Cape Peninsula
University of Technology

PREVALENCE OF HPV INDUCED LESIONS OF THE CERVIX AMONG GYNAECOLOGICAL CLINIC ATTENDEES IN NAMIBIA: ASSOCIATION OF RISK FACTORS AND CYTOMORPHOLOGIC FINDINGS

by

CHRISTO DELMé IZAAKS

Thesis submitted in fulfilment of the requirements for the degree

MTech: Biomedical Technology

in the Faculty of Health and Wellness Science

at the Cape Peninsula University of Technology

Supervisors: Prof. S. Khan
Prof. E.J. Truter

Bellville
Date submitted (February 2011)

Cape Peninsula
University of Technology

PREVALENCE OF HPV INDUCED LESIONS OF THE CERVIX AMONG GYNAECOLOGICAL CLINIC ATTENDEES IN NAMIBIA: ASSOCIATION OF RISK FACTORS AND CYTOMORPHOLOGIC FINDINGS

by

CHRISTO DELMé IZAAKS

Thesis submitted in fulfilment of the requirements for the degree

MTech: Biomedical Technology

in the Faculty of Health and Wellness Science

at the Cape Peninsula University of Technology

Supervisors: Prof. S. Khan
Prof. E.J. Truter

Bellville
Date submitted (February 2011)

DECLARATION

I, Christo Delmé Izaaks, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed	**Date**

ABSTRACT

Introduction: A prospective study was conducted across the spectrum of cervical aberrancies with the aim of assessing the distribution of HPV relating to the degree of cervical abnormalities using polymerase chain reaction (PCR) and $P16^{INK4A}$ assay as a marker for cervical disease progression. Patient demographics including their sexual, contraceptive and screening history were evaluated to determine whether subsidiary risk factors contribute towards the development of cervical lesions among Namibian women.

Methods: From Feb 2006 to March 2007, 187 women with abnormal cervical cytology were examined. Cervical smears were immunostained using the $P16^{INK4A}$ assay (Dakocytomation, Heidelberg, Germany). Brown discolourisation of the nucleus and/or cytoplasm of abnormal cells were considered positive for P16 immunoexpression. Absence of brown decolourisation in the nucleus or cytoplasm of abnormal cells was considered negative for P16 immunoexpression. DNA was successfully extracted from 182 specimens, and the respective samples were subjected to PCR using GP5+/6+ primers. Type-specific (HPV types 16 and 18) PCR were also applied. Patients' sociodemographics, sexual and reproductive history, HIV status, contraceptive use and Pap smear history were all recorded.

Results: Subject ages, number of partners, and age at first sexual encounter ranged from 15 to 49 years; one to 37 partners and 13 to 34 years respectively. Of the 187 cases, 60.4% expressed the P16 antigen. The distribution of epithelial lesions and P16 expression (recorded in brackets) was 28 (five) atypical squamous cells of undetermined significance (ASC-US), 96 (50) lower grade squamous intraepithelial lesion (LSIL), nine (seven) atypical squamous cells-cannot exclude HSIL (ASC-H) and 54 (51) higher grade squamous intraepithelial lesion (HSIL). Ninety-four percent of HSIL expressed P16. Fifty two percent of LSIL expressed P16. P16 expression declines from a peak 61% (25-34 year group) to a low 5% (45-49 year group). The presence of HPV-DNA by PCR was detected in 94.5% of the 182 samples. Type-specific PCR (HPV type 16 and 18) demonstrated a detection rate of 12.2% and 14.5% respectively. The main determinants of HPV-DNA in 172 patients were younger age and lifetime number of sexual partners. Younger age at first sexual encounter and HIV infection predominated in subjects that were HPV type 16 and/or 18 positive.

Conclusion: Using $P16^{INK4A}$ surrogate marker in conjunction with cervical cytology may prove valuable in identifying women who are at greater risk of developing cancer of the cervix.

Key words: Namibia, cervical cancer, $P16^{INK4A}$ stain, Human Papilloma Virus

ACKNOWLEDGEMENTS

I wish to thank:

- Our precious Lord and Saviour, Jesus Christ, for lavishing the determination and perseverance in writing this thesis.
- Prof S.Khan and Prof. E.J. Truter for their continued and steadfast support in writing this thesis.
- Cape Peninsula University of Technology (CPUT) for availing their facilities and resources for molecular testing.
- The postgraduates of the molecular laboratory at CPUT, in particular Michael Saayman, for his invaluable support.
- Prof W.Khan for her contribution towards this research.
- Namibia Institute of Pathology (NIP) for consenting to have the immunocytochemical testing done in the Histology department.
- Jurita van Wyk for donating a laptop on behalf of GlaxoSmithKline.
- The Cancer Association of Namibia for funding this research.
- The nursing staff at the various health clinics and health care centres in Windhoek for collecting and interviewing the patients.
- The staff of the Health Science department of the Polytechnic of Namibia for their supportive role.
- The National Research Foundation (NRF) for further funding of this research.
- My dear wife, Madelyn, for her inspiration.

The financial assistance of the National Research Foundation towards this research is acknowledged. Opinions expressed in this thesis and the conclusions arrived at, are those of the author, and are not necessarily to be attributed to the National Research Foundation.

DEDICATION

I wish to dedicate the thesis to my lovely wife, Madelyn, who has been so supportive and understanding. Without her loyal support this manuscript would not have been possible.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GLOSSARY

AdCa	Adenocarcinoma
AGUS	Atypical glandular cells of undetermined significance
AIDS	Acquired immunodeficiency syndrome
ALTS	ASC-US/LSIL triage study
AP-1	Activator protein 1
ART	Antiretroviral therapy
ASCCP	American Society for Colposcopy and Cervical Pathology
ASC-H	Atypical squamous cells, cannot rule out HSIL
ASC-US	Atypical squamous cells of undetermined significance
CC	Cervical carcinoma
CDK2	Cyclin-dependant kinase 2
CDK4	Cyclin-dependant kinase 4
CDK6	Cyclin-dependant kinase 6
CDKI	Cyclin-dependant kinase inhibitor
CI	Confidence interval
CIN 1	Cervical Intraepithelial Lesion grade I
CIN II	Cervical Intraepithelial Lesion grade II
CIN III	Cervical Intraepithelial Lesion grade III
CIS	Carcinoma in situ
COR	Crude odds ratio
DMPA	Depot medroxyprogesterone acetate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
HAART	Highly active antiretroviral therapy
HCG	Hyperchromatic crowded group
HPV	Human papilloma virus

HR-HPV	High risk Human Papilloma Virus
HSIL	Higher grade squamous intraepithelial lesion
HSV	Herpes simplex virus
LBC	Liquid based cytology
LCR	Lower control region
LEEP	Loop electrosurgical excision procedure
LSIL	Lower grade squamous intraepithelial lesion
NF-1	Nuclear factor 1
NIP	Namibia Institute of Pathology
NPV	Negative predictive value
ORF	Open reading frame
PCR	Polymerase chain reaction
PPV	Positive predictive value
pRb	Retinoblastoma protein
RNA	Ribonucleic acid
SCC	Squamous cell carcinoma
SIL	Squamous intraepithelial lesion
SOP	Standing operating procedure
STI	Sexually transmitted infection
TAE	Tris-acetate-EDTA
TBS	The Bethesda system
TSNA	Tobacco-specific *N*-nitrosamine
WHO	World Health Organisation

CHAPTER ONE
INTRODUCTION

Human Papilloma virus (HPV) is a major etiological agent in cancer of the cervix and is the second biggest cause of mortality due to cancer among females worldwide. Approximately 510 000 cases of cervical cancer are reported each year with almost 80% occurring in developing countries; 68 000 in Africa, 77 000 in Latin America and 245 000 in Asia (World Health Report, 2001). According to the World Health Organisation (WHO), the estimated deaths related to cervical cancer amount to 288 000 annually (World Health Report, 2001).

Previous epidemiological and molecular studies over the past two decades have convincingly demonstrated that high-risk HPVs and in particular HPV types 16 and 18 are a necessary but not sufficient cause of cervical neoplasia (Lazcane-Ponce *et al*., 2001). Many women, are however unaware of what exactly HPV is and its relationship to cervical cancer. Conversely, additional co-factors such as number of sexual partners, age at first sexual encounter, HIV status, oral contraceptive use and the male partners' sexual behaviour may further contribute to the development of cervical cancer. Many of these "additional co-factors" such as number of sexual partners, male sexual behaviour and young age at first sexual encounter are surrogate markers for HPV-not independent risk factors and make the acquisition of high-risk HPV more likely. Human Papilloma virus types 16 and 18 account for 70% of cervical cancers worldwide, however, Ferlay *et al*., (2004) found that in sub-Saharan Africa, HPV 16 and 18 accounted for 64% of cervical cancers, while HPV 45 accounted for almost 14%. In Central and South America, HPV 16 and 18 accounted for 65% of cervical cancers, and HPV 31 was found to account for over 7%. Winer *et al*., (2005) found that based on 42 studies conducted in 22 countries, the presence of HPV 16 and 18 in cervical cancer is 10% less in developing countries than in developed nations.

Most HPV infections of the cervix including oncogenic types regress spontaneously, especially among younger women. However, occasionally lesions do not regress and may advance to malignant progression with associated widespread metastasis (Lazcane-Ponce *et al.,* 2001). Fortunately, cervical cancer is a rare outcome of HPV infection among women, particularly if they are regularly screened for cancer using the Papanicolaou smear (Pap smear) and have appropriate follow up examinations for abnormalities. However, the likelihood of progression to cancer is higher and the time to progression shorter as the grade of dysplasia increases (Barron and Richardt, 1968). Yet the average time course from Cervical Intraepithelial Neoplasia (CIN) 3, (also known as severe dysplasia, a condition where precancerous or dysplastic changes are confined to the full thickness of the cells covering the cervix with no spread of disease) to invasive cancer, averages between 8.1 and

12.6 years (American College of Obstetricians and Gynaecologists, 2005). The detection of such neoplasia allows intervention procedures to prevent early invasive cervical cancer and thus reduces mortality.

Namibia, a small country situated in the South Western part of Africa has a high incidence of cervical cancer. Statistical data obtained from the Dr. AB May Cancer Centre in Windhoek, Namibia indicated that cervical cancer, as the fifth most common cancer in Namibia, is preceded only (in descending order) by non-Kaposi sarcoma skin cancers, Kaposi sarcoma, breast cancer and conjunctival cancer. With a population of just more than 2 million people (females 51.2%, males 48.8%), and with cervical abnormalities that is preventable, one cannot imagine that cervical cancer would be one of the leading cancers among women in Namibia. However, research has shown that the implementation of a well organised cervical screening programme significantly reduces the incidence and mortality of cervical cancer by up to 60% (Laara *et el*., 1987).

A presumptive cervical screening initiative (Pap smear screening) was unofficially introduced in all public health facilities in 1992 by the Health Ministry-Laboratory Services in Windhoek, and has since then demonstrated a reduction in the incidence and mortality of cervical cancer in Namibia. The inaccessibility of the people of Namibia to health care centres, particularly those who reside in rural areas, has further led to a primary health care approach being adopted in 1992 with the aim of making health care available to all Namibians. As one of the key challenges in the health care approach, the reproductive health policy which, as one of its output objectives focuses on the early detection of reproductive system cancers including cervical cancer (Pap smear screening) among women in Namibia, was launched. Despite the reduction in the incidence of cervical cancer, the rate of precancerous lesions of the cervix continued to increase due to a lack of well-delineated criteria with reference to the group of women eligible for cervical screening. This was further augmented by poorly constructed management regimens in terms of treatment and recommended follow-up in patients who presented with cervical precancer. Consequently, each health facility collects Pap smears based on what they think is feasible to them. Due to these limitations, the incidence of cervical disease, in particular the precancerous stages, continued to increase during this ten year period (1992-2002). As a result, the reproductive health segment was officially enforced in 2002 (National Policy for Reproductive Health, 2001). As part of the cancer prevention programme among women, health officials and in particular nurses who were not exposed to the Pap smear collection technique during their in-service-training, received special training on how to collect cervical smears. Unfortunately, in spite of the fact that the Pap smear collection technique was introduced into the current nursing curriculum, a considerable number of ‘’not representative’’ Pap smears are still being received. This further reduces the clinical sensitivity of the Pap smear in detecting cervical precursors, the effect,

which diminished the likelihood of a significant decline in cervical precancer and cancer. A standardised system for reporting cervical/vaginal cytology known as the Bethesda nomenclature of 1991 (Broder, 1992) combined with the Cervical Intraepithelial Neoplasia (CIN) classification was employed in this study. Following this classification, cervical smears were further reclassified using the present Bethesda terminology of 2001. Nursing personnel who are generally not well acquainted with the significance and importance of cervical cytological diagnosis, further failed to inform women who presented with cytologically diagnosed abnormal Pap smears of the appropriate recommendations. These women thus had no recommended follow up and this resulted in severe lesions which progressed to severe cervical disease. Patients also failed to collect their cytology results as required by their consulting physicians. This resulted in the progression of cervical lesions which eventually lead to invasive cervical cancer.

Individuals diagnosed with any type of cancer based on diagnostic and/or histological test results are referred to the Dr. A.B. May Cancer Centre situated in Windhoek for further diagnostic procedures and if necessary, palliative treatment. Statistical data that was retrieved from the Namibia Institute of Pathology (NIP) database indicated a steady increase in reported cases demonstrating cervical lesions ranging from CIN 1 with/without HPV, CIN 2 with/without HPV, CIN 3 with/without HPV and cervical cancer as 48, 77, 81 and 129 cases reported respectively in 2005 to 94, 90, 179 and 125 reported cases in 2006. These numbers are based on cytological assessment confirmed by histology. Statistics obtained from the database of Pathcare Laboratories and NIP indicated the following number of cases (bracketed) for 2006 which are solely based on cervical cytology: CIN 1 with/without HPV (525), CIN 2 with/without HPV (378) and CIN 3 with/without HPV (260). It can be argued that these huge discrepancies demonstrate that both cytology and histology are rather subjective parameters and a need for more sensitive and specific diagnostic tools is warranted.

Since 1993, cervical cancer has been considered to be an “AIDS-defining” disease in someone who tests sero-positive for Human Immunodeficiency virus (HIV), as this is one of the cancers that tend to occur at an increased rate in HIV infected patients. A recent study done by Parham *et al*., (2006) among 150 Zambian women found that 76% of women presenting for Anti Retroviral (ARV) treatment and who were infected with HIV, harboured cervical cell abnormalities of which 20% were found to be squamous cell carcinomas (SCC). Concomitantly, these figures may not be a true representation of truly abnormal lesions of the cervix, as most women may die of AIDS related disease (leading cause of mortality among people aged between 16-49 years in Namibia) before a cervical lesion can develop (Health Information Report of Namibia, 2001). Furthermore, patients who were too sick to obtain medical attention and those who succumbed to the disease in rural areas may have

failed to receive appropriate treatment, thus the true incidence of cervical cancer and its precursors may be much higher than has originally been anticipated.

As there is no national cervical screening programme in place in Namibia with particular reference to screening guidelines, cervical disease prevalence has indicated an increase. Based on guidelines set by the WHO, an effective cervical screening programme should include screening of at least 80% if not all of the women in the target population within a certain time period (10 years has been suggested). The selection for the target population is based on the incidence of cervical disease by age within groups and understanding the natural history of cancer of the cervix within these groups (Miller, 1992). The target population in the Namibian setting however has yet to be defined.

Figures for the number of women who have had a Pap smear performed have been obtained from the Namibia Institute of Pathology (NIP) and Pathcare databases. The two laboratories where Pap smear assessments are performed have indicated that an estimated 28 764 (4.6% x 10 years) women presented for Pap smear surveillance in 2006 and showed a gradual increase to 31 678 (5.1% x 10 years) in 2007 in women 15 years of age and older. This represents a small proportion of women who are in their reproductive years (620 000) and as such the current cervical screening initiative in Namibia is considered to be unsuccessful due to the aforementioned limitation. Consequently, cervical cancer incidence has not indicated a decrease and it is becoming evident that younger women in Namibia present with increasing numbers of cytologically diagnosed abnormal Pap smears. While the Pap smear is not designed to detect HPV but cervical abnormalities, these changes may indicate an underlying HPV infection. Carter *et al*., (2000) found that only 55%-70% of women with proven genital HPV infection show a detectable antibody response to the virus. This indicates that more than 30% of cervical lesions induced by HPV may persist and ultimately lead to cervical cancer.

Despite the success of a well organized cervical screening programme in developed countries, a considerable number of cervical cancers and precursor lesions still occur in women who have been adequately screened (Bosch and Harper, 2006). This indicates that the Pap smear has limitations with regard to sensitivity (the percentage of “true-positive” cases that are detected by the screening test) and specificity (the percentage of "true-negative" cases that are negative by the screening test) (Nanda *et al*., 2000).

Moreover, a high degree of intra-observer (differences in interpretation by an individual making observations of the same phenomenon at different times) and inter-observer (difference in the interpretation between different individuals performing the same visual task) variability associated with cytological and histological interpretation of precancerous lesions

as well as invasive cancer is a reality which exists. Nanda *et al*., (2000) concluded that the Pap smear has a sensitivity and specificity of 51% and 98% respectively, in detecting any grade of cervical intraepithelial lesion. This results in repeated cytological analysis, high medical cost and unnecessary invasive clinical procedures.

As there is now considerable evidence that HPV is the major etiologic agent in cervical cancer in spite of the fact that a high percentage of healthy women infected with HPV may never develop a lesion, more objective and clearly decisive biomarkers need to be implemented to improve detection of HPV related disease. In view of this, Human Papilloma virus can thus be used as a unique marker for identifying patients at risk for cervical neoplasia (Bibbo *et al*., 2002) i.e. HPV testing detects infection and not necessarily disease.

This study will include the use of a biomarker $P16^{INK4A}$ which has a high sensitivity and specificity particularly in borderline lesions of the cervix. Diagnostic adjuncts such as *p16*, a tumour suppressor gene that is expressed as a result of disruption of the pathways of *p53* and the product of the retinoblastoma gene (*pRb*) by high-risk Human Papilloma virus (HPV) oncoproteins E6 and E7, might have clinical value in detecting cervical lesions where expression of HPV oncogenic activity has taken place.

A molecular diagnostic assay such as polymerase chain reaction (PCR) is another approach for HPV detection. This technique is based on the detection of HPV DNA and can be used to identify specific HPV types. Polymerase chain reaction (PCR) analysis has a high sensitivity and is considered a useful tool for predicting which women with a history of high grade squamous intraepithelial lesions (HSILs) are at a greater risk of recurrence. Cuzick *et al*., (2006) suggested that if HPV DNA testing is used in conjunction with cytology, particularly in women 30 years and older, the relative insensitivity of a single Pap smear increases drastically. Its use as a primary screening parameter has also been suggested, with the Pap smear reserved for women who tested HPV positive however, this is controversial because of its low positive predictive value in younger women (<30 years old).

Finally, no studies have reported on the prevalence and determinants of HPV induced lesions of the cervix in Namibia. It is the goal of this study to use a specific marker that is able to identify cells in the early stages of the transformation process initiated by HPV integration. In this way, we would be able to determine the prevalence rate of HPV induced cervical lesions and the associated risk factors among Namibian women attending gynaecological facilities in Windhoek.

1.1 Research Objectives

The principal aims of this study were:

1) to determine the most probable factors linked to the higher prevalence of HPV induced lesions of the cervix using a questionnaire (see Appendix A);
2) to compare data obtained from this study with data where $P16^{INK4A}$ was used as a routine screening tool;
3) to investigate the possibility of using $P16^{INK4A}$ surrogate marker in addition to conventional Pap smear screening for the early detection of HPV induced lesions of the cervix in Namibian women thereby decreasing the risk of developing cervical cancer;
4) to determine the prevalence rate of cervical HPV infection among women in Namibia using PCR, thereby assessing the sensitivity and specificity of $P16^{INK4A}$ immunocytochemistry as a routine screening tool;
5) to improve the cytological detection of borderline lesions of the cervix such as ASC-US and LSIL, so as to facilitate aggressive management strategies in reducing the progression of cervical lesions.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Human Papilloma virus is a common sexually transmitted virus which causes infection in women and it has been estimated that at least 50% of adults have had a genital HPV infection at some stage of their reproductive life. The prevalence of HPV infection characteristically peaks soon after sexual activity (usually among women in their early 20's) with an infected partner and is associated with the number of sexual partners and other markers of sexual activity such as age at first sexual encounter, potential years of sexual activity and positive HIV status (Lazcano-Ponce *et al.,* 2001). The majority of infections are asymptomatic and transient, with the immune system clearing the detectable virus over time. However, in a small number of cases and usually over many years, HPV can cause changes in the cells of the cervix which can lead to cancer. In some studies, the average duration of detectable HPV infection has been noted to range from 8 to 13.5 months respectively for non-oncogenic (not giving rise to tumours or causing tumour formation) and oncogenic (having the potential to transform normal cells into tumour cells) types (Franco *et al*., 1999). Women with certain genetic and immune system co-factors may be more likely to harbour persistent high risk HPVs and are therefore at an increased risk for developing more severe and rapidly progressive cervical lesions (Herbert and Coffin, 2008).

Koutsky, (1997) found that up to 79% of women worldwide will be infected by HPV at some point in their life, 60% with short-term infection (detection of antibodies), 10-20% with persistent infection (detection of DNA), 1% with genital warts, and 4% will show cellular abnormalities associated with HPV infection. For most populations of women with mixed age groups, prevalence of HPV infection has been estimated at 5% to 15%, with HPV 16 found to be the most prevalent type worldwide (Jastreboff and Cymet, 2002). In a study of women aged between 18 to 40 years (among whom the prevalence of HPV infection was 39%) with no history of high-grade cervical intraepithelial neoplasia (CIN), high-risk HPVs were found to be more common (occurring in 26.7% of women) than low-risk HPVs (occurring in 14.7%) (Peyton *et al.,* 2001).

2.2 Morphology of Human Papilloma Virus

Human Papilloma virus belongs to the Papovaviridae family and contains a double stranded circular DNA genome with 7800-7900 base pairs (bp), a non-enveloped virion and an icosahedral capsid (Figure 1).

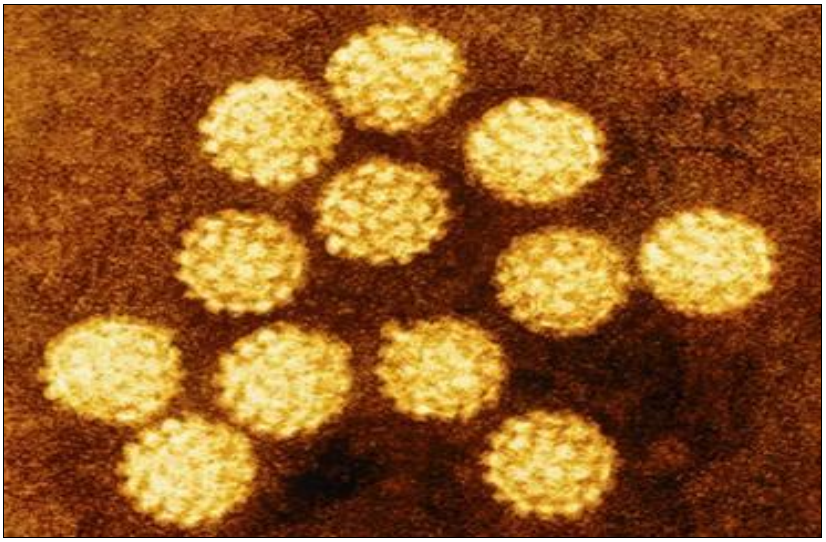

Figure 1: Colourised transmission electron micrograph of the HPV capsid (http://web.uct.ac.za/depts/mmi/stannard/papillo.html)

It is a relatively small (55 nm diameter) non-encapsulated virus of which the icosahedral capsid consists of 72 capsomers and a minimum of two capsid proteins, L1 and L2. The HPV genome (Figure 2) is split into three distinct regions namely, the non-coding long control region (LCR) or the upper regulatory region (URR), the early (E) gene region and the late (L) gene region. The long control region (about 400-1000 basepairs) contains converging binding sites that are important in the transcription of HPV, including activation of transcription factors AP-1 (activator protein) and NF-1 (nuclear factor). The LCR ensures that transcription occurs from the early and late regions, thus regulating the production of viral proteins and particles. The early region contains six open reading frames (ORF's); E1, E2, E4, E5, E6 and E7. These ORF'S are involved in viral replication and oncogenesis and encode all viral proteins except for the viral capsid proteins, which are encoded in the late region. The L1 and L 2 genes (in the late region) encode the major and minor capsid proteins, both of which are essential in the viral life cycle and responsible for encapsulating the virus (Jo and Kim, 2005).

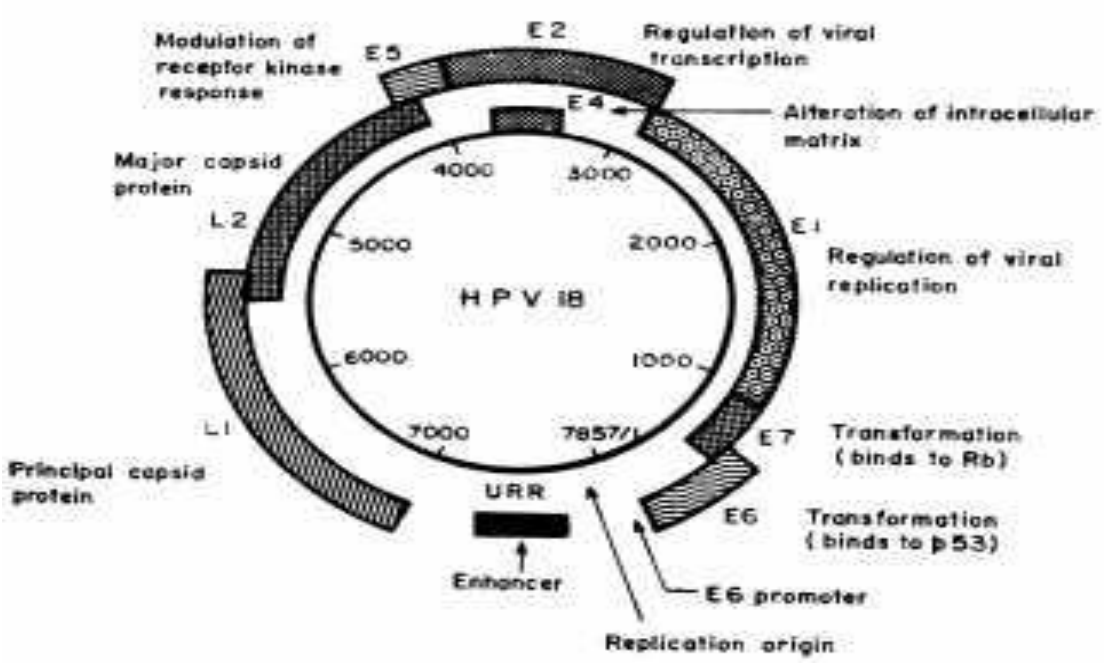

Figure 2: Diagrammatic representation of the circular HPV DNA genome (De Boer *et al*., 2005)

2.3 Classification of Human Papilloma Virus

More than 100 types of HPV have been identified, of which a third of these are associated with sexually transmitted genital infections (Koutsky and Kiviat, 1999). These viruses are further divided into two groups namely, the high risk (HR) (oncogenic) HPV viruses of which the most prevalent are HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56 with a high prevalence of HPV type 16 and the low risk (LR) (non-oncogenic) types which include HPV 6b, 11, 13, 41, 42, 44, 53, 54, 58, 61, 62, 66, 69, and others. Most of these viral infections are present for a short period of time and clear spontaneously over time however, as little as 20% of healthy women harbour HR-HPV and a small percentage may develop clinically significant dysplastic lesions or even cancer of the cervix. A study by Ho *et al*., (1998) to determine the natural history of genital HPV infection in college women, suggested that isolated incidences of HPV infections were detected even when no symptoms were present.

2.4 Transmission of Human Papilloma Virus

Human Papilloma viruses are transmitted sexually by direct skin-to-skin contact with an infected individual and can infect the epithelial cells in both mucosal surfaces and the skin. This potentially causes a range of epithelial proliferative lesions ranging from benign lesions, such as genital warts, to cancer. The virus is usually transmitted through vaginal, oral, or anal sexual contact and transmission can occur whether warts are present or not (McDermott-Webster, 1999). As genital HPV infections usually exhibit no clinical symptoms, the virus is frequently transmitted unknowingly. Human Papilloma viruses can remain dormant in the body for a considerable time, where no symptoms may appear for months or

even years after infection. It is believed that the virus gains access through defects in the epithelium that expose the basal epithelial cells to virion particles, thus holding the cell hostage. The virus can also be transmitted from mother to infant during childbirth, where the baby is born with subsequent formation of warts in the throat or voice box, however, this is a rare outcome of the infection (Jay and Moskicki, 2002). Sonex *et al*., (1999) have found the presence of HPV in finger nail brushings of patients with genital warts, indicating that it could be transmitted by finger-genital contact.

2.5 Mechanism of Human Papilloma Virus infection

Human Papilloma viruses are double-stranded DNA viruses that infect the squamous epithelium of the cervix and in particular the squamous columnar junction of the transformation zone. The transformation zone contains multipotent cells that by virtue of their anatomy and physiology render them susceptible to the virus. Human Papilloma virus infection may also be found in the larynx, anus, oesophagus, subungual mucosa (nail bed) and the conjunctiva (Kufe *et al*., 2003). The interval from exposure to development of a lesion may take a few weeks to several months, and even longer. The virus can gain access through an abraded area in the epithelium which exposes the basal layer to virus particles. Infection is generally stimulated by integrins which are cell surface receptors that mediate cell to cell attachment, cell to extracellular matrix attachment as well as signalling within the cell. As the cells containing the viral deoxyribonucleic acid (DNA) approach the top layers of the epithelium, the virus replicates and viral proteins are expressed in the upper layers of the stratum spinosum and stratum granulosum of the squamous epithelia (Stanley, 2002).

Some of the superficial epithelial cells in the condylomatous lesions (genital warts) characteristically display enlarged, hyperchromatic nuclei, with or without cytoplasmic halos, and the mature virus usually targets this cell population (Figure 3).

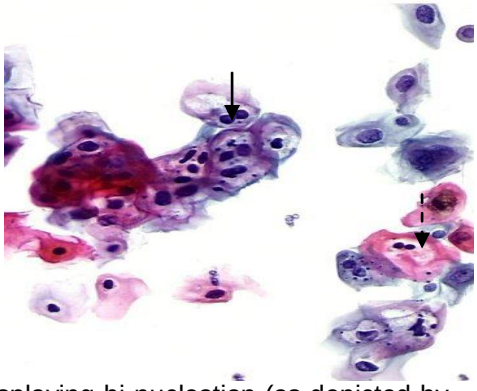

Figure 3: Typical koilocytes displaying bi-nucleation (as depicted by →) and perinuclear clearing (as depicted by - - ▶) in condylomata acuminata of the cervix (Frappart *et al*., 2003)

Disruption of the cell-cycle control mechanism in infected cells results in the expression of proteins such as KI-67, a cellular marker for cell growth. Evidence also exists that HPV

infection may occur in germinal or undifferentiated epithelial cells that give rise to both squamous and columnar components of the cervical mucosa (Hausen, 1999).

2.6 Replication of viral proteins

The viral proteins which are expressed early in HPV infection include E1, E2, E6 and E7. Initially, HPV binds to and infects the epithelial stem cells (basal cells) of the squamous epithelium where cells have lost their capacity to replicate. As a result, the viral genome becomes involved in the cell's genetic functions in order to compensate for the missing cellular functions that are necessary for viral DNA replication (Pan and Griep, 1994). The four aforementioned viral proteins are produced as the basal cells divide and begin to migrate towards the outside of the mucosa until it reaches the superficial layer of the mucosa. The viral replication protein E1, binds at the site where viral replication took place and controls replication of the DNA molecule, eventually resulting in the dissociation of the DNA strands (Iftner and Villa, 2003). The protein E2 on the other hand, is important for both viral replication and transcription, and alteration of this gene by viral integration or mutation results in intense transcription of the E6 and E7 oncogenes (Iftner and Villa, 2003). E7, as one of the major gene products, binds to and renders the retinoblastoma tumour suppressor protein (pRb) inactive, subsequently resulting in the amplification of the cell-cycle. It blocks expression of pRb, thereby releasing E2F transcription factors, promoting host cell and viral DNA synthesis. It further interacts with the CDK2 (cyclin-dependant kinase-2) protein, resulting in the prevention of cell cycle progression. Lastly, E6 covalently binds to the p53 protein where, through interaction with ubiquitin ligase the damaged portions of this complex are degraded by proteasomes. It further interacts with other cellular proteins and instigates telomerase activation leading to an indeterminate cell division. Thus, HPV E6 and E7 genes seem to have concomitant functions, the latter being the enhancer of DNA replication and cell growth, while E6 counteracts apoptotic cell death and ascertaining continuity of badly damaged and mutated cells. As a result, cells are free to divide, eventually leading to the stratification of the skin which is characteristic of a genital wart. Moreover, HPV defers the production of the L1 and L2 capsid proteins until the skin cells have finally matured, the latter exfoliates and thus become inaccessible to immune cells (Kufe *et al*., 2003). An immune response against L1 and L2 proteins takes time to appear, and does not occur in all individuals infected by HPV.

Malignant transformation of cervical epithelial cells occurs when integration of the circular HPV DNA into the host cell DNA has disrupted the E2 gene, resulting in E6 and E7 overexpression. Expression of the E2 gene ceases after viral integration and the specific gene is split into two equal parts. Since E2 inhibits E6 and E7, the lack of E2 after integration

results in exceptionally high levels of E6 and E7 proteins, leading to a high proportion of unbridled cell division as can be seen in cervical cancer (Iftner and Villa, 2003).

2.7 Detection of Human Papilloma Virus infection

Genital HPVs infect the cervix, leading to a variety of lesions that range from genital warts (condylomata acuminata), lower grade squamous intraepithelial lesions (LSILs) which is a lesion that is confined to the lower/basal third of the squamous epithelium, higher grade squamous intraepithelial lesions (HSILs) which is a lesion where abnormal cells occupy at least the lower third of the squamous epithelium and cervical cancer (CC). The former is usually associated with HPV types 6 and 11, albeit the low risk type (Jay and Moskicki, 2000), whereas the low and high grade CIN lesions are associated with both the low and high-risk HPV types.

Condylomata acuminata are overt lesions and appear as soft, moist, pink or flesh-coloured swellings usually observed in the genital area. They can be elevated or flat, single or multiple, small or large and sometimes cauliflower shaped that extends into fingerlike projections (papillae). These lesions can also appear on the vulva, in or around the vagina (Figure 4) or anus, on the cervix, and on the penis, scrotum, groin or thigh. These warts are usually painless, but they may cause itching or irritation. After sexual contact with an infected person, genital warts may appear within weeks or months or may not appear at all.

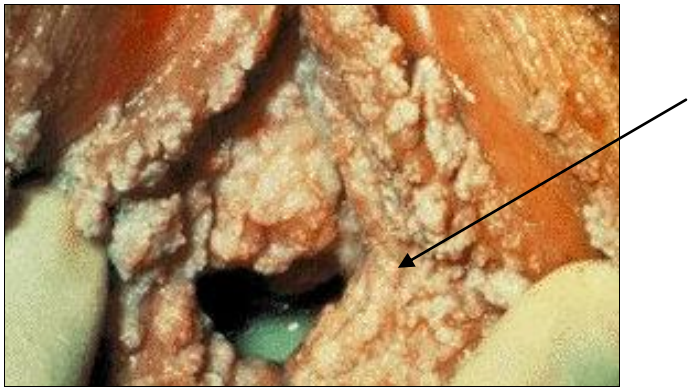

Figure 4: Clinical manifestation of condylomata acuminata (as indicated by the arrow) of the vulva (http://www.manbir-online.com/std/hiv.25.htm)

Flat condylomata present as non-papillary lesions, are more difficult to detect and may be readily seen only after swabbing with acetic acid and colposcopic examination, in which they appear as white, flat, shiny lesions (Figure 5). Flat condylomata appear to be characteristic of high grade intraepithelial neoplasia and even invasive cancer.

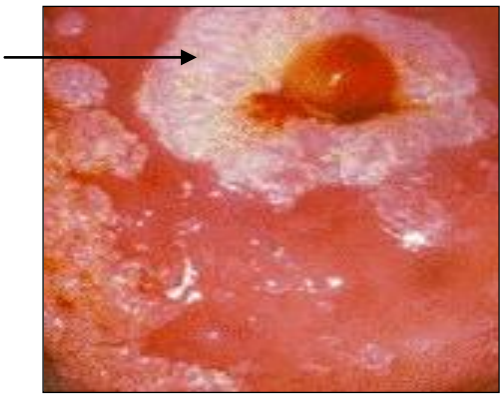

Figure 5: Representation of condylomata acuminata of the cervix (as indicated by the arrow) after acetic acid swabbing (http://www.manbir-online.com/std/hiv.25.htm)

The Papanicolaou (Pap) smear, which involves microscopical analysis of stained exfoliated genital cells, detects koilocytosis and other signs of HPV related disease (Figure 3).

The Pap smear is not sensitive enough and may not detect cancers in some women who present with abnormal cervical lesions. Nanda *et al.,* (2000) found that only 20%-50% of women with precancerous lesions are correctly identified. It is therefore important that the Pap smear is repeated as recommended by the American Society for Colposcopy and Cervical Pathology (ASCCP) to ensure that precancerous cells are not missed.

Low grade abnormalities on cervical cytology represent acute HPV infection. The cytopathologic effect of this viral infection can be seen in the squamous epithelial cells of the cervix and include cellular characteristics such as koilocytosis, nuclear atypia, delayed maturation; hyperkeratosis and parakeratosis (Sedlacek, 1999). Woodman *et al.*, (2001) found that 20%-33% of women who test positive for HPV infection (using polymerase chain reaction (PCR) amplification), will eventually develop abnormal cervical cytology. High grade cervical intraepithelial lesions are characterised by squamous epithelial cells displaying nuclear crowding, pleomorphism, loss of polarity, presence of mitotic figures as well as an increase in the number of abnormal cells as compared to LSILs.

Visual screening of the cervix depends on the interpretation of the cervix with the naked eye. An acetic acid solution is applied to the cervix which results in the white colourisation of tissue and abnormal cells when exposed to the solution (Figure 6). Visual inspection after swabbing with Lugol's iodine, also known as Schiller's test is another approach where colourisation of precancerous lesions of the cervix appears as well-defined, thick, yellow-or brown-shaded areas (Sankaranarayanan *et al*., 2003).

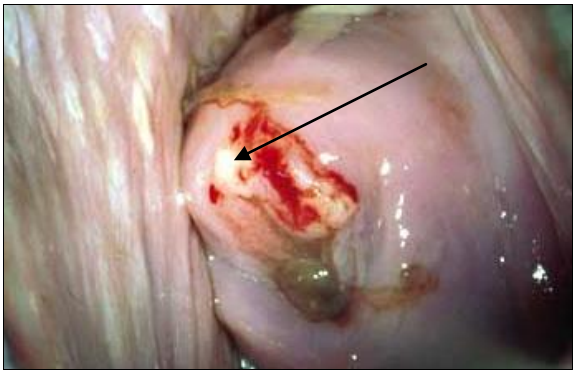

Figure 6: Acetowhite lesion of the cervix illustrating a biopsy confirmed CIN 2 lesion (as indicated by the arrow) (Sankaranarayanan *et al*., 2003)

A technique currently being investigated in cervical cytology is the use of immunocytochemical and biological markers that can be used concurrently with conventional cytology or even replace it. Such markers should be able to maintain a very high sensitivity and specificity and could be considered to be the key to identify cervical lesions that have been truly induced by HPV and in particular HR-HPVs. The cellular surrogate marker $P16^{INK4A}$, which utilises the tumour suppressor gene *p16*, not expressed in normal non-transformed cells but rather expressed by host cells due to viral oncogene expression, seems to be an important tool in gynaecologic cytology (Bibbo *et al*., 2002).

The most sensitive and specific method for detecting HPV infection is HPV DNA by either PCR amplification or hybrid capture, the latter which is based on the formation of hybrids between HPV DNA and ribonucleic acid (RNA) probes and is designed to detect high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, or 68) HPV types. Results are reported as either positive or negative and individual HPV types are not identified. Polymerase chain reaction (PCR) amplification for HPV DNA detection relies on the identification of HPV viral DNA. This technique is able to detect more true-positive precancerous lesions among women in their 30s and 40s as compared to the Pap smear (Schiffman *et al*., 2000).

There are two ways in which to perform the PCR in order to detect HPV DNA namely type-specific PCR or broad-spectrum PCR. With type-specific PCR, primers are chosen that will only amplify a single genotype of HPV. Broad-spectrum primers, GP5+/6+ are able to detect 20 different types of HPV (Iftner and Villa, 2003). Human Papilloma virus positivity has a very high sensitivity in demonstrating high grade cervical lesions however, the positive predictive value is relatively poor especially in younger women in whom the infection is very common. The technique's high sensitivity and its ability to produce consistent and reliable results make it a very promising screening tool.

2.8 Identifying progressive precancerous lesions

An elevated expression of the E6 and E7 oncogenes is affiliated with HSIL and CC and their effect can result in uncontrolled cell growth (Hausen, 1999). Demobilization of retinoblastoma protein (pRb) through HR-HPV E7 leads to the overexpression of the cyclin dependant kinase inhibitor (CDKI) P16 (Sano *et al*., 1998). Using specific monoclonal antibodies and $P16^{INK4A}$ immunostaining, Klaes *et al*., (2001) demonstrated overexpression of P16 specificity in CIN lesions associated with high risk (oncogenic) HPV and in CC. No P16 expression was observed in normal and inflammatory lesions of the cervix. Klaes *et al*., (2001) further found strong P16 immunoexpression in 60% of LSIL lesions, except for those associated with low-risk HPV types, and in all cases of HSIL. Human Papilloma virus DNA was detected in 56% of CIN 2 lesions whereas HR-HPV was detected in 85% of CIN 3 lesions and in 88% of CC lesions. Low risk Human Papilloma viruses (LR-HPVs) were not detected in any of these advanced lesions. Bibbo *et al.,* (2002) found P16 expression in 73% of cases in women diagnosed with LSIL and in 96% of cases in women diagnosed as HSIL. These findings are encouraging however the robustness and sensitivity of the assay is still to be established. The value of such an assay is that it is able to measure HPV gene expression, unlike the HPV DNA assay that is only able to detect the presence of viral genomes and not their activity.

2.9 Prevention of Human Papilloma Virus infection

The surest way to eliminate the risk for acquiring genital HPV infection and associated diseases is sexual abstinence or lifelong monogamy. However, it is difficult to determine whether a partner who has been sexually active in the past, is currently infected. An important preventative measure women can take to protect themselves from developing CC or precursor lesions, is to have regular Pap smear evaluations (Janicek and Averette, 2001). Avoiding skin to skin contact with an infected individual has also proved to be an effective strategy to prevent HPV infection.

Human Papilloma virus (HPV) infection can occur in both male and female genital areas that are not protected by a latex condom during sexual intercourse. Condom use thus may reduce the chances of transmitting HPV. Human Papilloma virus may shed beyond the covered area, thus condoms do not provide adequate protection as they do for some other pathogens such as HIV and gonorrhoea (Stone *et al*., 1999).

The most effective tool for HPV transmission is undoubtedly vaccination with an HPV vaccine containing the types which are most responsible for progressive cervical lesions. Prophylactic vaccines that offer protection against both high risk and low risk type HPVs are expected to considerably reduce the burden of HPV associated disease. A bivalent vaccine

is designed to protect against the two most common HR-HPV types namely 16 and 18. A quadrivalent vaccine is designed to protect against HPV types 16 and 18 as well as the most common low risk types 6 and 11 (Speck and Tyring, 2006). Furthermore, it is important to educate individuals about HPV prevalence and its natural history, with emphasis on how common the infection is, and to provide reassurance that CC is a rare outcome of HPV infection.

2.10 Mechanism of neoplastic transformation

The mechanism by which HPV infection can cause neoplastic transformation has been progressively studied and consists of different components (Figure 7). The first is direct effects of viral oncoproteins on the cell cycle, through mediation of E6 and E7 oncoproteins of oncogenic (high risk) HPVs and the pRb proteins respectively (Kufe *et al.,* 2003). E6 and E7 proteins are emphatically attracted to p53 and pRb, with the resultant expression of these two viral oncogenes in HPV-transformed cancer cells. The viral E7 protein attaches to the tumour suppressor protein pRb, and E2F-like transcription factors are released from this complex. The attachment interferes with the normal function of these cellular proteins, culminating into an elevated proliferation rate and genetic imbalance. Consequently, the transcription of Rb-regulated genes is stimulated which in turn promotes the G1/S-phase advancement of the cell cycle. E7 inactivates the pRb through mutation or deletion of the gene or through the addition of a phosphate group mediated by the upregulation of CDK 4 and CDK 6 (Klaes *et al*., 2001).

In benign lesions, the activity of CDK4 and CDK6 is strongly influenced by several CDKIs including P16. This tumour suppressor protein is inactivated in many cancers through mutation, deletion or modification of the gene, leading to a reduced or absent expression of the P16 gene product. This action causes an enhanced activity of CDK4 and CDK6 proteins, consequently leading to premature phosphorylation and inactivation of pRb. Once the pRb protein is demobilized, the affected cells are released from growth-suppressor stimuli via mediation of the P16 protein. These cells continue to proliferate even in the presence of high levels of P16. Since P16 expression is under a negative feedback control mechanism, attenuated pRb production results in an increased expression of the P16 protein (Klaes *et al*., 2001).

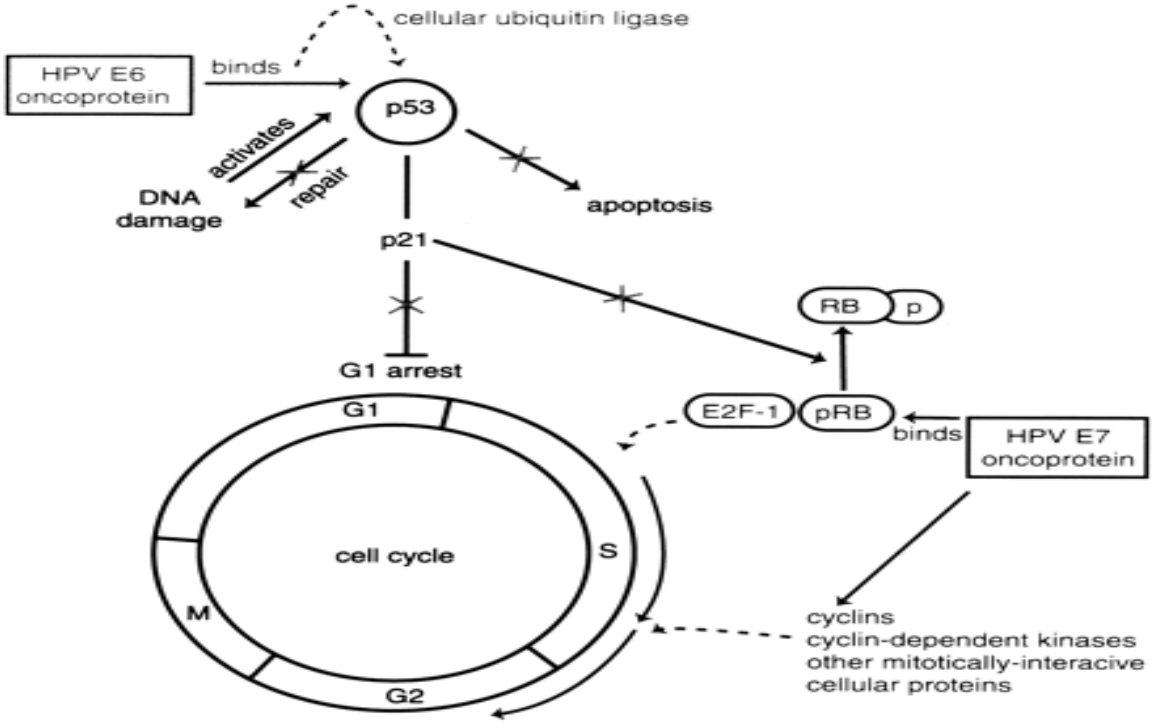

Figure 7: Pathogenesis of oncogenic HPV. HPV E6 and E7 genes encode multifunctional proteins that bind primarily to cellular p53 and pRb proteins, disrupt their functions, and alter cell cycle regulatory pathways, leading to cellular transformation (Burd, 2003)

2.11 Cytomorphological findings and screening guidelines

Cytological findings of the cervix are diagnosed according to the revised Bethesda system of 1991 combined with the CIN classification. These changes are graded according to morphology into categories that are subsequently managed, depending on the severity of the lesion. The publication of comprehensive evidence-based guidelines by the ASCCP (established in 2002), provides unambiguous and focused guidelines on managing abnormal cervical lesions (Wright *et al*., 2002).

2.11.1 Atypical squamous cells of undetermined significance (ASC-US)

Atypical squamous cells of undetermined significance (ASC-US) are cellular deviations that are more pronounced than reactive changes but lack cellular features of a definite squamous intraepithelial lesion (SIL). These changes usually appear in mature superficial or intermediate squamous cells and can also be seen in metaplastic cells. The following cellular characteristics can be observed: The nucleus is 2½-3 times the size of an intermediate cell nucleus with nuclear aberrancies occasionally found in cells that contain a dense orangeophilic cytoplasm. The nucleus/cytoplasmic ratio may be slightly elevated with mild variation in the nuclei shape and size. Chromatin is unevenly distributed which is embedded in a well-defined nucleus (Solomon and Nayar, 2004). Women presenting with a Pap smear result of ASC-US, have a 5%-17% chance of having a CIN 2, 3 lesion which should be confirmed by biopsy and should then be offered 3 management options of their condition.

These options include repeating cytology in 4-6 months, colposcopic-directed biopsy and HPV DNA testing (Wright *et al*., 2002).

2.11.1.1 ASC-US in special circumstances

Postmenopausal women who do not have a history of hormonal treatment and present a result of ASC-US, should receive a course of intravaginal oestrogen and a repeat Pap smear, one week after completion of the treatment. If the same result persists, colposcopy is advised however, if the Pap smear is negative, a repeat smear after four to six months is recommended. In developed countries, immunosuppressed women with a result of ASC-US should be referred for immediate colposcopy (Wright *et al*., 2002) however, low-resource settings may not necessarily follow the same approach.

2.11.1.2 ASC-suggesting a lower grade squamous intraepithelial lesion

Atypical changes occur mostly in superficial and intermediate cells but may also be observed in the parabasal and metaplastic cells. These lesions are managed as for LSIL. Parakeratotic cells are a common manifestation of ASC-suggesting LSIL and cells usually appear single-lying or in sheets. Such cells have a dense eosinophilic cytoplasm with normal to enlarged nuclei. Some bizarre forms may occur and care should be exercised to distinguish these changes from squamous cell carcinoma. Binucleation may be found within cells. Nuclei can have an irregular outline with dense chromatin pattern. Perinuclear clearing can also be found and should be distinguished from typical koilocytotic changes that occur in cellular changes associated with HPV (Frappart *et al*., 2003).

2.11.1.3 Atypical squamous cells- cannot exclude HSIL (ASC-H)

Atypical squamous cells are characterized by the absence of isolated, overt cellular characteristics of HSIL in cells. Cellular features generally appear either in single lying cells or in cohesive clusters of squamous epithelial cells displaying nuclear enlargement approximately 1½ to 2½ times the size of immature squamous metaplastic cells' nuclei. Atypical squamous cells are commonly embedded in streams of cervical mucus. The nuclear to cytoplasmic ratio remain elevated and may equalize that of a HSIL. Nuclear hyperchromasia and anisokaryosis are common manifestations of an ASC-H classified cervical lesion (Solomon and Nayar, 2001). Immediate colposcopy is recommended for women presenting with ASC-H, as up to 94% of such lesions are actually a CIN 2, 3 lesion in nature (Wright *et al*., 2002). If no lesion is noted on colposcopy, the cytology and colposcopic results should be reviewed and histology advised. A follow-up repeat Pap smear should be considered after 6-12 months or HPV DNA testing if histology results are being delayed (Wright *et al*., 2002).

2.11.2 Low-grade squamous intraepithelial lesions (LSIL)

Low grade squamous intraepithelial lesions encompass condylomata, mild dysplasia and cervical intraepithelial neoplasia (CIN 1) (Figure 8) are confined to the lower third of the squamous epithelium (deepest third of the surface layer of the cervix). Cellular changes occur predominantly in the intermediate and superficial cells and include the following: Cells are either arranged single or in sheets with well defined cell borders. The nuclei are at least 3 times the size of an intermediate cell nucleus. The nuclear/cytoplasmic ratio is increased to some extent with moderate variation in nuclear size, number and shape. Binucleation or multinucleation is frequent with moderate hyperchromasia. The chromatin pattern is finely to coarsely granular and may be uniform or degenerated giving it a smudgy appearance when associated with HPV. Nuclear contour outlines are somewhat irregular but may well-defined. Nucleoli are rare or inconspicuous if present. Cells have clearly delineated cytoplasmic borders. Characteristic perinuclear clearing (koilocytosis) as depicted by a clear halo around the nucleus with a dense peripheral rim of cytoplasm coupled with nuclear variations are diagnostic of a LSIL (Solomon and Nayar, 2004). It is estimated that 15%-30% of women with LSIL as diagnosed by Pap smear will have a CIN 2, 3 lesion on subsequent biopsy (Wright *et al*., 2002).

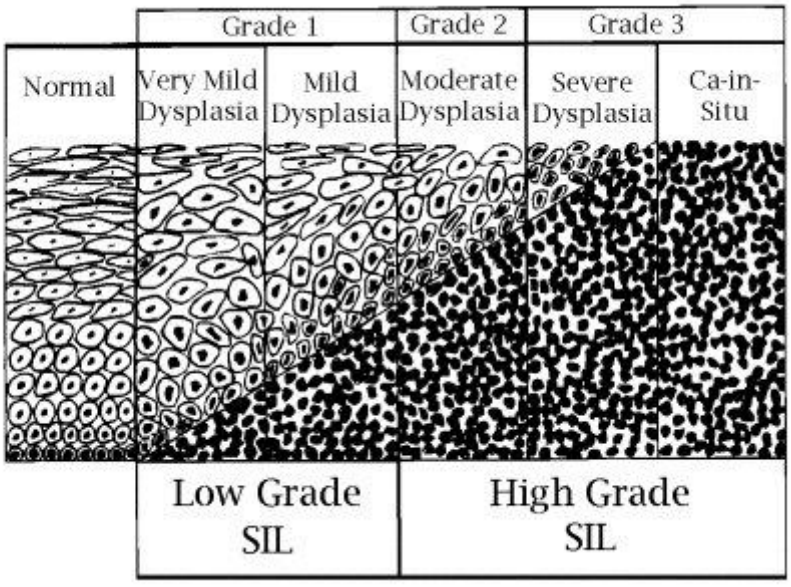

Figure 8: Precursor lesions of cervical carcinoma illustrating the differentiation of the squamous epithelium (http://www.sh.lsuhsc.edu/fammed/OutpatientManual/PapSmear.htm**)**

Due to the high risk of losing these patients, immediate colposcopy is recommended in developed countries. However, a more conservative approach is followed in Namibia where the Pap smear is repeated after three months. Should the repeat Pap smear remain positive for LSIL, colposcopy and a subsequent biopsy is advised. Being cognizant of the fact the immune system will clear the infection in up to 18 months, the specific management regimen

for LSIL classified cervical lesions should probably be redefined in the Namibian setting. According to Guido *et al*., (2000), 83% of patients with an initial Pap smear result of LSIL, test positive for HR-HPV by the hybrid capture II (HC II) assay. Based on this, HPV DNA testing by HC II is not advised to direct further management due to the low positive predictive nature of this test in women with LSIL lesions. Treatment regimens for a histologically confirmed LSIL lesion include ablation or resection of the transitional zone (Solomon *et al.,* 2001).

2.11.2.1 LSIL in special circumstances

Postmenopausal women diagnosed with LSIL can be kept under surveillance by repeat Pap smear screening every 4 to 6 months or HPV DNA testing after 12 months following initial screening. Similarly, those who are not currently on oestrogen therapy can receive a dose of intravaginal oestrogen one week prior to taking the Pap smear. Adolescents can have a repeat Pap smear taken in 6-12 months and HPV DNA testing in 12 months. A result of ASC-US or more advanced lesions or the presence of HR-HPV should prompt immediate colposcopy and biopsy (Wright *et al.,* 2002).

2.11.3 Higher grade squamous intraepithelial lesion (HSIL)

Higher grade squamous intraepithelial lesions (HSILs) include moderate dysplasia, severe dysplasia, carcinoma *in situ* (CIS) and CIN 2, 3. Abnormal cells occupy at least the lower third of the epithelium (deepest third of the surface layer of the cervix) to the point where the full thickness of the epithelium is made up of undifferentiated abnormal cells as seen in CIS. These cells appear either single lying, in sheets or in syncitial-like aggregates. Spindle cells and Indian-file arrangement may be very prominent. Cells are usually of the immature type and increasing numbers of abnormal cells can be found. The overall size of these cells is smaller than cells found in LSILs. The nuclei are enlarged and hyperchromatic with a marked increase in nuclear/cytoplasmic ratio. The nuclear membrane is irregular with a fine to coarse, evenly distributed chromatin pattern. Nucleoli are generally absent. The cytoplasm can be lacy, delicate, or dense, metaplastic, or abnormally keratinised (Solomon and Nayar, 2004).

An initial diagnosis of HSIL accounts for only 0.5% of all Pap smear results. Three quarters of these results are CIN 2, 3 or more pronounced lesions (Kinney *et al.,* 1998). There is no doubt that such cases require immediate colposcopic evaluation and a biopsy. A biopsy confirmed treatment option is the same as for LSIL. Should the biopsy fail to disclose a CIN 2, 3 lesion, endocervical sampling can be omitted (Wright *et al.,* 2002).

2.11.3.1 HSIL in special circumstances

Management of pregnant women with a diagnosis of HSIL include colposcopy, often without biopsy or defer management until after delivery. Endocervical sampling should be avoided at all cost and if the colposcopy result is unclear, a repeat Pap smear should be performed after 6-12 months as the transformation zone becomes more visible as the pregnancy advances (Wright *et al*., 2002).

2.11.4 Atypical glandular cells of undetermined significance (AGUS)

The 2001 Bethesda system replaces the term ''atypical glandular cells of undetermined significance'' with atypical glandular cells (AGC). These cells show either endometrial or endocervical differentiation, with nuclear atypia that surpasses reactive or reparative changes, but lack prominent features of invasive adenocarcinoma (De May, 1996). If possible, the diagnosis of AGC should be classified according to whether the cells are derived from the endocervix or endometrium. Atypical endocervical cells should be further sub-classified as to whether they favour a reactive or neoplastic process. Briefly, cellular changes include arrangement of cells in groups or sheets and strips, the latter arrangement which is more prominent in cells from the endocervix. The nuclei are enlarged with approximately three to five times the area of normal endocervical cells' nuclei and slightly hyperchromatic with variation in size and shape. Chromatin is fine to moderately coarse and nucleoli may be present. Mild hyperchromasia is commonly observed in the nucleus. The cytoplasm may be abundant and distinct with an elevated N/C ratio. Atypical endocervical cells favouring a neoplastic process present as cells that are arranged in sheets or strips with discernable crowding of the nucleus. Rosette formation in abnormal cells may at times be observed. The nuclei appear enlarged with mild hyperchromasia. The N/C ratio is elevated with poorly defined cell borders and scanty cytoplasm. Mitotic figures may occasionally be seen. Atypical endometrial cells are arranged in small clusters of five to ten cells. The nuclei are somewhat enlarged with mild hyperchromasia. Cell borders are poorly defined with occasional vacuolization observed in the cytoplasm. Nucleoli may be present (Solomon and Nayar, 2004). Traditionally, a repeat Pap smear, colposcopy and endocervical sampling should be used to evaluate such lesions however, as the sensitivity of these lesions with cervical cytology is between 50%-72%, colposcopy with endocervical sampling is recommended (Duska *et al.,* 1998). This procedure is applicable to women 35 years and older, and those with inexplicable vaginal bleeding should have additional endometrial sampling as well (Wright *et al*., 2002).

2.11.5 Squamous cell carcinoma (SCC)

This type of malignant or cancerous cells arises from the squamous epithelium where cells revert to a more primitive state, giving up their commitment to mature and ceasing the need

to reproduce. As they invade through the basement membrane into the cervical stroma, nuclear and cytoplasmic changes occur which can be observed in the cell morphology. Pleomorphic malignant cells are arranged isolated or in clusters, sometimes with markedly atypical or elongated nuclei. Keratinised or necrotic cells with bizarre shapes may be found. Spindle shaped cells as well as cell-in-cell arrangement (cannibalism) may be seen. Coarse and irregular chromatin clumping are prominent. A tumour diathesis (necrotic debris, old blood and inflammation) may be present in the smear background (Frappart *et al.,* 2003).

2.11.6 Adenocarcinoma (AdCa)

2.11.6.1 Invasive endocervical adenocarcinoma

This tumour entity arises from the endocervix (glandular epithelium) adjacent to the squamo-columnar junction and the cytological pattern is characterised by the presence of an abundance of well preserved cells that appear either single, in flat sheets or in clusters. The propensity to maintain their columnar shape contributes to the appearance of cells derived from the endocervix. The cells typically form two-dimensional rosettes or sheets. Individual cells usually have elongated, hyperchromatic and enlarged nuclei with irregular chromatin distribution. Multinucleation and nuclear crowding may be seen. Prominent macronucleoli may be present. The cytoplasm appears to be more acidophilic and finely vacuolated. A tumour diathesis may be evident (Solomon and Nayar, 2004).

2.11.6.2 Endometrial adenocarcinoma

This malignancy derives from the endometrium and abnormal cells are usually spontaneously exfoliated. Abnormal cells tend to be fewer with three-dimensional cellular arrangements (balls). Single-lying cells are more rounded with smaller and rounder nuclei. The nuclei may vary in size with loss of polarity. The chromatin pattern is irregularly distributed and parachromatin clearing may be found. The cytoplasm is scanty and vacuolated and stains typically cyanophilic depending on the stain used. Intracytoplasmic neutrophils are commonly found in the cytoplasm of abnormal cells. Small and distinct nucleoli are often noted, becoming larger as the tumour progresses. A watery, finely granular background is often present (Solomon and Nayar, 2004). All invasive lesions, whether it may be invasive squamous cell carcinoma or adenocarcinoma, should be referred to the appropriate specialist for treatment of the patient. A colposcopic evaluation followed by a biopsy is recommended. Aggressive treatment options can follow once the result is histologically confirmed (Clouse, 2004).

2.12 Risk factors

2.12.1 Background

It has been well established that persistent infection with HR-HPV is the major cause of cervical neoplasia. Although HPV infection is necessary for the transformation of cervical cancer and its precursors, it is by no means the sole cause. In fact, most HPV infections are temporary and will clear in almost 80% of women within 12-18 months (Jo and Kim, 2005). This suggests that additional factors are involved in the carcinogenesis of HPV related diseases of the cervix. Franco *et al*., (1999) found that HPV induced lesions of the cervix is strongly influenced by measures of sexual activity which include age at first sexual encounter, history of multiple sexual partners and the sexual behaviour of the male partner. They concluded that partners of patients with penile cancer are at an increased risk of CC later in their life. Other acknowledged risk factors include long term oral contraceptive use, high parity, immunosuppression, poor diet as well as smoking (Jo and Kim, 2005).

2.12.2 Sociodemographic factors

Human Papilloma virus infection is most common in sexually active women and can be detected within 12 months after sexual contact with an infected partner. Schneider *et al*., (2000) found that HPV prevalence in the general population can be as high as 5% to 7% among women 32 years and older. Persistence of HPV infection is associated with high risk types and is more common in women 30 years and older than women younger than 24 years, indicating that older women who are HPV positive may represent a subset of women who have difficulty in clearing the infection.

Women who are not married seem to be at a higher risk for HPV infection probably as they are less likely to have regular Pap smears. Unmarried women may mistakenly see themselves at lower risk for CC or related HPV induced lesions and do not consider having a routine Pap smear done. They may appear to be more involved in promiscuous sexual activity and this can lead to a higher prevalence of HPV infection and related lesions. Even though married women may not be necessary monogamous, they will have more Pap smears done because of interaction with their gynaecologist during parturition (Ferrante *et al*., 2000).

Similarly, women in a rural setting may not have the advantage of attending health facilities as timeously as they would like to do. Their counterparts in an urban setting might enjoy more socio-economic advantages and have greater access to health facilities (Ferrante *et al.,* 2000).

Alcohol use is found to be a risk factor for both HPV DNA positivity and related diseases in young women as it is usually related to risky sexual behaviour (Burkett *et al*., 1992).

Excessive alcohol use may increase the risk of HPV persistence in that it may inhibit adequate uptake of folate (Zhang *et al*., 1999).

Smoking has long been recognized as a co-factor that can promote development or progression of cervical neoplasia among women infected with HPV (Kjellberg *et al.,* 2000). Several mechanisms have been suggested to explain the association between smoking and lesions of the cervix. Tobacco has the ability to launch carcinogenic effects in accessory organs such as the pancreas, kidney and bladder, not directly exposed to cigarette smoking. Likewise it is possible to detect nicotine derivatives like cotinine and tobacco specific N-nitrosamines (TSNAs) in the cervical mucous secretion of active and passive smokers, exerting their detrimental effect on the DNA of cervical tissue (Szarewski and Cuzick, 1998). Smoking potentially hinders the production of an effective immune response against viral infections, thus resulting in a decrease in the number of Langerhans cells and other indicators of immune activity. The presence of systemic nicotine may further alter the metabolism of female sex hormones. Minkoff *et al*., (2004) found that smoking increases the risk of HPV infection in HIV-infected women and may increase their risk of HPV-related cervical disease**.**

2.12.3 Sexual, reproductive and contraceptive history

Long-term epidemiologic studies have supported the link between sexual activity as a predisposing factor related to HPV infection. Indeed, the greater the number of sexual partners, the greater the risk of having acquired HPV infection. However, having sexual intercourse with only one partner may also be associated with HPV infection. Ley *et al.,* (1991) found that more than 20% of women with one lifetime male sexual partner were infected with HPV, when compared to a HPV prevalence of 69% among women with 10 or more sexual partners. A longitudinal study of HPV infection in adolescent and young adult women, subsequently showed that having a new sexual partner resulted in a 10-fold increased risk for acquiring HPV infection (Moscicki *et al*., 2001).

Age also seems to be an important factor, as HPV infection is consistently found to be most common in sexually active women younger than 25 years of age (Koutsky, 1997). Even though the overall connotation with age may be as a result of riskier sexual behaviour (i.e. more sexual partners, non-condom use), proof exists that young adult women are more susceptible to HPV infection than more mature women for biological reasons. Early sexual activity may hasten the process of cervical maturation, as adolescents with multiple partners appear to have more developed cervices than non-sexually active juveniles. Although the acquisition of HPV infection is generally related to sexual activity, clearance of HPV, and

disease advancement or retrogression, is primarily determined by the host cellular defense mechanism (Scott *et al*., 2001).

Human Immunodeficiency virus infected women have been reported to have a higher incidence of cervical abnormalities, more pronounced lesions, higher-grade lesions and higher lesion recurrence than HIV negative women. Mandelblatt *et al.*, (1999) found that HIV infection is a cofactor in the association between HPV and cervical neoplasia.

Other risk factors for invasive CC include having an uncircumcised male partner, high parity (more than three pregnancies), prolonged oral contraceptive use, and infection with Herpes Simplex virus (HSV) or *Chlamydia trachomatis* (Munk *et al*., 1997).

Interestingly, several studies have emphatically recognised the importance of condom use in preventing persistent infection. Based on a meta-analysis of 20 studies, the risk of developing HPV-related disease of the cervix can be reduced by condom use (Manhart and Koutsky, 2002). Even though molecular studies are unable to demonstrate the mechanism by which condoms prevent HPV induced cervical aberrations, it has been theorised that using condoms may decrease the amount of virus transmitted. In turn, by reducing the infectious viral load, condoms may lessen the likelihood of developing an HPV-related lesion or assist in regression of the infection (Hogewoning *et al*., 2003). Thus, although condoms do not prevent all infections, their use remains paramount in preventing persistent infections and promoting HPV-related disease resolution.

The role of oral contraception remains ill-defined despite evidence in support of a biological mechanism. Moreno *et al.,* (2002) found that prolonged use of steroid contraceptive hormones increases the risk of HPV-related cervical carcinogenesis. Smith *et al*., (2003) found that long-term (more than 5 years) Depo Medroxyprogesterone Acetate (DMPA) injectable contraceptive use amongst women with persistent HPV infection, may increase the risk of CIS and invasive SCC.

No consistent relationship has been established between HPV transformed cervical lesions and reproductive characteristics. However, Schiffman *et al*., (2000) observed a three-fold increase of CIN lesions among women with four or more births when compared to nulliparous women. Munoz *et al*., (2004) found a two-fold increased risk of CIN 3 lesions associated with high parity (six or more live births) in Colombian women. No clear biologic mechanism for this phenomenon has been found, however injury to the cervix during child delivery has been suggested.

2.12.4 Cytological screening history

Cervical screening should start as early as 18 years of age or at first sexual encounter (Sawaya *et al.,* 2001). A few European countries recommend that cervical screening should start at age 21 in women whereas the Finnish Cancer Association recommends that cervical screening commence at age 30 in women, purely because of the low incidence of CC in this country. No cervical screening guidelines exist in Namibia thus making Pap smear screening ineffective in reducing cancer mortality and morbidity in this country. This results in a lack of knowledge about the disease, lack of familiarity with the concept of preventative health care, topographical and economic inaccessibility of services, poor quality of services, and a lack of encouragement from families and communities.

The majority of women in India and Southern Africa who are less educated and have low socioeconomic status and have less contact with the health care system are less likely to participate in screening. This is consistent with results from similar studies conducted in developed country settings. Women in Southern Africa who do not have access to screening services tend to be older (45 years or older), poorer, less educated (less than ten years of education), unemployed or working in the rural areas, living in nonpermanent habitation without a partner, not familiar with Pap smear screening procedures, and do not attend family-planning services regularly (Bradley *et al.,* 2004).

2.13 Aim of the study

The main aim of this study is to investigate the possibility of using $P16^{INK4A}$ surrogate marker in addition to conventional Pap smear screening for the early detection of HPV induced lesions of the cervix in Namibian women thereby decreasing the risk of developing cervical cancer. This could be the key to patient management as medical officers in Namibia have little experience in dealing with cervical lesions induced by HPV. This study will further determine which risk factors are prevalent in individuals harbouring oncogenic HPV. Through premature detection of precancerous cervical lesions having the potential to progress to more severe lesions including cervical cancer, we hope to minimize the prevalence rate of cervical cancer to the point where it will be the least prevalent cancer among women in Namibia.

CHAPTER 3
MATERIALS AND METHODS

3.1. Study Design

An analytical study for the prevalence of HPV induced lesions associated with cytomorphologic findings and related risk factors was conducted on women residing at the time of the study in Windhoek, Namibia between February 2006 and March 2007.

3.2 Data Collection

Data was collected from all women between the ages of 15-49 years (n=3852) who attended state owned health clinics and hospital wards in the Windhoek area for cervical screening, including clinics managed by the Cancer Association of Namibia. Of these, 3740 women consented to be included in this study. Data sheets on patient demographics, sexual and reproductive history and Pap smear screening history of participants in the form of questionnaires (see Appendix A) were obtained from women between the ages of 15-49 years.

3.3 Study Subjects

From February 2006 to March 2007, 3852 women presenting for cervical screening at ten gynaecological clinics in the Windhoek area, were approached to participate in the study. Women who were 15 to 49 years of age were eligible if they had no history of hysterectomy or current pregnancy at the time of the study. Briefly, subjects consented by way of their respective signature and/or thumbprint. Ethical approval was acquired from the ethics review committee the Ministry of Health and Social Services in Windhoek, Namibia (see Appendix B). After signing the consent forms (see Appendix C), subjects were interviewed and questionnaires were completed by participants.

Study participation among eligible study subjects was high, with a total of 112 refusals (2.1% of the total eligible subjects who were invited to participate). Of the 112 refusals, 34 (14.22%) were women with abnormally diagnosed Pap smears. The reason for refusal was primarily due to a lack of available time to undergo these interviews. A total number of 239 participants were selected from the study population based on abnormal cytological findings by Pap smear evaluation according to the 1991 Bethesda nomenclature combined with the CIN classification. Re-categorization of the reviewed cases was performed using the Bethesda system of 2001. Subsequently women who were found not to be eligible (n=34) were excluded. A control group was selected from women between the ages of 15-49 years who resided in the Windhoek area with histories of normal Pap smears. These women presented for routine pelvic examination, including family planning and an annual check-up. Additionally, control subjects were required to be born in the same region similar to the

women with abnormally diagnosed Pap smears. Medical records of control subjects were reviewed to ensure that potential controls have documented normal Pap smears in addition to meeting the above study enrolment criteria.

3.4 Pelvic examination and cervical cell samples

Pelvic examination was routinely done on both case and control subjects and recorded, after which women with gross clinical abnormalities of the cervix were referred to a colposcopist/gynaecologist for further assessment and management. A cervical Pap smear (see 3.7) was performed by experienced clinicians and nurses using an Ayre spatula or cytobrush to collect a standardised sample of ectocervical and endocervical cells. Cells were then smeared onto two glass slides and fixed with either commercially available ether/alcohol (Fencott Spray fix) or a 50% ethanol solution, after which it was forwarded to the laboratory for microscopic assessment. Only one Pap smear of each study subject was processed and interpreted by a cytotechnologist, according to the CIN classification combined with the 1991 Bethesda nomenclature. All slides were identified by a unique identification number. The interpretation of Pap smears was performed by the cytoscreener at the Namibia Institute of Pathology laboratory, Cytology Division. If the initial cervical smear of a study participant was found to have a cytological diagnosis ranging from ASC-US, AGUS, LSIL, ASC- suggesting LSIL, HSIL, SCC, and AdCa, the particular slide was reviewed by a second cytoscreener. Only after mutual agreement by both screeners on the cytodiagnosis obtained, were the results released. Any differences in diagnosis between the two cytoscreeners were resolved by an anatomical pathologist based at the Namibia Institute of Pathology (NIP).

In addition to the study slides, 10% of cervical smears which were reported as negative for epithelial cell abnormalities were included to ensure a level of quality assurance and to serve as a control for observer bias.

3.5 Interviews

All study subjects were interviewed by trained health care workers who focused on relevant risk factors for the development of HPV induced lesions of the cervix. The interviews were scheduled and conducted before any medical procedure, including Pap smear collection was performed. After obtaining informed consent from the participants, interviewees were asked questions about their sexual histories, sexually transmitted infections, HIV status, reproductive histories, cervical screening practices, contraceptive use and cigarette smoking. Demographic data was also obtained. All interviews were carried out in English, however if participants did not grasp the questions, a skilled interpreter was always available. If questionnaires were not completed by study subjects, the researcher personally conducted interviews for the subsequent completion of relevant questionnaires by participants. The

latter arrangement took place when study subjects underwent colposcopic examination for cytologically diagnosed abnormal cervical lesions. Medical records were assessed to validate responses about sexually transmitted infections, HIV status and Pap smear screening, parity and contraceptive use. Throughout all interviews, study participants were informed of their right to withdraw at any given time during the study, should they wish to do so. To increase the likelihood of obtaining accurate responses regarding sexual behaviour and HIV status, subjects were continuously reminded during the interview that this is purely a scientific study and that all data collected will be handled with strict confidentiality.

3.6 Questionnaires

The questionnaires (see Appendix A), which the previously trained healthcare workers had used earlier in the study, included questions on socio-demographic variables such as current place of residence, age and marital status, sexual, reproductive and contraceptive history, HIV status, smoking practices, history of alcohol consumption and cervical screening history. Each duly completed and signed questionnaire was co-signed by the specific interviewer and allocated a unique identification number. Participants who failed to complete the questionnaire were approached by the researcher on their specific follow up dates and invited to participate in this study. The researcher followed the same procedure as for all other study subjects. Participants who already completed questionnaires at the respective sites were approached by the researcher to complete the questionnaire for the second time. This generally occurred at a colposcopy clinic during their scheduled follow up visits where a repeat questionnaire was completed by study subjects after which both questionnaires were compared for correctness of information provided.

3.7 Papanicolaou Staining Method

The Papanicolaou staining method is the most widely used staining method for cervical cancer screening and is based on the affinity of cellular components for certain dyes. It consists of three cytoplasmic dyes namely orange G, eosin Y and light green as well as haematoxylin, which is a nuclear dye. The nucleus of a cell has an affinity for basic dyes such as haematoxylin whereas the cytoplasm of the cell is more likely to attract acidic (cytoplasmic) dyes. The Papanicolaou staining method can stain cells blue or blue-green (basophilia, cyanophilia), pink (acidophilia, eosinophilia), orange (orangeophilia), or indeterminate (grey-blue).

3.7.1 Preparation of solutions and stains (see Appendix D)

Final volumes of different concentrations of ethanol were prepared and added to staining troughs before staining commenced. Harris' haematoxylin was also made up to the desired concentration after which it was filtered before use. Scott's tap water was prepared in

accordance with standing operating procedures (SOPs) available in the cytology department at the NIP laboratory.

Prefixed cervical smears were received from the respective hospital wards and health centres and was prepared as follows for microscopical evaluation:

1. Slides were agitated in 70% ethanol solution for one minute.
2. Slides were agitated in 50% ethanol solution for one minute.
3. Slides were agitated in distilled water for one minute.
4. Slides were stained in artificially ripened Harris haematoxylin solution for 1 minute depending on the strength of the haematoxylin.
5. Slides were rinsed in running tap water for one minute.
6. Slides were placed in Scott's tap water for one minute.
7. Slides were placed in running tap water for one minute to stop blueing effect.
8. Slides were placed in 50% ethanol solution for one minute.
9. Slides were placed in 70% ethanol solution for one minute.
10. Slides were placed in 95% ethanol solution for one minute.
11. Slides were placed in commercially prepared OG6 stain for 2 minutes.
12. Slides were washed in two changes of 95% ethanol for one minute in each solution.
13. Slides were placed in commercially prepared EA50 staining solution for 3 minutes.
14. Slides were placed in two changes of 95% ethanol solution for one minute in each solution.
15. Slides were placed in three changes on absolute ethanol for one minute in each solution.
16. Slides were cleared in three changes of xylene for one minute in each solution.
17. Slides were mounted using permanent mounting media such as DPX mountant, (code No.1935000KF-Merck Chemicals).
18. Microscopic assessment was performed.

3.8 Bethesda Nomenclature and Reporting

Cervical smears, after being stained by the Papanicolaou method, were diagnosed microscopically using the Bethesda nomenclature of 1991 combined with the CIN classification. The examined cases were further re-grouped according to the Bethesda terminology of 2001.

The Bethesda system (TBS) is a complete diagnostic system that provides uniform diagnostic terminology to improve communication between laboratory and clinician, and from laboratory to laboratory. This terminology is based on current understanding of the pathogenesis and biology of cervical neoplasia. In short, it provides a uniform system of cytopathology reporting. This system addresses four points namely: 1) the need for a

standardised system of nomenclature so that results are comparable among various laboratories, 2) a clear statement of specimen adequacy (satisfactory, or limited, or unsatisfactory), 3) a general categorisation for triage (within normal limits, benign cellular changes, epithelial cell abnormalities, other malignant neoplasms and hormonal evaluation), 4) and the appropriateness of making recommendations for further evaluation if clinically indicated. Benign cellular changes are further divided into infection and reactive changes, whereas epithelial cell abnormalities are divided into squamous, glandular and other. Squamous cell abnormalities include atypical cells of undetermined significance (ASC-US), lower grade squamous intraepithelial lesions (LSIL) which include cervical intraepithelial neoplasia Grade 1 (CIN 1/mild dysplasia) and Human Papilloma virus (HPV) changes, higher Grade squamous intraepithelial lesions which include cervical intraepithelial neoplasia Grade 2 (CIN 2/ moderate dysplasia) and cervical intraepithelial neoplasia Grade 3 (CIN 3/ severe dysplasia), and squamous cell carcinoma (SCC). Glandular cell abnormalities include lesions ranging from the presence of cytologically benign endometrial cells found in a smear from postmenopausal women to adenocarcinoma (AdCa). A category of atypical glandular cells of uncertain significance (AGUS) is also included. Suggested guidelines for patient management include a six monthly follow up in the case of ASC-US and repeat testing if the condition persists. A three month follow up is suggested in case of a LSIL/HPV and if the condition persists, a biopsy is advised. In the case of HSIL, an immediate colposcopy is advised.

3.9 P16^{INK4A} cytology immunostaining

Destaining/Decolourisation of slides

This procedure was followed when a cytological diagnosis of ASC-US, AGC, ASC-suggesting LSIL, LSIL, HSIL, SCC, AIS, endocervical and endometrial AdCa as well as extra-uterine AdCa was made and no additional slide was received for subsequent immunocytochemical staining.

If an additional slide was received, it was rinsed in a solution of 50% ethanol to remove the fixative after which the slide was washed in running tap water for 10-15 minutes.

Destaining solution (see Appendix E) was prepared and slides were processed as follows:

Procedure

1. Slides were soaked in xylene until coverslip falls off.
2. Slides were placed in two changes of xylene to remove all traces of mounting medium.

3. Slides were placed into decreasing concentrations of ethanol i.e. 100%, 95%, 80%, 70%, 50% for one minute in each solution to remove all cytoplasmic stains.
4. Slides were placed in water for one minute.
5. Slides were placed in destaining solution to remove the nuclear stain and monitored every 5-10 minutes under the microscope until all nuclear stain has been removed.
6. Slides were placed in running water for 10-15 minutes to eliminate the acid.
7. Immuno-processing was performed.

$P16^{INK4A}$ Assay

The $P16^{INK4A}$ Cytology Kit is a qualitative, immunocytochemical assay for the detection of overexpression of the cyclin-dependent kinase inhibitor, P16 protein, in cervical cytological specimens. These kits are designed for use on cytology specimens prepared either in the conventional way or by the use of liquid-based cytology (LBC). The $P16^{INK4A}$ Cytology Kit contains reagents required to complete a two-phase immunocytochemical staining procedure specially optimised for cervical cytological specimens. Following incubation with the ready-to-use primary mouse monoclonal antibody (clone E6H4) to human P16 protein, this kit employs a ready-to-use optimised visualisation reagent based on dextran technology. The $P16^{INK4A}$ immunostaining procedure produces a coloured reaction product which precipitates at the P16 antigen site. Brown staining of cells (cytoplasm and nucleus) indicates P16 overexpression. Interpretation of results must be made within the context of the patient's history and other diagnostic tests by a certified professional.

This procedure was used for all cervical smears found to be positive on microscopical examination for lesions ranging from ASC-US, AGC, ASC- suggesting LSIL, LSIL, HSIL, SCC, AIS, endocervical and endometrial AdCa as well as extra-uterine AdCa to determine whether the abnormality was induced by HR-HPV.

The procedure was as follows:

Reagent Preparation (see Appendix F)
The following reagents were prepared prior to immunostaining: Wash Buffer, Epitope Retrieval Solution and Substrate-Chromogen Solution.

Alcohol incubation and rehydration
Slides were placed in a 50% v/v ethanol bath and incubated for 10 (±3) minutes. Excess liquid was decanted and specimens were rinsed in distilled or deionised water. Excess water was removed and slides were placed in diluted Wash Buffer for a minimum of 30 seconds.

Staining protocol

1. Epitope retrieval

Slides were placed in diluted Wash Buffer for a minimum of 30 seconds. Staining jars, e.g. Coplin jars, were filled with the diluted Epitope Retrieval Solution. Staining jars containing diluted Epitope Retrieval Solution were placed in a water bath. The water bath was heated to 95-99ºC and the Epitope Retrieval Solution in the staining jar was immersed in the water bath. The Coplin jar was covered with a lid to prevent evaporation and to stabilise the temperature. Cytological slides were immersed into the preheated Epitope Retrieval Solution in the staining jar. The temperature of the water bath and Epitope Retrieval Solution were brought back to 95-99ºC. Slides were incubated for 10 (±1) minutes at 95-99ºC. The entire jar with slides was removed from the water bath and was allowed to cool in the Epitope Retrieval Solution for 20 (±1) minutes at room temperature. The Epitope Retrieval Solution was decanted and specimens were rinsed in the diluted Wash Buffer. The slides were then soaked in Wash Buffer for 5 minutes.

2. Peroxidase-Blocking Reagent

Excess buffer was poured off. Using a lintless tissue (such as a Kimwipe or gauze pad), the specimen was carefully wiped to remove any remaining liquid and to keep reagents within the prescribed area.

NOTE: Due to risk of edge artefacts it is not recommended to use the Dacocytomation Pen in the $P16^{INK4A}$ staining procedure of cervical cytological specimens.

Specimens were covered with 200 µl ready-to-use Peroxidase-Blocking Reagent.

Slides were incubated for 5 (±1) minutes.

Slides were gently rinsed with Wash Buffer and then placed in a fresh Wash Buffer bath for 5-10 minutes.

3. Primary Antibody

Excess buffer was poured off and slides were wiped as described above.

Specimens were covered with 200 µl of ready-to-use Mouse Anti-Human P16 protein and incubated for 30 (±1) minutes. Slides were gently rinsed with Wash Buffer from a wash bottle and placed in a fresh Wash Buffer bath for 5-10 minutes.

4. Visualization Reagent

Excess buffer was poured off and slides were wiped as described above.

Specimens were covered with 200 µl of ready-to-use Visualization Reagent and incubated for 30 (±1) minutes.

Slides were rinsed as in Step 3. Baths were changed and rinsing was repeated twice.

5. Substrate-Chromogen Solution (DAB)

Slides were wiped as described above.

Specimen were covered with 200 µl of Substrate-Chromogen Solution and incubated for 5 (±1) minutes. Chromogen solution was tapped off and incubation was repeated with fresh chromogen solution **–** no washing was performed between the steps.

Slides were gently rinsed with Wash Buffer from a wash bottle and placed in distilled water for 5-10 minutes. Substrate-Chromogen Solution (DAB) waste was collected in a hazardous materials container for proper disposal.

6. Counterstain

Slides were progressively stained in a bath of Harris' haematoxylin for 3 minutes and gently rinsed with running tap water for 5 minutes to ensure that all residual haematoxylin has been removed.

Slides were rinsed in distilled water.

7. Mounting

Specimens were mounted and coverslipped with a permanent mounting medium such as DPX mounting medium (code No.1935000KF-Merck Chemicals).

3.9.1 Quality Control

Positive Control: Controls were processed in the same manner as the patient sample(s). Positive controls were indicative of correctly prepared specimens and proper staining techniques. One positive control for each set of test conditions was included in each staining run.

Known positive controls (histologically confirmed HSIL encompassing CIN III cervical smears) were utilised for monitoring the correct performance of processed specimens and test reagents. When these positive controls failed to demonstrate appropriate positive staining, results of the test specimens were considered invalid.

Negative Control: Negative controls (benign cervical smears diagnosed as negative for intraepithelial lesion or malignancy) were processed in an identical manner as for patient sample(s) with each staining run and were used to verify the specificity of the primary antibody as well as to provide an indication of the background staining. The variety of different cell types present in most cervical cytological specimens offered internal negative control sites (this was verified by the screener).

3.9.2 Interpretation of Results

The $P16^{INK4A}$ immunostaining procedure caused a coloured reaction product to precipitate at the P16 antigen site. Brown staining of cells (cytoplasm and nucleus) indicated P16 overexpression. At least 5 abnormal cells or 25% of abnormal cells, whichever is the greatest, displaying P16 overexpression were considered as positive for P16 immunoexpression.

3.10 DNA isolation and agarose gel electrophoresis

Slides were placed in staining racks and immersed in Xylene AR (400 ml) for 3-4 days to remove coverslips. Slides were then placed in two changes of 100% ethanol for 10s each, followed by immersion in 80% ethanol (10s), 60% ethanol (10s) and 40% ethanol (10s). This was followed by incubation in double distilled water (rehydration) for 10s. The fifteen negatively diagnosed cervical smears of patients that were used as negative controls in the $P16^{INK4A}$ procedure were included for PCR optimisation. Cellular material from slides were gently scraped off with a sterile scalpel blade and collected into a clean, sterile 2.0 ml Eppendorf tube containing 100 µl lysis buffer [4 M urea, 200 mM Tris, 20 mM NaCl, 200 mM EDTA, pH 7.4 (25ºC)] to which 40 µl recombinant PCR grade Proteinase K (20 µg/ml) was added. The cell suspension was thoroughly mixed and incubated overnight at 37ºC. DNA was extracted using the High Pure PCR Template Preparation kit (Roche Diagnostics GmbH Mannheim, Germany) according to the instructions of the manufacturer. Sterile distilled water as a negative control was included in every run. Initial gel electrophoresis to determine the presence of genomic DNA was performed on all samples before specific amplification took place.

3.11 PCR and agarose gel electrophoresis

Primer sets GP5+ and GP6+ (Rughooputh *et al*., 2006) with an amplicon size of 150 basepairs (bp) were used to identify any genital HPV infection that is found in the L1 region of the HPV genome. GP5+ and GP6+ positive samples were selected and further processed for the HPV 16 and HPV 18 genes that is situated in the E7 667-686 and 753-774 region of the HPV genome respectively. An amplicon size of 108 bp and 191 bp were expected for the HPV 16 and HPV 18 amplicon, respectively.

The sequences of the respective primers were:

Table 1: Primer sequences for PCR amplification

Primer Type	**Primer Sequence**
GP5+	5´ TTT GTT ACT GTG GTA GAT ACT AC 3´
GP6+	5´ GAA AAA TAA ACT GTA AAT CAT ATT C 3´
HPV 16 forward primer	5´ GAT GAA ATA GAT GGT CCA GC 3´
HPV 16 reverse primer	5´ GCT TTG TAG GCA CAA CCG AAG C 3´
HPV 18 forward primer	5´ TGA AAT TCC GGT TGA ACC TTC 3´
HPV 18 reverse primer	5´ GGT CGT CTG CTG AGC TTT CT 3´

A modified PCR master mix, constituted of the following components, was prepared and used in the amplification of primer sets GP5+ and GP6+: 10 µl 5xGo Flexi Buffer, 5 µl GP5+ primer (10 µM), 5 µl GP6+ primer (10 µM), 1 µl dNTP mix (10 mM), 4 µl $MgCl_2$ (25 mM), 14.75 µl sterile H_2O and 0.25 µl Go Taq polymerase. The reaction volume was 50 µl and was made up of a 10 µl sample DNA template and 40 µl modified PCR master mix. Double distilled H_2O as a negative control was included to monitor for contamination that might occur. Thirty amplification cycles were carried out under the following conditions: Initial denaturation at 94ºC for 2 minutes, 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 40ºC for 45 seconds, extension at 72ºC for 45 seconds and final extension at 72ºC for 5 minutes.

The PCR analysis for HPV 16 and HPV 18 E7 region were performed with the specific primer sets according to the method as described by Grainge *et al*., 2005. PCR analysis for the presence of HPV 16 and HPV 18 amplicons was performed using the HPV 16 and HPV 18 master mix containing 10 µl of isolated and purified DNA and quantified as follows: HPV 16 master mix (10 µl 5xGo Flexi Buffer, 5 µl HPV 16 forward primer (10 µM), 5 µl HPV 16 reverse primer (10 µM), 1 µl dNTP mix (10 mM), 4 µl $MgCl_2$ (25 mM), 14.75 µl sterile H_2O and 0.25 µl Go Taq polymerase); HPV 18 master mix (10 µl 5x Go Flexi Buffer, 5 µl HPV 18 forward primer (10 µM), 5 µl HPV 18 reverse primer (10 µM), 1 µl dNTP mix (10 mM), 4 µl $MgCl_2$ (25 mM), 14.75 µl sterile H_2O and 0.25 µl Go Taq polymerase). Forty amplification cycles were performed under the following conditions: Initial denaturation at 94ºC for 2 minutes, 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 40ºC for 45 seconds (HPV type 16) and at 42ºC for 45 seconds (HPV type 18), extension at 72ºC for 45 seconds and final extension at 72ºC for 5 minutes. Individual samples (10 µl) were mixed with 5 µl gel loading buffer (see Appendix F) and transferred to a 2% agarose gel containing 2 µl of a 10 mg/ml ethidium bromide solution. The gel was suspended in 1x Tris Acetic Acid EDTA pH 7.0 (TAE) buffer (see Appendix G) and ran for 80 minutes at 100V after which it was

visualised at a wavelength of 260 nm by ultraviolet transillumination for the presence of the HPV amplicon.

3.12 Statistical analysis and data management

All completed interviews were carefully reviewed by the study coordinator for completeness before entering data into Microsoft Excel data files. Data was quality controlled for range and cross-file field checked by comparing information on questionnaires to information on corresponding cytology request forms. Randomly selected questionnaires were double-checked for coding and errors.

In addition to simple descriptive statistics, comparisons of categorical variables (variable with no numerical meaning) were analysed by χ^2 tests. P values ≤0.05 were considered statistically significant. Crude odds ratio (OR) at 95% confidence intervals (CIs) were obtained from multiple logistic regression models to evaluate the association between HPV induced lesions and categorical variables. Cohen's Kappa values (significant p value closer to 1) were used to measure the agreement between sexually transmitted infections (STIs) and HIV infection combined with McNemar symmetry Chi-square (significant $p<0.05$) for any disagreement between these variables. In addition, Cohen's Kappa values as well as McNemar's test of symmetry were further applied to assess the agreement between HPV positives and HPV negatives based on their reaction using PCR analysis and $P16^{INK4A}$ immunostaining. Systat software (version 11, Systat Software Inc (SSI), San Jose, California, USA) was applied for all data analysis.

3.13 Limitations

Potential limitations of the study include the survey, most notably in Windhoek, which had a relatively low participation rate. As Pap smear screening is not widespread in Namibia, it is not, perhaps, surprising that a small proportion of women (14.22%) refused to participate in the study. Most European (white) women prefer to visit a private gynaecological practise and as little as 10% were found to visit state health facilities and this does not represent a statistically significant proportion which would have added greater value to our study. The proportion of tribal study subjects was not large enough to allow broad generalisability to all Namibian groups.

We did not collect data on the sexual behaviour of male partners of the participants; therefore we cannot assess the possible effects of male behaviour as a risk factor for HPV induced lesions of the cervix among females.

Histological confirmation as the confirmatory tool or gold standard for abnormal cytological diagnosis ranging from ASC-US, ASC- suggesting LSIL, AGUS, and LSIL was not obtained and may further limit the sensitivity of the P16 immunoassay test procedure applied.

CHAPTER 4
RESULTS

4.1 Cytology results

Cervical smears were collected from 3852 women who attended state owned gynaecological clinics and hospital wards, of which 3839 women completed an interviewer-administered questionnaire. Of this study sample (n=3839), 205 women with cervical abnormalities by Pap smear were selected and their respective samples were subjected to P16 immunostaining. Once P16 immunostaining was done, the respective cases were subjected to DNA extraction and subsequent PCR amplification. Results obtained from the PCR amplification process, were compared with the immunostaining results and discrepancies were addressed.

Initially, the case distribution of epithelial cell abnormalities was 1, 67, 87, 14 and 36 for SCC, HSIL, LSIL, ASC-suggesting LSIL and ASC, respectively. Using PCR as the gold standard, 18 cases displayed a result negative for the presence of any kind of genital HPV but positive for the presence of the P16 protein. The respective Pap smears were subsequently retrieved from the Namibia Institute of Pathology (NIP) slide storage facility and microscopically reassessed. Reassessments were performed to evaluate the reliability of the initial cytological diagnosis. Cytological diagnosis obtained during the reassessment was compared with the original cytological diagnosis. Histological diagnosis on the 205 cases, if available, was obtained using the NIP data base.

Of the 67 cases cytologically diagnosed as HSIL (CIN 2/3), 54 had confirmed biopsies while in 11 of the 18 discrepant lesions the biopsy diagnosis differed from the cytological diagnosis. The differences as indicated by the corresponding histological slides ranged from cervicitis in 6 cases and CIN 1 lesions in the remaining 5 cases. Microscopic review of the Pap smears from the 11 participants with a histological diagnosis of cervicitis and CIN1 demonstrated cellular changes associated with HSIL in all cases, thus indicating that the colposcopy may have failed to locate or the biopsies failed to sample a high-grade lesion in each patient. Re-assessment of the two HSIL classified cases with no histological assessment demonstrated cellular changes associated with active repair. In addition, one discrepant case classified as SCC revealed benign cellular changes on the corresponding histological section. Cytological review of the respective case demonstrated cellular changes associated with high endocervical sampling.

Microscopic re-assessment on the remaining six of the 18 discrepant Pap smears revealed that two cases initially diagnosed as ASC was found to be benign (reactive cellular changes) while four cases initially diagnosed as LSIL demonstrated ASC in three cases and benign (reactive) cellular changes in the remaining one case. No biopsy or colposcopy was

performed on these cases. On the other hand, colposcopic biopsies were performed on 33 of the 87 LSIL classified lesions and demonstrated concordance in 27 of these cases. Six of the respective 33 biopsied LSIL cervical lesions differed in diagnosis from their cervical counterparts and demonstrated reactive cellular changes. This variability in the cytological diagnosis was considered to be an indication that cytological assessments are frequently subjective and may be influenced by the screener's experience. As a result, the respective 18 discrepant cases were excluded from the study. Also, cervical lesions which demonstrated discordance in either their corresponding histological assessment or microscopic review were rejected.

A final sample included 187 study subjects and 205 matched controls. Study subjects and controls were found to be relatively similar demographically as expected because of the matching criteria. The mean age of the patients was 33 years for both women in the control and study subject groups.

4.2 Cervical cytology

4.2.1 Cervical cytology criteria

Cytological diagnoses were obtained using the revised Bethesda system of 1991 combined with the CIN classification. To ensure consistency and uniformity in reporting, pre-screened cervical slides were re-screened and reclassified based on TBS of 2001. As part of our internal quality control program, all previously screened cytologically diagnosed abnormal cervical smears were microscopically re-assessed by a second cytotechnologist. Of the 187 conventional Pap smears diagnosed, 28 (15%) were classified as ASC-US, 96 (51.3%) as LSIL, nine (4.8%) as ASC-H and 54 (28.9%) were classified as HSIL.

Generally, benign cellular changes were easily discernable from "atypical/abnormal cellular changes" as depicted by their cellular morphology. Cellular changes associated with cervical lesions classified as ASC-US (Figure 9) referred to cells that demonstrated three essential features namely: squamous differentiation, increased nuclear: cytoplasmic ratio and lastly mild nuclear hyperchromasia with subsequent irregular distribution of the chromatin, chromatin smudging and/or multinucleation. Cellular changes associated with ASC-suggesting LSIL were almost similar to changes associated with ASC-US lesions except for the presence of distinct round to oval shaped squamous epithelial cells. The cells looked like large metaplastic or small intermediate cells and were about one third the size of a superficial cell. Cells associated with ASC-H lesions (Figure 10) were generally sparse and occurred either single or in small groups of less than 10 cells per group. The cells' sizes were that of metaplastic cells with a dense cytoplasm and the nuclei 1.5 to 2.5 times larger than the size of an intermediate cell's nucleus. Lower grade lesions incorporated cellular changes

previously termed “HPV” (koilocytosis) and mild dysplasia or CIN I (Figure 11). Cellular changes were predominantly confined to intermediate and superficial cells. These cells generally appeared either single and/or in sheets with a well-demarcated cytoplasm. Characteristically, the size of the nuclei in the cells was more than three times the size of a normal intermediate cell’s nucleus. Koilocytosis which include a sharply delineated perinuclear halo and a thin cytoplasmic rim were also seen in single lying intermediate cells (Figure 12). Cellular changes associated with HSILs (Figure 13) were seen in smaller and less mature types of cells and were mostly noted in groups of cells. The respective cells displayed hyperchromasia in the nucleus coupled with variation in the size and shape of the nuclei. Characteristically, the nuclear: cytoplasmic (N/C) ratio remained very high in these types of cells.

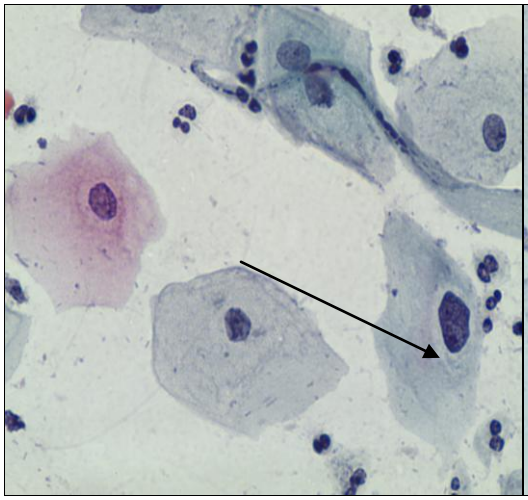

Figure 9: Cytological manifestation of an ASC-US classified cervical lesion (as depicted by ⟶) (x400 magnification)

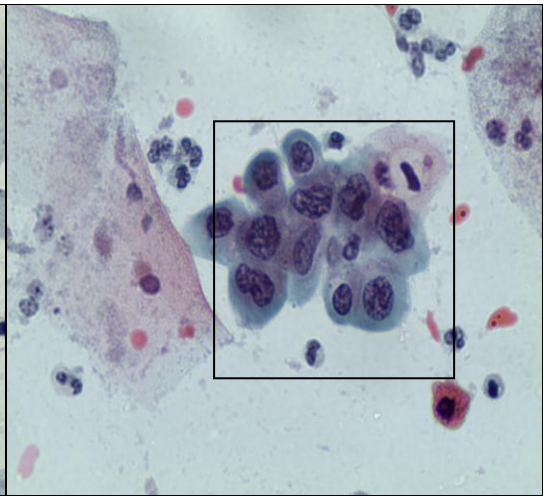

Figure 10: Cells with metaplastic cytoplasm seen in ASC-H (x400 magnification)

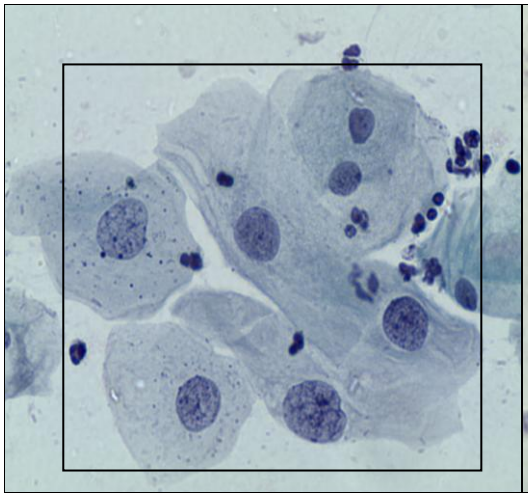

Figure 11: Single lying dysplastic cells seen in LSIL (x400 magnification)

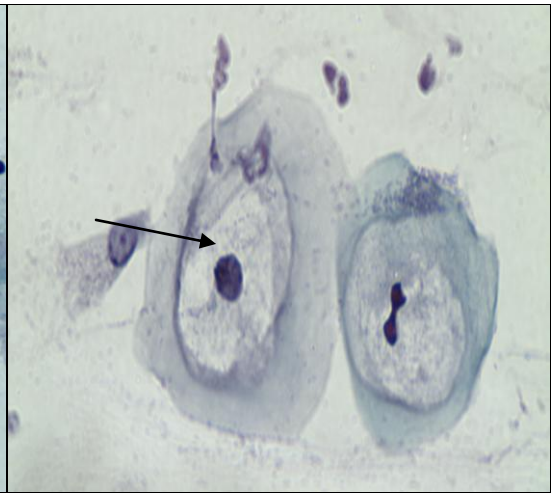

Figure 12: Cytological manifestation of LSIL depicting condylomatous changes (as depicted by ⟶) (x400 magnification)

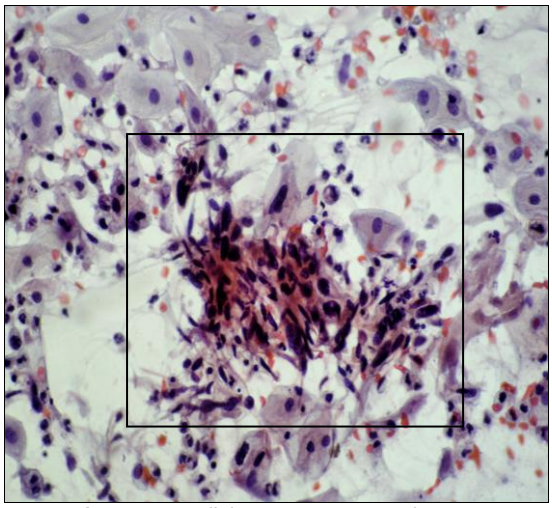

Figure 13: Cellular aggregate seen in HSIL

4.2.2 Distribution of abnormal cervical lesions

Of the total number of ASC-US lesions (n=28), 42.9% was predominantly found in women aged 25-29 years with an absence of ASC-US lesions observed among women aged 15-19 years (Table 2). As little as 3/96 (3.1%) of the total number of LSIL lesions which mainly include condylomatous changes were found in women aged 15-19 years. No other significant pathology was detected in this age group.

Of the total number of LSILs (n=96), 50/96 (52.1%) were most commonly seen in women aged 25-34 years, with a peak in women 30-34 years of age. The lowest prevalence of LSIL lesions (2/96) was found in women aged 45-49 years. Similarly, a peak prevalence of HSILs (CIN II/III with the presence/absence of HPV associated cellular changes) was seen in women 30-34 years of age (33.3%), progressively declining to a low 5/54 (9.3%) among women 45-49 years. Prevalence of HSIL lesions was the lowest among women aged 15-19 years as expected with only 1.9% of women aged 20-24 years displaying cellular changes associated with HSIL. A peak prevalence of 44.4% (4/9) for ASC-H lesions was found in women 30-34 years of age. Most notably, the distribution of abnormal cervical lesions peaked among women aged 25-34 years with 55.1% (101/187) of women reported to have abnormal cervical lesions in this particular age group.

Table 2: Prevalence of abnormal cervical cytology among women in Windhoek, Namibia

AGE	ASC-US	LSIL	ASC-H	HSIL	Number per age group
15-19	0	3	0	0	3
20-24	3	17	0	1	21
25-29	12	22	1	12	47
30-34	4	28	4	18	54
35-39	3	12	3	11	29
40-44	4	12	1	7	24
45-49	2	2	0	5	9
Total	**28**	**96**	**9**	**54**	**187**

Moreover, HPV cellular changes in association with CIN I were observed in 56/96 (58.3%) of lesions classified as LSIL with 28/96 (29.2%) of these lesions displaying mainly koilocytosis with the absence of cellular changes associated with cervical intraepithelial neoplasia grade I (CIN I). Among the lesions classified as HSIL (n=54), HPV associated cellular changes were observed in eight (50%) of CIN II (n=16) and nine (23.7%) of CIN III (n=38) lesions.

4.3 $P16^{INK4A}$ immunocytochemical staining

4.3.1 Immunocytochemical patterns

P16 staining as indicated by a brown colourisation of either the nuclei and/or cytoplasm was predominantly diffuse in HSILs and ASC-H lesions. Strong P16 overexpression was observed in both the nuclei and cytoplasm of abnormal cells seen in ASC-H (Figure 14) and HSILs. A positive P16 staining was significant in cellular aggregates of HSILs (Figure 15), with P16 immunoexpression most commonly found in the nuclei and cytoplasm of single lying dysplastic cells seen in LSILs.

Sporadic P16 staining was primarily observed in the nuclei of cells classified as ASC-US (Figure 16) and to a lesser extent in LSILs. Sporadic staining of bacteria, red blood cells and mucoid substances were readily identified. Cervical smears classified as “negative for intraepithelial lesion or malignancy (NILM) did not display P16 expression as were the fifteen controls with benign diagnoses. Strong and diffuse P16 staining was observed in all fifteen positive controls.

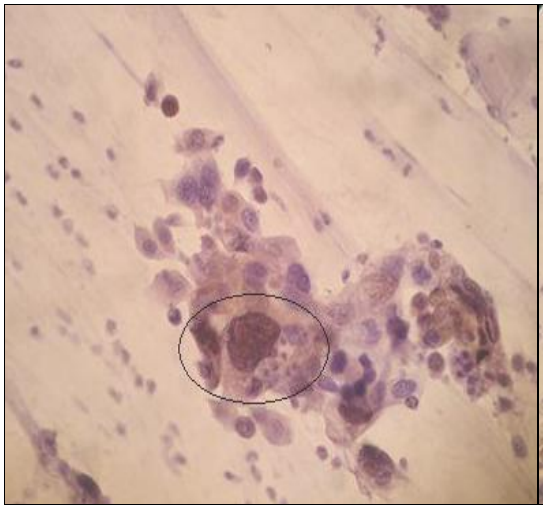

Figure 14: Positive $P16^{INK4A}$ immunoexpression seen In ASC-H (x400 magnification)

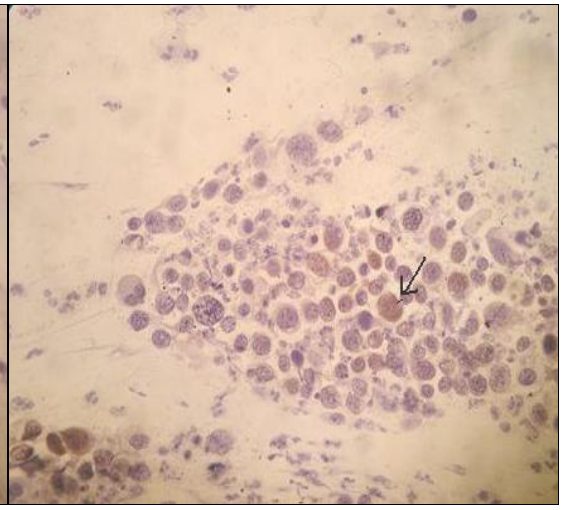

Figure 15: Positive $P16^{INK4A}$ staining (as depicted by ⟶) in a cytologically diagnosed HSIL (CIN III) (x200 magnification)

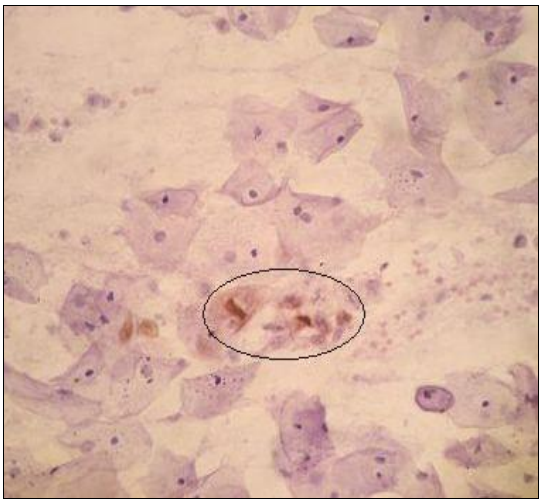

Figure 16: Positive $P16^{INK4A}$ staining in a cytologically diagnosed ASC-US lesion (x200 magnification)

4.3.2 Distribution of lesions based on immunocytochemistry

Figure 17 depicts the distribution of P16 expression in abnormal cervical lesions. The overall prevalence of P16 expression was found in 60.4% (113/187) of abnormal cervical lesions. The P16 protein was detected in five of 28 (17.9%) and 50 of 96 (52.1%) of ASC-US and LSIL classified lesions respectively. Concomitantly, P16 expression increased with the degree of abnormality to 77.8% (7 of 9) and 94.4% (51 of 54) in the ASC-H and HSIL groups, respectively. Squamous and glandular epithelial cells displaying benign cellular features were P16 negative. On the other hand, the distribution of P16 non-immunoreactivity was 82.1% (23/28), 47.9% (46/96), 22.2% (2/9) and 5.6% (3/54) in ASC-US, LSIL, ASC-H and HSIL classified lesions respectively.

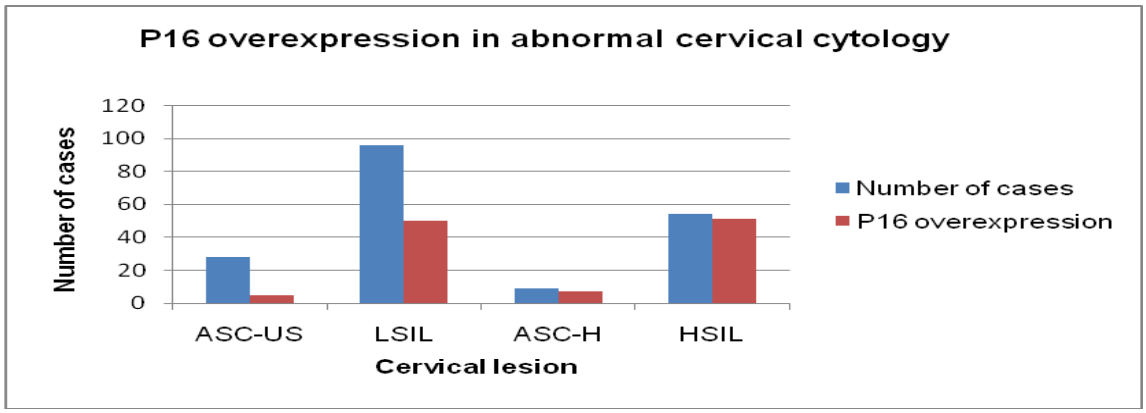

Figure 17: P16 expression in abnormal cervical cytology

Furthermore, P16 immunoexpression was detected in 9/113 (8.0%) of women aged 20-24 years after which it significantly increased to a peak of 69/113 (61.1%) among women aged 25-34 years. The highest incidence of positive P16 immunoexpression was observed among women aged 30-34 years. P16 staining declined to 17/113 (15.0%) in women aged 35-39 years to a low 6/113 (5.3%) among women aged 44-49 years (Figure 18). Expression of the P16 protein was completely absent among women aged 15-19 years.

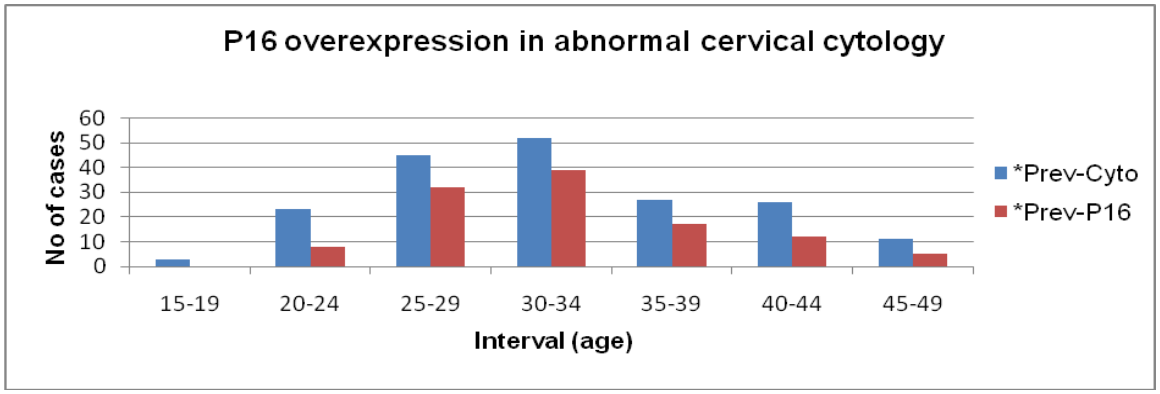

Figure 18: P16 detection link to age in cytologically classified abnormal cervical lesions
*Prevalence of cytologically classified condylomata acuminata denoted as Prev-Cyto.
*Prevalence of cytologically classified abnormal cervical lesions demonstrating P16 overexpression denoted as Prev-P16.

4.4 Human Papilloma Virus detection by PCR

Of the 187 cytologically-classified abnormal cervical smears, five samples were not processed for PCR testing due to insufficient volumes, probably as a result of evaporation of eluted DNA, and as such were omitted. Accordingly, 182 cytologically-classified abnormal cervical smears were subjected to HPV detection by PCR. Human Papilloma virus (HPV) detection by PCR using the GP5+/6+ primer pairs yielded positive results in 172/182 (94.5%) of the study subjects. An amplicon size of 150 bp was considered positive for the presence of any kind of general genital HPV (Figure 19).

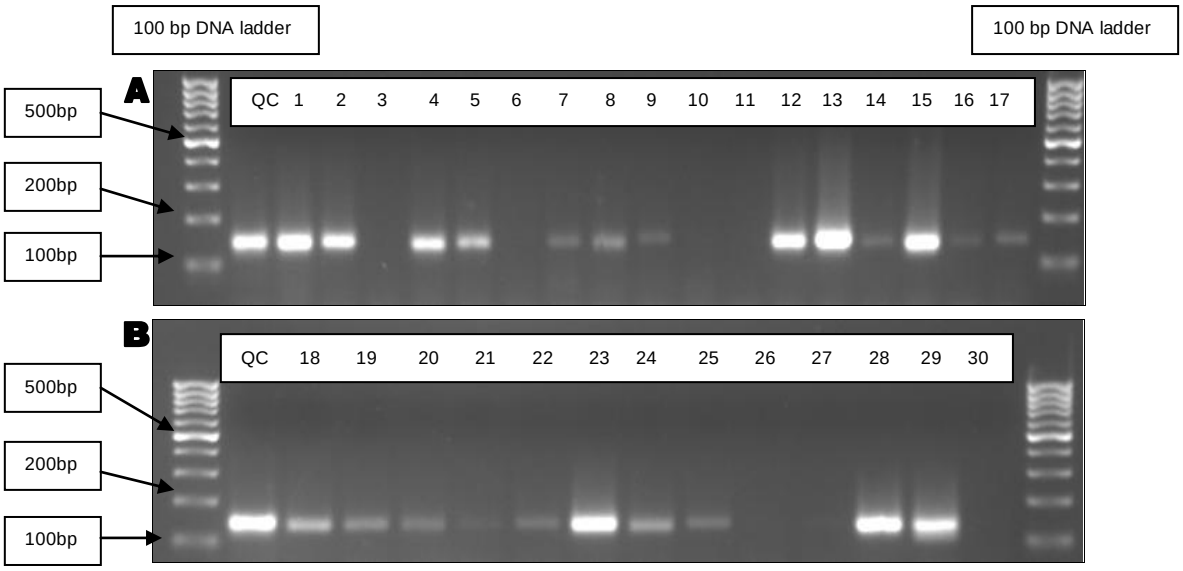

Figure 19: Representative agarose gel pictures (A-B) of PCR analysis (amplified by GP5+/6+ primer pairs) seen in (A) sample 1-17 and (B) sample 18-30
* Lane 1- 100 bp DNA ladder
*QC (Quality control) denotes a known positive control

Among the 27 samples classified as ASC-US, 25 (92.6%) were positive for PCR amplification for GP5+/6+ genital HPV. The proportion of participants positive for HPV increased significantly with increasing levels of cervical abnormality; 51 (98.1%) of the lesions diagnosed cytologically as HSIL (n=52), were positive for GP5+/6+ genital HPV compared to 87 (92.6%) of women diagnosed cytologically as LSIL (n=94). Interestingly, HPV detection by PCR was observed in nine (100%) of lesions classified as ASC-H (n=9). The prevalence of HPV (amplified by GP5+/6+ primer pairs) by age group was 24 (14%) in women 15-24 years of age, 88 (51.2%) in women 25-34 years of age and 63 (36.6%) in women 35 years and older.

To test whether expression of the P16 protein in the respective lesions was associated with the presence of HR-HPV, we subjected GP5+/6+ positive samples to type-specific PCR analysis (HPV types 16 and 18). An amplicon size of 108 bp and 191 bp was considered positive for the presence of HPV types 16 (Figure 20) and 18 (Figure 21) respectively.

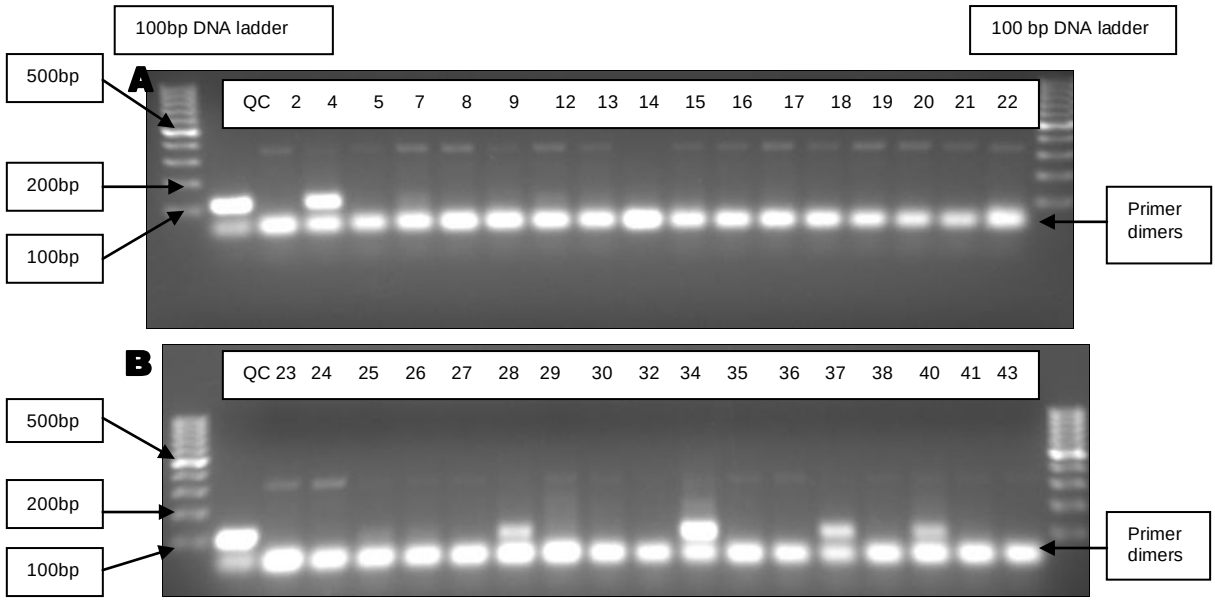

Figure 20: Representative gel pictures (A, B) of PCR analysis (amplified by HPV 16 primer pairs).
* Lane 1- 100 bp DNA ladder
*QC (Quality control) denotes a known positive control

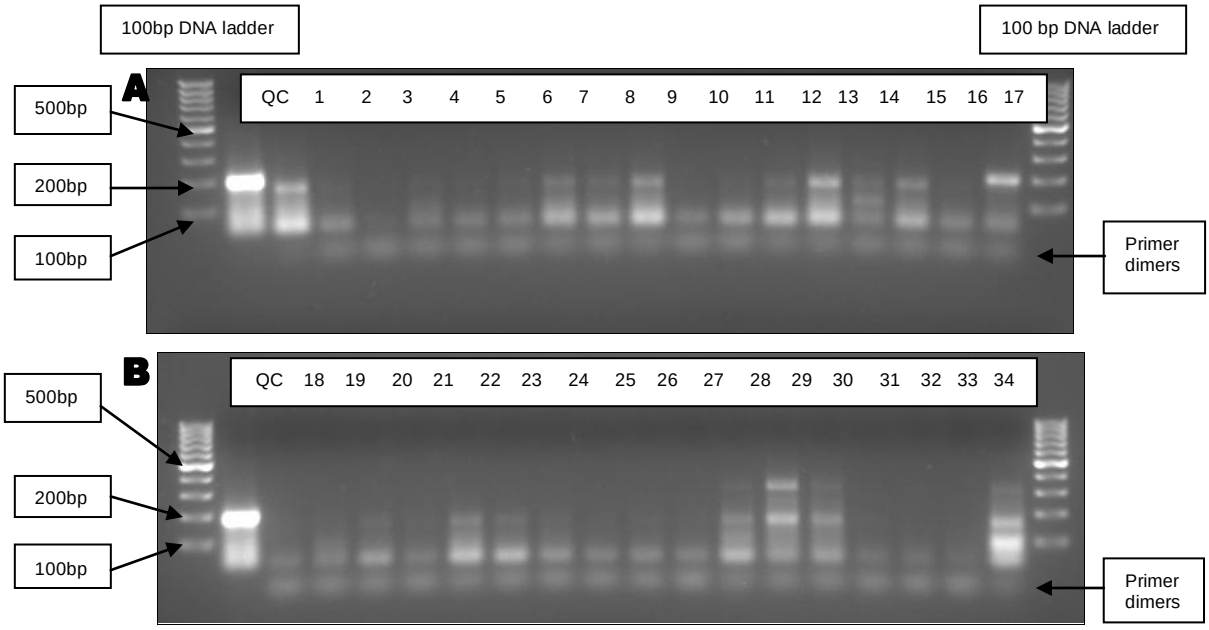

Figure 21: Representative gel pictures (A, B) of PCR analysis (amplified by HPV 18 primer pairs).
* Lane 1- 100 bp DNA ladder
*QC (Quality control) denotes a known positive control

Of the 172 samples which tested positive for PCR amplification in GP5+/6+, 54/172 (31.4%) were positive for HPV type 16 and/or 18. The samples of HPV positive for either types 16 and 18 accounted for 21 (12.2 %) and 25 (14.5%) of all HPV infections respectively. The samples of HPV positive for both types 16 and 18 were found in eight (4.7%) of the female participants.

Generally, the distribution of type specific HPVs (16 and 18) varied among the screened participants (Figure 22). Of the selected 25 samples classified as ASC-US positive for GP5+/6+, seven (28%) were associated with the presence of either HPV types 16 or 18. Concurrently, the HPV 16 genome was observed in 4% (one case) of an ASC-US classified lesion while HPV type 18 was noted in 24% (6 cases) of such lesions. No incidence of both HPV types 16 and 18 were observed in ASC-US classified lesions.

Human Papilloma Virus types 16 and 18 were further detected in 27.6% (24 cases) of LSIL classified lesions (n=87) positive for GP5+/6+. Of these lesions, 8.0% (seven cases) demonstrated the presence of HPV type 16 whereas HPV type 18 was observed in 15.0% (13 cases) of these lesions. More than 4% (four cases) of LSIL diagnosed lesions demonstrated infection with both HPV types 16 and 18.

The presence of HR-HPV (types 16 and 18) amplicons were observed in two (22.2%) of ASC-H and 21 (41.2%) of HSIL classified lesion (s). Interestingly, HPV type 16 was detected in 12 (23.5%) of HSIL classified lesions with HPV type 18 seen in six (11.8%) of these lesions. Concurrent infection of both HPV types 16 and 18 was seen in one (11.1%) and three (5.9%) of ASC-H and HSIL classified lesion (s), respectively.

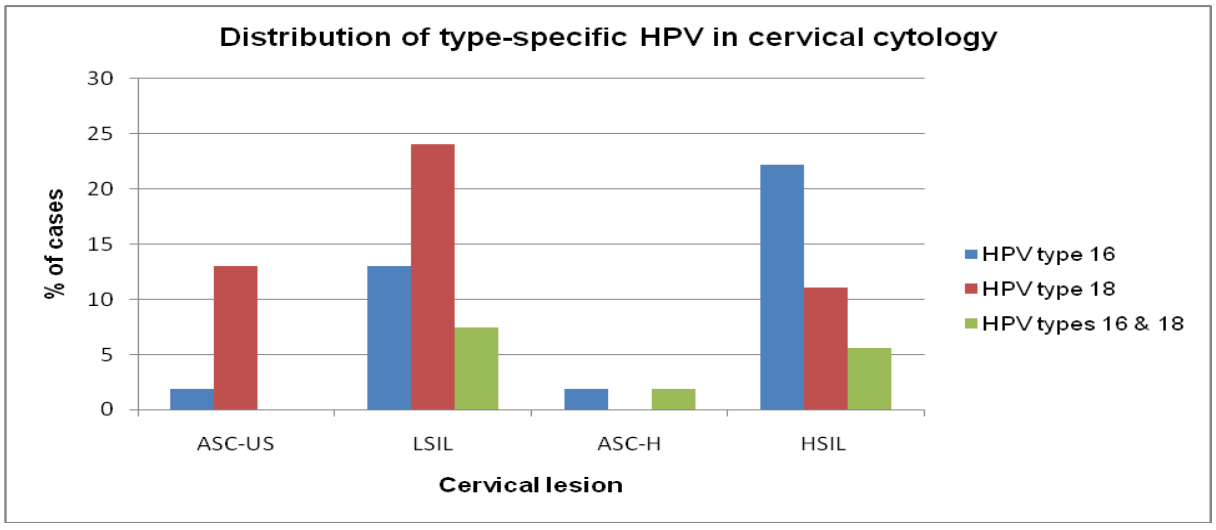

Figure 22: Distribution of HPV types 16 and 18 in cervical cytology

Furthermore, HR-HPV types 16 and 18 were detected in six (11.1%) of women less than 25 years old after which detection rapidly increased to 35 (64.8%) of women 25-34 years old; 30-34 years being the highest peak (Figure 23). However, a sudden decline was observed among women 35 years and older. As was expected, HPV 16 and 18 predominated among women 30-34 years old with a peak prevalence of nine (16.6%) and 11 (20.4%) for HPV 16 and 18 respectively. Notably, prevalence of HPV 16 was slightly elevated among women 20-24 years old.

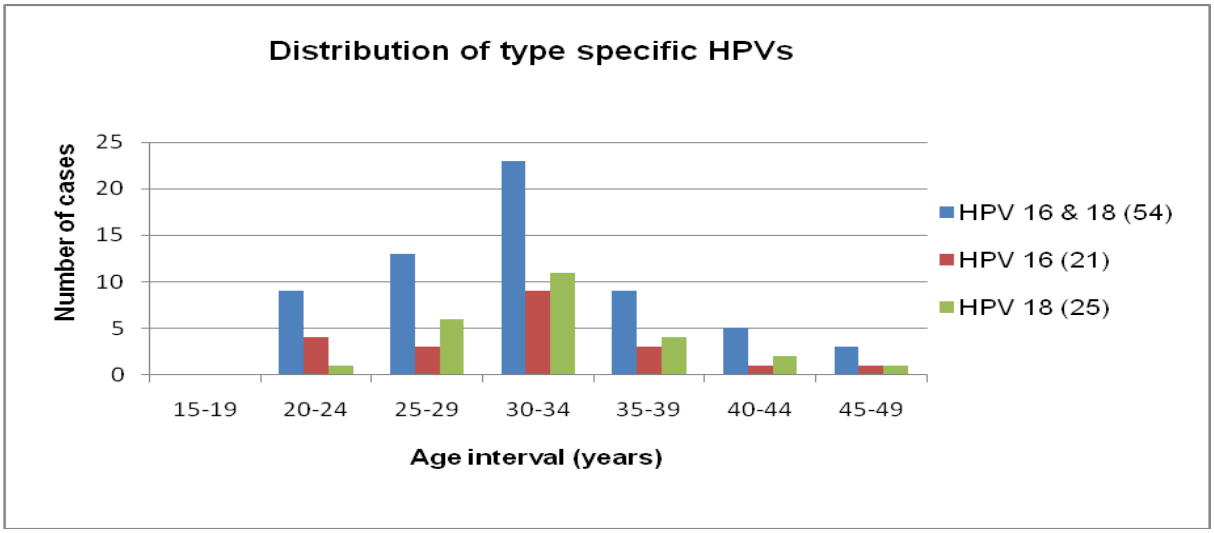

Figure 23: Distribution of HR-HPVs (types 16 & 18) among women in Namibia

4.5 Comparison of Human Papilloma virus detection by PCR and P16 immunostaining

Table 3 demonstrates the comparison of P16 immunocytochemical and PCR (amplified by GP5+/6+ primer sets) test results for 182 patients who had cytologically classified abnormal Pap smears. Of the 52 cytologically diagnosed HSIL (CIN II/III) specimens with confirmed biopsies, 92.3% (48/52) were positive for the presence of the P16 protein and HPV by PCR analysis. Two HSIL-classified lesions that was positive for HPV amplification by PCR was negative for the $P16^{INK4A}$ immunoassay. One HSIL classified cervical lesion was negative for both P16 and HPV DNA detection by PCR. Forty nine (52.1%) of the specimens displaying LSIL (n=94) on cytology were positive for both P16 and PCR as compared to 38/94 (40.4%) that were negative for P16 and positive for HPV detection by PCR. Seven (7.4%) of the cytologically classified LSIL lesions were negative for both P16 and HPV by PCR analysis. Of the 27 ASC-US classified lesions, four (14.8%) were positive for both P16 and PCR with 21 (77.8%) found to be positive for HPV amplification by PCR but lacking the P16 protein. Two (7.4%) ASC-US categorised lesions that tested negative for P16 immunoexpression were negative for HPV DNA detection by PCR. Seven (77.8%) of ASC-H classified samples (n=9) that were positive for HPV DNA detection by PCR expressed the P16 protein.

Table 3: Results of P16 immunocytochemical and HPV DNA by PCR testing on Papanicolaou smears of 182 patients with abnormal cervical cytology

Parameter	ASC-US	LSIL	ASC-H	HSIL	Total[†]
$P16^{INK4A}$+; PCR+	4	49	7	48	**108 (59.3)**
$P16^{INK4A}$+; PCR-	0	0	0	0	**0 (0)**
$P16^{INK4A}$–; PCR+	21	38	2	3	**64 (35.2)**
$P16^{INK4A}$–; PCR-	2	7	0	1	**10 (5.5)**
Total[†]	**27 (14.8)**	**94 (51.6)**	**9 (4.9)**	**52 (28.6)**	**182 (100)**

[†] Totals are given as number (percentage).

Table 4 demonstrates the comparison of P16 immunocytochemical and type specific PCR. Of the 25 ASC-US cytology specimens positive for GP5+/6+, two (8%) were positive for both the P16 protein and the presence of HPV types 16 and 18. Of these, P16 expression was strongly associated with the presence of HPV type 16 in one (4%) case and HPV type 18 in one (4%) case. Human Papilloma virus types 16 and 18 were not observed in two (8%) P16 positive ASC-US classified lesions. Sixteen (64%) of ASC-US classified lesions that were negative for either HPV types 16 and 18 showed negative expression of the P16 protein. In three P16 negative cases (12%), HPV type 18 was present.

Amplifiable DNA of HPV type 16 was detected in six (6.9%) of LSIL classified lesions expressing the P16 protein (n=87). In one (1.1%) HPV 16 positive case, P16 was not expressed. Expression of the P16 protein was strongly associated in 32 (36.8%) of HPV types 16 and 18 negative LSIL categorised lesions. Thirty three (37.9%) of LSIL classified lesions negative for HPV types 16 and 18 lacked the P16 protein. Likewise, P16 expression strongly correlated with seven (8%) HPV type 18 positive LSIL classified lesions. In three (3.4%) LSIL classified lesions positive for HPV DNA type 18 by PCR, the P16 protein was not detected. Infection with both HPV types 16 and 18 were observed in three (3.4%) P16 positive LSIL classified lesions. Two (2.3%) LSIL classified lesions displaying the presence of both HPV types 16 and 18, were negative for the P16 protein.

Expression of the P16 protein was strongly associated with the presence of HPV type 16 in two (22.2%) ASC-H classified cervical lesion (n=9). Four ASC-H classified lesions (44.4%) exhibiting P16 expression were negative for either HPV types 16 and 18 by PCR. One (11.1%) of ASC-H classified lesion negative for either HPV types 16 and 18 lacked the P16 protein. Infection with both HPV types 16 and 18 was completely absent in P16 positive ASC-H classified lesions.

In 10 (19.6%) HSIL classified lesions, P16 expression was strongly associated with the presence of HPV type 16 amplifiable DNA (n=51). Thirty (58.8%) HSIL classified lesions exhibiting P16 expression were negative for either HPV types 16 and 18 by PCR. A positivity rate for P16 expression and detectable HPV type 18 was observed in six (11.8%) HSIL classified lesions. Lack of correlation of P16 underexpression and presence of HPV type 18 by PCR was observed in two (3.9%) of HSIL classified lesions. The presence of both HPV types 16 and 18 was demonstrated in three (5.9%) P16 positive HSIL classified lesions.

Accordingly, a positivity rate for P16 expression and detectable HPV types 16 and 18 of 8% and 33.3% was observed in ASC-US and ASC-H classified lesions, respectively. The number of cases negative for both the $P16^{INK4A}$ immunoassay and type specific PCR ranged

from 64.0% to 37.9%, 11.1% and 0% in ASC-US, LSIL, ASC-H and HSIL respectively. Cohen's Kappa values for measures of agreement were calculated and found to be statistically insignificant (Kappa= 0.122), suggesting that agreement is most probably attributable to chance. The chi-square statistic for the McNemar symmetry is 57.76 with 1df (degrees of freedom) and p<0.0005. The null hypothesis that P16 overexpression and HPV types 16 & 18 reactivity and HR-HPV positivity is independent (Pearson chi-square= 5.399; p= 0.020), was rejected.

Table 4: Type specific PCR and P16 immunocytochemical results for HPV detection among patients with abnormal cervical cytology

Parameter	ASC-US	LSIL	ASC-H	HSIL	Total†
HPV16+; $P16^{INK4A}$+	1	6	2	10	**19 (11)**
HPV16-; $P16^{INK4A}$-	13	17	0	0	**30 (17.4)**
HPV16+; $P16^{INK4A}$-	1	1	0	0	**2 (1.2)**
HPV16-; $P16^{INK4A}$+	1	17	3	22	**43 (25)**
HPV18+; $P16^{INK4A}$+	1	7	1	6	**15 (8.7)**
HPV18-; $P16^{INK4A}$-	3	16	1	0	**20 (11.6)**
HPV18+;$P16^{INK4A}$-	4	3	1	2	**10 (5.8)**
HPV18-;$P16^{INK4A}$+	1	15	1	8	**25 (14.5)**
HPV16&18+; $P16^{INK4A}$+	0	3	0	3	**6 (3.5)**
HPV16&18+; $P16^{INK4A}$-	0	2	0	0	**2 (1.2)**
Total†	**25 (14.5)**	**87 (50.6)**	**9 (5.2)**	**51 (29.7)**	**172 (100)**

† Totals are given as number (percentage).

4.6 Comparison of discordant results with PCR results

The proportion of samples positive for HPV types 16 and 18 in LSIL classified lesions were significantly lower (23%) as compared to lesions where P16 immunoreactivity (55.2%) was observed. In addition, HSIL classified lesions demonstrated a similar trend with a P16 positivity rate observed in 96.1% of cases as compared to 41.2% of lesions which demonstrated the presence of oncogenic HPV types 16 and 18.

Using HPV detection by PCR as the gold standard, screening characteristics (sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV)) were calculated on the selected sample of the Namibian cohort (Table 5).

Table 5: Sensitivity, specificity, PPV, and NPV of P16 immunostaining in the Namibian population

Cut point	Outcome	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
$P16^{INK4A}$	LSIL	56.0	25.0	76.5	11.4
$P16^{INK4A}$	HSIL	90.4	50.0	98.0	16.7
$P16^{INK4A}$ + Cyto	LSIL	97.1	11.8	80.5	22.2

The sensitivity (55.7%) and specificity (25.0%) of the $P16^{INK4A}$ immunoassay for the detection of HPV induced cervical lesions was found to be very low in LSIL classified lesions. However, the sensitivity can be increased to 97.1% when HPV associated cellular changes for the detection of HPV was used in association with P16 immunoexpression. Unfortunately, the specificity declined considerably (11.8%) when this combination model was utilized.

The sensitivity in HSIL classified lesions increased significantly to 90.4% but the specificity remained at 50% when P16 expression *per se* was used as the indicator of HPV induced cervical lesions. The PPV (defined as the probability of disease among patients with a positive test) for LSIL and HSIL classified lesions remained high with the proportion of patients that was genuinely positive for P16 expression estimated to be 76.5% and 98.0% in LSIL and HSIL classified lesions respectively. In addition, the PPV for P16 expression in LSIL classified lesions increased to 80.5% when condylomatous changes combined with a P16 expression was used for the detection of HPV induced cervical lesions. The NPV (defined as the probability of no disease among patients with a negative test) for both LSIL and HSIL classified lesions remained under 20% with a disease prevalence in HSIL classified lesions of 96.3% based on a positive P16 reaction. A disease prevalence of 81.4% based on P16 immunoexpression for the detection of HPV associated cervical lesions was observed in LSIL classified lesions.

4.7 Risk factors

4.7.1 Characteristics of the target population

Table 6 summarizes the risk factors of study participants and their association with cytological abnormalities. Most participants were born in the Khomas region (49.8% for control subjects and 53% for study subjects)-data not shown. Participants ranged in age from 18-49 years for control subjects and 19-49 years for study subjects with a mean age of 33 years in both control and study subjects.

Table 6: Characteristics of women participating in the HPV prevalence project in Namibia

Risk factor		Test group (n=182)	%	Control group (n=205)	%
Age:	15-19	3	1.6	3	1.5
	20-24	23	12.6	24	11.7
	25-29	43	23.6	52	25.4
	30-34	51	28.0	60	29.3
	35-39	26	14.3	29	14.1
	40-44	25	13.7	31	15.1
	45-49	11	6.0	14	6.8
Residence:	Urban	180	98.9	204	99.5
	Rural	2	1.1	1	0.5
Marital Status:	Single	140	76.9	83	40.5
	Married	32	17.6	119	58.0
	Divorced	5	2.7	2	1.0
	Widowed	5	2.7	1	0.5
Number of births:	0 -2	111	61.0	142	69.3
	3-4	54	29.7	56	27.3
	≥5	17	9.3	7	3.4
Contraception :	Oral	18	9.9	48	23.4
	Injectable	78	42.9	65	31.7
	None	86	47.3	92	44.9
Smoking :	Yes	17	9.3	27	13.2
	No	165	90.7	178	86.8
Drinking :	Yes	40	22.0	59	28.8
	No	142	78.0	146	71.2
Chronic medication :	Yes	32	17.6	24	11.7
	No	150	82.4	181	88.3
HIV Status :	Reactive	64	35.2	7	3.4
	Non-reactive	69	37.9	161	78.5
	Unknown	49	26.9	37	18.1
Access to local health facilities:	Yes	180	98.9	192	93.7
	No	2	1.1	13	6.3
History of STDs in the past year :	Yes	51	28.0	11	5.4
	No	131	72.0	194	94.6
Age at first sexual debut:	≤15	9	4.9	6	2.9
	16-19	107	58.8	98	47.8
	≥20	66	36.3	101	49.3
Number of sexual partners :	1	38	20.9	80	39.0
	2	47	25.8	41	20.0
	>3	97	53.3	84	41.0
Pap smear screening :	Annually	23	12.6	69	33.7
	First time	55	30.3	95	46.3
	Vaginal problems	104	57.1	41	20.0

The majority of participants (98.9%) had access to local health facilities and do not reside in rural areas. Access to health facilities were based on a 10km radius from the nearest clinic or health facility in Windhoek. Anybody that resided further than the prescribed range was considered not to have access to local health facilities. Study subjects and controls differed sharply in marital status, HIV status, age at first sexual encounter, number of sexual partners and cervical screening history (Table 6). More than 75% of study subjects have never been married compared to a little more than 40% of women in the control group. The distribution of

women having more than 5 pregnancies remained low among women in both the control (3.4%) and study subjects (9.3%) groups.

A history of injectable contraceptive use was notable among 42.9% of study subjects with 9.9% of study participants reporting the use of birth control pills (oral contraceptives). Women in the control group expressed a similar trend with more than 31% of women reporting the use of injectable contraceptives. More than 47% of study subjects reported ever using injectable or oral contraceptives while in 10%, use of condoms by their male partners were encouraged. More than 9% of participants in the study subject group reported having smoked cigarettes, 7% were current smokers. Alcohol use was confined to 22.0% of study subjects compared to more than 28% of women in the control group; as little as 2% of study subjects reported to be casual drinkers (less than 3 glasses of wine per week).

Chronic medication use among study subjects (17.6%) was predominantly limited to HIV infected individuals who were currently on the national Anti-Retroviral Therapy (ART) programme with 6% of participants reporting use of chronic medication for other conditions such as high blood pressure, allergies, tuberculosis and asthma. Participants in the study group who tested positive for HIV, as seen on their green hospital passport or otherwise reported by the respective individual, accounted for more than 35% of women. Most notably, the highest rate of HIV positivity was observed among women aged 40-49 years (49.4%) with a lowest incidence of HIV positivity (17%) among women 20-24 years of age. Women aged 25-29 years reported a HIV positivity rate of more than 40% followed by a 32% HIV positivity rate among women aged 30-39 years (data not shown). Women in the control group differed significantly with regard to their HIV status with only 3.4% of women reported to be HIV positive. This is clearly an indication that HIV status does play a role in an increased HPV prevalence rate among sexually active women. Sexually transmitted infections were found to be limited to 28.0% of study subjects compared to 5.4% of women in the control group. The most prevalent sexually transmitted disease identified among study subjects was vaginal trichomoniases. Herpes Simplex Virus (HSV) and gonorrhoea were recorded in only 1% of study subjects. The measures of agreement between STIs and HIV reactivity were not statistically significant ($P = .234$). On the other hand, the degree of disagreement was statistically significant ($P < .001$) when applying a binomial distribution (McNemar test). No strong correlation was found between HPV prevalence and STIs among women of the study subject group.

Measures of sexual activity as depicted by age at first sexual encounter, number of sexual partners and years of sexual activity varied among study and control groups. The majority of study subjects reported to have more than 3 sexual partners (53.3%), age at first sexual

encounter between 16 and 19 years (58.8%) and an average of 13.5 years of sexual activity (median, 13; range, 1-32). In contrast, 41% of women in the control group reported to have more than 3 sexual partners with over 49% of women having had their first sexual contact at the age of 20 years and older. The average number of sexual partners was 2.52 (median, 2, range 1-9) for control subjects and 3.3 (median, 3; range, 1-37) for study subjects. The mean age at first sexual encounter was 19.9 years (median, 19; range, 10-31) for control subjects and 19.5 years (median, 18; range, 13-29) for study subjects.

Most participants (57.1%) in the study group attended gynaecological facilities only when they encountered vaginal problems such as abnormal vaginal bleeding, offensive vaginal discharge or lower abdominal pains; 30.3% never had a Pap smear in their lifetime. The mean age of women who presented for cervical screening based on a history of vaginal problems and/or first time attendants was 36.3 years (median, 32) for control subjects and 35 years (median, 33) for study subjects. Of the 30.3% of women in the study subject group who never had a Pap smear, five (2.6%) of them had their first Pap smear as a result of a postnatal follow up. Most notably, 46.3% of women in the control group never had a Pap smear in their lifetime while 20% presented for cytology screening as a result of vaginal problems they experienced. Of the 46.3% of women in the control group who never had a Pap smear taken before, 11.7% of them had it as a result of a routine postnatal visit. The mean age at first sexual encounter for first time attendants and women who presented for cervical (Pap smear) screening due to vaginal problems was 21.7 years (median, 19) for control subjects and 19 years (median, 18) for study subjects.

We further investigated the risk factors for HPV acquisition among study participants by comparing concordant HPV DNA results with the cytological results. Table 7 summarizes the results that were obtained. Women aged 25-34 years had a peak prevalence rate of more than 50% for HPV detection with more than 70% of women reported to be single or never been married. The majority of candidates have had 0-2 pregnancies (crude odds ratio (COR) = 3.2), demonstrating that the number of pregnancies that resulted in live births cannot be considered as a predictor of HPV acquisition in the study sample. More than 40% of women tested positive for HPV demonstrated injectable contraceptive use (crude odds ratio (COR) = 1.8). The distribution of oral contraceptive use in women remained consistent with over 10% of women reporting a history of oral contraceptive use. More than 40% of study subjects reported ever having used injectable or oral contraceptives. Less than 10% of participants reported use of condoms by either themselves or their respective male partners.

Chronic medication use was confined to more than 25% of individuals with ART medications that predominated, followed by chronic medication for tuberculosis, high blood pressure and

asthma. No strong correlation for HIV status was found between HPV DNA detection and cervical cytology. A very good agreement was observed in the Pap smear screening history with more than 50% of subjects presenting for cervical screening only when vaginal problems arose.

Age at first sexual encounter remained consistent with more than 50% of individuals having had their first sexual encounter between the ages of 16-19 years (crude odds ratio (COR) = 7.8). Nearly 60% of individuals reported to have more than 3 sexual partners in their lifetime (crude odds ratio (COR) = 2.4). The mean years of sexual activity was the highest among women 25-34 years of age estimated at 11.1 years (median, 11; range 3-19).

Table 7: Risk factors for HPV prevalence among Namibian women

Risk factor		HPV DNA positivity % (n=172)	HPV Cytology % (n=66)
Age:	15-19	1.9	4.5
	20-24	15.5	12.1
	25-29	23.9	22.7
	30-34	26.5	30.3
	35-39	16.8	16.7
	40-44	11.6	10.6
	45-49	3.9	3.0
Residence:	Urban	93.6	92.1
	Rural	6.4	7.9
Marital Status:	Single	72.3	77.3
	Married	23.2	16.7
	Divorced	1.9	4.5
	Widowed	2.6	1.5
Number of births:	1-2	60.6	53.0
	3-4	27.7	31.8
	≥5	11.6	15.2
Contraception :	Oral	14.8	13.6
	Injectable	43.2	40.9
	None	41.9	46.5
Smoking :	Yes	9.7	3.0
	No	89.3	97.0
Drinking :	Yes	23.9	16.7
	No	76.1	83.3
Chronic medication :	Yes	25.2	25.8
	No	74.8	74.2
HIV Status :	Reactive	32.9	45.4
	Non-reactive	39.3	25.8
	Unknown	27.7	28.8
Access to local health facilities:	Yes	97.0	98.0
	No	3.0	2.0
History of STDs in past year:	Yes	29.5	28.8
	No	70.5	71.2
Age at first sexual debut:	≤15	9.4	9.1
	16-19	57.6	56.1
	≥20	33.0	34.8
Number of sexual partners:	1	11.2	10.6
	2	29.8	31.8
	≥3	59.0	57.6
Pap screening:	Annually	20.6	12.1
	First time	29.0	34.8
	Vaginal problems	50.4	53.0

4.7.2 Risk factors associated with HR-HPV types 16 and 18

Table 8 demonstrates the most prevalent risk factors associated with the presence of oncogenic HPV types 16 and 18. Generally, risk factors associated with the distribution of either HPV types 16 and/or 18 was coherent and uniform among participants and no significant differences in the prevalence of type-specific risk factors were found. The mean age of women harbouring either HPV type 16 or 18 was 32 years (range 21-47 years, SD= 6.657). Unmarried women (85.7%) convincingly demonstrated to be at a higher risk of contracting HR-HPV type (s) 16 and/or 18. Women aged 25-34 years displayed a higher risk

of acquiring HPV types 16 and 18 as were the rate of HIV positivity (55.2%) among this age group. Women also demonstrated a higher rate of injectable contraception use (30.4%) as compared to oral contraceptive use (21.4%). Chronic medication histories which mainly include ARV treatment were confined to 52.3% of individuals that were HIV positive. Sexually transmitted infections were also observed in rather increasing numbers (32.1%) but no direct link was found between HR-HPV acquisition and other STIs. Younger age at first sexual encounter became even more evident with 76.6% of women indicating to have had their first sexual encounter between the ages of 16-19 years. Women with more than two sexual partners (59.7%) also appeared to be at a higher risk of acquiring HPV type (s) 16 and/or 18.

Table 8: Risk factors for HPV detection among women in Namibia for oncogenic HPV types 16 and 18

Risk factor		HPV positive (n=172) %	HPV type 16 (n=21) %	HPV type 18 (n=25) %
Age:	15-19	1.9	0	0
	20-24	15.5	17.2	6.1
	25-29	23.9	17.2	24.1
	30-34	26.5	34.5	39.4
	35-39	16.8	13.8	15.2
	40-44	11.6	6.9	9.1
	45-49	3.9	3.4	6.1
Residence:	Urban	93.6	96.6	90.9
	Rural	6.4	3.4	9.1
Marital Status:	Single	72.3	82.8	84.8
	Married	23.2	17.2	15.2
	Divorced	1.9	0	0
	Widowed	2.6	0	0
Number of births:	0-2	60.6	51.7	45.5
	3-4	27.7	37.9	33.3
	≥5	11.6	10.3	21.2
Contraception :	Oral	14.8	20.7	21.2
	Injectable	43.2	44.8	27.3
	None	41.9	34.5	51.5
Smoking :	Yes	9.7	24.1	12.1
	No	89.3	75.9	87.9
Drinking :	Yes	23.9	37.9	24.2
	No	76.1	62.1	75.8
Chronic medication :	Yes	25.2	27.6	18.2
	No	74.8	72.4	81.8
HIV Status :	Reactive	32.9	34.5	42.4
	Non-reactive	39.3	44.8	30.3
	Unknown	27.7	20.7	27.3
Access to local health facilities:	Yes	97.0	96.6	90.9
	No	3.0	3.4	9.1
History of STDs in past year:	Yes	29.5	24.1	30.3
	No	70.5	75.9	69.7
Age at first sexual debut:	≤15	9.4	3.4	9.1
	16-19	57.6	82.8	63.6
	≥20	33.0	13.8	27.3
Number of sexual partners:	1	11.2	10.3	12.1
	2	29.8	20.7	21.2
	≥3	59.0	69.0	66.7
Pap screening:	Annually	20.6	13.8	18.2
	First time	29.0	24.1	18.2
	Vaginal problems	50.4	62.1	63.6

CHAPTER 5
DISCUSSION

The reliability of both cytological and histological diagnosis in identifying cervical abnormalities is a common global problem, leading to unnecessary high costs and inappropriate patient management in the health sector. Based on these limitations, a need for more sensitive and alternative approaches such as biomarkers should be considered. Ideally, a potential biomarker should be affordable and have demonstrated the ability to detect cervical lesions induced by high risk Human Papilloma Viruses destined to progress to more severe dysplastic lesions. To assess the consistency of $P16^{INK4A}$ as a biomarker, we evaluated abnormally diagnosed cervical smears subjected to P16 immunostaining.

The results of this study demonstrated the value of the $P16^{INK4A}$ biomarker as a means of measuring HPV disease progression in cervical lesions. In line with our hypothesis, the results confirm that the detection of the P16 tumour suppressor protein in Pap smears are linked to an enhanced expression of high-risk HPV oncogenes observed during cervical carcinogenesis. Our findings further suggest that identification of ASC-US and LSIL classified cervical lesions induced by HPV provides information which may be useful in specific management strategies. It also indicates the need for appropriately formulated cervical screening guidelines for Namibia. Concurrent with our findings, the risk for progression of cervical abnormalities induced by HPV to more severe dysplastic lesions (including invasive cervical cancer) may also be associated with several factors including younger age (< 30 years of age), sexual history of participants and cervical screening practices. These results also demonstrate the need to implement adjunctive testing for improved controlling of cervical carcinogenesis. In this way, the sensitivity of cytologically-based detection methods is augmented. The clinical usefulness of the $P16^{INK4A}$ biomarker in addition to Pap smear screening reiterates the construction of proper health care policies pertaining to women's gynaecological health.

The interpretation of the overt cellular features associated with the majority of abnormally classified cervical lesions did not present a problem in this study and specific criteria as described by De May, (1996) (Chapter 2, Section 2.11, p17-22) were applied. Since ASC was considered a very frequent diagnosis in Pap smear screening, comprising approximately 5% of cervical screening diagnosis (Cox, 2005), TBS of 2001 propounded the sub-classification of atypical squamous cells (ASC) into ASC-US and ASC-H. We reported the presence of ASC-classified cervical lesions in 19.8% of Namibian women with cervical abnormalities with 15% of lesions with an ASC-US interpretation. Several authors (Solomon *et al*., 2001 and Davey *et al*., 2000) agree that atypical squamous cells in ASC-US classified cervical lesions presented as isolated, single-lying superficial and/or intermediate squamous

epithelial cells, and are rarely arranged in sheets. Specific cellular features confined to these lesions include a slightly elevated ratio of the nucleus to the cytoplasm (N/C) coupled with mild hyperchromasia of the nucleus and irregular chromatin distribution. Consistent with our study, Frappart *et al*., (2003) demonstrated the presence of single-lying squamous epithelial cells displaying binucleation and mild nuclear enlargement to be associated with an ASC-US interpretation. Concurrent with these specific findings, Frappart *et al*., (2003) further observed the presence of ill-defined nuclear membranes and dense chromatin distribution in the nucleus to be associated with ASC-US (cannot rule out LSIL). We meticulously assessed cellular features in well preserved squamous epithelial cells, taking into consideration the patient's age and previous Pap smear screening history should the need arise to ascertain an abnormal cytological diagnosis. Similar to our findings, Saad *et al*., (2006), who conducted a retrospective study on 800 gynaecological smears, acknowledged the presence of extensive inflammation, cellular degeneration and aberrant nuclear changes due to air-drying as potential pitfalls for an ASC-US interpretation. We endeavoured to minimise misclassification through data integration where the clinical history of the patient coupled with the presence of inflammatory mediators such as infections, and other associated factors were cooperatively considered. The presence of clearly delineated cellular features in well-preserved squamous cells with pale, round nuclei and a regular chromatin distribution denotes a benign diagnosis.

Hyperchromatic crowded groups (HCGs), a term initially proposed by De May, (1996), (designated as clusters of endocervical cells and/or aggregates of inflammatory, parabasal and endometrial cells) were readily identified and rarely presented a false positive diagnosis. Chivukula *et al*., (2007), who conducted a study among 601 women demonstrating either or no cellular abnormalities, reported the presence of three-dimensional clusters consistent with HCGs in 28% of ASC-US classified lesions, suggesting that their occurrence represent adequacy of the transformation zone thereby ensuing the detection of cervical cell abnormalities. Nuclear aberrations confined to miniature squamous epithelial cells with an eosinophilic cytoplasm (either arranged singly or in sheets) were at times observed in perimenopausal women (ages 45-49 years), eliciting a diagnosis of ASC-US viz ASC-H. These cells are referred to as atypical parakeratosis, described in the Bethesda system of 1991 as small squamous epithelial cells displaying cellular pleomorphism, including elongated and caudate shapes demonstrating an elevated N/C ratio with nuclear hyperchromasia. Chivukula *et al*., (2006), who reviewed 161 cervical smears of American women over a 2 year period with a cytological diagnosis of ASC-H, reported that 31% of ASC-H classified cervical lesions demonstrating similar groups with cyanophilic cytoplasm were reclassified as reactive changes after a biopsy was histologically evaluated. They postulated that lack of well-delineated criteria resulted in poor interobserver reproducibility

among Pap smear screening facilities, eventually leading to false positive diagnosis. Since the diagnosis of atypical parakeratosis is not included into the current Bethesda glossary, the term is used in an apropos manner for clarification.

Abramovich *et al*., (2003), who retrospectively reviewed 355 Pap smears of women with a histological diagnosis of atypical parakeratosis, demonstrated that at least 40% of women with atypical parakeratotic cells on their Pap smear may harbour an underlying cervical lesion. This emphasized the importance of specific and well-defined cellular criteria as it relates to the respective cervical abnormality. Vooijs *et al*., (1985) reinforced this in the event of an atypical parakeratosis interpretation, showing that two smears should be obtained through forceful scraping of the cervix to remove the top layer of parakeratotic cells in order to obtain a representative collection of the cells underneath. This ensured that possible epithelial cell abnormalities including invasive cervical cancer would not be missed. Keating *et al*., (2001), who reviewed 770 cervical smears of American women at the Brigham and Women's hospital (BWH), reported a high degree of nuclear contour irregularities in squamous metaplastic cells among perimenopausal and postmenopausal women as opposed to premenopausal women, suggesting that air-drying due to a delayed fixation with alcohol and cellular atrophy were more likely to be associated with this trait. Despite the cellular changes associated with air-drying including cellular degeneration, cytoplasmic eosinophilia and nuclear hypochromasia coupled with the absence of chromatin material, the interpretation of these Pap smears remains challenging. Well-defined cytological criteria in intact squamous epithelial cells using the size and shape of a well-demarcated intermediate cell for comparison proved valuable in precluding false negative diagnosis.

Previous studies (Massad *et al*., 2003 and Sawaya *et al*., 2001) reported an ASC-US interpretation in postmenopausal women to be punctiliously considered especially when nuclear variations are significant in an atrophic cell population. We observed similar changes in Pap smears collected from postmenopausal women and followed the approach of recommending a course of vaginal oestrogen therapy to be the applied after which the smear is repeated. In this way, the squamous epithelial cells differentiated into a more mature type of squamous epithelium, allowing for the proper identification of an ASC-US interpretation using the specific cellular criteria. Abati *et al*., (1998), who conducted a cytological review on 90 cervical smears, reported that only 11% of postmenopausal women with ASC-US associated atrophy demonstrated true ASC-US lesions after the women have been exposed to oestrogen treatment. However, Slawson *et al*., (1993) in his review of 191 cervical smears from American women with an ASC-US interpretation showed that 60% of women have cervical abnormalities on subsequent biopsy ranging from 85% for a LSIL interpretation to 15% for a HSIL interpretation. We did not examine for cervical abnormalities based on

histology correlations and speculate that these differences in cytology-histology analogues most likely originated from poor interobserver subjectivity, reinforcing the crucial need for adjunctive diagnostic procedures (Klaes *et al*., 2001). Slawson *et al*., (1993) further proposed that nuclear enlargement *per se* is not adequate in considering an ASC-US interpretation in postmenopausal women and suggested that the specific cytological criteria associated with an ASC-US interpretation should be applied collectively to avoid an overdiagnosis.

Another major concern for an erroneous ASC-US interpretation was the effect of intrauterine devices (IUDs) on the cervical epithelium. These pessaries actively exerted their effect by eliciting an immune response in the uterus leading to the desquamation of cytological abnormal cells from the cervix, retaining their potential to secrete mucus. Similar to our study, Risse *et al*., (1981), who conducted a study on 102 cervical smears of Dutch women, reported that over 7% of these women with IUDs, demonstrated cellular changes associated with an ASC-US interpretation on their respective Pap smears. On the other hand, Gupta, (1982), who conducted a study among 99 ASC-US classified patients with a history of an IUD *in situ,* reported cellular atypia in squamous epithelial cells not associated with pessary use. We do take cognisance that IUDs may induce cellular changes resembling ASC-US. However, the patient history must be considered in making a final diagnosis.

Atypical squamous cells-cannot exclude HSIL, commonly known as atypical squamous metaplasia is a new term that was introduced into TBS of 2001, encompassing small cells with an elevated N/C ratio, loosely arranged or a disordered arrangement in a so-called “crowded sheet” pattern. The latter arrangement is most commonly encountered in tissue biopsies characterised by groups of small cells reflecting loss of polarity and a tendency to overlap (Alli and Ali, 2003). In our study, cellular features associated with ASC-H classified lesions were generally assessed in single isolated cells. Nevertheless, assessing morphological features associated with ASC-H in thick tissue fragments posed a problem and consequently lead to a cytological diagnosis of HSIL viz ASC-H. This difficulty was further enhanced by the presence of inflammatory components such as the presence of numerous neutrophil leucocytes coupled with eosinophilic staining of parabasal cells and irregular distribution of chromatin material in these cells’ nuclei. Additionally, reparative cellular changes which include multinucleation, prominent macronucleoli and chromatin condensation were further recognised as potential causes for a false positive result. Boon *et al*., (1991), who conducted a study on the effects of the cytobrush on cervical samples, recognized similar structures and suggested that rigorous scraping of the endocervix with an endocervical brush resulted in the presence of such fragments. We propose that poor ecto/endocervical sampling performed by unskilled health workers resulted in the incomplete strata distribution of cells presenting as tissue fragments. These disturbances emphatically

demonstrate the need for documented training procedures in Pap smear collection and processing. In this way, standardised training can be applied to health officials involved in Pap smear collection and processing and specific shortfalls can be identified. This will minimise inadequate cervical sampling, consequently leading to better cervical yield and an overall improvement in the detection rate of cervical abnormalities. Consequently, cervical cells appear to be better preserved and screening errors will be reduced. We also endeavoured to minimise diagnostic pitfalls by assessing specific cellular features associated with the respective cervical abnormalities in single abnormal cells if present. Consistent with the guidelines stipulated by the ASCCP, the management of an ASC-H classified patient which included colposcopy followed by a biopsy was not influenced by a false HSIL diagnosis as both lesions have similar treatment modalities.

Our attempt to minimise any discordances were further enhanced by concealing critical diagnostic information from the cytotechnologists who executed the screening of cervical smears. In this regard, selected cervical smears were given to two cytotechnologists for microscopic assessment and diagnosis. The diagnoses from both screeners were then collected and compared for consistency and accord. Differences in the interpretation of cervical smears were redirected to the histopathologist who then re-evaluated the specific smear and make a final decision.

Lower-grade squamous intraepithelial lesions conceptually include cellular changes associated with mild dysplasia or CIN I and condylomata acuminata. The interpretation of CIN I and HPV-associated cellular changes did not bear different clinical significances as both had similar treatment regimens, thus supporting the common LSIL classification. De May, (1996) demonstrated the presence of either single lying or 2-dimensional arrangement of well-preserved “mature” or superficial squamous epithelial cells with nuclear enlargement approximating at least three times the size of a normal intermediate cells’ nuclei and coarsely granular chromatin arrangement as typical of a CIN I classified cervical lesion. On the other hand, Frappart *et al*., (2003) reported enlargement of the nucleus coupled with nuclear hyperchromasia and binucleation in parabasal cells to be associated with a LSIL classified lesion. These variations may be related to several factors including the number of clearly delineated abnormal cells apportioned by the different laboratory settings. Renshaw *et al*., (2005) determined that the potential to miss a true LSIL classified cervical lesion is higher in smears containing fewer abnormal cells (<50 abnormal cells per slide) as opposed to those with an abnormal cell count of between 101 and 250. This reiterates the dire need for adjunctive screening modalities in order to improve the sensitivity of the Pap smear in detecting cervical abnormalities. In our study, we observed the presence of loosely arranged single lying and well-preserved intermediate and or superficial-type squamous epithelial cells

with nuclear enlargement not less than 3 times the size of an intermediate cell's nucleus. We did not employ any threshold measures in terms of the number of abnormal cells.

Koilocytosis (as denoted by the presence of well preserved superficial cells or immature squamous cells with recognizable halos surrounding the nucleus and peripheral condensation of the cytoplasm) was common in identifying LSIL associated with HPV. We recognized that nuclear hyperchromasia coupled with nuclear pleomorphism in association with perinuclear clearing demonstrated the presence of koilocytes characteristic of HPV-induced lesions. Frappart *et al*., (2003) recognized the presence of so-called pseudokoilocytes with or without cytoplasmic clearing as a possible artefact or occasionally related to navicular cells commonly found in pregnant women. Cellular features that include ellipsoid-shaped squamous cells with a glycogen-filled cytoplasm presenting as refractile areas and convoluted boundaries were paramount in identifying navicular cells. Jovanovic *et al*., (1995) recognized the importance of squamous atypia in postmenopausal women commonly associated with pseudokoilocytosis and proposed that pseudokoilocytes in postmenopausal women should be differentiated from true koilocytes based on the following cellular criteria: nuclear chromatin distribution should appear to be evenly proportioned with mild nuclear enlargement and delicate cytoplasmic staining coupled with equable cytoplasmic halos.

Our findings further demonstrated pronounced cellular changes from cervical smears collected from four HIV-patients characterised by marked anisonucleosis contained within an eosinophilic cytoplasm, nuclear hyperchromasia, extensive inflammation and accompanying bacterial or fungal infection. Harris *et al*., (2007), who conducted a study among women in South Africa, found that HIV positive women presented with cervical cancer 15 years earlier than their HIV-negative counterparts. As Harris *et al*., (2007) reported, we concur that these cervical changes originate from the effects of the Human Immunodeficiency Virus in reducing immunological functions.

Occasionally, it was difficult to classify borderline features into either HSIL or LSIL. Cellular characteristics favouring a HSIL include numerous abnormal cells with a much higher N/C ratio, more prominent nuclear aberrations and irregular distribution and coarsening of the chromatin. Typically, the abnormality in HSIL classified lesions occur more commonly in parabasal and basal layers as opposed to LSIL where cellular abnormalities are found in the intermediate and superficial-type cells. The cytoplasm of LSIL classified lesions are well-demarcated and more abundant giving cells their polygonal shape. In HSIL classified lesions, the cytoplasm appears to be more of the immature type with a delicate and fragile intensity,

and an overall cell size smaller than those of a LSIL classified cervical lesion (Kurman and Solomon, 1994).

Interpretation of clearly delineated HSIL features in cervical cytology remains challenging with typical abnormal changes occurring essentially in the less mature cell population. In our study, the cellular pattern was characterised by the presence of cells occurring isolated, in sheets or in syncytial groups which demonstrated nuclear pleomorphism with nuclear hyperchromasia and a high N/C ratio. Nuclear indentations or grooves were prominent which were further accompanied by unevenness of the nuclear membrane and a fine-to-coarse-to-smoothly granular chromatin distribution. The cytoplasmic appearance of abnormal cells remained erratic with a "lacy" and delicate or densely packed metaplastic appearance (Wright *et al*., 2002). In spite of well-described criteria, Kang *et al*., (2001) demonstrated the presence of immature squamous metaplastic cells as characterised by the appearance of parabasal/basal cells with densely packed cytoplasm and an increased N/C ratio accompanied by oval-shaped nuclei commonly mimicking HSIL classified lesions. We observed similar cellular features potentially eliciting an HSIL diagnosis. However, differentiating the cellular criteria reported by Wright *et al*., (2002) from those reported by Kang *et al*., (2001) remain paramount in avoiding a false positive diagnosis. Murali *et al*., (2010) further demonstrated the presence of 3-dimensional arrangement of cells with mild anisonucleosis and an elevated N/C ratio, nuclear contour variations and a finely distributed chromatin pattern frequently associated with an HSIL classification in early pre-menopausal women. We generally tried to avoid these common pitfalls by requesting a repeat Pap smear after topical vaginal oestrogen treatment. At the same time, high endocervical sampling obtained through brushing of the endocervical canal by an endocervical brush and as defined by the presence of syncytial-like groups with an evenly distributed chromatin pattern, nuclear hyperchromasia and inconspicuous nucleoli were also regarded as a common source for misclassification (Fiorella *et al*., 1994). In one case, we observed these cellular features accompanied by a cyanophilic cytoplasm on the Pap smear of a pre-menopausal woman and requested a follow-up smear after vaginal oestrogen treatment. Most clinical settings in this study, however, use a modified Ayre spatula in collecting cervical material for microscopic assessment thus minimising the risk of this endocervical artefact. Chivukula *et al*., (2007) suggested that the presence of cilia and peg cells tremendously assist in affirming a benign diagnosis.

The presence of benign endometrial cells generally encountered prior to or towards the end of a women's menstrual period may be misclassified as a neoplasia especially if accompanied by degenerative changes and nuclear hyperchromasia. De May, (1996) recognized the importance of hyperchromatic crowded groups as an indicator of specimen

adequacy, their presence which enhances the sensitivity in detecting epithelial cell abnormalities. Typical neoplastic features, included highly irregularly-shaped cells accompanied by severe nuclear variation, hyperchromasia and irregular chromatin distribution were generally absent thus reducing the chances of considering a carcinoma of the endocervix or endometrium. Small cells resembling syncytial-like groups with/without nucleoli commonly mimic HSIL involving the endocervical glands. Chivukula *et al*., (2007) reported the presence of similar groups of cells misclassified as HSILs and suggested careful microscopic consideration under high resolution. Distinctive cellular characteristics such as extreme variations in the nucleus with reference to size, shape and haphazard chromatin distribution as well as the absence of nucleoli seem vital in classifying HSILs (Renshaw *et al*., 2006). Similar to Renshaw *et al*., (2005) we observed clusters of small cells with rounded and well-preserved contours which are associated with HSILs. However, we recognised the most common diagnostic pitfalls and strived to evaluate distinctive cytological criteria in single lying abnormal cells.

Additionally, monolayered sheets of cells that fold on themselves coupled with ill-defined cellular borders, round to oval nuclei with mild chromatin irregularities and slight nuclear hyperchromasia and prominent nucleoli were observed in one cervical smear. This generated a false positive response of a HSIL especially in the presence of a purulent inflammatory background and was referred for colposcopy. Tissue fragments demonstrated similar cellular characteristics however nuclear aberrations were not as pronounced and a discordant histological diagnosis of repair was reported. Colgan *et al*., (2001), who assessed the consistency rate among different laboratory settings with reference to diagnostic pitfalls relating to a diagnosis of typical repair, demonstrated that 0.47%-5.41% of cytoscreeners (cytotechnologists and cytopathologists) reported a false negative diagnosis ranging from LSIL to HSIL to invasive cervical carcinoma as opposed to a false negative rate of 24%-62% for a diagnosis of repair. We appreciate the need for histological confirmation as the gold standard, in particular, with an HSIL interpretation even though we are aware that interobserver's reproducibility needs to be optimized.

Similarly, degenerated endocervical cells associated with microglandular hyperplasia encountered in streaks of mucus were yet another problematic arrangement in our study, potentially leading to misclassification into HSIL. Generally, these cells resemble immature squamous metaplastic cells with a dense and often vacuolated cytoplasm and slight nuclear hyperchromasia. Shidham *et al*., (2004) who reviewed 48 cases of histologically confirmed microglandular hyperplasia, found that 85% of cases were reported as "negative for intraepithelial lesion or malignancy" with 5 cases with slight nuclear variations associated with "atypical squamous cells-cannot exclude a high-grade squamous intraepithelial lesion."

Koss *et al*., (2006) noted that there are no unique cytological criteria that will enable a diagnosis of microglandular hyperplasia and proposed endocervical brush artefacts as a common source for these findings. Nauth, (2007) suggested that hormonal overstimulation possibly through the use of oral contraception contributed to the proliferation of the endocervical glands leading to the desquamation of cell clusters expressing mild nuclear enlargement, infrequent cytoplasmic vacuolization and cellular degeneration. Recognition of cytologically associated changes should be made under high resolution microscopy and where possible in collaboration with the patients' contraceptive history.

No cervical screening program is currently in place in Namibia and the obvious need for such an initiative is strongly recommended against the background of the high HIV incidence which potentially increases cervical abnormalities. Concurrently, we observed a prevalence rate of 15% as ASC-US, 51.3% as LSIL, 4.8% as ASC-H and 28.9% as HSIL- classified cervical lesions. Okonda *et al*., (2009), who conducted a retrospective review on 12 188 cervical smears of women in Swaziland, reported an incidence rate of 19.8% as ASC-US, 9.0% as LSIL, 8.8% as ASC-H and 4.6% as HSIL. The number of cervical abnormalities in Windhoek may be an underestimation of women in the capital as only women attending public health facilities were included. Our study was also limited to women in their reproductive years (15-49 years) and as such may not be a true reflection of the number of cervical aberrancies in Namibia. Due to the lack of a well organised screening program, vulnerable groups may have been left out and this figure could potentially be much higher. Parham *et al*., (2006), who conducted a study among 150 Zambian women, demonstrated the presence of abnormal cervical lesions in 23.3%, 32.6% and 20% for LSIL, HSIL and possible squamous cell carcinoma, respectively. These figures demonstrate the aggressiveness of the HIV-associated cervical cancer incidence which may increase further in the absence of a well structured cervical screening program. Another study by Van Bogaert *et al*., (2001) among 22 160 South African women in a rural setting of the Transkei, reported a prevalence of cervical abnormalities of 34.7% for ASC-US, 8.3% for LSILs and 2.4% for HSILs. Consequently, these figures could drop drastically if a preventative screening program was in place.

The age distribution for cervical abnormalities was analysed in this study. While it is believed that cervical abnormalities are more commonly encountered among elderly women, our study frequently observed aberrancies in sexually active young women between the ages of 25-34 years. This specific age group may be more susceptible to multiple sexual influences, which in turn may increase their risk for HPV exposure. Contrary to our findings, Okonda *et al*., (2009) reported the highest incidence of cervical abnormalities among Swazi women at the age of 20-29 years. Van Bogaert *et al*., (2001) also found that more that 20% of cervical

abnormalities were detected among South African women before the age of 30 years. These findings corroborated with those of De May, (1996) and Nauth, (2007) where HSILs and LSILs were frequently observed among younger women.

Typically, a 70% reduction rate of cervical cancer can be expected in the presence of a well organised screening program (Wright *et al*., 2006). Nanda *et al*., (2000) who conducted a meta-analysis on 94 screening studies, reported a sensitivity of cervical cytology to be between 30%-87% and a specificity ranging from 86%-100%. Most of these studies were conducted before the advent of liquid based cytology (LBC) and the application of endocervical brushing however, based on a recent analysis by Cuzick *et al*., (2006) involving European and North American screening, the sensitivity of cytology for HSILs and CC were approximately 53% and ranging from 19% among German women to 77% among British women. Fahey *at al*., (1995) reported a false negative rate of between 20%-30% for conventional cytology while Clavel *et al*., (2001) proposed that the sensitivity of liquid based cytology could be improved by 15%-20% when compared to conventional cytology. Davey *et al*., (2006), who conducted a comparative study, found there was no remarkable improvement in sensitivity when LBC was used and suggested that randomized trials be used to determine the sensitivity of conventional cytology and LBC. To the contrary, Ronco *et al*., (2006), who conducted a study among Italian women, reported no significant differences in the sensitivity of conventional cytology as compared to LBC. It appears as if LBC as a newer technology is unable to improve the sensitivity of cervical cytology and therefore alternative or ancillary, non-cytology based screening modalities are needed.

By using an immunocytochemical staining method, we demonstrated the advantages of P16 staining of cervical smears allowing interpretations ranging from ASC-US, LSIL, ASC-H and HSIL. In this study, 187 abnormally diagnosed cervical smears were subjected to P16 immunostaining. Distinctive morphological characteristics coupled with immunocytochemical staining intensity were collaboratively regarded as predictors for cervical disease progression. Presence of strong positive immunostaining in the nucleus of abnormal cells was found in 55.5% and 83.3% of ASC-H and HSIL classified lesions respectively, suggesting that immuno-intensity in the nucleus may be indicative of cervical disease progression. Six HSIL classified cases demonstrated strong immunostaining in both the nucleus and cytoplasm. Differences exist among authors with regard to the stain location and whether it is the nucleus and/or cytoplasm and the number of P16 positive cells (Sano *et al*., 1998 and Negri *et al*., 2004) with several researchers not considering staining distribution and positive P16 immunostaining cell count as reflective of disease severity (Carozzi *et al*., 2006 and Wentzensen *et al*., 2005). We demonstrated strong P16 immunostaining in the nucleus of abnormal cells in 26% of LSIL classified lesions but there was also varying

intensity in both nucleus and cytoplasm of other cases of LSIL. We employed a threshold of at least five abnormal cells or 25% of abnormal cells exhibiting P16 immunoexpression to confirm immunoreactivity. Similarly, Sahebali *et al*., (2004) considered the number of cells displaying P16 immunoreactive as predictive of cervical disease severity as opposed to Duncan *et al*., (2007) and Wentzensen *et al*., (2005) who suggested that the number of positive cells is insignificant as it relates to the cervical abnormality. A recent review by Tsoumpou *et al*., (2009) does not dispute that $P16^{INK4A}$ immunostaining correlates with cervical disease progression but concluded that inconsistent scoring of P16 expression in cells may further promote subjective interpretation. Although P16 expression is related to the severity of cervical neoplasia, several studies (Guo *et al*., 2004 and Nieh *et al*., 2003) demonstrated that a lack of consensus for P16 positivity in equivocal and low-grade cervical lesions may question its reliability in these groups. Conversely, benign cellular changes demonstrating no evidence of HPV infection will not display P16 expression.

Moreover, two ASC-US classified cases demonstrated strong P16 expression, suggesting that these lesions may have the potential to progress to severe dysplastic lesions. Nieh *et al*., (2003), who reviewed 85 cervical smears of Taiwanese women, proposed that strong P16 immunoreactivity in ASC-US categorized Pap smears may be predictive of progressive change in the future. Although the presence of inflammatory components and streaks of mucus may affect visualization, it did not have a significant impact on the P16 immunoreactivity. In our study, P16 immunoreactivity was clearly observed and background elements did not affect the detection rate of cells displaying P16 immunoreactivity.

We also recognised the predictive values based on data obtained in this study for the purpose of improving sensitivity in detecting HPV-induced cervical abnormalities. In our study, the positive predictive value (PPV) as defined by the number of cases with a positive result correctly identified for this small proportion of women was low when screening by cytology was used however, a remarkable increase in the PPV was observed when P16 immunostaining combined with cytology was applied in detecting a HSIL lesion. Similarly, the sensitivity in detecting a HSIL lesion increased considerably when cervical cytology followed by P16 immunostaining was applied. Compared to a cytological diagnosis of LSIL, the PPV remains modest, suggesting that some potential lesions induced by HR-HPVs may have been missed. This does not imply that the respective lesions may regress but merely identifying potentially delayed P16 expression.

A review of the literature suggests that P16 as a cyclin-dependant kinase inhibitor exhibits elevated expression in cervical abnormalities caused by chronic infection with HR-HPVs. Persistent infection with any strain of oncogenic HPV types results in the arrest of the pRb-

E2F complex after which the release of one of the viral proteins, E7, impairs the function of the retinoblastoma tumour suppressor protein. The retinoblastoma protein fails to bind to E2F transcription factors and cell replication proceeds continuously and uncontrollably, eventually leading to the synthesis of activated P16 protein within the cell. This P16 protein now impairs phosphorylation of the Cdk4/6-Cyclin D complex causing the inactive pRb to become activated allowing association with the E2F transcription factor (Klaes *et al*., 2001). This non-phosphorylated pRb state has been focused on by several investigations targeting the G1/S control of the cell cycle. These include strategies in gene therapy aimed at suppressing the Cyclin-Cdk activity and cell death (Bibbo *et al*., 2002). Concomitantly, elevated expression of the viral oncogenes in HPV transformed cells is thus an indication of P16 expression suggesting the disease process in cervical cancer. On the other hand, reduced expression of the P16 protein may be inversely related to the absence of potential HPV transformed cellular changes. However, oncogenic expression of viral proteins may not have taken place at the time of measurement (Wang *et al*., 2004). Similarly, P16 can be regarded as a more reliable predictor of the malignant potential of the HPV (Klaes *et al*., 2001) compared to HR-HPV DNA detection which is less cost-effective and specific in detecting HPV types having the potential to elicit an oncogenic response particularly in women < 30 years of age.

Our results demonstrate good concordance between P16 expression and severity of the cervical lesion as related to HPV status. Our results correlate with those of Klaes *et al*., (2001), as we observed strong P16 expression in 51 (94.4%) of 54 higher grade squamous intraepithelial lesions (HSILs), suggesting that expression of the *p16* gene commonly occurs in cervical carcinogenesis. Conversely, cervical lesions that were not induced by HR-HPVs do not display P16 expression. Bibbo *et al*., (2002) reported strong P16 expression in 96.16% of HSILs which is similar to our observations. While Klaes *et al*., (2001) reported P16 expression in 92 (100%) of 92 HSIL lesions, our results correspond with those of Hu *et al*., (2005) in which P16 immunoexpression was detected in 97.1% of HSIL classified lesions. In 3 of 54 HSIL classified lesions, no P16 immunoreactivity was observed, suggesting that viral expression may not have taken place at the time of assessment. However, this does not necessarily imply that these lesions will regress. Sensitivity for P16 expression in HSIL classified lesions were found to be high combined with a high specificity for HPV negative benign cells. This suggests that $P16^{INK4A}$ might be a useful marker in identifying potentially malignant transformation of cervical cells.

The concordance between the P16 protein expression and high grade cervical abnormality is corroborated by Bibbo *et al*., (2002), Klaes *et al*., (2001) and Hu *et al*., (2005) who demonstrated that most, if not all, HSILs were $P16^{INK4A}$ positive. Among the patients with a cytological diagnosis of LSIL (CIN I with/without HPV), 50 (52.1%) of 96 scored positive for

P16 expression, which is in agreement with results reported by Pientong *et al*., (2004) where P16 expression was observed in 19/35 (54.3%) LSIL classified lesions. Hu *et al*., (2005) observed P16 immunoreactivity in 57.9% of LSIL lesions which is similar to our results. Bibbo *et al*., (2002) recognised the importance of using $P16^{INK4A}$ on liquid based cytology specimens and reported a $P16^{INK4A}$ positivity in 14 (73.7%) of 19 LSIL lesions. Moreover, Klaes *et al.*, (2001) reported P16 immunoreactivity in 40 (85%) of 47 LSIL classified lesions. Both Bibbo *et al*., (2002) and Klaes *et al*., (2001) used histologically proven samples as opposed to conventional cervical cytology without histological confirmation used in our study. This suggests that interobserver reproducibility may have been better controlled for in their studies. Since their analysis was based on histologically proven cervical smears, $P16^{INK4A}$ staining may have been established on the histological diagnosis. In this regard, positive P16 immunoexpression was ascertained for when the cytological diagnosis correlated with the histological assessment and as such brown colourization of either the nucleus and/or cytoplasm was confirmed. Our study is rooted on conventional cervical cytology and did not incorporate histologically proven LSIL classified lesions.

Contrary to Nieh *et al*., (2003) and Pientong *et al*., (2004) in which the P16 protein was detected in 60.6% and 52.5% of their respective cohorts, we demonstrated P16 expression in 5 of 28 (17.9%) of ASC-US classified lesions. Both authors used biopsy-proven ASC-US classified cervical lesions of which the P16 immunoreactivity may have been based on their corresponding histological diagnosis. Observer reproducibility, where P16 expression was only confirmed if the histological diagnosis correlated with the cytological diagnosis, may have been controlled in their studies and is thus a possible source of underlying bias in their study. Our study focused on the presence of the P16 protein in ASC-US classified Pap smears with or without histological correlation. This methodology may result in inherent bias that exists among cytology screeners who base P16 immunoreactivity in cells on their cytology diagnosis. Inevitably, it results in false positive P16 immunostaining in otherwise P16 negative abnormal cervical cells, eventually leading to an exaggeration of P16 immunoexpression. By applying a threshold value of P16 positivity in abnormal cells, objective identification of the samples can be secured. The failure to detect P16 expression in seven ASC-US classified cases may have occurred because of the vigorous washing procedure of cervical smears performed prior to immunotesting. This consequently resulted in abnormal cells being washed off the slide. Alternatively, viral expression may not have taken place before the smears were subjected to immunostaining.

Bibbo *et al*., (2002) required a threshold of more than 10 abnormal cells per slide displaying P16 immunoreactivity in either their nucleus and/or cytoplasm. Their criteria may have intensified their report in that P16 expression was correlated with the number of abnormal

cells expressing the P16 antigen. Inaccuracies can be substantially reduced in this way. Similar to Hu *et al*., (2005), we used a threshold of 5 or more abnormal cells per slide displaying P16 immunostaining to ascertain a positive immunoreaction. We recognize that fewer cells expressing the P16 protein may inadvertently culminate in reduced specificity fortified by the presence of squamous metaplastic cells however, P16 staining was invariably evaluated in cytologically abnormal cells.

Interpretation of positive immunoreactivity presented a problem in this study particularly since ethanol fixed smears counterstained with conventional haematoxylin were used. The application of this counterstain produces a poor chromatic contrast which ultimately leads to difficulty in assessing P16 immunoreactivity particularly in squamous metaplastic cells and cells displaying the HPV cytopathic effect. We attempted to overcome this problem by assessing P16 immunoexpression in cells displaying the specific features associated with the respective lesion. This may further necessitate the need for developing alternative counterstains that will not disrupt the distribution of chromatin material in cells. Additionally, cells were covered by excess mucus, its presence inhibiting the optimal dispersion of primary and/or secondary antibodies. This consequently resulted in false negative P16 immunoexpression in abnormal cells and may have reduced the sensitivity of the $P16^{INK4A}$ assay. We recognise potential pitfalls that can lead to erroneous expression of the P16 antigen. Microscopic assessment of P16 activity, at times, resulted in exaggeration of immunoexpression in cells. We strived to identify P16 expression exclusively in cytologically abnormal cells while attempting to unequivocally recognise cytologically benign cells. Evaluating the cyto-morphological features of cells displaying P16 immunostaining was of utmost importance in assessing the P16 status of specimens. Noticeably, proliferating squamous metaplastic cells expressed P16 immunoreactivity and were recognised as having the propensity to stain P16 reactive. Nevertheless, false positive P16 immunoexpression in conventional cervical cytology has yet to be fully elucidated.

Our study found that all cases positive for the P16 protein were positive for the detection of any type of HPV. However, contrary to what was expected, not all HPV positive samples reflected positivity for the P16 protein. We propose several explanations for this observation. One reason could be that the consensus primers used, detected 20 different HPV types of which 6 low-risk HPVs (6, 11, 40, 42, 43 and 44) and 14 high-risk HPVs (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) were included. Conversely, most cervical abnormalities induced by chronic HR-HPV exposure demonstrated the P16 antigen, indicating the presence of only oncogenic HPV types. Iftner *et al*., (2003) in their evaluation of the reproducibility and sensitivity of the different molecular screening assays for HPV, noticed that PCR-based methods using MY09/011 degenerate primers have a greater efficiency in

discriminating a much broader spectrum of HPV types amounting to 39 different HPV types. We, therefore, anticipated a much higher yield of multiple HPV infection in the study population when the MY09/011 primer pairs were utilized. Iftner *et al*., (2003) further observed a high sensitivity rate of 96% for both GP5+/6+ primer pairs when compared to the utility of the MY09/011 primers however, Kappa values for the respective primer pairs were 0.8, suggesting that there was not perfect agreement in detection. In fact, GP5+/6+ primers detected far fewer multiple infections than MY09/011 primers with additional differences in detection of HPV types 35, 53 and 61. Realising that the former primer pairs have a slightly higher sensitivity, our results correlated well with an elevated P16 immunoexpression.

Castle *et al*., (2002) who conducted a comparative study on 2978 clinical specimens using AmpliTaq DNA polymerases and compared it with MY-taq, found no significant differences in the sensitivity of the aforementioned polymerases. They proposed that both pre-analytical and analytical procedures with reference to DNA extraction, specimen collection and storage and the application of different Taq polymerases in the PCR may affect the sensitivity and specificity of the assay. We used the Go-Taq DNA polymerase specifically designed to maximise specificity thereby reducing the formation of primer-dimers. In contrast, Rughooputh *et al*., (2006), who conducted a study on archival tissues from cervical cancer patients in Mauritius, reported a high sensitivity of both primer pairs when compared to each other however they found an additional seven positive samples using GP5+/6+ primer pairs which tested negative using MY09/011 primer pairs. This clearly demonstrates that sensitivity in HPV DNA detection may be influenced by the integrity of the DNA in the respective samples. A need to standardize protocols is warranted.

Incidence of HPV infection among women with abnormal cervical lesions was 94.5% in the Namibian cohort. Similarly, Schiffman *et al.*, (2000) who examined the prevalence of cervical HPV among 8582 Costa Rican women reported a positive HPV infection rate of over 85%. Similar to ours, their cohort included all women attending health care facilities for gynaecological examination and subsequent Pap smear evaluation. Contradictory to our findings, Allan *et al*., (2008) detected HPV in 58.7% of South African women with abnormal cervical lesions. A similar study conducted by Kay *et al*., (2003) on South African women reported that 94% of cervical cancer patients and 88% of patients with CIN classified lesions harboured HPV infection. Women harbouring these HPV-related cervical abnormalities may appreciate the analogy of disease relating to contributing factors.

All HSIL (CIN II/III with/without HPV) categorised Pap smears but one out of 52 were positive for HPV detection based on PCR analysis (using GP5+/6+ primers) with only 40.4% of HSIL classified lesions testing positive for HR-HPV types 16 and 18. Moreover, the molecular

testing applied was restricted to the detection of only HR-HPV types 16 and 18, thus potential HR-HPV induced cervical lesions may have been missed. This is considered to be a limitation in this study and further studies are required to determine the prevalence rate for all high-risk HPV types among Namibian women. Fifty eight percent of the HSIL classified lesions which demonstrated P16 immunoreactivity were found to be negative for the presence of HR-HPV types 16 and 18. This contrasts with Klaes *et al*., (2001), where P16 immunoreactivity among HR-HPV positive samples were observed in 75% of HSIL classified lesions, suggesting that these lesions may have been induced by other oncogenic HPV types.

In the present cohort, ASC-H classified cervical lesions demonstrated a 100% prevalence rate of HPV DNA detection by PCR. The greater occurrence of HPV positivity in ASC-H classified lesions indicates that these lesions may have a high predictive value for HSIL in the general population of Namibia. Generally, these lesions are managed through the use of referred colposcopy and subsequent biopsy. Registered nurses received the Pap smear results and subsequently informed the patients of their diagnosis after which a medical doctor was consulted. Based on the doctor's discretion, the patient was referred to a gynaecologist who then performed a biopsy following the colposcopy. The biopsy specimen was sent to the state laboratory for analysis by a qualified histopathologist and further palliative treatment was done based on histological results. Management of these cases can become expensive especially in terms of procedures such as loop electrosurgical excision procedure (LEEP) as well as the travel arrangements by the patient. Michelow *et al*., (2007), who conducted a retrospective study on cervical smears of 25 South African women with an ASC-H interpretation, proposed that 50%-80% of women harbouring an ASC-H lesion will be exposed to unnecessary colposcopy and highlighted the application of HPV testing as an adjunctive test. Similarly, Saad *et al*., (2006) reinforced the need for HPV testing by selecting individuals with an ASC-H interpretation who were required to have colposcopic screening. Additionally, we believe that duplicate smears may further enhance the detection of the lesions while recognising that it may not be practical in all circumstances. Larger population-based studies are warranted to examine the sensitivity of duplicate smears in detecting higher grade lesions.

Human Papilloma virus genotyping were studied as an ancillary test in ASC-H classified cervical lesions. In our study, HPV types 16 and 18 genotypes were observed in 44.4% of women with an ASC-H interpretation. In one study, reporting rates for HR-HPV was high amounting to 85.6% in ASC-H (Sherman *et al*., 2002). Similar to our findings, Saad *et al*., (2006) reported a 31% prevalence rate of HR-HPV types among women with an ASC-H interpretation. They proposed that variability in the interpretation of cervical smears among

different cytoscreeners may have contributed to the low prevalence rate. Even though HPV types 16 and 18 may be the most common high risk types present in cervical cancer lesions (>70%), we concluded that potential ASC-H lesions have a very high progression rate.

In our study, eighty seven (91.5%) of LSIL classified lesions were found to be positive for GP5+/6+ compared with only 57.14% reported by Pientong *et al*., (2004). This variation could most likely be attributed to the biotinylated primers (GP5+/6+) used in our study which target the polymorphic L1 gene for PCR amplification, resulting in the detection of 20 different HPV types including both low and high risk HPVs. The presence of cellular changes associated with LSIL among women with a high HPV positivity seemed limiting because most of the lesions were transient with approximately 15%-30% of lesions having the potential to progress to HSIL. Consecutive cytological smears would thus not be advisable for women harbouring LSILs since a considerable number (53%-76%) of repeat cytology will exhibit concurrent cytological abnormalities. This in turn leads to advancement of the already abnormality to more severe lesions that should have been detected earlier. For this reason, all LSIL classified cervical lesions should be referred for colposcopic examination. Klaes *et al*., (2001) who examined the effect of the $P16^{INK4A}$ biomarker in cytologically diagnosed abnormal cervical smears reported the presence of the HPV genome in 64% of LSILs. This explains the variation in the number of HPV positive LSILs and correlates well with other studies as regards the poor reproducibility of cytology and to a lesser extent, histology (Colgan *et al*., 2001 and Klaes *et al*., 2001).

Human Papilloma virus genotyping among LSIL classified lesions revealed a positive rate of 23.0% for HPV types 16 and 18. Contrary to this, Pientong *et al*., (2004) demonstrated the presence of HR-HPV types in 51% of LSILs. This apparent greater detection rate was the result of the MY09/011 degenerate primers combined with the 5' –biotinylated-labelled hybridization probes. On the other hand, Klaes *et al*., (2001) reported the presence of HR-HPV types in 32% of samples classified as LSIL. While it appears that the prevalence rate in our study remains low, we recognise that our study only detected HPV types 16 and 18 as opposed to the findings of other investigators (Klaes *et al*., 2001 and Pientong *et al*., 2004) who targeted the presence of all detectable oncogenic HPV types. In our study, type specific primers demonstrated a combined acquisition rate for HPV types 16 and 18 in 4.5% of LSILs. The latter observation validates the need for future studies to assess overall prevalence rates of all oncogenic HPV types and establish whether potential LSIL lesions may have been induced by other low-risk HPV types.

The incorporation of the ASC-US classification into TBS has resulted in the management of minimally abnormal cells to become dubious especially when ASC-US diagnosis is being

overstated in cytology. Management algorithms which include repeat Pap smears every six months followed by colposcopy and subsequent biopsy in the case of severe cytological abnormalities have been proposed and are currently being used in several European and African countries including Namibia. This can lead to unforeseen expenses as patients have to travel for a follow up smear every six months however, we believe that this option is probably more cost-effective as women will only be referred for colposcopy once persistent cytological abnormalities are observed. A constraint that we anticipate in this model is that poor recordkeeping procedures may hinder the efficacy of such management strategies leading to overtreatment and financial implications. Shermann *et al*., (2002) who conducted the ASC-US/LSIL triage study (ALTS) with its focus on determining the most efficient ways of managing minor abnormalities so commonly observed on a Pap smear, concluded that HPV testing was sensitive enough to detect precancerous cervical lesions. Likewise, Walboomers *et al*., (1999) reinforced the need to integrate HPV DNA testing in the management of ASC-US lesions. Wright *et al*., (2006) who conducted a retrospective study on cervical smears of 1 290 women in Washington with an ASC-US interpretation, reported a HPV positivity rate of 34% with 4% with equivocal results. In their review, they concluded that 3%-36% of ASC-US classified lesions will harbour an underlying high-grade lesion thereby supporting the need for reflex HPV testing. In this way, women who are HR-HPV negative with an ASC-US lesion can be reaffirmed about their status and allowed to enter an annual examination regimen. In our study, the prevalence of women with an ASC-US diagnosis for HR-HPV was 28% with 20% of ASC-US lesions demonstrating the presence of HPV type 18. Similarly, Pientong *et al*., (2004) observed a positivity rate of 37.5% for HR-HPVs in ASC-US classified lesions. Wong *et al*., (2008), who conducted a study on cervical smears of 94 women with an ASC-US interpretation, found a positive rate of 48% for the presence of HR-HPVs among women diagnosed as ASC-US with 38% of the cases representing the possible presence of HPV types 16 and 18. Possible scenarios include the application of the Invader 2 assay having 4 HR-HPV probe sets that are capable of detecting fourteen different HR-HPV types. The high prevalence of HR-HPV types 16 and 18 in ASC-US classified lesions is disturbing and additional risk factors may be implicated in the development of cervical abnormalities. Kinney *et al*., (1998) demonstrated the presence of HR-HPV DNA in 20% of ASC-US categorised cervical smears. Clearly these findings attest the need for closer follow up of those women harbouring ASC-US lesions and a critical need for a larger population based investigation.

Recognition of the causal role of HPV in the carcinogenesis of cervical cancer and its precursors has shifted the emphasis on the role of subsidiary factors that are implicated in the development of cervical cancer and its precursors. Overall, risk for HPV infection among Namibian women has been found to be associated with younger age, a single marital status, injectable progestogen contraceptive use, HIV positivity status, younger age at first sexual

debut, increasing number of sexual partners and infrequent Pap smear screening. A majority of the risk factors were recognised by less than half of our participants. The risk of acquiring HPV infection decreased significantly with increasing age agreeing with Figueroa *et al*., (1995) who argued in favour of a biological effect such as increased immunity to HPV with progressive aging. Risk factors for HPV associated cervical disease progression have not been determined among women in Namibia. Human Papilloma Virus detection is strongly associated with age with more than 50% of women between the ages of 25-34 years demonstrating the presence of the HPV genome with a progressive decline to 6% among women 45-49 years of age. The greatest peak of HPV detection was noticed among women aged 30-34 years of age declining in women 45-49 years, supporting the notion that HPV acquisition decreases with increasing age (Wright *et al*., 2006). The highest peak of LSIL classified lesions were observed among women 25-34 years of age suggesting that these lesions have an elevated risk of progressing to higher grade lesions. Likewise, a peak prevalence of HR-HPV types 16 and 18 were observed among women in this age category. Lazcane-Ponce *et al*., (2001) demonstrated a high prevalence of HPV among women < 25 years of age with 17% of them infected, concluding that younger age may indeed serve as a predictor for HPV acquisition. A high rate of ASC-US lesions (43%) were found among women aged 25-29 years old which correlated with Wright *et al*., (2006) who reported that up to 40% of ASC-US classified lesions have the potential to progress to a HSIL. This suggests that the rate of potential higher grade lesions originating from an ASC-US could be greatly reduced through the implementation of a standardised cervical screening program. Cox, (1999), who evaluated 527 patients with an ASC-US diagnosis, reported that 77% of women younger than 20 years of age demonstrated the presence of the HPV genome as opposed to 58% of women > 25 years of age. Among women aged 25 years and younger, a high rate of LSIL lesions were found with 11% of women having an ASC-US lesion. From our data, it appears that women between the ages of 25-34 years have the highest rate of cervical abnormalities. No studies, as yet, have determined the age distribution related to P16 expression. Correspondingly, P16 expression correlated well with the presence of HR-HPV and was found to be the highest among women 30-34 years of age. Older women seemed to be at a lower risk of P16 expression suggesting that these women may have been exposed to the HPV earlier in their lifetime, instigating an effective immune response against the viral oncogenes.

We explored risk factors in different age groups and found that early age at first sexual encounter was the strongest risk factor in all age groups as documented by Munoz *et al*., (2004). The decreased age at first sexual debut could lead to an increased prevalence of cervical abnormality, possibly as a result of longer exposure to the infection and/or increased vulnerability of the undeveloped transitional zone. A peak prevalence of HPV associated

disease was observed in women 25-34 years of age, indicating that younger women who initiated sexual activity at a very young age tend to be more prone to HPV infection and subsequent cervical abnormality. The significance of establishing cervical immaturity as a potential risk factor for HPV infection may lie in interventions using hormonal manipulation and/or alternative methods to speed up the process of squamous cell metaplasia, thereby reducing the risk of HPV infections and persistence. If cervical immaturity proves to be associated with HPV infection and persistence, then young women who initiate sexual activity at a very young age may benefit from regular Pap smear screening which should start in early adolescence. We are postulating that each patient who presented with a cytologically diagnosed abnormal Pap smear may be the result of cervical screening failure.

Similar to a study done by Giuliano *et al*., (1999), our study showed that HPV infection was associated with an unmarried status. The high rate of HPV acquisition may be due to either an amplified rate of new partners or to partner infidelity. Contrary to our findings, Zhao *et al*., (2006), who conducted a study on 8798 unscreened women in rural China, found that 97% of participants were married. This possibly occurred because of a much older cohort (35-50 years). We propose that older age (35 years and upwards) is proportionally related to cohabitating lifestyles in seeking to commit themselves to one partner. These results suggest that cervical screening should be redirected to focus more on unmarried women having a cohabitating as well as a non-cohabitating lifestyle.

In the present cohort, lifetime number of sexual partners (mean ±3.488) was found to be the highest among women aged 25-34 years which is consistent with the findings of Moscicki *et al*., (2001). An increasing rate of sexual partners is directly related to a higher HPV positivity stratified by cervical disease severity. Most women between the ages of 25-34 years had at least 3 sexual partners during their lifetime suggesting that a greater risk of HPV exposure is anticipated. Similarly, Cooper *et al*., (2007), who conducted a study among women in South Africa, found that women having more than 3 sexual partners were at a greater risk for cervical abnormalities correlating with a higher HPV positivity rate. Contrary to this, a higher prevalence of HPV infections in older women was reported, suggesting that these women's sexual behaviour inversely correspond with an elevated risk of HPV infection (Chan *et al*., 2009). While most women younger than 25 years reported to being monogamous, women older than 40 years were cynical about having more than one sexual partner. Their responsiveness to a higher rate of sexual partners correlated to their current cervical disease status. Younger women seemed to be more receptive to sex education and considered the information relating to women's reproductive health vital in the aetiology of HPV. Interestingly, potentially high risk women (25-34 years of age) reported a greater number of sexual partners with one person having had 37 sexual partners. These women appear to be

very concerned about their current marital status and as such remain proactive in getting somebody to share their life with irrespective of whether their appeal will have a negative impact on their health. It is also noteworthy that responses from participants to some questions such as number of sexual partners may not necessarily be reliable as they may feel embarrassed that they may be judged by the interviewee.

Current parenteral contraceptive use by injection as opposed to oral contraceptive use was most commonly associated with HPV associated lesions of the cervix. As reported by Coker *et al.*, (2001), hormonal contraceptives may influence persistence and perhaps progression of HPV infections to clinical significant lesions of the cervix. Consistent with Coker *et al*., (2001), no association between HPV infection and cervical abnormality was found in patients who used oral contraceptives. Contrary to this, Kjellberg *et al*., (2000) and Moreno *et al*., (2002) found a very strong link between oral contraception and HPV induced cervical lesions. We postulate that most of our study participants had not been on oral contraception for a considerable time to exert an effect as was the case for those in Kjellberg *et al*., (2000) and Moreno *et al*., (2002) who targeted a much older population with a history of oral contraceptive use for more than 10 years.

We are in agreement with Munk *et al*., (1997) in that high gravidity (more than 3 births) can be associated with an increased risk of HPV related cervical disease. A possible explanation could be that during childbirth the cervix is exposed to extreme trauma, resulting in weakening of the stratum and thus exposing the epithelial layer to viral penetration.

No strong correlation between disease prevalence and condom use was demonstrated in our study however, several publications have underscored the importance of condom use in reducing the spread of HPV related disease of the cervix (Coker *et al*., 2001, Hogewoning *et al*., 2003, Stone *et al*., 1999). In our study, condom use was particularly confined to HIV positive individuals' possibly because of the fear of transmitting the infection to their sexual partner (s). A presupposition amongst participants existed that use of condoms (barrier contraception) by either participants or their male partners was only necessitated if no hormonal contraception was available, to prevent unwanted pregnancies. In our study, use of condoms was perceived by a large number of participants as a barrier to enjoyable sexual contact, thereby failing to satisfy their respective partners. Nonetheless, participants believed that HIV positive individuals should use barrier contraception while involved in sexual relationships.

Although smoking has been recognised as a risk factor in the carcinogenesis of cervical cancer, we found no strong correlation in our study which is consistent with reports by

Lazcane-Ponce *et al*., (2001). The adverse effects due to cigarette smoking were insignificant in our study as most Namibian women detest the idea of smoking. This relates to practices in their cultures where smoking among females is considered as being discourteous to the community. Smoking practices among coloured and white women differed considerably from their black counterparts however, our study was restricted to assess for smoking among all women harbouring abnormal cervical lesions excluding ethnical considerations associated with smoking. This warrants the need for further testing in smokers and non-smokers relating to HPV acquisition in women. Contrary to our findings, Minkoff *et al*., (2004) and Szarewski and Cuzick, (1998) found a positive association between cigarette smoking and HPV related cervical abnormalities however the explanation for this observation is not clearly understood. While excessive alcohol use may also be considered as a link to riskier sexual behaviour with concomitant suppression of the immune system, we found no strong correlation between alcohol use and HPV acquisition.

While HPV infection is very common, it is more prevalent among HIV positive women. A high prevalence of HIV positivity among women 25-34 years of age was identified (40.8%), underscoring the importance of HPV acquisition and persistence in immune suppressed women. Jay *et al*., (2002) concluded that HIV-seropositive women are at a greater risk to develop progressive cervical lesions with a lesser probability of full recovery. By extrapolation, 17.8% (9) of women never tested for HIV will be HIV reactive, based on the Ante Natal Clinic (ANC) sentinel survey that was performed by the Ministry of Health-Namibia in 2008 on all women attending antenatal clinics and hospital wards during the gestation period. The highest peak of HIV prevalence was observed in women 40-44 years of age, suggesting that these women may have been exposed to multiple sexual partners or greater number of sexual partners. The sexual behaviour of black Namibian women may also be influenced by their cultural norms culminating in a higher incidence of HIV reactivity, henceforth leading to an elevated risk of HPV infection. No strong correlation was found between cervical HPV and sexually transmitted infections which is in agreement with the findings of Lazcano-Ponce *et al*., (2001). Interestingly, a positive HIV reaction was observed in the majority of HR-HPV types 16 and 18 negative ASC-US and LSIL classified lesions displaying P16 immunoreactivity, suggesting that we may be dealing with a new subset of clinically significant cervical lesions. This finding strongly correlates with McKenzie *et al*., (2010) in which HIV-positive women were more commonly infected with HR-HPVs other than HPV types 16 and 18, thus supporting the need for further studies. This may be the key to identify progression of cervical lesions in immunocompromised women.

Ter Meulen *et a*l., (2006) reported an exponential risk of HPV acquisition among HIV positive women. Furthermore, risk for cervical abnormalities has been found to be strongly

associated with an HIV positive status and a higher number of HPV type 16 compared to HPV type 18 cases. Clifford *et al*., (2006), who conducted a meta-analysis of 5 600 study subjects, reported that 41% of HIV positive women harboured more than one type of HPV. In our study, most HIV positive women presented with severe dysplastic lesions. Realising that HIV can be spread through unprotected sexual intercourse with an infected partner; some women regard the use of hormonal contraceptives as a means of protection against HIV infection. Consequently, they become re-infected with the virus and in turn are exposed to co-infection with the HPV. Several studies have emphasized the development of cervical precancerous lesions in HIV- positive individuals compared with HIV- negative women (Ter Meulen *et al*., 2006 and Clifford *et al*., 2006). We demonstrated a high prevalence of HSILs (40%) among women 30-34 years of age with an even higher rate of LSILs among women 25-29 years of age. In the Namibian setting, HIV positive women do not receive any reflex testing with respect to either Pap smear evaluation or HPV DNA testing and are generally referred for gynaecological examination when cervical lesions are already in an advanced stage. This burdens the already challenged health system as a result of preventable colposcopic evaluation and subsequent palliative treatment. Conclusive evidence also suggest that women with a very low CD4 cell count (<200 cells/mm^3) have a greater chance of developing a precancerous lesion, suggesting that the immune response may be depleted (Clifford *et al*., 2006). We did not examine the immune status of the HIV positive women.

With respect to the use of chronic medication in particular HAART, we found no strong correlation between HPV acquisition and cervical disease progression. Distinct from our study, Harris *et al*., (2005) did not record a reduction in the prevalence of HPV-associated lesions among HIV-positive Italian women on HAART.

Finally, the majority of participants presented with abnormal cervical lesions due to inadequate Pap smear screening histories with a small proportion of women having had a Pap smear for the first time. In our study, women who had a Pap smear for the first time were in their early reproductive years and appeared familiar with the importance of a Pap smear. Clearly, it demonstrated the acute need for education in imparting knowledge about Pap smear screening, particularly to all sexually active Namibian women. Nanda *et al*., (2000), who conducted a meta-analysis of 12 studies to demonstrate the effectiveness of both conventional cytology and LBC, reported a sensitivity rate ranging from 30%-87% for detecting cervical abnormalities with a specificity rate ranging from 86%-100% for a single Pap smear. They concluded that even though the Pap smear is effective in detecting cervical abnormalities with a subsequent reduction in cervical cancer, cytological review can be found to be rather subjective. Similarly, we propose that a repeat Pap smear may further increase the sensitivity and specificity in detecting cervical abnormalities. In our study, some women

argued that Pap smear screening was only warranted if they experienced abnormal cervical pathophysiology. These candidates seem to underestimate the burden of cervical abnormalities and its relation to HPV. The need for proper information dissemination is thus essential to educate individuals with respect to HPV infections and cervical disease progression as well as the importance of regular Pap smear screening. The implementation of a national cervical screening program is thus essential in the Namibian setting as several studies have shown a reduction rate of 60%-90% in cervical cancer and 20%-60% in cervical cancer mortality. Interestingly, most participants in the younger age group (20-29 years) presented for Pap smear screening on an annual basis however, they did not demonstrate a lower incidence of cervical abnormalities, suggesting that recommended follow-ups were not adhered to. Another explanation is that specimens were not representative of the transformation zone, the latter which are implicated in the initiation of a cervical abnormality. Medical professionals involved in the collection of cervical smears need to be educated on the appropriate procedure for collecting cervical cells for cytological evaluation and standardised procedures should be put in place to avoid poor collection techniques. An intriguing finding in our study was that first time attendees did not reflect a higher rate of cervical abnormalities as was expected. A plausible explanation could be that these women were exposed to the HPV earlier, thus resulting in immunity being acquired. This inevitably lessened their chances of developing cervical abnormalities. Additionally, potential barriers for infrequent screening identified include older age, poor socio-economic status, educational background and negligence.

In our study, the type distribution in high grade cervical lesions was more confined as compared to LSIL with HPV type 16 detected in 19.6% of HSIL classified lesions. Conversely, HPV type 18 emerged as the more common type among ASC-US and LSIL classified lesions. In a similar study (Allan *et al*., 2008), HPV detection ranged from 42% in women with ASC-US, 70% in women with LSIL to 83% in women with HSIL. In addition, they observed a higher rate of HPV type 16 in the cervical lesions as opposed to our findings in which HPV type 18 was more commonly found among ASC-US and LSIL categorised lesions. Distinct from Allan *et al*., (2008) where HPV type 16 and 18 were found in 16% of abnormal cervical lesions, we demonstrated the presence of the respective HR-HPV types in 31.4% of abnormally classified cervical lesions. Similarly, Kay *et al*., (2003) observed a high prevalence of HPV type 16 among women in South Africa which is in agreement with studies in that HPV type 16 is the most common virus detected in the cervix irrespective of the cytological diagnosis.

The decreased incidence of HPV type 18 in HSILs was noted in earlier studies suggesting that HPV type 18 induces cervical lesions and elicits a more aggressive behaviour with a

short progression time in HSILs. This finding correlated with a high prevalence in ASC-US and LSIL classified lesions as found in our study suggesting that these lesions may already harbour an underlying higher grade lesion (Wright *et al*., 2006). It was noteworthy that HPV 18- associated cervical lesions progressed at a faster rate than those caused by HPV type 16. Interestingly, women with LSIL infected with the HPV type 18 virus are approximately 4 years younger than those with ASC-US classified lesions who have been infected with the same virus. This finding could potentially constitute a specific moiety caused by HPV.

The prevalence of oncogenic HPV types could not be determined as we only employed HPV type 16 and 18 primers to detect the rate of oncogenic virus infection. Using GP5+/6+ primers, we demonstrated a prevalence of HPV infection in 92.6%, 92.6% and 98.1% of ASC-US, LSIL and HSIL, respectively compared with Allan *et al*., (2008) who reported HPV infection in 42%, 70% and 83% of ASC-US, LSIL and HSILs. Based on this, it can be argued that a higher rate of HPV infection correlated with a more severe cervical lesion. Furthermore, we observed an increase in the number of concomitant infections with both HR-HPV types 16 and 18 with increasing abnormal cytology. Similar studies demonstrated a much greater sensitivity in detecting multiple HPV infections as opposed to the GP5+/6+ consensus primers applied in our study (Wong *et al*., 2008 and Iftner *et al*., 2007). We anticipated a much higher frequency of multiple infections and, therefore, may need further studies to focus on the prevalence rate of HPV infections using broad spectrum primers.

Similarly, P16 expression rated higher among women with multiple HPV infections correlating well with cervical disease severity. Strong P16 expression was observed in women having multiple infections suggesting that P16 expression may be directly related to the number of HPV infections in a particular lesion. Needless to say, 35 (37.2%) of 94 LSIL classified lesions scored positive for HPV detection by PCR and negative for P16 expression, demonstrating that these lesions were not associated with malignant transformation induced by HR-HPVs; instead they harboured non-oncogenic HPVs. Allan *et al*., (2008) further demonstrated HPV infection in 41.7%, 70.2% and 83% of ASC-US, LSIL and HSIL classified lesions respectively.

We explored the prevalence rate of oncogenic HPV types 16 and 18, the most common types associated with cervical cancer pathogenesis and identified HPV 16 and 18 genomes in 12.2% and 14.5% of study participants distributively. These findings were proportionately similar to Ter Meulen *et al*., (2006) who found the presence of HPV types 16 and 18 genomes in 13.2% and 17.5% of Tanzanian women, respectively. Contrary to this, Kay *et al*., (2003) demonstrated the presence of HPV type 16 in 82% of participants with only 10% of women infected with HPV type 18. In our study, HPV type 18 was most commonly found in

ASC-US and LSIL classified lesions with HPV 16 as the most prevailing type seen in HSIL classified lesions, suggesting that HPV type 16 infected individuals were at a higher relative risk of developing HSILs. Gravitt and Jamshidi, (2005) confirmed the presence of HPV types 16 and 18 in 25% of LSIL classified lesions which is similar to the 27.6% positivity rate found in our study. Likewise, 32%, 5.7%, 11.1% and 0% of ASC-US, LSIL, ASC-H and HSIL classified lesions, respectively scored positive for the detection of HPV types 16 or 18 but lacked the P16 protein. A possible explanation could be that not all high risk HPV types have the same potential for cell cycle disruption that can lead to the expression of the P16 protein. Moreover, the latency period that exists until the P16 protein is detected may further contribute to a delayed expression of the P16 protein. Our results demonstrate immunoreactivity in all HR-HPV induced HSIL lesions, proposing that this condition may hold the key in identifying integration of HR-HPVs in cervical tissue.

Several studies (Burd, 2003, Jastreboff and Cymet, 2002) have underscored the relationship between HPV positivity and severity of disease with persistent exposure to the HPV associated with severe cervical abnormalities. Distinctly, cervical cancer screening in Namibia is mostly performed when women avail themselves for screening which is further characterised by a lack of inadequate monitoring. As a result, potential high-risk groups may be missed. Serious consideration should be given to information dissemination in terms of Pap smear screening in Namibia which could lead to an even greater reduction in the incidence of cervical cancer.

Our findings clearly support the use of $P16^{INK4A}$ as a diagnostic marker in cervical disease progression. Although we have demonstrated good correlation of P16 expression in HSIL classified lesions, we believe that HPV testing may further enhance its utility in LSIL and ASC-US classified lesions. Similarly, Ronco *et al*., (2010), who assessed the efficacy of HPV testing compared to conventional cytology in 94 370 women, reported a greater detection rate of HSILs in women 35 years and older when HPV testing was used. They concluded that HPV testing in younger women (25-34 years) may not be advisable as the majority of HR-HPV infections in this group of women will resolve spontaneously. Accordingly, the use of $P16^{INK4A}$ as a biomarker in women may be more appropriate in detecting cervical abnormalities as has been corroborated by Klaes *et al*., (2001) and Bibbo *et al*., (2002) who have underscored the robustness of P16 immunostaining in cervical cell samples.

We recognise several limitations in this study. Firstly, results may be confounded as most women attend health care facilities as a result of gynaecological problems, with only a small proportion attending gynaecological clinics annually or as recommended. Candidates with potential HPV-induced cervical lesions may have been missed as some women fail to visit

health facilities due to the absence of apparent clinical symptoms. Furthermore, this study may be underrepresentative in Namibian women as most white women and women with medical aid insurance preferably visit private gynaecological facilities. As a result, the findings may not be extrapolated to the whole population.

Secondly, type specific primers used may not reflect the true status of the cohort as the presence of other oncogenic HPV types was not determined. These results may bias interpretation as only the two most common HR-HPV types were tested for, excluding the possibility of detecting other HR-HPV types. This presented a weakness and possibly restricting our inference in establishing the true incidence of HR-HPV induced lesions of the cervix in Namibian women. A need for further assessment is warranted to determine the distribution of all HR-HPVs in cervical specimens from Namibian women.

Thirdly, histological evaluation as the preferred diagnostic tool to validate cytological diagnosis was not performed on all specimens in particularly with respect to LSIL and ASC-US classified lesions. This presented a dilemma as some true lower grade lesions may have been potentially misclassified as ASC-US and vice versa. Even though histological confirmation is not required for first time ASC-US classified lesions, follow-up smears displaying “same” or “worse” diagnosis were treated as if this was a first time aberrant diagnosis due to improper and inadequate record keeping procedures. Additionally, LSIL and ASC-US classified lesions presented a subset of issues in that detection and correlation were not as corroborative as was hoped. However, we could not assess the robustness of the $P16^{INK4A}$ assay in these lesions as molecular testing for all known HR-HPV types was not done.

The results obtained at enrolment were based on cervical samples and were restricted to the cytological diagnosis only. This is considered as a potential limitation of this study as it may be affected by interobserver reproducibility of results. We collected histologically proven diagnosis for most HSIL classified lesions, where possible, to improve sensitivity however, since current cervical screening guidelines in Namibia do not advise for a biopsy for LSIL and ASC-US classified cervical lesions, histological confirmation was not obtained. Conversely, P16 immunostaining was applied to cervical smears with/without a biopsy-proven diagnosis.

We also considered the potential of bias in the selection criteria and reported a low refusal rate amongst candidates. Control groups were proportionally similar to the study group with reference to their ages and sexual history thus eliminating the possibility of bias based on the selection criteria. We endeavoured to minimize for information bias by administering standardised questionnaires to both control and study subjects; their cervical status being

concealed from one another. This was further strengthened by re-administering the same questionnaire for completion to randomly selected individuals in both control and study groups during their follow-up visits.

As a final point, the utility of novel immunoperoxidase markers, including BD ProEx C (TriPath Imaging, Inc., Burlington, NC) and HPVL1 (Cytoactive Diagnostics, GmBH, Pirmasens, Germany) in future large-scale, well-constructed studies will further strengthen our understanding of the mechanism of cervical carcinogenesis and thus have significant preventative and public health outcomes. Using these markers along with cytology could be valuable for the identification of patients at risk of developing progressive lesions and can contribute towards a greater appreciation of the pathogenesis of cervical lesions.

Conclusion

The following conclusions can be derived from our findings:

Firstly, our figures show that HR-HPV types 16 and 18 were mainly observed in HSIL-classified cervical lesions. The direct relationship of cervical disease progression stratified by HPV positivity is clearly shown. Remarkably, a second peak of HR-HPV types 16 and 18 were observed in ASC-US classified lesions. These women tended to be at a younger age (25-34 years), commencing sexual activity early in their adult life (16-19 years). Conversely, management of women with ASC-US classified lesions needs to be reviewed to minimize the progression to HSILs and eventually cervical cancer. Reinforcing current strategies such as repetitive Pap smear screening after six months of initial collection with subsequent P16 immunostaining may allow for the categorization of a distinct entity with mild cytological abnormalities destined for colposcopic evaluation and subsequent biopsy.

Secondly, our findings demonstrate that expression of the P16 protein is proportionately similar to the severity of the cervical lesion. The $P16^{INK4A}$ biomarker discriminates between cervical lesions having the potential to progress to severe dysplastic lesions (i.e. those that are induced by persistent exposure to HR-HPVs and those that will regress). Hypothetically, P16 immunoreactivity should be present in all HR-HPV positive samples however, we are uncertain as to why some P16 positive cases were negative for HR-HPV types 16 and 18. We suggest that these cases will be positive for HR-HPV types other than types 16 and 18. Alternatively, P16 expression may have been induced by other means such as the replacement of a hydrogen group with a methyl group however, elevated P16 activity through disruption of the pRB pathway has been clearly described in the pathogenesis of cervical cancer. Nevertheless, our findings clearly reinforce the usefulness of the $P16^{INK4A}$ biomarker in detecting borderline lesions such as ASC-US and LSILs having an aggressive behaviour.

In this way, unanticipated health care costs such as colposcopy could be reduced, consequently resulting in a reduction in the mortality rate of cervical cancer in Namibia.

Finally, our findings emphasize the compelling need for a national cervical screening programme to be implemented. Based on the findings in this study, women between the ages of 25-34 years old who engage in sexual activities early in their life are at the greatest risk for developing cervical lesions. Noticeably, these women test positive for a high number of HSILs and are identified as the target group as per the WHO guidelines. It is also noteworthy that repetitive screening considerably reduces the rate of cervical abnormalities. As such, we endorse the implementation of a well organised, structured cervical screening program in Namibia that adheres to strict quality assurance measures and have proper monitoring and assessment mechanisms to measure the incidence and mortality of cervical disease.

Our results indicated that current vaccine strategies may not be effective in Namibia to prevent HPV-induced lesions as only 31.4% of women demonstrated the presence of HPV types 16 and 18. Though we acknowledge that the vaccines have crossover activity with reference to other HR-HPV types, larger population-based studies are warranted to determine the efficacy of the vaccines.

To recapitulate we conclude that $P16^{INK4A}$ does have the potential to detect precancerous cervical lesions induced by HPV and that advancement of abnormal lesions of the cervix can be monitored by the $P16^{INK4A}$ assay. Its utility in the field of gynaecologic cytopathology may further serve as a guide in reconstructing appropriate management strategies for women who present with cervical aberrancies. Taking the associated risk factors into account, specific target groups who are at a higher risk of developing progressive cervical lesions can be identified. In this way, unique screening guidelines can be formulated and implemented that may further enhance the sensitivity of the Pap smear. This will ultimately lessen the cost of cervical screening practices and lead to a reduced rate of cervical cancer globally.

REFERENCES

- Abati, A., Jaffurs, W., Wilder, A.M. 1998. Squamous atypia in the atrophic cervical vaginal smear: a new look at an old problem. *Cancer*, 84 (4): 218-225.

- Abramovich, C.M., Wasman, J.K., Siekkinen, P., Abdul-Karim, F.D. 2003. Histopathologic correlation of atypical parakeratosis diagnosed on cervicovaginal cytology. *Acta Cytologica*, 47: 405-409.

- Allan, B., Marais D.J., Hoffman, M., Shapiro, S., Williamson, A. 2008. Cervical Human Papilloma virus infection (HPV) in South African women: implications for HPV screening and vaccine strategies. *Journal of Clinical Microbiology*, 46 (2): 740-742.

- Alli, P.M & Ali, S.Z. 2003. Atypical squamous cells of undetermined significance-rule out high grade squamous intraepithelial lesion: cytopathologic characteristics and clinical correlates. *Diagnostic Cytopathology*, 28: 308-312.

- American College of Obstetricians and Gynecologists. Practice Bulletin Number 66. 2005. Management of abnormal cervical cytology and histology. *Obstetrics & Gynecology,* 106:645-664.

- Barron, B.A and Richart, R.M.1968. A statistical model of the natural history of cervical carcinoma based on a prospective study of 557 cases. *Journal of the National Cancer Institute*, 41:1343-1353.

- Bibbo, M., Klump, W.J., DeCecco, J., Kovatich, A.J. 2002. Procedure for immunocytochemical detection of $P16^{INK4A}$ antigen in thin-layer, liquid-based specimens. *Acta Cytologica*, 46 (1): 25-29.

- Boon, M. E., Zeppa, P., Ouwerkerk-Noordam, E., Kok, L.P. 1991. Exploiting the "toothpick effect" of the cytobrush by plastic embedding of cervical samples. *Acta Cytologica*, 35:57-63.

- Bosch, X and Harper, D. 2006. Prevention strategies of cervical cancer in the HPV vaccine era. *Gynecologic Oncology*, 103: 21-24.

- Bradley, J.R.L and Denny, L. 2004. Widening the cervical cancer screening net in a South African township: Who are the underserved? *Healthcare for Women International*, 25 (3): 227–241.

- Burd, E. M. 2003. Human Papilloma virus and cervical cancer. *Clinical Microbiology Reviews*, 16 (1): 1-17.

- Burkett, B.J., Peterson, C.M., Birch, L.M., Brennan, C., Nuckols, M.L., Ward, B.E., Crum, C.P. 1992. The relationship between contraceptives, sexual practices, and cervical Human Papilloma virus infection among a college population. *Journal of Clinical Epidemiology*, 45 (11): 1295-1302.

- Carozzi, F., Cecchini, S., Confortini, M., Becattini, V., Cariaggi, M.P., Pontenani, G., Sani, C., Ciatto, S. 2006. Role of P16 (INK4A) expression in identifying CIN 2 or more severe lesions among HPV-positive patients referred for colposcopy after abnormal cytology. *Cancer Cytopathology*, 108 (2): 119 – 123.

- Carter, J.J., Koutsky, L.A., Hughes, J.P., Lee, S.K., Kuypers, J., Kiviat, N., Galloway, D.A. 2000. Comparison of Human Papilloma virus types 16, 18, and 6 capsid antibody responses following incident infection. *Journal of Infectious Diseases,* 181: 1911-1919

- Castle, P.E., Schiffman, M., Gravitt, P. E., Kendall, H., Fishman, S., Dong, H., Hildesheim, A., Herrero, R., Bratti, M.C., Sherman, M.E., Lorincz, A., Schussler, J.E., Burk, R.D. 2002. Comparisons of HPV DNA detection by MY09/11 PCR methods. *Journal of Medical Virology*, 68(3):417-23.

- Chivukula, M., Austin, R.M., Sidham, V.B. 2007. Evaluation and significance of hyperchromatic crowded groups (HCG) in liquid-based paps. *Cyto Journal*, 4:2.

- Chivukula, M and Shidham, V.B. 2006. ASC-H in Pap test--definitive categorization of cytomorphological spectrum. *Cyto Journal*, 3:14.

- Chan, P.K.S., Chand, A.R., Yu, M.Y., Li, W., Chan, M.Y.M., Yeung, A.C.M., Cheung, T., Yau, T., Wond, S., Yau, C., No, H. 2009. Age distribution of Human Papilloma virus infection and cervical neoplasia reflects caveats of cervical screening policies. *International Journal of Cancer*, 126 (1): 297-301.

- Clavel, C., Masure, M., Bory, J.P., Putaud, I., Mangeonjean, C., Lorenzato, M., Nazeyrollas, P., Gabriel, R., Quereux, C., Birembaut, P. 2001. Human Papilloma virus testing in primary screening for the detection of high-grade cervical lesions: a study of 7932 women. *British Journal of Cancer* 84 (12): 1616–1623.

- Clifford, G., Goncalves, M.A., Franceschi, s. 2006. Human Papilloma virus types among women infected with HIV: A meta-analysis. *AIDS* 20(18):2337-44.

- Clouse, A.L. 2004. Mastering the intricacies of abnormal cervical cytology. *Patient Care,* 38: 15-22.

- Coker, A.L., Sanders, L.C., Bond, S.M., Gerasimova, T., Pirisi, L. 2001. Hormonal and barrier methods of contraception, oncogenic Human Papilloma viruses, and cervical squamous intraepithelial lesion development. *Journal of Women's Health & Gender-Based Medicine,* 10 (5): 441-449.

- Colgan, T.J., Woodhouse, S.L., Styer, P.E., Kennedy, M., Davey, D.D. 2001. Reperative changes and the false-positive/false-negative Papanicolaou test: a study from the College of American Pathologists interlaboratory comparison program in cervicovaginal cytology. *Archives of Pathology & Laboratory Medicine*, 125 (1): 134-140.

- Cooper, D., Hoffman, M., Carrara, H., Rosenberg, L., Kelly, J., Stander, I., Denny, L., Williamson, A., Shapiro, S. 2007. Determinants of sexual activity and its relation to cervical cancer risk among South African Women. *BioMed Central for Public health*, 7: 341.

- Cox, J.T. 1999. Evaluating the role of HPV testing for women with equivocal Papanicolaou test findings. *The Journal of the American Medical Association*, 281: 1645-1647.

- Cuzick, J., Clavel, C., Petry, K.U., Meijer, C.J., Hoyer, H., Ratnam, S., Szarewski, A., Birembaut, P., Kulasingam, S., Sasieni, P., Iftner, T. 2006. Overview of the European and North American studies on HPV testing in primary cervical cancer screening. *International Journal of Cancer*, 119 (5): 1095-1101.

- Davey, E., Barratt, A., Irwig, L., Chan, S.F., Macaskill, P., Mannes, P., Saville, A.M. 2006. Effect of study design and quality on unsatisfactory rates, cytology classifications, and accuracy in liquid-based versus conventional cervical cytology: a systematic review. *The Lancet*, 367:122-32.

- Davey, D.D., Woodhouse, S., Styer, P., Stastny, J., Mody, D. 2000. Atypical Epithelial Cells and Specimen Adequacy. *Archives of Pathology & Laboratory Medicine*, 124 (2): 203-211.

- De Boer, M.A., Peters, L.A., Aziz, M.F., Siregar, B., Cornain, S., Vrede, M.A., Jordanova, E.S., Fleuren, G.J. 2005. Human Papilloma virus type 18 variants: histopathology and E6/E7 polymorphisms in three countries. *International Journal of Cancer,* 14 (3): 422-425.

- De May, Richard M.1996. *The Art and Science of Cytopathology: Exfoliative cytology.* Vol 1. Hong Kong: Carlson and Rogers.

- Duncan, L., Jacob, S., Hubbard, E. 2007. Evaluation of P16INK4A as a Diagnostic Tool in the Triage of Pap Smears Demonstrating Atypical Squamous Cells of Undetermined Significance. *Cancer Cytopathology*, 114 (1): 34-48.

- Duska, L.R., Flynn, C.F., Chen, A., Whall-Strojwas, D.,Goodman, A. 1998. Clinical evaluation of atypical glandular cells of undetermined significance on cervical cytology. *Obstetrics and Gynecology,* 91: 278-282.

- Fahey, M., Irwig, L., Macaskill, P. 1995. Meta-analysis of Pap tests accuracy. *American Journal of Epidemiology*, 141 (7): 680-689.

- Ferlay, J., Bray, F., Pisani, P., Parkin, D.M. GLOBOCAN 2002: Cancer Incidence, Mortality, and Prevalence Worldwide. IARC CancerBase No. 5, Version 2.0. Lyon: IARC Press, 2004.

- Ferrante, J.M., Gonzalez,E.C., Roetzheim, R.G., Pal, N., Woodard, L. 2000. Clinical and demographic predictors of late-stage cervical cancer. *Archives of Family Medicine, The Journal of the American Medical Association,* 9: 439-445.

- Figueroa, J.P., Ward, E., Luthi, T.E., Vermund, S.H., Brathwaite, A.R., Burk, R.D. 1995. Prevalence of Human Papilloma virus among STD clinic attenders in Jamaica: association of younger age and increased sexual activity. *Sexually Transmitted Diseases,* 22 (2): 114-118.

- Fiorella, R.M., Casafrancisco, D., Yokata, S., Kragel, P.J. 1994. Artifactual endocervical atypia induced by endocervical brush collection. *Diagnostic Cytopathology*, 11: 79-83.

- Franco, E.L., Villa, L.L., Sobrinho, J.P., Prado, J.M., Rousseau, M.C., Désy, M., Rohan, T.E. 1999. Epidemiology of acquisition and clearance of cervical Human Papilloma virus infection in women from a high-risk area for cervical cancer. *Journal of Infectious Diseases,* 180 (5):1415-1423.

- Frappart, L., Fontanière, B., Lucas, E and Sankaranarayanan, R. 2003. *Histopathology and Cytopathology of the uterine cervix - digital atlas*. Lyon: International Agency for Research on Cancer (IARC).

- Grainge, M.J., Seth, R., Guo, L., Neal, K.R., Coupland, C., Vryenhoef, P., Johnson, J., Jenkins, D. 2005. Cervical Human Papilloma virus screening among older women. *Emerging Infectious Diseases*, 11(11): 1680- 1685.

- Gravitt, P. E and Jamshidi, R. 2005. Diagnosis and management of oncogenic cervical Human Papilloma virus infection. *Infectious Disease Clinics of North America, 19:439*-458.

- Guido, R.S., Schiffmann, M., Solomon, D., Burke, L (ALTS group). 2000. Human Papilloma virus testing for triage of women with cytologic evidence of low-grade squamous intraepithelial lesions. Baseline data from a randomized trial. *Journal of the National Cancer Institute*, 92: 397-402.

- Gupta, P.K. 1982. Intrauterine contraceptive devices: Vaginal cytology, pathologic changes and clinical implications. *Acta Cytologica;* 26:571-613.

- Giuliano, A.R., Papenfuss, M., Schneider, A., Nour, M., Hatch, K. 1999. Risk factors for high-risk type human papillomavirus infection among Mexican-American women. *Cancer Epidemiology, Biomarkers and Prevention,* 8: 615–620.

- Harris, T.G., Burk, R.D., Palefsky, J.M. 2005. Incidence of cervical squamous intraepithelial lesions associated with HIV serostatus, CD4 cell counts, and human papillomavirus test results. *The Journal of the American Medical Association*, 293:1471-1476.

- Harris, N. S., Fowler, M.G., Sansom, S. L., Ruffo, N., Lampe, M.A. 2007. Use of enhanced perinatal human immunodeficiency virus surveillance methods to assess antiretroviral use and perinatal human immunodeficiency virus transmission in the United States. *American Journal of Obstetrics & Gynecology,* 197(3):S33-S41.

- Hausen, H. 1999. Immortalization of human cells and their malignant conversion by high risk Human Papilloma virus genotypes. *Seminars in Cancer Biology,* 9: 405-411.

- Herbert, J. and Coffin, J. 2008. Reducing patient risk for Human Papilloma virus infection and cervical cancer. *JAOA. Clinical Practice,* 108 (2): 65-70.

- Ho, G.Y., Bierman, R., Beardsley, L. N., Chang, C.J., Burk, R.D.1998. Natural history of cervicovaginal Papilloma virus infection in young women. *New England Journal of Medicine,* 338 (7):423-428, February 12.

- Hogewoning, C. J.,Bleeker, M.C., van den Brule, A.J, Voorhorst, F.J., Snijders, P.J., Berkhof, J., Westenend, P.J., Meijer, C.J. 2003. Condom use promotes regression of cervical intraepithelial neoplasia and clearance of Human Papilloma virus: a randomized clinical trial. *International Journal of Cancer*, 107 (5): 811–816.

- Hu, L., Guo, M., He, Z., Thornton, J., McDaniel, L.S., Hughson, M.D. 2005. Human Papilloma virus genotyping and P16INK4A expression in cervical intraepithelial neoplasia of adolescents. *Modern Pathology,* 18: 267–273

- Iftner, T and Villa, L.L. Human Papilloma virus Technologies. 2003. *Journal of the National Cancer Institute Monogaphs,* 31:80-88.

- Janicek, M and Averette, H. 2001. Cervical Cancer: Prevention, diagnosis and therapeutics. *CA: A Cancer Journal for clinicians*, 51 (2): 92-114.

- Jastreboff, A.M and Cymet, T. 2002. Role of the Human Papilloma virus in the development of cervical intraepithelial neoplasia and malignancy. *Postgraduate Medical Journal,* 78: 225-228.

- Jay, N. and Moskicki, A.B. 2000. Human Papilloma virus infections in women with HIV disease: Prevalence, risk, and management. *AIDS Reader*, 10: 659-68.

- Jo, H and Kim, J.W. 2005. Implications of HPV infection in uterine cervical cancer. *Cancer Therapy*, 3: 419-434.

- Jovanovic, A.S., McLachlin, C.M., Shen, L., Welch, W.R., Crum, C.P. 1995. Postmenopausal squamous atypia: a spectrum including "pseudo-koilocytosis". *Modern Pathology,* 8: 408-412.

- Kang, G.H., Min, K., Shim, Y.H., Kim, K.R. 2001. Papillary immature metaplasia of the uterine cervix: a Report of 5 cases with an emphasis on the different diagnosis from reactive squamous metaplasia, high-grade squamous intraepithelial lesions and papillary squamous cell carcinoma. *Korean Journal for Medical Science*, 16 (6): 762-768.

- Kay, P., Soeters, R., Nevin, J., Denny, L., Dehaeck, C., Williamson, A. 2003. High prevalence of HPV 16 in South African women with cancer of the cervix and cervical intraepithelial neoplasia. *Journal of Medical Virology,* 71:265-273.

- Keating, J.T., Ince, T., Crum, C.P. 2001. Surrogate Biomarkers of HPV Infection in Cervical Neoplasia Screening and Diagnosis. *Advances in Anatomic Pathology*. 8 (2): 83-92.

- Kjellberg, L., Hallmans, G., Ahren, A.M., Johansson, R., Bergman, F., Wadell, G., Angstrom, T., Dilliner J. 2000. Smoking, diet, pregnancy and oral contraceptive use as risk factors for cervical intra-epithelial neoplasia in relation to Human Papilloma virus infection. *British Journal of Cancer,* 82 (7): 1332-1338.

- Kinney, W.K., Manos, M.M., Hurley, L.B., Ransley, J.E. 1998. Where's the high-grade cervical neoplasia? The importance of minimally abnormal Papanicolaou diagnosis. *Obstetics and Gynecology,* 91: 973-976.

- Klaes, R., Friedrich, T., Spitkovsky, D., Ridder, R., Rudy, W., Petry, U., Dallenbach-Hellweg, G., Schmidt, D.,von Knebel Doeberitz, M. 2001. Overexpression of P16INK4A as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. *International Journal of Cancer*, 92: 276-284.

- Koutsky, L. 1997. Epidemiology of genital Human Papilloma virus infection. *American Journal of Medicine*, 102: 3– 8.

- Koutsky, L.A and Kiviat, N. B. 1999. *Genital Human Papilloma virus,* Sexually Transmitted Diseases. 3rd ed. New York: McGraw-Hill.

- Koss, L.G., Melamed, M.R. 2006. *Koss' Diagnostic cytology and its histopathological bases.* Vol. 2. 5th ed. Philadelphia: Lippincott Williams and Wilkins.

- Kufe, D.W., Pollock, R.E., Weichselbaum, R.R. 2003. *Papilloma viruses and Cervical Neoplasia.* Hamilton: BC Decker.

- Kurman, R.J and Solomon, D. 1994. The Bethesda system for reporting cervical/vaginal cytologic diagnoses: definitions, criteria, and explanatory notes for terminology and specimen adequacy. New York, N.Y.: Springer-Verlag.

- Laara, E., Day, N.B., Hakama, M. 1987. Trends in mortality from cervical cancer in the Nordic countries. Association with organized screening programmes. *The Lancet* 1:1247-1249.

- Lazcane-Ponce, E., Herrero, R., Munoz, N., Cruz, A., Shah, V.S., Alonso, P., Hernandez, P., Salmeron, J., Hernandez, M. 2001. Epidemiology of HPV infection among Mexican women with normal cervical cytology. *International Journal of Cancer*, 91: 412-420.

- Ley, C., Bauer, H.M., Reingold, A., Schiffman, M.H., Chambers, J.C., Tashiro, C.J., Manos, M.M. 1991. Determinants of genital Human Papilloma virus infection in young women. *Journal of the National Cancer Institute*, 83: 997–1003.

- Mandelblatt, J.S., Kanetsky, P., Eggert, L and Gold, K. 1999. Is HIV Infection a Cofactor for Cervical Squamous Cell Neoplasia? *Cancer Epidemiology Biomarkers & Prevention,* 8: 97-106.

- Manhart, L.E and Koutsky, L.A. 2002. Do condoms prevent genital HPV infection, external genital warts, or cervical neoplasia? A meta-analysis. *Sexually Transmitted Diseases*, 29: 725–735.

- Manbir online diseases and conditions. 1997. *Genital warts and HPV*. http://www.manbir-online.com/std/hiv.25.htm [25 February 2008].

- Massad, L.S., Behbarht, K., Collins, Y.C. 2003. Histologic findings from cervix among older women with abnormal cervical cytology. *Gynecologic Oncology*, 88: 340-344.

- McKenzie, N.D., Kobertz, E.N., Hnatyszyn, J., Twiggs, L.B., Lucci, J.A. 2010. Women with HIV are more commonly infected with non-16 and -18 high risk HPV types. *Gynecologic Oncology*, 116 (3): 572-577.

- McDermott-Webster, M.1999. The HPV epidemic. *American Journal of Nursing,* 99: 24L-24N.

- Michelow, P., Naidoo, C., Omar, T., Wanblad, C., Njoli, A., Dubb, M. 2007. The use of P16INK4A in further defining atypical squamous cells cannot exclude high grade lesions on cervical cytology (ASC-H): results of a pilot study. *The South African Journal of Epidemiology and Infection*, 22 (4): 109-112.

- Miller AB. 1992. Cervical Cancer Screening Programmes: Managerial Guidelines. Geneva: World Health Organization.

- Minkoff, H., Feldman, J.G., Strickler, H.D., Watts, D.H., Bacon, M.C., Levine, A., Palefsky, J.M., Burk, R., Cohen, M.H., Anastos, K. 2004. Relationship between smoking and Human Papilloma virus infections in HIV-infected and -uninfected women. *Journal of Infectious Diseases,* 189: 1821-1828.

- Moreno, V., Bosch, F.X., Munoz, N., Meijer, C.J., Shah, K.V., Walboomers, J.M., Herrero, R., and Franceschi, S. 2002. Effects of oral contraceptives on risk of cervical cancer in women with Human Papilloma virus infection: the IARC multicentric case-control study. *The Lancet,* 359: 1085-1092.

- Moscicki, A.B., Hills, N., Shiboski, S., Powell, K., Jay, N., Hanson, E., Miller, S., Clayton, L., Farhat, S., Broering, J., Darragh, T., Palefsky, J. 2001. Risks for incident Human Papilloma virus infection and low-grade squamous intraepithelial lesion development in young females. *The Journal of the American Medical Association*, 285: 2995–3002.

- Munoz, N., Mendez, F., Posso, H., Molano, M., van den Brule, A.J., Ronderos, M., Meijer, C., and Munoz, A. 2004. Incidence, duration, and determinants of cervical Human Papilloma virus infection in a cohort of Colombian women with normal cytological results. *Journal of Infectious Diseases*, 190 (12): 2077-2087.

- Munk, C., Svare, E.I., Poll, P., Bock, J.E., Kjaer, S.K. 1997. History of genital warts in 10,838 women 20 to 29 years of age from the general population. Risk factors and association with Papanicolaou smear history. *Sexually Transmitted Diseases*, 24: 567–572.

- Murali, R., Loughman, N.T., Pagliuso, J., McKenzie, P.R., Watson, G.F., Earls, P., Russel, P. 2010. Cytological features of transitional cell metaplasia of the lower female genital tract. *Pathology*, 42 (2): 113-118.

- Nanda, K., McCrory, D.C., Myers, E.R., Bastian, L.A., Hasselblad, V., Hickey, J.D., and Matchar, D.B. 2000. Accuracy of the Papanicolaou test in screening for and follow-up of cervical cytological abnormalities: a systematic review. *Annals of Internal Medicine,* 132 (10): 810-819.

- Nauth, H.F. 2007. *Gynecologic cytology*. Vol 1. Stuttgart, Germany*:* Georg Thieme Verlag.

- Negri, G., Vittadello, F., Romano, F. 2004. P16INK4A expression and progression of low grade squamous intraepithelial neoplasia of the uterine cervix. *Virchows Archives,* 445: 616-620.

- Nieh, S., Chen, S., Chu, T., Lai, H., Fu, E. 2003. Expression of P16INK4A in Papanicolaou smears containing atypical squamous cells of undetermined significance from the uterine cervix. *Gynecologic Oncology,* 91:201–208.

- Okonda, S., Wright, C., Michelow, P. 2009. The status of cervical cytology in Swaziland, Southern Africa: A descriptive study. *Cytojournal*, 6: 14.

- Pan, H and Griep, A.E. 1994. Altered cell cycle regulation in the lens of HPV-16 E6 or E7 transgenic mice: implications for tumor suppressor gene function in development. *Genes and Development*, 8: 1285–1299.

- Parham, G.P., Sahasrabuddhe, V.V., Mwanahamuntu, M.H., Shepherd, B.E., Hicks, M.L., Stringer, E.M., Vermund, S.H. 2006. Prevalence and predictors of squamous intraepithelial lesions of the cervix in HIV-infected women in Lusaka, Zambia. *Gynecologic Oncology,* 103 (3): 1017-1022.

- Peyton, C.L., Gravitt, P.E., Hunt, W.C., Hundley, R.S., Zhao, M., Apple, R.J., Wheeler, C.M. 2001. Determinants of genital Human Papilloma virus detection in a US population. *The Journal of Infectious Diseases*, 183: 1554–1564.

- Pientong, C., Ekalaksananan, T., Kongyingyoes, B., Kritpetcharat, O., Swadpanich, U., Pengsa, P., Yuenyao, P. 2004, Immunocytochemical staining of P16INK4A protein from conventional Pap test and its association with Human Papilloma virus infection. *Diagnostic Cytopathology,* 31 (4): 235-242.

- Renshaw, A.A., Prey, M.U., Hodes, L. 2005. Cytologic features of high-grade squamous intraepithelial lesion in conventional slides: what is the difference between cases that perform well and those that perform poorly. *Archives of Pathology and Laboratory Medicine*, 129: 733-735.

- Renshaw, A.A., Schulte, M.A., Plott, E. 2006. Cytologic features of high-grade squamous intraepithelial lesions in thin-prep Papanicolaou test slides: Comparison of cases that performed well in the College of American Pathologists interlaboratory comparison program in cervicovaginal cytology. *Archives of Pathology and Laboratory Medicine,* 128: 746-748.

- Republic of Namibia. Ministry of Health and Social Services. 2001. 1995-2001. Health Information System Epidemiological Reports. Windhoek: Ministry of Health and Social Services.

- Republic of Namibia. 2001. National *Policy for Reproductive Health*. Windhoek: Solitaire Press

- Risse, E. K. J., Beerthuizen, R.J. C. M. , Vooijs, G.P. 1981. Cytologic and histologic findings in women using an IUD. *Obstetrics and Gynecology*, 58:569-73.

- Ronco, G., Segnan, N., Giorgi-Rossi, P., Zappa, M., Casadei, G.P., Carozzi, F., Dalla Palma, P., Del Mistro, A., Folicaldi, S., Gillio-Tos, A., Nardo, G., Naldoni, C., Schincaglia, P., Zorzi, M., Confortini, M., Cuzick, J. 2006. New Technologies for Cervical Cancer Working Group. Human Papilloma virus testing and liquid-based cytology: results at recruitment from the new technologies for cervical cancer randomized controlled trial. *Journal of the National Cancer Institute,* 98:765–774

- Ronco, G., Giorgi-Rossi, P., Carozzi, F., Confortini, M., Palma, P.D., Del Mistro, A., Ghiringhello, B., Girlando, S., Gillio-Tos, A., De Marco, L., Naldoni, C., Pierotti, P., Rizzolo, R., Schincaglia, P., Zorzi, M., Zappa, M., Segnan, N., Cuzick, J. 2010. Efficacy of Human Papilloma virus testing for the detection of invasive cervical cancers and cervical intraepithelial neoplasia: a randomised controlled trial. *The Lancet Oncology*, 11 (3): 249-257.

- Rughooputh, S., Eddoo, R., Manraj, S., Jeebun, N., Greenwell, P. 2006. Detection of Human Papilloma virus from archival tissues in cervical cancer patients in Mauritius. *Journal of Clinical Virology,* 35: 173-178.

- Saad, R.S., Kanbour-Shakir, A., Lu, E. 2006. Cytomorphologic analysis and histological correlation of high-grade squamous intraepithelial lesions in postmenopausal women. *Diagnostic Cytopathology,* 34: 467-471.

- Sahebali,S., Depuydt, C., Segers, K. 2004. P16INK4A as an adjunct marker in liquid based cervical cytology. *International Journal of Cancer*, 108: 871-876.

- Sankaranarayanan, R., Wesley, R., Thara, S., Dhakad, N., Chandralekha, B., Sebastian, P., Chithrathara, K., Parkin, D.M., Nair, M.K. 2003. Test characteristics of visual inspection with 4% acetic acid (VIA) and Lugol's iodine (VILI) in cervical cancer screening in Kerala, India. *International Journal of Cancer*, 106 (3): 404-408.

- Sano, T., Oyama, T., Kashiwabara, K., Fukuda, T., Nakajima, T. 1998. Expression of P16 protein is associated with Human Papilloma virus oncogenic potential in cervical and genital lesions. *American Journal of Pathology,* 153 (6): 1741-1717.

- Sawaya, G.F., Brown, A.D., Washington, A.E., and Garber, A.M. 2001. Current approaches to cervical cancer screening. *New England Journal of Medicine* 344 (21): 1603-1607.

- Schiffman, M.,Herrero ,R., Hildesheim ,A., Sherman, M.E., Bratti, M., Wacholder, M., Alfaro, M., Hutchinson, M., Morales, J., Greenberg, M., and Lorincs, A.T. 2000. HPV DNA testing in cervical cancer screening: Results from women in a high-risk province of Costa Rica. *The Journal of the American Medical Association*, 283: 87-93.

- Schneider, A., Hoyer, H., Lotz, B., Leistritza, S., Kühne-Heid, R., Nindl, I., Müller, B. 2000. Screening for high-grade cervical intra-epithelial neoplasia and cancer by testing for high-risk HPV, routine cytology or colposcopy. *International Journal of Cancer*, 89: 529–534.

- Scott, M., Nakagawa, M., Moscicki, A.B. 2001. Cell-mediated immune response to Human Papilloma virus infection. *Clinical and Diagnostics Laboratory Immunology*, 8: 209 –220.

- Sedlacek, T. 1999. Advances in the diagnosis and treatment of Human Papilloma virus infections. *Clinical Obstetrics and Gynecology*, 42: 206-220.

- Shidham, V. B., Rao, R.N., Machhi, J., Chavan, A. 2004. Microglandular Hyperplasia has a cytomorphological spectrum overlappping with Atypical Squamous Cells - cannot exclude High-grade Squamous Intraepithelial Lesion (ASC-H). *Diagnostic Cytopathology,* 30: 57-61.

- Sherman, M.E., Schiffman, M., Cox, J.T. 2002. Effects of age and human papilloma viral load on colposcopy triage: data from the randomized Atypical Squamous Cells of Undetermined Significance/Low-Grade Squamous Intraepithelial Lesion Triage Study (ALTS). *Journal of the National Cancer Institute*, 94:102.

- Slawson, D.C., Bennett, J.H., Herman, J.M. 1993. Follow-up Papanicolaou smear for cervical atypia: are we missing significant disease? A HARNET Study. *Journal of Family Practice*, 36:289.

- Smith, J.S., Green, J., de Gonzales, B., Appleby, P., Plummer, M., Franceschi, S., Beral, V. 2003. Cervical cancer and use of hormonal contraceptives: a systematic review. *The Lancet*, 361: 1159-1167.

- Solomon D and Nayar R. 2004. *The Bethesda system for reporting cervical cytology*. 2nd ed. New York: Springer-Verlag.

- Solomon, D., Schiffman, M., Tarone, R., Guido, R.S., Burke, L. 2001. Comparison of three management strategies for patients with atypical squamous cells of undetermined significance: baseline results from a randomized trial. *Journal of the National Cancer Institute,* 93: 293-299.

- Sonnex, C., Struass, S., Gray, J.J. 1999. Sexually transmitted infections. *British Medical Journal*, 75: 317-319.

- South Africa. University of Cape Town. *Papilloma virus*. 1995. http://web.uct.ac.za/depts/mmi/stannard/papillo.html [12 January 2008].

- Speck, L.M and Tyring, S.K. 2006. Vaccines for the prevention of Human Papilloma virus infections. *Skin Therapy Letter*, 11: 1-3.

- Stanley, M.A. 2002. Prognostic factors and new therapeutic approaches to cervical cancer. *Virus Research*, 89 (2): 241-248.

- Stone, K.M., Timyan J., Thomas, E.L. 1999. *Barrier methods for the prevention of sexually transmitted diseases,* 3rd ed, Sexually transmitted diseases, New York: McGraw-Hill.

- Szarewski, A and Cuzick, J. 1998. Smoking and cervical neoplasia**:** a review of the evidence**.** *Journal of Epidemiology and Biostatistics,* 3: 229–256.

- Ter Meulen, J., Eberhardt, H.C., Luande, J., Mgaya, H.N., Chang-Claude, J., Mtiro, H., Mhina, M., Kashaija, P., Yu, X., Meinhardt, G., Gissmannl, L., Pawlita, M. 2006. Human Papilloma virus (HPV) infection, HIV infection and cervical cancer in Tanzania, East Africa. *International Journal of Cancer*, 51: 515-521.

- Tsoumpou, I., Arbyn, M., Kyrgiou, M., Wentzensen, N., Koliopoulos, N., Martin-Hirsch, P., Malamou-Mitsi, V., Paraskevaidis, E. 2009. P16INK4A immunostaining in cytological and histological specimens from the uterine cervix: a systematic review and meta-analysis. *Cancer Treatment Reviews*, 35 (3): 210-220.

- Van Bogaert, L.J., Knapp, D.C. 2001. Opportunistic testing of medically underserved women for cervical cancer in South Africa. *Acta Cytologica*, 45 (3): 313-316.

- Vooijs, P.G., Elias, A., van der Graaf, Y., Veling, S. 1985. Relationship between the diagnosis of epithelial abnormalities and the composition of cervical smears. *Acta Cytologica*, 29 (3): 323-8.

- Walboomers, J., Jacobs, M., Manos, M. 1999. Human Papilloma virus is a necessary cause of invasive cervical cancer worldwide. *Journal of Pathology*, 189: 12-19.

- Wang, H.L and Lu, D.W. 2004. Detection of Human Papilloma virus DNA and expression of p16, Rb, and p53 proteins in small cell carcinomas of the uterine cervix *American Journal of Surgical Pathology,* 28 (7):901-8.

- Wentzensen, N., Bergeron, C.H., Cas, F., Eschenbach, D., Vinokurowa, S., von Knebel Doeberitz, M. 2005. Evaluation of a nuclear score for P16INK4A stained cervical squamous cells in liquid based cytology samples. *Cancer Cytopathology*, 105: 461-467.

- Winer, R.L., Clifford, G.M., Franceschi, S., Sellors, J.W. 2005. Worldwide comparisons of HPV types in invasive cervical cancer and cervical intraepithelial neoplasia Grade 3 (CIN 3). Poster presented at: International Papilloma virus Conference, Vancouver.

- Wong, A.K., Chan, R.C.K., Nichols, W.S., Bose, S. 2008. Human Papilloma virus (HPV) in Atypical Squamous Cervical Cytology: the Invader HPV Test as a New Screening Assay. *Journal of Clinical Microbiology,* 46 (3): 869–875.

- Woodman, C.B.J., Collins, S., Winter, H., Bailey, A., Ellis, J., Prior, P., Yates, M., Rollason, T., Young, L.S. 2001. Natural history of cervical Human Papilloma virus infection in young women: a longitudinal cohort study. *The Lancet,* 357: 1831-1836.

- World Health Report 2001.

- Wright, T.C Jr., Cox, J.T., Massad, L.S., Twiggs, L.B., Wilkinson, E.J. 2002. 2001 consensus guidelines for the management of women with cervical cytological abnormalities. *The Journal of the American Medical Association,* 287: 2120-2129.

- Wright, J.D., Rader, J.S., Davila, R., Powell, M.A., Mutch, D.G., Gao, F., Gibb, R.K. 2006. Human Papilloma virus triage for young women with atypical squamous cells of undetermined significance. *Obstetrics & Gynecology*, 107: 822-829.

- Zhang, S., Hunter, D.J., Hankinson, S.E., Giovannucci, E.L., Bernard, A.R., Graham, A.C., Frank, E.S., Walter, C.W. 1999. A prospective study of folate intake and the risk of breast cancer. *The Journal of the American Medical Association*, 281: 1632–1637.

- Zhao, F.H., Forman, M.R., Belinson, J., Shen, Y.H., Graubard, B.I., Patel, A.C., Rong, S.D., Pretorius, R.G., Qiao, Y.L. 2006. Risk factors for HPV infection and cervical cancer among unscreened women in a high-risk area of China. *International Journal of Cancer*, 15: 118 (2): 442-448.

APPENDICES

[MHSS Protocol number: 17/3/5]

APPENDIX A: QUESTIONNAIRE

Research Protocol: Prevalence of HPV induced lesions of the cervix among gynaecological clinic attendees in Namibia: association of risk factors and cytomorphological findings

PRINCIPAL INVESTIGATORS:
Name: C. Izaaks[1], Associate Professor S. Khan[2] and Professor E. Truter[2]
Email: Christo.Izaaks@nip.com.na, KhanS@cput.ac.za, TruterE@cput.ac.za
Tel: 061-2954053, 021-9596902, 021-9596570
Investigator's Affiliation: Namibia Institute of Pathology (NIP)[1], Cape Peninsula University of Technology (CPUT)[2]

Ref no. CG.....

To be completed by the participant

1. Age of individual ……..
2. Usual place of residence ……
3. Now residing in …….
4. Marital status ……..
5. Number of pregnancies ……..
6. Contraception used ……
7. Do you smoke ……..
8. Do you drink ……
9. Do you use any chronic medication ……
10. What is your HIV status ……..
11. Do you have access to local health facilities ……..
12. Any sexually transmitted diseases in the past year……
13. Age at first sexual encounter ……..
14. Number of lifetime sexual partners ……
15. Frequency of Pap smear screening: Routine (annually) …….
    Only when vaginal problems arise ……...

Signature of participant if consent is given ……
Name of participant ……..
Date: ……

Name of interviewer.....

FOOTNOTE: Women infected with human immunodeficiency virus (HIV) are 10 times more likely to have an abnormal Pap smear than HIV negative women and are therefore at higher risk for developing cervical cancer. It is for this reason that they are encouraged to go for a Papanicolaou test (Pap smear) more regularly, the latter which is able to detect the disease early. This procedure takes no more than a minute and allows any cellular changes to be detected early and treated, long before any cancer can develop.

APPENDIX B: ETHICAL APPROVAL FORM

[MHSS Protocol number: 17/3/5]

REPUBLIC OF NAMIBIA

Ministry of Health and Social Services

Private Bag 13198 **Ministerial Building** **Tel: (061) 2032507**
Windhoek **Harvey Street** **Fax: (061) 227607**
Namibia Windhoek **E-mail: amuheua@mhss.gov.na**
Enquiries: Mr. A. Kulobone **Ref.:** 17/3/5 **Date:** 18 January 2006

OFFICE OF THE PERMANENT SECRETARY

Mr Christo. Delme Izaaks
NIP Laboratories
Windhoek

Dear Mr Izaaks

Re: Prevalence of HPV induced lesion of cervix among gynae clinic attendees in Namibia: association of risk factors and cytomorphologic findings.

1. Reference is made to your application to conduct the above-mentioned study.

2. The proposal has been evaluated and found to have merit.

3. Kindly be informed that approval has been granted under the following conditions:

3.1 A quarterly progress report is to be submitted to the Ministry's Research Unit;
3.2 Preliminary findings are to be submitted to the Ministry before the final report;
3.3 Final report to be submitted upon completion of the study;
3.4 Separate permission to be sought from the Ministry for the publication of the findings.

Wishing you success with your project.

Yours sincerely,

DR. K. SHANGULA
PERMANENT SECRETARY

Forward with Health for all Namibians by the Year 2000 and Beyond!

APPENDIX C: CONSENT FORM

Research Protocol: Prevalence of HPV induced lesions of the cervix among gynaecological clinic attendees in Namibia: association of risk factors and cytomorphological findings

PRINCIPAL INVESTIGATORS:
Name: **C. Izaaks**[1], Associate Professor S. Khan[2] and Professor E. Truter[2]
Email: Christo.Izaaks@nip.com.na, KhanS@cput.ac.za, TruterE@cput.ac.za
Tel: 061-2954053, 021-9596902, 021-9596570
Investigator's Affiliation: Namibia Institute of Pathology (NIP)[1], Cape Peninsula University of Technology (CPUT)[2]

Procedure

A prospective analytical study is to be conducted in Windhoek-Namibia over a twelve month period, among females aged 15-49 years visiting public health facilities and clinics for Pap smear screening. Two cervical smears will be collected from you and sent to the laboratory for cytological assessment. If found to be cytologically abnormal, the second unit will be processed using the $P16^{INK4A}$ marker. Cervical smears with an abnormal cytological diagnosis will be subjected to HPV DNA testing. Interviews will be conducted by health officials, after which self administered questionnaires will be completed by you which include your personal details, demographic background, sexual history, smoking habits and general health conditions. Free and voluntary informed and written consent must be obtained from you, for this information to be eligible for use in this study. You will have free access to the results at any time and no special conditions are attached hereto. A unique identification number will be allocated to you to protect your identity and rest assured that all information in this study will be handled with the strictest confidentiality. No person except the researcher will have access to the data obtained. All data are electronically filed for safe protection. If you agree, your cervical smear including your paper records will be destroyed upon completion of the study unless you desire to have it.

Purpose and benefits

The information obtained from this study may not improve your personal situation but may help to protect thousands of other people. The study will be submitted as a thesis for a Masters degree by the researcher.

Risks, Stress or Discomfort

Should you at any time of the interview experience discomfort or feel that you are not prepared to disclose some information, you have the right to stop the interviewer, retaining your right to withdraw at any time from the study.

Finally, we would like to review your medical record for information about your health history and treatment.

Signed (signature of parent if a minor).....
Date.....

APPENDIX D: PREPERATION OF STAINS AND SOLUTIONS

99% ethanol AR stock (actual concentration) solution is used to obtain the following concentrations:

50% ethanol v/v

$$\frac{\text{Required concentration X Volume required}}{\text{Actual concentration}}$$

$$\frac{50 \text{x} 1000 \text{ ml}}{99}$$

505 ml

A measuring cylinder is filled to a final volume of 1000 ml with de-ionized water after which it is thoroughly mixed and poured into a staining trough.

70% ethanol v/v

$$\frac{\text{Required concentration X Volume required}}{\text{Actual concentration}}$$

$$\frac{70 \text{x} 1000 \text{ ml}}{99}$$

707 ml

A measuring cylinder is filled to a final volume of 1000 ml with de-ionized water after which it is thoroughly mix and poured into a staining trough.

95% ethanol v/v

$$\frac{\text{Required concentration X Volume required}}{\text{Actual concentration}}$$

$$\frac{95 \text{X} 1000 \text{ ml}}{99}$$

960 ml

A measuring cylinder is filled to a final volume of 1000 ml with de-ionized water after which it is thoroughly mixed and poured into a staining trough.

Scott's tap water

1. Weigh 20g magnesium sulphate in sterile holder.
2. Weigh 2g sodium bicarbonate in sterile holder.
3. Measure 1000 ml de-ionized water in sterile volumetric flask.
4. Add the pre-weighed magnesium sulphate and sodium bicarbonate to the de-ionized water in a volumetric flask and mix thoroughly.
5. Solution is now ready for use.

Harris haematoxylin

1. Weigh the following reagents to the desired mass: haematoxylin powder- 5g, mercuric oxide- 2.5g and aluminium sulphate- 100g.
2. Dissolve the haematoxylin powder in 50 ml of 95% ethanol.
3. Dissolve the aluminium sulphate in 1000 ml de-ionized water in a volumetric flask, place on open Bunsen flame and bring to boil.
4. Remove flask from flame, add the haematoxylin solution slowly and bring to boil again.
5. Remove flask and add the mercuric oxide immediately to the solution.
6. Mix until solution assumes a dark purple colour.
7. Plunge the flask into a cool water bath.
8. When solution has cooled down, filter into well-stoppered dark bottle.
9. Add 4% glacial acetic acid (v/v) to the haematoxylin solution and use at full strength.
10. Filter haematoxylin solution each time before use.

APPENDIX E: DESTAINING SOLUTION

1. Measure 10 ml hydrochloric acid (5N) concentrate reagent grade in a volumetric flask using an electronic pipette.
2. Measure 1000 ml of 70% ethanol in a 1000 ml volumetric flask.
3. Add the hydrochloric acid to the ethanol and mix thoroughly.
4. Solution is now ready for use.

APPENDIX F: IMMUNOCYTOCHEMICAL REAGENT PREPARATION

E.1 Wash Buffer

Dilute a sufficient quantity of Dacocytomation Wash Buffer, Code No. S3006 1:10 using distilled or de-ionized water. Unused diluted buffer may be stored at 2-8ºC for one month. Discard diluted buffer if cloudy in appearance.

E.2 Epitope Retrieval Solution

Dilute a sufficient quantity of Vial 6 (Epitope Retrieval Solution) 1:10 using distilled or de-ionized water. Unused diluted solution may be stored at 2-8ºC for one month. Discard diluted solution if cloudy in appearance.

E.3 Substrate-Chromogen Solution (DAB)

The following procedure yields 1 ml of Substrate-Chromogen Solution:

1. Transfer 1 ml of DAB Buffered Substrate to a reagent vial.
2. Add one drop (25-30 µl) of DAB Chromogen to solution in a reagent vial and mix thoroughly.

Prepared Substrate-Chromogen Substrate is stable for approximately 5 days when stored at 2-8ºC. This solution should be thoroughly mixed prior to staining. Any precipitate occurring will not affect the staining.

NOTE: The addition of excess DAB Chromogen to DAB Buffered Substrate will result in the deterioration of the positive signal.

APPENDIX G: PREPERATION OF REAGENTS FOR PCR AMPLIFICATION

EDTA (0.5 M, pH 8.0)

Add 186.1g of disodium EDTA-2H_2O to 800 ml of H_2O. Stir vigorously on a magnetic stirrer. Adjust pH to 8.0 with NaOH (~20g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to ~8.0 by the addition of NaOH.

Tris-Cl (1M)

Dissolve 121.1g of Tris base in 800 ml of H_2O. Adjust the pH to the desired value by adding concentrated HCl.

pH	HCl
7.4	70 ml
7.5	60 ml
8.0	42 ml

Allow the solution to cool to room temperature before making the final adjustments to the pH. Adjust the volume of the solution to 1 litre with H_2O. Dispense into aliquots and sterilize by autoclaving. If the 1M solution has a yellow colour, discard it and obtain Tris of better quality. The pH of Tris solutions is temperature-dependant and decreases ~0.03 pH units for each 1ºC increase in temperature.

Loading Buffer

0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol or sucrose
in 30% (w/v) glycerol in distilled H_2O

50 X TAE

242g Tris
57.1 ml glacial acetic acid (GAA)
100 ml of 0.5 M EDTA
Adjust volume to 1 litre with distilled H_2O.
Sterilize by autoclaving.
Add 40 ml of 50 X TAE to 1960 ml distilled H_2O to obtain a 1 X TAE solution.
Sterilize by autoclaving.