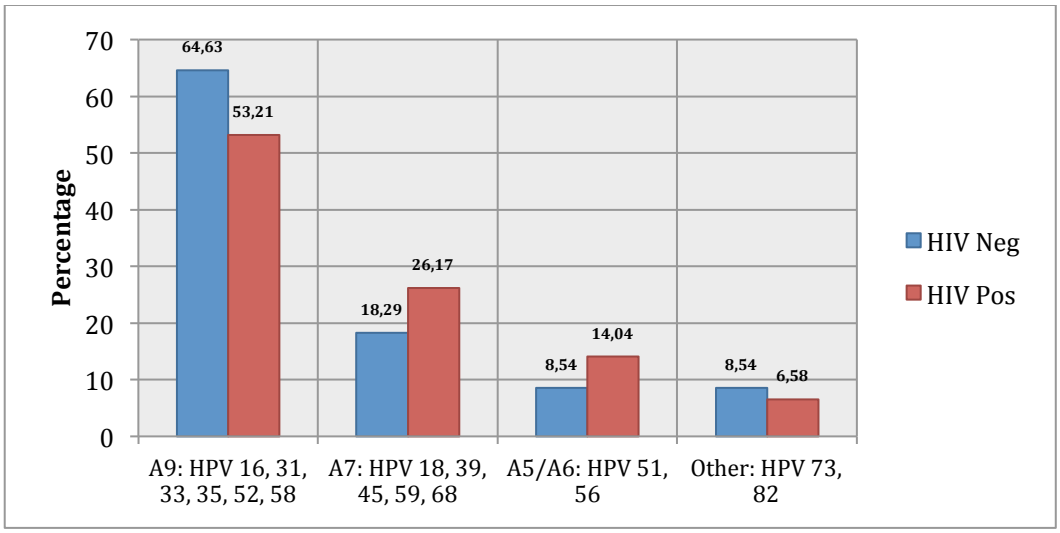


Figure 2.13: Phylogenetic distribution of all HrHPV infections detected

If one applies the same principle of selecting the most oncogenic HPV type mentioned in the previous section, infections from alpha-9 viruses were similar between the two groups. There were more viruses from alpha-7 phylogenetic subgroups among HIV-positive patients (see Figure 2.14) and this seemed to increase as immunity deteriorated (see Figure 2.15).

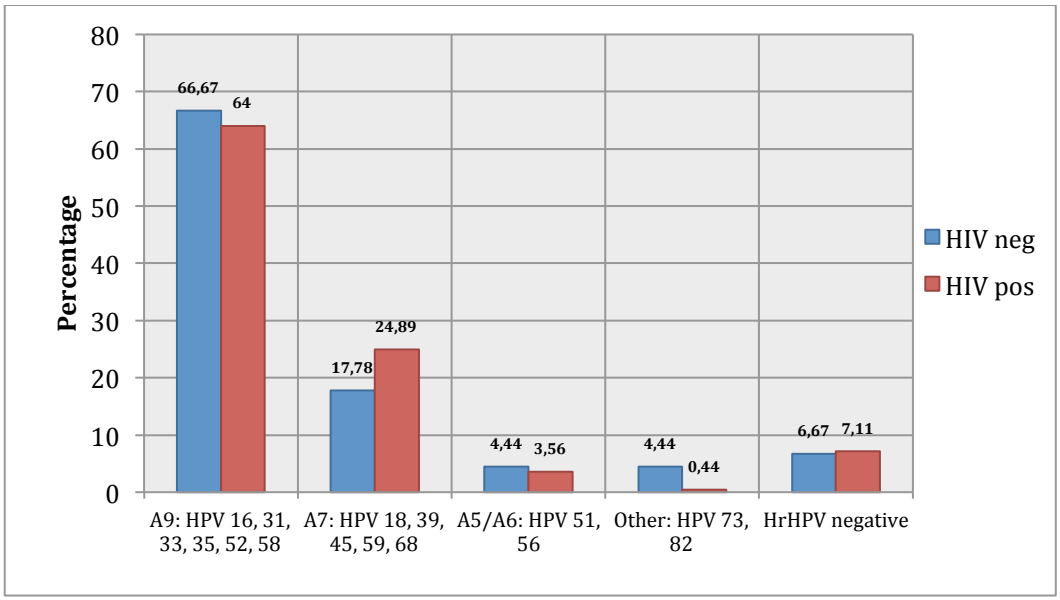


Figure 2.14: Phylogenetic distribution of most single oncogenic HrHPV infections reported

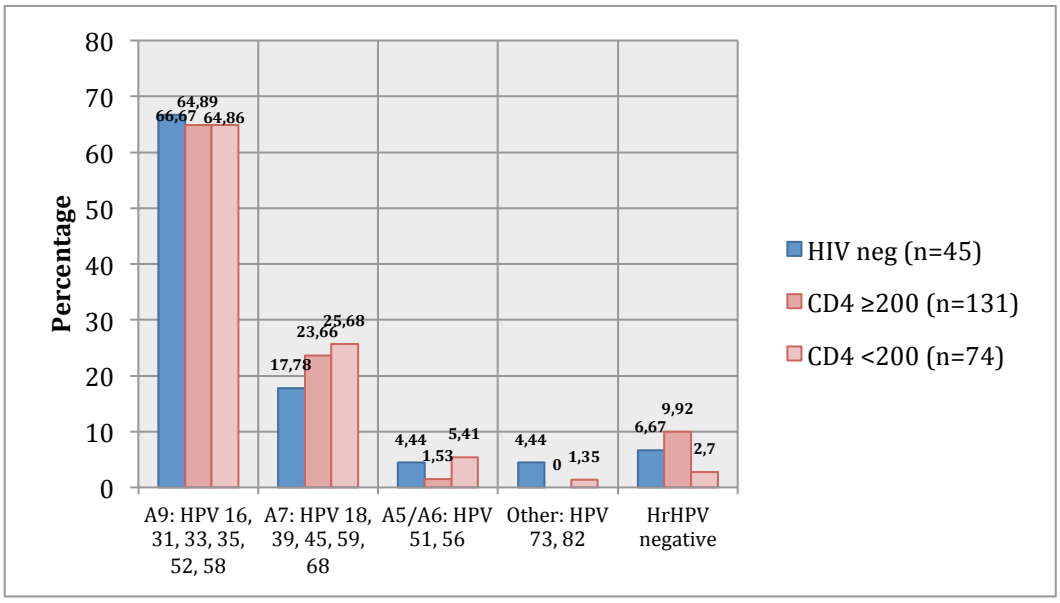


Figure 2.15: Phylogenetic distribution of most single oncogenic HrHPV infections reported in relation to immune competence

2.5. Discussion

2.5.1. Background

The findings from this cohort of South African women, on the distribution of HPV types among patients with histology, confirmed CIN II/III in relation to different levels of immunity are very important. In this study, the positive predictive value for CIN II or worse for patients referred with HSIL on cytology was 83.6%. This highlights the importance of using histopathologically confirmed cervical lesions as the endpoint in the study of cervical disease.[33]

The effectiveness of conventional cytology as a screening test varies between developed- and less-developed countries. When using LSIL as a positive screening threshold, the sensitivity for CIN II or worse ranges between 47% and 95% and the specificity between 60% and 96%. HPV testing combined with cytology will markedly increase the sensitivity for premalignant cervical lesions.[1]

Information on age and CIN development is not clearly established.[9] The average age of patients with CIN II/III was similar for HIV positive (36.4 years) and HIV negative (38.3 years) patients (p-value=0.186). A large multicentre study performed in South Africa reported an average age of patients presenting with HSIL to be just below 38 years.[34] A slightly larger percentage HIV-positive patients were 40 years and younger in this study.

More than 80% of patients in this study were HIV infected. Although not an AIDS-defining disease like invasive cervical cancer, CIN is regarded as an HIV-related disease.[8] The HIV status of all patients was known, reflecting positively on a high uptake of voluntary HIV testing after appropriate counselling. The large percentage of patients infected with HIV highlights the burden that the HIV/AIDS pandemic places on South Africa's healthcare system. Despite the risk of CIN among HIV-infected women, the risk of genital HIV shedding is significantly elevated in the presence of CIN, leading to a higher possibility of HIV transmission.[8]

2.5.2. HPV prevalence

The prevalence of any HPV in the current study was 96.7%. A recent meta-analysis reported the global prevalence of all HPV types in women with CIN II/III as ranging from 86% to 93%.[35] In 2012 the prevalence in Africa was reported as 89% for CIN II and 83% for CIN III.[35]

The prevalence of one or more HrHPV types (93%) was similar to a Botswana study (92%), but higher than the prevalence reported from Kenya (82%) and South Africa (75%).[10,11,36] A study from Spain found a 100% HrHPV prevalence among the 18 HIV-positive patients presenting with HSIL on cytology.[13]

The prevalence of HrHPV was higher among patients with a CD4 cell count less than 200 (97.3%) than those patients with a CD4 cell count of 200 or more (90.1%). This is considerably higher than the overall prevalence (84.1%) of HPV infections in HIV-positive women with HSIL as reported by Clifford et al.[37]

2.5.3. Single and multiple HPV type infections

Compared to the HIV-negative cohort of patients from the study by McDonald et al., HIV-negative patients in this study had more multiple HrHPV type infections (49% versus 20%).[36] Women infected with HIV are often co-infected with multiple types, as well as a broader spectrum of HPV types.[19,20,38] There were more infections with multiple HPV types were higher in this study than reported by Guan et al.[35] The 73.3% of HIV-positive patients co-infected with multiple HrHPV types are much higher than reported by other studies.[11,18,29]

A recent study in Kenya by Luchters et al. on the association between HIV infection and the type distribution of HPV types, confirmed previous findings that HrHPV is more common in HIV positive women, compared with HIV negative women.[39] Although most patients in their study had normal cytology, data from the group of patients with CIN II/III in the current study confirmed this observation. The difference between the two groups in the current study

was less pronounced. HIV-infected patients had on average almost twice as many HPV infections by different types compared to the HIV-non-infected group. Looking specifically at the prevalence of HrHPV, it was found to be around one and a half times more frequent in the HIV-positive group. A possible explanation for HIV-positive women simultaneously infected with multiple HPV types may likely be related to a similar route of transmission of the HIV and HPV viruses. Because of immune suppression, HPV infection cannot be cleared and there is likely reactivation of latent infection.[39]

Although less remarkable, there was also a significant increase in the average number of any HPV type infection, as well as both low- and HrHPV type infections among patients with a CD4 count less than 200, compared to those with a CD4 count 200 or more. This data clearly indicates that HIV-positive patients with CIN II/III are likely to be infected simultaneously with more HPV types, including HrHPV and LrHPV, than HIV-negative patients. This trend continues as immunity deteriorates, as indicated by a lower CD4 count.

2.5.4. Specific HPV types

In both HIV-negative- and HIV-positive groups, HPV 16 was the most prevalent HrHPV type, with almost one third of patients infected. Disregarding HIV status, the most common HrHPV types identified were, in decreasing order of prevalence, HPV 16, 58, 35, 52, 51, 45 and 31. This distribution differed from the meta-analysis, compared to both global and African data, on HPV type distribution in patients with HSIL on cytology, published in 2006.[32] The prevalence of HrHPV among patients with CIN II or CIN III, as illustrated by a more recent meta-analysis, were HPV 16 followed by HPV 52, 31, 58, 33, 18, 51, 45 and 35.[35] There has been a worldwide increase in the prevalence of HPV 52 and 58 over the past ten years.[18] Chen et al. suggested that the long-term risk for developing cervical cancer was higher for HPV 58 than other non-HPV 16 types.[40]

In HIV-non-infected patients the most prevalent HrHPV type was HPV 16, followed by HPV 52, 31, 35 and 58. This distribution of HrHPV types was different from the type distribution reported by McDonald et al., where HrHPV types 16, 35, 58, 33 and 45 were most prevalent among HIV-negative patients with CIN II/III.[36]

Apart from simultaneously infected with more HPV types, HIV-infected women may also harbour more non-HPV 16/18 infections.[38] Of all the HrHPV types detected, including patients with multiple HPV infections, non-HPV 16/18 accounted for around 53% and 57% of infections among HIV-positive and –negative patients respectively. There was no significant difference between specific HrHPV types covered by the bivalent and nine-valent vaccines comparing HIV positive and –negative patients. Disregarding cross-protection, the bivalent vaccine can potentially prevent up to 43% of infections in HIV negative patients and 47% of infections in HIV positive patients. The nine-valent vaccine has the potential of preventing around 78% of infections in both groups.

The most common HrHPV types in women co-infected with HIV were, in descending order of prevalence, HPV 16, 58, 35, 51, 52, 45, 18 and 31. In HIV-positive patients with premalignant cervical lesions, HPV 16 appears consistent as most prevalent HrHPV type in studies from various regions.[11,13,18,29,37] Guan et al. confirmed previous findings that HPV 31, 33 and 58 enhance the absolute risk for CIN III compared to other non-HPV 16 oncogenic types. The trend was similar for HPV 52 and 35.[35]

The prevalence of HPV 16 of just below 33% of the entire study population is comparable to the prevalence reported by Guan at al. for Africa (30.3%), which included both patients with CIN II/III and HSIL.[35] The prevalence of just below 33% is also comparable to the global prevalence (34.7% - 52%) reported by Clifford et al. for patients with HSIL.[32,35] However, the prevalence of HPV 16 (32.9%) among HIV-infected patients in this study is lower than other reports on HIV-positive patients from Europe, South Africa and Botswana (37.5% - 45%),

but also higher than reported by other Africa studies (26.5% - 29.4%).[11,13,18,19,28,41]

2.5.5. HPV, CD4 cell count and HAART use

The association between pre-malignant cervical lesions, CD4 cell count and HAART treatment is not yet clearly defined.[8] Teixeira et al. found that women with suppressed immunity, young age and HPV infection had an increased risk of developing cervical precancerous lesions.[9] Wilkerson and Prosser found a lower CD4 cell count increased the odds of having a squamous intraepithelial lesion.[42]

Findings from a large review on the impact of HAART on HPV and CIN did show a significant difference if women were on HAART in relation to HPV and disease clearance. This study was limited by short follow-up periods of up to 24 months and did not account for treatment compliance.[43] More recent studies, however, suggest that HrHPV infections can be cleared after a lengthy period of optimal HIV control and restoration of the immune function.[44-47]

Little is known about the effect of decreasing immunity as a result of HIV infection and the prevalence of specific HrHPV types. In this study, HPV 16 prevalence appeared constant regardless of HIV status and CD4 cell count and was the most prevalent HrHPV type detected. This finding supports previous theories that HPV 16 is less affected by the effect of immune surveillance, or the lack of it.[13,29] The prevalence of HPV 51, 56 and 73 was significantly higher in patients with CD4 counts less than 200/µl. Oncogenic HPV types that contribute towards the development of cervical cancer may differ between HIV-positive- and HIV-negative patients and with lower immunity. This data on type-specific HPV analysis is vital for the development of screening protocols and distribution of future HPV vaccines despite some cross-protection offered by current vaccines.[39]

Paramsothy and colleagues found an association between HAART treatment and HPV clearance among women with pre-existing cervical cytological

abnormalities.[48] De Vuyst et al. found lower HrHPV infection rates to be associated with a higher CD4 cell count and extended HAART usage.[11] Although no statistical significant difference was found between the groups in this study, the lower prevalence of HPV 16 among women treated with HAART for longer than six months might demonstrate a protective effect against new infections or improve clearance.

Mane et al. did not find a difference in the combinations of HrHPV type distribution in relation to HAART therapy, except for HPV 16.[29] Interestingly in this study, the prevalence of HPV 16 was lower among patients on HAART treatment between six and 12 months (18.92%) and patients on HAART treatment longer than 12 months (25.58%). HPV 16 was most prevalent (42.86%) among patients not yet qualifying for HAART treatment.

Although there was no statistical significant difference between these groups, the lower prevalence of HPV 16 among women who had been treated with HAART for longer than 6 months might demonstrate a protective effect against new infections or improve clearance. Patients who had been on HAART treatment for longer than 12 months had a lower prevalence of HPV 18, but a slightly higher prevalence of HPV 45, compared to patients initiating HAART treatment.

2.5.6. Strengths and limitations

This study is one of the first and largest in this population to compare HPV-type distribution in patients with histologically confirmed CIN II/III. The study provides important insight into the distribution of specific HrHPV types present in patients with CIN II/III and illustrates the effect of different levels of immunity in relation to CD4 cell count and HAART usage.

Limitations of the study include the fact that the patients who took part in this study were from the referral areas served by Steve Biko Academic Hospital and cannot be extrapolated to the rest of South Africa. The small number of HIV-non-

infected patients included in this study is also a limitation. Although important information was obtained regarding HrHPV distribution among patients on HAART, little can be said regarding the effect that HAART-induced immune reconstitution has on cervical lesion regression and persistent infections. A longitudinal study would be needed to evaluate this. This study reports only on HPV DNA detected on the cervical surface and highlights the need to determine the specific HPV type or types incorporated within the specific lesion.

2.6. CONCLUSION

In South Africa, burdened by the HIV pandemic, high numbers of high- and low-risk HPV-type infections are present in women with cervical pre-neoplasia. HPV-type distribution differs in HIV-infected patients. Administering the nine-valent HPV vaccine to women in our population might prevent as many as 80% of CIN II/III lesions. Women not yet requiring HAART or on HAART for longer than 12 months appear to be negatively associated with HPV 33, 59, and 82, and positively associated with HPV 73. Knowledge about the specific HPV-type distribution is crucial to direct development of future HPV vaccines and to guide HPV-based screening in both HIV-infected and –non-infected patients in this population.

2.7. REFERENCES

1. Hoppenot C, Stampler K, Dunton C. Cervical cancer screening in high- and low-resource countries: implications and new developments. Obstet Gynecol Surv. 2012;67(10):658-667.
2. Joshi SN, Gopalkrishna V, Kumar BK, Dutta S, Nyaynirgune P, Thakar M, et al. Cervical squamous intra-epithelial changes and human papillomavirus infection in women infected with human immunodeficiency virus in Pune, India. J Med Virol. 2005;76(4):470-475.
3. Holschneider CH. Cervical intraepithelial neoplasia: definition, incidence, and pathogenesis. In: UpToDate, Golf B (Ed). UpToDate, Waltham, MA, 2013.
4. Tweddel G, Heller P, Cunnane M, Multhaupt H, Roth K. The correlation between HIV seropositivity, cervical dysplasia, and HPV subtypes 6/11, 16/18, 31/33/35. Gynecol Oncol. 1994;52(2):161-164.
5. Gadducci A, Barsotti C, Cosio S, Domenici L, Riccardo Genazzani A. Smoking habit, immune suppression, oral contraceptive use, and hormone replacement therapy use and cervical carcinogenesis: a review of the literature. Gynecol Endocrinol. 2011 Aug;27(8):597-604.
6. Meeuwis KA, van Rossum MM, van de Kerkhof PC, Hoitsma AJ, Massuger LF, de Hullu JA. Skin cancer and (pre)malignancies of the female genital tract in renal transplant recipients. Transpl Int. 2010;23(2):191-199.
7. Carter JR, Ding Z, Rose BR. HPV infection and cervical disease: a review. Aust N Z J Obstet Gynaecol. 2011;51(2):103-108.
8. Pantanowitz L, Michelow P. Review of human immunodeficiency virus (HIV) and squamous lesions of the uterine cervix. Diagn Cytopathol. 2011;39(1):65-72.
9. Teixeira NC, Araújo AC, Correa CM, Lodi CT, Lima MI, Carvalho Nde O, et al. Prevalence and risk factors for cervical intraepithelial neoplasia among HIV-infected women. Braz J Infect Dis. 2012;16(2):164-169.
10. Adler DH, Kakinami L, Modisenyane T, Tshabangu N, Mohapi L, De Bruyn G, et al. Increased regression and decreased incidence of human

papillomavirus-related cervical lesions among HIV-infected women on HAART. AIDS. 2012;26(13):1645-1652.

11. De Vuyst H, Mugo NR, Chung MH, McKenzie KP, Nyongesa-Malava E, Tenet V, et al. Prevalence and determinants of human papillomavirus infection and cervical lesions in HIV-positive women in Kenya. Br J Cancer. 2012;107(9):1624-1630.
12. Joshi S, Sankaranarayanan R, Muwonge R, Kulkarni V, Somanathan T, Divate U. Screening of cervical neoplasia in HIV-infected women in India. AIDS. 2013;27(4):607-615.
13. Stuardo V, Agustí C, Godinez JM, Montoliu A, Torné A, Tarrats A, et al. Human papillomavirus infection in HIV-1 infected women in Catalonia (Spain): implications for prevention of cervical cancer. PLoS One. 2012;7(10):e47755.
14. Zimmermmann JB, Gobbi H, Alves MJ, Quirino MG, Melo VH. Langerhans cell density in cervical intraepithelial neoplasia associated with human papillomavirus infection in HIV-infected and HIV-noninfected Brazilian women. Int J Gynecol Cancer. 2012 Oct;22(8):1291-1296.
15. Massad LS, Xie X, Darragh TM, Minkoff H, Levine AM, D'Souza G, et al. Histologic correlates of glandular abnormalities in cervical cytology among women with human immunodeficiency virus. Obstet Gynecol. 2009;114(5):1063-1068.
16. Massad LS, Evans CT, Weber KM, Goderre JL, Hessol NA, Henry D. Changes in knowledge of cervical cancer prevention and human papillomavirus among women with human immunodeficiency virus. Obstet Gynecol. 2010;116(4):941-947.
17. Peedicayil A, Thiyagarajan K, Gnanamony M, Pulimood SA, Jeyaseelan V, Kannangai R, et al. Prevalence and risk factors for human papillomavirus and cervical intraepithelial neoplasia among HIV-positive women at a tertiary level hospital in India. J Low Genit Tract Dis. 2009;13(3):159-164.
18. Ramogola-Masire D, McGrath CM, Barnhart KT, Friedman HM, Zetola NM. Subtype distribution of human papillomavirus in HIV-infected women

with cervical intraepithelial neoplasia stages 2 and 3 in Botswana. Int J Gynecol Pathol. 2011;30(6):591-596.

19. Marais DJ, Passmore JA, Denny L, Sampson C, Allan BR, Williamson AL. Cervical and oral human papillomavirus types in HIV-1 positive and negative women with cervical disease in South Africa. J Med Virol. 2008;80(6):953-959.
20. Franceschi S, Ronco G. The prevention of cervical cancer in HIV-infected women. AIDS. 2010;24(16):2579-2580.
21. Burger EA, Kornør H, Klemp M, Lauvrak V, Kristiansen IS. HPV mRNA tests for the detection of cervical intraepithelial neoplasia: A systematic review. Gynecol Oncol. 2011;120(3):430-438.
22. Duerr A, Kieke B, Warren D, Shah K, Burk R, Peipert JF, et al. Human papillomavirus-associated cervical cytologic abnormalities among women with or at risk of infection with human immunodeficiency virus. Am J Obstet Gynecol 2001;184(4):584-590.
23. Levi JE, Fink MC, Canto CL, Carretiero N, Matsubara R, Linhares I, et al Human papillomavirus prevalence, viral load and cervical intraepithelial neoplasia in HIV-infected women. Braz J Infect Dis 2002;6(3):129-135.
24. Luque AE, Demeter LM, Reichman RC. Association of human papillomavirus infection and disease with magnitude of human immunodeficiency virus type 1 (HIV-1) RNA plasma level among women with HIV-1 infection. J Infect Dis 1999;179(6):1405-1409.
25. Palefsky JM, Holly EA. Chapter 6: Immunosuppression and Co-infection with HIV. J Natl Cancer Inst Monogr. 2003;31:41-6.
26. ACOG Committee on Practice Bulletins-Gynecology. ACOG Practice Bulletin No. 117: Gynecologic care for women with human immunodeficiency virus. Obstet Gynecol. 2010;116(6):1492-1509.
27. Luque AE, Hitti J, Mwachari C, Lane C, Messing S, Cohn SE, Adler D, et al. Prevalence of human papillomavirus genotypes in HIV-1-infected women in Seattle, USA and Nairobi, Kenya: results from the women's HIV interdisciplinary network (WHIN). Int J Infect Dis. 2010;14(9):e810-814.
28. Dols JA, Reid G, Brown JM, Tempelman H, Bontekoe TR, Quint WG, et al. HPV type distribution and cervical cytology among HIV-positive

Tanzanian and South African women. ISRN Obstet Gynecol. 2012;2012:514146.

29. Mane A, Nirmalkar A, Risbud AR, Vermund SH, Mehendale SM, Sahasrabuddhe VV. HPV genotype distribution in cervical intraepithelial neoplasia among HIV-infected women in Pune, India. PLoS One. 2012;7(6):e38731.
30. The South African antiretroviral treatment guidelines. Department of Health. 2010. Available at: http://www.uj.ac.za/EN/CorporateServices/ioha/Documentation/Documents/ART%20Guideline.pdf
31. Muñoz N, Bosch FX, de Sanjosé S, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 2003; 348: 518-527.
32. Clifford G, Franceschi S, Diaz M, Muñoz N, Villa LL. Chapter 3: HPV type-distribution in women with and without cervical neoplastic diseases. Vaccine 2006;S3/26-S3/34.
33. Kuhn L, Wang C, Tsai WY, Wright TC, Denny L. Efficacy of human papillomavirus-based screen-and-treat for cervical cancer prevention among HIV-infected women. AIDS. 2010;24(16):2553-61.
34. Fonn S, Bloch B, Mabina M, Carpenter S, Cronje H, Maise C, et al. Prevalence of pre-cancerous lesions and cervical cancer in South Africa – a multicentre study. SAMJ 2002;92(2):148-156.
35. Guan P, Howell-Jones R, Li N, Bruni L, de Sanjosé S, Franceschi S, et al. Human papillomavirus types in 115,789 HPV-positive women: a meta-analysis from cervical infection to cancer. Int J Cancer. 2012;131(10):2349-2359.
36. McDonald AC, Denny L, Wang C, Tsai WY, Wright TC Jr, Kuhn L. Distribution of high-risk human papillomavirus genotypes among HIV-negative women with and without cervical intraepithelial neoplasia in South Africa. PLoS One. 2012;7(9):e44332.
37. Clifford GM, Gonçalves MA, Franceschi S, HPV and HIV Study Group. Human papillomavirus types among women infected with HIV: a meta-analysis. AIDS. 2006;20(18):2337-2344.

38. Peedicayil A, Thiyagarajan K, Gnanamony M, Pulimood SA, Jeyaseelan V, Kannangai R, et al. Prevalence and risk factors for human papillomavirus and cervical intraepithelial neoplasia among HIV-positive women at a tertiary level hospital in India. J Low Genit Tract Dis. 2009;13(3):159-164.
39. Luchters SMF, Vanden Broeck D, Chersich MF, Nel A, Delva W, Mandaliya K, et al. Association of HIV infection with distribution and viral load of HPV types in Kenya: a servey with 820 female sex workers. BMC Infect Dis 2010;10:18.
40. Chen HC, Schiffman M, Lin CY, Pan MH, You SL, Chuang LC, et al. Persistence of type-specific human papillomavirus infection and increased long-term risk of cervical cancer. J Natl Cancer Inst. 2011;103(18):1387-1396.
41. Heard I, Cubie HA, Mesher D, Sasieni P, MACH-1 Study Group. Characteristics of HPV infection over time in European women who are HIV-1 positive. Br J Obstet Gynaecol. 2013;120(1):41-49.
42. Wilkerson M, Prosser R. A comparison of risk factors for atypical squamous cells and squamous intraepithelial lesions in a sample of US- and African-born HIV-positive women. AIDS Care. 2010;22(9):1115-1122.
43. Bratcher LF, Sahasrabuddhe VV. The impact of antiretroviral therapy on HPV and cervical intraepithelial neoplasia: current evidence and directions for future research. Infect Agent Cancer. 2010;5:8.
44. Fife K, Wu J, Squires K, Watts D, Andersen J, Brown DR. Prevalence and persistence of cervical human papillomavirus infection in HIV-positive women initiating highly active antiretroviral therapy. J Acquir Immune Defic Syndr. 2009;51(3):274–282.
45. Minkoff H, Zhong Y, Burk R, Palefsky JM, Xue X, Watts DH, et al. Influence of adherent and effective antiretroviral therapy use on human papillomavirus infection and squamous intraepithelial lesions in human immunodeficiency virus-positive women. J Infect Dis. 2010;201(5):681–690.
46. Konopnicki D, Manigart Y, Gilles C, Barlow P, de Marchin J, Feoli F, et al. Sustained viral suppression and higher CD4+ T-cell count reduces the risk

of persistent cervical high-risk human papillomavirus infection in HIV-positive women. J Infect Dis. 2013;207(11):1723–1729.

47. Blitz S, Baxter J, Raboud J Walmsley S, Rachlis A, Smaill F, et al. Canadian Women's HIV Study Group. Evaluation of HIV and HAART on the natural history of HPV infection and cervical cytopathology in HIV-positive and high risk HIV-negative women. J Infect Dis. 2013;208(3),454–462.

48. Paramsothy P, Jamieson DJ, Heilig CM, Schuman PC, Klein RS, Shah KV, et al. The effect of highly active antiretroviral therapy on human papillomavirus clearance and cervical cytology. Obstet Gynecol. 2009;113(1):26-31.

CHAPTER 3

HPV TYPE OR TYPES DEMONSTRATED WITHIN TISSUE SAMPLES OF HISTOLOGICALLY CONFIRMED CERVICAL INTRAEPITHELIAL LESIONS IN WOMEN WITH AND WITHOUT HIV-RELATED IMMUNE DEPLETION

3.1. INTRODUCTION

3.1.1. Cervical intraepithelial neoplasia (CIN) and human papillomavirus (HPV)

Better understanding of cervical carcinogenesis has led us to believe that invasive cervical cancer is preceded by human papillomavirus (HPV) infections. We do not have as good an understanding as to whether these infections are cleared by the host immune system or elude the immune system and become persistent.[1] A causal relationship between persistent HPV infections and the development of premalignant and malignant cervical disease is well known.[2]

Despite numerous HPV types illustrated on the cervical surface, the causal relationship between the specific oncogenic type and the resultant neoplasia is complex. If a single HPV type is detected from the surface of a cervical intraepithelial neoplasia (CIN) it is generally accepted as the lesion-causing virus. The difficulty arises in patients with multiple HPV type infections to ascribe a specific type to the individual CIN lesion.[3]

3.1.2. Multiple HPV infections

In patients infected with more than one HPV type, performing HPV DNA typing on cytological specimens from the cervical-vaginal surface gives a combined result of all, or the majority, of the viruses present. It is unable to differentiate if a specific virus type is present in the CIN lesion, normal epithelium, surface debris, other co-existing CIN lesions or as sexually deposited HPV DNA.[3,4]

The eight most common HPV types accounting for around 90% of cervical cancer cases worldwide in descending order of frequency are HPV 16, 18, 45, 31, 33, 52, 58 and 35.[5,6] A single HPV type is detected in around 95% of patients with invasive cervical cancer. These findings strengthen the belief that cervical lesions and cancer arise from a monoclonal event attributed to a single HPV genotype.[3,7] In patients with all grades CIN, multiple HPV type infections are present in up to

40%, and up to 60% for high-grade (CIN II/III) lesions.[3,6] As described in chapter 2, the prevalence of multiple HPV type infections for all patients in our cohort of patients with biopsy confirmed CIN II/III was 78.5%.

3.1.3. Single type oncogenesis

In spite of simultaneously being infected by multiple HPV types, a recent study found that 93% of specimens examined had only one HPV type within the CIN lesion. The conclusion was made that it is unlikely that more than one HPV type is found within a cell infected with HPV. In patients with multiple HPV types detected, colliding lesions caused by different viruses was the most likely explanation. The authors proposed that a high-grade pre-malignant lesion (CIN II/III) caused by a specific HPV type (most oncogenic) could progress to a monoclonal cervical cancer. This progression is most likely secondary to the oncogenic process induced by that specific HPV.[3] There is currently no evidence that could be found to show that this theory is also true in populations with a higher prevalence of HPV.

The biology of different HPV types varies in cell populations of differentiating squamous epithelia. Similarly, because of successive mutations and clonal expansion, neoplastic progression leads to histological heterogeneities. Therefore, little is known about the spatial and temporal modifications of specific HPV type infections at the same anatomical location.[8]

As a result of this inadequate knowledge, it is difficult to differentiate between subclinical and latent infections, and to identify regulatory modifications, successive mutations and epigenetic changes throughout the carcinogenic progression. Knowledge is also limited by available methodology. The best approach to overcoming these problems and to better understand disease development is by targeting a selected small group of cells.[8]

3.1.4. DNA isolation from formalin-fixed paraffin-embedded (FFPE) tissue

During the past few years much research has been focused on detecting HPV types in FFPE tissue. As the availability of sensitive analytical methods increases, FFPE tissue offers a vast source of information for retrospective epidemiological studies.[9] Although detecting high-risk HPV (HrHPV) DNA by nucleic acid amplification on cytological specimens forms part of cervical cancer screening, performing HPV typing on FFPE tissue is not yet widely used.[10]

Until recently, HPV detection in FFPE tissue was based mainly on immunohistochemistry for surrogate protein markers and in situ hybridisation for HPV DNA. The most commonly used surrogate marker of HPV-related dysplasia is $p16^{INK4a}$. Immunohistochemical techniques usually demonstrate high sensitivity but lower specificity, whereas HPV in situ hybridisation methods are more specific but less sensitive. In comparison to immunohistochemistry and in situ hybridisation, nucleic acid amplification testing offers better analytical sensitivity and specificity, and has the very important capability to distinguish between individual HPV types.[10-13]

Excessive fixation and lengthy storage can make it difficult to detect viral nucleic acids in FFPE tissue because of viral DNA/RNA degradation.[14] Fixating tissue with formalin produces cross-linking between proteins and between proteins and nucleic acids. As a result of the latter it is not easy to separate DNA from histones and to get pure nucleic acids at extraction.[14-16]

Sensitive molecular methods, like polymerase chain reaction (PCR), are necessary to accurately detect specific HPV types. Precise type-specific diagnosis of HPV by PCR is hindered by the vast amount of viral types with very different nucleotide sequences. HPV detection by means of PCR-based methods is well established and used in numerous clinical, epidemiological and natural history studies.[17]

3.1.5. HPV DNA detection

Several PCR-based techniques have been described to amplify and identify specific HPV types. Type-specific PCR primer sets can be used to detect separate HPV types, but requires performing multiple parallel assays for every sample, and type-specific PCR primers are not available for every individual HPV type. Another option would be to use general PCR primer sets, allowing amplification of a wide range of HPV types at the same time. Following the general amplification process, direct sequencing, restriction of fragment length polymorphism, or type-specific probe hybridisation can then be used to analyse the products.[17,18]

In this study, two independent reverse hybridization assays were used to detect HPV types. These assays are described in more detail under methods. The first system is known as the line blot assay (LBA). It uses a primer set, designated "PGMY" and based on the MY09/11 primer set, and within the L1 region of HPV amplifies 450-bp fragments. A total of 37 individual types can be identified by LBA.[17]

The second system, based on the SPF_{10} PCR primer set, is known as the "line probe assay" (LiPA). In contrast to the LBA, this method amplifies only 65-bp fragments in the HPV L1 region. Initially SPF_{10} amplimers are tested for HPV DNA in a microtiter plate general hybridisation assay and, if positive, analysed by SPF_{10} LiPA. This method can detect 25 different HPV types. Type-specific probes selected from the interprimer region of each PCR primer set is used in both LBA and LiPA.[17,19,20]

3.1.6. Motivation for the study

Detecting HPV types from the cervico-vaginal epithelium is unable to differentiate between specific HPV type associated with distinct lesions, HPV depositions or adjacent infections.[21] The main motivation for this study was to prove which (one) of the multiple types are actually responsible for the

dysplastic lesion. Second, the motivation was to investigate whether a single or limited HPV type/s oncogenesis could be demonstrated in this subpopulation with a very high number of persistent infections with oncogenic viral types, as has been suggested in populations with very different viral epidemiology.

Identifying the specific HPV types and the CIN lesions it is associated with will enable a more clear understanding of the natural history and behaviour of specific viral types. Separating lesion-associated HPV types from incidental HPV types can guide future preventative and therapeutic vaccines, assist in monitoring patients in the post-vaccine era and aid in the development of new assays to detect HPV DNA and RNA.[22]

3.2. OBJECTIVES AND HYPOTHESES

3.2.1. Objectives

The objectives of this study were to:

- Identify one or more HPV type/s within tissue samples of cervical intraepithelial neoplasia that are truly oncogenic;
- Determine which HPV types identified with DNA typing on the surface were also found within the tissue;
- Investigate the effect of the immune system integrity on tissue HPV genotyping; and
- Determine whether a single or multiple HPV types were present within the cervical lesion in this population.

3.2.2. Hypothesis

The hypotheses of this study were that:

- A single HPV type would be identified within the cervical lesion (CIN) for women simultaneously infected by multiple HPV types;

- There would be a difference in HPV tissue genotyping between HIV-infected- and non-infected women;
- Multiple HPV types found within cervical intraepithelial lesions would be restricted to HIV-positive women

3.3. MATERIALS AND METHODS

3.3.1. Study design

This is a descriptive study performed at the gynaecologic oncology unit, in collaboration with the department of medical virology and anatomical pathology at the University of Pretoria. This study consists of data from patients recruited from 1 July 2010 to 28 February 2011 that formed part of the study population described in Chapter 2. The DNA isolation and tissue HPV DNA typing was performed from 1 January 2014 to 28 February 2015. Patients included in the study were women aged 18 years and older, referred for treatment of high-grade squamous intraepithelial lesions (HSIL) on conventional cervical cytology (Papanicolaou smears) as part of the national screening programme.

3.3.2. Consent process and ethical considerations

Patients received counselling and an information document that explained the method and voluntary nature of the study. During counselling by trained nursing personnel, patients were motivated to undergo HIV testing as per standard departmental-management protocols. It was clearly explained to patients that testing was voluntary and not a prerequisite for treatment. Planned treatment was also explained: large loop excision of the transformation zone (LLETZ) or directed biopsies if malignancy was suspected. All patients were informed of their HIV results, if applicable. All patients tested for HIV received post-test counselling and were offered a CD4 cell count. These patients were referred to the appropriate antiretroviral therapy clinic for further management. This study was approved by the Research Ethics Committee of the Faculty of Health Sciences of the University of Pretoria (26/2010, 189/2012).

3.3.3. Patient recruitment

One-hundred-and-twenty-eight (128) consecutive patients referred with HSIL on cervical cytology were selected to participate in the study. The first 25 patients were used to refine the research methodology and excluded from the study. Only 94 of the 103 patients with confirmed CIN I to CIN III were included in the final study analysis. Of the nine patients excluded, two patients had invasive cervical cancer; three had cervicitis and two had no histological abnormalities. The histology was inadequate in two cases. CD4 cell count was recorded, if applicable. A total of 116 lesions were included form the 94 patients.

3.3.4. Sample collection and transport

Surface HPV testing, using a dry swab, was performed on all participating patients. After collection, the swabs were transported in phosphate buffered saline and 10% methanol solution to the Department of Medical Virology at the University of Pretoria, for HPV DNA testing.

Patients underwent colposcopic evaluation and a LLETZ procedure or biopsy as indicated, which were placed in buffered formalin. The samples were transported to the department of Anatomical Pathology at the University of Pretoria, where histological examination was performed.

3.3.5. Histopathological diagnosis and lesion selection for HPV typing

Tissue submitted from LLETZ biopsies was fixed in formalin, embedded in paraffin wax and stained with haematoxylin and eosin. Two primary observers, in terms of histological severity, graded the lesions. In cases of discrepancy, a third unbiased observer was consulted. The second grading was performed without knowledge of the initial diagnosis and was done on two separate occasions to evaluate both inter- and intra-observer variability. In cases where there was no correlation between the three pathologists, the most severe grading was used. The criteria used for the grading of cervical dysplasia were as follows: CIN I - dysplasia involving in the lower third of the cervical epithelium;

CIN II - dysplasia involving the lower two thirds of the epithelium with maturation and flattening of the surface epithelium still being present; CIN III - full epithelial thickness dysplasia with no evidence of maturation.[23] The section with the highest-grade precursor lesion was then selected from each patient. In patients where more than one lesion was identified, the two lesions with the highest grade were selected.

All the biopsy blocks were sectioned according to the sandwich cutting technique and extensive care was taken to prevent cross-contamination. The sections included a 4µm section for diagnosis, two 4µm sections for immunohistochemical staining (not included in this study) and four 8µm sections used to perform targeted-tissue-based HPV DNA (T-HPV) typing on. An independent pathologist evaluated all the specimens and identified the lesions on the different sections. Every lesion was separated from surrounding normal epithelial tissue and underlying cervical stroma and removed by manual micro-dissection. DNA isolation was performed on the tissue obtained from these sections.

3.3.6. Paraffin extraction and DNA isolation and quantification

The author performed DNA isolation manually using the Cobas® DNA sample preparation kit according to manufacturer's instructions as follows. The tissue collected from the lesions was placed in a microcentrifuge tube and 500µl Xylene was added. After the tissue was mixed well by vortexing, it was left to stand for 5 minutes at 20°C. This time period was followed by adding 500µl absolute ethanol and mixed by vortexing for 10 seconds and again left to stand for 5 minutes at 20°C. The tube was centrifuged at 18 000 x g for 2 minutes and the supernatant was removed and discarded. This procedure was repeated after the sample had been mixed with 1ml absolute ethanol. The tissue pellet was then dried for 10 minutes at 56°C.

The tissue pellet was resuspended in DNA tissue lysis buffer and 70µl of reconstituted Proteinase K was added. After the mixture had been vortexed for

30 seconds and it was assured that the tissue was fully emerged, it was placed in a 56°C dry heat block and incubated for 60 minutes. The specimens were then placed in a 90°C dry heat block and incubated for 60 minutes after mixing it for 10 seconds.

The tubes were allowed to cool to around 20°C and 200µl of DNA PBB was added and the tubes were incubated for 10 minutes followed by adding 100µl isopropanol. The lysate was then transferred into appropriately labelled filter tubes (FT) / collection tube (CT) unit and the FT/CT unit centrifuged for 1 minute at 8 000 x g. Each FT was then placed in a new CT and 500µl working DNA Wash Buffer I was added, followed by centrifuging the FT/CT unit at 8 000 x g for 1 minute. Adding 500µl working DNA Wash Buffer II to the FT after discarding the flow-through in each CT and placing the FT back into the CT and repeating centrifuging followed this procedure.

The FT was then placed into a new CT and centrifuged at 18 000 x g for 1 minute to dry the filter membrane. The FT was then placed into an elution tube followed by the addition of 100µl DNA EB to the centre of the of each FT membrane without touching the membrane left to incubate for 5 minutes at 20°C. The FT with elution tube was then centrifuged at 8 000 x g for 1 minute in order to collect the DNA Stock in the elution tube. DNA quantification was done before the specimens were sent via courier to the Netherlands for HPV typing.

The quantity of extracted DNA was determined using the Invitrogen Qubit® as per manufacturer's instructions. The mastermix was prepared using 1µl Qubit® dsDNA BR reagent and 200µl Qubit® dsDNA BR buffer. After this, 197µl of mastermix was added to 3µl of the DNA sample in a 0.5ml tube and vortexed for 10 seconds. Following a two-minute incubation period, the results were obtained after reading on the Invitrogen Qubit® 2.0 Fluorometer and recorded in ng/µl.

3.3.7. Surface HPV (S-HPV) DNA testing

S-HPV DNA analyses were performed by the department of Medical Virology, University of Pretoria. DNA extraction was accomplished by means of the DNA Isolation Kit (Roche Molecular Systems®, Branchburg, NJ) on the MagNa Pure automated extraction system. HPV linear array (LA) genotyping kit (Roche Molecular Systems®, Branchburg, NJ) was used to determine the HPV type. Fifteen high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82), three probable high-risk types (HPV 26, 53 and 66) and 19 low/undetermined risk types (HPV 6, 11, 40, 42, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, IS39 and CP6108) were tested for.[24]

3.3.8. Targeted-tissue-based HPV (T-HPV) DNA typing using LBA

In the initial protocol of this study it was proposed to use only LBA to detect HPV types because this method was used to detect the surface HPV reported. As a pilot series to refine the methodology, the first 25 consecutive samples were selected. After the CIN lesions were identified and micro-dissected, the DNA isolated and quantified, 10 of the samples were submitted for HPV DNA testing using LBA. The test yielded very poor results with only one sample testing positive for HPV 16.

In an attempt to improve results, different volumes (10µl, 25µl and 40µl) were mixed with PCR water to make up 50µl mixture that was added to the 50µl HPV master mixture to make up the final reaction volume of 100µl. Despite experimenting with the amount of isolated DNA no improvement occurred in the outcome, with none of the samples detecting specific HPV types.

In a further effort to improve HPV detection results, the dissection area was slightly enlarged to include a larger area of the identified lesions. The assumption was that the reason for not detecting HPV types using LBA might be the result of the low quantity of DNA obtained from FFPE tissue following micro-dissection. Despite a larger dissection area and higher DNA quantity, there were

still no type-specific results obtained after HPV DNA testing using LBA. The postulation was made that HPV detecting method was the reason for failure to detect specific HPV types and another method was explored. The samples were then tested using LiPA, which tested HPV DNA positive in all samples and detected specific HPV types as discussed under results.

3.3.9. Targeted-tissue-based HPV (T-HPV) DNA typing using LiPA

The broad spectrum SPF10 PCR amplifies a 65 bp fragment from the L1 region of the HPV genome. Amplimers were captured onto streptavidin-coated microtitre plates using biotinylated reverse primers. After denaturation of the PCR products by alkaline treatment, a DEIA was used to detect HPV-positive samples.

This method is able to detect more than 50 HPV types.[25] PCR DEIA-positive samples were used for subsequent genotyping of 25 mucosal HPV types (HPV 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68 or 73, 70, and 74). Because the interprimer regions of HPV 68 and 73 are identical the test cannot distinguish between them and, therefore, these two HPV types are reported as either 68 or 73.[26] DDL Diagnostic Laboratory in the Netherlands performed T-HPV DNA analyses.

3.3.10. Consensus HPV (C-HPV) types

These HPV types were not detected by a specific DNA typing method, but were recorded after the S-HPV and T-HPV results had been recorded. Only the HPV types detected by both methods were recorded. If there was a single HPV type present in the T-HPV it was recorded regardless of the S-HPV present as that specific type was evident of the lesion-associated HPV type. The main reason for employing this method was to identify the most likely HPV type causing the lesion between two different HPV typing methods.

3.3.11. Data analysis

Data was captured on Microsoft® Excel® datasheets, and analyses were performed using Stata® statistical software release 13 (StataCorp, College Station, TX). Data was mainly binary in nature and summary statistics were frequency, percentage, cross-tables and bar charts. Comparison between groups was carried out with Fisher's exact test for discrete outcomes. Testing was done at the 0.05 level of significance.

3.4. RESULTS

3.4.1. Population characteristics

A total of 116 lesions from 94 patients were included in the study. The ages of the 94 patients ranged between 22 and 66, with a mean age of 35.6 years. The mean ages of HIV-positive- and -negative patients were almost identical and there was no significant difference in relation to age between the two groups.

Sixty (51.72%) of the lesions included were classified as CIN I or CIN II (CIN I/II) and 56 (48.28%) lesions were classified as CIN III. Twenty (62.5%) CIN III lesions from HIV-negative patients and 36 (42.86%) CIN III lesions from HIV-positive patients were included. See Table 3.1. Because of the small number of CIN I lesions, CIN I and CIN II were combined to compare findings with CIN III.

Table 3.1: Study population characteristics

Study population			
	Total	HIV Negative	HIV Positive
Number of patients	94	23 (24.47%)	71 (75.53%)
Number of lesions	116	32 (27.59%)	84 (72.41%)
$CD_4 \geq 200$			50 (59.52%)
$CD_4 < 200$			34 (40.48%)
Mean age of patients (in years)	35.6	35.6	35.5
Histology			
	Total	HIV Negative	HIV Positive
CIN I	11 (9.48%)	2 (6.25%)	9 (10.71%)
CIN II	49 (42.24%)	10 (31.25%)	39 (46.43%)
CIN III	56 (48.28%)	20 (62.5%)	36 (42.86%)

3.4.2. Surface HPV (S-HPV)

A total of 172 HrHPV and 129 LrHPV among CIN I/II lesions and 132 HrHPV and 100 LrHPV among CIN III lesions, were detected. The prevalence of one or more HrHPV infection was 98.33% for all CIN I/II lesions; 100% and 97.92% in the HIV-negative- and -positive groups respectively. In CIN III lesions the prevalence for single or multiple HrHPV type infection was 96.43%, 95% and 97.22% for all lesions, HIV-negative groups and -positive groups respectively.

In CIN I/II lesions, 13.33% had a single HPV type infection compared to 30.36% in CIN III. In both CIN I/II and CIN III lesions, the HIV negative group had more single-type infections. (See Table 3.2.)

Table 3.2: Distribution of single and multiple HPV type infections

Surface HPV				
		Total	HIV Negative	HIV Positive
CIN I/II	Single types	8 (13.33%)	3 (25%)	5 (10.42%)
	Multiple types	52 (86.67%)	9 (75%)	43 (89.58%)
CIN III	Single types	17 (30.36%)	11 (55%)	6 (16.67%)
	Multiple types	39 (69.64%)	9 (45%)	30 (83.33%)
Targeted-tissue-based HPV				
		Total	HIV Negative	HIV Positive
CIN I/II	Single types	23 (38.33%)	5 (41.67%)	18 (37.5%)
	Multiple types	37 (61.67%)	7 (58.33%)	30 (62.5%)
CIN III	Single types	26 (46.43%)	10 (50%)	16 (44.44%)
	Multiple types	30 (53.57%)	10 (50%)	20 (55.56%)

Table 3.3 and 3.5 show the prevalence of all HrHPV types detected in lesions with different histological gradings – the difference in relation to HIV status and the results from different HPV detection sites. In order of decreasing frequency, the most prevalent HrHPV types among all CIN I/II lesions were HPV 16, 51, 58, 31, 33 and 35. HPV 16 was present in 38.33% of lesions, HPV 51 in 30% and HPV 58 in 28.33% of lesions. The most prevalent HPV types among the HIV-negative group were, in descending frequency, HPV 31, 58, 35, 16 and 52. In the HIV positive group, HPV 16 was followed by HPV 51, 33, 35 and 58.

The most prevalent surface HrHPV types present in all CIN III lesions were HPV 16, 51, 58, 18 and 31. HPV 16 was present in 33.93% of lesions. HPV 16 and/or 18 were present in just over 50% of all CIN III lesions. HPV 16, 31, 52 and 58 were most prevalent among the HIV-negative group. Among the HIV-positive group HPV 51 (41.66%) and 58 (33.33%) were more common than HPV 16 (27.78%). There was no significant difference between any of the individual HPV types in relation to HIV status.

Table 3.3: Prevalence of HrHPV type by HIV status over histological grading and detection region

HrHPV	CIN I/II				CIN III			
	Surface HrHPV type distribution							
	All	HIV neg	HIV pos		All	HIV neg	HIV pos	
	N=60 (%)	N=12 (%)	N=48 (%)	p-value	N=56 (%)	N=20 (%)	N=36 (%)	p-value
16	23 (38.33)	3 (25)	20 (41.67)	0.34	19 (33.93)	9 (45)	10 (27.78)	0.244
18	5 (8.33)	0 (0)	5 (10.42)	0.572	10 (17.86)	1 (5)	9 (25)	0.078
31	16 (26.67)	6 (50)	10 (20.83)	0.066	10 (17.86)	5 (25)	5 (13.89)	0.468
33	16 (26.67)	2 (16.67)	14 (29.17)	0.486	9 (16.07)	2 (10)	7 (19.44)	0.466
35	16 (26.67)	4 (33.33)	12 (25)	0.716	9 (16.07)	2 (10)	7 (19.44)	0.466
39	4 (6.67)	0 (0)	4 (8.33)	0.574	6 (10.71)	1 (5)	5 (13.89)	0.405
45	9 (15)	1 (8.33)	8 (16.67)	0.671	8 (14.29)	1 (5)	7 (19.44)	0.236
51	18 (30)	1 (8.33)	17 (35.42)	0.086	17 (30.36)	2 (10)	15 (41.67)	0.016
52	14 (23.33)	3 (25)	11 (22.92)	1	6 (10.71)	3 (15)	3 (8.33)	0.655
56	10 (16.67)	0 (0)	10 (20.83)	0.188	4 (7.14)	0 (0)	4 (11.11)	0.285
58	17 (28.33)	5 (41.67)	12 (25)	0.293	15 (26.79)	3 (15)	13 (33.33)	0.21
59	6 (10)	1 (8.33)	5 (10.42)	1	5 (8.93)	0 (0)	5 (13.89)	0.148
68	7 (11.67)	2 (16.67)	5 (10.42)	0.619	6 (10.71)	1 (5)	5 (13.89)	0.405
73	7 (11.67)	0 (0)	7 (14.58)	0.326	3 (5.36)	1 (5)	2 (5.56)	1
82	4 (6.67)	2 (16.67)	2 (4.17)	0.175	5 (8.93)	1 (5)	4 (11.11)	0.645
Hr neg	1 (1.67)	0 (0)	1 (2.08)		2 (3.57)	1 (5)	1 (2.78)	
	Tissue HrHPV type distribution							
	All	HIV neg	HIV pos		All	HIV neg	HIV pos	
	N=60 (%)	N=12 (%)	N=48 (%)	p-value	N=56 (%)	N=20 (%)	N=36 (%)	p-value
16	18 (30)	1 (8.33)	17 (35.42)	0.086	21 (37.5)	10 (50)	11 (30.56)	0.165
18	5 (8.33)	1 (8.33)	4 (8.33)	1	11 (19.64)	2 (10)	9 (25)	0.294
31	9 (15)	4 (33.33)	5 (10.42)	0.069	8 (14.29)	4 (20)	4 (11.11)	0.437
33	14 (23.33)	5 (41.67)	9 (18.75)	0.128	15 (26.79)	7 (35)	8 (22.22)	0.354
35	14 (23.33)	1 (8.33)	13 (27.08)	0.262	13 (23.21)	5 (25)	8 (22.22)	1
39	0 (0)	0 (0)	0 (0)		0 (0)	0 (0)	0 (0)	
45	3 (5)	0 (0)	3 (6.25)	1	5 (8.93)	2 (10)	3 (8.33)	1
51	14 (23.33)	2 (16.67)	12 (25)	0.713	17 (30.36)	6 (30)	11 (30.56)	1
52	21 (35)	3 (25)	18 (37.5)	0.513	16 (28.57)	7 (35)	9 (25)	0.54
56	5 (8.33)	1 (8.33)	4 (8.33)	1	2 (3.57)	0 (0)	2 (5.56)	0.532
58	8 (13.33)	1 (8.33)	7 (14.58)	1	6 (10.71)	2 (10)	4 (11.11)	1
59	3 (5)	2 (16.67)	1 (2.08)	0.099	0 (0)	0 (0)	0 (0)	
68/73	3 (5)	1 (8.33)	2 (4.17)	0.495	1 (1.79)	0 (0)	1 (2.78)	1
Hr neg	1 (1.67)	0 (0)	1 (2.08)		2 (3.57)	1 (5)	1 (2.78)	

3.4.3. Targeted-tissue-based HPV (T-HPV)

Among CIN I/II lesions, a total of 117 and 40 HrHPV and LrHPV were detected respectively. A total of 115 HrHPV and 42 LrHPV were present in CIN III lesions. A single HPV type infection was present in 49 (42.24%) of all lesions in the total group.

The single HPV type infection rate was 38.33% (23 of 60 lesions) for CIN I/II and 46.43% (26 of 56 lesions) for CIN III, as illustrated in Table 3.2. The prevalence of single or multiple HrHPV type infections in CIN I/II lesions was identical to that of surface HrHPV mentioned above; that is, 98.33% for all lesions; 100% and 97.92% in the HIV-negative- and -positive groups respectively.

Figure 3.1 illustrates the HrHPV type distribution in lesions with single T-HPV infections irrespective of CIN grading. HPV 52 (35%) was most prevalent among all CIN I/II lesions, as illustrated in tables 3 and 5, followed by HPV 16 (30%) and equally HPV 33, 35 and 51 (23.33%). The most prevalent type in the HIV-negative group with CIN I/II was HPV 33 (41.67%) and 31 (33.33%). In the HIV-positive group, HPV 16 (35.42%), 35 (28.05%) and 33 (18.75%) were the most prevalent types.

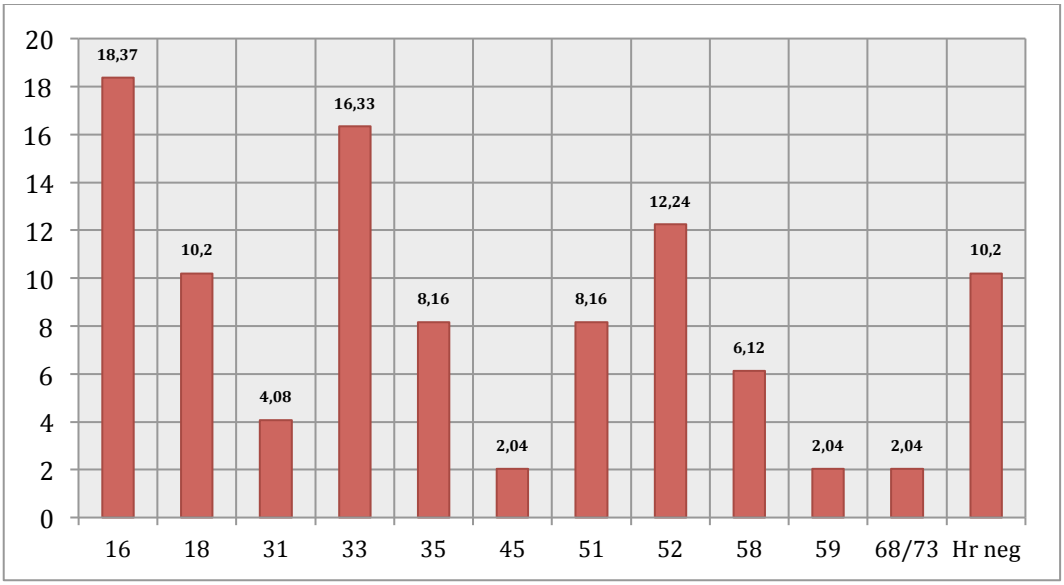


Figure 3.1: HrHPV type distribution in lesions with single T-HPV infections irrespective of CIN grading (n=49)

Among CIN III lesions, the most prevalent HrHPV type was HPV 16, present in 35.5% of lesions, followed by HPV 51 (30.36%), 52 (28.57%), 33 (26.79%) and 35 (23.21%). In CIN III lesions, in order of decreasing prevalence, the most common HrHPV types were: in the HIV negative group, HPV 16 (50%), 33 (35%), 52 (35%), 51 and 35 (both 30%); in the HIV positive group, HIV 16 and 51 equally in 30.56% of lesions, followed by HPV 18, 52, 33 and 35. As with S-HPV, no significant difference between any of the individual HPV types in relation to HIV status was found. Figures 3.2 and 3.3 illustrate the HrHPV type distribution of S-HPV and T-HPV from CIN I/II and CIN III lesions.

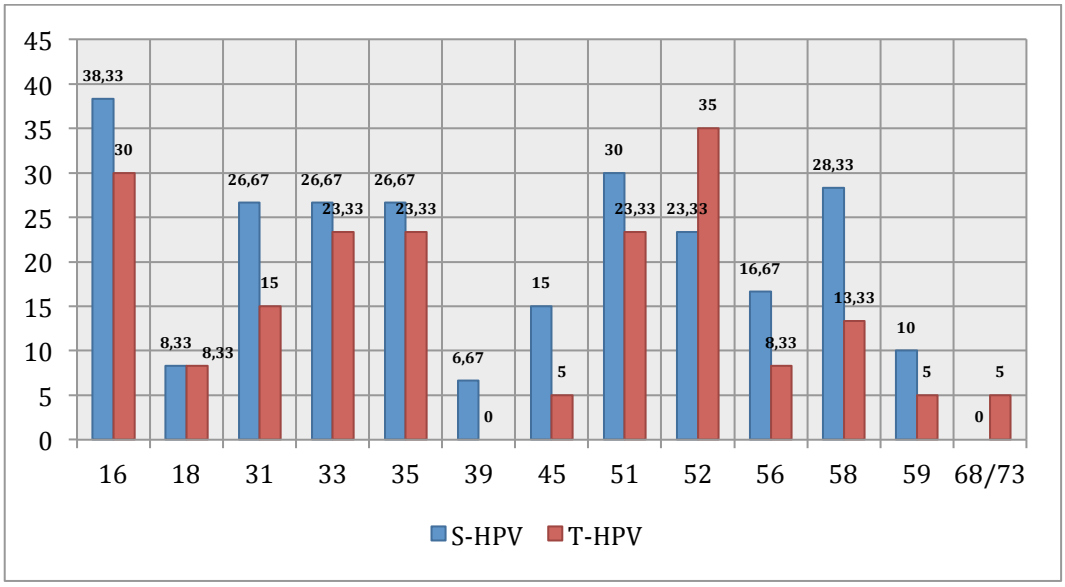


Figure 3.2: HrHPV type distribution of S-HPV and T-HPV in patients with CIN I/II

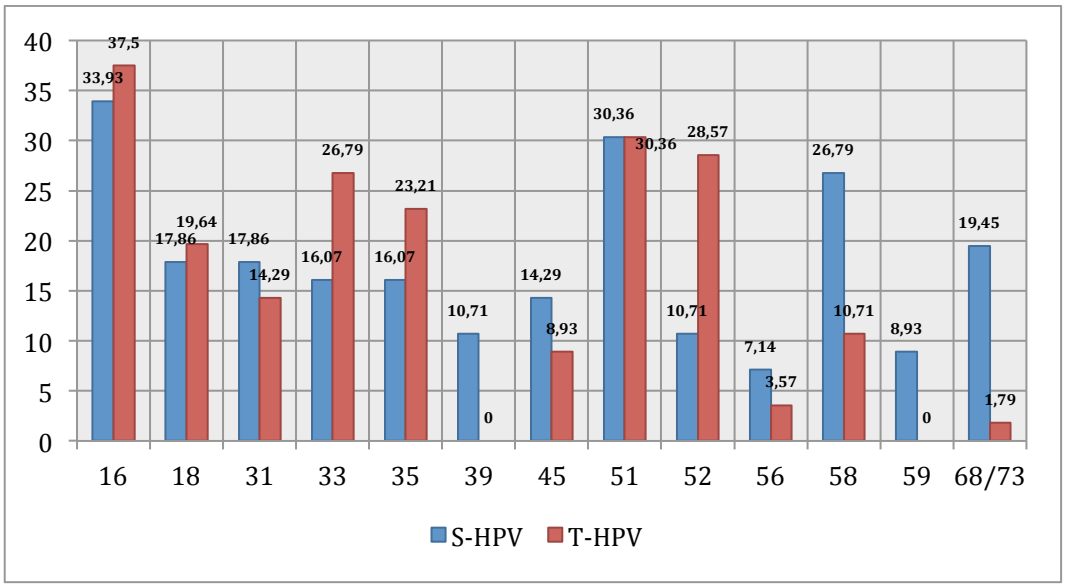


Figure 3.3: HrHPV type distribution of S-HPV and T-HPV in patients with CIN III

3.4.4. Consensus HPV (C-HPV) types

Correlating S-HPV with T-HPV, only two lesions had no viruses that were detected by both assays. In lesions where multiple types were detected on S-HPV, five lesions had identical multiple types on T-HPV. The number of viral types detected ranged from 2 to 4 HPV types.

In the 25 lesions with single HPV types detected on S-HPV, 14 lesions also had a single HPV detected on T-HPV. Thirteen of the 14 HPV types were identical. The one lesion that was different had HPV 69 on S-HPV and HPV 31 on T-HPV. For this specific lesion, HPV 31 was considered as the lesion-associated type.

Thirty-two lesions had more than one corresponding HPV type. Of these 32 lesions, 23 lesions had two corresponding HPV types, eight lesions had three types and one lesion five corresponding HPV types.

In 60 CIN I/II lesions, 89 HPVs were identified, of which 10 (11.23%) were not HrHPV types. The most prevalent HrHPV types in all lesions were HPV 16 (26.67%), 52 (18.33%), 35 (16.67%), 33 (15%) and 51 (15%). See tables 3.4 and 3.5.

From the 56 CIN III lesions, 70 viruses were identified. Nine (12.87%) of these viruses were not HrHPV types. HPV 16 was most prevalent with 30.36%, followed by HPV 52 (14.29%), 18 (12.5%), 51 (10.71%) and 35 (8.93%).

Table 3.4: Corresponding and single HrHPV types in relation to histological grading, HIV status and CD_4 cell count expressed as a percentage of the number of viruses

All patients

	Lesions	Virus	HrHPV types													
			16	18	31	33	35	39	45	51	52	56	58	59	68/73	Hr neg
CIN I/II	60	N=89	16	3	7	9	10	0	1	9	11	2	7	1	3	10
		%	17.98	3.37	7.87	10.11	11.24	0	1.12	10.11	12.36	2.25	7.87	1.12	3.37	11.23
CIN III	56	N=70	17	7	4	4	5	0	3	6	8	3	3	0	1	9
		%	24.29	10	5.71	5.71	7.14	0	4.29	8.57	11.43	4.29	4.29	0	1.43	12.87

HIV-negative patients

	Lesions	Virus	HrHPV types													
			16	18	31	33	35	39	45	51	52	56	58	59	68/73	Hr neg
CIN I/II	12	N=14	1	0	3	3	1	0	0	1	3	0	0	0	1	0
		%	7.14	0	21.43	21.43	7.14	0	0	7.14	21.43	0	0	0	7.14	0
CIN III	20	N=24	9	1	2	2	1	0	0	2	3	0	1	0	0	3
		%	37.5	4.17	8.33	8.33	4.17	0	0	8.33	12.5	0	4.17	0	0	12.5

HIV-positive patients

	Lesions	Virus	HrHPV types													
			16	18	31	33	35	39	45	51	52	56	58	59	68/73	Hr neg
CIN I/II	48	N=70	15	3	4	6	9	0	1	8	8	2	7	1	2	9
		%	20	4	5.33	8	12	0	1.33	10.67	10.67	2.67	9.33	1.33	2.67	12
CIN III	36	N=46	8	6	2	2	4	0	3	4	5	3	2	0	1	6
		%	17.39	13.04	4.35	4.35	8.7	0	6.52	8.7	10.87	6.52	4.35	0	2.17	13.04

HIV-positive patients in relation to CD_4 cell count

		Lesions	Virus	HrHPV types													
				16	18	31	33	35	39	45	51	52	56	58	59	68/73	Hr neg
CIN I/II	CD_4 ≥200	30	N=46	7	3	1	3	5	0	1	5	5	1	6	1	0	8
			%	15.23	6.52	2.17	6.52	10.87	0	2.17	10.87	10.87	2.17	13.05	2.17	0	17.39
	CD_4 <200	18	N=29	8	0	3	3	4	0	0	3	3	1	1	0	2	1
			%	27.59	0	10.34	10.34	13.79	0	0	10.34	10.34	3.45	3.45	0	6.9	3.45
CIN III	CD_4 ≥200	20	N=24	4	5	1	0	0	0	2	3	4	1	0	0	0	4
			%	16.67	20.83	4.17	0	0	0	8.33	12.5	16.67	4.17	0	0	0	16.67
	CD_4 <200	16	N=22	4	1	1	2	4	0	1	1	1	2	2	0	1	2
			%	18.18	4.55	4.55	9.09	18.18	0	4.55	4.55	4.55	9.09	9.09	0	4.55	9.09

Table 3.5: Sequence of five most prevalent HrHPV types for S-HPV, T-HPV and C-HPV detected in all patients, HIV-negative- and -positive patients

All patients											
CIN I/II						CIN III					
S-HPV	16	51	58	31	33	S-HPV	16	51	58	18	31
T-HPV	52	16	33	35	51	T-HPV	16	51	52	33	35
C-HPV	16	52	35	33	51	C-HPV	16	52	18	51	35
HIV-negative patients											
CIN I/II						CIN III					
S-HPV	31	58	35	16	52	S-HPV	16	31	52	58	33
T-HPV	33	31	52	51	59	T-HPV	16	33	52	51	35
C-HPV	31	33	52	16	35	C-HPV	16	52	31	33	51
HIV-positive patients											
CIN I/II						CIN III					
S-HPV	16	51	33	35	58	S-HPV	52	58	16	18	33
T-HPV	52	16	35	51	33	T-HPV	16	51	18	52	35
C-HPV	16	35	51	52	58	C-HPV	16	18	52	35	51

Among the lesions from HIV-negative patients HPV 16 (45%) and 58 (15%) were also the two most prevalent types. In lesions from patients co-infected with HIV, the most prevalent type was also HPV 16 (22.22%), but was followed by HPV 18 (16.67%), 52 (13.89%), 35 (11.11%) and 51 (11.11%). As immunity deteriorates in the HIV-positive group CIN III lesions, it appears as if the prevalence increases for HPV 33, 35, 56 and 58 (See Figure 3.4).

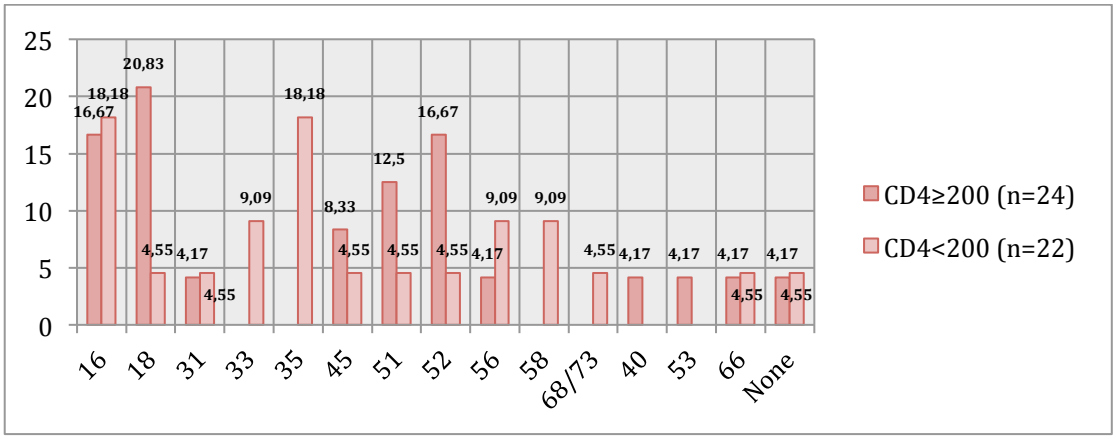


Figure 3.4: C-HPV distribution in HIV-positive patients in relation to CD_4 cell count (expressed as a percentage of the number of viruses)

3.5. DISCUSSION

According to our knowledge this is the first study on the African continent that has focused on detecting specific HPV types within CIN lesions. It is also the first study to include a subset of lesions from HIV-infected patients.

3.5.1. Study methodology

The most likely reason why the LBA method failed is because of fragmentation of nucleic acids occurring when the tissue is fixed with formalin. As the LiPA PCR method amplifies a smaller portion (65-bp versus 450-bp) of the viral genome, it is more effective for examining FFPE tissue.[27,28]

When isolating DNA from FFPE tissue heat can reverse DNA cross-links to some degree. Several different incubation duration and temperatures have been proposed to improve this.[29-31] In keeping with most of the protocols for nucleic acid extraction from FFPE tissue, the specimens were pretreated with xylene and ethanol to remove the paraffin wax. The difficulty with this method is that it is labor intensive, chemically toxic and there is a risk of unintentionally removing small tissue fragments during the process.[9]

Steinau et al. compared the xylene pretreatment method, followed by two washes with pure ethanol, to a high-heat treatment method. Instead of xylene the researchers added 180µl lysis buffer (from DNeasy kit) directly to the paraffin section, followed by incubation at 120°C for 20 minutes. Different temperatures were also used after adding proteinase K. Findings from this study showed more inadequate HPV results using linear array from specimens treated with xylene compared to those treated with the high-heat method.[9] Although different kits were used compared to this study, pretreating the samples with xylene might have contributed to the inadequate results using LBA.

3.5.2. Surface HPV (S-HPV) versus targeted-tissue-based HPV (T-HPV)

The current study was not designed to compare the two HPV detection methods. In order to directly compare these tests, samples should be obtained from the same area, whereas in this study the one method detected HPV on the cervico-vaginal surface and the other detected HPV in micro-dissected targeted-tissue-based lesions. However, when comparing single HPV types in lesions with single types on both tests, the correlation was excellent, with 92.9% (13 out of 14) showing identical viruses in both tests. These tests have been shown to be highly comparable if samples are collected from the same area.[17,32]

If more than one HPV type is present, comparing the two detection methods becomes more complicated. Despite the use of a preferential subset of type-specific primers, different HPV types compete for the same primers. If two different types are present in the sample in equimolar quantity, both of them will be likely to be amplified and identified. If, however, one of the types is present in much higher quantities than the other, it is likely that it will outcompete the minor type, leaving it undetected.[17,32] This is likely why the multiple types detected by both tests were not the same and why LiPA-detected types were undetected by LBA. Only two lesions in this study did not have corresponding viruses between the two methods.

Among CIN I/II lesions, HPV 16 was the most prevalent for S-HPV and HPV 52 for T-HPV. Although the order of prevalence differed slightly between the different methods, HPV 16, 33 and 51 constantly remained under the five most prevalent types regardless of the testing method. Although prevalent in the S-HPV, HPV 31 and 58 were not common in the T-HPV.

The two most prevalent types detected among CIN III lesions for S-HPV and T-HPV were HPV 16 and 51. None of the other types under the five most prevalent types were the same. Interestingly, although the sequence differed, the same five T-HPV types that were most prevalent among CIN I/II lesions were also detected among CIN III lesions.

3.5.3. Prevalence of single and multiple types in T-HPV

All lesions tested positive for one or more HPV type. Among all lesions, the prevalence for single HPV types were slightly higher in CIN III lesions compared to CIN I/II lesions (38.33% versus 46.43%). The prevalence of multiple types is higher than reported in other studies, although the micro-dissection method in these studies was more specific, using laser capture micro-dissection.[3,4,21]

In the current study there were many lesions with multiple types. Limited by the sampling methods, it is not possible to determine whether more than one virus present is causing the lesion. It is unclear whether there are colliding lesions with separate viruses, or if there is contamination from incidental infections on the surface or adjacent normal cervical epithelium.

3.5.4. Consensus HPV (C-HPV)

HPV 16 was the most prevalent C-HPV among CIN I/II lesions and as with S-HPV and T-HPV, HPV 16, 33 and 51 constantly remained among the five most prevalent types. The five most prevalent types (HPV 16, 33, 35, 51, 52) were the same for T-HPV and C-HPV.

The most prevalent type among all CIN III lesions was HPV 16. Compared to T-HPV from CIN III lesions, the prevalence increased for HPV 18, 35 and 52 in C-HPV and decreased for HPV 51.

3.5.5. HPV and HIV

Although the number of lesions from HIV-negative patients was small, there were more single type S-HPV and T-HPV infections comparing CIN I/II with CIN III lesions. In the HIV-negative group with CIN III, HPV 16, 33 and 52 were constantly among the five most prevalent types regardless of the HPV detection method.

Among lesions from HIV positive patients, HPV 16 and 18 were among the five most prevalent types. There was a shift in prevalence for HPV 18 as it increased from 4th most prevalent in S-HPV to 3rd and 2nd in T-HPV and C-HPV respectively. Although the sequence was different, the five most prevalent C-HPV types in the HIV positive group were the same as the C-HPV of all CIN III lesions (HPV 16, 18, 35, 51, 52).

3.5.6. Comparing data with literature

Published data illustrating the relationship of HPV DNA found within CIN are lacking.[3] No studies with the same study methods could be found to directly compare with data from this study. A study investigating molecular mapping of high-grade pre-malignant lesions found HPV 16 to be the main causal oncogenic type.[22]

After Quint et al. published their findings that a single HPV type causes a single high-grade lesion, two more studies were published using laser capture micro-dissection (LCM) to detect the specific HPV type causing the lesion.[3,4,21] Callegari et al. found HPV 58 (58%), 31 (14%) and 18 (5%) as the most prevalent types in their group of patients with CIN III or adenocarcinoma-in-situ (AIS).[21] found HPV 16 to be the main causal HPV type in their study, with 51% of patients with CIN II/III testing positive.[4] Comparing the C-HPV to their findings, Van Marel et al.'s

discovered that only 30% of CIN III was caused by HPV 16, followed by HPV 52 (14%) and HPV 18 (12%). If the theory is applied that every virus represents a lesion, these percentages drop even more. Irrespective of histological grade, among lesions with single T-HPV types, the most prevalent type was HPV 16, followed by HPV 33, 52, 18 and 35.

3.5.7. Limitations

The limitations of this study include the two different HPV detection methods used. If the same method was used it may have avoided a large number of viruses detected that were different among patients with multiple type infections. The second limitation is that the tissue-targeted micro-dissection of the lesion was possibly too large and that might explain the detection of multiple type infections in a large number of lesions. It is therefore very important to follow this study up with a similar study using LCM to determine if these are multiple adjacent lesions with one virus causing each of the lesions or if there are multiple HPV types present in one lesion.

Information regarding parity, sexual partners and other sexually transmitted diseases was not included. Such a lack of information might also explain certain differences between the groups. Lastly, the small number of lesions included from HIV-negative patients is a limitation and makes comparing the groups and drawing conclusions from results difficult. The large number of patients with HIV, however, reflects the referral population to the colposcopy clinic. Patients with HIV are generally younger and have more contact with the health system, allowing for more opportunity for screening for cervical dysplasia.

3.6. CONCLUSION

Findings from this study can neither confirm nor refute the theory that a single HPV type is responsible for either low- or high-grade lesions in patients. In this study, multiple HrHPV types were detected in many lesions (57.76%). Surface HPV testing can be utilized to accurately predict the most probable type in tissue

in patients with single type infections. In patients with multiple types on the surface, it is likely that multiple types will be present within the tissue of the lesion, but not all of these types were detected on the surface. Data from this study can be used to predict the most probable associated type in the lesions of these patients.

Data from this study does not prove oncogeneity, but only the association of specific HPV types with low- and high-grade lesions diagnosed on histology. Both viral prevalence and the potential of the virus to integrate into cellular DNA and change growth patterns will determine the likelihood of detecting the viral type in the tissue from cervical dysplastic lesions. Very high prevalence of many types of HrHPV in this population changes the epidemiology of CIN I/II and CIN III. In this study a different pattern of HrHPV presence within lesions is described from the patterns previously reported by other epidemiological studies.

3.7. REFERENCES

1. Castle PE, Stoler MH, Solomon D, Schiffman M. Relationship of community biopsy-diagnosed cervical intraepithelial neoplasia grade 2 (CIN2) to the quality control pathology reviewed diagnoses: an ALTS report. Am J Clin Pathol. 2007;127:805-815.
2. Bosch FX, Lorincz A, Muñoz N, Meijer CJ, Shah KV. The causal relation between human papillomavirus and cervical cancer. J Clin Pathol. 2002;55(4):244-265.
3. Quint W, Jenkins D, Molijn A, Struijk L, van de Sandt M, Doorbar J, et al. One virus, one lesion - individual components of CIN lesions contain a specific HPV type. J Pathol. 2012;227(1):62-71.
4. van der Marel J, Berkhof J, Ordi J, Torné A, Del Pino M, van Baars R, et al. Attributing oncogenic human papillomavirus genotypes to high-grade cervical neoplasia: which type causes the lesion? Am J Surg Pathol. 2015;39(4):496-504.
5. Clifford G, Franceschi S, Diaz M, Munoz N, Villa LL. Chapter 3: HPV type-distribution in women with and without cervical neoplastic diseases. Vaccine. 2006;S3/26-S3/34.
6. Smith JS, Lindsay L, Hoots B, Keys J, Franceschi S, Winer R, et al. Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: A meta-analysis update. Int J Cancer. 2007;121(3):621-632.
7. Koshiol J, Lindsay L, Pimenta JM, Poole C, Jenkins D, Smith JS. Persistent human papillomavirus infection and cervical neoplasia: a systematic review and meta-analysis. Am J Epidemiol. 2008;168(2):123-137.
8. Kalantari M, Garcia-Carranca A, Morales-Vazquez CD, Zuna R, Montiel DP, Calleja-Macias IE, et al. Laser capture microdissection of cervical human papillomavirus infections: copy number of the virus in cancerous and normal tissue and heterogeneous DNA methylation. Virology. 2009;390(2):261-267.

9. Steinau M, Patel SS, Unger ER. Efficient DNA extraction for HPV genotyping in formalin-fixed, paraffin-embedded tissues. J Mol Diagn. 2011;13(4):377-381.
10. Mills A, Balasubramaniam R, Longacre TA, Kong CS, Pinsky BA. Laboratory-developed L1 sequencing and type-specific, real-time polymerase chain reaction for the detection and typing of human papillomaviruses in formalin-fixed, paraffin-embedded tissues. Arch Pathol Lab Med. 2013;137(1):50-54.
11. Reimers N, Kasper HU, Weissenborn SJ, Stützer H, Preuss SF, Hoffmann TK, et al. Combined analysis of HPV- DNA, p16 and EGFR expression to predict prognosis in oropharyngeal cancer. Int J Cancer. 2007;120(8):1731–1738.
12. Shi W, Kato H, Perez-Ordonez B, Pintilie M, Huang S, Hui A, et al. Comparative prognostic value of HPV16 E6 mRNA compared with in situ hybridization for human oropharyngeal squamous carcinoma. J Clin Oncol. 2009;27(36):6213–6221.
13. Smeets SJ, Hesselink AT, Speel EJ, Haesevoets A, Snijders PJ, Pawlita M, et al. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. Int J Cancer. 2007;121(11):2465–2472.
14. Gilbert MT, Haselkorn T, Bunce M, Sanchez JJ, Lucas SB, Jewell LD, et al. The isolation of nucleic acids from fixed, paraffin-embedded tissues—which methods are useful when? PLoS One. 2007;2(6):e537.
15. Plenat F, Montagne K, Weinbreck N, Corby S, Champigneulle J, Antunes L, et al. Molecular consequences of fixation and tissue processing: the examples of nucleic acids and proteins. Ann Pathol. 2006;26(1):8–21.
16. Srinivasan M, Sedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol. 2002;161(6):1961–1971.
17. van Doorn LJ, Quint W, Kleter B, Molijn A, Colau B, Martin MT, et al. Genotyping of human papillomavirus in liquid cytology cervical specimens by the PGMY line blot assay and the SPF(10) line probe assay. J Clin Microbiol. 2002;40(3):979-983.

18. Baay MF1, Quint WG, Koudstaal J, Hollema H, Duk JM, Burger MP, et al. Comprehensive study of several general and type-specific primer pairs for detection of human papillomavirus DNA by PCR in paraffin-embedded cervical carcinomas. J Clin Microbiol. 1996;34(3):745-747.
19. Kleter B, van Doorn LJ, Schrauwen L, Molijn A, Sastrowijoto S, ter Schegget J, et al. Development and clinical evaluation of a highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus. J Clin Microbiol. 1999;37(8):2508-2517.
20. Kleter B, van Doorn LJ, ter Schegget J, Schrauwen L, van Krimpen K, Burger M, et al. Novel short-fragment PCR assay for highly sensitive broad-spectrum detection of anogenital human papillomaviruses. Am J Pathol. 1998;153(6):1731-1739.
21. Callegari ET, Tabrizi SN, Pyman J, Saville M, Cornall AM, Brotherton JM, et al. How best to interpret mixed human papillomavirus genotypes in high-grade cervical intraepithelial neoplasia lesions. Vaccine. 2014;32(32):4082-4088.
22. van der Marel J, Quint WGV, Schiffman M, van de Sandt MM, Zuna RE, Dunn ST, et al. Molecular mapping of high-grade cervical intraepithelial neoplasia shows etiological dominance of HPV16. Int J Cancer. 2012;131(6):E946-953.
23. Holschneider CH. Cervical intraepithelial neoplasia: definition, incidence, and pathogenesis. In: UpToDate, Golf B (Ed). UpToDate, Waltham, MA, 2013.
24. Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med. 2003;348(6):518-527.
25. van Doorn LJ, Molijn A, Kleter B, Quint W, Colau B. Highly effective detection of human papillomavirus 16 and 18 DNA by a testing algorithm combining broad-spectrum and type-specific PCR. J Clin Microbiol. 2006;44(9):3292-3298.
26. de Sanjose S, Quint WG, Alemany L, Geraets DT, Klaustermeier JE, Lloveras B, et al. Human papillomavirus genotype attribution in invasive

cervical cancer: a retrospective cross-sectional worldwide study. Lancet Oncol. 2010;11(11):1048-1056.

27. Ren ZP, Sallstrom J, Sundstrom C, Nistér M, Olsson Y. Recovering DNA and optimizing PCR conditions from microdissected formalin-fixed and paraffin-embedded materials. Pathobiology. 2000;68(4-5):215–217.
28. Kocjan BJ, Seme K, Poljak M. Comparison of the Abbott Real-Time High Risk HPV test and INNO-LiPA HPV Genotyping Extra test for the detection of human papillomaviruses in formalin-fixed, paraffin-embedded cervical cancer specimens. J Virol Methods. 2011;175(1):117–119.
29. Man YG, Moinfar F, Bratthauer GL, Kuhls EA, Tavassoli FA. An improved method for DNA extraction from paraffin sections. Pathol Res Pract. 2001;197(9):635–642.
30. Shi SR, Cote RJ, Wu L, Liu C, Dater R, Shi Y, et al. DNA Extraction from archival formalin-fixed paraffin-embedded tissue sections based on the antigen retrieval principle: heating under the influence of pH. J Histochem Cytochem. 2002;50(8):1005–1011.
31. Dedhia P, Tarale S, Dhongde G, Khadapkar R, Das B. Evaluation of DNA extraction methods and real time PCR optimization on formalin -fixed paraffin-imbedded tissues. Asian Pac J Cancer Prev. 2007;8(1):55–59.
32. van Hamont D, van Ham MA, Bakkers JM, Massuger LF, Melchers WJ. Evaluation of the SPF10-INNO LiPA human papillomavirus (HPV) genotyping test and the roche linear array HPV genotyping test. J Clin Microbiol. 2006;44(9):3122-3129.

CHAPTER 4

TARGETED-TISSUE-BASED HPV TYPES DETECTED WITHIN CIN LESIONS IN HIV-POSITIVE- AND -NEGATIVE WOMEN: REDUCING INTER- AND INTRA-OBSERVER VARIABILITY WITH $P16^{INK4A}$ and KI-67

4.1. INTRODUCTION

4.1.1. Cytological screening

The ideal predictors of disease progression from cervical intraepithelial neoplasia (CIN) to invasive cervical cancer are still lacking. Identifying specific biomarkers in pre-malignant lesions that can positively predict progression will have a major impact on the management of these patients, especially in identifying patients truly at risk of progression.

Cytological screening, which is dependent on cytomorphological evaluation of the outer layers of the cervix, is universally used to identify pre-malignant and malignant cervical disease.[1] Despite specificity of conventional cytology of at least 90%, sensitivity of this screening method remains poor.[2] With a sensitivity of around 55% for the detection of CIN grade II (CIN II) and worse, screening by means of cytology is less than ideal. Sensitivity of cytology can increase by performing high-risk human papillomavirus (HrHPV) testing on cervical scrapings.[1]

Cytology in comparison to HPV testing is less sensitive and has a lower negative predictive value (NPV) to identify CIN II or worse, at a single time point. The risk of developing high-grade lesions (CIN II or worse) following a negative HPV DNA test appears low, allowing for prolonged screening periods.[3]

4.1.2. Histology and grading of lesions

Histological markers of increased risk of progression include larger lesion size, multicentric involvement, glandular involvement and degree of dysplastic cells. The poor ability of a positive HPV test to predict the risk of developing invasive cervical cancer has led to alternative methods to triage women with positive HPV DNA and RNA tests.[3]

Proper management of patients with CIN relies on correct histopathological grading, seeing that CIN grade I (CIN I) is treated differently from CIN grade II and III (CIN III). Among pathologists there appears to be a lack of inter- and intra-observer reproducibility of CIN grading. Such a situation emphasizes the need for specific biomarkers to objectively assist in diagnosing genuine precursors of cervical cancer.[4]

4.1.3. Risk of lesion progression

The majority of CIN I lesions are linked to benign HPV replication. Between 70% and 80% of these CIN I lesions will regress spontaneously without treatment. Regression rates are even higher among adolescents and women younger than 25.[5-7] Within 12 months, between 0.2% and 4% of CIN III lesions will progress to invasive cancer.[8] A significant number of lesions will also persist or regress, which highlights the fact that not all high-grade CIN lesions are genuine pre-malignant lesions. Of all the pre-neoplastic cervical lesions, diagnosing CIN II tends to be the least reproducible and the biological behavior of CIN II is not clear. Within four to six years regression rates of up to 55% have been reported.[4,9]

4.1.4. HPV pathogenesis

In order to find specific biomarkers it is important to understand the pathogenesis of cervical cancer caused by HPV. Virions gain access through micro-abrasions in the epidermis and infect the basal epithelium.[4] Proteins E5, E6 and E7 are three transforming proteins encoded by oncogenic HPVs. E5 likely plays a role in early oncogenesis and works together with E6 and E7.[10]

HPV E7 proteins interact with the retinoblastoma tumour suppressor protein (pRb). pRb controls S-phase entry via its association with E2F transcription-factor family members. High-risk HPV E7 targets pRb for proteasomal degradations and E7 proteins cause abnormal activation of cyclin-dependent kinase 2 (cdk2). Cdk2 is associated with cyclin E and A, as well as cdk inhibitors ($p21^{CIPI}$ and $p27^{KIPI}$). E7 expression leads to abnormal expression of cyclin E and

A and ensures that differentiating keratinocytes are retained in a DNA synthesis competent state.[10]

HrHPV E6 proteins target p53 for ubiquitination (post-translational modification where ubiquitin is attached to a substrate protein), which is facilitated through cellular ubiquitin ligase E6AP and causes proteasomal degradation. By inducing supernumery centrosomes and multipolar mitoses in epithelial cells, HrHPV E6 and E7 cause mitotic defects and aneuploidy. Viral integration into the host genome is a significant event in malignant progression of cervical lesions as it leads to dysregulation of E6/E7 expression and diminished E2 action.[10]

4.1.5. Immunohistochemical (IHC) markers

Although the histopathological criterion to diagnose CIN is well defined, differentiating between low- and high-grade lesions may be difficult at times. This difficulty has led to a search for more objective methods to aid in diagnosing these lesions. Molecular studies have identified various markers in recent years, which include cellular proteins affected by viral oncoproteins, and cell cycle markers disrupted by various actions of the virus.[10]

One of the most extensively investigated biomarkers in cervical neoplasia is $p16^{INK4a}$ (p16).[4] p16 is a cyclin that has a kinase inhibitory function.[11] In normal cells, pRb binds to the transcription factor E2F and blocks gene transcription in this way. The p16 gene coding for the cyclin-dependent kinase is blocked and replication and proliferation of cells is blocked.[12]

As mentioned above, oncogenic protein E7 targets pRb and prevents binding of transcription factor E2F, leading to transcription of certain genes, one of which being the p16 gene.[13] Viral oncoprotein E7, therefore, causes an indirect overexpression of p16. Owing to these changes p16 can be used as a surrogate marker of active oncogenic HPV infections, grade of disease and might also be a marker of disease progression.[4,14-16]

Ki-67 is an antigen expressed in the nuclei of proliferating cells during the entire cell cycle, except for G0.[10,17] Although Ki-67 positivity in the middle and upper layers may indicate a high-grade lesion, it cannot differentiate these lesions from epithelial proliferation caused by reactive and inflammatory lesions. It is therefore advisable to use Ki-67 as an adjunct to other biomarkers.[10]

4.1.6. Motivation for the study

It is not ethically and morally justifiable to monitor patients with CIN III until it progresses to invasive cancer. Utilizing IHC markers reduces inter- and intra-observer variability in diagnosing true pre-malignant lesions. The assumption is made that CIN III lesions testing positive for both p16 and Ki-67 are the lesions most likely to progress and identifying the HPV types within these lesions might assist in identifying the more oncogenic types causing these lesions.

4.2. OBJECTIVES AND HYPOTHESES

4.2.1. Objectives

The objectives of this study were to:

- Correlate the histopathological criteria and specific immunohistochemical stainings with regard to grading of CIN lesions;
- Determine the HPV type distribution in these patients, especially those with lesions that have a high probability to progress to invasive cancer; and
- Determine if the HPV type distribution differs between human immunodeficiency virus (HIV) negative and –positive women and HIV-positive women with different levels of immune competence.

4.2.2. Hypotheses

The hypotheses of this study were that:

- There would be a positive correlation between IHC staining and histological grade of CIN lesions.
- As the hypothesis is made that lesions staining positive are likely to progress, the HPV type distribution would be similar to the distribution found in our population of women with invasive cervical cancer.
- HPV type distribution would be different among the HIV-infected subgroup compared to -non-infected patients.

4.3. MATERIALS AND METHODS

4.3.1. Study design

This is a descriptive study performed at the gynaecologic oncology unit and the department of anatomical pathology, University of Pretoria. Histological specimens included were collected from women aged 18 years and older, referred for treatment of high-grade squamous intraepithelial lesions (HSIL) on conventional cervical cytology (Papanicolaou smears) as part of the national screening programme. Patients were treated with a large loop excision of the transformation zone (LLETZ) in accordance with the unit's protocol of seeing and treating patients in a single visit.

A total of 94 consecutive LLETZ samples, collected between 1 July 2010 and 28 February 2011 were included. These were a consecutive subset of patients included and described in Chapter 2. The IHC staining, DNA isolation and tissue HPV DNA typing was performed from 1 January 2014 to 28 February 2015.The Research Ethics Committee of the Faculty of Health Sciences of the University of Pretoria (26/2010, 189/2012) approved this study.

4.3.2. Histology

Tissue submitted from LLETZ, fixed in formalin, embedded in paraffin wax and stained with haematoxylin and eosin were graded by two primary observers in terms of lesional severity. In cases of discrepancy, a third unbiased observer was consulted. The second grading was performed without knowledge of the initial diagnosis and was done on two separate occasions to evaluate both inter- and intra-observer variability. The section with the highest-grade precursor lesion was then selected from each patient and submitted for IHC stains. The IHC stainings were also scored blindly on two separate occasions to reduce intra-observer variability in the interpretation of the stains.

The criteria used for the grading of the histological slides were as follows:
CIN I - dysplasia involving the lower third of the cervical epithelium;
CIN II - dysplasia involving the lower two-thirds of the epithelium with maturation and flattening of the surface epithelium still present; and
CIN III – Full-epithelial-thickness dysplasia with no evidence of maturation.

4.3.3. Immunohistochemical (IHC) markers

IHC stains were performed using the following primary monoclonal antibodies: (a) mouse primary antibody against the $p16^{INK4a}$ protein (Ventana CINtec p16 histology), and (b) rabbit anti-human Ki-67 antigen mouse.

The ready-to-use K8000 kit of Dako was used on the Dako AutostainerLink 48 for immunostaining. Formalin-fixed paraffin embedded samples were cut into 3µm thickness tissue sections. The sections were dewaxed and the antigens retrieved at high pH (9.4) with the EnVision Flex Target retrieval solution at 96˚C for 20 minutes followed by the EnVisionFlex wash buffer rinse for 5 minutes. H_2O_2 blocking was then performed with EnVisionFlex peroxidise blocking reagent for 10 minutes and then rinsed with the buffer solution for 5 minutes. The primary antibody was then incubated for 30 minutes at room temperature and again rinsed for 5 minutes with the buffer. The labelled polymer,

EnVisionFlex horse radish peroxidise, was applied for 30 minutes followed by 5 minutes of buffer. The substrate working solution followed EnVisionFlex DAB chromogen for 10 minutes. A 5-minute buffer rinse was applied again. The sections were then counterstained with Mayer haematoxylin for 3 minutes and blued with tap water for 5 minutes. The sections were then dehydrated to xylene and mounted.

The IHC stains for p16 and Ki-67 were applied and scored by standard light microscopy. p16 has both nuclear and cytoplasmic expression.
Both of these staining patterns were considered as positive and were scored as follows: (0) no staining; (1) very occasional, single-cell staining;
(2) patchy, strong staining (often not continuous with basement membrane);
(3) diffuse and strong staining (continuous from basement membrane, extending upward). p16 was also correlated according to the CIN present in terms of the proportion of dysplastic epithelium present (as described above at histology).

Ki-67 has only nuclear staining. Staining might be observed in the lower third under normal conditions. Occasional positive basal or parabasal cells were reported as negative and only reported positive if positive cells were observed above the lower third of the epithelium.

4.3.4. DNA isolation

This method has been described in chapter 3.

4.3.5. HPV DNA typing

DDL Diagnostic Laboratory in the Netherlands performed HPV DNA analyses. As described in Chapter 3, the broad spectrum SPF10 PCR amplifies a 65 bp fragment from the L1 region of the HPV genome. Amplimers were captured onto streptavidin-coated microtitre plates using biotinylated reverse primers. After denaturation of the PCR products by alkaline treatment, a DEIA was used to detect HPV-positive samples.

This method is able to detect more than 50 HPV types. Amplimers from SPF10.[18] PCR DEIA-positive samples were used for subsequent genotyping of 25 mucosal HPV types (HPV6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68 or 73, 70, and 74). Because the interprimer regions of HPV 68 and 73 are identical the test cannot distinguish between them and they are therefore reported as either 68 or 73.[19]

The reason for using line blot assay and not linear array was discussed in Chapter 3.

4.3.6. Data capturing and analysis

Data was captured on Microsoft® Excel® datasheets, and analysis performed using Stata® statistical software release 13 (StataCorp, College Station, TX). Discrete data was mainly binary in nature and summary statistics were frequency, percentage, cross-tables and bar charts. Continuous data was summarized using descriptive statistics. Comparison between groups was done with Fisher's exact test for discrete outcomes. Testing was done at the 0.05 level of significance.

4.4. RESULTS

4.4.1. Study characteristics

A total of 94 histological specimens from 94 patients were included in the study. Table 4.1 illustrates the study group characteristics. HIV results and CD_4 results, if applicable, were available for all patients of whom 71 (75.5%) patients were HIV positive and 23 (24.5%) patients were HIV negative. CD_4 count was reported as more or equal to 200/µl, or less than 200/µl. The mean age for the study group was 35.6 years and was almost identical between the two groups. The median age for both groups was 33 years.

4.4.2. Histological diagnosis

Of the 94 patients included with HSIL on cytology, eight (8.51%) patients had CIN I on histology. Twenty two (95.65%) of the 23 HIV-negative patients had CIN II or CIN III, whereas 64 (90.14%) HIV-positive patients had CIN II or CIN III.

Table 4.1: Study population characteristics

Study population			
	Total	HIV Negative	HIV Positive
Study population	94	23 (24.5%)	71 (75.5%)
$CD_4 \geq 200$			43 (60.56%)
$CD_4 < 200$			28 (39.44%)
Mean Age (in years)	35.6	36.6	35.5
Histology			
	Total	HIV Negative	HIV Positive
CIN I	8 (8.51%)	1 (4.35%)	7 (9.86%)
CIN II	40 (42.55%)	7 (30.43%)	33 (46.48%)
CIN III	46 (48.94%)	15 (65.22%)	31 (43.66%)

4.4.3. IHC staining

Among women with CIN I, 50% of lesions had diffuse and strong staining for p16. Seventy-five percent of CIN II lesions and 91.31% of CIN III had diffuse positive results. P16 positivity significantly increased with histological grading (p=0.002). See Table 4.2.

Lesions from nine patients had a negative Ki-67 staining. Among women with CIN I, 50% had a positive Ki-67 stain compared to 90% of CIN II lesions and 97.83% of CIN III lesions. As with p16, positivity increased as histological grade of the lesions increased. (p=0.001) See Table 4.2.

Regardless of histological grading, 54 (57.45%) of 94 patients had dual positive staining for both p16 (diffuse and strong positive) and Ki-67. Three (37.5%) of the 8 patients with CIN I, 29 (72.5%) of the 40 patients with CIN II, and 41 (89.13%) of the 46 patients with confirmed CIN III on histology had positive staining for both p16 (diffuse and strong positive) and Ki-67.

Table 4.2: Immunohistochemical staining

Histological diagnosis and p16 staining					
	p16 Neg	p16 Single	p16 Patchy	p16 Diffuse	p-value
CIN I	3 (37.5%)	0	1 (12.5%)	4 (50.0%)	0.002*
CIN II	3 (7.5%)	1 (2.5%)	6 (15.0%)	30 (75.0%)	
CIN III	1 (2.17%)	3 (6.52%)	0	42 (91.31%)	
Histological diagnosis and Ki-67 staining					
	Ki-67 Neg		Ki-67 Pos		p-value
CIN I	4 (50.0%)		4 (50.0%)		0.001*
CIN II	4 (10.0%)		36 (90.0%)		
CIN III	1 (2.17%)		45 (97.83%)		
*statistically significant					

4.4.4. HPV prevalence

All 94 samples tested positive for HPV DNA. A total of 261 high- and low-risk HPVs were detected. For the entire group 196 HrHPV viruses were present. Among the 23 HIV-negative patients, 52 high-risk viruses were detected and 144 high-risk viruses among the 71 HIV-positive patients.

The prevalence for one or more HrHPV infections was: 93.62% (88 of 94 patients) for the entire study population; 86.95% (20 of 23 patients) for HIV negative patients and 95.78% (68 of 71 patients) for HIV positive patients. In women with CIN III on histology, the prevalence was 93.33% (14 of 15 patients) and 96.78% (30 of 31 patients) in HIV-negative- and -positive subgroups respectively. Of the 15 HIV-negative patients with CIN III, 7 (46.67%) had a

single HrHPV type infection. Slightly fewer (41.94%) HIV-infected patients had single HrHPV type infections. Table 4.3 illustrates the HrHPV type distribution for all patients as well as HIV-positive- and -negative patients in relation to histological diagnosis.

Table 4.3: HrHPV type distribution in relation to CIN grade and HIV status

Total population	Cases	HrHPV types													
		16	18	31	33	35	39	45	51	52	56	58	59	68/73	Hr neg
CIN I	N=8	4	1	3	4	3	0	2	3	3	0	2	0	0	0
	%	50	12.5	37.5	50	37.5	0	25	37.5	37.5	0	25	0	0	0
CIN II	N=40	10	3	5	9	11	0	1	10	13	4	4	2	1	4
	%	25	7.5	12.5	22.5	27.5	0	2.5	25	32.5	10	10	5	2.5	10
CIN III	N=46	18	10	7	11	12	0	4	14	13	2	6	0	1	2
	%	39.1	21.7	15.2	23.9	26.1	0	8.7	30.4	28.3	4.3	13	0	2.2	4.3

HIV Negative	Cases	HrHPV types													
		16	18	31	33	35	39	45	51	52	56	58	59	68/73	Hr neg
CIN I	N=1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	%	0	0	0	0	100	0	0	0	0	0	0	0	0	0
CIN II	N=7	1	1	3	4	0	0	0	2	2	1	1	1	0	2
	%	14.3	14.3	42.9	57.1	0	0	0	28.6	28.6	14.3	14.3	14.3	0	28.6
CIN III	N=15	7	2	3	5	5	0	1	5	5	0	2	0	0	1
	%	46.7	13.3	20	33.3	33.3	0	3.3	33.3	33.3	0	6.7	0	0	3.3

HIV Positive	Cases	HrHPV types													
		16	18	31	33	35	39	45	51	52	56	58	59	68/73	Hr neg
CIN I	N=7	4	1	3	4	2	0	2	3	3	0	2	0	0	0
	%	57.1	14.3	42.9	57.1	28.6	0	28.6	42.9	42.9	0	28.6	0	0	0
CIN II	N=33	9	2	2	5	11	0	1	8	11	3	3	1	1	2
	%	27.3	6.1	6.1	15.2	33.3	0	3	24.2	33.3	9.1	9.1	3	3	6.1
CIN III	N=31	11	8	4	6	7	0	3	9	8	2	4	0	1	1
	%	35.5	25.8	12.9	19.4	22.6	0	9.7	29.0	25.8	6.5	12.9	0	3.2	3.2

4.4.5. HrHPV prevalence in relation to histological grade

The most prevalent single and multiple HrHPV type infections detected among all patients, irrespective of histological grade, was HPV 16 (34.04%) followed by HPV 52 (30.85%), 51 (28.72%), 35 (27.66%) and 33 (25.53%). In all patients with CIN I/II, the most prevalent HPV type was HPV 52 (33.33%), followed by HPV 16 and 35 (both 29.17%) and HPV 33 and 51 (both 27.08%).

The most prevalent HrHPV type detected in all patients with CIN III with single- and multiple type infections was HPV 16 (39.13%). HPV 16 was followed by HPV 51 (30.43%), 52 (28.26%), 35 (26.09%) and 33 (23.91%).

Among HIV negative patients with CIN I/II, the most prevalent HPV types were HPV 33 (50%), 31 (37.5%), 51 and 52 (both 25%). In HIV-positive patients with CIN I/II, HPV 52 (35%) was the most prevalent of single and multiple HrHPV type infections, followed by HPV 16 (32.5%), 35 (32.5%), 51 (27.5%) and 33 (22.5%).

In the 15 HIV-negative women with CIN III, the most common single and multiple HrHPV types were HPV 16 (46.67%), 33, 35, 51 and 52 (each 33.33%). In the 31 HIV-positive patients with CIN III, HPV 16 (35.48%) was the most prevalent, followed by HPV 51 (29.03%), 18 (25.81%), 52 (25.81%) and 35 (22.58%).

4.4.6. Single HrHPV type distribution

The distribution of the single high-risk viruses is illustrated in Figure 4.1. Single HPV types were detected in 21 of the 48 patients with CIN I/II of whom 18 had a single HrHPV type. HPV 6, 11 and 66 were the three non-HrHPV detected. The most prevalent HrHPV type was HPV 35, followed by HPV 16 and 33.

In the 46 patients with CIN III, 20 patients had a single HPV type infection. Only one patient did not have a HrHPV type and was infected with HPV 66. HPV 16 was the most prevalent single HrHPV type in patients with CIN III.

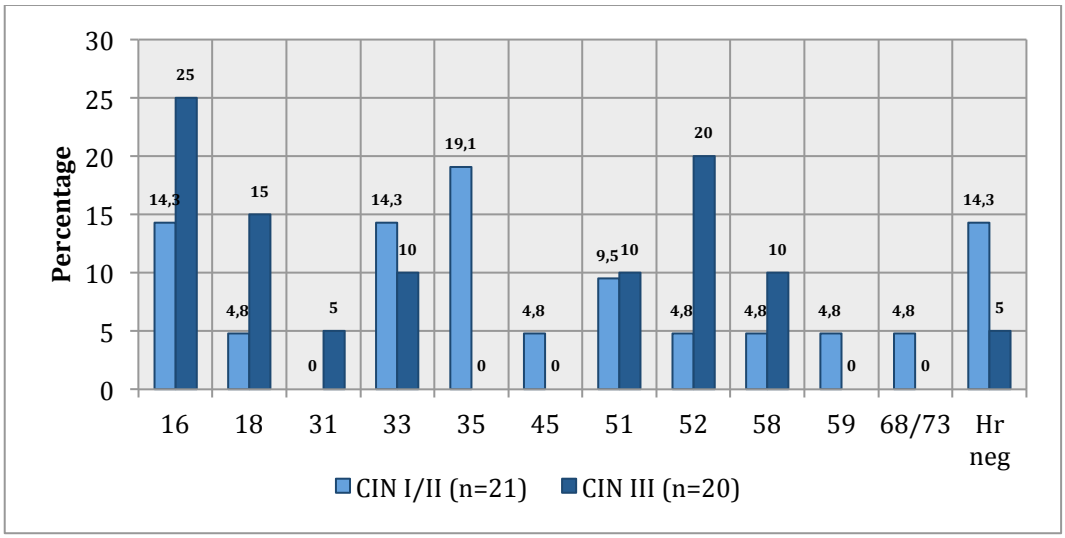


Figure 4.1: Single HrHPV type distribution in patients with CIN I/II and CIN III

4.4.7. CIN III, Ki-67 and p16 positive

Of the 46 patients with CIN III on histology, 41 patients had positive Ki-67 and p16 diffuse positive staining. These patients were analyzed regarding HPV type distribution. HrHPV infections were present in 39 patients of whom 17 had a single HrHPV type infection. A total of 82 high-risk viruses were detected. See Figure 4.2.

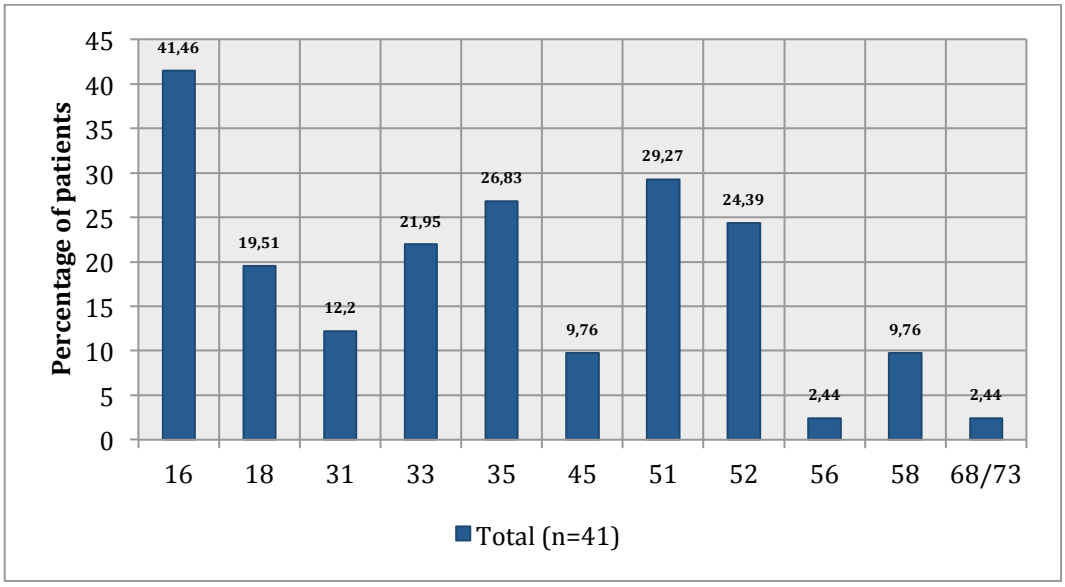


Figure 4.2: Single and multiple HrHPV distribution in patients with CIN III and Ki-67 and p16 positive staining

Of the 30 high-risk viruses present in the 14 HIV negative patients, the most prevalent was HPV 16, followed by HPV 35, 33, 51 and 52. The most common HrHPV type of the 52 high-risk viruses detected among the 27 HIV-positive patients was HPV 16, followed by HPV 51, 18, 35 and 52. (See Figure 4.3). Neither HPV 39 nor HPV 59 was present in any of the specimens tested.

Figure 4.4 illustrates the HrHPV distribution of the 52 viruses in relation to the CD_4 cell count of the 27 HIV-positive patients. In the group with a CD_4 cell count ≥ 200, HPV 16 and 51 were most prevalent, followed by HPV 18 and 52. In the group with a CD_4 cell count < 200, the most prevalent types were HPV 16, 35, 33 and 51.

Of the 18 patients with a single HPV type infection, only one patient had a non-HrHPV type infection and HPV 66 was detected in this patient. In the 17 patients with CIN III, dual positive staining and a single HrHPV type, the most prevalent types were HPV 16, 52, 18, 33 and 51. See Figure 4.5.

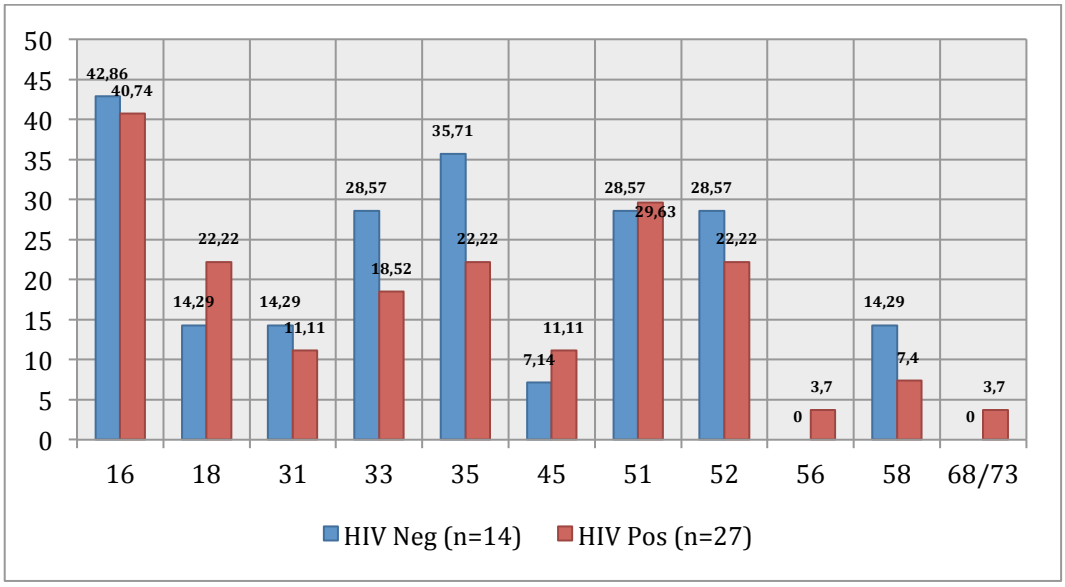


Figure 4.3: Single and multiple HrHPV distribution in HIV-negative- and -positive patients with CIN III and Ki-67 and p16 positive staining

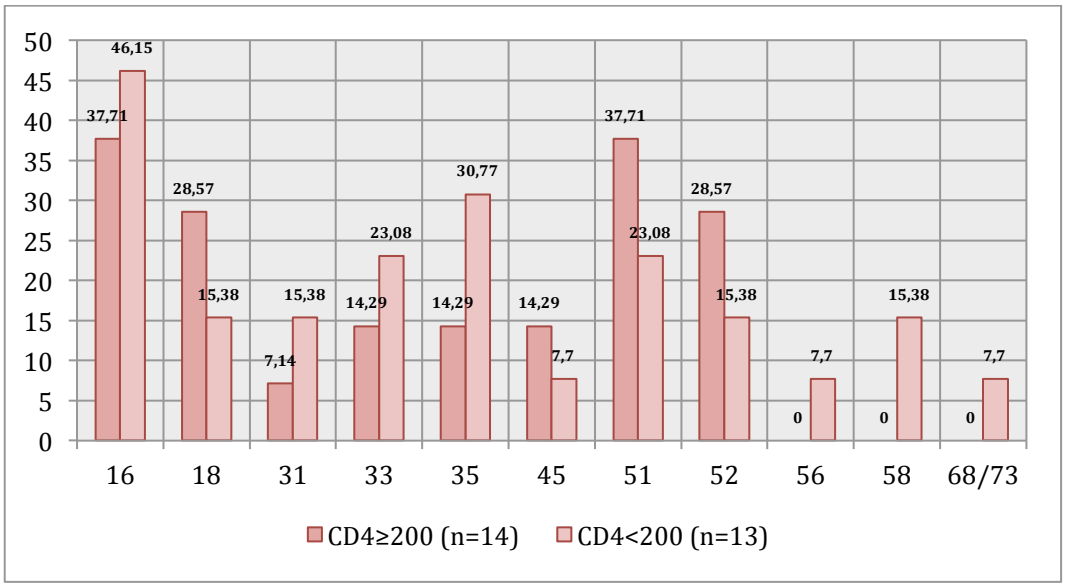


Figure 4.4: HrHPV distribution in HIV positive patients with CIN III and Ki-67 and p16 positive staining in relation to CD_4 cell count

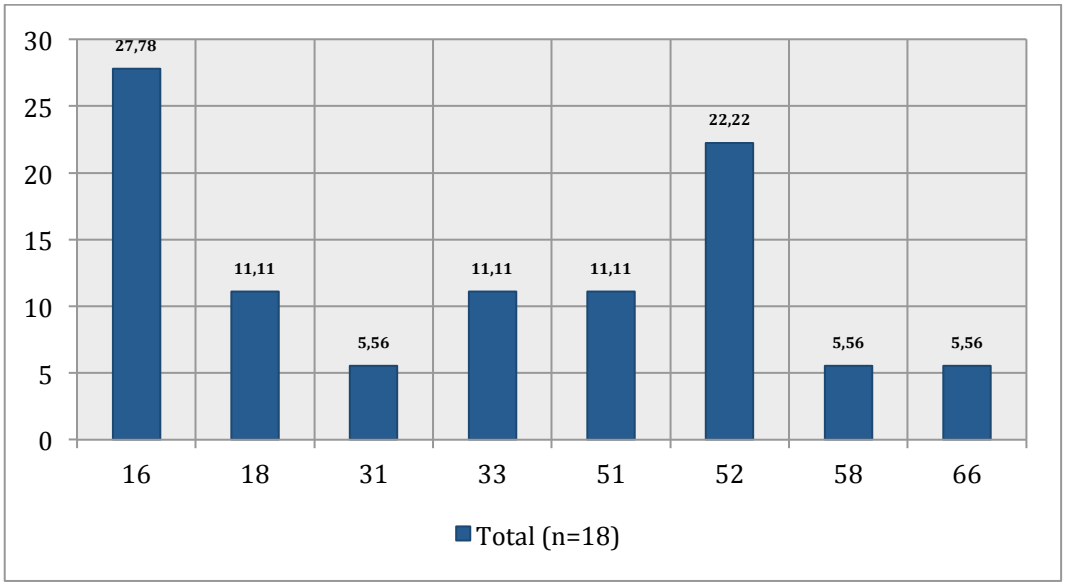


Figure 4.5: Single HrHPV type infections in patients with CIN III and Ki-67 and p16 positive staining

4.5. DISCUSSION

4.5.1. Immunohistochemical (IHC) markers

Overtreating women with early cervical dysplasic lesions may have a negative long-term effect on their fertility. It is therefore important to find indicators to show which lesions have a greater likelihood to progress.[20]

One meta-analysis showed diffuse p16 staining in 2% of normal biopsies, 38% of CIN I, 68% of CIN II and 82% in CIN III.[11] As with Ki-67 there was a significant correlation between p16 positivity and histological grading. In this study, p16 stained diffuse positive in 50% of CIN I, 75% of CIN II and 91% of CIN III. These findings are in agreement with other studies that reported a higher expression of Ki-67 and p16 in high-grade- compared to low-grade lesions.[21-23] Cheah et al. found p16 immunopositivity to be consistent with HrHPV integration in women with high-grade lesions and invasive squamous cell cancer, making it more useful to determine possible malignant progression than HrHPV detection alone.[24]

Hariri and Oster showed in their study that p16 had a very high negative predictive value in patients with CIN I of up to 96%. Over a follow-up period of at least five years, 25 out of 26 patients with CIN I had a good outcome.[25] Authors from another study concluded that although p16 is important in patients with high-grade lesions, it might be even more important in detecting women at risk already in the low-grade phase.[12] A large population based study showed a five-times increased risk for developing CIN II or more over a two-year follow-up period if biopsies showed CIN I or less at baseline with positive p16 staining.[26]

Ki-67 staining in this study had a positive correlation with histological grade, increasing from 50% for CIN I to almost 98% for CIN III. A number of other studies also found that the grade of epithelial dysplasia correlate with Ki-67 expression as it increases from CIN I to invasive carcinoma and it may also correlate with the presence of high-risk HPV DNA.[27,28] Ki-67 might also be a possible indicator of progression in low-grade lesions and be especially useful to distinguish atrophic epithelial changes from high-grade lesions.[29,30]

Combining biomarkers p16 and Ki-67 is more sensitive and specific for high-grade squamous and glandular lesions than either of them used in isolation.[17] Singh et al. also showed dual staining with p16 and Ki-67 and noticeably increased specificity without sensitivity being compromised for diagnosing CIN II/III or glandular lesions compared to HrHPV testing.[31] One study found a strong relationship between p16, Ki-67 and cyclin E to identify HPV-associated precursors and to differentiate normal squamous epithelium from pre-neoplastic lesions.[32]

In spite of the strong association between p16 and CIN grade, routine staining of cervical specimens is not yet included in everyday practice by histopathologists. The hesitance to make staining routine might be due to inconsistencies from the literature regarding correlating p16 positivity and integration, as well as uniformity in grading of p16 staining. Because there is no consistent use of antibodies in various studies it makes standardizing interpretation of staining intensities rather challenging.[4] Despite these difficulties, p16 and Ki-67 might

hold great potential in improving inter-observer consistency in diagnosing CIN lesions that require treatment.[33]

4.5.2. High-risk HPV prevalence

The prevalence of HrHPV was high in this study with 95.6% of patients with CIN III infected with one or more HrHPV types. The prevalence is, however, consistent with unpublished data from our unit, indicating a 93% prevalence of HrHPV infection in women with biopsy confirmed CIN II/III. Of note, all women in this study with CIN I had one or more HrHPV type infections.

The larger number of viruses detected in this population could indicate that the area of micro-dissection was too large and that surface HPV types were also detected. However, women in our population have a heavy burden of HPV infections, with around 45% of women without cytological abnormalities infected with one or more HrHPV type.[34] Therefore, because of the higher prevalence of HPV in our population, one can expect a higher number of viruses present compared to regions with lower infection rates.

A number of recent studies have proven that only one virus type causes each independent CIN lesion.[35-37] If one applies that concept to patients with CIN III with positive p16 and Ki-67 staining, the 82 HrHPV types detected from the micro-dissected tissue likely indicates 82 lesions. One can expect that these lesions might be more likely to progress to invasive cancer and, therefor, the HPV types involved are of importance.

4.5.3. Effect of HIV

There was a high HIV infection rate in this study population of 75%. This, however, is a true reflection of the patient population treated at our colposcopy clinic, as the majority of patients are HIV-infected. Although few conclusions can be drawn looking at the HPV type distribution compared to CD_4 count, the prevalence appeared to increase for HPV 16, 31, 33, 35, 56, 58 and 68/73 as immune function deteriorates.

4.5.4. Limitations

Limitations of the study included a relative small sample size. The small number of HIV-negative patients compared to HIV-positive patients is also a limitation as HIV-positive patients contribute considerably more to the study population and it makes comparing results between the two subgroups difficult. Apart from HIV status, other possible confounding factors like parity, number of sexual partners, etc. were not known and not included in the HPV DNA analysis. The sample area of microdissection was not specific enough and, therefore, led to detection of a high number of viruses, making interpretation difficult. This highlights the need to correlate these findings with a more accurate dissection, using laser capture microdissection LCM.

4.6. CONCLUSIONS

A statistically significant association was established in this study between p16 and Ki-67 immunopositivity and histological grade of CIN. Immunohistochemical staining is an important adjunct to decrease inter- and intra-observer variability in diagnosing cervical pre-malignant lesions. The vast majority of patients included had one or more HrHPV type detected with the most prevalent types in CIN III testing positive for p16 and Ki-67: HPV 16, 51, 35, 52 and 33. Compared to patients with invasive carcinoma HPV 51 and 52 are over represented in the CIN III group, but HPV 18 and 45 are underrepresented. A follow-up study using LCM is needed to verify these findings.

4.7. REFERENCES

1. Eijsink JJ, Yang N, Lendvai A, Klip HG, Volders HH, Buikema HJ, et al. Detection of cervical neoplasia by DNA methylation analysis in cervico-vaginal lavages, a feasibility study. Gynecol Oncol. 2011;120(2):280-283.
2. Coquillard G, Palao B, Patterson B. Quantification of Intracellular HPV E6/E7 mRNA Expression Increases the Specificity and Positive Predictive Value of Cervical Cancer Screening Compared to HPV DNA. Gynecol Oncol. 2011;120:89-93.
3. Monsonego J, Hudgens MG, Zerat L, Zerat JC, Syrjänen K, Halfon P, et al. Evaluation of oncogenic human papillomavirus RNA and DNA tests with liquid-based cytology in primary cervical cancer screening: the FASE study. Int J Cancer. 2011;129(3):691-701.
4. Martin CM, O'Leary JJ. Histology of cervical intraepithelial neoplasia and the role of biomarkers. Best Pract Res Clin Obstet Gynaecol. 2011;25(5):605-615.
5. Moscicki AB, Ma Y, Wibbelsman C, Darragh TM, Powers A, Farhat S, et al. Rate of and risks for regression of cervical intraepithelial neoplasia 2 in adolescents and young women. Obstet Gynecol. 2010;116(6):1373–1380.
6. Moscicki AB, Shiboski S, Hills NK, Powell KJ, Jay N, Hanson EN, et al. Regression of low-grade squamous intra-epithelial lesions in young women. Lancet. 2004;364(9446):1678–1683.
7. Cox JT, Schiffman M, Solomon D, & ASCUS-LSIL Triage Study (ALTS) Group. Prospective follow-up suggests similar risk of subsequent cervical intraepithelial neoplasia grade 2 or 3 among women with cervical intraepithelial neoplasia grade 1 or negative colposcopy and directed biopsy. Am J Obstet Gynecol. 2003;188(6):1406–1412.
8. Goldie SJ, Kohli M, Grima D, Weinstein MC, Wright TC, Bosch FX, et al. Projected clinical benefits and cost-effectiveness of a human papillomavirus 16/18 vaccine. J Natl Cancer Inst. 2004;96(8):604–615.

9. Castle PE, Schiffman M, Wheeler CM, Solomon D. Evidence for frequent regression of cervical intraepithelial neoplasia grade 2. Obstet Gynecol. 2009;113(1):18–25.
10. Kostopoulou EA, Koukoulis G. Immunohistochemistry in the diagnosis of squamous intraepithelial lesions of the uterine cervix. In: Vanden Broeck D, editor. Human papillomavirus and related diseases - from bench to bedside - a clinical perspective. InTech; 2012. p. 41-64. Available from: http://www.intechopen.com/books/human-papillomavirus-and-related-diseases-from-bench-to-bedside-a-clinical-perspective/immunohistochemistry-in-the-diagnosis-of-squamous-intraepithelial-lesions-of-the-uterine-cervix
11. Tsoumpou I, Arbyn M, Kyrgiou M, Wentzensen N, Koliopoulos G, Martin-Hirsch P, et al. p16(INK4a) immunostaining in cytological and histological specimens from the uterine cervix: a systematic review and meta-analysis. Cancer Treat Rev. 2009;35(3):210-220.
12. Capobianco G, Marras V, Wenger JM, Santeufemia DA, Ambrosini G, Lutzoni R, et al. P16 immunostaining and HPV testing in histological specimens from the uterine cervix. Eur J Gynaecol Oncol. 2013;34(3):227-230.
13. Wentzensen N, von Knebel Doeberitz M. Biomarkers in cervical cancer screening. Dis Markers. 2007;23(4):315-330.
14. Murphy N, Ring M, Heffron CC, King B, Killalea AG, Hughes C, et al. p16INK4A, CDC6, and MCM5: predictive biomarkers in cervical preinvasive neoplasia and cervical cancer. J Clin Pathol. 2005;58(5):525–534.
15. Wang SS, Trunk M, Schiffman M, Herrero R, Sherman ME, Burk RD, et al. Validation of p16INK4a as a marker of oncogenic human papillomavirus infection in cervical biopsies from a population based cohort in Costa Rica. Cancer Epidemiol Biomarkers Prev. 2004;13(8):1355–1360.
16. Carozzi F, Confortini M, Dalla Palma P, Del Mistro A, Gillio-Tos A, De Marco L, et al. Use of p16-INK4A overexpression to increase the specificity of human papillomavirus testing: a nested substudy of the NTCC randomised controlled trial. Lancet Oncol. 2008;9(10):937–945.

17. Samarawardana P, Singh M, Shroyer KR. Dual stain immunohistochemical localization of p16INK4A and ki-67: a synergistic approach to identify clinically significant cervical mucosal lesions. Appl Immunohistochem Mol Morphol. 2011;19(6):514-518.
18. van Doorn LJ, Molijn A, Kleter B, Quint W, Colau B. Highly effective detection of human papillomavirus 16 and 18 DNA by a testing algorithm combining broad-spectrum and type-specific PCR. J Clin Microbiol. 2006;44(9):3292-3298.
19. de Sanjose S, Quint WG, Alemany L, Geraets DT, Klaustermeier JE, Lloveras B, et al. Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. Lancet Oncol. 2010;11(11):1048-1056.
20. Mehlhorn G, Obermann E, Negri G, Bubendorf L, Mian C, Koch M, et al. HPV L1 detection discriminates cervical precancer from transient HPV infection: a prospective international multicenter study. Mod Pathol. 2013;26(7):967-974.
21. Nam EJ, Kim JW, Hong JW, Jang HS, Lee SY, Jang SY, et al. Expression of the p16INK4a and Ki-67 in relation to the grade of cervical intraepithelial neoplasia and high-risk human papillomavirus infection. J Gynecol Oncol. 2008;19(3):162-168.
22. Agoff SN, Lin P, Morihara J, Mao C, Kiviat NB, Koutsky LA. p16(INK4a) expression correlates with degree of cervical neoplasia: a comparison with Ki-67 expression and detection of high-risk HPV types. Mod Pathol. 2003;16(7):665-673.
23. Ungureanu C, Teleman S, Socolov D, Anton G, Mihailovici MS. Evaluation of p16INK4a and Ki-67 proteins expression in cervical intraepithelial neoplasia and their correlation with HPV-HR infection. Rev Med Chir Soc Med Nat Iasi. 2010;114(3):823-828.
24. Cheah PL1, Looi LM, Teoh KH, Mun KS, Nazarina AR. p16(INK4a) is a useful marker of human papillomavirus integration allowing risk stratification for cervical malignancies. Asian Pac J Cancer Prev. 2012;13(2):469-472.

25. Hariri J, Oster A. The negative predictive value of p16INK4a to assess the outcome of cervical intraepithelial neoplasia 1 in the uterine cervix. Int J Gynecol Pathol. 2007;26(3):223-228.
26. Pacchiarotti A1, Ferrari F, Bellardini P, Chini F, Collina G, Dalla Palma P, et al. Prognostic value of p16-INK4A protein in women with negative or CIN1 histology result: a follow-up study. Int J Cancer. 2014;134(4):897-904.
27. Kruse AJ, Baak JP, de Bruin PC, van de Goot FR, Kurten N. Relationship between the presence of oncogenic HPV DNA assessed by polymerase chain reaction and Ki-67 immunoquantitative features in cervical intraepithelial neoplasia. J Pathol. 2001;195(5):557-562.
28. Gatta LB, Berenzi A, Balzarini P, Dessy E, Angiero F, Alessandri G, et al. Diagnostic implications of L1, p16, and Ki-67 proteins and HPV DNA in low-grade cervical intraepithelial neoplasia. Int J Gynecol Pathol. 2011;30(6):597-604.
29. Kruse AJ, Baak JP, de Bruin PC, Jiwa M, Snijders WP, Boodt PJ, et al. Ki-67 immunoquantitation in cervical intraepithelial neoplasia (CIN): a sensitive marker for grading. J Pathol. 2001;193(1):48-54.
30. Crum CP, Rose P. Cervical squamous neoplasia. In: Crum CP, Lee KR, editors. Diagnostic Gynecologic and Obstetric Pathology. Elsevier; 2006. p. 267-354.
31. Singh M, Mockler D, Akalin A, Burke S, Shroyer A, Shroyer KR. Immunocytochemical colocalization of P16(INK4a) and Ki-67 predicts CIN2/3 and AIS/adenocarcinoma. Cancer Cytopathol. 2012;120(1):26-34.
32. Keating JT, Cviko A, Riethdorf S, Riethdorf L, Quade BJ, Sun D, et al. Ki-67, cyclin E, and p16INK4 are complimentary surrogate biomarkers for human papilloma virus-related cervical neoplasia. Am J Surg Pathol. 2001;25(7):884-891.
33. van Bogaert LJ. P16INK4a immunocytochemistry / immunohistochemistry: need for scoring uniformization to be clinically useful in gynecological pathology. Ann Diagn Pathol. 2012;16(5):422-426.

34. Van Aardt MC, Dreyer G, Richter KL, Becker P. Human papillomavirus-type distribution in South African women without cytological abnormalities. South Afr J Gynaecol Oncol. 2013;5(2):S21-S27

35. Callegari ET, Tabrizi SN, Pyman J, Saville M, Cornall AM, Brotherton JM, et al. How best to interpret mixed human papillomavirus genotypes in high-grade cervical intraepithelial neoplasia lesions. Vaccine. 2014;32(32):4082-4088.

36. Quint W, Jenkins D, Molijn A, Struijk L, van de Sandt M, Doorbar J, et al. One virus, one lesion - individual components of CIN lesions contain a specific HPV type. J Pathol. 2012;227(1):62-71.

37. van der Marel J, Berkhof J, Ordi J, Torné A, Del Pino M, van Baars R, et al. Attributing oncogenic human papillomavirus genotypes to high-grade cervical neoplasia: which type causes the lesion? Am J Surg Pathol. 2015;39(4):496-504.

CHAPTER 5

HPV TYPE DISTRIBUTION IN WOMEN WITH INVASIVE CERVICAL CANCER AND THE EFFECT OF HIV-INDUCED IMMUNE DYSFUNCTION

5.1. INTRODUCTION

5.1.1. Impact of cervical cancer

Among all South African women, cervical cancer is the second most common malignancy and the most common cancer in women between the ages of 15 and 44 years.[1] In South Africa, it is estimated that annually 7735 women are newly diagnosed with cervical cancer and 4248 women die as a result of it.[1] The age-standardized annual incidence rate of cervical cancer in sub-Saharan Africa and South Africa ranges between 31.0 and 40.3 per 100,000 women.[1-4] Globally, the mortality is as high as 50% and is attributed to poor screening processes, a delay in seeking medical attention and advanced disease at the time of diagnosis. This is especially true for countries with a high incidence of human immunodeficiency virus (HIV) infection.[5,6]

Cervical cancer has a huge impact on society, because death often occurs at a young age while women are raising families.[7] In sub-Saharan Africa, because of other important health issues like HIV/AIDS, malaria and tuberculosis taking precedence, matters related to cervical cancer have been neglected.[3] Contributing to this is a shortage of epidemiological data, limited financial and human resources and lacking cancer services and screening policies.[3] Unless current preventive policies change, an estimated 140,000 women will be newly diagnosed with cervical cancer by 2030. At least 95,000 women will die as a direct result.[8]

5.1.2. Human papillomavirus (HPV) and cervical cancer

Although infections with one or more HPV types occur in approximately 80% of sexually active adults, only a small percentage of infected women will develop cervical cancer.[9-11] Cervical neoplasia should be regarded as the end result of biological, genetic, immunological and/or environmental co-factors in women infected with high-risk HPV (HrHPV).[12]

Cancers associated with persistent HPV infections occur primarily at the transformation zones where one epithelial type changes into another. This mainly occurs at the cervix, anus and oropharynx. The cervical transformation zone is not static; as glandular epithelium is replaced by squamous epithelium it shifts towards the endocervical canal.[7]

The development of cervical cancer is usually a product of HPV transmission, a persistent infection, development of pre-malignant lesions following progression of a clone of persistently infected cells. Eventually, an invasive disease.[7] This malignant transformation, as discussed previously, is facilitated through HPV oncoproteins, E6 and E7, and their effect on p53 and retinoblastoma protein pathways.[13]

5.1.3. Oncogenic potential of HPV types

Papillomaviruses are categorised into 16 different genera, with the majority of HPV types classified as either alpha or beta genus. Alpha genus consists mainly of genital or mucosal HPV whereas HPV from the beta genus is primarily responsible for inconsistent skin infections.[14] In humans, HPV types associated with infection of the lower genital tract stem from five species; namely alpha-5, -6, -7, -9 and -10, with alpha-7 (HPV types 18, 39, 45, 59, 68, 70) and alpha-9 (HPV types 16, 31, 33, 35, 52, 58, 67) predominating.[15]

HPV can be categorized as either low-risk HPV (LrHPV) or high-risk HPV (Hr-HPV) types. This classification is based on epidemiological studies on the molecular and functional ability of specific HPV types' oncogenic potential.[16] Infection with one or more HrHPV types is the major trigger in the etiopathogenesis of cervical cancer, with HPV 16 and 18 found in roughly 70% of cases.[17] Universally HPV 16 and 18 are the subtypes most frequently found in invasive cervical cancer. However, cancer of the cervix is also associated with no less than 16 other types, with HPV 45, 31, 33, 58 and 52 being the most prevalent of these.[18]

5.1.4. Human immunodeficiency virus (HIV) and cervical cancer

In 2007, it was reported that around 67% of the estimated 33 million people around the globe infected with HIV reside in sub-Saharan Africa, and around three-quarters of AIDS-related deaths occur in the region.[19] Secondary to an HIV-mediated increase in persistent and recurrent HPV infections, women infected with HIV have a greater chance of developing cervical neoplasia.[20,21]

Comparing women in the general population with HIV-infected women, HIV-infected women may have more than a 20-fold increased risk for developing invasive cervical cancer.[22] Compared with other HrHPV types, HPV 16 infections and associated premalignant cervical changes appear to be less reliant on the immune status of a woman.[23] A meta-analysis found HPV 16 less frequently in HIV-infected women with normal Papanicolaou smears – low-grade as well as high-grade cervical disease – compared with non-HIV-infected women.[24]

5.1.5. Motivation for the study

Although HPV types associated with invasive cervical cancer are known for many countries and regions, there still remains a lack of knowledge of HPV type distribution in many areas worldwide. Especially in developing countries, the question of which HPV types are prevalent among women with invasive cervical cancer is still largely unanswered.[25]

Knowledge on the effect of HIV co-infection on the prevalence of specific HPV types is also clearly missing.[22,26,27] This is especially true for Africa, burdened by both HIV and cervical cancer. Although some studies have addressed the distribution of HPV types in HIV co-infected women with cervical cancer, similar studies, especially in Africa and South Africa, are needed. [22,26,27]

It is crucial to expand on current knowledge and better understand HPV type distribution among women with cervical cancer and to determine similarities and differences between our population, other geographical regions within

South Africa and the rest of the world. It is important in order to evaluate current and future vaccine efficacy and to guide future vaccine development and screening guidelines.[27]

5.2. OBJECTIVES AND HYPOTHESES

5.2.1. Objectives

The objectives of this study were to:

- Determine which HrHPV types are prevalent in women with cervical cancer in our population;
- Compare the HrHPV types associated with cervical cancer in HIV-infected women with non-HIV-infected women; and
- Compare findings from our population with data from Africa and other continents to determine possible similarities and differences.

5.2.2. Hypotheses

The hypotheses of this study were that:

- The distribution of the most prevalent HPV types in women with invasive cervical cancer would differ from other geographical regions.
- HrHPV types other than HPV 16 and 18 would be more prevalent in our population, especially among HIV-infected women.
- HPV types in our population associated with, and found in, cervical cancer would differ between women infected with HIV versus women not infected with HIV.

5.3. MATERIALS AND METHODS

5.3.1. Study design

This retrospective descriptive study was performed at the gynecologic oncology unit, University of Pretoria. It consisted of data obtained during 2 study periods. The first study period started in January 2003 and ended in December 2004. The second collection period was initiated in 2008 and lasted until July 2011. Patients included in the study were women 18 years or older, referred for staging and treatment of histologically confirmed invasive cervical cancer.

5.3.2. Consent process and ethical considerations

Patients received counseling and an information document that explained the method and voluntary nature of the study. During counseling by trained nursing personnel, patients were motivated to undergo HIV testing as per standard departmental management protocols, but it was explained clearly that testing was voluntary. Written informed consent and standard management protocols were the same for both study periods. This study was approved by the Research Ethics Committee of the Faculty of Health Sciences of the University of Pretoria (27/2008, 108/2008, 189/2012).

5.3.3. Patient recruitment

One-hundred-and-twelve consecutive women were invited to participate in the study during 2003 and 2004. Of these women 106 patients fulfilled the inclusion criteria. Starting in 2008, another 201 consecutive patients were recruited, of whom 193 patients were included in the study. Women finally included with proven invasive cervical cancer were 299. Fourteen patients were excluded, of whom 9 patients had endometrial cancers, 3 had cervical intraepithelial neoplasia grade III, 1 had carcinosarcoma, and 1 had only severe cervicitis on the final histological diagnoses. Patients excluded were treated according to standard departmental treatment protocols for the specific disease diagnosed.

5.3.4. Sample collection and transport

Tissue biopsies for histological confirmation of the diagnosis were taken in the out-patient clinic with punch biopsy forceps from each cervical tumor and placed in buffered formalin. The samples were transported to the Department of Anatomical Pathology at the University of Pretoria, where histological examination was performed.

During the initial study period, DNA sampling was performed using tampons and transported in phosphate-buffered saline and 10% methanol solution. During the second study period, HPV DNA analysis was performed on cervical tumor tissue preserved in a methanol-based buffer solution (PreTect [NorChip AS, Norway]).

5.3.5. HPV DNA testing

DNA extraction was accomplished during the first study period by means of the DNA Isolation Kit (Roche Molecular Systems, Branchburg, NJ) on the MagNa Pure automated extraction system. Human papillomavirus linear array genotyping kit (Roche Molecular Systems) was used to determine the HPV type. MagNa Pure extractions and linear array genotyping were performed at the University of Pretoria. Fifteen high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), 3 probable high-risk types (HPV 26, 53, and 66), and 19 low-/undetermined-risk types (HPV 6, 11, 40, 42, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, IS39, and CP6108) were tested for.[28]

NucliSENS manual extraction kit (bioMerieux, Marcy l'Etoile, France) was used for isolation of nucleic acid during the second study period. Human papillomavirus DNA analysis, testing for 39 individual HPV types (ie, HPV 6, 11, 16, 18, 26, 30, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82/ MM4, 82/IS39, and CP6108) and 6 rare HPV types (ie, HPV 32, 83, 84, 85, 86, and JC9710) as a pool, was performed on GP5+/6+ polymerase chain reaction products using reverse line

blot assay. Polymerase chain reaction toward the A-globin gene was included as DNA control for all HPV-negative samples.[29,30] NucliSens extraction, GP5+/6+ polymerase chain reaction and RLB genotyping were performed at NorChip AS, Norway.

5.3.6. Data capturing and analysis

Data were captured on Microsoft Excel datasheets, and analysis performed using Stata statistical software (StataCorp, College Station, TX). The distribution of HPV types was expressed in terms of frequencies, percentages, and 95% confidence intervals and displayed in table form and bar charts. The HIV groups were compared with respect to proportion of HPV infections using Fisher exact test at the 0.05 level of significance. The risk of HPV infection associated with HIV status was determined from the crude odds ratio along with its 95% confidence interval.

5.4. RESULTS

5.4.1. Age distribution and HIV status

Among the 299 patients included in the total study population, 154 (51.51%) were non-HIV infected, 77 (25.75%) were HIV infected and for 68 patients (22.74%) the HIV status was not known. Although the patients in the different study periods were similar, there were more HIV-positive patients in the second study period and more multiple-type infections in the first study period (Table 5.1).

The ages of women ranged from 23 to 89 years, with the largest group between the ages of 50 and 59 years. The mean age for the total study population was 50.7 years. The mean ages for non-HIV-infected women were 55.8 (SD, 12.5) years and 41.4 (SD, 11.4) years for women infected with HIV. The HIV-infected women were significantly younger than non-HIV-infected women (P for mean

age < 0.0001). Figure 5.1 illustrates the age distribution and HIV prevalence for the total study population.

Table 5.1: Comparison between patients included in study

		Study period 1	Study period 2	Total
Number of patients		106	193	299
Mean age		53.0	49.4	50.7
HIV	Unknown	31 (29.24%)	37 (19.17%)	68 (22.74%)
	Negative	58 (54.72%)	96 (49.74%)	154 (51.51%)
	Positive	17 (16.04%)	60 (31.08%)	77 (25.75%)
Histology	Squamous	102 (96.22%)	175 (90.67%)	277 (92.64%)
	Non-Squamous	4 (3.78%)	18 (9.33%)	22 (7.36%)
HIV neg	hrHPV neg	15.52%	12.5%	13.64%
	Single hrHPV	65.52%	85.41%	77.92%
	Multiple hrHPV	18.96%	2.09%	8.44%
HIV pos	hrHPV neg	11.76%	13.33%	12.99%
	Single hrHPV	41.18%	65.0%	59.74%
	Multiple hrHPV	47.06%	21.67%	27.27%

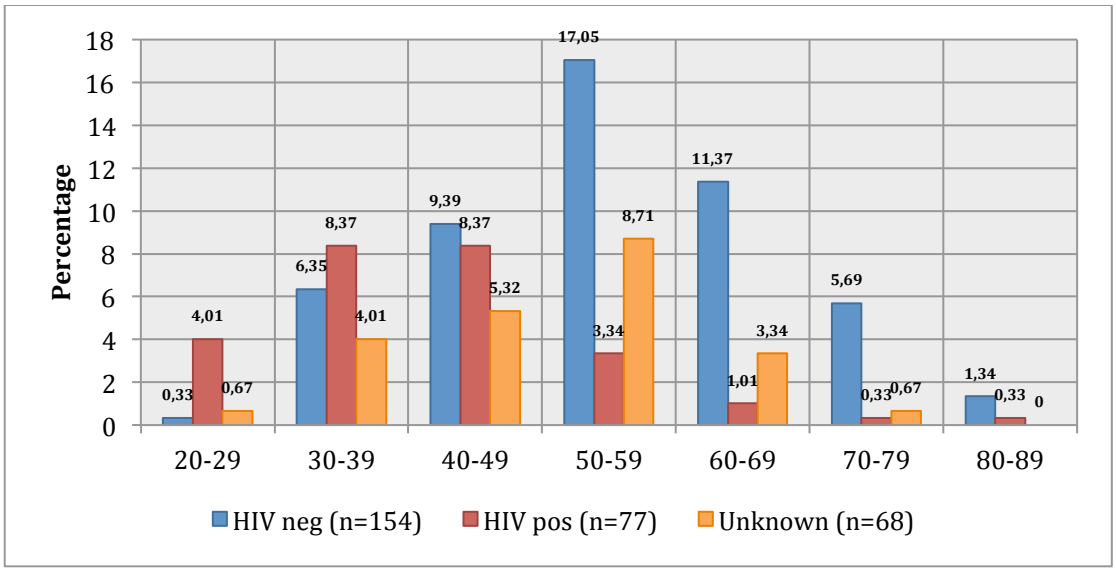


Figure 5.1: Age distribution and HIV prevalence for the study population

5.4.2. Histological distribution

The majority of patients had squamous cell carcinoma (277/299, 92.64%). Twenty-two patients (7.36%) had non-squamous cervical cancer. This number included 9 patients with adenocarcinoma, 10 patients with adenosquamous, and 3 patients with small cell neuroendocrine carcinoma. Of the 22 patients with non-squamous cervical cancer, 3 patients were HIV infected, 11 were non-HIV infected, and the remainder unknown.

5.4.3. HPV prevalence

Human papillomavirus DNA was demonstrated in 91.71% of all study samples. The prevalence of confirmed HPV infection was 90.91% for both non-HIV-infected- and HIV-infected women and 94.1% among women with unknown HIV status.

5.4.4. Single and multiple HrHPV type infections

In total, 264 (88.29%) of 299 tumors tested positive for HrHPV, and 194 (64.88%) of 299 were positive for a single HrHPV type. These 2 groups form the basis for describing the HPV-type contribution in this study (Table 5.2).

Table 5.2: Summary of HrHPV type infections amongst total population, single HrHPV type infections and distribution amongst HIV-infected- and -non-infected patients

HrHPV type distribution for total population						HrHPV type distribution for single type infections				
HPV type	HIV neg (n=154)	HIV pos (n=77)	p-value Fisher exact	Total (n=299)	Total (%)	HIV neg (n=106)	HIV pos (n=46)	p-value Fisher exact	Total (n=197)	Total (%)
16	74	32	0.635	136	45.5	55	21	0.597	97	49.2
18	22	18	0.212	53	17.7	16	9	0.485	32	16.2
31	5	2	0.463	7	2.3	4	1	1.000	5	2.6
33	8	8	0.158	18	6.0	6	1	0.676	8	4.1
35	12	7	0.798	26	8.7	10	3	0.755	18	9.1
39	1	1	0.794	3	1.0	0	0	-	0	0
45	9	13	**0.015***	32	10.7	5	7	**0.045***	19	9.6
51	4	2	1.000	7	2.3	0	1	1.000	1	0.5
52	8	2	0.413	15	5.0	5	1	0.668	7	3.6
56	1	0	0.192	3	1.0	1	0	1.000	1	0.5
58	5	8	0.082	16	5.4	2	0	1.000	5	2.6
59	0	1	0.234	2	0.7	0	0	-	0	0
68	2	0	0.264	4	1.3	0	0	-	0	0
73	1	1	0.105	5	1.7	0	1	1.000	1	0.5
82	2	1	1.000	3	1.0	0	0	-	0	0
Non-hrHPV	36	32	-	90	30.1	2	1	-	3	1.5
***Statistically significantly more prevalent amongst HIV-infected women**										

The prevalence of HrHPV infections was 86.4% among HIV-negative women and 87.0% among the HIV-positive group. The HIV-infected women had significantly more multiple HrHPV type infections (P = 0.001). Figure 5.2 illustrates the difference between HIV-positive- and HIV-negative women regarding the number of HrHPV types present in women with invasive cervical cancer. There were more multiple HrHPV type infections during the first study period. This is likely to be the result of different HPV testing methods. However, HIV-positive

patients had significantly more multiple HrHPV type infections in both the first (P = 0.049) and second (P = 0.0001) study periods.

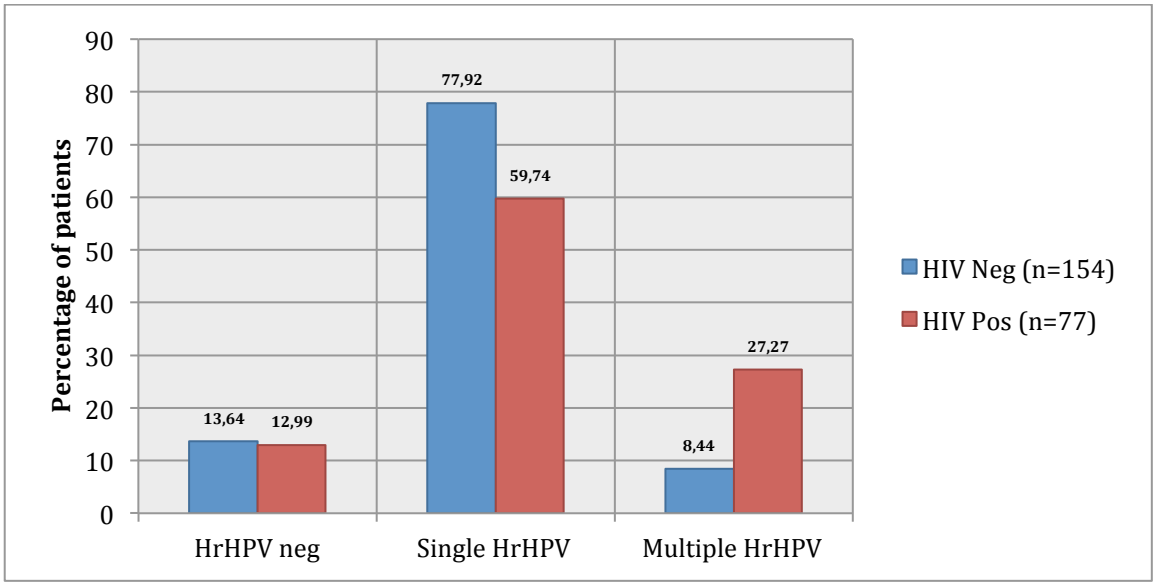


Figure 5.2: Absent-, single- and multiple HrHPV type infections in HIV-positive- and -negative patients (p=0.001)

5.4.5. Distribution of HrHPV types in order of prevalence for the total study population

Including women with single and multiple HPV-type infections, the most common HrHPV-type infection was HPV 16, followed by HPV 18, 45, 35, and 33. In the non-HIV-infected group in order of decreasing frequency the 5 most common HrHPV types were HPV 16, 18, 35, 45, and 33/52 (equally fifth most prevalent).

The most prevalent HrHPV type infections were slightly different in the HIV-infected group, with HPV 16 infections the most common, followed by HPV 18, 45, 33, and 58. Compared with HIV-negative women, a higher percentage of HIV-positive women were infected with HPV 45 (P = 0.015). Table 5.2 tabulates the prevalence of different HrHPV type infections among the total study population and women with single HrHPV type infections. Table 5.2 also shows the distribution among HIV-infected- and non-HIV-infected women. Figure 5.3

illustrates the distribution of HrHPV types and the difference between HIV-infected- and –non-infected patients.

The odds of specific HPV high-risk type infection when women were also infected with HIV were increased for HPV 33, 35, 45, 51, 58, 73 and 82. HPV 45 infection was significantly linked to HIV-infected women (OR 3.07, 95% CI 1.07 – 8.77). See Table 5.3.

Table 5.3: Odds ratio for HPV type infection if HIV positive

HPV type	Odds ratio	95% Confidence interval
16	0.89	0.47 – 1.67
18	0.97	0.43 – 2.21
31	0.95	0.14 – 6.21
33	2.80	0.85 – 9.22
35	1.10	0.36 – 3.40
39	0.79	0.31 – 19.93
45	3.07	1.07 – 8.77
51	1.79	0.26 – 12.29
58	1.45	0.38 – 5.54
73	1.44	0.05 – 37.89
82	3.42	0.25 – 47.76

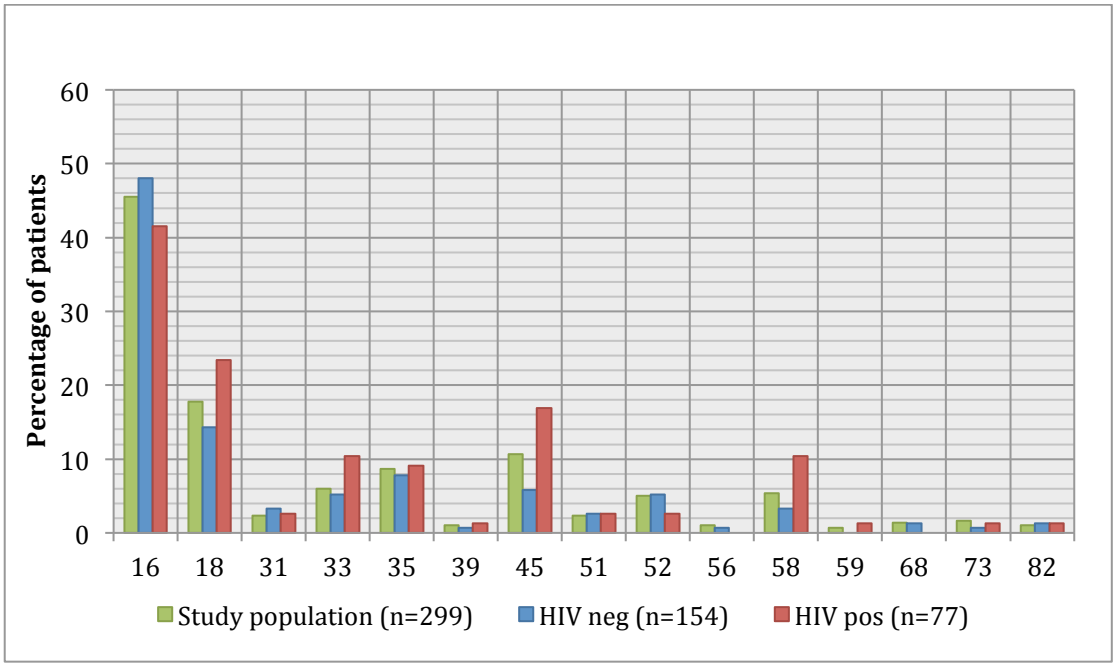


Figure 5.3: Distribution of HrHPV types and the difference between HIV-infected- and -non-infected patients

5.4.6. Distribution of non-HrHPV types

The most common probable HrHPV (PrHrHPV) type observed was HPV 53 and the most common LrHPV types were HPV 61, HPV 70, HPV 71 and HPV 81. The number of infections with HPV 6 and HPV 11 were very low.

Three patients were infected with a single LrHPV. These LrHPV types were HPV 69, 70 and 81. Figure 5.5 illustrates the distribution of non-HrHPV types and the difference between HIV-infected- and -non-infected patients. HPV 26, -40, -64, -67 and –IS39 were absent in all patients and not included in Figure 5.4.

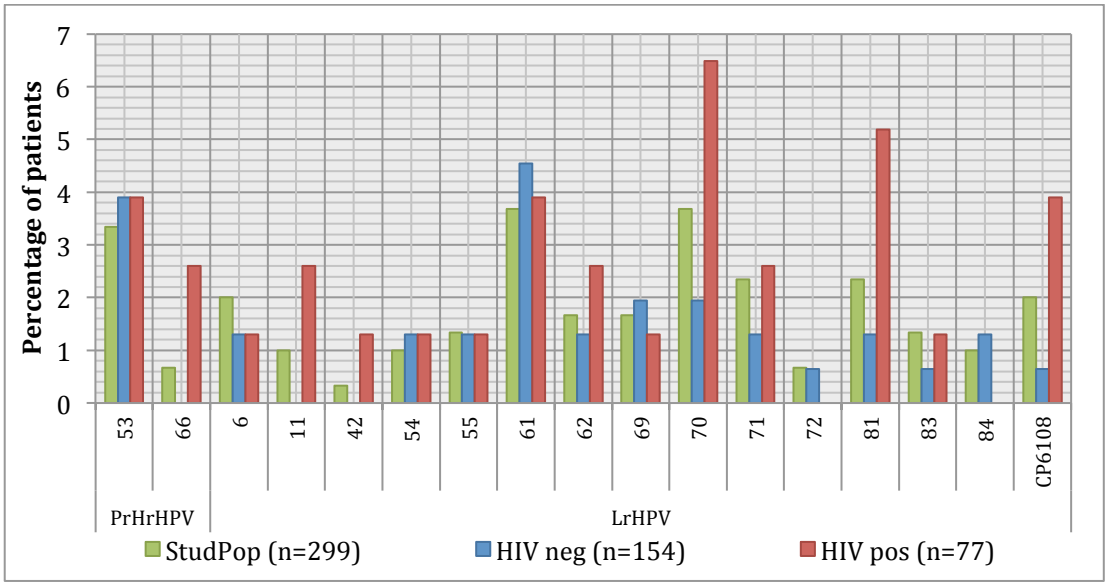


Figure 5.4: Distribution of all non-high-risk HPV types identified in all patients and the difference between HIV-infected- and -non-infected patients

5.4.7. Single HPV type infections

A single HPV infection was present in 197 patients (65.89%) and is illustrated in Table 5.2. The most prevalent single HPV types were in decreasing order HPV 16, 18, 45, 35, and 33. The distribution of single HPV type infections among HIV-infected- and non-HIV-infected women is illustrated in Figure 5.5. The prevalence of HPV 45 was more than 3 times higher in HIV-positive women with single HPV-type infections compared with HIV-negative women (P = 0.045).

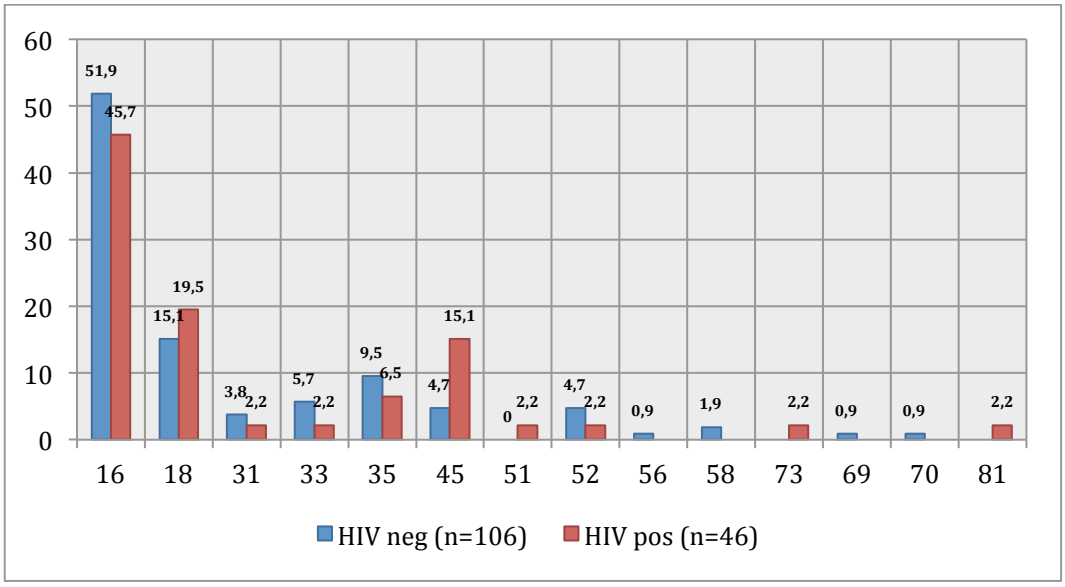


Figure 5.5: Single HPV type infections in all women and HIV-infected- and -non-infected women

Figure 5.6 illustrates the HrHPV type distribution for all women with HrHPV positive tumors (264/299; 88.29%) and for women with single HrHPV type positive tumors (194/299; 64.88%). To consider HPV types as oncogenic when present as part of multiple infections in patients with invasive cervical cancer seems inaccurate. This consideration probably overestimates the oncogenic potential and contribution to cancer cases. Single HrHPV type infection may be more important when wanting to prove oncogenicity of the specific virus. However, patients with invasive cervical cancer may still have acquired transient infections after they had developed invasive cancer.

Comparing single HrHPV type infections with all HrHPV type infections, it appears as if the prevalence remains similar for HPV 16, 31, and 35 between the two groups and HPV 18 and 45 slightly more representative among women with single and multiple HrHPV type infection. The other HrHPV types are markedly more representative among women with one or more HrHPV type infections. This finding is likely the result of different methods having been used to detect HPV DNA, with more multiple-type infections among tampon collections compared with biopsy samples. However, HPV DNA methods may detect transient HPV infection not associated with the disease.

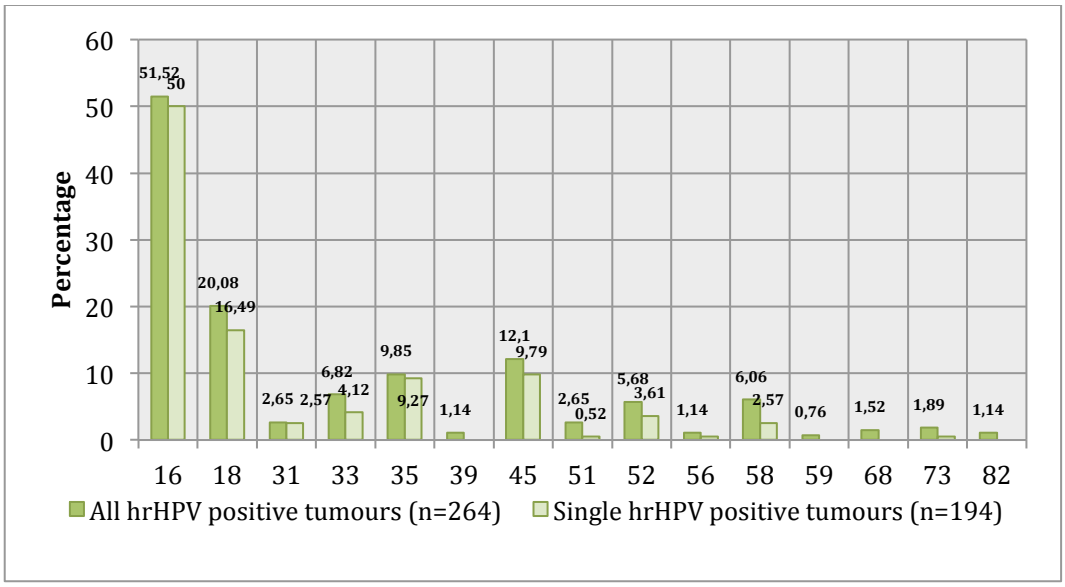


Figure 5.6: Type distribution among all HrHPV-positive tumors and single HPV-positive tumors

5.4.8. Most probable single oncogenic HPV type identified

As noted in previous chapters, the eight most common types of HPV identified in 89% of cervical cancer cases globally, in descending order of frequency, are HPV 16, 18, 45, 31, 33, 52, 58 and 35.[31] If a patient had multiple HrHPV types – for example, HPV 16, 31, 52 and 82, only the presumed most oncogenic virus (for example, HPV 16) was recorded.

In all three groups illustrated in Figure 5.7 the two most prevalent HPV types were the same as reported. However, there were some differences noted in the different groups. Among all women, HPV 31, ranked fourth worldwide, was the eighth most prevalent and HPV 35, ranked eighth worldwide, was fourth. Among HIV-negative women, HPV 35 ranked third, above HPV 45, and fourth among HIV-positive women. Four (1.34%) patients had high-risk types other than the top eight mentioned above and 33 (11.04%) patients were HrHPV negative.

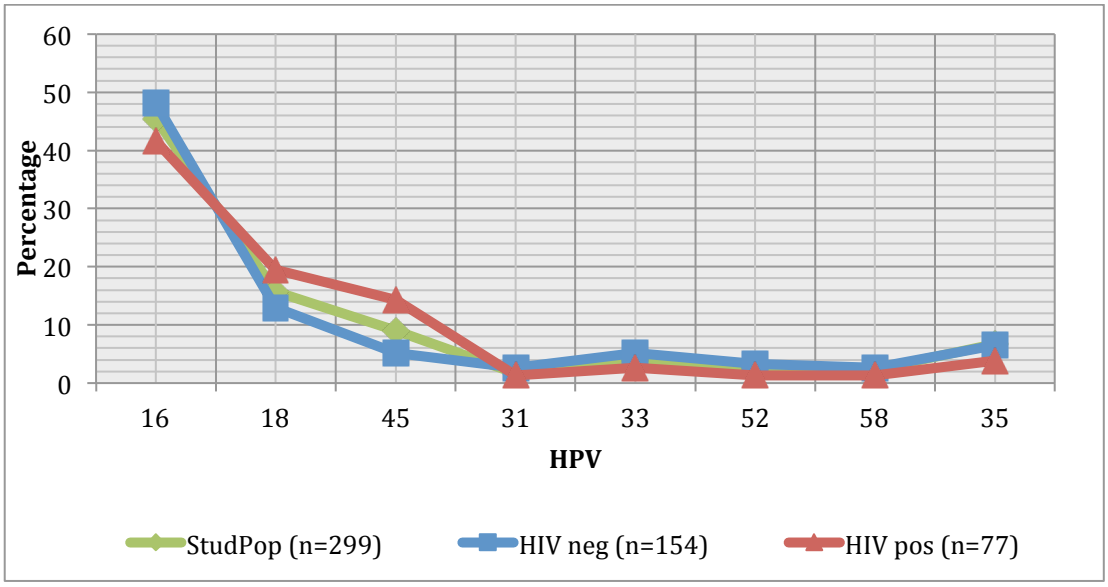


Figure 5.7: Most probable single oncogenic HPV type identified in all patients and HIV-infected and –non-infected patients

5.4.9. Phylogenetic distribution of HPV type infections

In women with single HPV type infections and most probable single HPV type infections, illustrated in Figure 5.8, infections with HPV alpha-9 species (HPV types 16, 31, 33, 35, 52, 58, 67) were most common. These HPV types were responsible for around 70% of infections, followed by infections with alpha-7 species (HPV types 18, 39, 45, 59, 68, 70) in around 27% of infections. In both groups, very few patients had infections with alpha-5, alpha-6 and other alpha species.

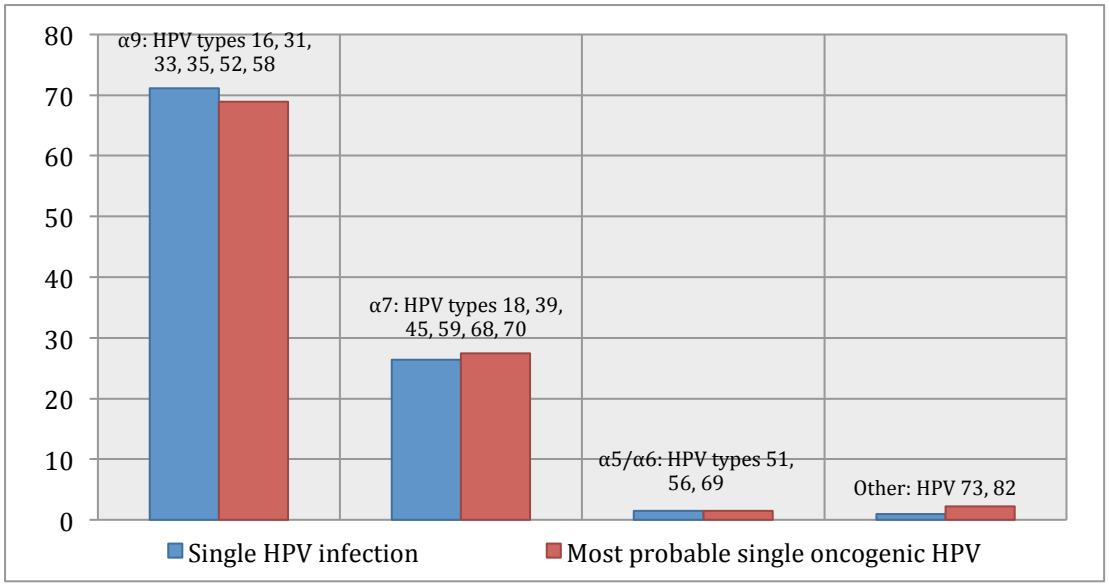


Figure 5.8: Phylogenetic distribution of HPV type infections of single type infections and most probable single oncogenic type infection

5.4.10. Histological subtypes and HrHPV type distribution

The most striking difference is that HPV 18 (54.55%) was most prevalent among women with non-squamous cell cervical cancer, followed by HPV 16 (13.64%). Among women with squamous cell cancer, HPV 16 (48.01%) was most prevalent followed by HPV 18 (14.8%). Despite a relatively small number of patients with non-squamous cell cervical cancer, there was a statistically significant difference in relation to the prevalence of HPV 16 ($P = 0.002$) and HPV 18 ($P < 0.001$) between the 2 groups. See Figure 5.9.

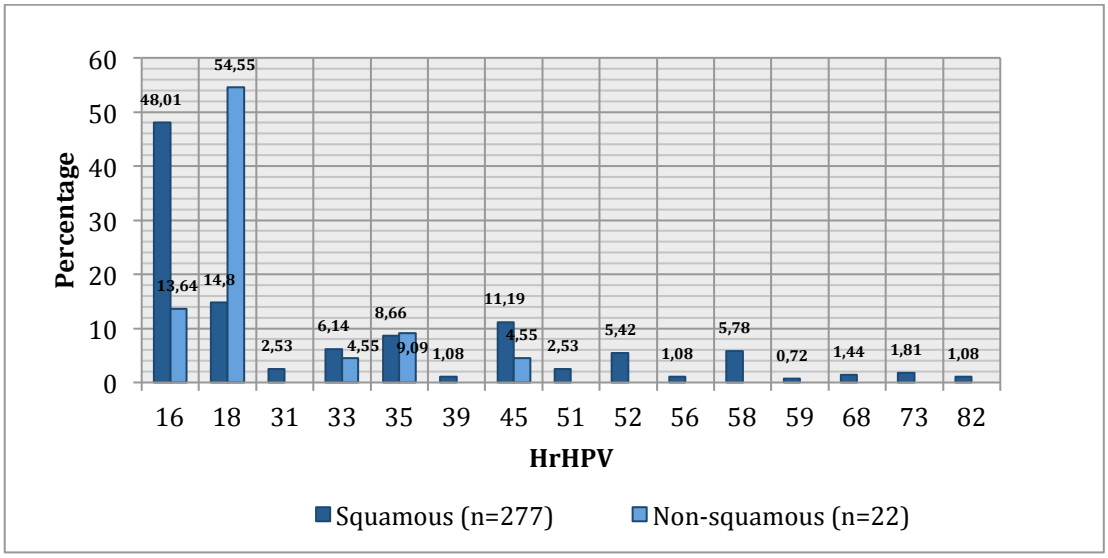


Figure 5.9: Distribution of HrHPV types in squamous and non-squamous cell cervical cancer

5.5. DISCUSSION

A growing number of studies focus on HPV prevalence in women with invasive cervical cancer in South and sub-Saharan Africa. This study adds valuable information toward understanding the interaction between HPV infections, HIV, and invasive cervical cancer in our population. To our knowledge, this is the largest study in our defined population to date.

5.5.1. Age distribution and HIV status

In this study, HIV-infected women with invasive cervical cancer were on average 14 years younger than non-HIV-infected women, which is comparable to previously reported South African data.[2] Eighty percent of women younger than 30 years were HIV positive. The prevalence of HIV infections was higher in this study (25.8%) than van Bogaert's (13.6%),[2] but similar to the South African sub-group (27.6%) reported by Denny et al.[4] The multicentre study by Denny et al.[4] included 570 women with histologically confirmed invasive cervical cancer from Ghana, Nigeria and South Africa. Approximately 83% of patients had squamous cell carcinoma.

The prevalence of HPV infections among women with invasive cervical cancer was similar to the reported prevalence of 90% from the meta-analysis by Li et al.[32] In contrast to other reports on HIV-positive women,[4,19] the prevalence of HPV infections in this study was the same (90.9%) for both HIV-infected and non-HIV-infected women. However, HIV-infected women had significantly more multiple HrHPV types compared with non-HIV-infected women (P = 0.001). The number of women with multiple HPV-type infections, especially among HIV-infected women, is more than the previously reported data from South Africa and Kenya for all HPV-type infections and considerably higher than that reported for Europe.[10,11,22] This might be because of tampon sampling.

5.5.2. All HPV type infections

Human papillomavirus types 16 and/or 18 were present in 63.21% of the entire population, but only 61.2% had either HPV 16 or 18. The prevalence of either HPV 16 or 18 is considerably lower than the globally reported 73%, but similar to South African data reported by Bruni et al.[1,32] Human papillomavirus types 16 and/or 18 were present in 69.9% of women, with single and multiple types reported by Denny et al.[4] and 69.2% reported by Louie et al.[3] for sub-Saharan African women with invasive cervical cancer. In this study, no significant difference was found between HIV-infected, non-HIV-infected, and HIV-unknown patients with regard to HPV 16 (P = 0.635) or HPV 18 (P = 0.212) infections. These findings are similar to those from Mozambique, South Africa, and Kenya.[22,26]

De Vuyst et al. also showed little difference in the prevalence of HPV 16 in cervical cancer specimens between HIV-positive and HIV-negative women.[17] These findings and findings from the current study reestablish confidence in possible effects of current HPV 16/18 vaccines on women infected with HIV.[22]

Comparing the most prevalent single and multiple HPV-type infections to the meta-analysis by Li et al.[32] (HPV 16, 18, 58, 33, 45, 31, 52, 35), HPV 45, 35, and

33 were more prevalent than HPV 58, reported as the third most common globally. However, the HPV type sequence of the current study compares better with the African sub-group in the meta-analysis. The top five HPV types are identical, except that HPV 33 and 35 are swopped around and that HPV 58 did not feature among the top eight most prevalent viruses.[32] The order of the five most prevalent HPV types in this study was exactly the same as reported by Denny et al.[4] The higher prevalence of HPV 35 does not appear to be related to HIV co-infection and is most likely a regional difference.

The odds ratio for a HPV 45 infection was triple for HIV-positive- compared with HIV-negative women (odds ratio, 3.07; 95% confidence interval, 1.07-8.77). In contrast to findings published by de Vuyst et al.,[22] HIV-positive patients in this study had three times higher infection rates for HPV 45 compared with HIV-negative women (P = 0.015). Human papillomavirus type 45 is therefore not only important among South African women but especially in HIV-infected South African women, who are currently not directly covered by HPV vaccines but only by some cross-protection.

5.5.3. Single HPV type infections

In women infected with a single HPV type, the sequence of the most common types were similar to the findings from a large European study,[11] except HPV 33 and 31 that were fourth and fifth most common. In sub-Saharan women, the most prevalent single HPV type infections were reported as HPV 16, 18, 35, 45, 33, and 52.[4] The percentage of women infected with HPV 16 or 18, however, was similar to findings by Denny et al.[4] Women in this study, co-infected with HIV and a single HPV type, had significantly more HPV 45 infections compared with non-HIV-infected women. The importance of HPV 45 infections in Africa was also highlighted by Ndiaye et al.[8] Current bivalent HPV vaccine demonstrated significant cross-protection against HPV 45 in clinical trials, which might be particularly important in this study's population.[13]

5.5.4. Phylogenetic distribution of HPV type infections

Around 75% of cervical cancers are attributed to HPV types classified as HPV alpha-9 species.[33] In this study, the majority (71.07%) of single HPV type infections belonged to the alpha-9 species group. Tjalma et al.[11] found that the prevalence of HPV types of the alpha-7 species is higher among invasive cervical cancer compared to high-grade pre-malignant lesions (CINII/III).[11] Wang et al.[34] found infection with HPV alpha-7 species to be a negative prognostic factor in patients with invasive cervical cancer undergoing primary radiotherapy.

5.5.5. Histological subtypes and HPV type distribution

In agreement with global data, HPV 16 infections in this study were significantly more prevalent in patients with squamous cell cancer (P = 0.002), whereas HPV 18 was the most prevalent HPV type among women with non-squamous cell carcinoma (P < 0.001).[11,32] Denny et al.[4] also found HPV 18 as most prevalent among women with adenocarcinoma in sub-Saharan Africa. Although HPV 45 is globally reported as the third most common HPV type infection in women with adenocarcinoma, in this study HPV 35 was more common among patients with non-squamous cell carcinoma. Because of the small sample size, the significance of this finding is questionable.

5.5.6. Limitations

Limitations of this study were the combination of 2 study populations and the absence of a standard method of testing samples for all women included. Although the two HPV tests may have had different specificity and cutoff values for single and multiple types, statistical analysis was not performed separately for the different HPV testing methods because of limited sample size. However, both study groups included highly sensitive methods to detect type-specific HPV DNA, and the main objective of this study was to compare HPV types in HIV-infected and non-HIV-infected patients and not the different HPV assays. Some

women did not have HIV results, which might have influenced findings. Although the center where the study was performed serves a large referral area, the study did not include patients from all regions of South Africa. The effect of CD4 cell count could not be evaluated because of lack of information. Lastly, because HIV-positive women were significantly younger than HIV-negative women, age may influence HPV prevalence.

5.6. CONCLUSION

Regardless of HIV status, HPV 16 and 18 were the most prevalent HrHPV types present among women with cervical cancer in this study. Disregarding cross-protection, current bivalent and quadrivalent HPV vaccines could directly prevent cervical cancer in 65% or more of women in this population. Human papillomavirus types 45 and 35 are important in the South African context and HPV 45 even more relevant among HIV-infected women. This study highlights the need for future vaccines to target HPV 45 and 35 in women infected with HIV. It is also important to take these findings into consideration when screening strategies for cervical cancer are being developed in our population, especially in HIV-infected women.

5.7. REFERENCES

1. Bruni L, Barrionuevo-Rosas L, Albero G, Aldea M, Serrano B, Valencia S, et al. ICO Information Centre on HPV and Cancer (HPV Information Centre). Human papillomavirus and related diseases in South Africa. Summary Report. Available at: http://www.hpvcentre.net/statistics/reports/XWX.pdf. Accessed March 17, 2014.
2. van Bogaert LJ. Age at diagnosis of preinvasive and invasive cervical neoplasia in South Africa: HIV-positive versus HIV-negative women. Int J Gynecol Cancer. 2011;21(2):363-366.
3. Louie KS, de Sanjose S, Mayaud P. Epidemiology and prevention of human papillomavirus and cervical cancer in sub-Saharan Africa: a comprehensive review. Trop Med Int Health. 2009;14(10):1287-1302.
4. Denny L, Adewole I, Anorlu R, Dreyer G, Moodley M, Smith T, et al. Human papillomavirus prevalence and distribution in invasive cervical cancer in sub-Saharan Africa. Int J Cancer. 2014;134:1389-1398.
5. South African Human Papillomavirus Advisory Board. Cervical cancer and human papillomavirus: South African guidelines for screening and testing. South Afr J Gynaecol Oncol. 2010;2(1):23-26.
6. Bratcher J, Palefsky J. Anogenital human papillomavirus infection and associated neoplasia in HIV-positive men and women. The PRN Notebook [serial on the Internet]. 2008 Sept [cited 2012 Jul 27];13:1-8. Available from: http://www.prn.org.
7, Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S. Human papillomavirus and cervical cancer. Lancet. 2007;370(9590):890-907.
8. Ndiaye C, Alemany L, Ndiaye N, Kamaté B, Diop Y, Odida M, et al. Human papillomavirus distribution in invasive cervical carcinoma in sub-Saharan Africa: could HIV explain the differences? Trop Med Int Health. 2012;17(12):1432-1440.

9. Alsbeih G, Ahmed R, Al-Harbi N, Venturina LA, Tulbah A, Balaraj K. Prevalence and genotypes' distribution of human papillomavirus in invasive cervical cancer in Saudi Arabia. Gynecol Oncol. 2011;121(3):522-526.
10. Mariani L, Monfulleda N, Alemany L, Vizza E, Marandino F, Vocaturo A, et al. Human Papilloma Virus prevalence and type-specific relative contribution in invasive cervical cancer specimens from Italy. BMC Cancer. 2010;10:259.
11. Tjalma WA, Fiander A, Reich O, Powell N, Nowakowski AM, Kirschner B, et al. Differences in human papillomavirus type distribution in high-grade cervical intraepithelial neoplasia and invasive cervical cancer in Europe. Int J Cancer. 2013;132(4):854-867.
12. Kim J, Kim BK, Lee CH, Seo SS, Park SY, Roh JW. Human papillomavirus genotypes and cofactors causing cervical intraepithelial neoplasia and cervical cancer in Korean women. Int J Gynecol Cancer. 2012;22(9):1570-1576.
13. Tay SK. Cervical cancer in the human papillomavirus vaccination era. Curr Opin Obstet Gynecol. 2012;24(1):3-7.
14. Lazarczyk M, Cassonnet P, Pons C, Jacob Y, Favre M. The EVER Proteins as a natural barrier against papillomaviruses: A new insight into the pathogenesis of human papillomavirus infections. Microbiol Mol Biol Rev. 2009;73(2):348-370.
15. Carter JR, Ding Z, Rose BR. HPV infection and cervical disease: a review. Aust N Z J Obstet Gynaecol. 2011;51(2):103-108.
16. Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med. 2003;348(6):518-527.
17. De Vuyst H, Gichangi P, Estambale B, Njuguna E, Franceschi S, Temmerman M. Human papillomavirus types in women with invasive cervical carcinoma by HIV status in Kenya. Int J Cancer. 2008;122(1):244-246.
18. Denny L. Cervical cancer treatment in Africa. Curr Opin Oncol. 2011;23(5):469-474.

19. Pantanowitz L, Michelow P. Review of human immunodeficiency virus (HIV) and squamous lesions of the uterine cervix. Diagn Cytopathol. 2011;39(1):65-72.
20. Maiman M. Cervical neoplasia in women with HIV infection. Oncology. 1994;8(8):83-94.
21. Didelot-Rousseau MN, Nagot N, Costes-Martineau V, Vallès X, Ouedraogo A, Konate I, et al. Human papillomavirus genotype distribution and cervical squamous intraepithelial lesions among high-risk women with and without HIV-1 infection in Burkina Faso. Br J Cancer. 2006;95(3):355-362.
22. De Vuyst H, Ndirangu G, Moodley M, Tenet V, Estambale B, Meijer CJ, et al. Prevalence of human papillomavirus in women with invasive cervical carcinoma by HIV status in Kenya and South Africa. Int J Cancer. 2012;131(4):949-955.
23. Strickler HD, Palefsky JM, Shah KV, Anastos K, Klein RS, Minkoff H, et al. Human papillomavirus type 16 and immune status in human immunodeficiency virus-seropositive women. J Natl Cancer Inst. 2003;95:1062-1071.
24. Clifford GM, Gonçalves MA, Franceschi S, HPV and HIV Study Group. Human papillomavirus types among women infected with HIV: a meta-analysis. AIDS. 2006;20(18):2337-2344.
25. de Sanjose S, Quint WG, Alemany L, Geraets DT, Klaustermeier JE, Lloveras B, et al. Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. Lancet Oncol. 2010;11(11):1048-1056.
26. Naucler P, Mabota da Costa F, da Costa JL, Ljungberg O, Bugalho A, Dillner J. Human papillomavirus type-specific risk of cervical cancer in a population with high human immunodeficiency virus prevalence: case-control study. J Gen Virol. 2011;92(Pt 12):2784-2791.
27. Odida M, de Sanjosé S, Quint W, Bosch XF, Klaustermeier J, Weiderpass E. Human Papillomavirus type distribution in invasive cervical cancer in Uganda. BMC Infect Dis. 2008;8:85.

28. Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med. 2003;348:518-527.
29. Snijders PJ, van den Brule AJ, Jacobs MV, et al. HPV DNA detection and typing in cervical scrapes by general primer GP5+/6+ PCR. In: Davy CEDJE, Doorbar J, eds. Methods in Molecular Medicine: Human Papillomaviruses-Methods and Protocols. Totowa, Ontario, Canada: Humana Press; 2005:101-114.
30. van den Brule AJ, Pol R, Fransen-Daalmeijer N, Schouls LM, Meijer CJ, Snijders PJ. GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. J Clin Microbiol. 2002;40:779-787.
31. Clifford G, Franceschi S, Diaz M, Muñoz N, Villa LL. Chapter 3: HPV type-distribution in women with and without cervical neoplastic diseases. Vaccine 2006;S3/26-S3/34.
32. Li N, Franceschi S, Howell-Jones R, Snijders PJ, Clifford GM. Human papillomavirus type distribution in 30,848 invasive cervical cancers worldwide: Variation by geographical region, histological type and year of publication. Int J Cancer. 2011;128(4):927-935.
33. Burk RD, Chen Z, Harari A, Smith BC, Kocjan BJ, Maver PJ, et al. Classification and nomenclature system for human Alphapapillomavirus variants: general features, nucleotide landmarks and assignment of HPV6 and HPV11 isolates to variant lineages. Acta Dermatovenerol Alp Panonica Adriat. 2011;20(3):113-123.
34. Wang CC, Lai CH, Huang HJ, Chao A, Chang CJ, Chang TC, et al. Clinical effect of human papillomavirus genotypes in patients with cervical cancer undergoing primary radiotherapy. Int J Radiat Oncol Biol Phys. 2010;78(4):1111-1120.

CHAPTER 6

SUMMARY

6.1. INTRODUCTION

The primary intention of this doctoral study was to investigate the causal relationship between high-risk HPV type infections and disease of the cervix. A second intention was to determine if HPV-related development of cervical neoplasia and HPV types associated with disease progression, from pre-malignant to malignant lesions, differ in relation to immune system integrity.

This final chapter summarises the important findings from the different studies that make up this thesis and to interpret the relationship between specific HrHPV type infections and cervical disease. The final conclusions from this doctoral study will be drawn and recommendations for further research set out.

6.2. SUMMARY OF RESEARCH FINDINGS

6.2.1. Human papillomavirus (HPV) prevalence of all HPV types

In women representative of the general population but without cytological abnormalities, HPV infections were highly prevalent and extremely common in women with acquired immunodeficiency syndrome (AIDS). The prevalence of any HPV type infection was around 67.1% in women representative of the general population with normal cytology and around 87.7% in women with AIDS without cytological abnormalities.

In patients with cervical intraepithelial neoplasia (CIN) grade II/III confirmed on histology, the prevalence of any HPV type detected from the cervical surface was 96.7%. The prevalence was similar in HIV-negative patients (97.8%) and HIV-positive patients (96.4%).

Any HPV type infection was detected in all the patients where HPV testing was performed on targeted tissue-based low- and high-grade lesions.

Human papillomavirus DNA was detected in 91.7% of all patients with invasive cervical cancer (ICC). The prevalence was the same (90.9%) for HIV-infected and HIV-non-infected patients.

6.2.2. The prevalence of high-risk HPV (HrHPV) type infections

The prevalence of HrHPV type infections was 44.9% in women with normal cervical cytology. The highest prevalence of HrHPV infections was seen in women between 20 and 24 years, followed by women aged from 25 to 29 years. Fifty-six percent of women without cytological abnormalities below the age of 30 were infected with at least one HrHPV types. No differentiation was made between HIV-infected and -non-infected women, which might explain the higher prevalence in this study. In women with AIDS who had a normal cervical cytology 78.5% were infected with one or more HrHPV types.

The prevalence of HrHPV type infections detected from the surface of all CIN II/III lesions was 93%. The prevalence was similar for HIV-negative (93.3%) and HIV-positive (92.9%) patients. In chapter 3 the prevalence of HrHPV infections detected from the surface of CIN I/II lesions was 98.3%, and 96.4% of CIN III lesions.

The prevalence of HrHPV types detected from targeted tissue-based testing within CIN I/II lesions was identical to the prevalence of HrHPV infections detected from the surface, namely 98.3% for all lesions; 100% and 97.9% in lesions from HIV-negative and -positive patients respectively. The prevalence of HrHPV type infections detected from targeted tissue-based testing in patients with CIN III with positive Ki-67 and p16 staining was 95.1%.

In patients with ICC, 88.3% of tumors tested positive for HrHPV. The prevalence of HrHPV infections was similar (86.4% vs 87%) for HIV-negative patients and HIV-positive patients.

6.2.3. Single and multiple HPV type infections

In women with normal cytology, 23.6% had a single HrHPV type infection and 21.4% were simultaneously infected with more than one HrHPV type. Although the single HrHPV infection rate was similar among women with AIDS (26.2%), more than half (52.3%) had multiple high-risk types.

Patients with HIV and CIN II/III also had significantly more multiple HrHPV types (73.3%) detected from the cervical surface compared to HIV-non-infected patients (48.9%). From the surface, a single HrHPV type was detected in 44.4% of HIV-negative patients and 19.6% of HIV-positive patients with CIN II/III.

The prevalence of single HPV type infections detected within targeted tissue-based CIN I/II lesions was 38.3% and 46.4% of CIN III lesions. Among Ki-67 and p16 positive CIN III lesions, the prevalence of a single HrHPV type detected from lesional tissue was 41.5%.

Of women with ICC, 64.8% tested positive for a single HrHPV type and 23.4% for multiple high-risk types. Among this group, HIV-infected patients also had significantly more multiple HrHPV type infections (p=0.001) compared to HIV-non-infected patients.

6.2.4. HrHPV type-distribution among various study groups

In women with normal cytology HPV 16 was the most prevalent HrHPV type, followed by HPV 51, 58 and 45. Among women with AIDS and normal cervical cytology, the prevalence of HPV 16 and/or 18 was 24.6%. HPV 16 ranked ninth out of the 10 most prevalent HrHPV types.

Among patients with CIN II/III, the most prevalent HPV type detected from the cervical surface was HPV 16, irrespective of HIV status. HPV 16 and/or 18 were present in 45.9% of patients. Including patients with multiple HPV type

infections, non-HPV 16/18 type infections accounted for around 53% and 57% of infections in HIV-positive and -negative patients respectively. Although HPV 16 was the most prevalent type in HIV-positive patients, patients with a CD4 cell count of less than 200µl had a significantly higher prevalence of HPV 51 (p=0.013), 56 (p=0.013) and 73 (p=0.001).

The most prevalent single HrHPV type detected from targeted tissue-based CIN I/II lesions were HPV 35, 16 and 33, and from CIN III, HPV 16, 52, 35 and 18. The most common HrHPV type detected from lesional tissue (Chapter 4) in all patients with CIN III with single and multiple HPV type infections was HPV 16 (39.3%). HPV 16 was also the most prevalent type for both HIV-infected (35.5%) and -non-infected (46.7%) patients.

In patients with ICC, including single and multiple HPV type infections, the most prevalent HrHPV type infection was HPV 16, followed by HPV 18, 45, 35 and 33. Compared to HIV-negative patients, a higher percentage of HIV-positive patients were infected with HPV 45 (p=0.015). The five most prevalent single HPV type infections were identical to single and multiple HPV type infections in patients with ICC. The prevalence of HPV 45 was three times higher in HIV-positive patients with single HPV type infections compared to HIV-negative women.

6.3. INTERPRETATION OF SPECIFIC HIGH-RISK HPV TYPE FINDINGS

The prevalence of specific HrHPV types in different stages of cervical disease was compared to determine the relationship between HrHPV type infections and disease of the cervix. Firstly, the prevalence of specific HrHPV types in women representative of the general population with normal cervical cytology was used (Chapter 1). Secondly, the consensus HPV (Chapter 3) in CIN III lesions was recorded as it is postulated to be the most likely lesion to cause the pre-malignant lesion. Lastly, the prevalence of the specific HrHPV type detected in patients with ICC was used (Chapter 5).

6.3.1. Type-specific HrHPV distribution in all patients

The HrHPV type-specific comparisons in all patients are illustrated in figures 6.1 to 6.4. Irrespective of HIV status, the prevalence steadily rose for HPV 16 and 18 infections from women with normal cervical cytology to ICC. Compared to women with normal cytology, HPV 45 infections were under-represented in CIN III lesions, but the prevalence clearly increased in patients with ICC.

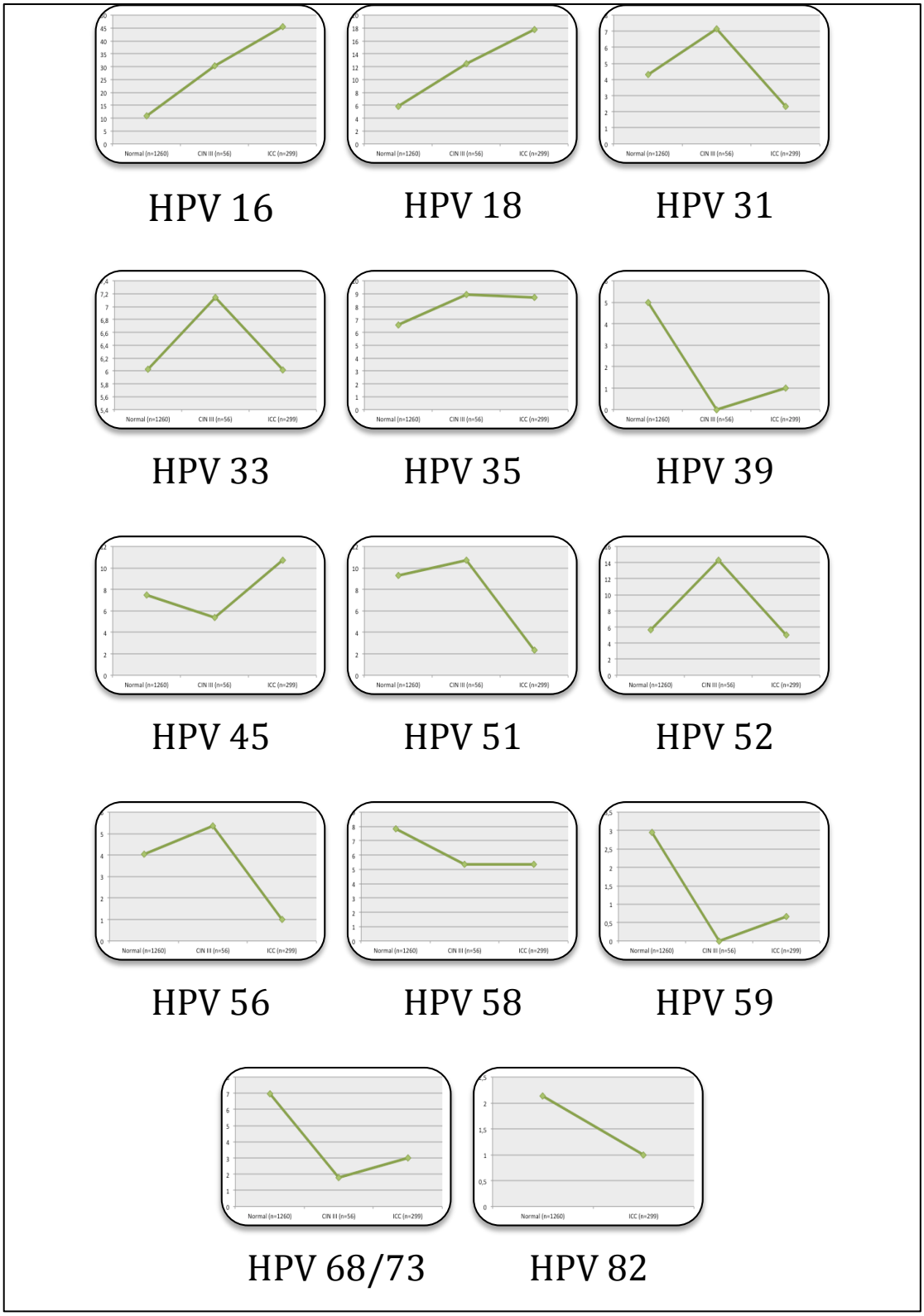


Figure 6.1: Prevalence of specific HrHPV types in general population with normal cytology (Normal), consensus HPV in CIN III (CIN III) lesions and invasive cervical cancer (ICC)

HPV 33 and 52 infections appeared over-represented in CIN III lesions, but the prevalence in ICC was similar to infections in women without cytological abnormalities. The prevalence of HPV 35 infections were higher in CIN III lesions and ICC compared to infections in women with normal cytology, although slightly higher in CIN III than in ICC.

Compared to women with normal cytology, HPV 31, 51 and 56 were over-represented in CIN III lesions, but under-represented in ICC. The prevalence of HPV 39, 59 and 68/73 was higher in ICC than CIN III lesions, but the prevalence in both was lower than in women with normal cytology.

There was a steady decline in the prevalence of HPV 58 from women with normal cytology to ICC. HPV 82 detection was not included in the detection method used for tissue-based DNA analysis and, therefor, the CIN III prevalence is not shown in the figures.

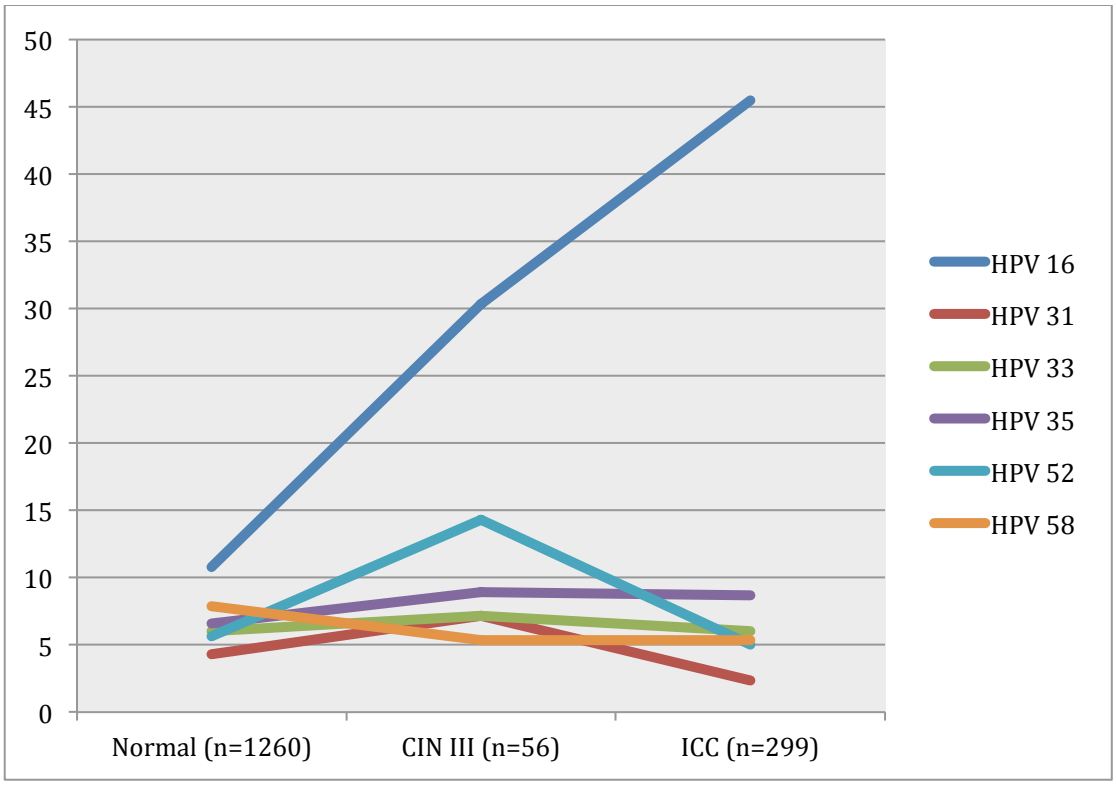


Figure 6.2: Prevalence of specific alpha-9 HrHPV types in general population with normal cytology (Normal), consensus HPV in CIN III (CIN III) lesions and invasive cervical cancer (ICC)

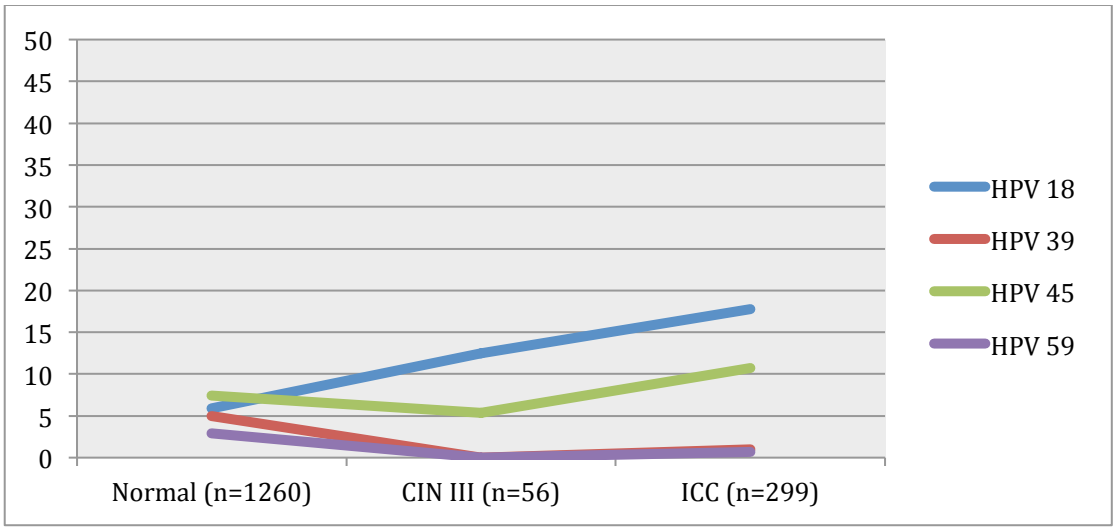


Figure 6.3: Prevalence of specific alpha-7 HrHPV types in general population with normal cytology (Normal), consensus HPV in CIN III (CIN III) lesions and invasive cervical cancer (ICC)

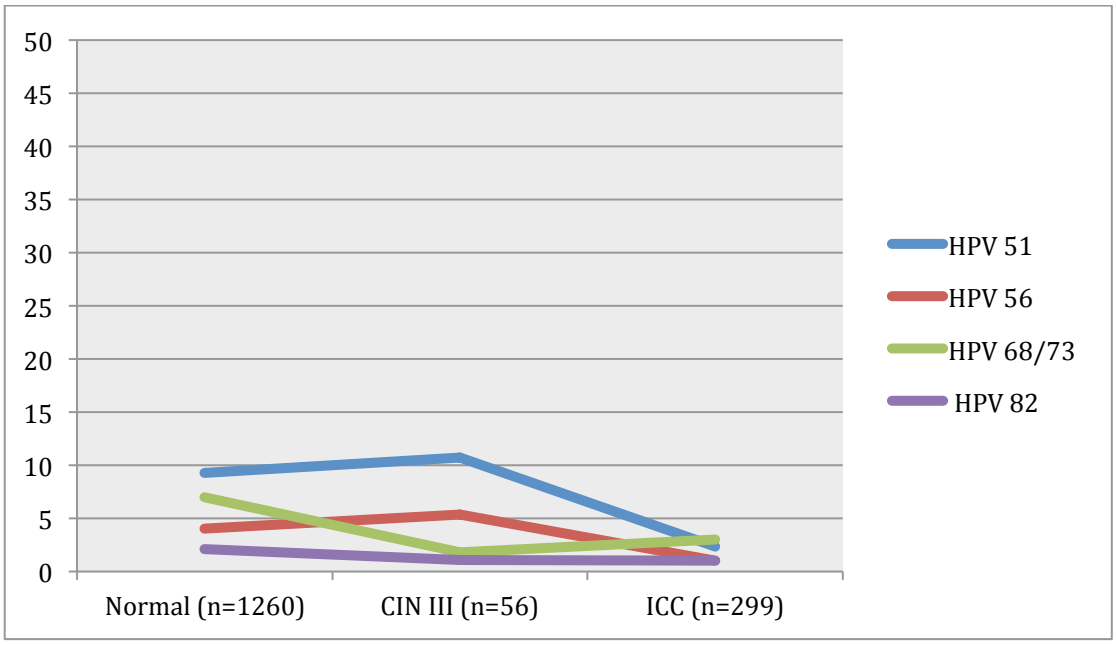


Figure 6.4: Prevalence of specific alpha-5, -6, and other HrHPV types in general population with normal cytology (Normal), consensus HPV in CIN III (CIN III) lesions and invasive cervical cancer (ICC)

6.3.2. Type-specific HrHPV distribution in HIV-negative patients compared to all patients

There was a difference in the distribution of HPV 16, 35, 45, 56 and 68/73 when HIV-negative patients were compared with all patients described above. The type-specific HrHPV distribution in HIV-negative patients is illustrated in figures 6.5 to 6.7.

The prevalence of HPV 18 was lower in CIN III lesions than in women with normal cytology. HPV 35 was under-represented in patients with CIN III and over-represented in ICC.

Both HPV 45 and 56 were absent in CIN III lesions and the prevalence was lower in ICC than in women with normal cytology. HPV 68/73 was not detected in CIN III lesions or ICC.

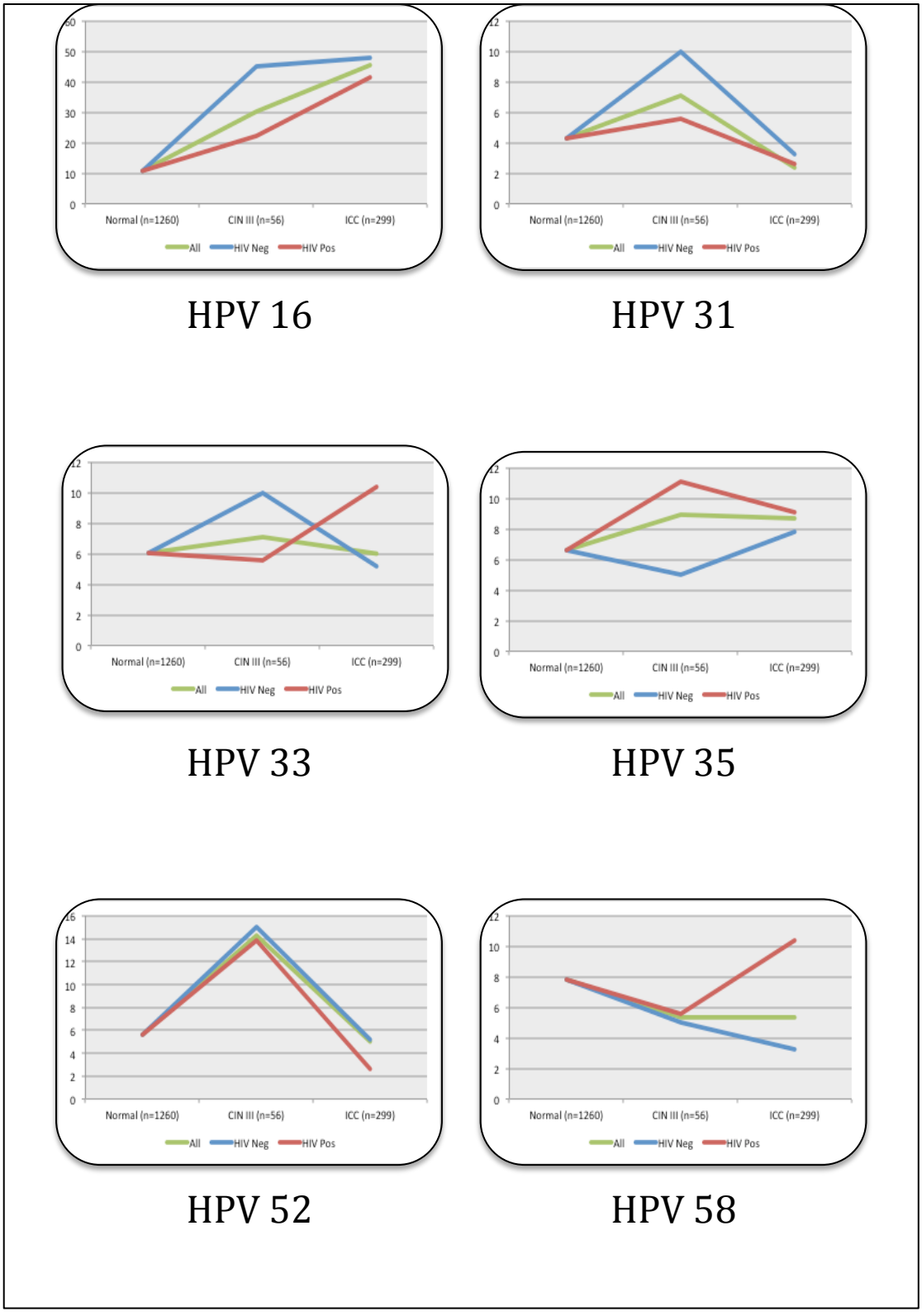


Figure 6.5: Prevalence of specific alpha-9 HrHPV types in general population with normal cytology (Normal), consensus HPV in CIN III (CIN III) lesions and invasive cervical cancer (ICC) in all patients, HIV-negative and -positive patients

6.3.3. Type-specific HrHPV distribution in HIV-positive patients compared to all patients

Comparing HIV-positive patients to all patients described above, there were differences seen in the distribution of HPV 33, 45, 58 and 68/73 in relation to cervical disease. The type-specific HrHPV distribution in HIV-positive patients is illustrated in figures 6.5 to 6.7.

The prevalence of HPV 33 was slightly lower in CIN III lesions compared to women with normal cytology, but higher in ICC than both. HPV 45 showed a steady increase in prevalence from normal cytology to ICC, similar to HPV 16 and 18.

In comparison to women with normal cytology, HPV 58 was under-represented in CIN III lesions and over-represented in ICC. HPV 68/73 showed a decline in prevalence from normal cytology to ICC.

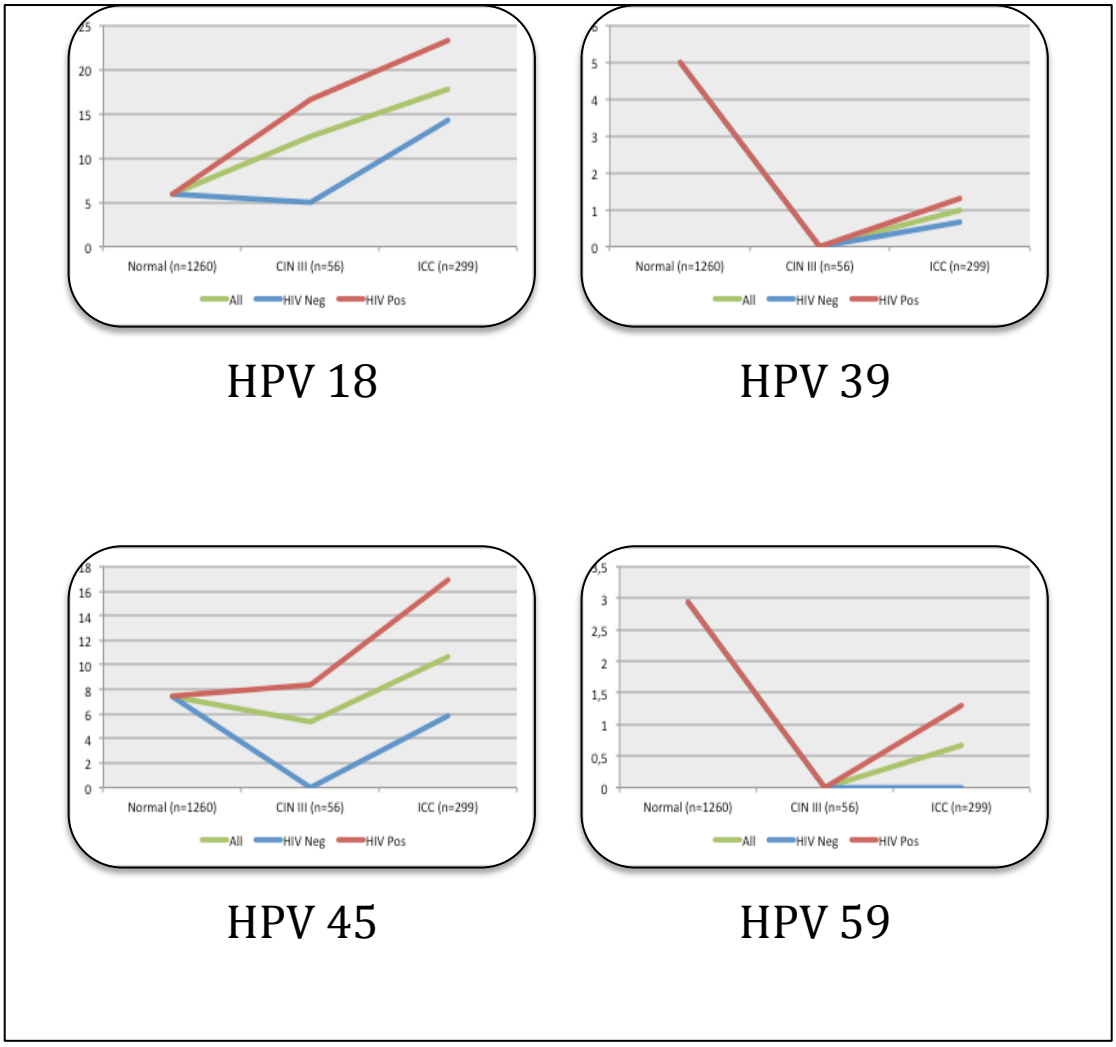


Figure 6.6: Prevalence of specific alpha-7 HrHPV types in general population with normal cytology (Normal), consensus HPV in CIN III (CIN III) lesions and invasive cervical cancer (ICC) in all patients, HIV-negative and -positive patients

6.3.4. Type-specific HrHPV distribution in HIV-positive patients compared to HIV-negative patients

The HrHPV types that showed similar distribution in prevalence patterns, in different stages of cervical disease, in HIV-infected- compared to -non-infected patients were HPV 16, 31, 39, 51 and 52. A difference was seen for HPV 18, 33, 35, 45, 56, 58, 59 and 68/73. See figures 6.5 to 6.7.

HPV 18 was less prevalent in CIN III lesions from HIV-negative patients compared to women with normal cytology. Whereas in HIV-positive patients there was a steady increase in the prevalence in relation to cervical disease. HPV 33 was over-represented in ICC compared to women with normal cytology in HIV-negative patients. In HIV-positive patients the result was exactly the opposite.

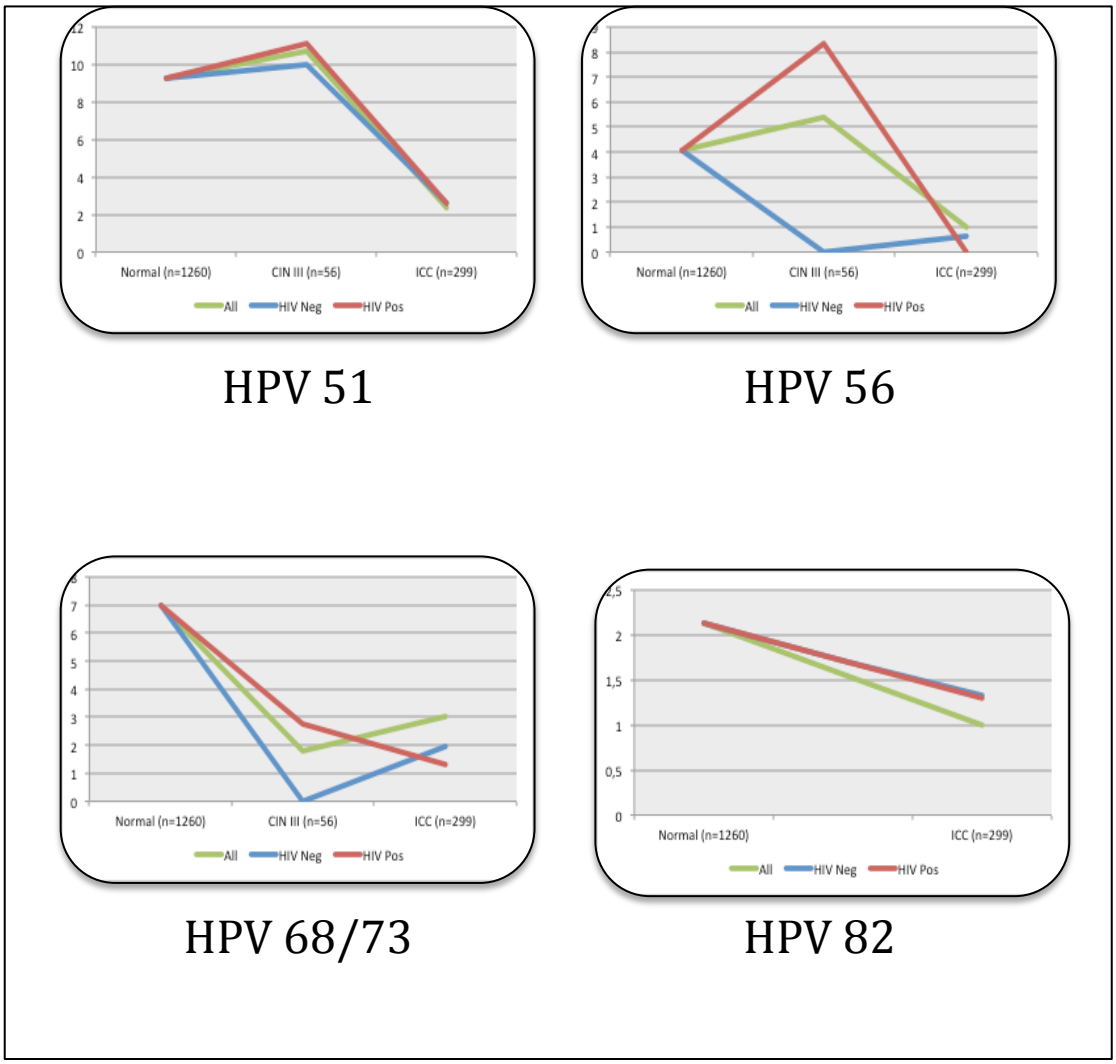


Figure 6.7: Prevalence of specific alpha-5, -6, and other HrHPV types in general population with normal cytology (Normal), consensus HPV in CIN III (CIN III) lesions and invasive cervical cancer (ICC) in all patients, HIV-negative and -positive patients

Compared to women with normal cytology, HPV 35 was under-represented in CIN III lesions from HIV-negative patients and over-represented in CIN III lesions from HIV-positive patients, but both had a higher prevalence in ICC than shown in women with normal cytology. In HIV-positive patients HPV 45 showed an increase in prevalence from normal cytology to ICC.

HPV 56 was not detected in CIN III lesions from HIV-negative patients and absent in ICC among HIV-positive patients. HPV 58 was under-represented in CIN III lesions from HIV-positive patients, but over-represented in ICC compared to women with normal cytology. In HIV-negative patients, HPV 59 and 68/73 were not detected in CIN III lesions or ICC.

6.3.5. Specific HrHPV types detected in cervical cancer compared to pre-malignant lesions

HPV types detected in patients with ICC described in Chapter 5 from the same referral regions as patients with pre-malignant lesions studied in chapters 3 and 4, showed that the five most prevalent HPV types for all patients with ICC to be HPV 16, 18, 45, 35 and 33. HPV 16, 18, 35, 45 and 33 were the most prevalent types among the HIV-negative subgroup and HPV 16, 18, 45, 33 and 58 among the HIV-positive subgroup.

Comparing the consensus HPV types detected in all patients with CIN III in Chapter 3 with the most prevalent types detected in ICC in Chapter 5, HPV 51 and 52 were over-represented in CIN III, whereas HPV 33 and 45 were under-represented. In HIV-negative patients only HPV 16 and 33 corresponded and in HIV-positive patients only HPV 16 and 18. In none of the groups, regardless of the HPV detection method did HPV 45 feature under the five most prevalent types in patients with pre-malignant lesions.

Comparing HPV types detected from lesional tissue in patients with CIN III reported in Chapter 4, there appeared to be correspondence regarding HPV 16,

33 and 35 for all patients and the HIV-negative patients. HPV 51 and 52 were over represented in the CIN III group, but HPV 18 and 45 were under-represented compared to patients with ICC. In HIV-infected patients, only HPV 16 and 18 were present in both the five most prevalent types detected in CIN III and ICC.

6.4. COMPARISON OF RESEARCH FINDINGS WITH AVAILABLE LITERATURE

Research findings from specific studies were discussed in the individual chapters. In order to avoid repetition, comparisons were only made with most relevant publications and key references.

6.4.1. Prevalence of all HPV and HrHPV infections in women with normal cytology

The prevalence of 67.1% of any HPV type infection is higher than any previously reported data. The global adjusted HPV prevalence among women with normal cytology is reported as 11.7% and ranges between 1.7% and 35.4%.[1] In Middle Africa the reported prevalence of any HPV infection among women with normal cytology is 8.7%. In Western Africa the prevalence ranges between 11.5% and 47.9%, and between 3.2% and 41.4% in Eastern Africa. The prevalence for South Africa, obtained from two Cape Town studies, is reported as ranging from 15.5% to 20.4% of women with normal cytology.[1,2]

Figure 6.8 illustrates the type-specific prevalence in women with normal cytology in different regions. Globally HPV 16 is most prevalent and 2nd most prevalent in South Africa. In the current study HPV 16 ranked 3rd after HPV 62 and 84, but was almost four times higher than the global prevalence. Even the 10th most prevalent HPV type infection (HPV 70; 7.1%) was more prevalent than the most prevalent type (HPV 16; 2.8%) reported in any other region.[3,4]

According to the meta-analysis of 1 million women with normal cytological findings published in 2010, the most prevalent HPV infections were HPV 16 (3.2%), 18 (1.4%), 52 (0.9%), 31 (0.8%) and 58 (0.7%).[5] In contrast to other regions HPV 18 was not among the 10 most prevalent types. HPV 45 was only seen under the 10 most prevalent types in the current and other South African studies. The worldwide prevalence of HPV 16 and/or 18 is reported as 3.9% in women with normal cytology. The infection rate in the current study (15.6%) is much higher than in other regions, ranging between 3.4% and 8.3%.[3,4] Data on HIV co-infection might explain the higher prevalence to some degree.

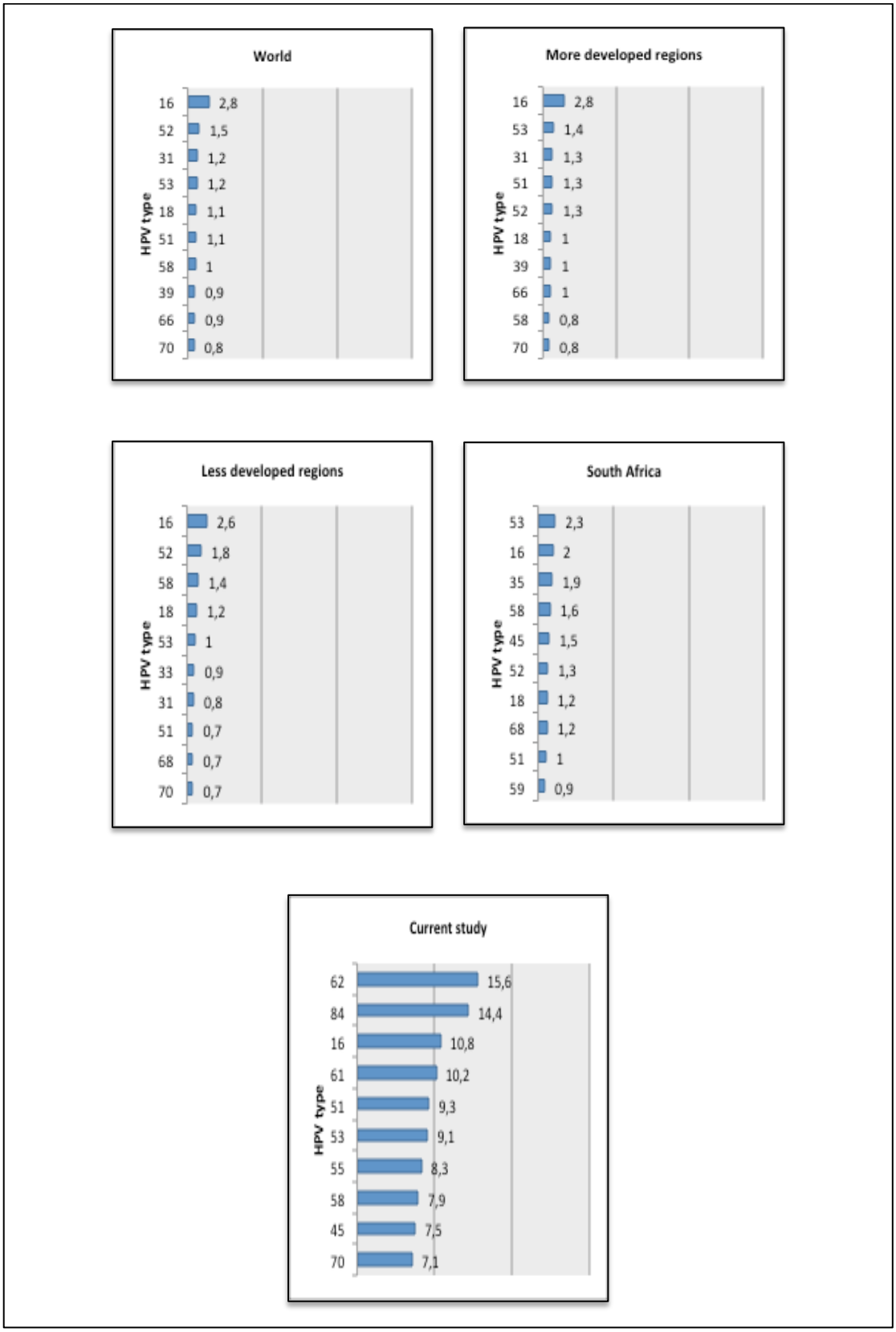


Figure 6.8: HPV type-specific prevalence in women with normal cytology in different regions

6.4.2. HPV in high-grade cervical dysplasia

The prevalence of any HPV infection in patients with CIN II/III was 96.7%. A meta-analysis published in 2012 that included more than 16000 women with CIN II/III reported the global prevalence between 86% and 93% and the prevalence in Africa between 83% and 89%. HPV prevalence increased in direct proportion to the severity of cervical dysplasia. In the meta-analysis, the most prevalent HrHPV types detected in patients with CIN II/III were HPV 16 (52.9%), 52 (12.6%), 31 (11.7%), 58 (9.9%), 33 (8.9%), 18 (8.1%), 51 (6.7%) and 45 (4.0%).[6]

As illustrated in Figure 6.9, the HPV type-distribution in high-grade lesions differs from region to region. Although HPV 16 is the most prevalent type in all regions, the prevalence was lower in the current study in comparison to global prevalence and more and less developed areas. HPV 16 contributed more to high-grade lesions compared to available South African data.[3,4]

The three most prevalent HPV types in the current study – HPV 16, 58 and 35 – were the same as reported for South Africa, but the 2nd and 3rd most prevalent types were swapped around. HPV 18 ranked 6th both globally and in more developed regions, and 4th in less developed regions. HPV 18 ranked 7th in South Africa and 10th in the current study. HPV 45 prevalence in high-grade lesions is low globally and in more developed regions. HPV 45 ranked 9th in less developed regions, 6th in South Africa and 7th in the current study.[3,4]

The number of studies reporting on HPV types detected specifically within high-grade CIN lesions is extremely limited. In the current study, HPV 16 (35.5%) was the most prevalent type detected within CIN III lesional tissue, followed by HPV 51 (30.4%), 52 (28.6%), 33 (26.8%) and 35 (23.2%).

Van der Marel et al. found HPV 16 (50.9%) most frequently within CIN II/III lesions where single and multiple types were detected. HPV 16 was followed by HPV 31 (15.9%), 33 (7.0%), 18 (6.2%), 52 (6.2%) and 58 (4.7%).[7] In the only

other similar study found, published by Callegari et al., within CIN III lesions HPV 16 (56.5%) was most prevalent, followed by HPV 31 (18.5%), 51 (5.4%), 18 (4.8%) and 33 (4.2%).[8] Although the study method was not identical, it is clear that non-HPV 16 types contribute more to CIN III lesions in the current study compared to the two available studies in the literature.

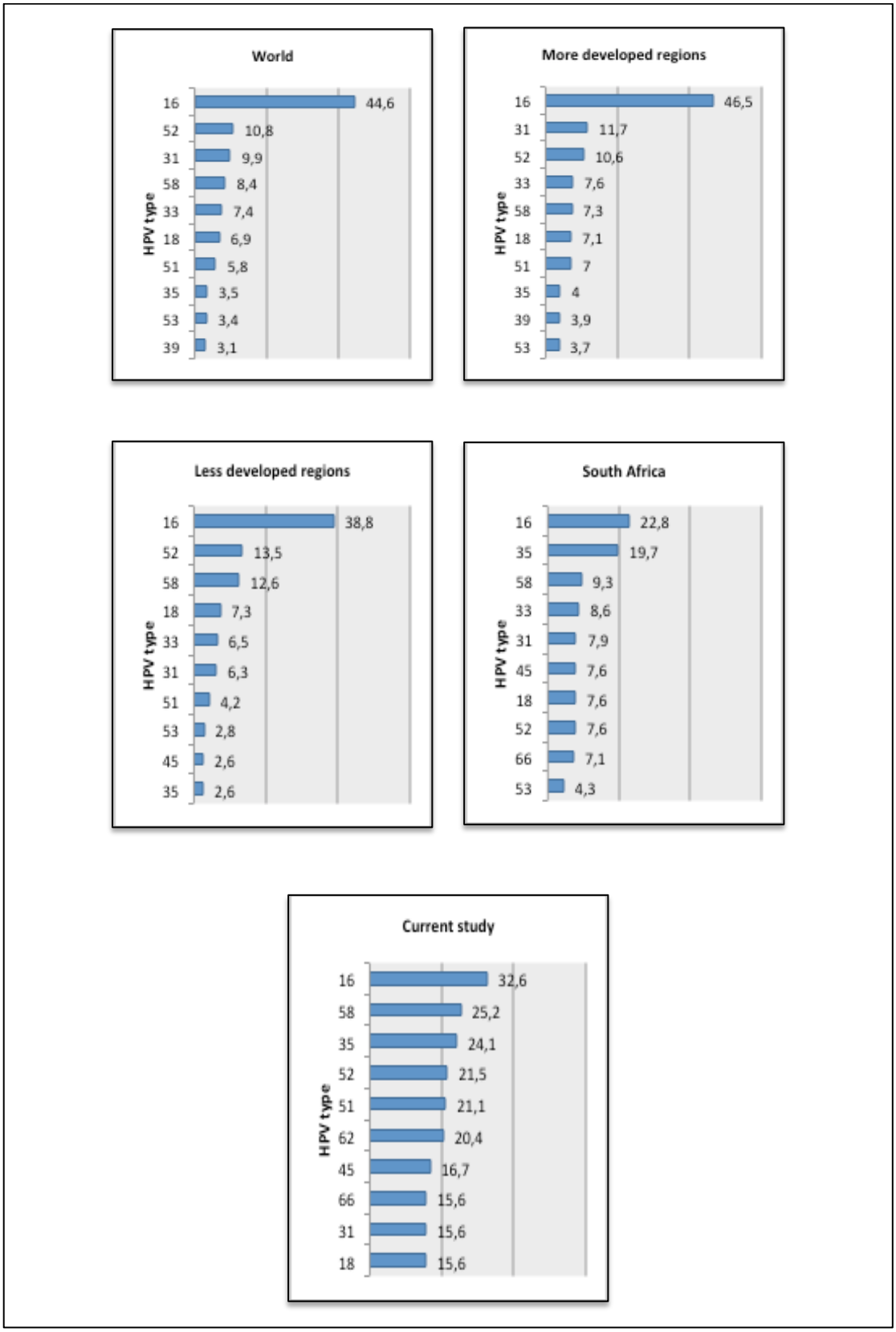


Figure 6.9: HPV type-specific prevalence in women with high-grade lesions in different regions

6.4.3. HPV and ICC

The prevalence of HPV infections in women with ICC in the current study (91.7%) is similar to the prevalence reported in the large review published in 2011.[9] Based on the most recently published data on HPV distribution in ICC, HPV 16 caused 55.4% of ICC worldwide, followed by HPV 18 (14.6%), 45 (4.8%), 33 (4.2%) and 58 (3.8%).[3]

In Africa, the prevalence of HPV 16 in ICC ranged between 38.5% and 81.8%. In the majority of studies from Africa, HPV 18 and 45 followed HPV 16, as was the case in the current study. The relative contribution from HPV 16 and/or 18 infections in the current study fell within the 56% to 91% range reported for sub-Saharan Africa. HPV 35 was 4th most prevalent in the current study, which was similar than almost half of the studies included in the African review.[2]

Comparing data from the current study with other regions, HPV 16 was less prevalent and HPV 18 more prevalent than any other region shown in Figure 6.10. The prevalence of HPV 16 and/or 18 was almost identical to South African data. The six most prevalent types were the same in the current study as they were in less developed regions, except in the current study HPV 33 ranked 5th and HPV 58 ranked 6th. Compared to the worldwide prevalence HPV 35 was more prevalent than HPV 33 and 58 and HPV 31 appeared less important in the current study population.[3,4]

6.4.4.Influence of HIV on HPV epidemiology

Most HIV-infected women will be co-infected with HPV and are more susceptible to HPV-associated cancers.[10] A recent South African study, published in 2015, reported the HPV prevalence irrespective of cervical abnormalities in HIV-positive women as 74.0% and 36.7% in HIV-negative women.[11]

A meta-analysis performed at the International Agency for Research by Clifford et al. assessed type-specific HPV prevalence in 716 HIV-positive women with ICC

in sub-Saharan Africa.[12] This publication was recently submitted for publication and is under review and includes patients with ICC from the current study. The prevalence of HPV in women with ICC in the current study of around 91% in both HIV-negative- and -positive women were almost identical as found by Clifford et al. These researchers reported HPV detection in 91.6% and 90.3% of HIV-positive- and -negative women with ICC respectively.[12]

The most prevalent single and multiple HrHPV types in HIV-infected patients with ICC in the current study were HPV 16 (41.5%), 18 (23.4%), 45 (16.8%), 33 (10.4%), 58 (10.4%) and 35 (9.1%). The HPV type-distribution is similar to the results from the meta-analysis that reported the most prevalent high-risk type as HPV 16 (41.1%), 18 (22.3%), 45 (14.6%), 35 (8.0%), 58 (7.8%), 31 (5.0%) and 33 (4.6%).[12]

The order of the four most prevalent single-type infections in the current study were identical to that found by Clifford et al. In HIV-infected patients in the

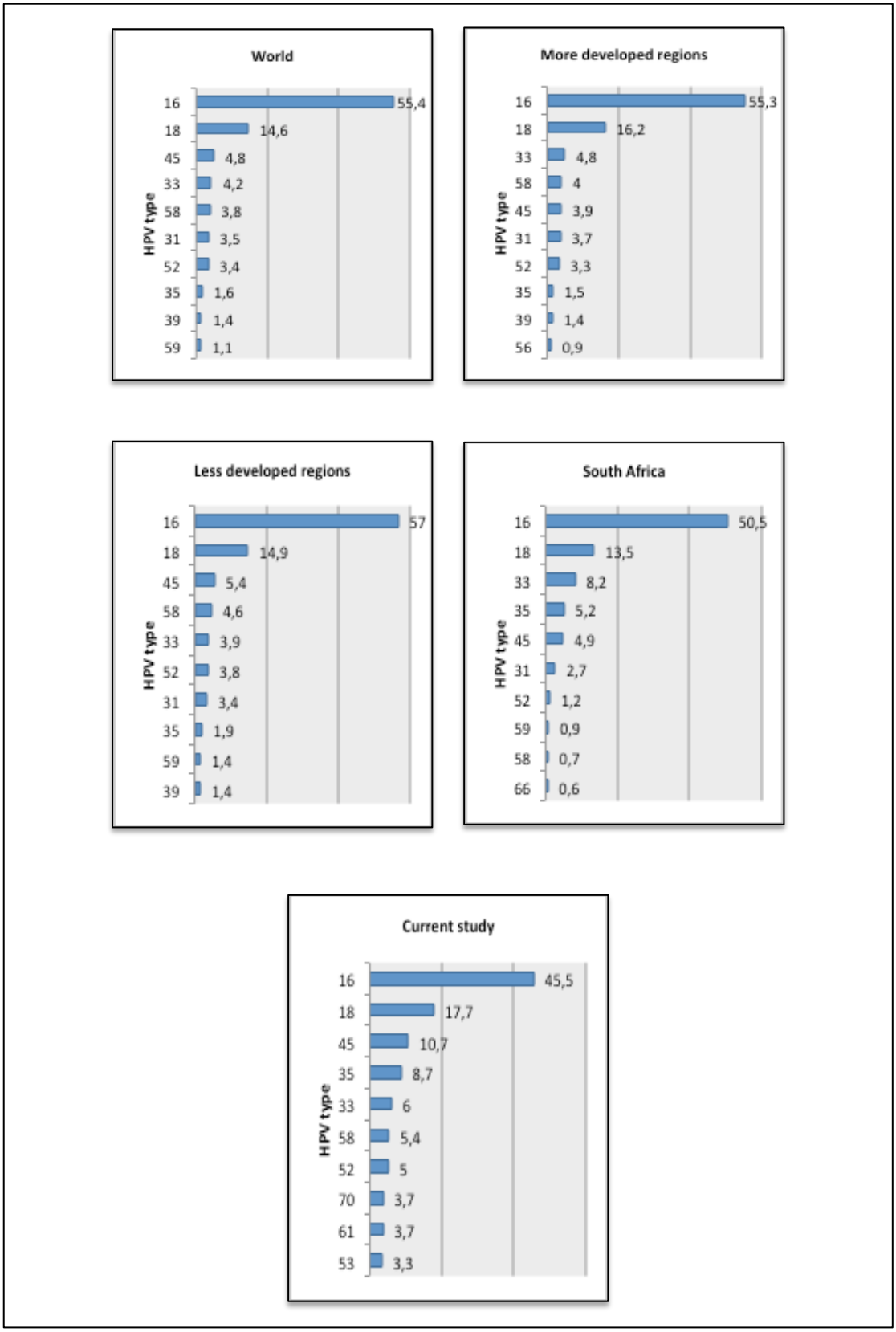


Figure 6.10: HPV type-specific prevalence in women with ICC in different regions

current study with single HPV infections, HPV 16 (45.9%) was most prevalent, followed by HPV 18 (19.5%), 45 (15.1%) and 35 (6.5%). The prevalence reported by the meta-analysis was lower for HPV 16 (38.6%) and 45 (10.8%), but similar for HPV 18 (19.5%) and 35 (4.5%). The authors concluded that although a lower portion of ICC in HIV-infected women was attributable to HPV 16, HPV 18 caused a higher proportion and therefore the HPV 16/18 vaccines may have a similar preventative effect in HIV-positive women.[12] Emerging HIV-related epidemiology of specifically types 33, 58 and possibly 56 should be monitored to determine type-specific shifts in future.

6.4.5. HPV type-distribution compared to literature

Figures 6.11 and 6.12 illustrate the HPV type-distribution graph for individual types compared to the type-distribution graph in the meta-analysis.[6] Prevalence is expressed as a percentage of HPV-positive samples by cervical disease grade. Viral types that showed a similar distribution curve as that reported by Guan et al. included HPV 16, 31, 45, 51, 52 and 56. HPV 18 was slightly less prevalent in CIN III compared to normal cytology reported by Guan et al. The distribution curves were reversed for HPV 33, 35 and 58. Although HPV 45 had a similar curve for both studies, in the current study the prevalence was higher in all cervical disease grades.

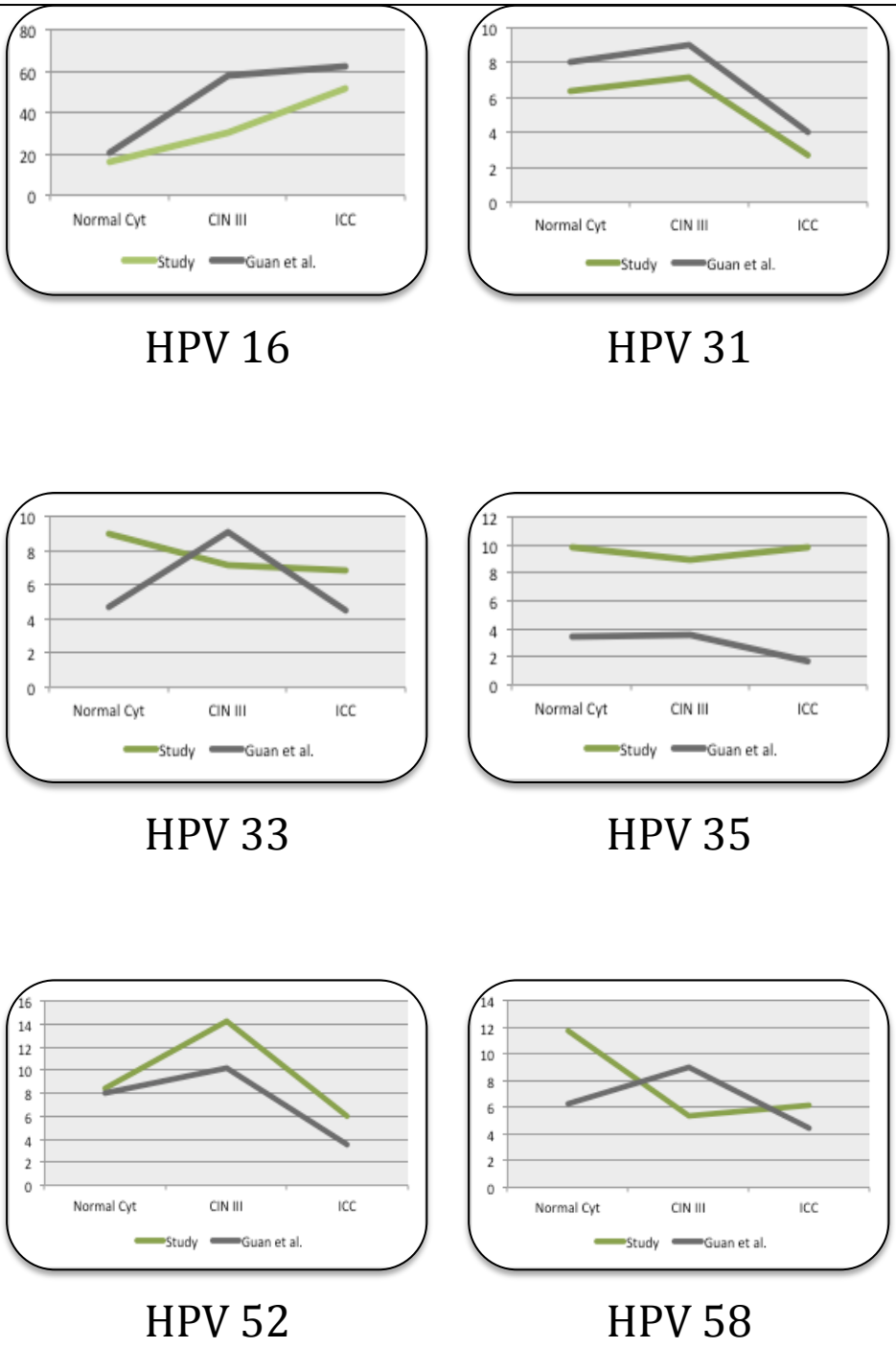


Figure 6.11: Positivity for alpha-9 HrHPV types as a proportion of HPV-positive samples by cervical disease grade

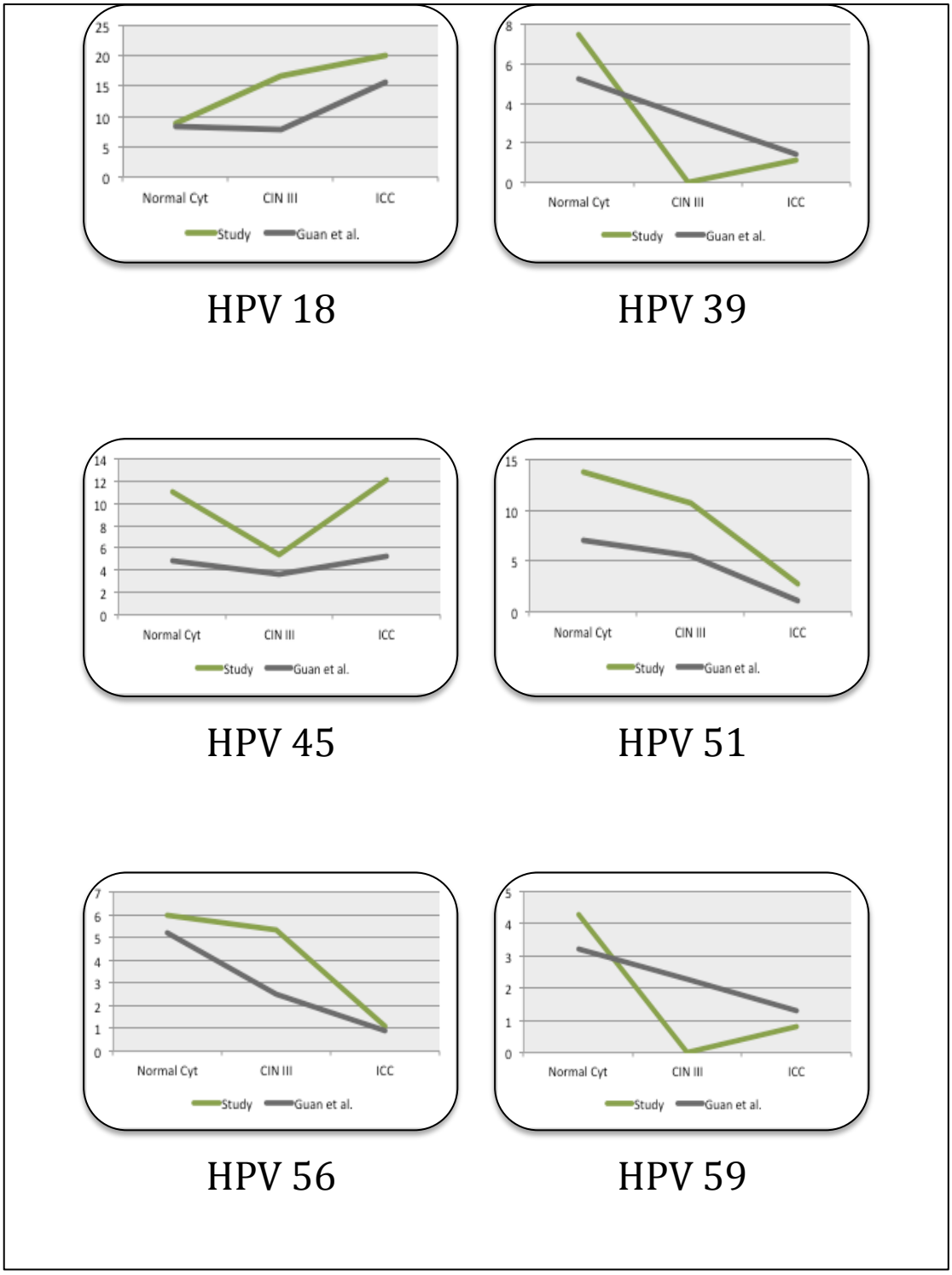


Figure 6.12: Positivity for non-alpha-9 HrHPV types as a proportion of HPV-positive samples by cervical disease grade

6.5. RELATIVE ONCOGENIC POTENTIAL OF HIGH-RISK HPV TYPES

The relationship between the prevalence of specific HrHPV types in normal cytology, CIN III and ICC is shown in Table 6.1 and figures 6.13 to 6.15.

Table 6.1: The relationship between the prevalence of high-risk viral types in normal cytology, CIN III and ICC

HPV type	ICC : Normal cytology		CIN III : Normal cytology		CIN III : ICC	
	Prevalence(%)	Ratio	Prevalence(%)	Ratio	Prevalence(%)	Ratio
16	45.5 : 10.8	4.2:1	30.4 : 10.8	2.8:1	45.5 : 30.4	1.5:1
18	17.7 : 5.9	3:1	12.5 : 5.9	2.1:1	17.7 : 12.5	1.4:1
31	2.3 : 4.3	0.5:1	7.1 : 4.3	1.7:1	2.3 : 7.1	0.3:1
33	6.0 : 6.0	1:1	7.1 : 6.0	1.2:1	6.0 : 7.1	0.8:1
35	8.7 : 6.6	1.3:1	8.9 : 6.6	1.3:1	8.7 : 8.9	1:1
39	1.0 : 5.0	0.2:1	0 : 5.0	Invalid	1.0 : 0	Invalid
45	10.7 : 7.5	0.9:1	5.4 : 7.5	0.7:1	10.7 : 5.4	2:1
51	2.3 : 9.3	0.2:1	10.7 : 9.3	1.2:1	2.3 : 10.7	0.2:1
52	5.0 : 5.6	0.9:1	14.3 : 5.6	2.6:1	5 : 14.3	0.3:1
56	1.0 : 4.1	0.2:1	5.4 : 4.1	1.3:1	1 : 5.4	0,2:1
58	5.4 : 7.9	0.7:1	5.4 : 7.9	0.7:1	5.4 : 5.4	1:1
59	0.7 : 2.9	0.2:1	0 : 2.9	Invalid	0.7 : 0	Invalid
68/73	3.0 : 7.0	0.4:1	1.8 : 7.0	0.3:1	1.8 : 3.0	0.6:1
82	1 : 2.1	0.5:1	Not included in test method for CIN III			

6.5.1. CIN III : Normal cytology

Compared to normal cytology HPV 16, 18, 31, 33, 35, 51, 52 and 56 were more frequently detected in CIN III. HPV 45, 58 and 68/73 were the only types to be more prevalent in women with normal cytology. See Figure 6.13.

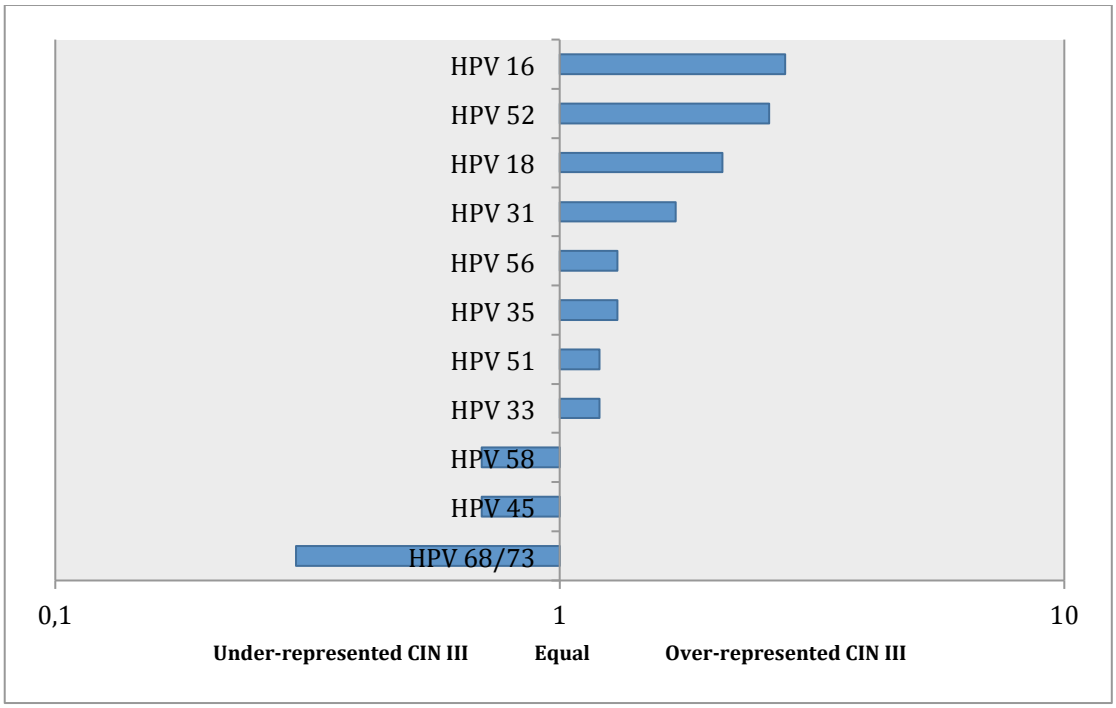


Figure 6.13: Relationship between the prevalence of high-risk viral types in normal cytology and CIN III

6.5.2. ICC : CIN III

HPV 16, 18 and 45 were the only types detected more frequently in ICC than in CIN III. The prevalence of HPV 35 and 58 was the same for ICC and CIN III. Compared to ICC, the prevalence of HPV 31, 33, 51, 52, 56 and 68/73 were higher in CIN III. See Figure 6.14.

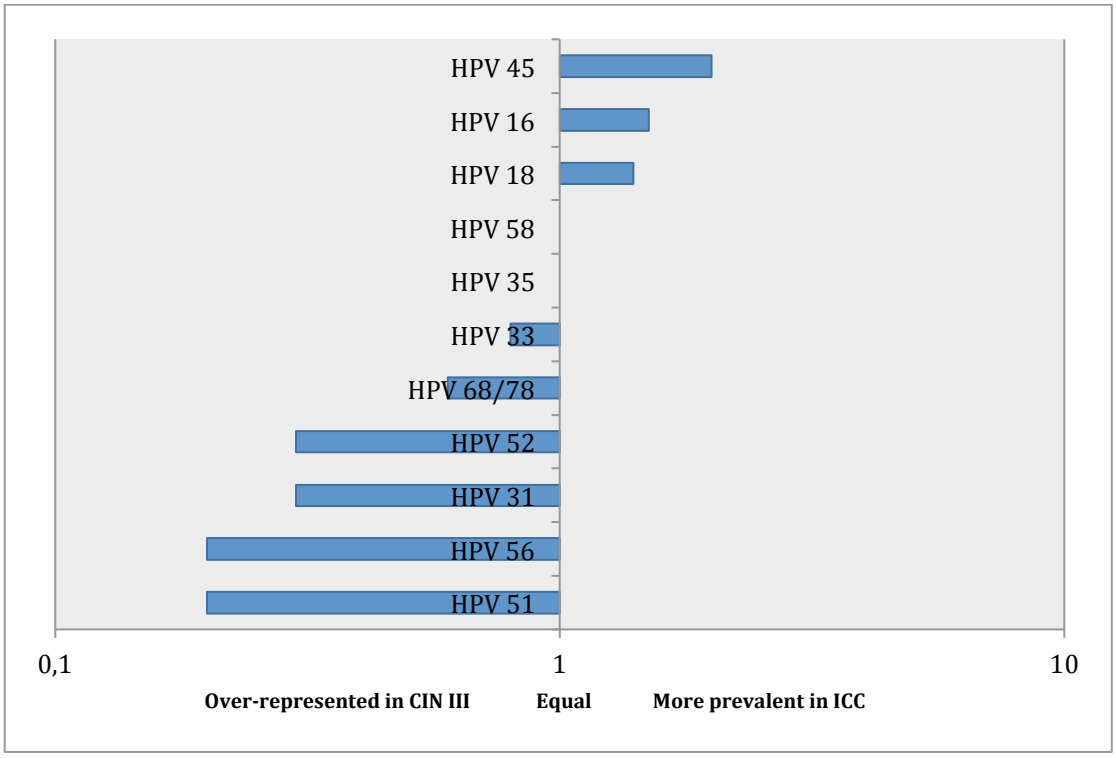


Figure 6.14: Relationship between the prevalence of high-risk viral types in ICC and CIN III

6.5.3. ICC: Normal cytology

The only types detected more frequently in ICC compared to normal cytology were HPV 16, 18 and 35. HPV 33 was equally prevalent in ICC and normal cytology. All the other high-risk types were more prevalent in normal cytology. See Figure 6.15.

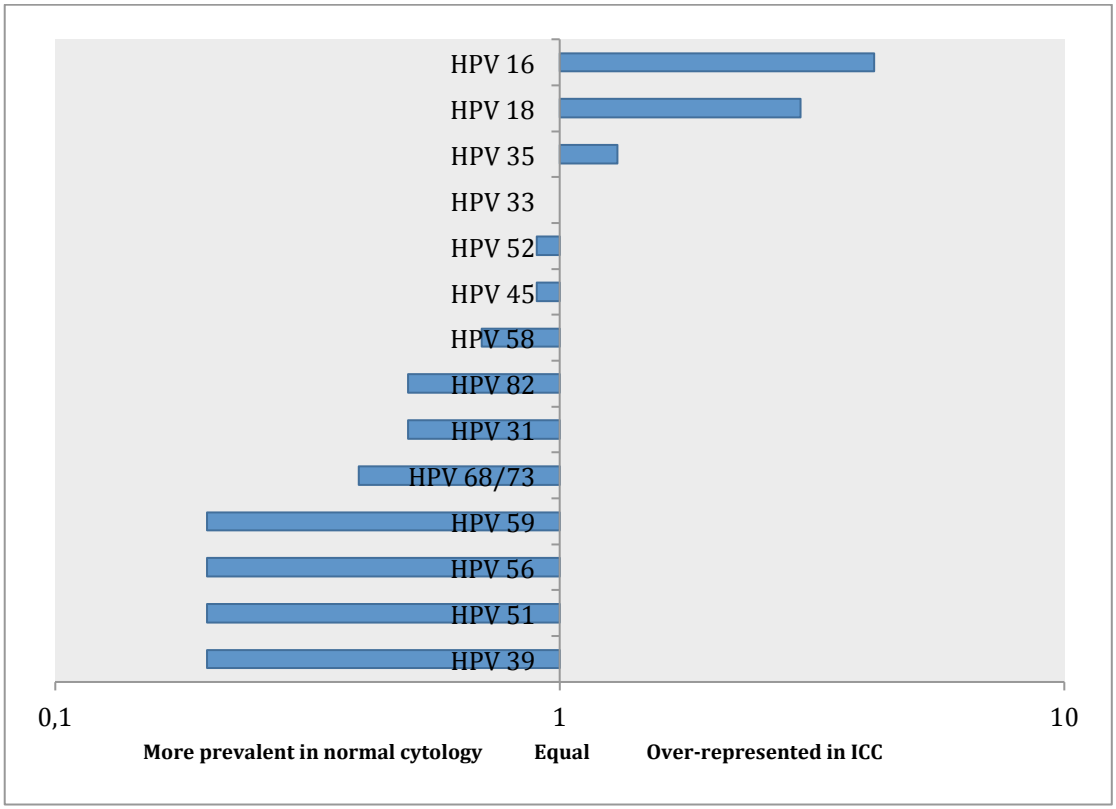


Figure 6.15: Relationship between the prevalence of high-risk viral types in ICC and normal cytology

The implications of these findings will be discussed under the next heading (Section 6.6.2).

6.6. IMPLICATIONS OF RESEARCH RESULTS FOR CERVICAL CANCER PREVENTION PROGRAMMES

6.6.1. Primary prevention with HPV vaccines

In a country burdened with high rates of HPV infections, cervical pre-neoplastic disease, and insufficient screening programmes, the answer most likely lies in primary prevention. There is limited data available on vaccination rates in South Africa. It is estimated that 50 000 (0.2%) individuals were vaccinated from December 2009 and November 2014 in the private health sector. A school-based vaccination program was implemented in April 2014, targeting around 500 000

girls older than 9 years in grade 4. Initial reports show excellent coverage of 91% and 93% of first and second round vaccinations. Coverage is estimated at roughly 39% for all children in South Africa born in 2004.[13]

To assess the possible impact that currently available HPV vaccines could have in women in our population, the contribution of different HrHPV types in different stages of cervical disease was evaluated.

Firstly, the prevalence of HPV 16 and/or 18 infections alone, without any other HrHPV co-infections, were reported. Secondly, the infection rates of the seven HrHPV types alone: HPV 16, 18, 31, 33, 45, 52 and 58, covered by the nine-valent vaccine were stated. Lastly, the infection rates resulting from other HrHPV types alone were shown, currently not specifically covered by vaccines. This distribution was illustrated for women with normal cytology, tissue-based types detected in CIN I/II and CIN III lesions, as well as patients with ICC.

As illustrated in Figure 6.16, in all women an HPV 16/18 vaccine should prevent at least 15% of infections in women without cytological abnormalities, between 10% and 17% of CIN lesions and around 50% of ICC. The nine-valent vaccine could potentially prevent 33% of HPV infections in women with normal cytology, between 68% and 86% of CIN lesions and up to 80% of ICC.

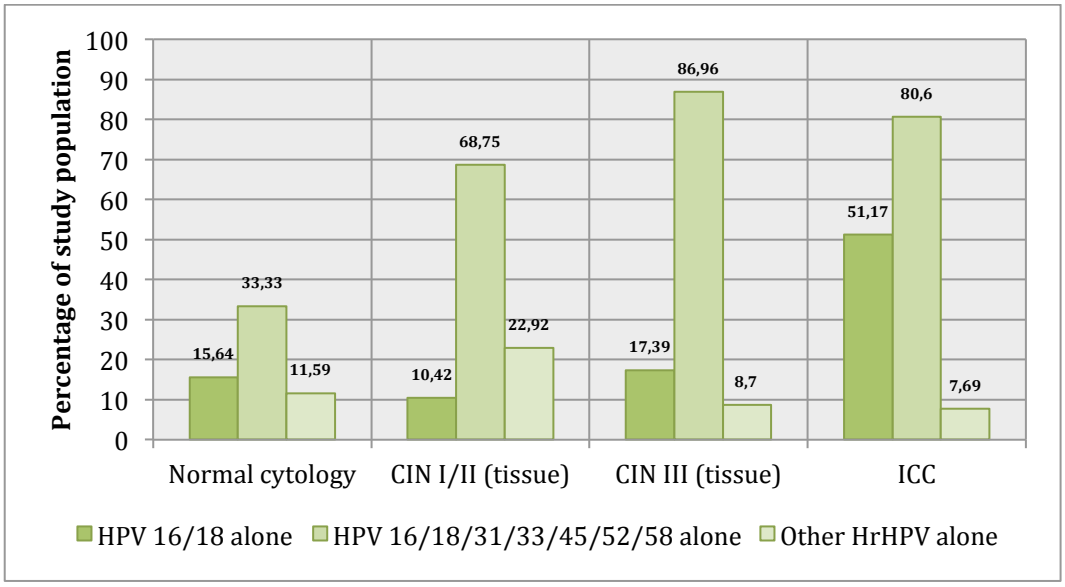


Figure 6.16: Potential preventative effect for HPV 16/18 vaccine and HPV 16/18/31/33/45/52/58 vaccine in different stages of cervical disease for all women

In HIV-negative patients, an HPV 16/18 vaccine should prevent at least 6% of CIN lesions and 55% of ICC. The nine-valent vaccine has the potential of preventing up to 80% CIN lesions and ICC. See Figure 6.17.

In HIV-positive patients, an HPV 16/18 vaccine might prevent at least 22% of CIN lesions and at least 45% of ICC. The nine-valent vaccine might prevent up to 90% of CIN lesions and around 80% of ICC. See Figure 6.17.

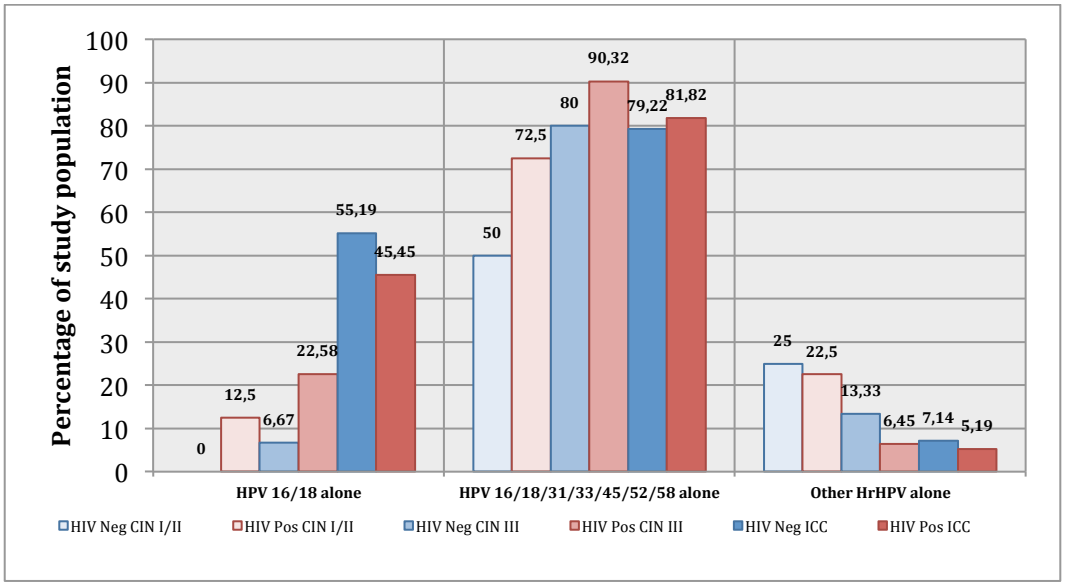


Figure 6.17: Potential preventative effect for HPV 16/18 vaccine and HPV 16/18/31/33/45/52/58 vaccine in different stages of cervical disease for HIV-negative and -positive women

6.6.2. Secondary prevention with HPV-based tests for CIN III or worse

Compared to normal cytology, a negative HrHPV test offers better reassurance against CIN III or worse. As testing for LrHPV does not predict CIN III or worse it should be omitted from cervical cancer screening.[14]

In 2013 Richter et al. detected HrHPV infections in 54.3% of South African women representative of the general population, irrespective of cervical abnormalities.[15] In the current study 46% of women with normal cytology tested positive for one or more high-risk type. Therefore, screening women in our population with HPV will detect a large number of women with positive HPV testing and normal cytology. It is, therefore, important to further identify women with HrHPV infections with a higher chance of developing CIN III or worse.

In the current study (as shown under heading 4) HPV 16, 18, and 35 were more prevalent in women with ICC than with normal cytology and HPV 33 equally present in ICC and normal cytology. HPV 16, 18, 31, 33, 35, 51, 52 and 56 were

more prevalent in CIN III than normal cytology, indicating a higher dysplastic potential of these viral types. Similarly to Guan at al., HPV 16, 18 and 45 were the only types more frequently detected in ICC than in women with CIN III.

In the meta-analysis by Guan et al., HPV 16, 18 and 45 were the only HrHPV types more prevalent in patients with ICC than in women with normal cytology.[6] These three types were also the only viral types detected more frequently in patients with ICC than CIN III, indicating the oncogenic potential of these high-risk viral types.

In a resource-poor setting it is necessary to consider alternative screening with selected HrHPV types to reduce the number of women with normal cytology and at low risk for future disease that will screen positive when all high-risk types are being screened for. Based on the findings set out immediately above a screening test to detect CIN III with a higher chance of progression to ICC and ICC should include at least HPV 16, 18, 35 and 45 and also possibly HPV 33 and 58.

6.7. CONCLUSION

The studied population of South African women differs significantly from published data from both developed regions and previously presented data from other regions from South Africa. The main difference is much higher prevalence of HrHPV and multiple type infection among all women but more so among HIV-positive subpopulation. We also described potential differences in the oncogenic importance of specific HPV types among immune depleted women never discussed before.

HPV type-specific differences in prevalence and in causing pre-invasive and invasive disease, as described previously, were confirmed for our country and are of huge importance in decisions regarding cancer prevention. Currently used vaccines (HPV 16/18) do not cover cervical cancer types sufficiently although up

to 65% of cases will be prevented. Nine-valent vaccines could prevent up to 80% of cases but will be improved by the addition of type 35. It is recommended that efforts for both vaccination and screening should be focused only on HPV alpha-9 and alpha-7 groups and firstly only on HPV 16, 18, 45 and 35.

6.8. FUTURE RESEARCH

A number of potential areas were identified for future research:

HPV type distribution in HIV-positive- and -negative women without cytological abnormalities. This information is important for better assessing the relationship between specific HPV types and diseases of the cervix, as this study did not have the HIV status of women included with normal cytology.

Comparing surface HPV with lesional HPV from CIN lesions with the use of laser-capture microdissection. This information is important for clarifying findings from chapters 3 and 4 on whether individual CIN lesions are caused by one or more HPV types.

Impact of vaccines on HPV epidemiology. The information on HPV types in vaccinated women is important for screening and following these women up in an appropriate way.

Cost analysis studies. The high prevalence of HrHPV types in women with normal cytology is important to consider before beginning HPV-based screening in this population and before investigating the possible impact screening might have on health care in South Africa.

6.9. REFERENCES

1. Forman D, de Martel C, Lacey CJ, Soerjomataram I, Lortet-Tieulent J, Bruni L, et al. Global burden of human papillomavirus and related diseases. Vaccine. 2012;30(Suppl5):F12-23.
2. De Vuyst H, Alemany L, Lacey C, Chibwesha CJ, Sahasrabuddhe V, Banura C, et al. The burden of human papillomavirus infections and related diseases in sub-Saharan Africa. Vaccine. 2013;31(Suppl5):F32-46.
3. Bruni L, Barrionuevo-Rosas L, Albero G, Aldea M, Serrano B, Valencia S, et al. ICO Information Centre on HPV and Cancer (HPV Information Centre). Human Papillomavirus and Related Diseases in the World. Summary Report 2015-04-08. [Data Accessed]
4. Bruni L, Barrionuevo-Rosas L, Albero G, Aldea M, Serrano B, Valencia S, et al. ICO Information Centre on HPV and Cancer (HPV Information Centre). Human Papillomavirus and Related Diseases in South Africa. Summary Report 2015-03-20. [Data Accessed]
5. Bruni L, Diaz M, Castellsagué X, Ferrer E, Bosch FX, de Sanjosé S. Cervical human papillomavirus prevalence in 5 continents: meta-analysis of 1 million women with normal cytological findings. J Infect Dis. 2010;202(12):1789-1799.
6. Guan P, Howell-Jones R, Li N, Bruni L, de Sanjosé S, Franceschi S, et al. Human papillomavirus types in 115,789 HPV-positive women: a meta-analysis from cervical infection to cancer. Int J Cancer. 2012;131(10):2349-2359.
7. van der Marel J, Berkhof J, Ordi J, Torné A, Del Pino M, van Baars R, et al. Attributing oncogenic human papillomavirus genotypes to high-grade cervical neoplasia: which type causes the lesion? Am J Surg Pathol. 2015;39(4):496-504.
8. Callegari ET, Tabrizi SN, Pyman J, Saville M, Cornall AM, Brotherton JM, et al. How best to interpret mixed human papillomavirus genotypes in high-grade cervical intraepithelial neoplasia lesions. Vaccine. 2014;32(32):4082-4088.

9. Li N, Franceschi S, Howell-Jones R, Snijders PJ, Clifford GM. Human papillomavirus type distribution in 30,848 invasive cervical cancers worldwide: Variation by geographical region, histological type and year of publication. Int J Cancer. 2011;128(4):927-935.
10. Bosch FX, Broker TR, Forman D, Moscicki AB, Gillison ML, Doorbar J, et al. Comprehensive control of human papillomavirus infections and related diseases. Vaccine. 2013;31(Suppl7):H1-31.
11. Mbulawa ZZ, Coetzee D, Williamson AL. Human papillomavirus prevalence in South African women and men according to age and human immunodeficiency virus status. BMC Infect Dis. 2015;15:459.
12. Clifford G, De Vuyst H, Tenet V, Tully S, Franceschi S. Effect of HIV infection in human papillomavirus types causing invasive cervical cancer in sub-Saharan Africa. Submitted for publication. (Under review)
13. Richter K. Implementation of HPV vaccination in South Africa. Public Health Association of South Africa. 2015 (cited 26 January 2016). Available from: https://www.phasa.org.za/implementation-hpv-vaccination-south-africa/
14. Thomsen LT, Frederiksen K, Munk C, Junge J, Castle PE, Iftner T, et al. High-risk and low-risk human papillomavirus and the absolute risk of cervical intraepithelial neoplasia or cancer. Obstet Gynecol. 2014;123(1):57-64.
15. Richter K, Becker P, Horton A, Dreyer G. Age-specific prevalence of cervical human papillomavirus infection and cytological abnormalities in women in Gauteng Province, South Africa. S Afr Med J. 2013;103(5):313-317.

ACKNOWLEDGEMENTS

First and foremost I want to thank my supervisor Professor Greta Dreyer. It has been an honour to be her first PhD student. I would not have been able to complete this thesis if it was not for the opportunities she created for me. She has been a tremendous mentor for me and her guidance and encouragement motivated me through difficult times. She has a passion for people and her desire to make a difference is contagious.

I would like to thank Professor Gerhard Lindeque and the members of the department of Obstetrics and Gynaecology for the incredible backing I received to perform this research. I would especially like to thank physicians and nurses who have been there to assist me when I recruited patients and collected data for my PhD thesis, as well as all the personnel at the different laboratories who were involved in testing samples to ensure accurate and reliable results.

I would like to thank Discovery Foundation for funding. Without their financial assistance this study would not have been possible. A special thank you to Professor Piet Becker for data analyses throughout this study.

I would like to thank my family and friends for all their unbelievable love and encouragement. For my parents, Marius and Ria, your prayers for me and unconditional love and backing were what sustained me thus far, and most of all, thank you to my loving, supportive, encouraging, and patient wife, Judy.

The patients of the gynaecologic oncology unit for participating in the various studies and to all women fighting cervical cancer, I dedicate this thesis to you.

Finally and most importantly I thank my God for carrying me through all the difficulties. I have experienced Your guidance every day and will keep on trusting You for my future. “I can do all things through Christ who strengthens me”.

PUBLICATIONS AND PRESENTATIONS

PRESENTATIONS

Carcinogenic and incidental HPV types in HIV positive VS negative SA women with cervical pre-neoplasia. Eurogin Congress. Lisbon, Portugal, 8-11 May 2011 – Oral presentation

Difference between HIV infected and HIV non-infected women with cervical pre-neoplasia: HPV serotypes associated with cervical intra-epithelial neoplasia. 14th Biennial Meeting of the International Gynecologic Cancer Society (IGCS). Vancouver Canada, 13-16 October 2012 – Oral presentation

A South African perspective on HPV type distribution in women without cytological abnormalities. University of Pretoria Faculty day 2013 – Poster presentation

Different oncogenic HPV types in HIV positive than negative South African women with cervical cancer. Eurogin Congress. Florence, Italy, 3-6 November 2013 - Oral presentation

Human papillomaviruses other than types 16 or 18 associated with more cervical cancer in HIV-infected South African women. AORTIC Conference, Durban, South Africa, 21-24 November 2013 - Poster presentation

HPV types associated with histologically confirmed CIN II/III among South African women with and without HIV. IGCS regional, Cape Town, South Africa, 16-18 May 2014 – Poster presentation

HPV type distribution in South African women with AIDS and normal cervical cytology. ESGO 2015, Nice, France, 24-27 October 2015 – Poster presentation

PUBLICATIONS IN PEER-REVIEWED JOURNALS FROM PHD STUDY

Van Aardt M, Dreyer G, Richter K, Becker P. Human papillomavirus-type distribution in South African women without cytological abnormalities: a peri-urban study. South Afr J Gynaecol Oncol. 2013;5(2):S21-S27.

Van Aardt MC, Dreyer G, Pienaar HF, Karlsen F, Hovland S, Richter KL, Becker P. Unique human papillomavirus-type distribution in South African women with invasive cervical cancer and the effect of human immunodeficiency virus infection. Int J Gynecol Cancer. 2015;25(5):919-925.

Accepted for publication

Van Aardt MC, Dreyer G, Snyman LC, Richter KL, Becker P, Mojaki SM. Oncogenic and incidental HPV types associated with histologically confirmed cervical intraepithelial neoplasia in HIV-positive- and –negative South African women. Accepted to S Afr Med J. Dec 2015

ETHICS APPROVAL

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

* **FWA** 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
* **IRB** 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.

Universiteit van Pretoria
University of Pretoria

Faculty of Health Sciences Research Ethics Committee
Fakulteit Gesondheidswetenskappe Navorsingsetiekkomitee

DATE: 6/10/2012

NUMBER	**189/2012**
TITLE OF THE PROTOCOL	The HPV type-related epidemiology and pathogenesis of cervical neoplasia in women with and without HIV-related immune depletion
PRINCIPAL INVESTIGATOR	Dr M.C. van Aardt **Dept:** Obstetrics and Gynaecology / Steve Biko Academic / University of Pretoria. **Cell:** 0827767818 **E-Mail:** mc@vanaardt.net
SUB INVESTIGATOR	Not Applicable
STUDY COORDINATOR	Not Applicable
SUPERVISOR	Prof. Greta Dreyer **E-Mail:** gretadreyer@mweb.co.za
STUDY DEGREE	PhD
SPONSOR COMPANY	Not applicable
CONTACT DEATAILS	Not Applicable
POSTAL ADDRESS	Not applicable
MEETING DATE	31/10/2012

The Protocol and Informed Consent Document were approved on 31/10/2012 by a properly constituted meeting of the Ethics Committee subject to the following conditions:

1. PhD Committee approval, and
2. The approval is valid for 4 years period [till the end of December 2016], and
3. The approval is conditional on the receipt of 6 monthly written Progress Reports, and
4. The approval is conditional on the research being conducted as stipulated by the details of the documents submitted to and approved by the Committee. In the event that a need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Members of the Research Ethics Committee:

Prof M J Bester	(female)BSc (Chemistry and Biochemistry); BSc (Hons)(Biochemistry); MSc(Biochemistry); PhD (Medical Biochemistry)
Prof R Delport	(female)BA et Scien, B Curationis (Hons) (Intensive care Nursing), M Sc (Physiology), PhD (Medicine), M Ed Computer Assisted Education
Dr NK Likibi	MBB HM – Representing Gauteng Department of Health) MPH
Dr MP Mathebula	(female)Deputy CEO: Steve Biko Academic Hospital; MBCHB, PDM, HM
Prof A Nienaber	(female) BA(Hons)(Wits); LLB; LLM; LLD(UP); PhD; Dipl.Datametrics(UNISA) – Legal advisor
Mrs MC Nzeku	(female) BSc(NUL); MSc(Biochem)(UCL, UK) – Community representative
Prof L M Ntlhe	MbChB (Natal) FCS (SA)
Snr Sr J Phatoli	(female) BCur(Eet.A); BTec(Oncology Nursing Science) – Nursing representative
Dr R Reynders	MBChB (Prêt), FCPaed (CMSA) MRCPCH (Lon) Cert Med. Onc (CMSA)
Dr T Rossouw	(female) MBChB (cum laude); M.Phil (Applied Ethics) (cum laude), MPH (Biostatistics and Epidemiology (cum laude), D.Phil
Dr L Schoeman	(female) B.Pharm, BA(Hons)(Psych), PhD – Chairperson: Subcommittee for students' research

Mr Y Sikweyiya	MPH; SARETI Fellowship in Research Ethics; SARETI ERCTP; BSc(Health Promotion)Postgraduate Dip (Health Promotion) – Community representative
Dr R Sommers	(female) MBChB; MMed(Int); MPharmMed – **Deputy Chairperson**
Prof TJP Swart	BChD, MSc (Odont), MChD (Oral Path), PGCHE – School of Dentistry representative
Prof C W van Staden	MBChB; MMed (Psych); MD; FCPsych; FTCL; UPLM - **Chairperson**

DR R SOMMERS; MBChB; MMed(Int); MPharmMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

♦Tel:012-3541330 ♦Fax:012-3541367 / 0866515924 ♦E-Mail: manda@med.up.ac.za
♦Web: //www.healthethics-up.co.za ♦H W Snyman Bld (South) Level 2-34 ♦ Private Bag x 323, Arcadia, Pta, S.A., 0007

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

19/09/2013

Approval Certificate
Amendment
(to be read in conjunction with the main approval certificate)

Ethics Reference No.: 189/2012

Title: The HPV type-related epidemiology and pathogenesis of cervical neoplasia in women with and without HIV-related immune depletion. Dept : Obstetrics and Gynaecology , University of Pretoria. Cell : 082 776 7818

Dear Dr M.C.van Aardt

The **Amendment** as described in the documents received on 22/08/2013 was approved by the Faculty of Health Sciences Research Ethics Committee on the 18/09/2013

Please note the following about your ethics amendment:
- Please remember to use your protocol number (189/2012) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committe may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics amendment is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

♦ Tel:012-3541330 ♦ Fax:012-3541367 Fax2Email: 0866515924 ♦ E-Mail: manda@med.up.ac.za
♦ Web: //www.healthethics-up.co.za ♦ H W Snyman Bld (South) Level 2-34 ♦ Private Bag x 323, Arcadia, Pta, S.A., 0007