AN INVESTIGATION CERVICAL CANCER, HUMAN PAPILLOMAVIRUS (HPV) INFECTION AND STEROID CONTRACEPTION

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ABSTRACT

PROJECT ONE

Introduction

HPV is detected in about 99.7% of cervical cancers. However, the HPV type distribution in South African women is unknown.

Objectives

To determine HPV-type distribution among women with cervical dysplasia in relation to oral contraceptive usage.

Methods

Prospective cross-sectional study of four groups of patients according to oral contraceptive usage: non-users, users of less than five years duration, users of between five years and ten years and users of more than ten years duration. Swabs of the cervix were analysed for HPV DNA using polymerase chain reaction method.

Results

A total of 124 women were recruited for the study. There were 75 HIV-infected patients (seroprevalence 61%). Of the 102(82%) HPV-positive patients, 79 patients had high-risk HPV DNA (78%). In terms of the four oral contraceptive groups, high-risk HPV DNA was detected in 70% (n=21), 79% (n=22), 90% (n=21) and 71% (n=15) of patients, respectively. The odds of having HPV DNA was six times higher for the combination of contraceptive users of less than 5 years duration/non-users (OR 5.9, 95% CI: 1.87 - 18.77).

There was no change when adjustment was made for age (OR 6.1, 95% CI: 1.9 - 19.4). HPV DNA types 16 and or 18 was present in a total of 21 patients (49%) (non-contraceptive users and users < 5years duration) versus 15 patients (42%) who used oral contraceptives of more than 5 years duration (p=0.524). HPV type 16 was the commonest HPV type detected (20.2%) and HPV type 58 was the next commonest high-risk HPV type (16.1%). HPV types 58 and 33 was detected in a much greater percentage of our population and HPV 16 in a much smaller percentage of our population compared with a non-South African population.

Conclusion

The findings of this study demonstrate an interesting distribution of HPV types in a South African population.

PROJECT TWO

Introduction

Various risk factors have been implicated in the causation of cervical cancer including human papillomavirus (HPV), the early genes (*E6* and *E7*) of which encode the main transforming proteins. Studies have suggested that steroid hormones may enhance the expression of these genes leading to loss of p53 gene-mediated cell apoptosis.

Methods

A total of 120 cervical tissue samples were obtained from patients with proven cervical cancer. Patients who used depo-medroxyprogesterone acetate steroid contraception were recruited as part of the study arm. Only HPV DNA type 16 samples were used for the study. Controls included three cell lines (CaSki, SiHa, & C33A) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal housekeeping gene. Of 120 patients, there were 111 patients with HPV type 16 identified. Of this number, RNA was present in 63 samples. There were 30 women (30/63) who used steroid contraception. In relation to patients who used contraception, HPV 16 *E6* gene expression was present in 79% (n = 23) and 88% (n = 30) of steroid users compared to nonusers, respectively. In total there were 25 patients (40%) with expression of the HPV 16 *E6*I* gene and 30 patients with expression of the *E6*II* gene. There were 57% of steroid users (n = 17) who had expression of the *E6*I/E6*II* gene, compared to 52% (n = 17) of nonusers (P = 0.800).

Conclusion

From a molecular level, this study reflects almost similar distribution of the HPV 16 E6/E6*1 and E6*11 and does not confirm the role of injectable progesterones in cervical carcinogenesis. Further studies with larger patient numbers are needed.

DECLARATION

This study represents work done by the author.

The research described in this thesis was performed in the Department of Obstetrics and Gynaecology, Inkosi Albert Luthuli Central Hospital, Durban, South Africa.

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PRESENTATION ARISING FROM THE PROJECT

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PUBLICATIONS ARISING FROM THE STUDY

1. **An investigation into oral contraceptive use, human papilomavirus (HPV)-type distribution and cervical intraepithelial neoplasia, Durban, South Africa. Eur J Gynaecol Oncol 2009 (accepted for publication).**
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3. **The role of steroid contraceptive hormones in the pathogenesis of invasive cervical cancer: a review. Int J Gynecol Cancer 2003; 13: 103-110.**
4. **Use of the nested reverse transcription-polymerase chain reaction for the detection of human papillomavirus 16 E6 transcriptional activity in cervical cancer: A technical perspective. Eur J Gynecol Oncol 2003; 25: 51 - 54**

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INTRODUCTION

Cervical cancer remains a major health problem affecting thousands of women across the globe. Being a preventable disease by virtue of the availability of a screening method to detect and treat precancerous lesions, it remains a mystery as to why so much morbidity and mortality is prevalent in modern society. A classic example of control of this disease is the Nordic countries where the incidence and mortality of cervical cancer has been reduced by about 80% with an established cervical cancer screening program. Lack of political will, inadequate or non-existent facilities, failure of the health care provider to educate women and failure of women to avail themselves for screening have contributed to the devastating effects affecting women predominantly in the productive years of their lives. The Human Papillomavirus (HPV) is a necessary pre-requisite for the development of precancerous and cancerous lesions not only of the cervix, but also of the lower female genital tract. However, co-factors are necessary for the progression to invasive cancer. Two commercially available vaccines against the human papillomavirus have been developed and are being provided to women as part of a government initiative in many developed countries. In the long term, it remains to be seen if the HPV vaccines will become an armamentarium of women's rights world-wide to prevent HPV-related diseases.

1.0 EPIDEMIOLOGY OF CERVICAL CANCER

Cervical cancer is the most common malignancy amongst women of developing countries and the second commonest cancer amongst women worldwide. In South Africa, the incidence of cervical cancer has been reported to be approximately 30 per 100 000 women (Sitas et al, 1993). Although the worldwide incidence and mortality of cervical cancer has declined over the last four decades (32.6 per 100 000 to 8.3 per 100 000 in the United States in the late 1940s), cervical cancer continues to be the leading cause of cancer–related mortality in the developing world (Brinton, 1992). Annually, there are about 500 000 new cervical cancer cases with a mortality of just over 250 000 women, representing about 10% of all female cancers. This is mainly due to the fact that about 85% of women in the developing world present with late-stage disease (59.3% stage III versus 5.2% stage 1B) (Cronje, 2004; Moodley et al, 2001 & Lomalisa et al, 2000). This pattern is similar in other developing regions such as Southeast Asia, Africa, Central America and the Caribbean, where the incidence rate of cervical cancer approaches 30 cases per 100 000 women (Fowler & Sayegh, 2005) . Estimates from Globocan 2002 have reported that there were 6742 cases of cervical cancer in South Africa, with mortality figures of 3681 and an age-standardized rate of 37.5. In comparison with breast cancers (Ferlay et al, 2004), there were 6018 cases of breast cancer in South Africa with a mortality of 2790 and an age-standardized rate of 35. This is in contrast to the incidence rate of cervical cancer in the United States of about 7.2 cases per 100 000 women with an overall mortality rate of 2.9 cases per 100 000 women (Fowler & Sayegh, 2005). This

disproportionate burden of cervical cancer is mainly due to the lack of well-organized screening programs (Womack et al, 2000). In many developing countries where cervical cancer screening exists, women's knowledge of cervical cancer and Papanicolaou (Pap) smears is very limited. Data from the United States Surveillance, Epidemiology and End-Results (SEER) program has demonstrated that survival amongst Black women with cervical cancer is far inferior to their White counterparts. The five-year survival rate for White women was 71% compared with 60% for Black women.

Cervical cancer has well recognized precancerous stages, which, if detected early by screening, can be treated to prevent the development of full-blown cancer. Unlike the developed countries such as the Nordic countries following the introduction of national screening in the 1960s, where the mortality has been reduced by about 80%, lack of screening facilities or inadequate screening facilities and poor uptake account for screening failures in the developing world (Wellensiek et al, 2002). The data also shows that the largest decline was in Iceland (84% from 1965 to 1982), where a large percentage of the population was screened. The pathogenesis of cervical cancer and its precursors, is causally linked to the high-risk human papillomaviruses (HPV). Human papillomaviruses are reported to be present in more than 99.7% of cervical cancers (Bosch, 2002). The most common high-risk HPV viruses found in cervical cancers are types 16 and 18 (70%). Human papillomaviruses are a necessary but insufficient cause of cervical cancer. Co-factors are therefore necessary for the expression of the malignant phenotype. It is estimated that only 5% of women in the developing world receive screening compared with 40% to 50% of women in the developed world (Sherris et al, 2001).

A further problemmatic aspect of cervical cancer is co-infection with the human immunodeficiency virus (HIV) epidemic in so many parts of the developing world. Although the HIV / Acquired Immunodeficiency Virus Syndrome (AIDS) pandemic is global in distribution, its maximum impact has been in Sub-Saharan Africa, where two-thirds of people (21 million) are reported to be living with HIV/AIDS (Ateka, 2000). By some estimates, up to 40% of patients with AIDS will develop some type of malignancy (Smith, 1998). In 1993, the Centres for Disease Control (CDC) expanded the case definition and included invasive cervical cancer as an AIDS-defining illness (CDC, 1993). Women who are infected with HIV are more likely to be infected with multiple HPV-types as well as to develop cervical intraepithelial neoplasia. They are also at greater risk of persistent HPV infection and lower regression of cervical intraepithelial neoplastic lesions (Schuman et al, 2003). Immunosuppression with CD4 counts of less than 200 cells/μL is associated with a 10-fold risk of developing cervical intraepithelial neoplasia (Heard et al, 2000). The seroprevalence of human immunodeficiency virus infection amongst antenatal attendees in KwaZulu Natal, South Africa was reported as 38.7% for 2003 (Moodley, 2006). Hence, in KwaZulu Natal, South Africa, the combination of deficient cervical cancer screening and the HIV epidemic predisposes many young women to cervical cancer with its attendant morbidity and mortality.

1.1 RISK FACTORS AND AETIOLOGY OF CERVICAL CANCER AND ITS PRECURSORS

1.1.1 PARITY

Although parity has been regarded as a risk factor for cervical cancer, it is the number of live births that has been consistently found to be of importance, irrespective and independent of other factors related to sexual activity such as number of partners. Brinton et al (1989) noted that the risk increased to 5.1 for women with 14 or more pregnancies related to live births, suggesting that hormonal factors and cervical trauma are putative mechanisms. It has also been suggested that the pregnancy-associated hormonal influence may have an impact on the HPV genomic elements which are responsive to progesterone (Pater et al, 1994). According to the data from the International Agency for Research on Cancer (IARC), there is a clear link between parity and HPV-positivity in that high parity in HPV-positive women increases the risk of cervical cancer even further (Munoz et al, 2002).

1.1.2 THE NUMBER OF SEXUAL PARTNERS AND FREQUENCY OF SEXUAL INTERCOURSE

Many studies have reported a link between the number of sexual partners and the risk of cervical cancer (Slattery et al, 1989; La Vecchia et al, 1986). There is three times the risk associated with ten or more partners compared with one partner (Brinton et al, 1987; Peters et al, 1986). Although Boyd & Doll (1964) did not find any significant association between frequency of intercourse and risk of cervical cancer, Herrero et al (1990a) reported a significant link between the frequency of coitus, multiple exposures prior to age 20 and risk of cervical cancer.

1.1.3 SMOKING

Smoking is a well recognized risk factor not only for precancerous and invasive cervical caners, but also for dysplasia of the vulva. This link has been alluded to in many epidemiological studies. Although initially the effect of smoking was thought to be linked to associated factors such as sexual behaviour, several studies which controlled for extraneous factors have still established a link between smoking and cervical cancer (Brinton et al. 1986; Clarke et al, 1982). Atalah et al (2001) reported an odds ration of 2.8 for smokers. The risks are highest for long-term or high- intensity smokers. More recent studies have shown a positive link between smoking and cervical cancer (Kjellberg et al, 2000) Thomas et al (2001) demonstrated that the odds of ever being a smoker was higher

in women with cervical carcinoma in-situ, invasive cervical carcinoma and cervical intraepithelial neoplasia grade 3. This implies that not only is smoking important as a causative agent, but has an important role in the later stages of disease pathogenesis. Castellsague and Munoz (2003) in a review of risk factors for cervical cancer reported an odds ratio of 2 to 5 for those who ever smoked among HPV-positive women. They also noted most studies which reported risk estimates according to intensity, duration or pack-years demonstrated an increased risk of cervical cancer with increasing exposure to tobacco smoking. Shields et al (2004) demonstrated a two-fold increased risk of precancerous and invasive cervical cancers in current or ever-smokers versus non-smokers. Syrjanen 2008 reported data from a cohort study of 3187 women to determine the influence of risk factors for cervical cancer. In multivariate analysis smoking was found to be an independent risk factor for cervical cancer because of the increased acquisition of high-risk HPV infections. The possible mechanisms involved include high levels of nicotine and cotine found in cervical mucus (Schiffman et al, 1987; Prokopczk, 1997), the immunosuppressive effects of smoking and the enhanced effects of infectious agents such as HPV (Barton et al, 1988). These immunosuppressive effects include the reduction of Langerhans cells in the cervix. Melikian et al (1999) reported the presence of benzo[a]pyrene and its metabolites in cervical mucus to be two times higher amongst smokers versus non-smokers. Yang et al (1996) demonstrated that the malignant transformation of immortalized endocervical cells occurs in the presence of cigarette condensate. Giuliano et al (2002) reported that smokers retain HPV infections significantly longer and have a lower chance of clearing HPV infection than non-smokers.

1.1.4 ROLE OF THE MALE PARTNER

Support for male partner contribution is derived from geographic clusters of cervical and penile cancers and elevated rates of cervical cancers among wives of men with penile cancer (Franco et al 1988; Graham et al 1979). Kessler (1977) showed in a follow-up study that wives of men previously married to cervical cancer patients had elevated rates of cervical neoplasia compared to a control group. Direct evidence for a male factor derives from studies in which the sexual histories of husbands of cervical cancer patients have been compared with those of control husbands (Brinton et al 1989; Buckley et al 1981). In all these studies, husbands of cervical cancer patients were likely to have histories of genital conditions, including venereal warts, gonorrhea and herpes simplex. Poor hygiene of the male partner has been thought to contribute to the aetiology of cervical cancer especially in relation to the issue of circumcision. Although initial studies showed a protective effect of circumcision (Terris & Oalmann, 1960), most other studies have shown no significant difference between case and control husbands (Boyd & Doll 1964; Rotkin 1967). The issue, however, remains to be resolved as Kjaer et al (1991) showed that wives of circumcised men were at a significantly lower risk (0.3). The issue of male circumcision also remains unresolved due to problems in assessment, with most studies showing no effect on cervical cancer risk (Brinton et al 1989).

1.1.5 ROLE OF DIETARY FACTORS

The protective effects of dietary factors such as beta-carotene, Vitamins A,C and E have been consistently reported (Garcia-Closas et al, 2005; Potischman and Brinton,1996 & Shannon et al, 2002). However, Kjellberg et al (2000), found no link between diet and risk of invasive cervical cancer or precancerous cervical lesions. Other dietary factors which have been shown to reduce the risk of invasive cervical cancer include tocopherols, folates and lycophene (Palan et al, 1991; Goodman et al, 2001 & van Eenwyk et al, 1991). The biological basis for the protective role of these dietary factors is thought to be due to their antioxidant actions on intracellular free radicals. Beta-carotene is thought to be a metabolic precursor to retinoic acid which modulates epithelial cell growth and differentiation (Potischman and Brinton, 1996). However, it is difficult to determine the exact role of dietary factors due to confounding by other risk factors known to be associated with the development of cervical cancer.

1.1.6 ROLE OF SEXUALLY TRANSMITTED INFECTIONS OTHER THAN HPV

Since HPV is present in virtually all cervical cancers (99.7%), all other agents are thought to act as co-factors with HPV in the pathogenesis of cervical intraepithelial and invasive neoplasias. These agents include the Herpes simplex Type 2 virus, *Chlamydia trachomatis, Neiseria gonorrhoea, Treponema pallidum*, cytomegalovirus, Epstein-Barr virus and bacterial vaginosis. Although many studies have reported an association of

these infectious agents with cervical cancer, some studies have reported unadjusted risks, resulting in difficulties in interpreting relationships independent of those associated with socioeconomic status and other risk factors. Brinton (1987) reported consistent increases in risk associated with histories of condyloma acuminate after adjusting for the number of sexual partners. However, genital warts are usually linked to HPV types 6 and 11 rather than HPV types 16 and 18 which are associated with cervical cancer (Koutsky et al, 1988). There has been a fairly consistent relationship between invasive cervical cancer and *Chlamydia trachomatis* (Koskela et al, 2000; Anttila et al, 2001). Smith et al (2002) reported a relative risk of 2.1 for *Chlamydia trachomatis* and HPV infections. Zur Hausen et al (1982) proposed that the HSV type 2 virus may be a co-factor in the pathogenesis of cervical cancer and that HPV sequences may be required to maintain the transformed phenotype. McDougall et al (1986) demonstrated that HSV can transform cells in culture and HSV type 2 proteins and HPV integrated DNA are found in cervical cancers. The IARC case-controlled studies have demonstrated an increased risk of 2-fold and 3-fold for invasive cervical cancers in HPV-positive women. However, other studies did not detect HSV type 2 virus consistently in cervical tumours (Lehtinen et al, 2002; Vonka et al, 1984). Inconsistent associations have been observed for other infections such as *Treponema pallidum*, cytomegalovirus, Epstein-Barr virus and bacterial vaginosis.

1.1.7 ROLE OF THE HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTION

In 2003, it was estimated that there were about 5 million new HIV infections and 3 million deaths from HIV-related illnesses. In the same year the joint United Nations Programme on HIV/AIDS concluded that there were about 40 million adults and children infected with HIV/AIDS. Majority of these infections were in persons living in sub-Saharan Africa, where majority of women do not have access to cervical cancer screening (Chirenje, 2004). The majority of these infections are in developing regions such as sub-Saharan Africa where cervical cancer is also most prevalent. Although the human immunodeficiency virus (HIV) / Acquired Immunodeficiency Virus Syndrome (AIDS) is a global phenomenon, the bulk of the disease occurs in developing regions of the world such as Sub-Saharan Africa (Ateka, 2000). By previous estimates, up to 40% of patients with AIDS will develop some type of malignancy (Smith et al, 1998). Women who are HIV-infected have a higher rate of developing human papillomavirus (HPV) infections, especially of the high-risk types. Such women are at greater risk of developing squamous intraepithelial neoplasia and invasive cervical cancers (Ellerbrock et al, 2000).

In areas ravaged by the HIV virus, the occurrence of female genital tract malignancies in association with the HIV virus has become a reality, creating new challenges for clinicians. The prevalence of HPV in HIV-infected and non-infected women has been shown to be 65% and 29%, respectively (Duerr et al, 2001). However, a more recent report by Levi et al (2002) documented the prevalence of HPV DNA in 98% of HIV-

infected women. Whilst HPV infection is usually transient in HIV non-infected women, HIV-infected women have higher rates of persistent infection and are less likely to clear high-risk HPVs than HIV non-infected women (20% versus 3%, respectively)(Sun et al, 1995). Jamieson et al (2002) reported increased prevalence of multiple HPV infections in HIV-infected compared with HIV non-infected women. HPV type 16 was the commonest high–risk type amongst HIV-infected women in the New York Cervical Disease Study (NYCDS) and Women's Interagency Study (WIHS) (Barkan et al, 1998) compared with HPV type 53 which was reported to be the commonest HPV type in the HIV Epidemiology Research Study (HERS) (Smith, 1998). Ahdieh et al (2001) reported higher HPV-positivity (75%) in HIV-infected women with CD4 counts of less than 200 cells/µL compared with women with CD4 counts >500 cells/µL (54%). Blossom et al (2007) conducted a cross-sectional study amongst Ugandan women to determine the prevalence of HIV infection, HPV infection and cervical abnormalities. Of the 106 women studied, the HPV and HIV prevalence was 46.2% and 34.9%, respectively. High-risk HPV genotypes 52, 58 and 16 were the commonest types detected. Only 18% of women had HPV types 16 and 18. There were 73% of HIV-infected women compared with 16% of HIV non-infected women with cervical cytological abnormalities ($p<0.0001$). Abnormal pap smears was significantly associated with HIV sero-positivity ($p<0.001$). Therefore, the majority of women had HPV types other than 16 and 18. HPV type 52 was also the commonest type reported by de Vuyst et al (2003) amongst Kenyan women. Lin et al (2006) also reported that types 16, 52 and 58 were the commonest types in South Taiwanese women. Women who are HIV-infected with severe immunosuppression are five times more likely than HIV non-infected women to have

lower genital tract neoplasia (Ferenczy et al, 2003). Ellerbrock et al (2000) demonstrated a 55% risk of developing cervical intraepithelial neoplasia in HIV-infected women over a two year follow-up period. Cervical dysplastic lesions are more likely to progress and recur after conventional treatment. Spitzer (1999) reported recurrent lesions in 87% of women at 36 months post treatment.

Some studies have supported the concept that there is an increased prevalence of invasive cervical cancer amongst HIV-infected women (Royansky et al, 1996; Franceschi et al, 2003 & Goedert, 2000), whereas other studies failed to establish this relationship (Newton et al, 2001 & Mbulaiteye et al, 2003). International HIV seroprevalence rates amongst women with cervical cancer vary from 1.6% in Hong Kong (Chan et al, 2004), 15% in Kenyan women (Gichangi et al, 2003) to 21% reported in Durban, South Africa (Moodley et al, 2001). In 1990, the prevalence for HIV infection amongst the antenatal population in the province of KwaZulu- Natal, South Africa, was 1.6% (Webb, 1997). In the same year the incidence of HIV infection in women with invasive cervical cancer in this province was 5%. In 1999 the prevalence of HIV in women attending antenatal clinics for this province was 32.5% (Ateka, 2000). Sitas (2000) reported that the relative risk of HIV with cervical cancer to be 1.6 (CI: 1.1 – 2.3) in South Africa.

In 1993 the Centres for Disease Control and Prevention (CDC) labelled invasive cervical cancer as an AIDS-defining illness based on limited data. From this time onwards, this issue has remained controversial. However, in 1998 the CDC Sentinel Hospital Surveillance System for HIV infections reported that the prevalence of invasive cervical

cancer for HIV-infected women to be 10.4 cases per 100 000 women compared with 6.2 cases per 100 000 HIV non-infected women (RR=1.7; 95% CI 1.1 – 2.5) (Chin et al, 1998). Similar elevated increases were reported by other studies (Fordyce et al, 2000; Franceschi et al, 1998). Franceschi et al (1998) in an Italian-based study reported a relative risk of 15.5 (95% CI 4.0 – 40.1) for cervical cancer amongst women with HIV/AIDS. In contrast Phelps et al (2001) reported a rate of invasive cervical cancers to be 5 per 1000 person-years amongst HIV-infected women compared with 0 per 1000 person-years amongst HIV non-infected women. This large multicentre study was conducted in the course of the HIV Epidemiology Research Study (HERS) and followed 871 HIV-infected women between 1993 and 2000 and reported five cervical cancers compared with no cancers amongst HIV non-infected women. Massad et al (2004) reported only one case of cervical cancer in the Women's Interagency HIV Study (WIHS) of 1661 HIV-infected and 8260 HIV non-infected women-years of follow-up.

Since the HIV epidemic has reached epidemic proportions in the developing world where invasive cervical cancer is also most prevalent, the relationship between HIV/AIDS and cervical cancer should be most apparent in these parts of the world. However, reports from African countries such as Kenya, Rwanda and Cote d'Ivore have not confirmed any positive link (Gichangi et al, 2002; Newton et al, 2001 and La Ruche et al, 1998). Although the prevalence of the HIV infection had increased threefold in Kenya over a period of a decade, there was no increase in the number of cervical cancers compared with that of other gynaecological cancers (Gichangi et al, 2002). Newton et al (2001) reported a link between HIV infection and non-Hodgkin's lymphoma but no increase in

invasive cervical cancer in Uganda. Parkin et al (1999) also did not find any increase in cases of cervical cancer in Uganda. It is postulated that the mortality amongst women from competing HIV/AIDS-related illnesses in the developing world is responsible for the short lifespan of women who do not live to an age to manifest with cervical cancers. Moodley et al (2001) reported that HIV-infected women presented at least 15 years earlier than HIV-negative women. Boccalon et al (1996) postulated that the HIV virus may influence the pathogenesis of HIV associated cervical pathology by molecular interaction between HIV and HPV genes as a result of up-regulation of the *E6/7* oncogenes by the HIV virus. Infection with both HIV and HPV may result in dysregulation of hormonal and cellular components of the immune system, leading to progression of the disease (Clark and Chetty, 2002). Studies have also reported high failure rates of preinvasive cervical lesions with standard therapy in HIV-infected women compared with HIV non-infected women (38-62% versus 15-18%, respectively) (Chirenje et al, 2003).

2.0 THE HUMAN PAPILLOMAVIRUS AND ITS LINK TO INTRAEPITHELIAL AND INVASIVE CERVICAL NEOPLASIA

2.1 HISTORICAL PERSPECTIVE

In 1907 the viral aetiology of common warts was established and first described by Giuseppe Ciuffo. It was only in the 1970s with the advent of molecular technology when HPV was studied in detail (Ciuffo, 1907; Meisles and Fortin, 1976). The transmission of the virus from man to man was determined by innoculation with a cell-free extract of wart tissue. Warts may infect several sites in the human body and can also infect several animal species. Rous and Beard (1935) reported the link between benign papillomas in rabbits and certain cancers. As molecular technology advanced it became possible to study the molecular link between papillomaviruses and cancer. Meisels and Morin (1981) further described the high prevalence of HPV and cervical dysplasia. De Villiers et al (1981) cloned the first genital human papillomaviruses. Zur Hausen (1982) also described the link between papillomaviruses and other lesions. HPV types 6 and 11 were described in association with benign condylomata acuminate. Subsequently, together with other investigators (Durst et al, 1983 and Boshart et al, 1984) HPV types 16 and 18 were cloned by zur Hausen and others from cervical cancers and the transcription of these HPV types in cervical cancers was confirmed. Since then many studies confirmed the link between papillomaviruses and cervical dysplasia or cancers. In 1988 zur Hausen reported that HPV types 16 and 18 were present in about 70% to 80% of high grade squamous intraepithelial and invasive cervical neoplasias. With the advent of

recombinant DNA technology and molecular cloning, many HPV types were discovered. To date more than 150 HPV types have been reported (zur Hausen, 2000).

2.2 EPIDEMIOLOGICAL EVIDENCE LINKING HPV AND CERVICAL NEOPLASIA

The highest rates of HPV infection is after the onset of sexual activity usually in the 20-30 year age interval. In most women HPV infection is self-limiting and as much as 90% of women will clear HPV infections over a period of time. The median duration of high-risk HPV infections is about one year and few months for low-risk infections (Schiffman and Kjaer, 2003). In populations at high risk of HPV infection, a second peak of HPV infection has been reported among postmenopausal women (Herrero et al, 2000). Of the women who develop persistent infections only 2-3% will develop cervical dysplasia (Clarke and Chetty, 2002). Results from epidemiological studies showed a consistent link between cervical cancers, pre-cancers and papillomaviruses. These studies included case series, case-controlled and cohort studies. Of the more than 100 HPV types described, about 40 have been shown to infect the genital tract (Woodman et al, 2007). Since some of the early studies utilized non-amplified DNA hybridization techniques the results were inconsistent. The point prevalence of HPV infection ranges from 14% to 35% (Ho et al, 1998). Munoz et al (1988) reported a wide range of 15% to 92% of the presence of HPV DNA in cervical tumour specimens. However, with the advent of the highly sensitive

polymerase chain reaction (PCR) technology, there emerged strong molecular evidence for the link between HPV and cervical neoplasias (Schiffman et al 1993; Munoz et al, 1992). The largest study of the prevalence of HPV in cervical cancers was the International Biological Study on Cervical Cancer which studied over 1000 women from many countries and utilized PCR technology (Bosch et al, 1995). This study reported a prevalence of 93%. A re-analysis of the specimens was performed and with the use of different primers the prevalence of HPV in cervical cancers increased to 99.7%. This high prevalence is consistent across the world even in areas of varying prevalence of cervical cancer and was demonstrated in both retrospective and prospective studies (Bosch, 2002).

Based on information pooled from 11 case-controlled studies (Munoz et al, 2003), it has been established that there are 15 high-risk HPV types which are oncogenic to the epithelium of the anogenital tract (16, 18, 26,31, 33, 35, 39, 45, 51, 52, 53,56, 58, 59, 66, 68, 73 and 82). Twelve types are regarded as low-risk HPV types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6108). The oncogenicity of all other HPV types is unknown. HPV types 16 and 18 have been identified in about 70% of cervical carcinomas (Clifford, 2003). Across the world HPV types 16 and 18 predominate in cervical carcinomas (Clifford, 2005).

The sequence of events leading to the development of invasive cervical carcinomas was subsequently established. It became evident that HPV infection was a necessary factor followed by the development of intraepithelial lesions and then cervical carcinomas. Herrero et al (2000) demonstrated that HPV infection was acquired after sexual activity

in young women. The prevalence of HPV infection then declined after the age of 30 years. Smith et al (2008) reported the age-specific prevalence of HPV in a meta-analysis of 346 160 women. It was noticed that HPV prevalence decreased with increasing age from peak prevalence in women younger than 25 years of age. In women between 25 and 35 years, the HPV prevalence differed in different geographical regions of the world from 15% in Northern Europe to 20% in Africa. In older women, there were inconsistent trends with a decrease or plateau noticed in most studies. Low-grade cervical squamous intraepithelial lesions (LGSIL) were commonest around the age of 29 and were associated with many HPV types. Its prevalence then declined after the age of 30 years. Low-risk HPV infection is associated with transient HPV types, whereas high-risk HPV types are associated with more persistent infections (Franco et al, 1995). High-risk HPVs are also associated with a greater risk of progression from atypical cells of undetermined significance (ASCUS) to high grade lesions and greater duration of infection compared to low-risk HPVs (Schlecht et al, 2003). High-grade cervical squamous intraepithelial lesions (HGSIL) were noted commonly between the age of 30 and 40 years. High-risk HPVs notably types 16 and 18 were dominant in HGSIL lesions and cervical cancers. The odds ratios described were 320 for HGSIL and 710 for cervical carcinomas. Sun et al (1997) reported that HIV-infected women have a higher incidence of high-risk HPV types in low-grade squamous intraepithelial lesions (LGSIL). Although about 60% of low-grade intraepithelial lesions regress in HIV non-infected women, this regression rate decreases to about 27% in HIV-infected women (Petry et al, 1994; Maiman et al, 1993). Langerhans cells are the antigen-presenting cells of the cervix. Spinillo et al (1993)

reported a significant reduction in Langerhans cells in CIN lesions among HIV-infected women compared to a matched control group.

2.3 CLASSIFICATION AND STRUCTURE OF PAPILLOMAVIRUSES

Previously the Papillomaviruses belonged to the Papillomavirus genus and with the Polyomavirus genus, constituted the Papovaridae family. They are now grouped independently as the papillomavidae family and are unrelated to polyomaviruses and SV40. The size and genomic organization of the two genera are different. Animal papillomaviruses are species-specific and have a predilection for epithelia at specific sites. These viruses share structural and functional similarities, including the ability for proliferation and transformation of the host epithelium. They are associated with dysplastic and neoplastic processes. The HPVs exist in a number of types called genotypes since their classification is based on the nucleotide of which about 40 infect the anogenital epithelium.

The Papillomaviruses are classified according to the DNA sequence homology in certain genes, especially the *L1* gene, which codes for the viral capsid. This classification has been ratified by the International Committee on the Taxonomy of Viruses (de Villiers et al, 2004). For a new HPV type to be confirmed, the total DNA from the virus must be cloned and the DNA sequence must be obtained from the *E6*, *E7* and *L1* genes. The *L1*, *E6* and *E7* genes of any type should have less than 90% identity with any other known type. A subtype is established if new isolates have a homology between 90 – 98% with

any known type. A variant is established if a new isolate has more than 98% homology. Papillomaviruses which share a 60% homology in the L1 region are grouped together in the α and π genera. The α genus includes all high-risk HPVs, low-risk HPVs, whereas the β papillomaviruses include viruses associated with Epidermodysplasia verruciformis. The genera are further sub-divided into species e.g., α9 includes 16, 31, 33, 35, 52 and 58. Depending on their tropism, they are divided into mucosal and cutaneous types. The phylogenetic tree representing the family of papillomaviruses is represented in Figure 1.

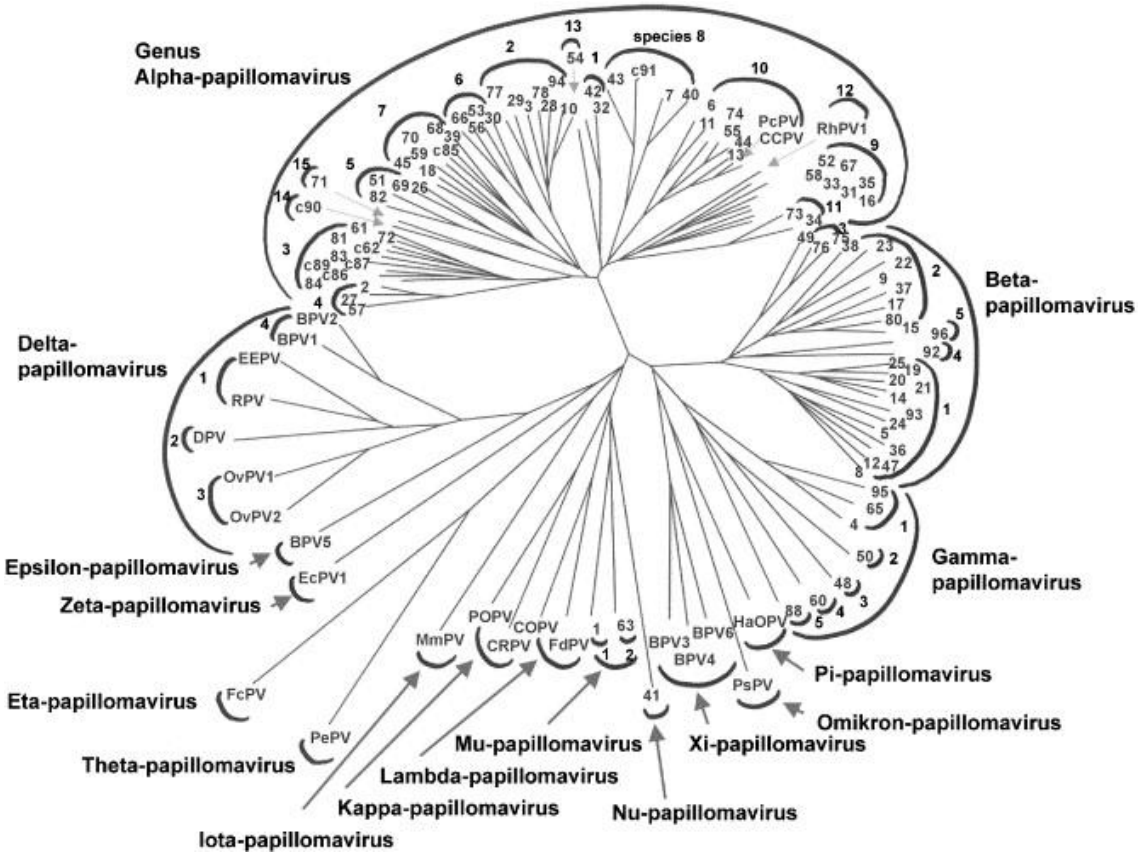


Figure 1: Phylogenetic tree of the HPV family of viruses

2.4 STRUCTURE OF THE HUMAN PAPILLOMAVIRUS

Human Papillomaviruses are small, double-stranded DNA viruses of about 55nm with an icosahedral protein capsid containing 72 capsomers. It has a circular genomic structure containing 7500 to 8000 base pairs.

The capsid has two structural proteins:

- The L1 protein which makes up 80% of the total viral protein with a molecular mass of 53 000 – 59000 daltons. This protein is the major capsid protein encoded by the *L1* gene.
- The L2 protein is a minor protein with a molecular weight of 70 000 daltons and is encoded by the *L2* gene.

2.5 THE HPV NUCLEIC ACID/GENOME

Within the capsid is the circular double-stranded supercoiled DNA genome of approximately 8 kilobases (Kb) in length with a molecular weight of 5×10^6 daltons. Only one strand of this genetic material serves as a template for DNA transcription or open reading frames (ORFs) (Cole et al, 1987). The genome is divided into early (E), late (L) and non-coding regions. The open reading frames are the coding regions and are classified as “early” or “late” depending on when gene function occurs in a specific time period in the life-cycle of the HPV infection. Early genes are expressed at the onset of the

infection and mediate specific gene functions which control viral DNA transcription, replication and cellular transformation. The E1 and E2 genes play a role in viral replication and maintenance of the genome. The linear representation of the HPV genome is represented in Figure 2.

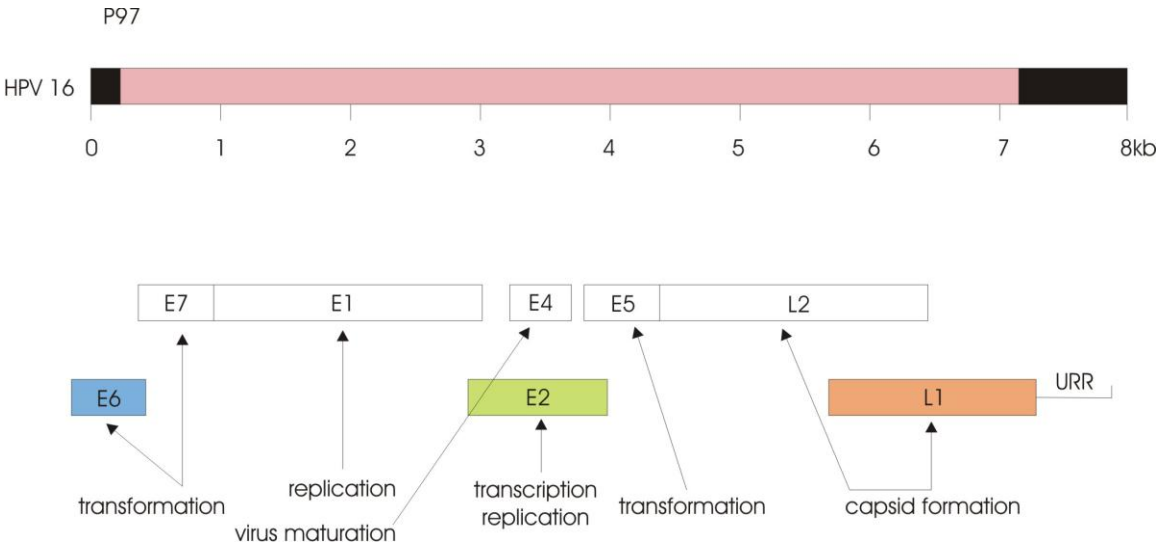


Figure 2: Linear arrangement of HPV 16 genome

The open reading frames (ORFs), E and L are preceded by a non-coding region, also referred to as the upstream regulatory region or long control region. Many early ORFs have been identified. The genome of the open reading frame (ORFs) is divided into:

2.5.1 ORFS WITH ONCOGENIC PROPERTIES

THE HPV E6 ONCOPROTEIN

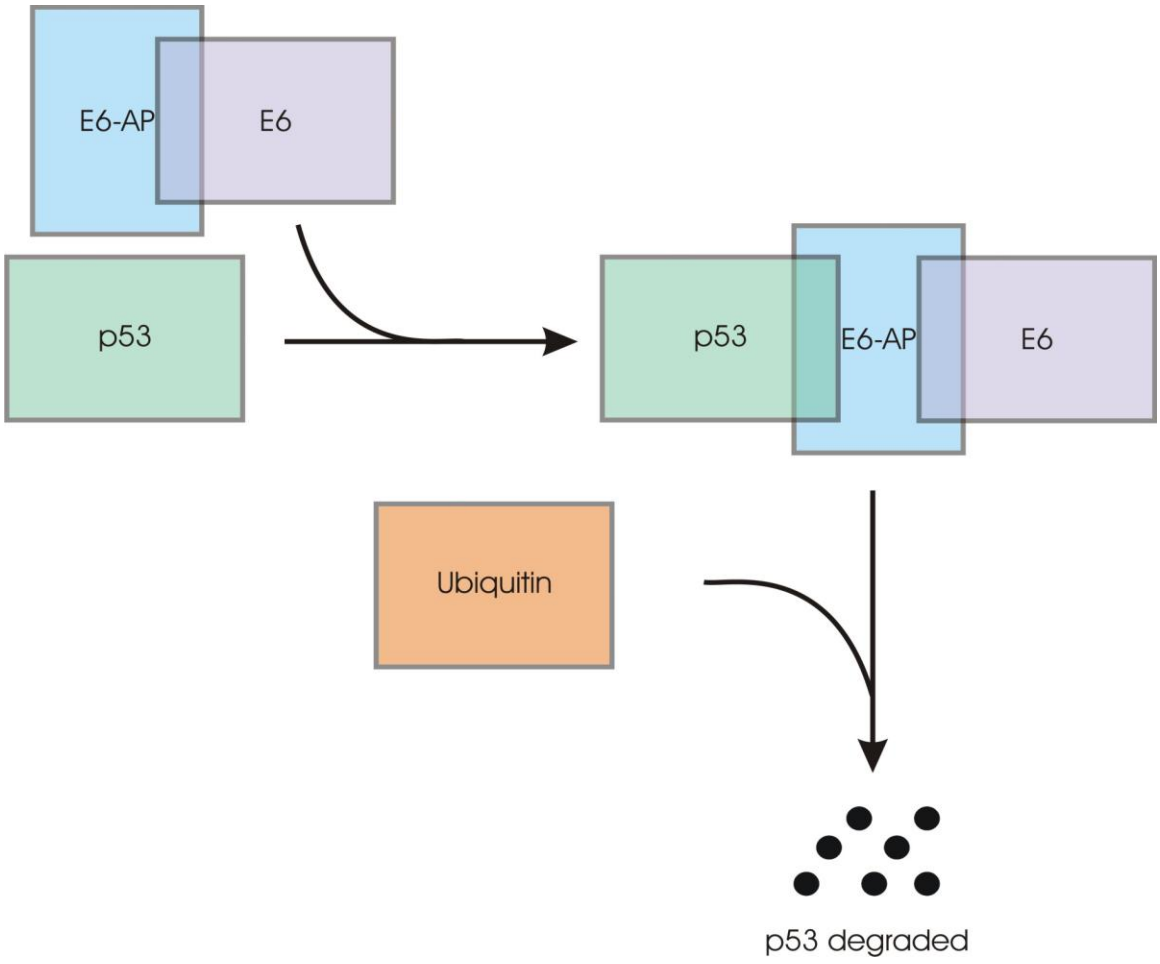
The E6 protein has a molecular weight of 16kD and is composed of 150 amino acids All papillomaviruses encode an E6 ORF downstream of the non-coding region. The E6 ORF encodes for a small protein of about 150 amino acids producing a product of 16-18kDa. Due to alternative splicing of the E6 transcripts, E6*I and E6*II proteins are produced. The full-length E6 protein has a zinc binding motif (cys-x-x-cys) which when bound to zinc ions is capable of binding to DNA. Co-operation with the E7 protein is necessary for its full oncogenic role. One of the first genes expressed during HPV infection is the E6 gene (Fehrmann & Laimins, 2003).

The high-risk HPVs are integrated into the host cell genome in cervical cancers. These genes therefore play a role in the initiation and progression of tumours. The *E6* gene of the high risk HPV virus deregulates the cell cycle via its interaction with the p53 tumour-

suppressor gene. The E6 protein product binds to the p53 gene product and forms a complex which then results in proteolysis of the p53 gene product (Scheffner et al, 1990).

The degradation occurs via an ubiquitin pathway that results in the reduction of the half-life of the p53 from 3 hours to 20 minutes. The shortening of the half-life of the p53 reduces its function. The first protein which was shown to interact with E6 is E6-associated protein (E6AP), which is an E3 ubiquitin ligase. The E6 degradation of p53 requires the cellular protein referred to as E6AP, which in combination with E6 serves as an E3 ubiquitin ligase (Scheffner et al, 1993; Huibregste et al, 1993). A separate process independent of the E6AP activity is the down regulation of p53 transcriptional control by high-risk E6 proteins. This occurs via a p53 co-activator CBP/p300 by E6. CBP/p300 regulates various signal-modulating events and plays a role in cell cycle inhibition and differentiation (Giles et al, 1998). The E6-E6AP has also been reported to promote degradation of the src family kinase Blk (Oda et al, 1999). These tyrosine kinases are important in signal transduction in proliferating cells and it is thought that E6 de-regulates these pathways affecting cell growth. Shai et al (**2008)** recently documented that K14Crep53 mice treated with oestrogen developed cervical cancer due to inactivation of the *p53* gene in he presence also of the HPV *E6* and *E7* oncogenes. The authors concluded that in hormone-responsive tissues, *p53* inactivation in association with HPV oncogenes is necessary for carcinogenesis, including mammary tumours.

Figure 3: HPV 16 *E6* oncogene and *p53* gene degradation



Many other protein-protein interactions of E6 are described e.g., ERC 55 and paxillin may affect vital areas of cellular function, the disruption of which may promote malignant transformation (Chen et al, 1995; Tong and Howley, 1997). Rapid tumour

formation has been shown to occur in p53 null mice, providing evidence for its tumour suppressor role. Key host regulatory factors such as cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDIs) (Southern et al, 1998; Thomas et al, 1998). The cell cycle arrest at the G1/S point occurs as a result of cyclin/kinase inhibition by p21 CDI. Loss of functional p53 due to E6 binding results in failure of G1/S arrest (Southern et al, 2000). The E6 gene product is also capable of activating the enzyme telomerase which is responsible for counteracting the shortening of the chromosome's telomeres. This natural shortening occurs in the process of aging and the E6 interaction prolongs the lifespan of the affected cell (Mantovani & Banks, 2001). The E6 protein also modulates immune function by regulating the transcription of genes involved in innate immunity. High-risk E6 interacts with two proteins involved in the innate response to viral infections: interferon regulatory factor-3 (IFR-3) and Toll-like receptor 9 (TLR9) (Ronco et al, 1998; Hasan et al, 2007). Viral infection or dsRNA activates IRF-3 leading to the transcription of interferon-β (Hiscott, 2007). Viral or bacterial dsDNA activates TLR9 which induces cytokine production involved in cellular defence (Muller et al, 2008). Loss of function of IRF-3 and TLR9 has been demonstrated in cell lines indicating that such HPV 16 E6 expression is involved in HPV-related cervical carcinogenesis. Both E6 and E7 can independently immortalize human cells.

THE HPV E7 ONCOPROTEIN

The E7 ORF encodes for a small protein of about 100 amino acids which weigh about 10kDa and is well conserved among different HPVs. The E7 oncogene is a key oncogene which exerts its activity by binding to the cellular proteins of the retinoblastoma (*pRB*) gene family. The *pRB* gene is regarded as a tumour-suppressor gene. The pRB protein is a phosphoprotein which in the phosphorylated state is required for the limitation of cell proliferation and suppresses the oncogenic properties of different HPV types (Brookstein & Lee, 1991). In conjunction with the E2F group of transcription factors, the *pRB* exerts control over cell replication (Boyer et al, 1996). The hypophosphorylation of the *pRB* results in uncontrolled release of transcriptionally active E2F, cell cycle progression into the S-phase of the cell cycle and loss of cell cycle-dependent regulation of E2F responsive genes (Chellapan et al, 1992; Weinberg 1995). Although the E7 protein is capable of transformation and immortalization in vitro, it requires E6 for HPV transformation and immortalization (Barbosa and Schlegel, 1989). The E7 protein interacts with various proteins involved in the regulation of cell-growth, especially the transition from G1 to S-phase of mitosis. The E7 protein de-regulates the cell cycle leading to increased cell proliferation, immortalization and transformation. This is achieved by the interaction of E7 with the proteins of the retinoblastoma tumour suppressor family (Rb, p130, histone deacetylases (HDAC), AP-1 transcription factors, TATA box proteins, cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors). The role ole of HPV 16 *E7* oncogene and *pRb* tumour-suppressor gene in cell-cycle regulation (infected cell and uninfected cell) is illustrated in Figures 4a and 4b.

Figure 4a: Role of HPV 16 *E7* oncogene and *pRb* tumour-suppressor gene in cell-cycle regulation (Uninfected cell)

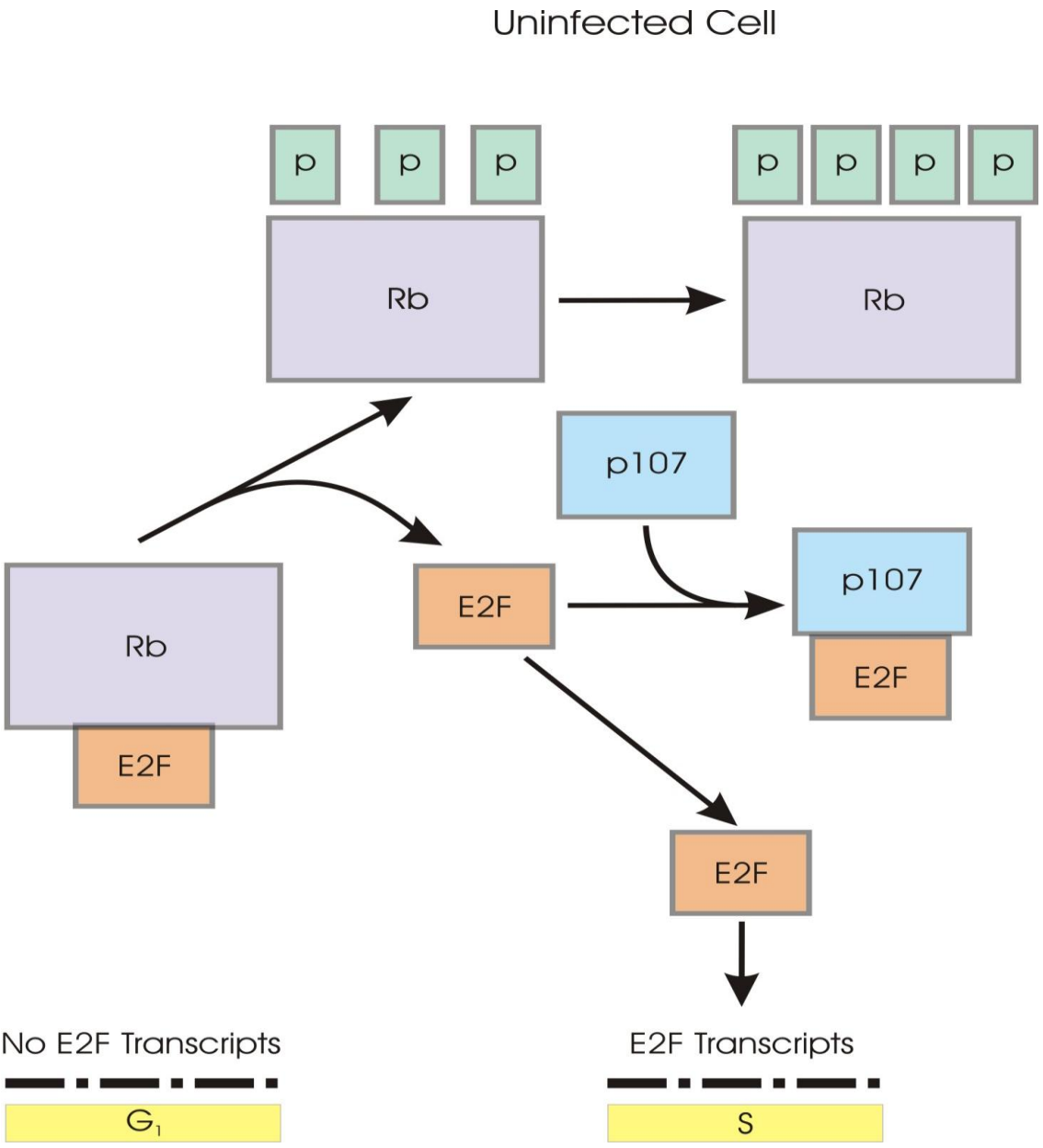
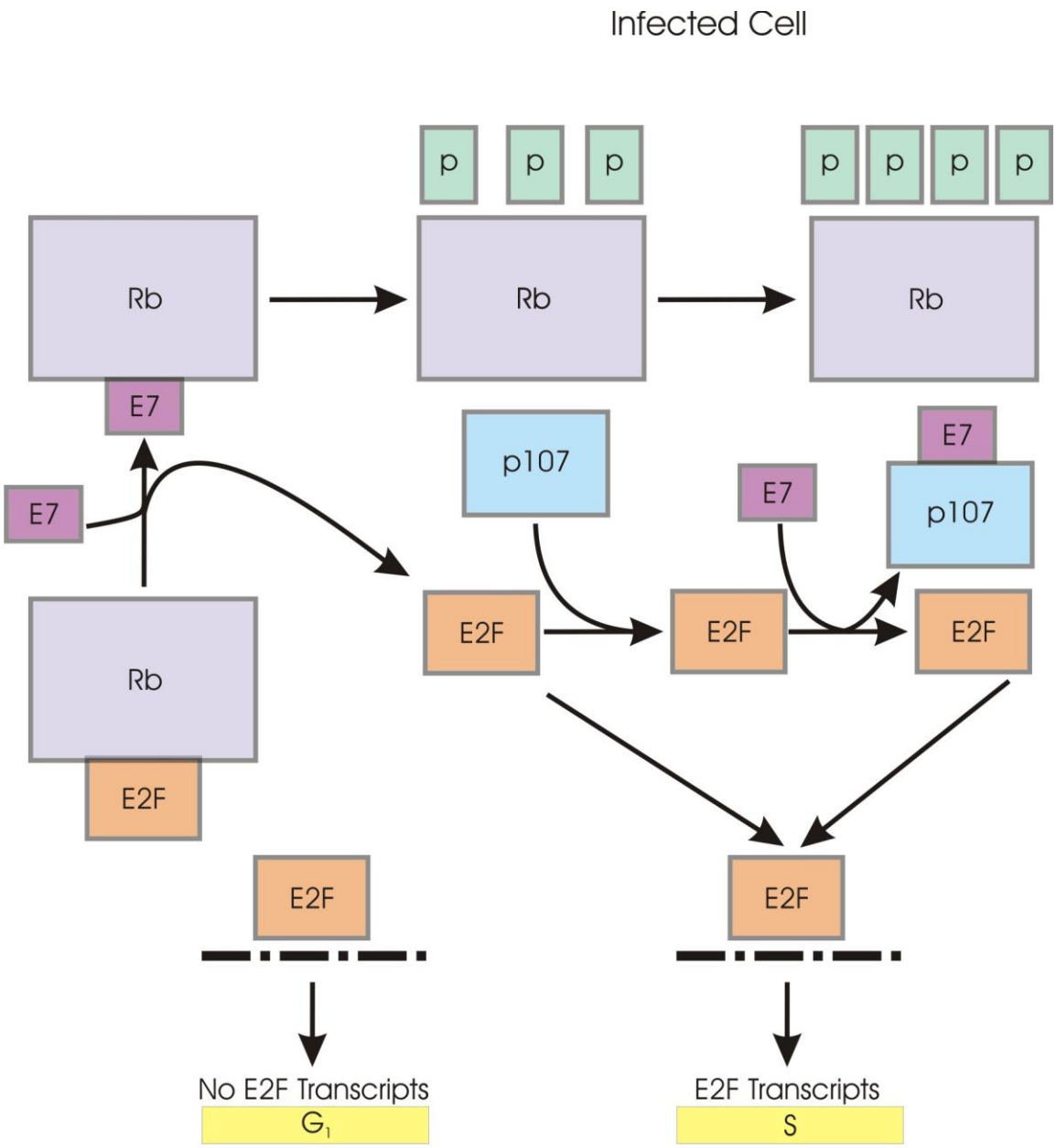


Figure 4b: Role of HPV 16 *E7* oncogene and *pRb* tumour-suppressor gene in cell-cycle regulation (Infected cell)



When the cell progresses from G0 to S-phase, Rb becomes hyperphosphorylated by G1 cyclin-CDKs, releasing E2F, which in turn activates genes involved in DNA synthesis and cell-cycle progression (Dyson, 1998). Binding of E7 to hypophosphorylated Rb

induces cells to enter premature S phase by disrupting Rb-E2F complexes (Patrick et al, 1994). The E7 gene product can also overcome G1/S cell cycle arrest induced by either p21 or p27 by binding to both of these proteins or with cyclins A and E (Demers et al, 1996; Hickman et al, 1994; Morozov et al, 1997). In contrast to the E6 gene product of low-risk HPVs which do not bind *p53*, the E7 gene product of the low-risk HPVs bind to p53 at a reduced affinity (Elbel et al, 1997). Human papillomaviruses play a role in the initiation of oncogenic events. Transcripts encoding for the E6 and E7 are initiated in the Upstream Regulatory Region (URR) of the viral genome.

Transcriptional activity of both E6 and E7 is under control of p97 which is suppressed mainly by the viral E2 product (Romanczuk et al, 1990). Transcription of the HPV 16 E6/E7 ORFs produces three different splice products due to alternate splicing using a common donor site at nucleotide 226 and two different splice acceptor sites at nucleotides 409 and 526. The full length transcript encodes for the functional E6 protein, whilst the E7 protein is most likely encoded by the E6*1 and E6*11 splice products (Smotkin & Wettstein, 1986; Smotkin et al,1989; Cornelissen et al, 1990). The major splice product is the E6*1 (Smotkin et al, 1989). Using specific primer pairs designated S3 and S4 in the reverse transcriptase-polymerase chain reaction (RT-PCR) to detect these splice products yields the following fragments: full-length product consisting of 525 base pairs (E6), E6*1 consisting of 343 base pairs and E6*11 consisting of 226 base pairs. Further testing using the S1/S2 primers in a nested system (nRT-PCR) with the products of the S3/S4 primers yield the following products: an E6 full-length fragment with 395 base pairs; E6 *1 fragment consisting of 213 base pairs and E6*1 consisting of

95 base pairs (Sotlar et al, 1998). It is thought that the detection of the E6*1 and E6*11 splice products are unequivocal proof of HPV 16 E6/E7 oncogene transcription (Cornelissen et al, 1990). The presence of the E6*1 spliced product is thought to correlate with lesion severity from cervical scrapes (Sotlar et al, 1998).

The amplification products of the HPV 16 genome in relation to the spliced products produced with the S3/4 and S1/2 primers and their respective positions is illustrated in Figure 5.

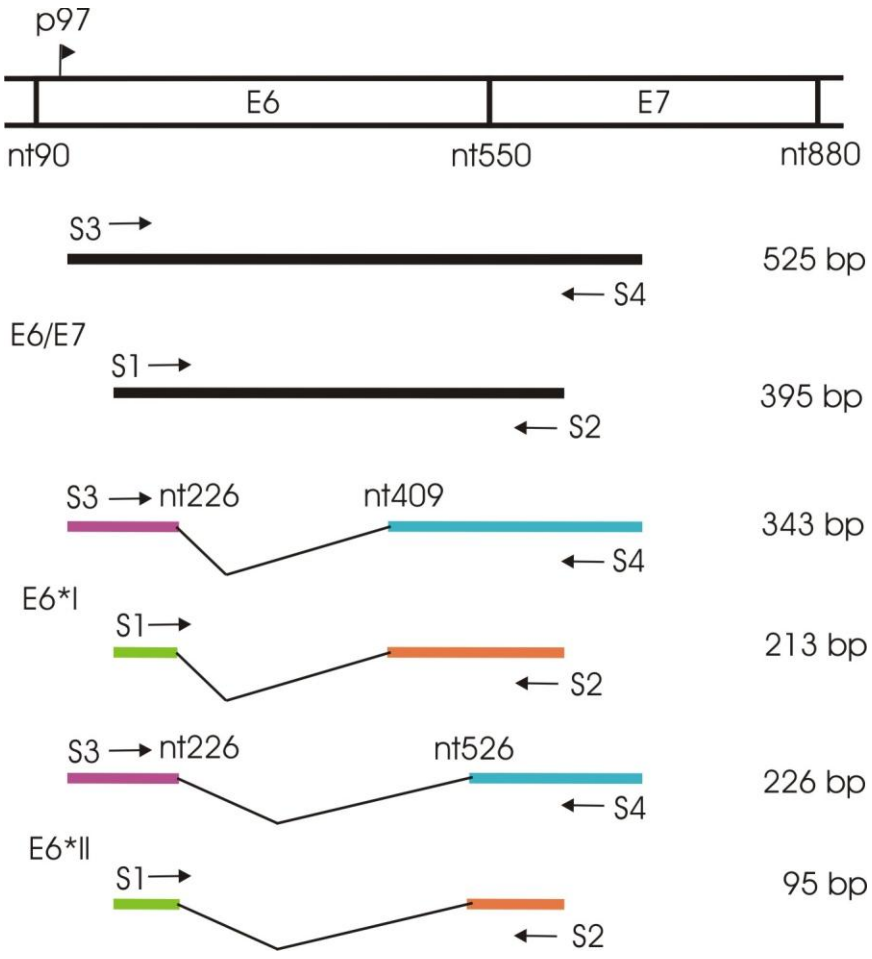


Figure 5: HPV 16 amplified products. Spliced products produced with the S3/4 and S1/2 primers and their positions

In vitro experiments have demonstrated that HPV16 E7 targets insulin-like growth factor binding protein-3 (IGFBP-3) which regulates the mitogenic activity of IGF-1. The IGFBP-3 is a product of the p53-inducible gene which blocks cell proliferation and induces apoptosis (Zwerschke et al, 2000). The induction of apoptosis by the IGFBP-3 is blocked by HPV E7 which facilitates proteolytic cleavage of IGFBP-3.

The E6 and E7 genes play a vital role in cervical cancer genesis as is evidenced by the constant observation that in HGSIL and cervical cancers, the HPV genome is integrated into host chromosomes (Daniel et al, 1995). The E6 and E7 genes are always retained and the proteins expressed (Cone et al, 1992). Both the E6 and E7 transcripts are expressed in low levels in basal cells with an increase in expression in the more differentiated upper cell layers of the epithelium. In high grade lesions and invasive cancers the E6 and E7 transcripts are expressed in all layers of the epithelium (Stoler et al, 1992). The pathological pathway of HPV infections depend on the physical state of HPV DNA. High-risk HPV are more likely to integrate within the human genome, whereas low-risk HPVs are usually maintained as extrachromosomal circular episomes (Arends et al, 1998).

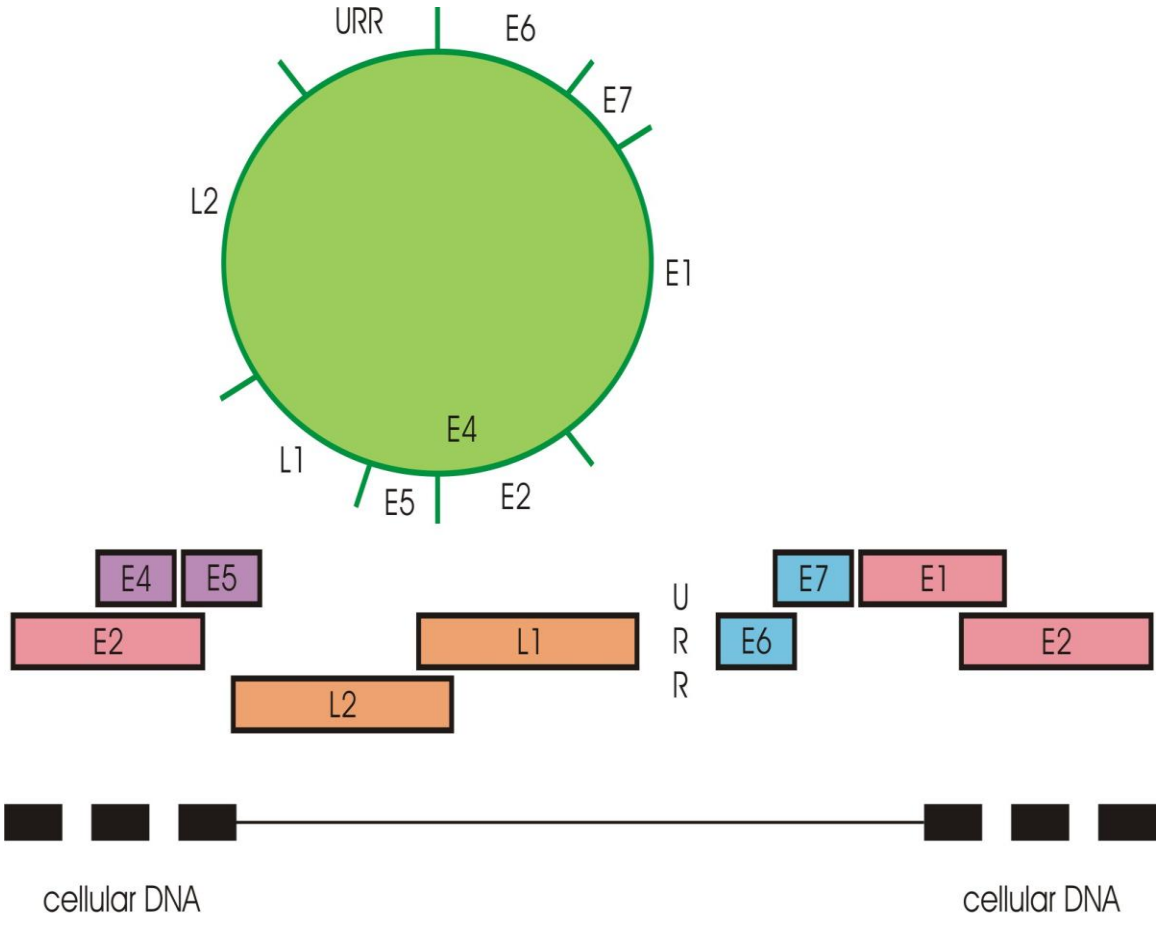
ROLE OF VIRAL INTEGRATION IN TUMOUR GENESIS

There are two forms of the HPV with regards to its physical state: the episomal form and the integrated form. The virus initiates infection as an episome with low DNA copy number in the basal cells. High copy number is associated with replication. With differentiation and viral replication, high viral copy numbers are found in the superficial

epidermis (Bedell et al, 1991). In precursor lesions of the cervix, the HPV exists as an episomal form. However, in invasive cancers HPV integration into the host genome has been reported (Matsukura et al, 1989) (Figure 6).

Viral integration may take place at various sites such as chromosomal break points, cellular oncogenes or fragile sites. The HPV viruses seem capable of recognizing specific DNA sequences in the genome and integrate in the human DNA, making surrounding areas of the genome unstable and allow genes in these regions access to mutagenic agents. Although integration may be random, disruption of the cellular HPV is consistently within the E1 or E2 ORF, resulting in loss of one or both genes (Choo et al, 1987). During this integration process, the URR and E6 and E7 transforming genes remain conserved/intact with increased transcription of these genes reflective of integration and loss of E2. The replication cycle of HPV is depicted in Figure 6.

Figure 6: A linear representation of the genome of HPV 16 E1 and E2 ORF following HPV integration



However, some HPV-associated cancers contain only episomal forms, whereas other cancers contain a mixture of episomal and integrated forms (Kristiansen et al, 1994; Matsukura et al, 1989). Transcription of the episomal form can regulate HPV function of the integrated virus disrupted in the E1 and E2 genes. From in vitro studies it has been demonstrated that 62% of tumours have integrated HPV, 16% have episomal forms and 22% have both episomal and integrated forms. Almost all tumours with episomal or mixed forms with HPV 16 DNA had intact E1 and E2 genes. In contrast, 50% of tumours

with integrated HPV 16 DNA had disrupted E2 significantly associated with decreased disease-free survival rate (17% versus 70.4%). Szostek et al (2008) recently reported on the physical state of HPV 16 among 42 women with SIL and 23 women with invasive cervical cancer. In the cancer group, the free episomal viral genome was not detected. Twenty six percent of samples from this group contained a mixture of the episomal and integrated forms of viral DNA. In women with SIL, the free episomal form was predominant.

2.5.2 HPV REGULATORY PROTEINS

THE E1 AND E2 PROTEINS

Viral HPV DNA is integrated into the host cell genome (Cullen et al, 1991). The E1 nucleotide gene sequence is a highly conserved region amongst various HPV types. The E1 ORF codes for many proteins of molecular weights ranging from 68 to 85kDa. The E1 and E2 genes play a role in viral replication and maintenance of the genome. The E1 gene has helicase and adenosine triphosphatase (ATPase) activity which catalyses the unwinding of the DNA structure and also brings the DNA polymerase to the origin of the replication where E1 is complexed with the E2 protein to initiate replication (Ustav et al, 1998). The E1 binding site is located between two A+T-rich sequences, which demonstrate variability in length and little sequence conservation between HPV types. These sequences are in between three binding sites for the E2 protein whose purpose is to stabilize the binding of E1 and promote viral DNA replication (Wilson et al, 2002).

The E2 ORF codes for proteins which are necessary for viral DNA replication and regulation of transcription. It therefore acts as a transcriptional regulator by binding to a specific DNA sequence (ACCGNNNNNNCGGT) in the viral URR which constitutes the E2 binding site (Dostatni et al, 1988; McBride et al, 1989). The disruption of the E1 and E2 genes results in de-regulated expression of the viral E6 and E7 (Munger & Phelps, 1993). This is achieved by preventing the formation of the complex of transcription at the p97 promoter. Increase in E6 and E7 expression produces HPV transformed cells which are less likely to undergo programmed cell death/apoptosis (Sanchez-Perez et al, 1997). The E2 proteins modulate the viral enhancer and the E6 promoter. A separate E2 protein comprising a fusion of the E8 ORF with part of the E2 protein has been reported by Zobel et al, 2003. This complex is able to repress transcription and DNA replication and therefore plays a role in maintenance of the latent state observed in the basal epithelial cells.

THE E4 ORF

The exact role of the E4 is unknown. The E4 ORF resides in the early region of the genome, but is expressed in the late stage of the cell cycle. The E4 ORF encodes a protein localized in the cytoplasm in cutaneous warts (Breitburd et al, 1987). It is postulated that the E4 protein may play a role in virion assembly (Doorbar et al, 1998). The E4 ORF in conjunction with kearatin may facilitate collapse of the cytokeratin

network and viral release or may play a role in G2 arrest and HPV DNA replication. The E4 protein is regarded as a 'late' protein localized to the upper layers of the epithelium.

THE E 5 ORF

The E5 gene product is a small protein of about 10kDa localized mainly within the intracellular compartments such as Golgi apparatus. Its main function is thought to be mitogenic stimulation of cells by interacting with signal transduction pathways. The E5 proteins of HPV types 6 and 16 increase the proliferative capacity of human keratinocytes via interaction with epidermal growth factor receptor (EGFR) resulting in stimulation of DNA synthesis. In a recent study of the role of the E5 gene using immortalized human keratinocytes (HaCaT cells), it was demonstrated that E5 was necessary for the formation of bi-nucleated cells which is a common precursor of precancerous lesions (Hu et al, 2008). Co-expression of HPV 16 E6/E7 enhanced the proliferative capacity of the E5-induced bi-nucleated cells.

2.5.3 CAPSID ANTIGEN GENES

The mature HPV particle has an icosahedral outer capsid coat composed of two proteins: the L1 protein and the L2 protein.

The L1 protein is regarded as a major protein and comprises 80% of the total viral protein. It has a molecular mass of 53 000-59 000 kDa. The L2 protein is a minor component with a molecular mass of 70 000 kDa. *In vitro* studies have shown that there

is cross reactivity between the L1 regions of HPV 6, 16 and BPV. In contrast there is less reactivity in the L2 region. Part of the L2 region, namely 210 amino acids from the N-terminus and 30 amino acids from the C-terminus, are conserved. The L1 and L2 proteins have a nuclear target signal and move from the cytoplasm to the nucleus where capsomeres are synthesized and virions are assembled (Zhou et al, 1990). The L1 protein serves as a major target for vaccine research and has resulted in the successful development of two commercially available vaccines (Gardasil ® & Cervarix®). These two vaccines are based on the development of virus-like particles (VLP) against the HPV 16 and 18 L1 proteins.

THE UPSTREAM REGULATORY REGION (URR)

The URR is also known as the long control region or the non-coding region of HPVs. It is present between the early and late regions. This region contains regulatory sequences and the origin of DNA replication, two transcriptional start sites and promoter elements for DNA polymerase II. This element has variation in length amongst the papillomaviruses. The URR also has enhancer elements which mediate transactivation of transcription and also contains glucocorticoid receptor complex elements. The promoter site viz. p97 transcription initiator of HPV 16 is located in the URR. In experiments designed to analyse the activity of isolated regulatory sequences, it has been shown that glucocorticoid hormones are significant activators of the promoter (von Knebel Doeberitz et al, 1991). Binding of progesterone and glucocorticoids to this region increases E6 and E7 transcription (Crook et al, 1991). The URR also contains keratinocyte-enhancer

elements by which HPV 16 are thought to be tropic for squamous epithelia (Cripe et al, 1987). The HPV type 16 enhancer localized to a 232 bp fragment is epithelial-specific. This fragment contains binding sites for AP-1 (transcription factor-binding site), nuclear factor 1 (NF-1) and transcriptional enhancer factors (TEF-1 and TEF-2) (Chong et al, 1991). The AP-1 factor confers cell-type specific transcription of HPV genes.

2.6 REPLICATION CYCLE OF THE HUMAN PAPILLOMAVIRUS

The infectious cycle of the human papillomavirus parallels that of the target cell which in the case of the cervix is the squame. Papillomaviruses replicate and assemble exclusively within the nucleus. Viral growth is dependent upon a cycle of keratinocyte differentiation. The HPV enters the epithelium probably via microabrasions in the epithelium and infects the basal cells. HPV genomes are established as extrachromosomal elements within the nucleus and replicate in synchrony with cellular DNA. After cell division, the daughter cells migrate towards the supra-basal compartment, within which the uninfected keratinocytes initiate terminal differentiation. The HPV-infected cells enter the S-phase of the cell cycle with resultant amplification of viral replication products (Munoz et al, 2006). In the lower layers of the epithelium, cell proliferation and episomal maintenance occurs. Early stages of viral growth include amplification of viral copy number from 1-10 to 50-100 virus episomes/cell (Middleton et al, 2003). Thereafter the virus and cell replicate without the amplification of viral number. The HPV encode for only one DNA replication enzyme, E1 and is otherwise fully dependent on host

cellular machinery. However, this replication can only occur in mitotically active cells. To overcome this problem, the HPV reactivates cellular DNA synthesis in non-dividing cells, inhibit apoptosis and inhibit the program of cellular differentiation, allowing for viral replication. There is strict control of the HPV E6 and E7 oncogenes with very little E6/E7 levels detectable. Viral gene expression is limited to the keratinocyte or cells which have the capability for squamous maturation. High levels of viral protein expression (E6/E7 and late genes) occurs only in the upper layers of the stratum spinosum and granulosum of the squamous epithelium (Middleton et al, 2003). In the upper layers, viral gene expression is up-regulated resulting in the release of thousands of viral episomes. Here, the late viral proteins L1, L2 and E4 are synthesized in conjunction with viral assembly. Disintegration of the epithelial cells as a result of natural turnover facilitates release of infectious virions (Fehrmann & Laimins, 2003). The time from viral infection to release is about three weeks which is about the time taken for differentiation of the basal cell.

2.7 IMMUNOLOGY OF HPV INFECTIONS

Not all women with HPV infection will develop disease. It has been noted that persistent HPV infection is necessary for the development of neoplastic cervical lesions (Chakrabarti and Krishna, 2003). Both the innate and adaptive immune systems are required for the defence against HPV infections. The innate system comprises phagocytes, cytokines and complement. The adaptive system comprises antibodies and

cytotoxic effector cells (T and B cells). T-cells are capable of recognizing antigens processed into short peptides and bound to the major histocompatibility complex (MHC) proteins and are presented as membrane-bound receptor complexes on the surface of cells. The two major subsets of T-cells are the CD8 and CD4 cells. CD8 T-cells recognize antigens presented by the class II MHC and CD4 cells recognize antigens presented by the class I MHC. The activation of the CD4 cells results in the production of cytokines. There is reduction in expression of HLA class I molecules in cervical cancer associated with a decrease in CD8 T cells (Hilders, et al 1994). Genital warts which do not regress do not have immune cells at the site of infection. The first line of defence is the innate system which interfaces with the pathogen and destroys it without the development of any memory. However, the innate system activates the adaptive system which produces effector cells to maintain memory of the pathogen. Whilst antibodies clear the pathogen, the cell mediated responses clear the viral-infected cells. Unlike many other micro-organisms which are destroyed by the human body before disease manifests, the HPV is adept at evading the host immune mechanisms. At the onset of initial infection, mucosotropic virions remain at the site of infection and do not induce lysis of the infected epithelial cells and hence there is no inflammation. The antigenic capsid proteins are not expressed until differentiation has reached the superficial layers of the epithelium. The innate immune system is down-regulated by interactions of the E6 and E7 early HPV genes and the innate immune system, in respect of interferon and nuclear factor-κß signals (Nees et al, 2001; Kanodia et al, 2007). Type 1 interferons, namely IFN-α and IFN-β have anti-viral, anti-proliferative, anti-angiogenic and immunostimulatory functions which serve as a bridge between the innate and adaptive immunity (Le Bon and

Tough, 2002). There is poor recruitment of effector cells to the cervical epithelium. Systemic viraemia does not occur. HPV-infected keratinocytes are also resistant to lysis by natural killer cells although they can be destroyed by cytokine-activated NK cells and macrophages. HPV infection is associated with the failure of antigen presentation to the MHC I and II complexes. Only about fifty percent of women exposed to HPV infection will develop an immune response (Stanley, 2003). Failure to induce a satisfactory immune response is due to deficient activation of the innate immunity and ineffective priming of the adaptive mechanisms resulting in viral persistence. Thus the basic mechanisms to trigger the immune response in cervical epithelium are non-existent (Stanley, 2006).

Human papillomavirus infections are predominantly an intraepithelial phenomenon and therefore the Langerhans cells, which are the antigen-presenting cells of the squamous epithelium, should be able to detect the HPV virus. Antigen presenting cells have special properties to prime tumour-specific T cells in the T cell-dependent areas of the lymph nodes (Paglia and Guzman, 1998). It seems as if the Langerhans cells are not activated by the viral capsids. This is in contrast to the stromal dendritic cells which are activated by viral capsids. Since the HPV virus remains within the epithelium, this activation does not occur. Although the innate immune response is immediate, antibody conversion against high-risk HPVs takes almost 6 months to 1 year. Antibody levels, however, remain stable over many years and it has been demonstrated that innoculation with viral like particles result in a rapid IgM and IgA antibody response followed later by appearance of stable IgG antibodies which can be detected in cervical mucus.

Antibody production prevents the spread of infection and repeat infections (Stanley, 2001). In natural infections, cell-mediated immune responses result in clearance of genital warts and intraepithelial lesions (Coleman et al, 1994). This occurs with sero-conversion and the formation of antibodies to major capsid proteins. However, in humans the seroconversion is either low or does not occur at all. The majority of women clear HPV infection and this clearance is dependent on whether the HPV is of the high-risk or low-risk type. High-risk HPV 16 takes about 8-14 months to clear (Giuliano et al, 2002). In 10% of women, HPV infection is not cleared and persistent infection develops. Of this 10%, about 2-3% of women develop SIL and progression to invasive cervical cancer (Stanley, 2008). The development of such neoplastic cervical lesions is associated with the expression of the HPV E6 and E7 proteins at cellular level and resistance to innate and adaptive immunity. Although interferon- β is capable of clearing episomal HPV, it cannot clear integrated HPV (Pett et al, 2006). Immune defence mechanisms against HPV E2 and E6 are deficient in cervical neoplastic lesions even if cytotoxic T-cells are produced (Kobayashi et al, 2004).

In summary: the HPV virus evades the innate immune system and delays the activation of the adaptive immune system. The dendritic cells are exposed to low levels of viral proteins for a lengthy period of time resulting in lack of local defence mechanisms and the establishment of viral infection. The establishment of persistent HPV infection results in the expression of the E6 and E7 proteins and the lack of a cell-mediated response favouring the progression to SIL and invasive cancers.

3.0 CLINICAL CORRELATES OF HPV TYPES

3.1 CUTANEOUS HPVs IN IMMUNO-COMPETANT POPULATION

Viruses which belong to this group produce infections that are not oncogenic. The viral types correlate with the location of the lesion, clinical features and histological features (Figure 1). HPV types 1 and 4 are associated with plantar warts and HPV type 2 with childhood verrucae and dorsal limb skin (Reid et al, 1988).

3.2 CUTANEOUS HPVs IN IMMUNO-COMPROMISED INDIVIDUALS

There are about 50% of known HPV types which have been isolated from the skin of immuno-compromised persons (Obalek et al, 1986). The main sources are from patients with a rare autosomal recessive disease known as Epidermodysplasia verruciformis. These lesions usually persist for life and can lead to the development of squamous cancers (Pfister, 1987). More than 35 HPV types have been isolated from SIL lesions in immuno-compromised persons, with types HPV types 5 and 8 present in 90% of these malignancies (Reid et al, 1988).

3.3 HPVs AFFECTING THE AERO-DIGESTIVE AND ANOGENITAL MUCOSAE

More than a third of the known HPV types are mucosotropic with involvement of the mucous membranes or anogenital skin. The anogenital HPV types are divided into 4 groups based on clinicopathological and DNA homology:

3.3.1 LOW-RISK HPV TYPES

HPV Types 6 and 11 are responsible for papillomas of the upper respiratory tract and benign exophytic warts of the genitalia of the internal and external genitalia. The Zur Hausen laboratory was the first to demonstrate that most condyloma acuminate contained DNA sequences of HPV 6 and 11. Human papillomavirus types 6 and 11 were first cloned from condylomata acuminate and laryngeal papillomas in 1981 and 1982, respectively (de Villiers et al, 2004). They also account for about 20% of low-grade squamous intraepithelial lesions (flat condylomas). In addition to verrucous carcinoma, there is evidence of their association with genital malignancy. HPV types 42, 43 and 44 occur in a small group of LGSIL, vulval and penile lesions, but not in invasive cancers (Reid et al, 1990). The alpha genus of papillomaviruse comprises five species of low-risk HPVs. They include: species 1 (HPV 32 and 42), species 3 (HPV 61, 62, 72, 81, 84, 86, 87 and 89), species 4 (HPV 2a, 27 and 57), species 8 (HPV 7, 40, 43 and 91) and species 10 (HPV 6, 11, 13, 44, 55 and 74). Human papillomavirus types 6 and 11 are found in

LGSIL. A meta-analysis of 55 studies recently reported HPV 6 to be found in 8.1% of HPV-positive LGSIL and HPV 11 in 3.2% of such cases (Clifford et al, 2005).

3.3.2 HIGH-RISK HUMAN PAPILLOMAVIRUSES

The high-risk HPVs include types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82 (Munoz et al, 2003). It is without any doubt that high-risk HPVs are the causative agent for cervical cancer and the WHO has declared HPV types 16,18, 31, 33, 39, 45, 51, 56, 58, 59 and 66 and class I-cancer causing agents (Bosch et al, 2002; Cogliano et al, 2005). A pooled analysis of 3000 cases from the IARC studies and a meta-analysis of about 10 000 cases have reported the distribution of HPV types in cervical cancer. From both series, the eight most common HPV types, in descending order of frequency were, HPV 16, 18, 45, 31, 33, 52, 58 and 35 (Munoz et al, 2004; Clifford et al, 2003). These types accounted for about 90% of cervical cancers worldwide. Human papillomavirus types 16 and 18 are the most common types in both squamous cell carcinomas and adenocarcinomas of the cervix, accounting for 70% of squamous cell carcinomas and 86% of adenocarcinomas (de Cremoux et al, 2009; Munoz et al, 2004; Castellsague et al, 2006). Human papillomavirus type 16 alone is responsible for about 58.9% of cervical cancers (Munoz et al, 2003). Cervical cancer occurs with higher frequency with specific HPV types (HPV 16, 18, 31, 33, 35 and 45), increasing viral loads and concomitant infection with multiple HPV types (Munoz et al, 2003; Swan et al, 1999).

3.3.2.1 HPV TYPE 16 VIRUS

Human papillomavirus type 16 was cloned from cervical cancers and subsequently from benign condylomas and female lower genital tract neoplasms. In women with normal cytology, the prevalence of HPV type 16 ranges from 2.3% to 3.5% (Bosch et al, 2008). However, Gupta et al (2008) in a study from India, reported HPV 16 prevalence of 10.1% from a sample of 769 cytologically normal women. Worldwide, the prevalence of HPV 16 in HGSIL and invasive cancer ranges from 33.3% to 46% and 52% to 57.9%, respectively (Bosch et al, 2008). An odds ratio of 573.5 was reported for risk of detecting HPV 16 in cervical cancers in a Mexican study (Illades-Aguiar et al, 2009). Human papillomavirus type 16 has been detected in other lesions of men and women: 71% of vulval intraepithelial neoplastic lesions, 67-88% of vaginal intraepithelial neoplastic lesions, 60-88% of penile intraepithelial neoplastic lesions, 76% of anal intraepithelial neoplastic lesions and 72-87% of oropharynx and tonsilar lesions (Giuliano et al, 2008; Lowy et al, 2008). The distribution of HPV 16 in cytologically normal women varies geographically from 8% in Sub-Saharan women to 21% in European women (Clifford et al, 2005). The HPV 16 genome has two major promoters, the p97 promoter and the p670 promoter (Smotkin and Wettstein, 1986; Grassmann et al, 1996). The p97 promoter lies upstream and is responsible for early gene expression. The p670 promoter lies within the E7 ORF and is responsible for late gene expression. Minor promoters of the HPV 16 genome such as the p97 promoter have been described, although its function remains unknown (Rosenstierne et al, 2003).

3.3.2.2 HPV TYPE 18 VIRUS

High-risk HPV 18 virus has been isolated from invasive cervical cancers and is common in adenocarcinomas (Wilczynski et al, 1988). In a French study, HPV 18 was more frequently associated with adenocarcinoma (40.6%) than HPV 16 (10.4%) (de Cremoux et al, 2008). In cytologically normal women, HPV 18 has been detected in 0.7% to 1.8% of cases (Bosch et al, 2008). In women with HGSIL and invasive cervical cancers, HPV 18 has been detected in 5.4% to 10.4% and 14.9% to 21.6% of cases (Bosch et al, 2008). An odds ratio of 804.4 was reported for the detection of HPV 18 in the Mexican study by Illades-Agiuar et al (2008). The odds ratio of finding HPV 18 in vulval and vaginal intraepithelial lesions and invasive lesions is 3.8 and 1.5 to 12.00, respectively (Giuliano et al, 2008). HPV 18 is the second most common HPV type to be detected in anal cancers (9%) (Giuliano et al, 2008). Invasive cancers with predominant HPV 18 tend to occur in younger women, have more frequent metastases, higher recurrence rates and higher tumour grades.

4.0 VULNERABILITY OF THE HOST TO CERVICAL NEOPLASIA

Host factors are thought to play a role in the genesis of cervical cancer. The MHC proteins are vital for immune recognition and host resistance in the pathogenesis of tumours. Abnormal expression of both classes of the MHC occurs during neoplastic transformation. Serologically typed HLA-DQw3 has been linked to the development of cervical cancer in certain populations (Wank and Thomssen, 1991). Mehal et al (1994) have demonstrated a non-significant rise in the prevalence of HLADQw3 in squamous cell carcinomas compared with a group with normal histology. HLA DR 13 haplotypes DRB1*1301 have been shown to decrease the risk of cervical cancer (Hildesheim and Wang, 2002).

The *p53* gene is regarded as the "guardian" of the genome in the protection against cancers. The binding affinity of the HPV E6 protein to the p53 gene product determines the degradation of the p53 gene product by E6 protein product. The HPV 16 and 18 gene product bind with high affinity to the p53 gene product. Gene sequence variations or polymorphisms may influence the degradation process. A common polymorphism, p53 polymorphism occurs as either a Proline or Arginine at the p72 codon (Kutler et al, 2003; Fernandes et al, 2008). The HPV 16 E6 protein is capable of degrading a p53 molecule with an Arginine at codon 72 in comparison to a molecule with a Proline at codon 72 which is resistant to E6-mediated degradation. Women homozygous for p53 gene with an

Arginine at codon 72 are 7 times more likely to develop cervical cancer than a woman with a Proline residue at this position. Recently, Bhattacharya and Sengupta (2007), reported that p53 codon 72 polymorphism is only significant in the presence of HLA-DQB1 and HLA-B*07 homozygosity in comparison with normal women (p=0.006).

4.1 THE CELL CYCLE AND ITS ASSOCIATION WITH HUMAN PAPILLOMAVIRUS INFECTION

Somatic cells pass sequentially through defined stages during DNA and mitosis referred to as the cell cycle. The sequence of events is carefully controlled and each phase depends on the previous phase. Each phase of the cell cycle is monitored by check-points which determine progress into the next phase. In eukaryotic cells this process lasts for about 24 hours and comprises: G1 phase (12 hours); S phase (6 hours); G2 phase (6 hours); M phase (mitosis) which terminates with cell division and lasts for about 30 minutes. The progress through each step is facilitated by a number of phosphorylation steps involving protein complexes. These complexes are activated in a co-ordinated manner and initiate specific events such as DNA replication, chromosome segregation and cell division. The protein complexes include a catalytic cyclin-dependent kinase (CDK) sub-unit and an activating regulatory sub-unit known as cyclins. Various cyclins and CDKs control different stages of the cell cycle. The progression of the cell through a specific stage of the cell cycle consists of activation of these complexes followed by deactivation of these complexes when the phase is complete. The complexes are also involved in negative regulatory mechanisms, which inhibit cyclins and CDKs, including

cyclin-dependent kinase inhibitors (CDIs). The relationship between HPV 16 E6/E7 oncoproteins and cell-cycle events is illustrated in Figure 7.

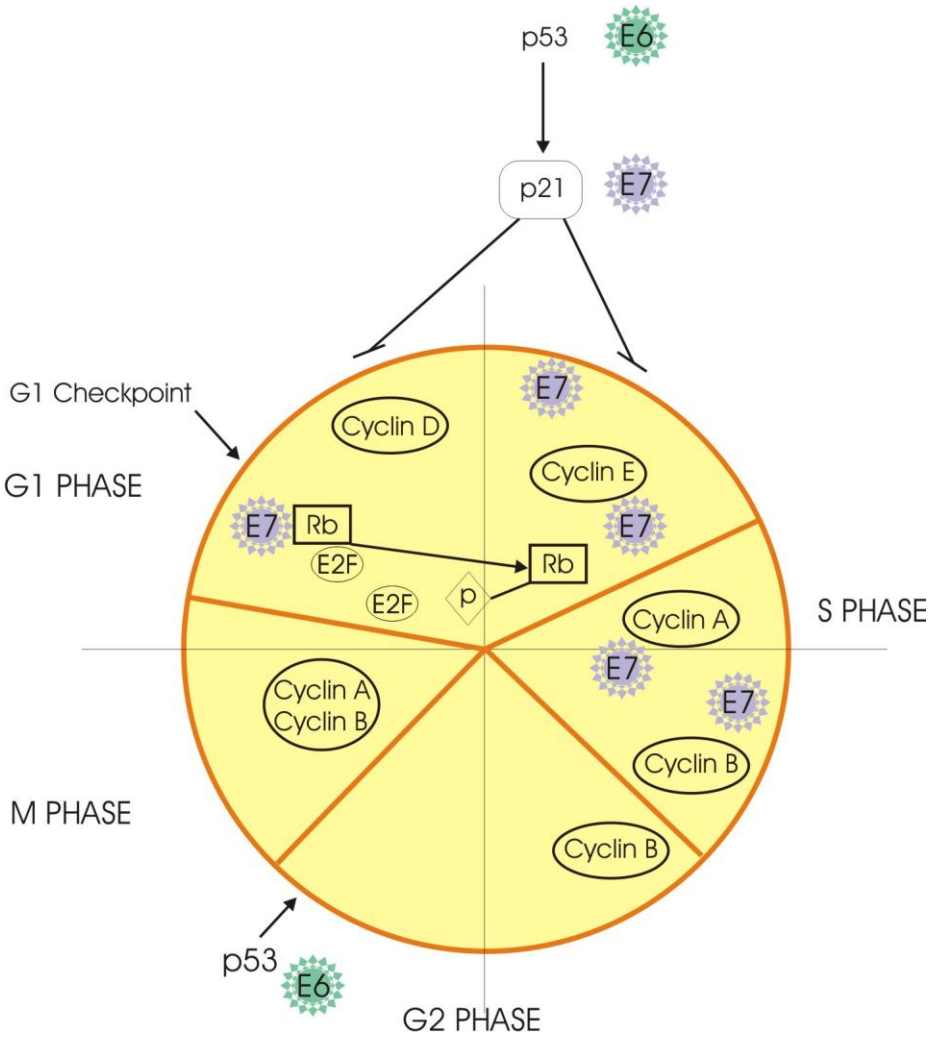


Figure 7: HPV 16 E6/E7 oncoproteins and cell-cycle interactions

4.2 CELL-CYCLE PROTEINS

4.2.1 CYCKLIN-DEPENDENT KINASES

CDKs are a component of a family of serine/threonine kinases, which have specific functions when activated. The activation involves binding of the CDKs with its regulatory sub-unit. This may require phosphorylation by a CDK activating kinase (CAK). There are about 9 CDKs identified (CDK1 to CDK9). Each of the CDKs contain 300 amino acid residues and has 75% homology (Grana and Reddy, 1995).

4.2.2 CYCLINS

There are about 17 different mammalian cyclins identified viz: (A,B1,B2,C,D,D1,D2,D3,E1,E2,F,G1,G2,H,I,K,T1 AND T2). The functional activity of all cyclins is not yet established. They vary in size from 35kDa to 90kDa. Cyclin levels vary during the cell cycle. The G1 phase cyclins, D1 and E, are involved in the G1 phase and transition into the S-phase. Cyclin A is a S phase cyclin required for DNA synthesis and is also thought to be involved in the late G2 phase. Cyclin B is a mitotic cyclin (Arellano and Moreno, et al 1997). Cyclin H/CDK 7, cyclin C/CDK 8 and cyclin T/CDK 9 form components of the transcription mechanism and regulate transcription elongation through phosphorylation of RNA polymerase II (Rickert et al, 1996).

4.2.3 CYCLIN-DEPENDENT KINASE INHIBITORS

There are two groups of CDKs: the p21 $^{(waf/C1P1/SDI\ 1)}$ family and INK inhibitors (Pavletich, 1999). The p21 is known as the "universal inhibitor", because it interacts with a number of cyclin and CDK complexes. It also binds with proliferating nuclear cell antigen (PNCA) to inhibit DNA replication. Other members of this family include $p27^{(K1P1)}$ and p57 $^{(K1P2)}$ which bind to various cyclin/CDK complexes. The INK 4 family members include p16 (INK 4a), p15 INK 4c, p19 INK 4d and its protein interacts specifically with CDK4 and CDK 6, preventing these CDKs from complexing with the D group of cyclins (Hirai et al, 1995).

4.2.4 CELL-CYCLE PHASES

4.2.4.1 G1/S PHASE

The passage of the cell cycle through the G1/S phase is controlled by cyclins D, E and A. The main target of cyclin D/CDK4/ CDK6 is the pRB tumour suppressor gene product. When phosphorylated by cyclin D, the pRB/cyclin D1 complex releases E2F thereby initiating the synthesis of genes for S phase entry and DNA synthesis. The E2F is a crucial transcription factor required for the activation of genes which encode proteins for DNA synthesis and cell cycle progression. The release of E2F allows the cell to pass through the check-point and commence DNA replication in the S phase of the cycle. Cyclin A complexes with CDK2 and forms the main S phase complex required for on-

going DNA replication. Various cell cycle regulators such as p14 (ARF), p15 (INK4b) and p16(INK4a) are required for G1 cell cycle arrest. Feng et al (2007) demonstrated that senescence markers p15(INK4b), p16(INK4a) and p14(ARF) are overexpressed in both dysplasia and carcinoma of the cervix.

4.2.4.2 G2/M PHASE

Cyclins B and CDK1 are the main regulators of mitosis and are mitosis-forming factors to initiate mitosis and drive the cell to interphase.

4.2.4.3 CELL-CYCLE CHECKPOINTS

Checkpoints are surveillance mechanisms to prevent replication of damaged DNA. There are two major checkpoints at the G1/S and G2/M interface which are capable of cell-cycle arrest to allow damaged DNA to be repaired or removed by apoptosis.

G1/S checkpoint:

The *p53* tumour suppressor gene prevents entry of cells with damaged DNA into the S phase of the cell cycle. In normal situations, the p53 protein is present in low concentrations. The level of the wild type *p53* gene increases following insult to the genetic structure. When DNA is damaged the p53 stabilizes and its concentration increases leading to arrest in both the G1/G2 phases. The *p53* gene transactivates p21

which in turn binds to and inactivates various cyclin/CDK complexes, preventing phosphorylation of pRB and release of E2F causing arrest at the G1 phase. Cells are therefore prevented from entering the S phase and allow time for DNA repair or apoptosis. This mechanism is not functional in tumour formation due to loss of the p53 function or changes in cyclins/CDIs/CDK complexes. The hallmark of cancer is the deregulation of p53 and pRB by deletion or mutation or by targeting genes (Sherr, 2000). The E6/E7 gene products are capable of performing this function (Kisseljov et al, 2008).

G2/M checkpoints:

Chromosomal abnormalities which escape the G1/S phase of the cell cycle may be detected in the G2/M phase. Arrest of the cell due to DNA damage is facilitated by the inhibition of cdc25 and prevention of the activation of cyclin B/cdc2. The p53 gene product prevents the G2/M transition by reducing the levels of cyclin B protein and lowering the activity of cyclin B promoter.

5.0 THE ROLE OF HPV IN RELATION TO THE CELL CYCLE

The HPV disrupts both the G1/S and G2/M cell-cycle checkpoints to facilitate their own replication. They also interfere with function of various host cell regulatory proteins, cyclins, CDIs and CDKs, which in turn allows replication of abnormal cellular DNA and the accumulation of genetic abnormalities. The HPV E6 oncoproteins causes degradation of the wild-type *p53* gene such that the half-life is reduced from 1-4 hours to 15-30 minutes in immortalized keratinocytes (Hubbert et al, 1992). The binding of the E6 oncoprotein to functional p53 gene product results in failure of G1/S arrest. The level of *p53* gene does not increase in cells infected with HPV where there is expression of E6. The E6 may also degrade the p21 cyclin to disrupt the G1/S checkpoint. Structural and numerical chromosomal abnormalities result from deregulation of the *p53* gene function which is also responsible for the mitotic spindle G2 checkpoint.

The E7 oncoprotein disrupts the cell cycle by binding to and inactivating the retinoblastoma gene product (Munger et al, 1989; Weinberg, 1995). The result is the release of active E2F and loss of cell-cycle-dependent regulation of E2F responsive genes (Chellapan et al, 1992). The E7 gene product can overcome G1/S cell cycle arrest induced by binding to p21 p27 proteins. In vitro experiments have demonstrated that HPV E2 expression is associated with S phase arrest and a 5-fold increase in viral genome copy number. Decreased levels of *p53* in cells expressing high levels of E2 (Frattini et al, 1997). A recent study of cell cycle proteins amongst women with cervical cancer in comparison with women with normal cervical tissues concluded that

aberrations involving p27 (KIPI), cyclin E, CDK4, P16 (INK4a) are early events in HPV 16 and 18-associated cervical cancers, whereas cyclin D1 and p53 pathway abnormalities are late events (Bahnassy et al, 2007). Further, by immunohistochemistry, p53, p27 (KIPI), and Ki-67 are independent prognostic factors that may help in prognosticating outcome of cervical cancer patients. In a study of the role of HIV infection in relation to HPV infection, it was found that there is significant altered expression of regulatory and cell cycle proteins due to HIV-1 infection occurring in the background of HPV infection (Nicol et al, 2008). Epithelia expressing vascular endothelial growth factor (VEGF) and p27 were significantly increased with HIV-infected/HPV positive infection compared to HPV infection without HIV-infection among women with SIL lesions. It was noted that the effect was additive to the HPV infection. Modulation of p27, VEGF and maybe pRB expression may explain why HIV infection is associated with an increased risk of cervical cancer in women co-infected by HPV, since it is thought that HIV may directly promote cancer development by interfering with cellular functions leading to viral persistence and progression to neoplasia. Recently, Clere et al (2007) demonstrated that HPV 16 E6 oncoprotein participates in the genesis of cervical cancer and angiogenesis by inducing VEGF transcription from the promoter in a *p53*-independent manner.

6.0 THE *p53* GENE AND ITS ROLE IN CERVICAL CANCER

6.1 HISTORY

The *p53* gene was identified in 1979 by David Lane, Arnold Levine and Lloyd Old. At the outset, it was thought to be an oncogene until in 1989, Bert Vogelstein recognized it as being a tumour suppressor gene (Levine et al, 2004). Maltzman and Czyzyk (1984) first demonstrated that the p53 protein was responsive to DNA damage from UV radiation. In 1993, *p53* was voted as molecule of the year by *Science* magazine (Beijnen, 1993). About fifty percent of all human cancers are related to a mutation in the gene including cervix, breast, colon lung, liver, prostate, bladder and skin. There are over 570 mutations of *p53* described in various human cancers (Hainaut et al, 1998).

The *p53* gene is about 20 kilobase long consisting of eleven exons and is located on the short arm of chromosome 17p13.1. Human *p53* is 393 amino acids long and has seven domains of which a Proline-rich domain is important for apoptotic activity. This gene codes for a 2.6 Kb mRNA molecule which contains a large 3' untranslated region which is involved in the stabilization of the mRNA. The normal allele of this gene encodes a 53kD nuclear phosphoprotein which is involved in cell-cycle regulation. There seems to be three independent pathways by which the p53 gene is activated: DNA damage by agents such as ionizing radiation interfering with key enzymes (ATM and Chk2); aberrant growth signals and ATR kinase activation by agents such as chemotherapeutic

drugs (Vogelstein et al, 2000). Within the cell the p53 protein binds DNA, which in turn stimulates another gene to produce a protein called p21. The p21 protein interacts with a cell-division protein (CDK2). Complexing of p21 and CDK2 prevent the cell from passing through the next stage of cell division. Mutant *p53* cannot bind DNA effectively and as a result the p21 protein cannot serve as a signal to stop cell division, resulting in cells dividing uncontrollably with formation of tumours.

The anti-cancer mechanisms of *p53* include: ability to activate DNA repair proteins when DNA has sustained injury; to maintain the cell cycle at the G1/S checkpoint when DNA damage is recognized and to initiate apoptosis if DNA cannot be repaired. In normal cells, *p53* is inactive and bound to MDM2 or HDM2 (human). Activation of *p53* is induced by various agents such as oncogenes, drugs and UV light. Damage of DNA is detected at checkpoints and induces proteins to phosphorylate *p53* at sites close to or even within the MDM2-binding region. Activation of *p53* by oncogenes is mediated by p14ARF or via stimulation of transcription proteins which bind to MDM2 to inhibit its activity. Activation of the *p53* gene results in activation of other genes including the gene encoding for p21. Activated p21 binds to G1-S/CDK and S/CDK complexes to inhibit their activity and thereby inducing cell-cycle arrest. The *p53* gene maintains the retinoblastoma protein in a hypophosphorylated state at the G1 phase or checkpoint and prevents the mutated cell from entering the S phase of the cell cycle to proliferate.

Mutations of the *p53* gene are known to occur in the conserved codons or four ‘hotspots’ (exons 5-8) of the gene. These mutations are common in a variety of cancers

demonstrating *p53* mutation. More than 50% of human cancers exhibit mutations in this gene. These mutations could be inversions or small mutations caused by endogenous or exogenous agents. Other mechanisms of inactivation of the *p53* gene include loss of alleles at the *p53* locus, deletions, insertions or silencing of the p53 protein by formation of complexes with viral or cellular proteins (Hainaut et al, 1998). Although mutations arise somatically, familial inheritance is also described such as the Li-Fraumeni syndrome.

Expression of human *p53* gene is controlled by two promoters: p1 which is situated at the 5' end of the first exon, and p2, mapping to the first intron. The expression of *p53* is regulated during the cell cycle. The protein accumulates in the cytoplasm during the G1 phase, enters the nucleus at the early S phase and remains here for a short period of time. The suppressor activity of the mutant type requires nuclear localization of the proteins. The level of the wild type *p53* in normal cells is very low and has a short half-life and is therefore undetectable by immunohistochemistry. In transformed cells these levels rises rapidly and is linked to an increase in transcription of *p53*-responsive genes, induction of growth arrest, DNA repair and programmed cell death. Mutations lead to a more stable product with a longer half-life that can be detected by immunohistochemistry. The mutant gene is a proto-oncogene whereas the wild type gene is a tumour-suppressor gene. Loss of the *p53* gene function occurs early in malignancy.

6.2 THE *p53* GENE AND CERVICAL CANCER

The oncogenic or high risk HPV types produce two proteins, E6 and E7, which stimulate transformation of cells (Narisawa-Saito and Kiyono, 2007). These proteins bind to the p53 gene product and the retinoblastoma gene product, respectively and promote degradation of the p53 gene product by ubiquitin-dependent proteolytic pathway. The *E6* gene product promotes the degradation of p53 through its interaction with E6AP, an E3 ubiquitin ligase in comparison to E7 which binds to the pRb protein and disrupts its complex formation with E2F transcription factors. Understanding the *E6* and *E7*-induced carcinogenesis is important as RNA technology using RNA interference (RNAi) target therapy has been shown to induce simultaneous *E6* and *E7* suppression and apoptosis in HPV16-related cancers by activating cellular p53, p21 and Rb (Sima et al, 2008) .Loss of the normal functioning *p53* and *pRB* leads to deregulation of the cell cycle, allowing accumulation of genetic mutations and cell cycle progression after DNA damage (Kanda and Kukimoto, 2006). Although some mutations such as missense mutations have been described in HPV-negative tumours, the existence of true HPV negative cervical cancers have been disputed by some authors (Shai et al, 2008; Southern & Herrington, 1998; Walboomers et al, 1999). Some HPV-positive tumours have demonstrated *p53* mutations (Fujita et al, 1993) and likewise, mutations are not always present in HPV negative tumours (Hgan et al, 1997).

However, Kim et al (2000) investigated the possibility of HPV 16 E6 oncoprotein-induced carcinogenesis by *p53* - independent pathways. They constructed several plasmid

vectors expressing wild-type (wt) or mutant (mt) E6 proteins. RKO cells that express wt *p53* were stably transfected with these plasmids and challenged with DNA damaging agents. The level of *p53* was significantly increased by DNA damaging agents in control cells and cells transfected with plasmids expressing mt E6 that do not bind to *p53*. The *p53* gene did not increase in cells transfected with plasmids expressing mt E6 that do bind to *p53*. They also investigated the oncogenic effect of these various E6 proteins and determined the mutation frequency of the hprt locus in control cells and cells expressing different E6 proteins. They found that cells expressing wt E6 and mt E6 (capable or incapable of binding to *p53*) showed notable increases in the mutation frequency at hprt locus compared with that of control cells. The elevation of mutation frequency in cells expressing mt E6 was similar to that in cells expressing wt E6. These data indicate that E6-induced mutagenicity is induced not only via *p53* inactivation, but also via p53-independent pathways.

High mobility group proteins of A type (HMGA) are a family of nonhistonic architectural transcription factors made up of three groups: HMGA 1a, HMGA 1b and HMGA2 (Reeves, 2004). Their function is to bind DNA and facilitate the assembly or disassembly of protein transcription complexes referred to as enhanceosomes and to control the transcription of genes. The enhanceosomes are essential for the expression of HPV 18 E6/E7 transcripts (Bouallaga et al, 2003). The HMGAs are expressed in the early phases of cell proliferation and differentiation. In normal tissues, HMGA expression is at very low levels but is over-expressed in tumours of epithelial origin. In the development of tumours, the HMGAs are reported to possess transforming activity and gene

transcriptional activity (Reeves et al, 2001). HMGA1 is capable of binding to p53 and to impair its oncosuppressor properties through inhibition of HipK2, which is one of the kinases phosphorylating p53 on serine 46 (Pierantoni et al, 2006). The Notch pathway serves as a tuner of cell proliferation and deregulation of which contributes to cancer genesis (Mellone et al, 2008). There are reports of increased Notch 1 expression in cervical cancer and link with HPV and the need for Notch 1 to maintain HPV E6/E7 expression (Zagouras et al, 1995; Talora et al, 2002). Whilst p53 might induce Notch 1 expression, HPV oncoproteins are thought to be involved in the repression of Notch 1 oncosuppression in cervical cancer (Yugawa et al, 2007). In a series of elegant experiments Mellone et al (2008) demonstrated that: HMGA1 expression in cervical cancer cells is maintained by HPV E6/E7 proteins; the repression of HMGA1 inhibits cell proliferation and facilitates p53 reactivation; HMGA1 is necessary for the full expression of HPV 18 E6/E7 oncoproteins, forming an autoregulatory loop between HPV E6/E, transcription and HMGA1.

7.0 THE ROLE OF TELOMERASE ACTIVITY IN HPV-RELATED CERVICAL CANCER

Telomerase is a ribonucleoprotein which extends to the telomeric ends of linear chromosomes in eukaryotes. In somatic cells it remains quiescent but becomes active in embryonic development and stem cells. The RNA fragment of telomerase, TERT, serves as a template for repetitive DNA in telomeres, TTAGGG, in human cells. Another sub-unit of telomerase is dyskerin, whereas, hTERT serves as a catalytic unit of telomerase

(Cohen et al, 2007). The hTERT can be detected in cancers and immortalized cells. High-risk HPV E6 can activate telomerase in epithelial cells and E6AP is necessary for hTERT regulation by E6 (Gewin et al, 2004). The E6/E6AP complex targets p53 for ubiquitination and degradation via NFX1-91 which serves as a transcriptional repressor of hTERT (Gewin et al, 2004). Expression of a splice variant of NFX1-91, viz, NFX1-123 is necessary for telomerase activity (Katzenellenbogen et al, 2007). The oncogene c-myc is important for HPV-associated and non-HPV associated activation of hTERT. During DNA replication, loss of genetic material is possible. To avoid losing genetic material, the ends of the chromosome are capped with repetitive telomeric DNA and proteins referred to as shelterin (de Lange, 2005). Telomeres shorten during each cell division and as the telomeres shorten, cells are signalled to senesce. In the absence of this process, severe DNA damages could occur. Such DNA damage forms the basis of cellular dysregulation seen in cancers.

8.0 THE ROLE OF DNA METHYLATION IN CERVICAL CANCER

The introduction of cytosine residues at CpG dinucleotides is referred to as methylation of DNA and plays a role in transcriptional silencing. Methylation of HPV DNA was described many years ago (Burnett and Sleeman, 1984). However, the significance of methylation was not fully understood until recently. Methylation in HPV 16 and 18 genomes occur mainly in the LCR regions and L1 ORF (Zheng and Baker, 2006). Human papillomavirus E6 and E7 gene transcription commences at the E6 promoter p97. The

activity of the promoter is stimulated by an enhancer. When HPV integrates into host DNA, a region downstream of p97 becomes a transcriptional stimulator (Stunkel et al, 2000). Repression of transcription by methylation of DNA at CpG dinucleotides target HPV, resulting in transcriptional repression of HPV genomes. Both hypomethylation and CpG hymethylation occur during carcinogenesis. CpG hypermethylation represses gene expression by favouring proteins to bind to methylated DNA, which in turn induce histone deacetylases to condense chromatin (Hendrich and Bird, 2000). It has been shown by *in vitro* and *in vivo* experiments that HPV 16 genomes are targeted by CpG methylation resulting in repression of transcription (Badal et al, 2003). The authors studied HPV from normal smears as well as invasive tumours and cell lines and demonstrated that methylated regions are asymmetrically distributed over the genome, methylation occurs at CpGs overlapping with promoters and enhancers and that methylation decreases with the progression to cervical cancer.

9.0 MICROSTAELLITE INSTABILITY AND CERVICAL CANCER

Microsatellites are simple, short repetitive DNA sequences located in the non-coding region (introns) of the human genome. They are usually about 1 to 4 bases in length and exist as di-, tri- and tetranucleotides. Dinucleotide repeats are the most common type (Sherman and Kurman, 1998). Since these genetic materials are located within non-coding regions of the genome, microsatellites are referred to as “junk” DNA and are thought to represent an accumulation of uncorrected errors in DNA replication.

Microsatellites consist of variations or polymorphisms which are characteristic for an individual and are the same in different cells of the same individual. They are inherited in a stable fashion and remain highly conserved from one generation to the next. Microsatellites are found near chromosomal telomeres and centromeres.

Loeb, (1994) described increased chromosomal abnormalities and mutations in cancer and hypothesized that cancer has a mutator phenotype. Microsatellites are thought to be prone to replication errors. Tumours which possess somatic alterations in the length of their microsatellite loci are known as MSI+ (microsatellite instability positive) or RER+ (replication error positive). Usually, the replication errors are repaired by precise mechanisms. However, defective mismatch repair genes and mutations in cancer result in defective proteins which cannot correct the replication error. The polymerase chain reaction using radioactive-labelled primers are used to detect specific microsatellite sequences in paired DNA samples of normal and tumour tissue. Microsatellite instability is thought to be present when there is an alteration in the size of at least two of the microsatellite loci in tumour DNA when compared with normal DNA (Ohwada et al, 1999).

Microsatellite instability has been described in cervical cancers (Kisseljov et al, 1996; Helland et al, 1997; Nishimura et al, 2000) as well as in CIN lesions (Wong et al, 2003). Kisseljov et al, (1996) studied microsatellite instability in chromosome 6 in cervical carcinomas and found microsatellite instability in 11% to 23% of patients. Wang et al, (2006) recently reported microsatellite instability to be present in 25.4% of cervical

cancers, 48.4% of endometrial cancers and 21.9% of ovarian cancers. Although cervical cancer is predominantly HPV-driven, it also requires an accumulation of genetic alterations for carcinogenesis. Wani and Nair, (2003) reported that microsatellite instability does not play a major role in cervical cancers.

Chung et al, (2001) studied microsatellite instability in cervical cancers and performed immunohistochemical staining to determine the expression of major DNA mismatch repair genes, hMSH2 and hMLH1. Seven cases (14%) demonstrated a high frequency of microsatellite instability at 2 or more loci. High-frequency microsatellite instability correlated with advanced stage disease ($p<0.05$) and reduced overall survival ($p=0.059$). In contrast, Wong et al, (2003) found an increasing trend of high-frequency microsatellite instability with higher stage cervical cancer ($p=0.035$), but high-frequency microsatellite instability was not associated with poor prognosis.

Rodriguez et al (1998) concluded that microsatellite instability is very infrequent in cervical cancers and occurs independently of HPV status. Nishimura et al, (2000) detected microsatellite instability in 23% of invasive cervical cancers and loss of heterozygosity (LOH) of chromosome 3p in 15% of cervical cancers. They regarded LOH to be an early event and microsatellite instability to be a late event in cervical carcinogenesis. Clarke et al (2003) in a study of microsatellite instability among women with early stage cervical cancer, reported that there was a low rate of microsatellite instability (9/40 tumours), but significant rates of loss of heterozygosity (36% at D3S1255 and D2S123).

10.0 THE ROLE OF STEROID CONTRACEPTION, HUMAN PAPILLOMAVIRUS AND CERVICAL NEOPLASIA

10.1 INTRODUCTION

Human papillomavirus DNA can be detected in almost all cervical cancers (99.7%). However, HPV infection itself is insufficient to produce a malignant transformation by itself because high-risk HPV infection is common in women with normal cervical cytology and most of these women do not develop neoplasia of the uterine cervix. Other factors or co-factors are necessary for the neoplastic manifestation of high-risk HPV infections. Steroid contraceptive hormones are a group of natural or synthetic compounds used by about 100 000 million women worldwide (Abma et al, 1997). Advances in the development of steroid contraceptives have resulted in a reduction in dose, change in progestogen type and side-effect profile. Although many side-effects, adverse effects and concerns have been advanced concerning steroid contraception, they remain the most reliable, reversible and effective pharmacological agents in empowering women for fertility regulation. Of the many serious side-effects, the suspected association with the development of cancer has raised many concerns. However, prior to prohibiting the use of such agents, a careful analysis of the evidence and risk-benefit ratios need to be determined before any change in clinical practice is advocated.

The causality of cancer is further compounded by the presence of several variables such as: the lack of a suitable animal model; long lag time from exposure to clinical cancer;

low prevalence of cancers in young females and role of genetic, geographic and environmental carcinogens. Although steroid contraceptives undergo prior extensive testing in animal models, extrapolation of adverse effects from animals to human species does not necessarily provide evidence of causality in humans. However, steroid hormones are postulated to play a role in cervical carcinogenesis since the transformation zone is sensitive to estrogens and is the site of cervical neoplasia (Elson et al, 2000). Further, 16α-hydroxyestrone causes DNA damage which could result in an accumulation of mutations which act synergistically with HPV to induce cervical carcinogenesis (Telang et al, 1992; Newfield et al, 1998). Evidence suggests that current and recent users of steroid contraception have an increased risk of cervical cancer, which declines after cessation of use of such contraceptives (Veljkovic and Veljkovic, 2010).

10.2 THE BENEFITS OF STEROID CONTRACEPTION

In contrast to the adverse effects, there are significant non-contraceptive benefits of steroid contraception as well as documented evidence of protection against cancers such as endometrial, ovarian cancer and colorectal cancer. Some of the non-contraceptive benefits include: regulation of menses; reduction in menstral blood loss and dysmenorhoea, prevention of pregnancy, reduction of iron-deficiency anaemia, benign breast disease, pelvic inflammatory disease. The benefits of cancer-protection persist sometime after discontinuation of oral steroid contraception (Diczfalusy, 1992; Bertram, 2004; La Vecchia & Bosetti, 2004). The protection against ovarian disease including various histological types of ovarian cancer is about 40% and the benefits persist for at

least 10 years after cessation of use and probably up to 15 to 20 years (La Vecchia et al, 2001; Bosetti et al, 2002; Lech and Ostrowska, 2006). Oral contraceptives reduce the risk of endometrial cancer by about 50% (La Vecchia, 2001) and the reduced risk has been shown to persist for 10 years to 15 years after cessation of use. In a study from China, endometrial cancer cases where found to use oral contraceptives less frequently (Xu et al, 2004). A hormonal influence on colorectal cancer has been suggested and demonstrated in various studies (Fernandez et al, 2000; IARC, 1999; Levi et al, 2003). It has been noted that there was an excess risk of colorectal cancer among nuns (Fernandez et al, 2000). Levi et al (2003) reported a relative risk of 0.8 for ever use of oral contraceptives in a Swiss case-control study.

10.3 CANCERS LINKED TO STEROID CONTRACEPTION

The three cancers linked to the use of steroid contraception include: Hepatocellular carcinoma, breast cancer and cervical cancers/dysplasia (La Vecchia et al, 2001). A detailed review of steroid contraception in relation to breast and hepatocellular cancers is beyond the scope of this presentation. An association with hepatocellular carcinoma has been reported with long-term use of oral contraceptives in the absence of hepatitis B viral infection (La Vecchia et al, 2001) and chronic liver disease (IARC, 2005). The Collaborative Group on Hormonal Factors in Breast Cancer, (1996), reanalyzed data of 53 297 cases of breast cancers and 100 239 controls from 54 epidemiological studies. It was found that there was an increase in the relative risk of breast cancer of 1.24, but that the risk approached that of never users 10 years after cessation of use. Breast cancers

diagnosed among such cases were found to be less advanced. Dose, duration and type of compound had no effect on breast cancer risk. Vessey et al (2003) found no increased breast cancer mortality and oral contraceptive use after several decades of follow-up. Similar results were reported by Norman et al (2003) including users of newer formulations of oral contraceptives (Dumeaux et al, 2003).

10.4 REVIEW OF PUBLISHED DATA LINKING STEROID CONTRACEPTION TO CERVICAL NEOPLASIA

10.4.1 EVIDENCE FROM COHORT STUDIES

An increased prevalence of cervical neoplasia was first reported in cohort studies. In 1977, Meisels et al, reported on a study of 84 540 women with normal cervical cytology and 2017 women with mild and moderate dysplasia. A significant correlation was found between the use of oral contraceptive pills and mild/ moderate dysplasia.). Most cohort studies (Andolsek et al 1983; Beral et al 1988; Vessey et al 1983a) that have examined this relationship are difficult to interpret because of limited information on potential confounding factors. An analysis of data from the Royal College of General Practitioners Oral Contraception Study published in 2007 reported the risks and benefits of oral contraception in association with many cancers (Hannaford et al, 2007). The dataset consisted of 339 000 woman- years and 744 000 woman-years of observation between non-users and ever-users of the pill, respectively. There was a statistically significant trend of increasing risk of cervical cancer and cancers of the central nervous system and

pituitary gland with increasing duration of pill-usage. However, an increased risk between oral contraceptive use and precursor cervical dysplastic lesions has been noted. Also, few women had developed invasive cancer during the course of these studies. Vessey et al (1983a) reported results from a study of women who had used oral contraceptives in a 10-year follow-up for the Oxford Family Planning Association and noted that there was a rising trend of cervical neoplasia in the 10-year follow-up. A comparison was made with the incidence of dysplasia among women who used the intrauterine contraceptive device (IUCD). The incidence of dysplasia was 0.28/1000 women-years among 3162 IUCD users and 0.31/1000 women-years for oral contraceptive users. There were only 12 recorded cases of dysplasia and therefore no statistical analysis could be performed. A similar trial was conducted by the New Zealand Contraception and Health Study Group (1994) and found no difference in the incidence of dysplasia between oral contraceptive users and women who used the IUCD.

A recent review of the factors which affect mortality of the long term follow up of women from the Oxford Family Planning Association contraceptive study foundthat mortality rate ratio for cervical cancer amongst women who used oral contraceptives was increased (rate ratio 7.3) but there was very wide confidence intervals (1.2 – 305) (Vessey et al, **2010**).

Gram et al (1992) in a prospective follow-up study of 6622 women, found a relative risk for oral contraceptive users of 1.5 (95% CI: 1.1 – 2.1) and 1.4 (95% CI: 1.0 – 1.8) for past users of the pill compared to non-users of the pill. Adjustments were made for age,

marital status, smoking and alcohol intake. Ramcharan (1974), reported results of the Walnut Creek Contraceptive Drug Study comprising 17942 women observed for 37 373 women-years. The analysis did not take into account age at first birth, sexual activity and number of partners and showed no statistical significant association of dysplasia among oral contraceptive users. Andolsek et al (1983) found similar increase in rate of cervical neoplasia as Vessey et al (1983a) with extended months of usage, although the follow-up period was limited (average 4.5 years). In both these studies invasive cancers occurred in the users of the oral contraceptive pill. Notwithstanding the confounding variables, Vessey et al (1983b) subsequently showed that oral contraceptive users had different sexual histories than the group which used the intrauterine device (comparison group).

Stern et al (1977) reported on a prospective study of 300 women with cervical dysplasia compared to 300 women with normal cytology. Oral contraception was prescribed to the study group and followed for 7 years. There was no dysplasia among women with normal cytology who did not take the pill. In comparison, there was a significant conversion from dysplasia to carcinoma in-situ in the group of pill-users. Peritz et al (1977) reported a four-fold increase in risk of cervical carcinoma with contraceptive pill use of more than four years duration. Beral et al (1988) reported an almost double risk of cervical cancer for pill users compared to non-users, with the risk rising to four times after ten years of use. Syrjanen et al (2006) followed up a cohort of 3187 women to determine the acquisition of high-risk HPV and cervical dysplasia. On multivariate analysis contraceptive pill use was not predictive of HPV acquisition or cervical dysplasia as sexual behaviour is different among users and non-users.

A recent publication by Longatto-Filho et al (**2010)** documented the relationship between all form of hormonal contraceptive use, the length of their use as risk-factors for high-risk HPV infections or cervical intraepithelial neoplasia (CIN). This cohort study consisted of over 12 000 women from Brazil and Argentina. It was found that the duration of hormonal contraceptive use was not significantly related to high-grade CIN lesions ($p=0.069$), low-grade CIN lesions ($p=0.781$) or ASCUS ($p=0.231$). Using multiple logistic regression methods, it was concluded that the duration and time of hormonal contraceptive use were not independent risk-factors for high-risk HPV infections or high-grade CIN lesions.

In contrast, Cibula et al (**2010)** documented an increased risk of cervical cancer and long term oral contraceptive use. Quoting the meta-analysis of the International Collaboration of Epidemiological Studies of Cervical cancer comprising data from 16573 women with cervical cancer and 35509 women without cervical cancer, it was found that the risk of cervical cancer increased with increasing duration of oral contraceptive use (RR= 1.90; CI: 1.69 – 2.13) for 5 or more years of use. It was found that the risk decreased after stopping pill use and returned to that of non-users after 10 years.

10.4.2 EVIDENCE FROM CASE-CONTROLLED STUDIES

The majority of case-controlled studies show a positive association between oral contraceptive use and cervical neoplasia.

In 1972, Worth and Boyes reported results of a case-controlled study of 308 women and noted that there were no differences in the incidence of carcinoma between cases and controls irrespective of the type of formulation used. Similar results of a lack of association after controlling for confounding factors were reported by Zondervan et al (1996) and de Vet et al (1993). Studies which have shown a positive link with contraceptive usage, reported that duration of contraceptive use was the crucial variable. It has been suggested that the excess risk of cervical dysplasia may be due to the effect of HPV infection. Coker et al (2001) adjusted for HPV infection and found no link between oral contraceptive use and cervical dysplasia. This is in contrast to the findings of Ylitalo et al (1999) who reported a four times greater risk of dysplasia among current pill consumers compared with non-users. In well controlled studies, the relative risk ranges from 1.3 to 1.8 for users of 5 years or more.

In other studies (Ebeling et al 1987; Mandelson et al 1990; Parazzini et al 1990) recent users were at higher risk than non-recent users, reflecting a late effect of oral contraceptives. Hoyo et al (2004) in a more recent study found that oral contraceptive use of more than five years duration was associated with an elevated risk of carcinoma in-situ compared to non-users (OR 1.4, 95% CI: 0.8 – 2.5). More importantly, the elevated risk

was noted if women commenced oral contraceptive use before the age of 24 years. McFarlane-Anderson et al (2008) conducted a case-controlled study of the risk of oral contraceptives and cervical dysplasia and cancer among Jamaican women amongst whom both the oral contraceptive usage and the background risk of cervical cancer is high. After adjusting for age and number of sexual partners, use of oral contraceptives was associated with cervical dysplasia (OR 1.92, CI 1.11 – 3.34; p=0.02) and severity of dysplasia (OR 2.22, CI: 1.05 – 4.66); p=0.036).

Although one study (Ebeling et al, 1987) showed an effect of age at first use i.e. higher risk if began before age 25, most other studies (Brinton et al 1986; Brock et al 1989; Parazzini et al 1990) showed no effect of varying effects by age at first use or interval since last use. Other variables that have been examined include the number of partners and history of genital infections. A higher oral contraceptive-associated risk was found among women with multiple sexual partners (Parazzini et al 1990) whilst another study (Brinton et al 1986) found that the effects of oral contraceptives to be greatest amongst women with histories of genital infections. The inference here is that sexually transmitted agents may interact with oral contraceptives in the pathogenesis of cervical neoplasia. An example of this interaction is that between steroids and the human papillomavirus (zur Hausen 1982). Brinton et al (1990) in a case-controlled study performed in Panama, Costa Rica, Colombia and Mexico found no difference in the occurrence of cervical cancer after confounding factors were adjusted for.

The role of cervical cancer screening seems to play an important role in the effect of oral contraceptive use. It is felt that oral contraceptive users were more likely to have their disease detected at an earlier stage because of the availability of screening facilities. The transition time of precursor lesions in one study (Stern et al, 1977) showed that the probability of progression from cervical dysplasia to carcinoma–in–situ was 0.30 in oral contraceptive users compared to 0.05 in non-users. However, these estimates were based on 10 and 3 progressions, respectively.

Other case controlled studies that have revealed an increased risk of greater than 1 (range 1.2 – 5.7) include those by Cuzick et al (1989); Slattery et al (1989) and Clarke et al (1985). A large multinational WHO (World Health Organization) collaborative study of neoplasia and steroid contraceptives was performed involving 11 participating centres in 9 countries (WHO 1993). The analysis was performed to assess the risk of invasive cervical cancer and oral contraceptives. Factors that were taken into account included information on prior use of oral contraceptives, screening for cervical cancer and suspected risk factors for cervical cancer from 2361 cases and 13644 controls. It was found that the relative risk of invasive squamous cell cancer was estimated to be 1.31 with a 95% confidence interval that excluded one. The risk of this disease increased significantly with duration of use after 4 to 5 years from first exposure and declined with time after cessation of use to that in non-users in about 8 years. There were no sources of bias or confounding factors identified to offer plausible explanations for these findings. A limitation of this study was the lack of information on HPV although other sexually transmitted agents (Herpes simplex virus and Cytomegalovirus) were not confounding

factors. The significant findings of this study were observed in women with and without prior cervical Papanicolaou screening.

The case-controlled study reported by Moreno et al (2000) showed that the long term use (> 5 years) of hormone contraceptives increases the risk of cervical cancer by up to 4 fold in women with HPV 16 DNA compared to women negative for HPV DNA. A subsequent report by Moreno et al (2002) (IARC) pooled data from eight case-control studies of patients with HPV-positive cervical cancer and two studies of patients with carcinoma in-situ. In comparison with non-users, women who used oral contraceptives for less than 5 years did not have an increase in cervical cancer (OR 0.73; 95% CI: 0.52 – 1.03). The odds ratios for women who used oral contraception for between 5 and 9 years and 10 years and greater were 2.82 (95% CI: 1.46 – 5.42) and 4.03 (95% CI: 2.09 – 8.02), respectively. A report from Manchester, United Kingdom (Deacon et al, 2000) found an increased risk of borderline significance for high grade squamous intraepithelial lesions among women who used oral contraceptives for 8 years or more. One of the strongest links between current and long-term pill users has been reported by Smith et al (2003) for adenocarcinoma in-situ and adenocarcinoma.

Twenty eight eligible studies comprising 12 551 women with cervical cancer were evaluated. Current pill users and pill users of 6 years or more duration had 12 fold and 6 fold increased risk for adenocarcinoma in situ and an increase risk of adenocarcinoma, respectively. Similar relative risks were noted for squamous and adenocarcinomas. Green et al (2003) conducted a systematic review of 19 epidemiological studies in relation to

genital HPV infection and oral contraception to determine if genital HPV infection was more common with oral contraceptive users. All studies measured prevalent HPV infection at one point in time with no distinction between recent or persistent infection. There was no evidence for a strong negative or positive association between HPV positivity and ever-use or long duration of use of oral contraceptives. The authors were of the opinion that in view of the heterogeneity between studies and the possibility of bias and confounding, that their results should be interpreted with caution and further studies concerning the issue were needed.

A recent re-analysis of a large collaborative study of 16573 women with cervical cancer and 35509 women without cervical cancer from 24 epidemiological studies found an increased risk of cervical cancer with hormonal contraceptive use. Ten years of contraceptive use from the age of 20-30 years was associated with an increased cumulative incidence of cervical cancer by age 50 years of 7.3-8.3 per 1000 in less-developed countries and by 3.8-4.5 per 1000 in more developed countries (Appleby et al, 2007). The risk of adenocarcinoma was also increased especially in women younger than age 35 years. It was noted that these findings were derived from countries where cervical cancer screening was sub-optimal or non-existent at the time the studies were performed. It is thought that the additional absolute risk of cervical cancer due to combined oral contraceptives in well-screened populations is likely to be lower (Sasieni, 2007). Castellsague and Munoz (2003) summarized 6 studies from around the world which were restricted to HPV DNA-positive women.

The Eastern US Study (Lacey et al, 1999) reported an odds ratio of 17.1 (95% CI: 1.5 – 188.2) for current users versus never users of the pill. These findings were applicable only to adenocarcinoma in situ. The Costa Rican study (Hildesheim et al, 2001) reported a 3.1-fold increased risk for pill users of 5 years or more duration compared with never users among women with two or less pregnancies. The pooled analysis of studies performed by the IARC (Moreno et al, 2002) found that even though the risk of carcinoma in situ and invasive cervical cancer was moderately associated with cancer (OR=1.4), a strong dose-response relationship was evident with increasing number of years of use. There was no increase in risk of disease for oral contraceptive users for up to 4 years of use. The risk of invasive cervical cancer was four-fold for users of the pill of more than 5 years and 3-fold for carcinoma in situ. Castle et al (2002) in a prospective study found no association between oral contraceptive users and HGSIL or cervical cancer. However, limitations of the study include: only one measurement of contraceptive use at the outset of the study; shorter follow-up times for pill-users; treatment of CIN 1 and CIN 2 in the control group who used the pill and in whom progress to CIN 3 or cancer could have occurred in the absence of treatment and lack of information about the duration of pill use. In the largest clinical trial of hormone replacement therapy(HRT) (Women's Health Initiative), women aged 50-79 years were randomized to receive conjugated equine estrogen (0.625 mg) with medroxy-progesterone acetate (2.5mg) daily (8506) or placebo (n=8102). Cervical cytological smears were performed every 3 years (Anderson et al, 2003). The study showed that the incidence of cervical cancer did not significantly differ between the treated and control groups (hazard ratio=1.4, 95% CI: 0.5 – 4.4). It was concluded that the study was of too

short a duration and did not have sufficient statistical power to determine the effect of HRT in cervical cancer

Although Chang (1989) reported an increased rate of HPV infection among pill users, most studies have failed to confirm this association (Vaccarella et al, 2006). Limited data is available concerning the risk of cervical cancer with the use of the oestradiol transdermal patches (Faculty of Family Planning and Reproductive Health Care Clinical Effectiveness Unit, 2004).

In conclusion, the collaborative re-analysis showed that women using the oral contraceptive pill have a small increased risk of cervical cancer which begins to decline after they stop taking the pill and returns to normal ten years after stopping the pills. In the long term the small increased risk of cervical and breast cancer is outweighed by the established reductions in ovarian and endometrial cancers (IARC, 2008).

10.5 ROLE OF PROGESTERONE-ONLY CONTRACEPTIVE AGENTS IN THE PATHOGENESIS OF CERVICAL NEOPLASIA

The evidence for the oncogenic potential of progestins, in general were established in the 1970s. In the early 1970s the Food and Drug Administration of the United States of America requested two drug companies to withdraw preparations containing medroxyprogesterone acetate from the market after it was found that they were associated with a significantly greater number of mammary nodules in beagle dogs treated with 1,

10 and 25 times the human dose than the control dogs (Preston, 1971). Similar nodules removed from beagle dogs receiving other progestins showed histologic signs of malignancy. However, it was then considered that beagle dogs are poor subjects for the administration of sex steroids. They are unusually sensitive to progestins.

It has been established that certain nucleotide sequences of the human papillomavirus (HPV) type 16 offer responsiveness to glucocorticoids and progesterone (Strahle et al, 1987). In the study by Pater et al (1990) it was shown that the HPV was capable of oncogenic transformation of baby rat kidney cells in the presence of progesterone (norgestrel) but not in the presence of oestrogen. Intact and integrated HPV type 16 deoxyribonucleic acid (DNA) was present and expressed in all five progesterone – transformed colonies that were examined. These cell lines were also capable of anchorage-independent growth and induced tumours in syngeneic animals. This oncogenic transformation was further demonstrated in the presence of the *ras* oncogene from the active contraceptive pills but not from the inert pills. A similar finding was reported by Cook et al (1988) which showed oncogenic transformation of baby mouse kidney cells with a combination of HPV 16 and *ras* oncogene in the presence of the progesterone R5020. The role of HPV E6/E7 on the expression of messenger HPV RNA in relation to administration of estrogens and progesterones was studied inCaSki and SiHa cell lines (Ruutu et al, 2006). It was found that progesterones increased cell proliferation in both cell lines, an effect antagonised by RU486. Estrogens protected the cells from apoptosis, an effect not antagonised by Tamoxifen. Transcription levels of HPV E6/E7 mRNA were not increased by estrogens and progesterones. Estrogens may

acts via an antiapoptotic mechanism to allow growth of cells infected with high-risk HPVs.

In pregnancy progesterone is thought to enhance the expression of the HPV gene, resulting in an increase in the viral copy number and increased multiplication of virus-transformed cells (Chan et al, 1989). It has been shown that there is increased prevalence of the HPV in pregnancy (Schneider et al, 1983). The work by Mittal et al (1993) showed that the anti-progestin RU 486 inhibits the induction of HPV 16 gene expression in cervical keratinocytes directly through hormone response elements in the regulatory region of the viral genome. This lends further evidence to the role of progesterone hormones in the pathogenesis of cervical neoplasia.

The results of the World Health Organization Collaborative Study of Neoplasia (WHO 1985) showed an overall relative risk of 1.2 in women who had used the long -acting depo-medroxyprogesterone acetate. Further, a risk estimate of 9 was reported from one participating centre (Chile). Another case-controlled study from four Latin American countries (Herrero et al, 1990b) showed a relative risk of 2.4 in users of over five years duration and that the risk was enhanced after five years and ten years since last use and first use, respectively. Forty-five percent of women from this study used depo-medroxyprogesterone acetate, whilst fifty-five percent used norethisterone enantate. Hoyo et al (2004) reported that when compared to women who did not use injectable depo-medroxyprogesterone acetate, women who used this agent for five years or more had an elevated risk of cervical carcinoma in-situ, especially if use was

commenced before age 24 (OR 1.9, 95% CI: 0.7 – 4.8). McFarlane-Anderson et al (2008) studied women attending a colposcopy clinic to determine the risk of cervical dysplasia and cancer. There were 10% of women who used depo-medroxyprogesterone acetate injectable contraception. Depo-medroxyprogesterone acetate use with age and number of sexual partners as covariates, was associated with dysplasia and severity of disease (OR 2.43, 95% CI: 1.39 – 4.57).

In contrast to the above studies, La Vecchia (1994) and a WHO report (WHO 1993) concluded that there was no association between progestagen-only steroids and cervical cancer, possibly accounting for lower cervical cancer risks amongst countries such as Thailand where 12% of women use such agents. However, HPV DNA status in these studies was unknown but is nevertheless important as steroid contraception may not increase the risk of cervical cancer in the absence of HPV. Coker et al (2001) studied the influence of various hormonal methods of contraception including the progesterone only implant (Levonorgesterel, Norplant: Wyeth-Ayerst, Phildelphia, PA). There was no increased risk of cervical dysplasia after controlling for age, coitarche and high-risk HPV-positivity. Misra et al (2003) reported the cytological effects of Norplant I among Indian women followed up for up to 5 years. There was an elevated incidence of SIL in the first year after insertion. This incidence then declined and no SIL was noticed 3 years post insertion. The authors concluded that over a five year period Norplant-I is safe from an oncological point of view.

The risk of persistent HPV infections (24 months) and abnormal cervical cytology was studied by Maucort-Boulch et al, (2010). There was an increased likelihood of persistence of HPV infections in association with current use of injectable contraceptive use (OR=1.15; 95% CI: 1.01 – 1.32). Non-regression of CIN lesions and persistence of any HPV type was reported by Moscicki et al (2010) (Hazard ratio 0.85; 95% CI:0.75 – 0.97). In a case-controlled study by Harris et al (2009), it was found that use of DMPA injectable contraception was associated with the presence of oncogenic HPV infections and that there was an inverse relationship with CIN 2/3 or greater (adjusted OR 4.7; 95% CI: 1.4 – 15.8). Using logistic regression models, Castle et al **(2005)** showed that current progesterone injectable contraceptive users were at elevated risk of developing CIN 3 compared to non-users (OR= 1.6; CI: 1.2 – 2.1).

11.0 POSTULATED MECHANISMS OF STEROID-RELATED CERVICAL CARCINOGENESIS AND THE LINK BETWEEN STEROID CONTRACEPTION AND HUMAN PAPILLOMAVIRUS INFECTION

Clinical and experimental evidence indicate that HPV infection by itself does not lead to the development of cervical cancer. By the polymerase chain reaction technique up to 84% of women with normal cervical smears carry HPV 16 DNA (Herrington, 1994). It therefore appears that co-factors are necessary for the complete manifestation of HPV oncogenicity (Delvenne et al, 2007). Persistent HPV infection is associated with cervical neoplasia and although it has been suggested (Castellsague and Munoz, 2003) that steroid

hormones might induce the reactivation or persistence of HPV, the data available does not confirm this (IARC, 1999). The role of HPV as a link to cervical cancer has been reported by Munoz et al (1992) and Walboomers et al (1999). Such studies have shown that high-risk HPV types can be detected in over 99% of patients with invasive cervical cancer. The role of contraceptive steroids in association with HPV has recently been reported by Moreno et al (2002) and described in the previous section. *In vitro* work by Arbeit et al (1996) in a controlled study demonstrated that the high risk HPV type 16 was able to stimulate the development of vaginal and cervical squamous cell carcinomas in transgenic mice which were exposed to slow release pellets of 17 beta- oestradiol in the presence of the human keratin –14 promoter (K14- HPV 16 transgenic mice). Squamous cell carcinomas developed in a multi-stage pathway only in transgenic mice and not in non-transgenic mice. The mice were treated with 0.72 milligrams of oestradiol for 60 days. In an interesting publication by Chung et al (2009), it was found that when mice were exposed to the estrogen alpha receptor inhibitor, ICI 182 780 and raloxifene, there was clearance of both precursor lesions and carcinomas of the cervix and vagina. These findings again might point to the hormonal basis of cervical precursor and invasive lesions.

Kumar et al (1996) showed that dexamethasone significantly increased the expression of the viral E6/E7 oncogene mRNA from intact HPV in primary human ectocervical cells in in-situ hybridization assays. This action is thought to be mediated through the HPV 16 glucocorticoid - response elements. The HPV E6 and E7 open reading frames can be regulated by glucorticoids (Mitrani-Rosenbaum et al, 1989). The 1 kb enhancer/promoter

of both HPV 16 and 18 contain response elements for glucocorticoids and progesterones (Arbeit et al, 1996). For the binding of the glucocorticoid hormone consensus sequences involving the motif 5' – TGTTCT – 3' have been postulated.

The glucocorticoid-response elements (GRE) involve a part of this motif with the perfect palindrome being represented by the sequence 5"-AGAACANNNTGTTCT –3' (Strahle et al, 1987). Within the 12-base pair incomplete palindrome of the sequence 5' – TGTTCT – 3", the 15– base pair segment has 9, 10 and 11 base pairs in common with the tyrosine aminotransferase gene motif II and GRE in the human metallothionein IIA gene promoter, respectively. This 12-base pair palindrome is considered to be relevant for glucocortoid receptor binding (Strahle et al, 1987). Transient expression experiments have proven that glucocorticoids lead to significant activation of the viral promoter (von Knebel Doeberitz et al, 1991).

In summary therefore, steroid glucocorticoids and progesterone hormones activate the expression of HPV 16 via the interaction of the glucocorticoid receptor with three glucocorticoid response elements in the HPV 16 regulatory region.

Further work by Mitrani-Rosenbaum et al (1989) showed that oestrogen treated SiHa cervical cells with 1 micromolar beta oestradiol stimulated the transcription of HPV 16 mRNA. There was an eight-fold induction after 16 hours of oestrogen treatment. Similar findings were reported by other workers (Green & Chambon, 1987; Kumar et al, 1987; Martinez et al, 1987). Progesterone has been shown to enhance the expression of HPV 16

E6/E7 oncogene transcription in CaSki and HEp-2 cell lines (Yuan et al, 1999). CaSki cell lines are cervical cancer cell lines containing integrated HPV 16 DNA while Hep-2 cell lines were transfected with HPV 16 DNA.

The role of steroids (dexamethasone) has been demonstrated in *in vitro* experiments showing that the growth rate of steroid treated cell lines correlated consistently with the expression of the HPV *E6 and E7* genes (von Knebel Doeberitz et al, 1991). This supports their role in the maintenance of the proliferative phenotype of cervical carcinoma cells. It has been reported that the cervical cancer cell-line CaSki which contains integrated HPV 16 DNA when exposed to prolonged progesterone treatment enhances their colony forming efficiency on plastic surfaces and on soft agar (Yuan et al, 1999). In addition dexamethasone treatment prior to irradiation reduces *p53* gene expression (Kamradt et al, 2000).

Kanai et al (1998) showed that in neoplastic lesions the expression of oestrogen receptors is markedly decreased in comparison to progesterone receptors which were increased. Steroid hormones induce the loss of normal growth control and abnormal cell cycle regulatory mechanisms. As a support for the above theory, a study by Hildesheim et al, (1990) showed that women who gave a history of recent or long-term (4 years) contraceptive use were at 2.3 and 2.9-fold increased risks of HPV positivity, respectively.

The exact role of the hormone receptors remain to be elucidated. In a study using a mouse model to study the effects of HPV 16-associated cervical cancers, Chung et al

(2008) reported that HPV 16 E7 and exogenous estrogen does not promote atypical squamous metaplasia in the absence of the α estrogen receptors.

A report by Madeleine et al (2001) described a population-based case-control study to examine the role of human papillomavirus (HPV) and oral contraceptive (OC) use in the etiology of adenocarcinoma in situ of the cervix (ACIS). One hundred and fifty women diagnosed with ACIS and 651 randomly selected control women completed in-person interviews. The presence of HPV DNA in archival ACIS specimens was determined by E6 and L1 consensus PCR. The overall prevalence of HPV DNA was 86.6%, with 39.0% positive for HPV-16 DNA, 52.4% positive for HPV-18 DNA and 13.4% positive for more than one HPV type. The age-adjusted relative risk of ACIS associated with HPV-18 seropositivity was 3.3 (95% confidence interval 2.2-4.9). No increased risk was associated with antibodies to HPV-16 L1. Among women born after 1945 the relative risk increased with the duration of OC use, with the highest risk for 12 or more years of use (odds ratio, 5.5; 95% confidence interval, 2.1-14.6) relative to non-users. The detection of HPV DNA in 86.6% of ACIS and the strong association of ACIS with HPV-18 L1 seropositivity underscore the importance of HPV, particularly HPV-18, in the aetiology of ACIS. In addition, long-term OC use may contribute to the pathogenesis of these tumors in some women.

However, to date there is no molecular evidence from human studies explaining the role of HPV and steroid contraception in the genesis of cervical cancer. Both estrogen and progesterone receptors have been described in cervical epithelium and it has been demonstrated that in HGSIL lesions high levels of hormone receptors, especially

progesterone receptors are expressed (Monsonego et al, 1991). It is thought that these receptors signal pathways which may 'synergise' with the cellular effects of high-risk HPV oncogenes (Elson et al, 2000). Steroid hormones may sensitise the transformation zone by altering the immuno-surveillance mechanisms such as antigen-presenting cell function (Ramoue et al, 2003). Elevated degrees of cervical ectropion and ectopy associated with the use of estrogen-containing steroid formulations may increase the susceptibility to infections, including HPV (Jacobson et al, 2000). Denny et al (1999) studied the extent of cervical epithelial disruption and ectopy associated with the use of depo-medroxyprogesterone acetate. In comparison to non-users of injectable depo-medroxyprogesterone acetate, there was no significant increase in epithelial disruption or ectopy among users (39% versus 38%).

A further role for hormones comes from the studies of von Knebel Doeberitz, et al (1994) and von Knebel Doeberitz (1997) which showed that dexamethasone treatment of various cervical carcinoma cell lines prevents the transcriptional activation of *p53*-regulated genes. Consequently, these cells display significantly relaxed G1/S cell cycle control and reduced activation of apoptosis upon genotoxic damage. Other evidence for the role of steroid hormones (McMillan et al, 1988) showed that steroid hormones reduce the number of antigen presenting molecules (MHC class II) on epithelial cells which under the influence of certain cytokines (IFN gamma or IL-2) express increased levels of MHC class II molecules. They also reduce the number of MHC class I molecules on dexamethasone-treated epithelial cells. This may reduce the immune surveillance of persistently HPV-infected keratinocytes increasing the chance for

genetically damaged HPV cells to find their way into dysplasia or neoplasia. Various researchers have shown that the HPV E16 oncoprotein binds to the p53 gene product and stimulate its degradation by ubiquitin-dependent protease systems (Sherr, 2000; Duensing et al, 2000). Steroid hormones are thought to increase the expression/transcription of the HPV E6 and E7 oncogenes, which in-turn, bind to and degrade the p53 gene product leading to apoptotic failure and cellular proliferation (de Villiers, 2003; Hubbert et al, 1992; Kessis et al, 1993).

Gavric-Lovrec and Takac (2010) published their findings regarding the link between use of various contraceptives, HPV 16 and 18 and prevalence of histologically-proven CIN lesions. The presence of HPV DNA was detected by in situ hybridization techniques. It was found that irrespective of type of contraceptive use and the presence of HPV types 16 and 18 infections.

The evidence provided above demonstrates a link between steroid hormones, the human papillomavirus and *p53* gene function. However, to date, there has been no molecular research performed in human subjects to prove the existence of transcriptional differences of the HPV *E6* oncogene in patients with cervical cancer who have used steroid contraception compared to women who have not used such agents.

12.0 THE IMPLICATIONS OF THE EVIDENCE PROVIDED FOR CLINICAL PRACTICE WITH REGARDS TO STEROID CONTRACEPTION

The oncology sub-committee of the World Health Organization referred to as the International Agency for Research in Cancer (IARC) has a working group of international experts who review the role of various agents/exposures in cancer. The consensus statements are published in the form of *Monographs* which critically review and evaluate the published scientific evidence on human carcinogenic hazards. More than 400 potentially carcinogenic agents and exposures have been evaluated and identified in 91 volumes of the monographs. Various national and international health authorities use the IARC monographs as scientific basis for the prevention of cancer. Volume 91 of July 2005 (http://monographs.iarc.fr) evaluated and summarized the status of steroid contraception in the role of cancers. In terms of the risks of various agents, four groups are described in the monographs. Group 1 states that the agent is carcinogenic to humans. This category is only used when there is sufficient evidence of carcinogenicity in humans. In June 2005, the IARC working group, after an evaluation of all available literature, classified steroid hormone contraception as a Group 1 direct cancer-causing agent to humans. The evidence was based mainly on epidemiological studies, some with conflicting results. It is important to note here that cervical cancer is prevalent mainly in the developing parts of the world where also unwanted pregnancies and maternal mortality is highest. To date, over 100 000 million women use steroid contraception as a means of fertility regulation. On the balance it seems as if the benefits of steroid

contraception outweigh the risks and the IARC has clearly stated that no change in clinical practice should be made. However, the patient should be counselled about the risks and benefits (Schneider et al, 2005). This is despite the fact that although regular cervical cytological screening is advised, it may not be readily available in many parts of the developing world.

13.0 HPV VACCINES AND THE FUTURE

In view of the low sensitivity of Papanicolaou smears there is a need to screen frequently and cover a sufficient percentage of the population to make any significant impact on the reduction in the prevalence of cervical cancer. This, however, is not feasible and not cost-effective especially in developing parts of the world where screening is most needed. Prevention of intraepithelial neoplasia should therefore be the target. Vaccine development programmes have been in existence from about 1933 and successful vaccines have become a routine part of health care programmes. It has been recognized that the L1 protein coat of HPVs could self-assemble into viral-like particles (VLPs) when expressed in recombinant eukaryotic systems. The antigenicity induced by these VLPs is almost identical to that induced by native virions. Vaccination with VLPs induces the production of neutralizing antibodies against HPV far greater than that recorded in natural infections (Schiller et al, 2008). Since HPV types are serotypically distinct, the VLPs induce type-specific protection against HPV infection (Christensen et al, 1994). Thus, vaccine development has focused on HPV types 16 and 18, which are the commonest HPV types present in cervical cancers. Vaccination against HPV 16 is

thought to confer almost two-thirds protection against cervical cancer. The two commercially developed vaccines include Cervarix™ (Bivalent HPV 16 and 18, GlaxoSmithKline Biologicals, Rixensart, Belgium) and Gardasil™ (Quadrivalent HPV 6, 11, 16 & 18, Merck and Co, Inc West Point, Pennsylvania, USA). HPV 16 and 18 are responsible for about 70% of all cervical cancers and these vaccines are expected to provide protection against an equivalent amount of cervical cancers.

The vaccines are delivered by intramuscular injection at 0, 2 and 6 month intervals. Many trials have been conducted in women at risk of HPV infection but who are disease- free at the onset of the trial. The first randomized trial was conducted using a HPV 16 VLP (Koutsky et al, 2002). Thereafter, trials were conducted with both vaccine types (Harper et al, 2004; Villa et al, 2005; Harper et al, 2006; Mao et al, 2006). Evidence from phase II and III trials of vaccine research show that both vaccines protect against persistent HPV 16 & 18 infections as well as HPV 16 & 18 –related diseases. Complete protection in vaccine recipients against new and persistent infections have been demonstrated compared with a significant rate of HPV infection among placebo recipients. Subsequently, two phase III, randomized, placebo-controlled trials of Gardasil, Females United to Unilaterally Reduce Endo/Ectocervical Disease (FUTURE) 1 and FUTURE 2 were performed (Schiller et al, 2008). The FUTURE 1 trial determined the efficacy of the vaccine to prevent HPV infection and genital warts, whereas FUTURE 2 was designed to determine the efficacy of the vaccine to prevent HPV-related CIN2/3, VIN and AIN. The vaccine was shown to be 100% effective in preventing CIN 2/3 as well as external genital lesions such as warts. A significant number of cases (59) were seen in the placebo arm of

the study. Combined analysis of the Gardasil trials and the HPV 16 monovalent vaccine trial has been reported (Ault, 2007). The protection against HPV vaccine type for CIN 3 was 98% (95% CI: 89-100). The efficacy was 100% (95% CI: 31-55) for the protection against adenocarcinoma in-situ. Gardasil was reported to be 100% protective (95% CI: 72-100) against incident vulval and vaginal high-grade dysplasias.

Cross-reaction between HPV type 16 and HPV 31 as well as between HPV 18 and HPV 45 has been described (Harper et al, 2006). The vaccine was shown to have an efficacy of 94% (95% CI: 63 – 100) for HPV 45 and 5% (95% CI: 12 – 78) for HPV 31 (Harper et al, 2006). Data from the FUTURE I/II trials reported at scientific conferences has provided evidence that the quadrivalent vaccine has partial prophylactic efficacy against incident CIN 2/3 caused by HPVs not covered by the vaccine. The benefit of such cross-reactivity would increase the coverage of a HPV vaccine developed primarily against HPV types 16 and 18.

Although the initial vaccine trials were conducted among women between the ages of 16 and 25 years, bridging studies conducted among boys and girls aged 9 and 15 years as well as older women (24 – 45 years for Gardasil ® and ages 26 – 55 years for Cervarix ®) have demonstrated efficacy of the vaccine across a wide age range (Schwartz et al, 2006). Cervarix ® has been approved from age 10 years to 45 years on the basis of bridging results. The first priority of vaccination programs should be the vaccination of adolescent girls prior to the onset of sexual activity. Young adult women who have not commenced sexual activity would also be expected to derive benefit from vaccination since the

bridging studies have demonstrated evidence of such benefit. It is thought that sexually active women would also benefit from vaccination as only 1% of such women is likely to be infected with all four HPV types at once. In the European Union, Gardasil is approved for boys between the ages of 9 to 15 years. Women in turn would benefit from vaccination of males by producing a larger degree of herd immunity (Garnett, 2005). HPV 16 accounts for about 50% of all cervical cancers and therefore vaccines will need to contain more HPV types in order to reduce a greater percentage of cervical cancers. It is therefore crucial that for every country which intends to implement a vaccination program the frequency of the various HPV types in both dysplastic and invasive cervical cancers needs to be determined.

Implementation of a vaccination program requires the on-going availability of a screening program in order to detect dysplastic lesions due to HPV types not included in the vaccine or vaccine failures. Education of women about HPV, HPV-related diseases and HPV vaccination will play a major role in the prevention of cervical cancer. In a study to assess women's knowledge, attitudes and behaviour related to HPV and HPV vaccination in Ontario, Canada, it was found that women had moderate levels of knowledge of HPV-related issues (Lenehan et al, 2008). However, based on the information provided, women expressed willingness to support preventative measures such as vaccination. In the developing world this situation is expected to be far worse as knowledge is expected to be more deficient. A major challenge therefore exists in low-resource settings where screening and the implementation of vaccines will take time to

materialize. This is further compounded by the epidemic of HIV in low-resource settings wherein the immunogenicity of HPV vaccines remains to be established.

SUMMARY OF THE LITERATURE

Steroid contraception is thought to be a co-factor in the pathogenesis of HPV-induced cervical dysplasias and cancers. Although the exact mechanism is not clearly elucidated, the current thinking is that steroids enhance the expression of high-risk HPV oncogenes, which in turn lead to the degradation of the p53 and pRb tumour-suppressor gene products. Protection against HPV types which are responsible for pre-invasive and invasive cancers of the lower female genital tract by vaccine technology should lead to a reduction of such diseases irrespective of the co-factors at play. HPV types may be population-dependent and an attempt to establish the HPV types in a given population is necessary to ensure adequate population coverage. The aims of the research is therefore two-fold: to determine the HPV types prevalent in groups of women who have used oral contraception for varying durations and to determine the interaction of the injectable progesterone contraceptive steroids and the HPV oncogene expression.

14.0 PART ONE OF THE PROJECT

AN INVESTIGATION INTO ORAL CONTRACEPTIVE USAGE, HUMAN PAPILLOMAVIRUS (HPV)-TYPE DISTRIBUTION AND CERVICAL INTRAEPITHELIAL NEOPLASIA, DURBAN, SOUTH AFRICA

14.1 HYPOTHESIS AND AIMS

THERE IS NO DIFFERENCE IN HPV TYPE-DISTRIBUTION AMONGST WOMEN IN DURBAN, SOUTH AFRICA, WITH CERVICAL DYSPLASIA IN COMPARISON TO THAT PUBLISHED IN THE LITERATURE FOR FIRST WORLD COUNTRIES IRRESPECTIVE OF ORAL CONTRACEPTIVE USAGE

a. To determine the prevalence of the HPV types among women with varying duration of oral contraceptive usage,
b. To determine the relationship between oral contraceptive usage, HPV-type distribution and cervical intraepithelial neoplasia

14.2 PATIENTS AND METHODS

After institutional ethical approval was granted, patients were prospectively recruited from the colposcopy clinics of King Edward VIII and Inkosi Albert Luthuli Central Hospitals, Durban, South Africa. This aspect of the work was performed in 2009. Only patients who met the criteria to study the objectives of this study were recruited. Patients

were divided into four groups: group 1- no contraceptive usage, group 2- oral contraceptive use of less than 5 years duration, group 3- oral contraceptive use of between 5 and 10 years duration and group 4- oral contraceptive use of more than 10 years duration. Patients were recruited on a case-control basis. The study was performed in the course of normal clinical duties. Therefore all women with abnormal Papanicolaou smears warranting colposcopy were selected. After the history and examination, they were subjected to colposcopic examination by myself and biopsies were taken in addition to the normal process of Lancet laboratory provided a medium for the transport of the swab specimens to their laboratory so that contamination and handling errors were avoided.treatment by the Large Loop excision of the Transformation Zone technique (LLETZ). All data including the nature of the abnormal Papanicolaou smear findings, HIV status and contraceptive usage were recorded on a questionnaire. After counselling, bloods were taken for HIV status. Prior to colposcopy examination, a swab was taken of the ectocervix and endocervix for HPV DNA typing. Colposcopy and treatment in the form of large loop excision of the transformation zone (LLETZ) was performed**.** At the follow-up visit patients were counselled regarding their HIV status and bloods were taken for CD4 counts where relevant. Human papillomavirus DNA typing was performed using the Roche Linear Array Polymerase Chain Reaction (PCR) genotyping test (Roche Molecular diagnostics, Pleasanton, CA) at the Lancet laboratory, South Africa. At present, lancet laboratory is the only laboratory which offers HPV typing for commercial use. Laboratory personnel assisted in the HPV typing using the Roche Linear Array PCR technique. Correlation was made regarding the Papanicoloau smear result, histology findings, contraceptive usage, HPV DNA status and HIV serostatus.

14.3 STATISTICAL METHODS

The sample size was determined by a professional statistician and was based on HPV types in published literature. At the time of this project, no data existed for HPV types in South African women. An association between contraception use and high risk HPV DNA types with other variables was evaluated by calculating odds ratios and by chi-square tests or Fisher's exact tests where appropriate. Logistic regression was then used where required to adjust the association for confounding by age. Statistical analysis was done using Stats Statistical Software: Release 10.

14.4 RESULTS

A total of 124 women were recruited for the study. There were four groups of patients according to oral contraceptive usage: non-users, users of less than 5 years duration, users of between 5 and 10 years duration and users of more than 10 years duration. The mean age of all patients was 39.2 years (range 22 – 78 years). The mean age of patients according to these four groups was: 38.3 years, 39 years, 36.3 years and 44 years, respectively. Table I illustrates the demographic factors for all 124 patients. There were 104 patients (84%) with high-grade squamous intraepithelial lesions (HGSIL) detected on Papanicolaou smear findings, whilst 20 patients (16%) had low-grade squamous intraepithelial lesions (LGSIL). The distribution of patients with HGSIL according to the four contraceptive groups was: 28 (88%), 24 (80%), 26 (84%) and 26 (84%), respectively.

Of the total of 124 patients, there were 75 patients who were HIV-infected resulting in a HIV sero-prevalence of 61%. Two patients declined HIV testing. For each of the four contraceptive groups, the HIV sero-prevalence was 78% (n=25), 55% (n=16), 67% (n=20) and 45% (n=14), respectively. The CD4 counts for the 75 patients are illustrated in table I.

Table I: Demographic factors for 124 patients

	Contraception										
	None		< 5 yrs		5-10 yrs		>10yrs		Total		p value
	n	%	n	%	N	%	n	%	n	%	
Age(mean)	38.3	(7.8)	38.7	(9.6)	36.3	(10.4)	43.8	(10.3)	39.2	(9.8)	0.01
Parity											
< 3	23	72%	19	63%	21	68%	17	55%	80	65%	
>= 3	9	28%	11	37%	10	32%	14	45%	44	35%	0.5
Number of partners											
< 3	21	66%	20	67%	19	61%	26	84%	86	69%	
>= 3	11	34%	10	33%	12	39%	5	16%	38	31%	0.2
Marital Status											
Married	5	16%	6	20%	6	19%	12	39%	29	23%	
Not married	27	84%	24	80%	25	81%	19	61%	95	77%	0.2
Partner Circumcised											
Yes	6	19%	9	30%	9	29%	10	32%	34	27%	
No	26	81%	21	70%	22	71%	21	68%	90	73%	0.6
HIV											
Positive	25	78%	16	55%	20	67%	14	45%	75	61%	
Negative	7	22%	13	45%	10	33%	17	55%	47	39%	0.045
CD4 group											
< 200	16	64%	9	56%	11	55%	6	43%	42	56%	
200-350	7	28%	2	13%	4	20%	4	29%	17	23%	0.4
> 350	2	8%	5	31%	5	25%	4	29%	16	21%	
Pap smear											
HGSIL	28	88%	24	80%	26	84%	26	84%	104	84%	
Other	4	13%	6	20%	5	16%	5	16%	20	16%	0.9
Smoking											
Yes	1	3%	4	13%	2	7%	6	19%	13	11%	
No	31	97%	26	87%	28	93%	25	81%	110	89%	0.2
Warts											
Yes	4	13%	4	13%	3	10%	0	0%	11	9%	
NO	28	88%	26	87%	28	90%	31	100%	113	91%	0.2
Colposcopy											
HGSIL	30	94%	29	97%	30	97%	29	94%	118	95%	
Other	2	6%	1	3%	1	3%	2	6%	6	5%	0.9
Treatment Dysplasia											
LLETZ	32	100%	30	100%	31	100%	30	97%	123	99%	
Hysterectomy	0	0%	0	0%	0	0%	1	3%	1	1%	0.7
Histology Results											
HGSIL	23	72%	21	70%	17	55%	19	61%	80	65%	
Other	7	22%	7	23%	2	6%	4	13%	20	16%	
No abnormality	2	6%	1	3%	3	10%	2	6%	8	6%	
Not graded	0	0%	1	3%	7	23%	6	19%	14	11%	

Of these patients, 56% (n=42) had CD4 counts of less than 200 cells/µL. All these patients were receiving anti-retroviral therapy. The majority of patients (n=123; 99%) were treated with large loop excision of the transformation zone (LLETZ) as a single-step procedure at the time of colposcopy. One patient had biopsy-confirmed HGSIL followed by a hysterectomy as per patient request and history of menorrhagia. There were 8 patients (6%) whose histology results revealed no dysplasia and 14 patients who had dysplasia, the grade of which could not be determined on histology due to cautery artefact and /or traction distortion. All 22 patients were followed up with repeat Papanicolaou smears with no evidence of dysplasia at the 6 month follow-up visit.

Figure 8: High-risk HPV type distribution

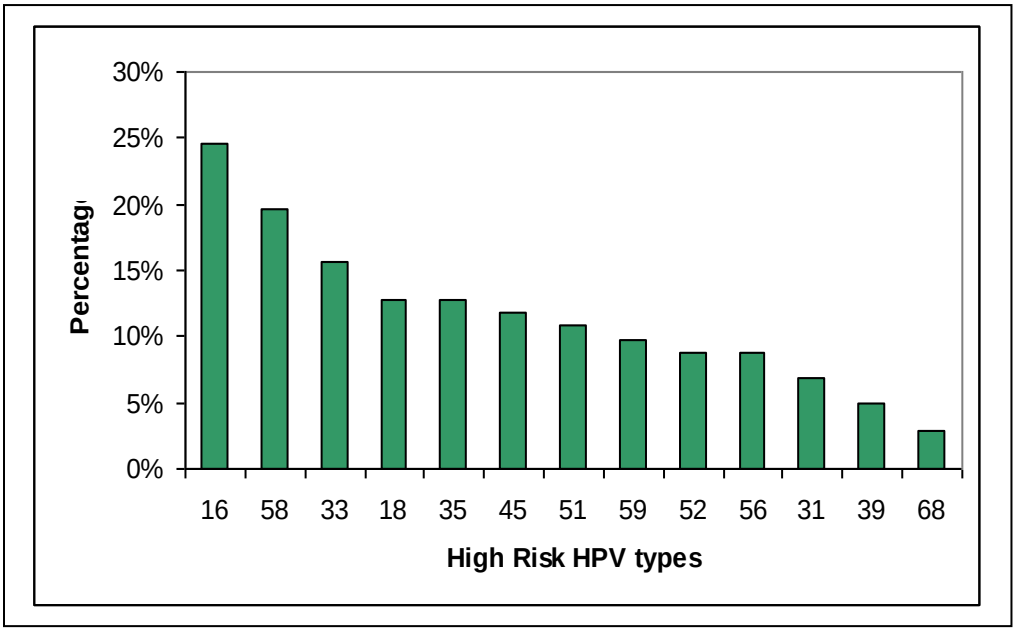
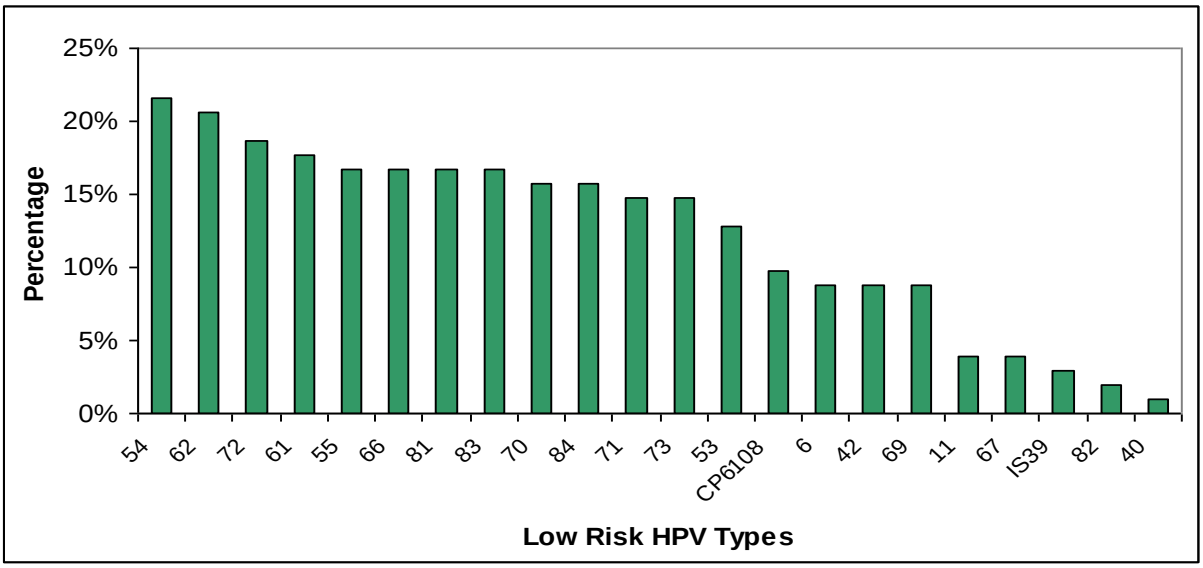


Figure 9: Low-risk HPV type distribution



With regards to HPV DNA detection, there were 102 patients who were HPV positive (82%). All 22 patients who were HPV DNA negative were retested to exclude a false negative result these patients and remained HPV negative. Of the 102 patients there were 79 patients who had high-risk HPV DNA (78%). The prevalence of high-risk and low-risk HPV DNA is illustrated in Figures 8 and 9, respectively. In terms of the four oral contraceptive groups, high-risk HPV DNA was detected in 70% (n=21), 79% (n=22), 90% (n=21) and 71% (n=15) of patients, respectively. The distribution of HPV DNA negative results for the four contraceptive groups was: 2, 2, 8 and 10 patients. High-risk and low-risk HPV DNA was detected in 52 and 23 HIV-infected patients compared with 26 and 21 HIV non-infected patients, respectively. There was no difference in the detection of high-risk HPV DNA between HIV-infected and HIV non-infected women

(p=0.1). There was also no difference in the detection of high-risk HPV DNA and low-risk HPV DNA according to CD4 counts (p=0.9). High-risk HPV DNA and low-risk HPV DNA according to CD4 counts is illustrated in Table II.

CD4 group	High risk HPV		Low-risk HPV		Total	P value
< 200	29	81%	7	19%	36	
200-350	12	80%	3	20%	15	
> 350	11	85%	2	15%	13	0.9

Table II: CD4 counts versus high-risk and Low-risk HPV status

If the group of non-contraceptive users and users of less than 5 years duration are combined and compared with users of between 5-10 years and more than 10 years duration, then the distribution of high-risk HPV DNA was 74.1% (43/58) and 82% (36/44), respectively (p=0.4). The odds of having HPV DNA was six times higher for the combination of contraceptive users of less than 5 years duration/non-users (OR 5.9, 95% CI: 1.87 – 18.77). Using logistic regression analysis and adjusting for age, there was no change when adjustment was made for age (OR 6.1, 95% CI: 1.9 – 19.4). The distribution of HPV DNA versus age and high-risk HPV DNA and low-risk HPV DNA versus age is illustrated in Tables III & IV, respectively.

Table III: HPV DNA status versus Age

Age group 9years)	HPV negative	HPV positive	Total
< 30	5 (33%)	10 (67%)	15
30 – 39	6 (11%)	48 (89%)	54
≥ 40	11 (20%)	44 (80%)	55
Total	22	102	122

Table IV: High-risk and Low-risk HPV versus age

Age group (years)	Low-risk HPV	High-risk HPV	Total
< 30	2 (20%)	8 (80%)	10
30 – 39	9(19%)	39 (81%)	48
≥ 40	12 (27%)	32 (73%)	44
Total	23	79	102

HPV DNA types 16 and/ or 18 was present in a total of 21 patients (49%) (non-contraceptive users and users < 5years duration) versus 15 patients (42%) who used oral contraceptives of more than 5 years duration (p=0.524). In other words, the odds of having HPV DNA types 16 and 18 were similar by duration of contraceptive use. Only three patients had HPV DNA type 16 alone whilst 5 patients had HPV DNA 16 in association with a second HPV type. There were 36 patients who had HPV 16 and 18 in addition to other HPV types. There were 13 patients with only 2 HPV types other than types 16 and 18. The majority of patients therefore had multiples HPV types.

There were 20 patients who had a single HPV type detected. These single types included types 35, 16, 6, 45, 62, 33, 69, 81, 58, 72, 83, 54, 51, 31, 82 and 52. The prevalence of

HPV types 31, 33 and 45 was 7%, 16% and 12%, respectively. Although HPV type 16 was the commonest HPV type detected (20.2%), HPV type 58 was the next commonest high-risk HPV type and was detected in 16.1% of patients. The odds of having HPV type 33 was 2-fold if the patient was HIV-infected in comparison to patients who were HIV non-infected (OR 2.04, CI: 0.6 – 9.2). The five most common high-risk HPV types in relation to HIV status is reflected in table V.

Table V: High-risk HPV distribution in relation to HIV status

High-risk HPV	HIV-infected women (%)	HIV non-infected women (%)
16	20	21
58	19	11
33	16	8
18	14	4
45	45	6

Table VI and Figure 10 illustrate a comparison of HPV types in sub-Saharan Africa with the HPV types obtained in this study (Clifford et al, 2003).

HPV Type	Review paper Sub Saharan Africa Percentage	% of HPV + Index study n=102 Percentage	p value
16	48%	25%	<0.001
18	19%	13%	0.03
45	15%	12%	0.2
33	3%	16%	<0.001
58	3%	20%	<0.001

Sign rank test p = 0.14

Table VII: Comparison of high-risk HPV types worldwide (Clifford, 2003) and index study

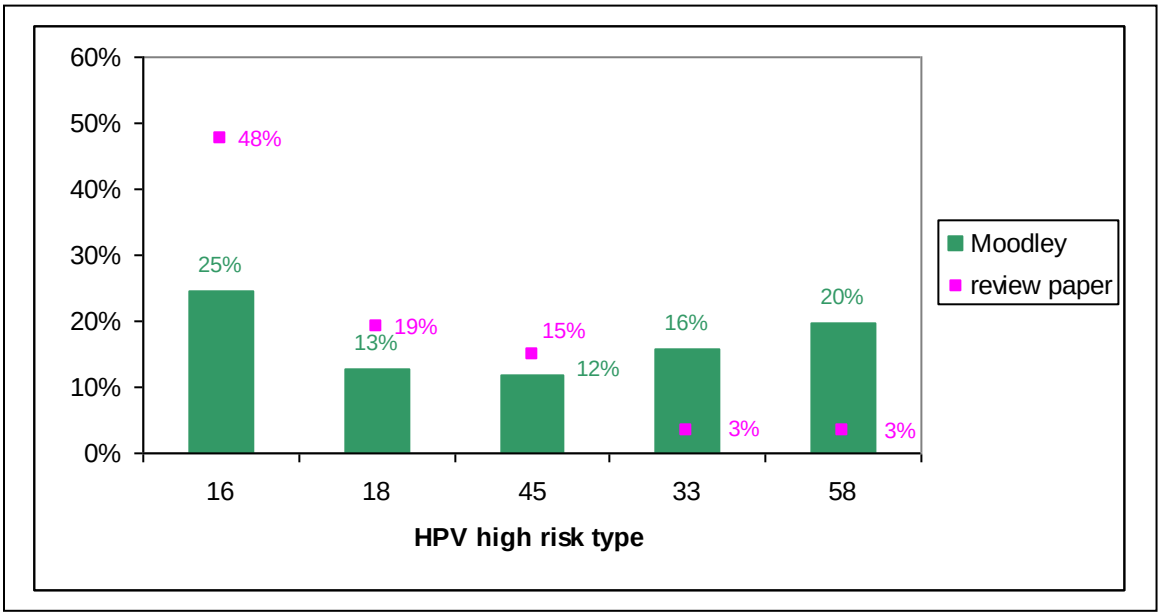


Figure 10: Comparison worldwide high-risk HPV prevalence (Clifford, 2003) and index study

14.5 DISCUSSION

Epidemiological research has supported the role of HPV as a necessary aetiological agent in the development of cervical dysplasia and cervical cancer (Walboomers et al, 1999; zur Hausen, 1982). Further, HPV is also implicated in the development of intraepithelial lesions and invasive cancers of the lower female genital tract and genital and anal cancers in males and females (IARC, 2007; Nielsen et al, 2007; Micali, 2006). The evidence for the role of HPV is also borne out in HPV vaccine trials which have demonstrated 100% efficacy in the protection against HPV types 6, 11, 16,18-related vulval and vaginal intraepithelial neoplasias (Joura et al, 2007). It is reported that at any given point in time about 10.4% of women across the world with normal cervical cytology will harbour the human papillomavirus (Clifford et al, 2006).

Steroid contraception has been epidemiologically linked as a co-factor in the development of cervical cancer and pre-neoplastic cervical lesions. Although these data have been inconsistent, many studies have reported an increased risk (Moodley et al, 2003; Green et al, 2003). However, epidemiological studies have provided a strong and statistically significant association, especially among long-term users of oral contraception (Green et al, 2003; Delgado-Rodriguez, 1992). There is a consistent association between long-term oral contraceptive users of more than 5 years duration and cervical neoplasia (Smith et al, 2003). Increased risk for the use of oral contraception and cervical cancer is also reported for adenocarcinoma in-situ and invasive adenocarcinomas. Oral contraceptive users of 6 years or more duration are reported to

have a 6-fold increased risk of adenocarcinoma in-situ (Lacey et al, 1999). Data from the IARC consisting of pooled analysis of case-control studies have reported that HPV-positive women who have used oral contraception were 42% more likely to develop carcinoma in-situ and cervical cancer than never users (OR 1.42; 95% CI: 1.0 – 2.0) (Moreno et al, 2002). Biological plausibility has been provided because of increased acquisition, persistence and progression of HPV-infected epithelia to cervical cancer as well as the presence of hormone receptors in the cervix (Moodley et al, 2003; de Villiers 2003). It is postulated that steroid contraceptives increase the expression of the *E6/E7* oncogenic HPV genes of the high-risk HPV viruses, which in turn enhance the degradation of the p53 tumour suppressor gene product (Moodley et al, 2003).

In contrast, the report from the Royal College of General Practitioner's Oral Contraception study found no association with cervical cancer and that steroids, may in fact, have a net public gain (Hannaford et al, 2007). This study had commenced in 1968 and included datasets of cancers and observation of women by their general practitioners. In comparison to never users, ever-users had a statistically significant reduction in the risk of any cancer (adjusted relative risk 0.88, CI: 0.83 – 0.94). Statistical significant reductions were found for cancers of the large bowel or rectum, uterine body and ovaries. A small non-significant increase was found for cancers of the lung, cervix and central nervous system. A molecular study of the interaction between steroid contraception, HPV, *E6* HPV 16 gene expression and cervical cancer in our setting revealed no increase in expression between steroid users and controls (Moodley et al, 2003).

If steroid contraception influences the development of cervical neoplasias via HPV or high-risk HPV, then an analysis of the type distribution of HPV among oral contraceptive users of varying duration would be relevant. To our knowledge, no such report has been documented in South Africa. The high HIV sero-prevalence of 61% in this study is reflective of a sexually active younger population of women in the study. Just over half of patients had CD4 counts less than 200 cells/µL but were receiving anti-retroviral therapy. Most of these women are referred by the HIV clinics with an abnormal Papanicolaou smear for colposcopy.

The finding of 82% HPV prevalence in this study among women with abnormal cytology is similar to that reported for Africa (85%), Americas (83%), Europe (88%) (Bosch et al, 2008). In contrast, HPV prevalence among cytologically normal women across the world include reports of 22% in African women, 20.4% in women from the Americas, 11.3% among North American women, 8.0% in Asian women and 8.1% in European women (Bosch et al, 2008). In a meta-analysis of HPV in 3230 HIV-infected women with normal cytology, HPV prevalence was reported to be 57% in African women compared with 31% in Asian women, 32% in European women, 31% in North American women and 57% in women from South/Central America (Clifford et al, 2006). In a study to determine the natural history of high-risk HPV and cervical disease in Cape Town, South Africa, the prevalence of high-risk HPV in a cohort study of HIV-infected women was 68% (n=400) (Denny et al, 2008). The prevalence of high-risk HPV among the HIV-infected women was 51% (52/102) and 78% among all women in this study. A difference was found in the detection of high-risk HPV DNA if HIV status or CD4 counts were compared, perhaps because of the smaller number of women who had HPV (n=102). The

five most prevalent HPV types and high-risk HPV types in this study were HPV 54, 62, 16, 58 and 72 and HR-HPV 16, 58, 33, 18 and 35, respectively, in order of decreasing frequency. In contrast, the five most common high-risk HPV types from the study in Cape Town, South Africa were HPV 16, 52, 53, 35 and 18, in order of decreasing frequency. In a meta-analysis of high-risk HPV types among African women with HGSIL, HPV type 16 was the commonest (41%) followed by HPV type 31 (10.1%). This study showed that HPV 16 was the commonest high-risk HPV type detected, but in only 20.2% of women, followed by HPV 58 (16.1%). HPV type 31 occurred in 5.6% of women in our study. Figure 3 illustrates a comparison of high-risk HPV types in this population of women with that of non-South Africans (Clifford et al, 2003). Table V reflects the differences in high-risk HPV distribution between these two populations and it can be noted that HPV types 58 and 33 occur in a much greater percentage of our population and HPV 16 in a much smaller percentage of this population compared with the non-South African population.

It would have been expected that the reason for finding a greater percentage of high-risk HPV in the combined groups of women who did not use oral contraception and users of less than 5 years duration was due to an age factor. However, logistic regression analysis considering the age factor revealed that age did not account for the observed difference. Human papillomavirus type 16 alone was detected in only 3 women, whilst HPV types 16 and 18 were detected in 36 women in this study. However, the occurrence of multiple HPV types among most women is similar to that described in many other studies (Clifford et al, 2006; Denny et al, 2008). HPV DNA is detected in virtually 100% of women with cervical carcinomas (Walboomers 1999). The type- specific distribution

among African women with carcinomas include HPV 16 (55%), 18 (16%), 33 (7.6%), 45 (6.5), 31 (2.9%), 58 (2.7%) and 52 (1.5%). The findings of the HPV-type distribution in this study are important in understanding the potential effects of HPV vaccination against HPV types 16 and 18. This study did not include women with cervical cancers and it is possible that HPV 16 and 18 in cervical cancers might still reflect types 16 and 18 to be the commonest types in our population. However, a polyvalent vaccine designed to cover HPV types such as 58 and 33 might improve the coverage and therefore impact in reducing the prevalence of cervical cancer in an African/ South African population of women.

In conclusion, although the number of patients in this study is small, the information on HPV type distribution adds to the database of HPV type distribution in an African population. It is also accepted that the HPV types detected among women with established cervical intraepithelial lesions may not reflect the HPV types which were initially present and promoted the development of such lesions. The role of steroid contraception in relation to HPV remains to be proven by prospective laboratory-based research.

15.0 PART TWO OF THE PROJECT

AN INVESTIGATION INTO HPV 16 *E6* ONCOGENE- EXPRESSION AND USE OF INJECTABLE MEDROXY-PROGESTERONE STEROID CONTRACEPTIVES AMONG WOMEN WITH INVASIVE CERVICAL CANCER

15.1 HYPOTHESIS OF THE STUDY

Steroid hormones enhance/increase the expression of the human papillomavirus type 16 *E6, E6**1 and *E6**11 gene expression in women with cervical cancer who have used injectable progesterone contraception compared with women with invasive cervical cancer who have not used injectable contraception.

The literature search has demonstrated evidence that the increased expression of these genes lead to degradation of the p53 tumour-suppressor gene product predisposing patients to cervical dysplasia and cancer.

15.2 AIMS

1. To compare human papillomavirus type 16 *E6* oncogene- expression in women with cervical cancer in relation to the use of injectable medroxyprogesterone acetate.
2. To compare human papillomavirus type 16 *E6**1 and *E6**11 spliced variant expression in women with cervical cancer in relation to injectable medroxyprogesterone acetate contraceptive usage.
3. To compare human papillomavirus type 16 *E6, E6**1 and *E6**11 oncogene expression with clinical factors such as duration of contraceptive use and clinical stage of disease.

15.3 MATERIALS AND METHODS

All patients recruited for this study were managed at King Edward VIII Hospital, Durban, South Africa. Institutional ethical approval was obtained prior to commencement of the project. All patients were counselled regarding the nature of the project and the contribution required of them. The nature of this counselling was built into the proposal. Only patients who tested negative for the human immunodeficiency virus infection were recruited for this study. This was to avoid issues of transportation, permission to export the samples to the United Kingdom where the study was done and exposure to the laboratory staff. Patients for this study were recruited on a case-control basis, i.e. confirmed cases of cervical cancer

amongst patient who used depo medroxyprogesterone acetate contraception in the study arm and patients who did not use this form of contraception with cervical cancer in the control arm. The sample size was determined by a professional statistician and was based on the probability of finding the E6/E6*1 and E6*11 genes.

The entire project was performed by my self from the stage of recruitment, counselling of patients, collection of tissue samples to laboratory investigations. A total of 120 cervical tissue samples were obtained from patients with proven cervical cancer. Two punch biopsy specimens of the tumour tissue were taken. These specimens were snap-frozen and transported in liquid nitrogen to the laboratory. The biopsies were taken adjacent to each other from the cervical tumour. One specimen was used for the typing of human papillomavirus, whilst the second specimen was used for the main project if the tumour tissue contained HPV type 16. This second specimen was stored at -80^0 celcius until the final project was performed. The laboratory work was performed by my self in the laboratory of the Biological and Life Sciences department of the University of Liverpool, UK under the supervision of Professor CS Herington.The laboratory methods utilised were as follows:

15.4 LABORATORY METHODS

15.4.1 TYPING OF CERVICAL TISSUE SPECIMENS FOR HPV 16;

15.4.2 GROWTH OF CELL LINES *IN VITRO;*

15.4.3 EXTRACTION OF RIBONUCLEIC ACID (RNA) FROM TISSUE SPECIMENS;

15.4.4 ASSESSMENT OF THE QUALITY AND QUANTITY OF RNA EXTRACTED BY SPECTROPHOTOMETRY;

15.4.5 REVERSE TRANSCRIPTION OF RNA TO SYNTHESISE cDNA USING REVERSE TRANSCRIPTASE ENZYME;

15.4.6 MOCK REVERSE TRANSCRIPTION OF RNA WITHOUT REVERSE TRANSCRIPTASE ENZYME TO DIFFERENTIATE RNA FROM GENOMIC DEOXYRIBONUCLEIC ACID (DNA);

15.4.7 POLYMERASE CHAIN REACTION OF PRODUCTS FROM 16.5) AND 16.6) ABOVE USING SPECIFIC PRIMER PAIRS TO THE *E6* ONCOGENE;

15.4.8 NESTED POLYMERASE CHAIN REACTION OF PRODUCTS FROM 16.7) ABOVE USING SPECIFIC PRIMER PAIRS TO DETERMINE THE EXPRESSION OF HPV 16 *E6*I* AND *E6*II* SPLICED VARIANTS;

15.4.9 GEL ELECTROPHORESIS TO DETERMINE THE EXPRESSION OF THE *E6, E6*I* AND *E6*II* ONCOGENES IN BOTH GROUPS OF PATIENTS;

15.4.10 COMPARISON OF THE PRESENCE OR ABSENCE OF BANDS WITH

THE USE OF THE S3/S4 PRIMERS AND S1/S2 PRIMERS IN RELATION TO THE USAGE OF STEROID CONTRACEPTION

15.4.1 TYPING OF THE CERVICAL CANCER TISSUE SPECIMENS FOR HUMAN PAPILLOMAVIRUS TYPE 16

Reagents:

1. Buffer A

10 mmol/l Tris	(pH 7.4)	1.21 g/l
100 mmol/l NaCl		5.84 g/l
10 mmol/l EDTA	(pH 8.0)	3.72 g/l

2. RNAse 15 μg/ml add fresh before each extraction.
3. Proteinase K

   15 μg/ml Buffer A
4. 10% SDS

   10 g SDS up to 100 ml with distilled water. Do not autoclave.
5. 6 mol/ l NaCl

   35g NaCl/100 ml with distilled water
6. Chloroform / Isoamyl alcohol

   Mix in ratio of 24:1
7. Chloroform (Merck)

8. 70% ethanol
9. TE Buffer

10 mmol/l Tris (pH 7.4)	1.21 g/l
1 mmol/l EDTA (pH 8.0)	0.273 g/l

15.4.1.1 DNA EXTRACTION

The method used for DNA extraction was a modification of the procedure described by Miller et al (1997) and involved the following steps:

1. Cervical tissue was sliced very thinly with scalpel blade and added to 5 ml buffer A.
2 Proteinase K (15 µg/ml), RNAse (15 µg/ml) and 10% SDS (800µl/ml buffer A) was added. (To 5 ml Buffer A 4 ml SDS was added). This was incubated overnight at 37^{o} celcius and mixed occasionally when possible. An additional 15 µg proteinase K was added and temperature was raised to 50 ^{0} celcius if all tissue was not digested.
3 6 mol/l NaCl was added to a final concentration of 1 mol/l NaCl. Diluted NaCl 1:6. (To the 9 ml of the above, 3.5 ml 6 mol/l NaCl was added).
4 This was gently shaken by inversion for 10 minutes at 22 ^{0} celcius (room temperature).
5 The mixture was centrifuged at 2000g for 15 minutes at 22 ^{0} celcius and supernatant removed to a polypropylene tube.

6 Equal quantities of chloroform / isoamylalcohol were added gently mixed by inversion for 5 minutes.

7 This was centrifuged at 3000g for 10 minutes at 8 ^{0} celcius and the supernatant was decanted into a clean polypropylene tube.

8 Equal quantities of chloroform was added and mixed gently by inversion for 5 minutes.

9 This was centrifuged at 3000g for 10 minutes at 8 ^{0} celcius and the supernatant removed to a clean clear glass tube.

10 Equal quantities of isopropanol was added and inverted gently until DNA strands were precipitated out of solution.

11 The DNA was hooked out with a sealed hooked Pasteur pipette. This was washed for 2 minutes in 70% ice cold ethanol by dipping the DNA into it. The DNA was transferred into a 200 – 400 µl TE buffer.

15.4.1.2 HPV TYPING

Tumour DNA was analysed for the presence of HPV by polymerase chain amplification using the LI consensus primers MY 11 and MY 09, and the cycling conditions recommended by Manos et al (1994). The sequences of these primers are as follows:

PRIMER 1 (MY 11)

5’ – GCMCAGGGWCATAAYAATGG - 3’

PRIMER 2 (MY09)

5’ – CGTCCMARRGGAWACTGATC – 3’

M = A + C R = A + G W = A + T Y = C + T

(Primer 1 is a sense strand sequence which binds to the complimentary anti-sense DNA strand running from 3’ to 5’. Primer 2 is an anti-sense sequence and binds to the complimentary sense DNA strand running from 5’ to 3’. These primers are degenerate at the positions indicated, at which one or other nucleotide is substituted to render them complimentary to the DNA of different HPV types).

PCR Procedure

Tumour DNA from patients in the study was amplified as follows:

Each PCR reaction contained 16µL DNA (ranging from 9ng to 75ng), 5 µl of *Taq* polymerase buffer, 2 mmol/L $MgCl_2$ 1 µmol/L of each primer, 200 µmol/L of each dNTP and 1.25 units of *Taq* polymerase made up to a final volume of 50 µl with sterile de-ionized water. The mixture (excluding DNA) was then aliquoted to

each reaction tube using a positive displacement pipette. The DNA was boiled for 10 minutes and chilled on ice prior to being added to the PCR mixture. After spinning briefly, a few drops of mineral oil were added to each tube. Each run included a reaction mix without DNA to monitor for contamination of reagents

The PCR cycling conditions consisted of:

93^0 celcius for 1 minute (denaturation)

55^0 celcius for 1 minute (annealing)

72^0 celcius for 1 minute (extension)

A final 5 minute extension at 72^0 celcius completed the reaction.

Sequencing:

The PCR product was then cycle sequenced using the MY 09 primer and the Big Dye™ Cycle Sequencing Kit (Applied Biosystems, Foster City, Ca, USA) on an Applied Biosystems Prism 310 Automated Genetic Analyser. The sequences were analysed using the Sequence Navigator Software (v. 1.0.1 – Applied Biosystems) and aligned manually to the following Gen Bank sequence: HPV 16 (V89348)

Amplified tumour DNA was classified as HPV 16 when there was a 96 - 100% match with a 28 base region specific to the HPV 16 type and an approximately 95% match with the remaining amplified L1 sequence. In the case of non-

amplification with the L1 consensus primers, 3 repeat amplifications attempts were carried out.

15.4.2 GROWTH OF CELL LINES *IN- VITRO*

Three cell lines were grown in culture. The CaSki and SiHa cell lines are both human cervical carcinoma cell lines which carry transcriptionall active human papillomavirus 16 genomes. The C33A cell line does not contain the HPV genome. These cell lines were used as positive (CaSki/SiHa) and negative (C33A) controls to detect false positive and false negative results.

These cell lines were stored at -190^0 celcius. They were transported on dry ice to the laboratory where the cultures were grown. Fetal bovine serum (GIBCOBRL® Technologies) stored at -20^0 celcius was placed in a water bath and allowed to thaw. Once thawed, it was transferred in a hood under which all subsequent steps were performed. Fifty milliliters of fetal bovine serum was added to 500 ml of Dulbecco's Modified Eagles Medium (MEM)(4^0C) (GibcoBRL® Lifetechnologies) to give a 10% solution. Five milliliters of Streptomycin/Penicillin was added to the mixture.

Then 5 ml of this mixture was transferred to 2 culture flasks (25 cm^2). The culture chamber was then transferred to an incubator at 37^0 celcius supplemented with carbon

dioxide and incubated. The cultures were assessed the next day and were found to grow to confluence.

The media from the flasks were removed (cells attached to the walls of the flasks) and 2 ml EDTA was added to each of these flasks and re-incubated at 37^0 celcius for 5 minutes. The cultures were examined under the microscope and loose cells were noted to be present. Six millitres of the volume from each flask was transferred to a universal tube. These tubes were centrifuged at 4 g for 3 minutes. Pellets of cells were present at the bottom of the tubes. The media was removed and 2 ml Dulbecco's modified eagles medium was added to each tube. One milliliter of the solution from each tube was transferred to a 75 ml flask.

Extraction of RNA was then performed from these cell pellets as per tissue protocol described below.

15.4.3 EXTRACTION OF RNA FROM CELL LINES AND CERVICAL TUMOUR TISSUE

15.4.3.1 CELL LINES

Once the confluence of growth was noted, the growth media was removed. Thereafter, 2.5 ml of TRIZOL (GibcoBRL®, Life technologies) RNA isolation solution was added (1ml/10cm^2). The flask was gently shaken from side to side and the TRIZOL was pipetted out of the flask repeatedly to loosen cells from the wall of the flask. One millitre of this solution was used to extract RNA as per tissue extraction protocol.

15.4.3.2 EXTRACTION OF RNA FROM CERVICAL TUMOUR TISSUE

1. Cervical tissue samples were homogenized in 1 ml of TRIZOL RNA extraction reagent (GibcoBRL® Lifetechnologies).
2. This mixture was incubated at 15 – 30^0 celcius for 5 minutes.
3. 0.2 ml of chloroform was added for 1 ml per trizol.
4. The tubes containing the samples were shaken vigourously for 15 seconds **(Photograph 1, Appendix).**
5. The mixture was then incubated at 15 – 30 ^{0} celcius for 2 – 3 minutes.

6. The samples were centrifuged at 9750 g for 15 minutes at 2 – 8^0 celcius. Following this step the mixture separated into a lower red phenol phase and an upper clear colourless aqueous phase **(Photograph 2, Appendix)**. This aqueous phase contains the RNA material.
7. The aqueous phase was transferred to a fresh tube.
8. 0.5 ml of isopropyl alcohol per 1 ml trizol was added to the tubes with the aqueous phase.
9. This mixture was incubated at 15 – 30^0 celcius for 10 minutes.
10. The mixture was centrifuged at 9750 g for 10 minutes at 2 – 8^0 celcius. The RNA appears as a pellet at the bottom of the tube.
11. The supernatant was removed.
12. 1 ml of 75% ethanol was added to each sample. The samples were vortexed.
13. The samples were centrifuged at 7500g for 5 minutes at 2 – 8^0 celcius.
14. The ethanol was removed.
15. The RNA pellets were air-dried.
16. The RNA samples were stored at –80^0 celcius till the next step was performed.

15.4.4 ASSESSMENT OF THE QUALITY AND QUANTITY OF RNA EXTRACTED BY SPECTROPHOTOMETRY

Prior to spectrophotometric determination of RNA quantity, the RNA pellets were resuspended in PCR water (SIGMA ALDRICH®) (Small pellets = 30 µl water, Medium size pellets = 40 µl water and large size pellets = 50 µl water).

RNA quantitation was performed using a spectrophotometric machine (Philips Unicam 8700®, Ltd, UK). For quantitating the amount of RNA, readings were taken at wavelengths of 260 nanometers (nm) and 280 nm. The reading at 260 nm allowed for the calculation of the concentration of nucleic acid in the sample. An optical density (OD) of 1 corresponds to approximately 40 µg/ml of single stranded RNA. The ratio of the readings at 260 nm and 280 nm (OD_{260} / OD_{280}) provided an estimate of the purity of the nucleic acid. Pure preparations of RNA had ratios of 2.0.

1. The quvette from the spectrophotometer was cleaned by washing with PCR water repeatedly.
2. 79 µl of PCR water was transferred into the quvette.
3. The baseline was determined.
4. 1 µl of resuspended RNA sample was added to the 79 µl PCR water in the quvette.
5. The OD 260 and OD 280 were determined.

The amount of RNA was determined according to the following formula:

OD_{260} X 40 X dilution X volume resuspension

= quantity in micrograms (µg)

The concentration of the RNA was calculated according to the following formula:

Amount of RNA (µg) / volume resuspension = µg/µl

15.4.5 REVERSE TRANSCRIPTION OF RNA TO SYNTHESIZE cDNA (SINGLE-STRAND DNA) USING REVERSE TRANSCRIPTASE ENZYME

cDNA was synthesized for all samples including the positive cell line controls (Caski, SiHa) and the negative control (C33A) cell line control.

Reagents used for cDNA synthesis included:

a. Superscript reverse transcriptase enzyme (GibcoBRL® Lifetechnologies)
b. 5 X first strand buffer (GibcoBRL® Lifetechnologies)
c. 0,1 Molar DTT (Dithiothreitol) (GibcoBRL® Lifetechnologies)
d. 5 mM deoxynucleotides (dATP, dCTP, dGTP, dTTP) (GibcoBRL® Lifetechnologies)
e. PCR water (SIGMA ALDRICH®)
f. Human Placental Ribonuclease Inhibitor (HPRI) (Amersham Pharmacia biotech®)

Method:

cDNA synthesis was performed in a final volume of 20 µl using eppendorf tubes.

1. 1µl HPRI (10U/µl)
2. 2 µl Oligo-DT 16 (0.5µg/ul)
3. x µl RNA (2µg)
4. PCR water –final volume to 11µl

The above mixture was heated at 70^0 celcius for 10 minutes and then transferred onto ice for 2 minutes.

The following reagents were added to the above mixture:

1. 8 µl 5X free strand buffer
2. 4 µl 0.1 M dNTP's
3. 2 µl reverse transcriptase enzyme

This mixture was heated at 37^0 celcius for 1 hour.

The products of this reaction were stored at -20^0 celcius till the next step.

15.4.6 MOCK REVERSE TRANSCRIPTION OF RNA WITHOUT REVERSE TRANSCRIPTASE ENZYME TO DIFFERENTIATE RNA FROM GENOMIC DEOXYRIBONUCLEIC ACID (DNA)

The same procedure as described above was repeated on all samples including the positive and negative cell line controls but **without** the superscript/reverse transcriptase

enzyme. The aim of this step was to differentiate true HPV RNA from genomic DNA by PCR described below.

15.4.7 POLYMERASE CHAIN REACTION OF PRODUCTS FROM 15.5) AND 15.6) ABOVE USING SPECIFIC PRIMER PAIRS TO THE E6 ONCOGENE

Polymerase chain reactions were performed using primers specific for nucleotides 142 to 161 (primer S3) and nucleotides 666 to 647 (primer S4) of the human papillomavirus type 16 (outer primers). This was necessary to detect the *E6* unspliced transcript with 525 base pairs.

PCR was performed for all samples including the 2 positive cell line controls (Caski, SiHa) and the negative control (C33A) cell line as well as Glyceraldehyde Phosphate Dehydrogenase (GAPDH). The GAPDH was used as an internal standard to test the presence and quality of RNA. This is also necessary to differentiate a true negative (HPV negative; GAPDH positive) from a false negative (HPV negative; GAPDH negative).

The specific sequences are illustrated below:

S3

5’ – ACA GTT ATG CAC AGA GCT GC – 3’

S4

5’ – CTC CTC CTC TGA GCT GTC ATT – 3’

OPTIMISING PRIMERS CYCLING POLYMERASE CHAIN REACTION

PCR reactions of reverse transcribed cDNA were performed on a Hybaid Omnigene® (Middlesex, UK) GeneAmp PCR system in a final volume of 49 µl.

The eppendorf tubes from the PCR reactions were removed at various cycles (ie. 18, 20, 25, 30 and 35) and temperatures. The aim was to establish the cycle number and temperature which gives the optimal band on gel and to prevent cycling going to saturation. From the cycling, cycle number 35 produced an optimal band.

The cycling pattern was established and consisted of:

Denaturation x 1 cycle at 95^0 celcius for 1 minute;

Annealing at 56^0 celcius for 45 seconds;

Elongation at 72^0 celcius for 1 minute.

The above was repeated X 30 cycles.

The first cycle was preceded by 5 minutes of denaturation at 95^0 celcius and the last cycle was followed by a 5- minute incubation at 72^0 celcius.

Components used for the PCR reaction included:

1. cDNA from all samples including controls
2. PCR water
3. 10 X PCR Buffer (GibcoBRL®Lifetechnologies)
4. 50mM magnesium Chloride (GibcoBRL® Lifetechnologies)
5. 1% W-1 (GibcoBRL® Lifetechnologies)
6. 1mm dNTP's (GibcoBRL® Lifetechnologies)
7. S3 /S4 primers (GibcoBRL® Lifetechnologies)
8. *Taq* DNA Polymerase (5U/µl) (GibcoBRL® Lifetechnologies)

The procedure performed was as follows

1. A premix consisting of 2µL cDNA and 17.5 µL PCR water was made.
2. A second premix consisting of 5µL Buffer, 5µL S3 primer, 5µL S4 primer, 4µL magnesium chloride and 10 µL 1mM dNTP's.
3. 29 µL of the second premix was added to the first premix.
4. This second premix was inserted in the PCR machine and heated to 95^0 celcius for 2 minutes according to the "hotstart" method.
5. Once the 2 minute point was reached, *Taq* polymerase (0,5 µL; 2,5U) was added to each eppendorf tube and the reactions were subjected to PCR cycling as described above.

Preparation of an agarose gel for electrophoresis was performed to determine if specific bands were present with the use of the S3/S4 primers:

Reagents used:

1. 1 X TAE solution
2. Agarose 1,5% 1,2 grams
3. Ethidium Bromide 1 µL

Procedure:

1. 80 ml of 1 X TAE was poured into a flask to which 1.2 grams of Agarose (weighed) was added. This mixture was heated till the solution turned clear and no bubbles were present.
2. The solution was allowed to cool following which 1 µL Ethidium bromide was added
3. This solution was poured into gel chamber and the combs inserted.
4. Once set, the combs were removed

Loading PCR products to the gel:

Reagents:

1. 1,3 µL Molecular weight marker X174 DNA Hae III DIGEST (Sigma® Aldrich Chemical Co)
2. 16,7 µL PCR product
3. 2µL Bromophenol blue dye (Loading Buffer)

A 20 µL soultion of the above preparation was added into each well of the gel and the gel was run with the aid of an electric current.

The gel was examined under Ultraviolet light to detect the presence of PCR products and to confirm that the PCR reaction was working by examining the positive and negative controls.

The molecular weight marker was specifically chosen because of its ability to identify the following size base pairs that can be amplified up for both the S3/S4 and S1/S2 primers in this project.

The sizes of the base pairs are as follows

1,353	
1,078	271
872	234
603	194

310	118
281	72

Once the presence of appropriate size products was confirmed and the PCR reaction was noted to be 'working', the nested PCR was performed as described below.

15.4.8 NESTED POLYMERASE CHAIN REACTION OF PRODUCTS FROM 15.4.7) ABOVE USING SPECIFIC PRIMER PAIRS TO DETERMINE THE EXPRESSION OF HPV 16 *E6*I* AND *E6*II* SPLICED VARIANTS

The nested PCR was performed on all PCR products from reactions with and without reverse transcriptase during the cDNA synthesis.

The specific primer pairs used was the S1 primer specific for nucleotides 192 to 211 and S2 specific for nucleotides 586 to 567 (inner primers). This was necessary to detect the spliced variants viz, *E6*I* (213 base pairs) and *E6*II* (95 base pairs) of the *E6* HPV oncogene.

The sequences for these primers are as follows:

S1: 5' – GTG TGT ACT GCA AGC AAC AG – 3'

S2: 5' - GCA ATG TAG GTG TAT CTC CA – 3'

The procedure for the nested PCR was as follows:

1. A dilution of the PCR product from the S3/S4 reaction was made by adding 1 µL of PCR product to 99 µL of PCR water for each sample including the positive and negative cell line controls. This product served as the template for the nested PCR.
2. A premix was made consisting of 2 µL (2µL of 1:100 solution) of the dilute PCR product and 17.5 µL PCR water.
3. A second premix consisting of 5µL Buffer, 4µL Magnesium, 10µL 1 mM dNTP's, 5µL S1 primer and 5µL S2 primer.
4. 29µL of the second premix was added to the first premix and placed into the PCR machine heated to 95^0 celcius for 2 minutes according to the "hotstart" protocol.
5. At this point, *Taq* polymerase enzyme (0.5 µL/ 2,5U) was added and the PCR run as per cycling described previously.

15.4.9 GEL ELECTROPHORESIS TO DETERMINE THE EXPRESSION OF THE HPV 16 *E6, E6*I* AND *E6*II* ONCOGENES IN BOTH GROUPS OF PATIENTS

Preparation of the gel and electrophoresis was performed as described for the S3/S4 PCR products obtained. These nested PCR products were examined under UV light to detect the presence or absence of the various bands.

15.4.9 COMPARISON OF THE PRESENCE OR ABSENCE OF BANDS WITH THE USE OF THE S3/S4 PRIMERS AND S1/S2 PRIMERS IN PATIENTS WHO USED STEROIDS AND THOSE WHO DID NOT USE STEROIDS AND THEN SUBJECTED TO STATISTICAL ANALYSIS.

All bands detected with the use of the various primers were counted and tabulated with respect to patients who used steroid contraception and those who did not use steroid contraception.

16.0 STATISTICAL METHODS

The student t-test was used to analyse: duration of contraceptive use; interval since last contraceptive use and the duration of contraceptive use versus *E6 /E6*I /E6*II* gene expression. Fisher's exact was used to analyse contraceptive use versus *E6* gene expression, stage versus *E6 /E6*I /E6*II* gene expression and histology versus *E6 /E6*I /E6*II* gene expression. The two sample t-test was used to analyse age versus contraceptive use and age versus contraceptive use versus *E6 /E6*I /E6*II* gene expression. The Pearson chi-square test was used to analyse the odds of *E6 /E6*I / E6*II* gene expression versus the use of steroids (depo-medroxyprogesterone acetate).

17.0 RESULTS

17.1 PATIENT DEMOGRAPHICS

17.2 CLINICAL DATA

A total of 120 patients were recruited for the study. With the exception of 4 Asian patients, all other patients were of African racial origin. The parity of patients ranged from 0–7 with the median parity being 3. The mean age of all patients was 52 years (range 17–76 years) (Figure 11). The majority of patients (60) presented with abnormal vaginal bleeding whilst 30 patients also gave a history of an offensive vaginal discharge. Overall there were 14% (15) of patients who were married and only 20% (22) of patients had had a pap smear performed. The mean number of partners for all patients was 2 (SD 1). The majority of patients (80%) lived in rural areas whilst only 20% of patients lived in urban areas. Only patients who tested negative for the human immunodeficiency virus (HIV) infection were recruited for the study.

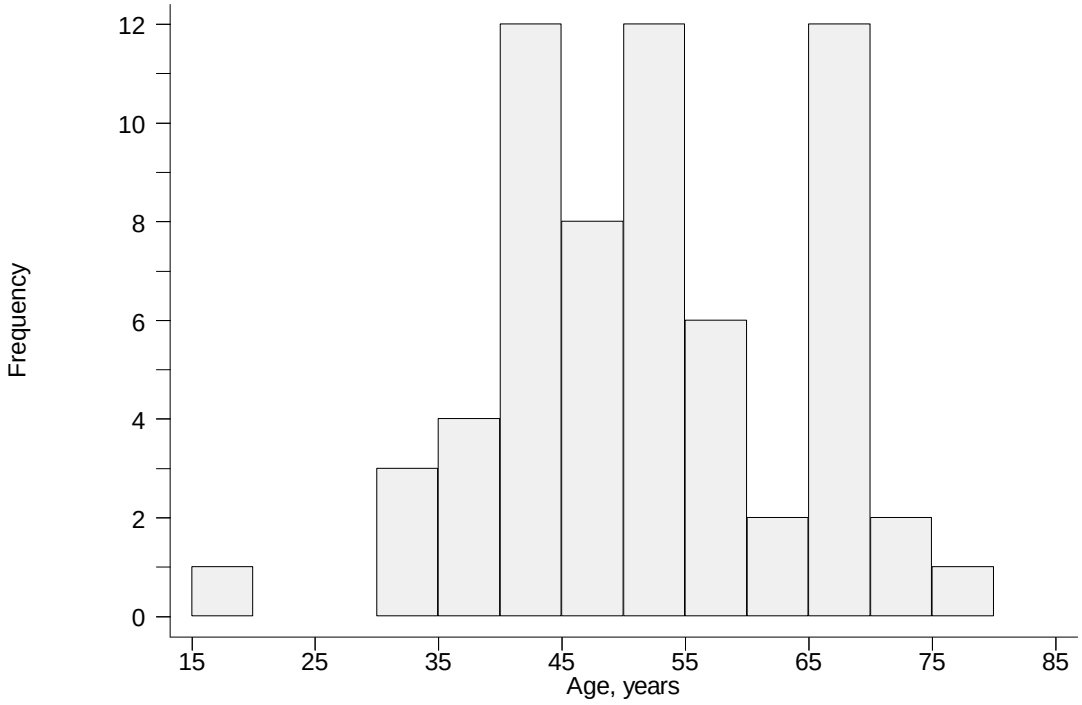


FIGURE 11: Age-distribution of patients

17.3 CONTRACEPTIVE DATA

Of the total of 120 patients recruited for the study, there were 48 patients (40%) who used depo-medroxyprogesterone acetate injectable steroid contraception. However, RNA was extracted in only 86 patients. Of this, there was a further 23 samples with genomic DNA identified by the mock "cDNA" synthesis step. Of the remaining 63 samples with RNA, there were a total of 30 patients (47%) who used depo- medroxyprogesterone acetate injectable contraception. The mean ages of depo- medroxyprogesterone acetate contraceptive users and non-users was 45 years and 59 years, respectively ($p<0.001$). Of

the patients who used contraception, the duration of contraceptive use extended from less than 1 year to 10 years (mean 6 years SD 2.3 years). The median interval since last use of depo-medroxyprogesterone acetate was 5 years (SD 3.5 years) (Table VII).

17.4 SMOKING

There were only 5 patients who admitted to having smoked cigarettes. There were no current smokers or patients who consumed alcohol.

17.5 RNA EXTRACTION

Of the total of 111 cervical tissue samples with confirmed HPV 16 infection, RNA was extracted in 86 samples. Of this, there were 23 samples which contained genomic DNA as determined by mock “cDNA synthesis” without reverse transcriptase enzyme, leaving 63 samples for analysis.

	age	par	contracep	duration	histolgy	grade	interval	stage
1.	64	6	No contra	0	1	2	.	6
2.	65	5	No contra	0	2	4	.	4
3.	43	2	Depo	3	1	2	1	6
4.	49	2	Depo	7	2	4	9	4
5.	69	6	No contra	0	1	3	.	6
6.	56	3	No contra	0	1	2	.	4
7.	52	3	Depo	5	1	2	12	5
8.	38	2	No contra	0	1	3	.	4
9.	48	2	Depo	6	1	2	8	6
10.	68	6	No contra	0	1	2	.	6
11.	46	4	No contra	0	1	2	.	6
12.	47	6	No contra	0	1	2	.	6
13.	69	3	No contra	0	3	4	.	4
14.	45	5	No contra	0	1	2	.	6
15.	43	3	Depo	8	1	2	6	3
16.	59	5	No contra	0	1	2	.	6
17.	54	3	Depo	4	1	2	12	4
18.	34	4	No contra	0	1	3	.	4
19.	71	6	No contra	0	1	3	.	6
20.	42	2	Depo	9	1	2	1	2
21.	65	4	No contra	0	1	2	.	6
22.	65	5	No contra	0	1	2	.	6
23.	67	5	No contra	0	1	2	.	6
24.	76	7	No contra	0	1	2	.	2
25.	36	2	Depo	3	1	2	4	4
26.	65	5	No contra	0	1	3	.	6
27.	69	6	No contra	0	1	2	.	5
28.	56	4	Depo	6	1	2	8	6
29.	17	1	Depo	0	1	3	.	6
30.	56	6	Depo	3	1	2	6	2
31.	45	2	Depo	6	1	2	7	6
32.	41	2	Depo	7	1	2	4	4
33.	36	2	Depo	9	1	2	1	6
34.	60	7	No contra	0	1	3	.	8
35.	53	3	Depo	5	1	2	7	6
36.	44	3	Depo	5	1	4	4	4
37.	54	5	Depo	7	1	3	1	2
38.	40	4	No contra	0	1	2	.	4
39.	43	3	Depo	3	1	2	5	6
40.	66	6	No contra	0	1	3	.	6
41.	57	4	No contra	0	1	3	.	6
42.	54	4	No contra	0	1	2	.	6
43.	41	2	Depo	9	1	2	4	6
44.	42	2	Depo	7	1	3	3	6
45.	69	5	No contra	0	1	3	.	6
46.	72	4	No contra	0	1	2	.	6
47.	51	0	Depo	6	1	2	2	4
48.	67	5	No contra	0	5	4	.	4
49.	42	5	Depo	8	1	2	4	5
50.	53	5	No contra	0	1	3	.	6
51.	45	3	No contra	0	1	2	.	6
52.	43	3	No contra	0	1	2	.	6
53.	41	3	Depo	10	1	2	2	6
54.	30	2	Depo	7	1	2	3	6
55.	52	2	No contra	0	1	2	.	3
56.	51	3	Depo	5	1	2	10	3
57.	37	4	No contra	0	1	4	.	6
58.	54	2	Depo	6	1	2	5	6
59.	59	5	No contra	0	1	2	.	6
60.	52	3	Depo	6	1	2	11	6
61.	46	2	Depo	5	1	2	8	4
62.	34	2	Depo	10	4	4	1	4
63.	50	3	Depo	3	1	2	11	4

TABLE VIII: DEMOGRAPHIC DATA FOR 63 PATIENTS

KEY TO TABLE VII:

HISTOLOGY

Squamous cell carcinoma – 1

Adenocarcinoma - 2

Undifferentiated carcinoma – 3

Adenoid cystic carcinoma – 4

Adenocarcinoma – 5

Par = Parity

Contracep = contraception

GRADE

2 = Moderately differentiated

3 = Poorly differentiated

4 = Unknown

17.6 HISTOLOGY FOR 63 PATIENTS

Of the total of **120** samples, there were 108 samples (90%) with squamous cell carcinoma, 3 (2.5%) with adenocarcinoma, 5 (4.1%) with adenosquamous carcinoma, 2 (1.7%) adenoidcystic carcinoma and 2 (1.7%) undifferentiated carcinoma of the cervix. The histological findings of the **63** study patients with cervical carcinomas are illustrated in Table VIII.

TABLE IX: HISTOLOGY FOR 63 PATIENTS

HISTOLOGY	NUMBER	%
SQUAMOUS	58	92
ADENOCARCINOMA	2	3
ADENOID-CYSTIC	1	2
ADENO-SQUAMOUS	1	2
UNDIFFERENTIATED	1	1

HISTOLOGICAL GRADES

The histological grades of patients with cervical tumours are shown in table IX.

TABLE IX: HISTOLOGICAL GRADES FOR 63 CERVICAL CARCINOMAS

GRADE	NUMBER	%
MODERATELY DIFFERENTIATED	43	68
POORLY DIFFERENTIATED	13	21
UNKNOWN DIFFERENTIATION	7	11

17.7 CLINICAL STAGES FOR 63 PATIENTS

The majority of patients in the study presented with late stage disease (72/120).

The clinical stages for the remaining 63 patients recorded as per recommendations of the International Federation of Gynaecology and Obstetrics (FIGO) are reflected in Table X.

TABLE X: CLINICAL STAGES FOR 63 PATIENTS

CLINICAL STAGE	NUMBER	%
1B1	4	6.35
1B2	3	4.76
IIA	16	25.40
IIB	3	4.76
IIIB	36	57.14
IVB	1	1.59

17.8 EXPRESSION OF HPV 16 *E6, E6*I* & *E6*II* ONCOGENES FOR 63 PATIENTS

Of the total of 63 patients, there were 53 patients who had expression of Human Papillomavirus (HPV) type 16 *E6* full-length gene expression. There were 25 and 30 patients with expression of the HPV *E6*I* and *E6*II* genes, respectively. There were 21 patients with both the HPV 16 *E6*I* and *E6*II* genes. In relation to patients who used contraception, HPV 16 *E6* gene expression was present in 77% (n=23) and 91% (n=30) of users and non-users of steroid contraception, respectively (Table XI).

TABLE XI: EXPRESSION OF HPV 16 *E6* ONCOGENE IN RELATION TO CONTRACEPTIVE USE

CONTRACEPTIVE USE	***E6* GENE PRESENT**	***E6* GENE ABSENT**
	n (%)	n (%)
YES	23 (77)	7 (23)
NO	30 (91)	3 (9)

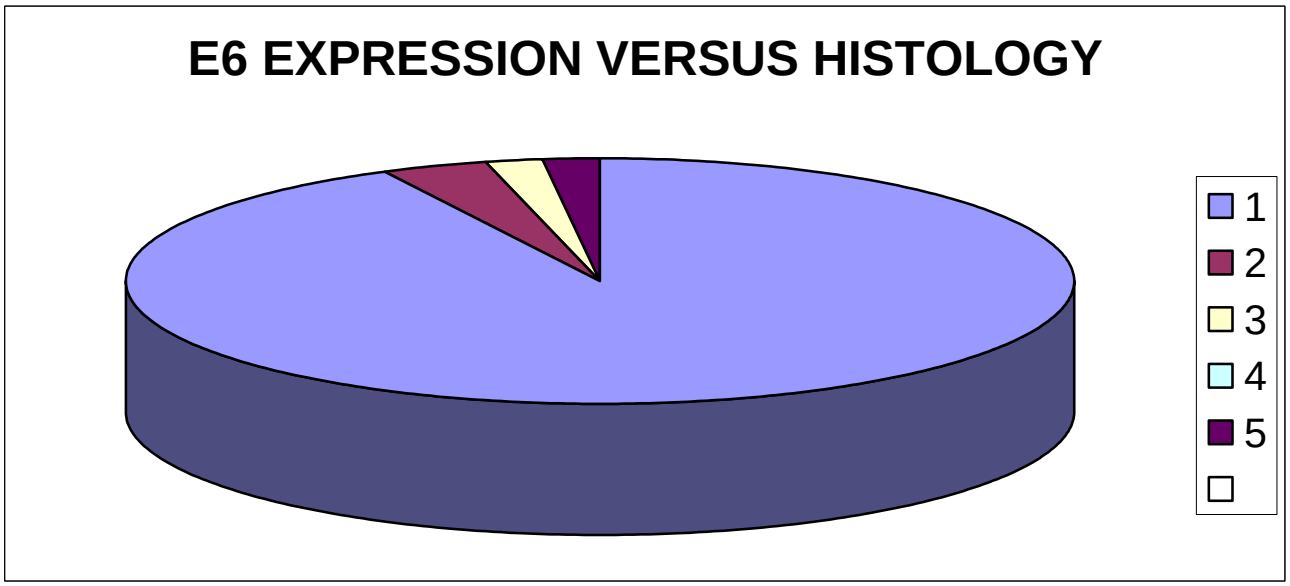
In the group of patients who used steroid contraception, the mean ages of patients **with** and **without** *E6* gene expression was 44.7 and 45.3 years, respectively (p=0.89). Of the non-contraceptive users, the mean age of patients **with** and **without** *E6* expression was 59 years (SD 12 years) and 49 years (SD 11 years), respectively (p=0.19). Of the patients **with** the HPV *E6* gene expression, the mean interval since last use of medroxyprogesterone acetate was 5.0 years (SD 3.3 years), whilst amongst the group **without** expression of the HPV E6 gene, the mean interval since last use of medroxyprogesterone acetate was 4.4 years (SD 3.9 years) (p=0.13). Amongst the patients who had expression of the HPV *E6* gene, the mean duration of contraceptive use was 2.6 years (SD 3,4 years) in contrast to the group without E6 expression where the mean duration of contraceptive use was 4.2 years (SD 3.4 years) (p=0.16).

HPV 16 *E6* ONCOGENE- EXPRESSION VERSUS HISTOLOGY

The histology of the 53 patients with HPV type 16 *E6* expression is depicted in figure 12.

The majority of patients had squamous cell carcinomas.

FIGURE 12: HPV 16 *E6* EXPRESSION VERSUS HISTOLOGICAL TYPES



1=SQUAMOUS (48) 2=ADENOCARCINOMA (2) 3=ADENOID- CYSTIC (1)

4=ADENOSQUAMOUS (1) 5=UNDIFFERENTIATED (1)

There were 21 patients who had expression of both the *E6*1* and *E6*11* genes. The presence of both HPV 16 *E6*I* and *E6*II* gene versus histological types is depicted in Table XII.

TABLE XII: HISTOLOGY VERSUS HPV 16 *E6*I* & HPV 16 *E6*II* ONCOGENE EXPRESSION

HISTOLOGICAL TYPES	**HPV 16 *E6*I/II* EXPRESSION**
SQUAMOUS	19
ADENOCARCINOMA	0
ADENOIDCYSTIC	1
ADENOSQUAMOUS	0
UNDIFFERENTIATED	1

17.9 CLINICAL STAGE VERSUS HPV 16 *E6* ONCOGENE- EXPRESSION

The gel image for the molecular weight marker and bands obtained is illustrated in **Gel Image 1** (Appendix). Of all patients with early stage disease, 78% (n=23) had expression of the HPV *E6* gene in contrast to late stage disease where 88% (n=40) had HPV *E6* gene expression (p=0.48). If steroid contraceptive use is taken into consideration, then the relative risk (RR) of having the HPV *E6* gene expression was 0.80 (CI 0.54 – 1.20) for contraceptive users compared to non-users for early stage disease. Whereas, in late stage disease the relative risk for the HPV 16 *E6*- gene expression in contraceptive users versus non-users was 0.85 (0.63 – 1.14). The overall relative risk of the HPV *E6* gene expression in steroid contraceptive users versus non-users was 0.83 (CI 0.66 – 1.06) for both early and late stage disease (p= 0.82). The gel image for the RT-PCR products obtained using the S3/4 primers for samples and cell-lines is illustrated in **Gel Images 2/3 (Appendix).**

17.10 EXPRESSION OF THE HPV TYPE 16 *E6*I/E6*II* ONCOGENES IN STEROID USERS AND NON-STEROID USERS

In total there were 25 patients (40%) with expression of the *E6*I* gene and 30 patients with expression of the *E6*II* gene. Of all the patients who had expression of the *E6*I / E6*II* gene, 57% (17/30) used depo-medroxyprogesterone acetate steroid contraception compared to 52% (17/33) who did not use steroid contraception (p=0.800) (Table XIII).

Table XIV illustrates *E6 / E6*I* and *E6*II* gene expression for individual patients in relation to contraceptive use. The gel image demonstrating the bands obtained for both samples and cell-lines using the nested RT-PCR system with the S1/2 primers is illustrated in **Gel Images 4/5/6/7/8 (Appendix).** The RT- PCR and nested RT-PCR on the cell-lines (SiHa & CaSki) using specific primers (S1/S2 & S3/S4) resulted in products *E6* (full-length gene); *E6*1* and *E6*11* genes (**Gel image 8, Appendix).** As expected, no products were seen using the negative control as well as C33A cell line.

TABLE XIII: Contraceptive use versus HPV 16 E6*I /E6*II oncogene expression

CONTRACEPTIVE USE	***E6*I/II* ABSENT**	***E6*I/II* PRESENT**	**p VALUE**
	n (%)	n (%)	
YES	13 (43)	17 (57)	
NO	16 (48)	17 (52)	0.800

Of all patients with *E6*I / E6*II* gene expression, the mean age was 52 years (SD 12 years) compared to patients without the *E6*I / E6*II* gene expression where the mean age was 47 years (SD 12 years) (p=0.14). Of all patients with expression of the *E6*I / E6*II* gene, the mean duration of contraceptive use was 5.9 years (SD 2.7 years). In early- stage disease, the relative risk of having expression of the *E6*I / E6*II* gene in contraceptive users versus non-contraceptive users was 1.03 (CI 0.49 – 2.16). Whereas in late stage disease, the relative risk of *E6*I / E6*II* gene expression was 1.14 (CI 0.63 – 2. 02) for contraceptive users compared to non-contraceptive users. Overall, the relative risk

of HPV *E6*I / E6*II* gene expression in contraceptive users versus non-contraceptive users for early and late stage disease was 1.10 (CI 0.69 – 1.74) (p=0.83) (Table XIII). There were 13 patients (57%) with early stage disease who had expression of the *E6*I / E6*II* genes compared to 53% of patients (n=21) with late stage disease who had expression of the HPV *E6*I / E6*II* gene (p=0.80) (Table XIV). The full-length *E6* oncogene was detected in 29 patients using both the S3/4 as well as the S1/2 primers. However, the nested PCR method using S1/S2 primers detected 54 patients with the *E6*I & E6*II* transcripts in comparison to classical PCR where only 31 such transcripts were detected.

	contraception	*E6*	*E61*	*E611*
1.	No contraception	1	2	2
2.	No contraception	1	2	2
3.	Depo	1	2	2
4.	Depo	1	2	2
5.	No contraception	1	2	2
6.	No contraception	1	1	2
7.	Depo	1	1	1
8.	No contraception	1	2	2
9.	Depo	1	2	2
10.	No contraception	1	1	1
11.	No contraception	1	1	1
12.	No contraception	1	2	2
13.	No contraception	1	2	1
14.	No contraception	1	2	2
15.	Depo	1	2	2
16.	No contraception	1	2	2
17.	Depo	1	1	1
18.	No contraception	1	2	2
19.	No contraception	1	2	2
20.	Depo	1	1	1
21.	No contraception	1	2	2
22.	No contraception	1	1	1
23.	No contraception	1	1	1
24.	No contraception	1	1	2
25.	Depo	1	1	1
26.	No contraception	1	1	1
27.	No contraception	1	1	1
28.	Depo	1	1	1
29.	Depo	1	2	1
30.	Depo	1	2	2
31.	Depo	1	1	1
32.	Depo	1	1	1
33.	Depo	1	2	2
34.	No contraception	1	2	2
35.	Depo	1	2	2
36.	Depo	1	2	2
37.	Depo	1	2	2
38.	No contraception	1	1	1
39.	Depo	1	2	2
40.	No contraception	1	2	1
41.	No contraception	1	2	1
42.	No contraception	1	2	2
43.	Depo	1	2	1
44.	Depo	1	2	2
45.	No contraception	1	2	2
46.	No contraception	1	2	2
47.	Depo	1	2	2
48.	No contraception	1	2	2
49.	Depo	1	2	2
50.	No contraception	1	1	2
51.	No contraception	1	2	2
52.	No contraception	1	1	1
53.	Depo	1	1	1
54.	Depo	2	1	1
55.	No contraception	2	1	1
56.	Depo	2	1	1
57.	No contraception	2	1	1
58.	Depo	2	2	1
59.	No contraception	2	2	1
60.	Depo	2	1	2
61.	Depo	2	2	1
62.	Depo	2	2	1
63.	Depo	2	1	1

TABLE XIV: Contraceptive use versus hpv 16 *E6, E6*I* & *E6*II* expression.

KEY TO TABLE XIV:

1= EXPRESSION PRESENT

2= EXPRESSION ABSENT

DEPO = MEDROXYPROGESTERONE ACETATE INJECTABLE

CONTRACEPTION

17.11 EXPRESSION OF THE HPV 16 *E6*I/E6*II* IN RELATION TO STAGE

The expression of the HPV spliced products are depicted in Table XV.

TABLE XV: Expression of hpv *E6*1/E6*II* in early stage versus late stage disease

E6*I/ E6*II	**EARLY STAGE**	**LATE STAGE**
	n (%)	n (%)
PRESENT	13 (57)	21 (53)
ABSENT	10 (43.4)	19 (47.5)

18.0 DISCUSSION

Cervical cancer comprises approximately 12% of all cancers in women worldwide. Whilst it is the fifth commonest cancer amongst women in developed countries, it remains the commonest cancer of women in the developing countries. The global estimates are 452 000 new cases and more than 234 000 deaths from cervical cancer in the year 2000. About 80% of these cases occur in women from developing countries. Although cervical cancer is both preventable and treatable, a comprehensive screening program is not available in South Africa. In developed countries, it has been shown that such screening programs can achieve up to an 80% reduction in the incidence and mortality from cervical cancer. Also, the majority of patients in developing countries present with late stage disease (Moodley et al, 2001).

Human papillomavirus types 16 and 18 are most closely linked to cervical cancer. The oncogenic types of human papillomavirus i.e., 16 and 18, elaborates two proteins viz. E6 and E7 which stimulates the neoplastic transformation of cells. These proteins bind to the p53 gene product and retinoblastoma gene (*RB*) product, respectively. The E6 protein of types 16 and 18 HPV promotes the degradation of the p53 protein product by an ubiquitin-dependent proteolytic pathway. Loss of the normal functioning *p53* and *pRB* gene product leads to deregulation of the cell cycle allowing accumulation of genetic mutations and cell-cycle progression after DNA damage. Clinical and experimental evidence indicate that HPV infection by itself does not lead to the development of

cervical cancer. It therefore appears that co-factors are necessary for the full expression of HPV oncogenicity. *In vitro* work by Arbeit et al (1996) in a controlled study showed that the high risk HPV type 16 was able to stimulate the development of vaginal and cervical squamous cell carcinomas in transgenic mice which were exposed to slow-release pellets of 17 beta-oestradiol in the presence of the human keratin–14 promoter (K14- HPV 16 transgenic mice).

Steroid hormones are thought to enhance the expression of the HPV *E6* oncogene, which in turn inactivates the *p53* tumour suppressor gene leading to tumour formation and proliferation. In practical terms it is difficult to examine the relationship between the *p53* gene and HPV in cervical cancers as the half-life of the *p53* gene is very short. Indirect evidence can be gained by studying the expression/transcription of the HPV *E6* genes amongst steroid users keeping in mind the evidence demonstrating the degradation of the *p53* gene product by the HPV *E6* gene product. To date, the current study is the only known study in human subjects to investigate the transcriptional differences of the HPV *E6* oncogene in patients with cervical cancer exposed to steroid contraception.

All patients recruited for this study tested negative for the human immunodeficiency virus infection by an Elisa blood test. This was necessary for several reasons: to prevent exposure to personnel during the project; to avoid problems with transport of tissue to the University of Liverpool, United Kingdom, where the research project

was performed and to prevent false results since it has been shown by Boccalon et al (1996), that the human immunodeficiency virus can upregulate the HPV *E6* oncogene.

Patients recruited for this study used medroxyprogesterone acetate injectable steroids for contraception as this is the most widely used contraception in patients in our setting due to low compliance with oral steroid contraception. In this study, with the exception of age, there was no difference in stage, histology and grade between steroid users and non-users. The mean duration of steroid use and mean interval since last use was 6 years and 5.5 years, respectively. In early and late stage disease, the relative risk of having expression of the *E6*I / E6*II* gene in contraceptive users versus non-contraceptive users was 1.03 and 1.14, respectively. This is in contrast to that described by Herrero et al, (1990b) who showed a relative risk of 2.4 in users of over five years duration and that the risk was enhanced after five years and ten years since last use and first use, respectively. This has been shown to be a crucial variable even when controlling for age and parity. The critical duration of use of steroids for an adverse effect is 5 years, whilst interval since last use of less than 8 years has been shown to be closely associated with the development of cervical cancer. Forty-five percent of women from this study used depo-medroxyprogesterone acetate injectable steroids.

Previous studies have shown that cervical cancer screening plays an important role in the effect of contraceptive use since users were thought to have their disease detected at an earlier stage because of the availability of screening

facilities. In a previous study (Moodley et al, 2001) performed over the same time period (18 months), it was found that only 6% of patients in the local population had ever had a Papanicolaou smear taken.

With regards to HPV type 16 *E6* oncogene expression, there was no significant difference in expression between users and non-users of steroid contraception. Of the patients who used steroids the duration of use was greater in those tissue samples without *E6* expression compared to samples with *E6* expression, although this difference was not statistically significant. Even when the interval of steroid use was considered there was no significant difference in *E6* expression. In patients with expression of the HPV *E6*I* (25 patients) and *E6*II* (30 patients) genes, the mean duration of steroid use was 5.9 years. There was no significant difference in the expression of these genes in steroid users compared to non-steroid users.

Notwithstanding the above findings, these results reflect the interaction between steroid hormones and gene expression in cervical cancer. Whereas all other studies including the large multinational WHO collaborative study (WHO, 1993) have shown an increased risk of cervical cancer with steroid use there has been no information regarding the association with the HPV. Besides the true non- differences in transcription between these groups, the small number of patients in these sub-groups may be a limiting factor. The findings of this study may be in keeping with the results of some

previous studies (La Vecchia 1994; WHO 1993) which have not shown a link between injectable progesterone steroid contraception and cervical cancer. However, HPV DNA status in these studies was unknown. This is crucial as steroids may not increase the risk of cevical neoplasia in the absence of HPV.

Overall, the relative risk of *E6*I* and *E6*II* expression in steroid users and non-users for early and late stage disease was 1.10. This implies that there was no difference in steroid use with regards to *E6* expression and disease stage. Again small patient numbers in these sub-groups may be a limiting factor.

With regards to the technical aspects of this study, HPV typing was performed using the PCR method with consensus primers. The PCR method has been known for its unprecedented sensitivity for DNA amplification. Thus, small sample collection is not a problem. The technique is also relatively simple and short and there is potential for automation. The disadvantages include: extreme sensitivity makes the process vulnerable to false positives resulting from contamination by PCR product or sample-to-sample in the laboratory; the enzymatic process is sensitive to inhibitors that may retard or block amplification and there is potential for the detection of viral DNA of unknown significance.

The type of primers used may also influence the outcome of HPV typing. In general, there are 2 types of primers that may be used: the type–specific and the consensus primers. The type-specific primers are designed to detect a specific HPV type whereas

the consensus primers facilitate the amplification of a broad spectrum of HPV types. One of the problems with the type-specific primers is that there is dependence on visualisation of ethidium bromide-stained gels which limits the sensitivities of these systems since only samples containing levels of HPV sufficient to generate a visible amount of PCR product can be identified. There is also a continual discovery of new HPV types and they may have a limited role in epidemiological studies. The consensus primers have the advantage of being able to amplify a wide range of genotypes. The primers used in this study for HPV typing involved consensus primers directed against the conserved regions of the HPV genome, i.e. the L1 open reading frames. These primers have been thought to be superior to primers which amplify within the *E6/E7* open reading frames and the amplification process is far better with the *L1* or *E1* consensus systems.

With regards to the RNA extraction method using the TRIZOL® reagent, this method proved unsuccessful in 25 tissue samples in which no RNA was extracted. At the outset there were 111 cervical tissue samples with HPV 16 proven by typing and following the RNA extraction step, there were 86 samples, of which 23 contained genomic DNA. This is in spite of similar samples from the same patients having proven HPV type 16 infection. However, this may reflect either the absence of RNA in the tissue samples or technical problems in the extraction process which seems difficult to explain since the same technique was used in samples with successful RNA extraction. Due to the small size of the tissue samples, the cervical tissue samples in the RNA extraction reagent were crushed using sterile plastic sticks. It is uncertain if this process could have destroyed

RNA in the samples, although this seems unlikely as the process was successful in 86 samples.

The nested polymerase chain reaction technique showed an added advantage when small patient numbers are studied. A larger percentage (43%) of HPV 16 *E6*I* & *E6*II* oncogene transcripts were detected using the S1/S2 primers in the nested polymerase chain reaction technique. This is comparable to the report by Sotlar et al (1998), who showed that the nested PCR system improved the yield 100-fold in detecting these transcripts compared to classical PCR. The same authors also found that a template of only 10 picograms of CaSki cDNA was sufficient for the detection of the 395 base-pair *E6*I* fragment using the combined primer pairs S3/4 (outer primers) and S1/2 (inner primers) in the nested RT-PCR technique. The S3/4 primers were also specific for HPV 16 in that HPV 18-specific sequences of HeLa cells were not amplified using this primer pair. They also showed that this method can be applied even to paraffin-embedded biopsies to differentiate between the presence of HPV genomic DNA and transcriptionally active viruses albeit with a reduced sensitivity compared to unfixed fresh tissue.

A study by Nakagawa et al (2000), using primers against conserved sequences among various HPV types in the *E6* ORF, found that the RT-PCR method detected 97% to 100% of *E6* full-length and spliced products. However, the study number only comprised 34 tissue samples of invasive cervical cancer. Thus far, the presence of the HPV *E6* and *E7* transcripts have been detected only in cervical cancer cell-lines and in a small number of

tissue samples from patients with proven invasive cervical cancers. This study documents not only the use of the nested RT-PCR method but also the study of the expression of the HPV 16 *E6 /E6*I /E6*II* genes at the molecular level of the HPV 16 *E6* transcripts in a large number of patients in relation to the use of steroid contraception. The nested RT-PCR method is also invaluable in studying the role of the HPV full-length and spliced - transcripts since it has been shown that the detection of these transcripts increased with the progression of disease from low-grade CIN to invasive carcinomas (Sotlar et al 1998).

Although the aim of this study was to investigate the role of steroid hormones (medroxyprogesterone acetate) in the link between human papillomaviruses and cervical cancer, it has been shown from a previous study (Moodley et al, 2001) that only 42% of patients in this local setting used contraceptive steroids. The intention therefore was to assess if there is a molecular link as suggested by previous reports between steroid use, HPV infection and cervical carcinogenesis only in those patients who have used steroid contraceptives in the setting of this study.

Various factors may have contributed to the negative correlation found in this study. Firstly, there were small patient numbers in the sub-groups with expression of the *E6**I and *E6**II gene. It is uncertain if a larger patient number would have altered the outcome or if these findings may reflect true non- transcriptional activity between the two groups of patients. However, this is the first series to describe such molecular interaction in human subjects. Sotlar et al (1998) documented that PCR amplification of full-length *E6/E7* sequences cannot differentiate between reverse-transcribed cDNA and genomic

DNA, whereas the detection of the *E6**I and *E6**II spliced-products are unequivocal proof of HPV transcriptional activity. The E6*I splice-product represents 97% of the mRNA population of HPV 16 containing human CaSki cells. This emphasizes the role of the nested RT-PCR method for detecting spliced products of the HPV type 16. It may therefore very well be that the negative correlation found in our study may in fact reflect true non-transcriptional activity of the HPV.

Secondly, the dose, type, duration and interval since last use as well as the mode of administration of steroid hormones in producing enhanced transcription of these oncogenic viruses remains unknown. In spite of this, if steroid hormones are the initiating factors in cervical carcinogenesis, some differences should be detected at the transcriptional level between users and non-users. Further, the dose or quantity needed as an initiating factor cannot be larger than what is currently present in steroid contraceptives. It may very well be that minute quantities of steroids are required at a critical time of the HPV infection to propagate the subsequent events leading to the phenotype recognized as cervical carcinoma. Madeleine et al (2001) reported a higher prevalence of HPV DNA amongst patients using oral contraception for more than 12 years in relation to adenocarcinoma in-situ of the cervix. In spite of the recency of this report, no molecular evidence is provided for the link between steroid hormones, HPV and cervical carcinogenesis.

Comparing the results of this study with that of Moreno et al (2002) it is evident that the presence of high-risk types of HPV are significant in patients with invasive

cervical cancers. Our findings of the lack of association of progesterone steroid contraceptive usage of less than 5 years duration with cervical cancer is in keeping with that of Moreno et al (2002) although their study reviewed women who used oral contraception. Therefore future studies amongst women using progesterone contraceptives for longer duration (5 to 10 years or longer) are required to establish if long-term steroid exposure plays a role in HPV-related cervical carcinogenesis. Although Moreno et al (2002) have shown elevated risks for long term steroid use, the HPV DNA negative controls may not have been true negatives i.e., although some women may have tested "negative" for HPV DNA in their study, these women may have been previously exposed to HPV which subsequently cleared since HPV infections are noted to be transient.

The large multi-national collaborative WHO study showed that the risk of cervical cancer increased significantly with duration of use after 4 to 5 years from first exposure to oral contraceptive steroids and declined with time after cessation of use to that of non-users in about 8 years (WHO, 1993). Herrero et al (1990) showed a relative risk of cervical cancer of 2.4 in users of progesterone injectable contraception over five years duration and that the risk was enhanced after five years and ten years since last use and first use, respectively. Since the mean duration of injectable steroids used in this study was around years, this may be a possible explanation as to why no statistically significandifferences were found. Thirdly, the relative role of oestrogens versus progesterones alone or in combination is largely unknown.

Notwithstanding these limitations, this study does not show a link between progesterone-only steroids and enhanced expression of the HPV type 16 *E6* oncogenes. The clinical implications of these findings are significant as many women especially in developing countries such as South Africa depend on injectable steroids as the major form of contraception. In this setting where problems such as unwanted pregnancies and the HIV epidemic are crucial issues, a reliable method such as the injectable steroids is vital. A further reason why a link between steroids and cervical neoplasia is important in developing countries is that the incidences of cervical neoplasias are very high and access to cervical screening is usually limited. Besides, there are other non-contraceptive benefits such as the prevention of iron-deficiency anaemia via the induction of amenorrhoea in many patients using injectable progesterone contaception.

19.0 CHAPTER FIVE

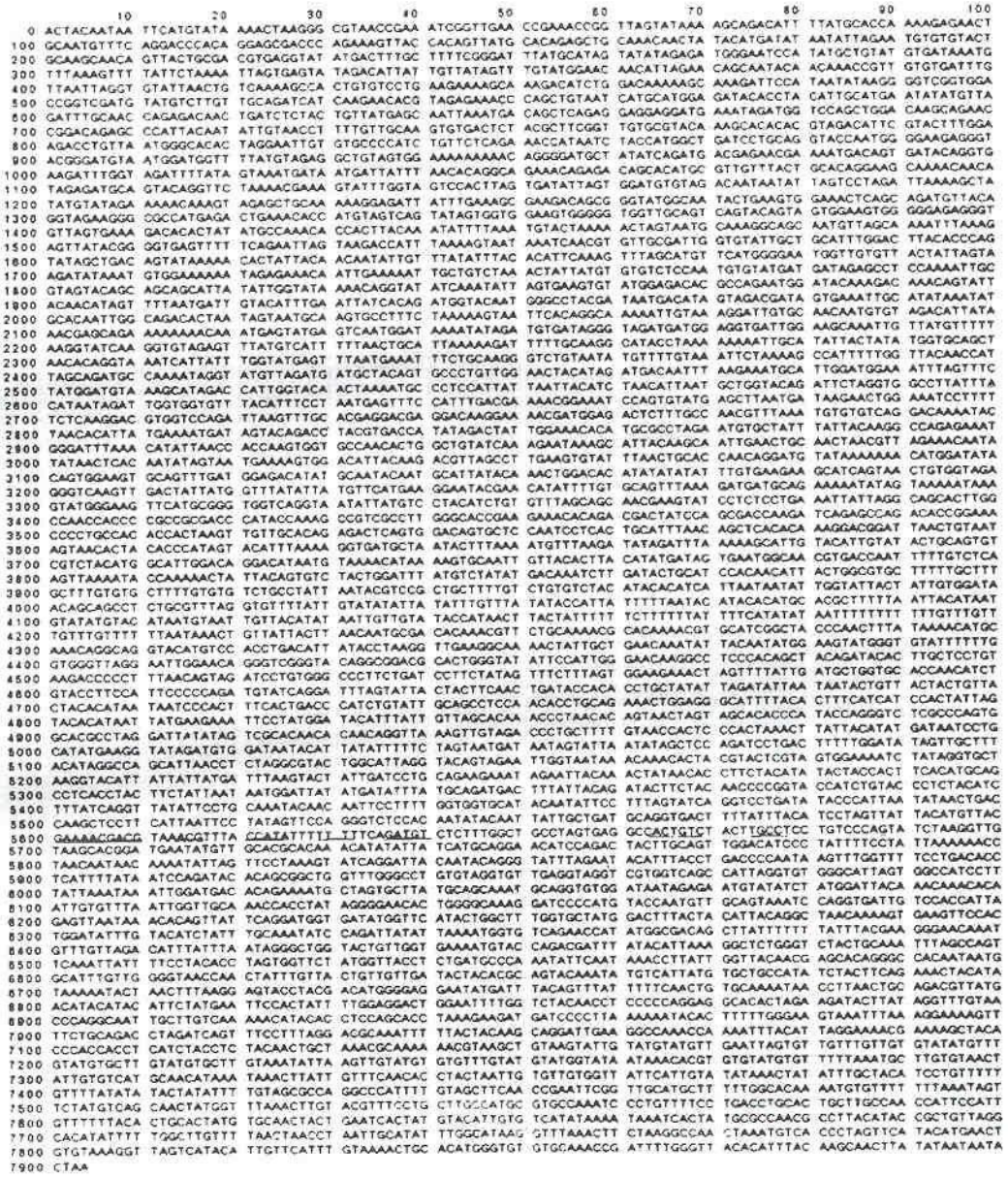
19.1 CONCLUSIONS AND RECOMMENDATIONS

Cervical cancer is the most common gynaecological malignancy amongst women in South Africa with an incidence quoted as high as 40/100 000. The majority of these women have poor recourse to cervical cancer screening facilities which is lacking in developing countries. Various factors have been implicated as causal or casual in the pathogenesis of cervical cancer. Although earlier studies have been reassuring with regards to the role of steroid contraception and cervical cancer later studies have shown concern with regards to these agents. However, many studies have not related these agents with other factors, especially sexually transmitted agents, mainly, the human papillomavirus types 16 or 18. Theoretically, it has been thought that steroids enhance the expression of the HPV 16 *E6* gene, which in turn degrades the *p53* gene which is regarded as the "guardian" of the genome by virtue of its tumour suppressor role. To date no such study has been performed amongst human subjects to support this theory.

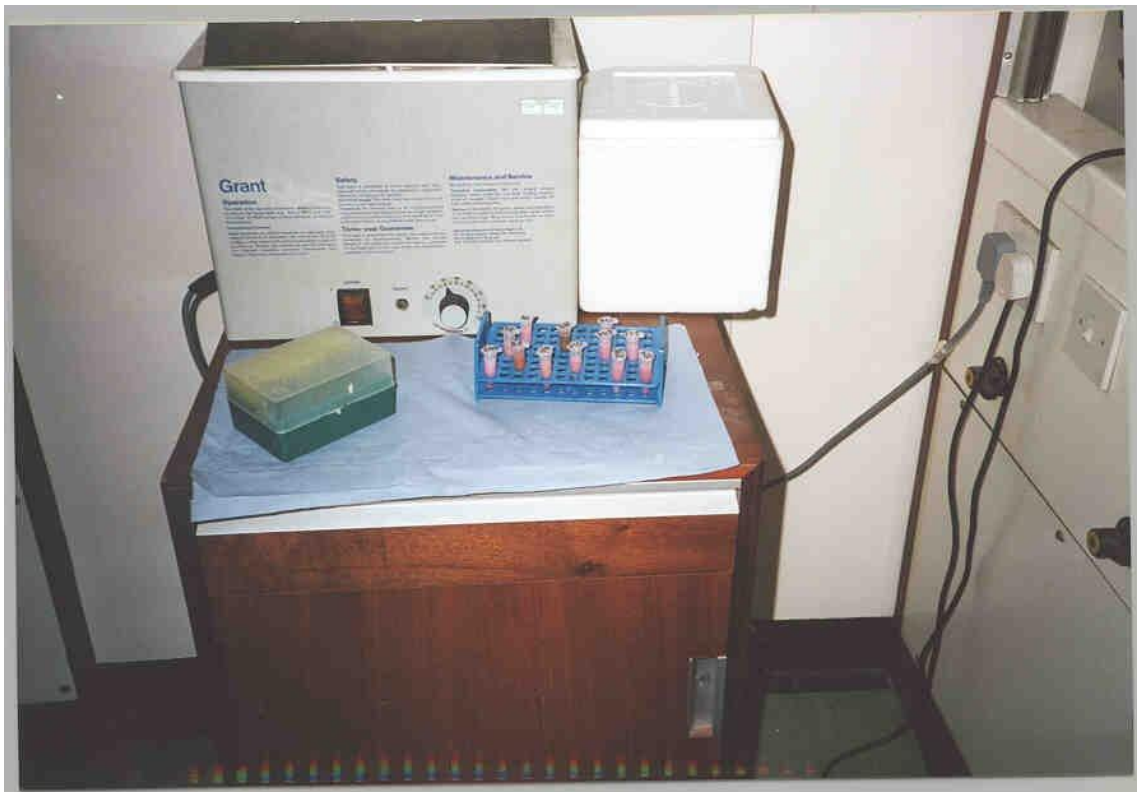
Epidemiologically and theoretically, it has been postulated that steroid contraception and human papillomavirus are causally related to cervical cancer. To date no such molecular study has been performed amongst human subjects to prove or disprove this theory. This study represents an original project, the first to be conducted amongst such large numbers

of human subjects with controls both in the study design as well as in the molecular aspects of the project. The findings from this project do not support a link between progesterone injectable contraceptive agents and cervical cancer. The limitation of this study is the small sample size of specimens eventually processed and analysed. In view of the nature of the task undertaken to accomplish this work, as well as the time and effort spent on this project, my recommendation would be that this project be repeated amongst a larger number of women using various types of steroid contraceptives of longer duration to what was studied in this project to confirm or refute the observed association between steroid contraceptive hormones and cervical cancer. This may be accomplished by a joint multi-national, multi-centre collaboration between clinicians and molecular biologists in this ever-expanding field.

APPENDIX



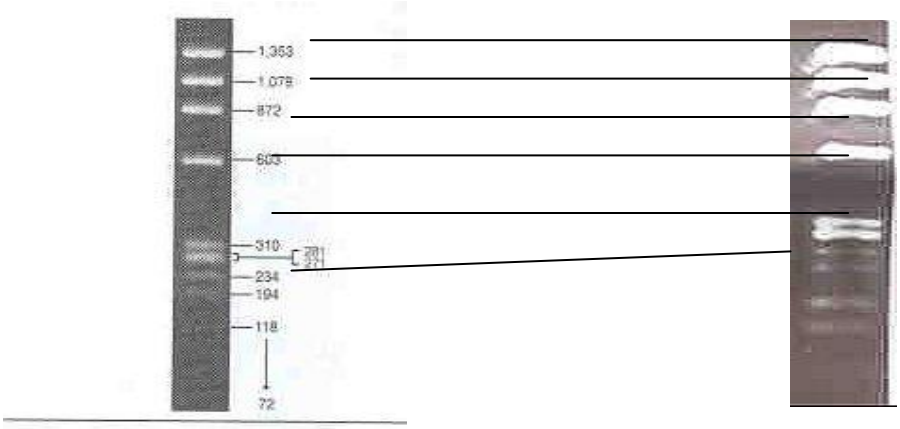
HPV 16: GENE SEQUENCE OF HPV 16 (Seedorf et al 1985)



PHOTOGRAPH 1: EPPENDORF TUBES IN RACK DURING RNA EXTRACTION PROCESS

PHOTOGRAPH 2:

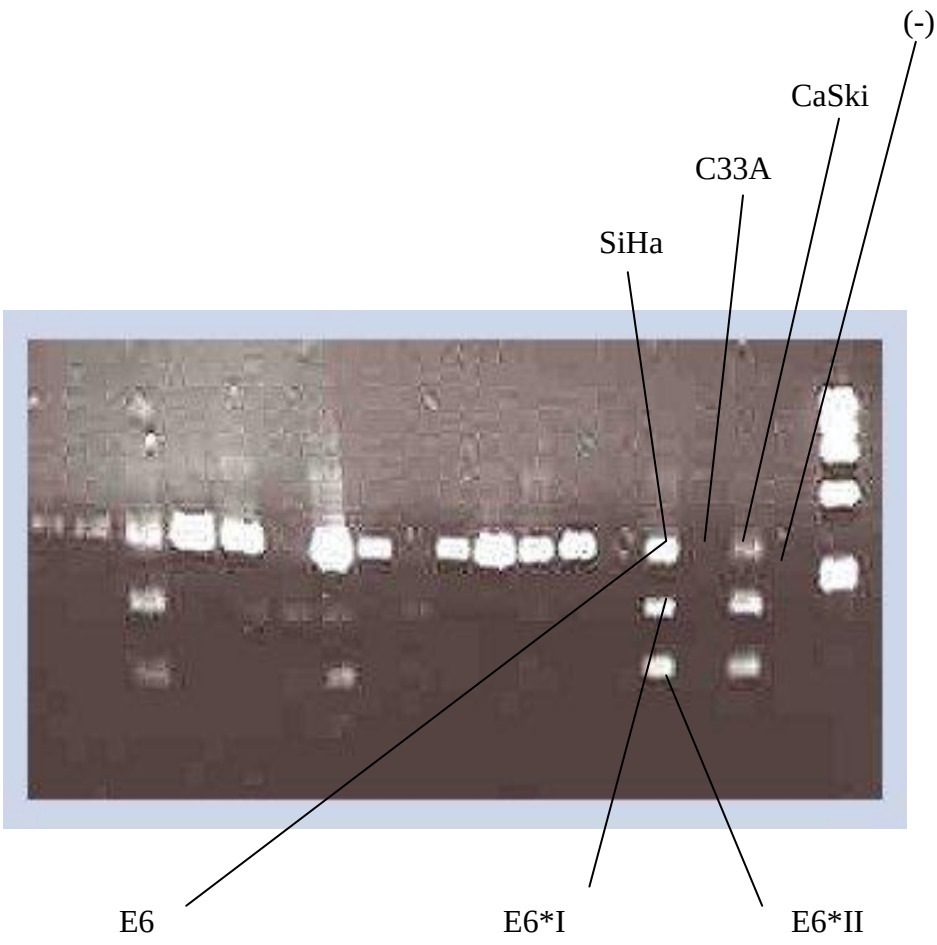
EPPENDORF TUBES DURING RNA EXTRACTION PROCESS CONTAINING UPPER CLEAR AQUEOUS PHASE & LOWER RED PHENOL PHASE



A **B**

GEL IMAGE 1: BAND SIZES A) MOLECULAR WEIGHT MARKER

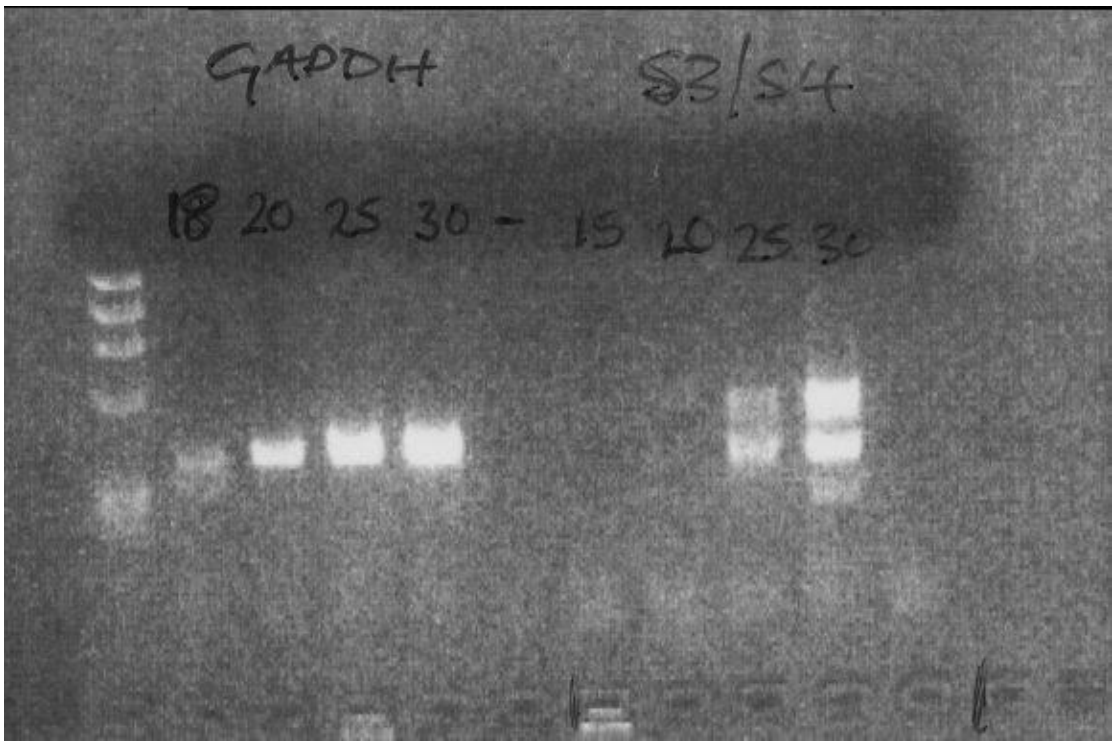
B) SAMPLE



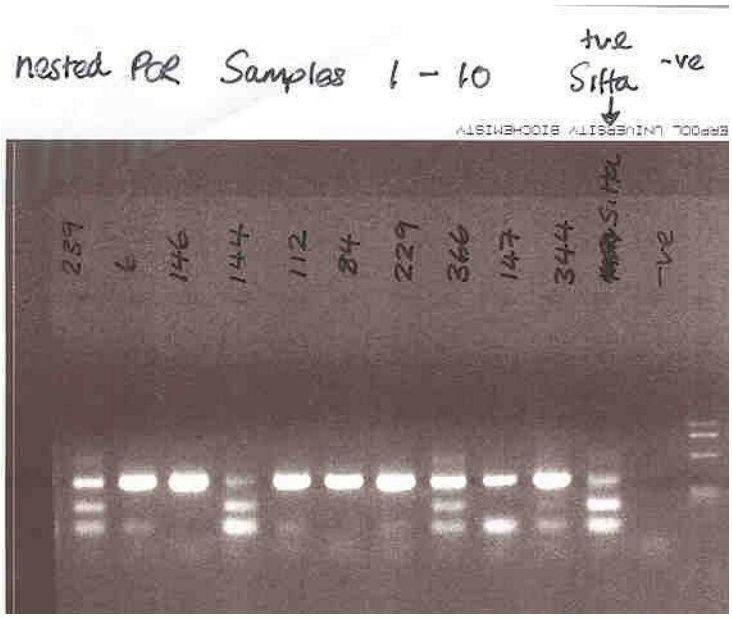
GEL IMAGE 2: RT-PCR PRODUCTS DEMONSTRATING *E6/E6*I/E6*II* GENES WITH S3/S4 PRIMERS SAMPLES & CELL LINES

Anticipated band sizes: ***E6*** **– 525 bp** ***E6*1*** **– 343bp** ***E6*11*** **– 226bp**

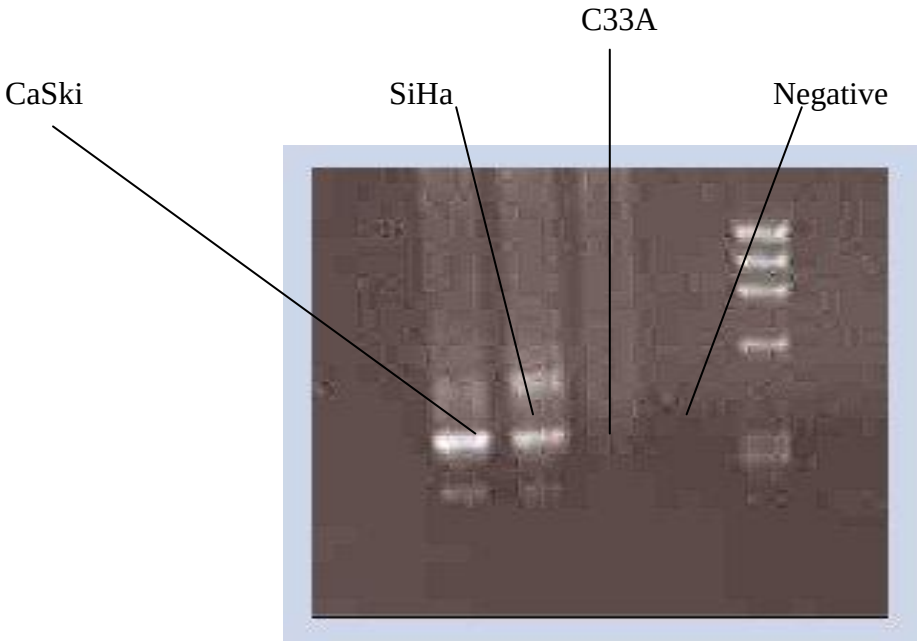
GEL IMAGE 3: RT-PCR PRODUCTS OF SAMPLES & CELL-LINES WITH S3/S4 PRIMERS



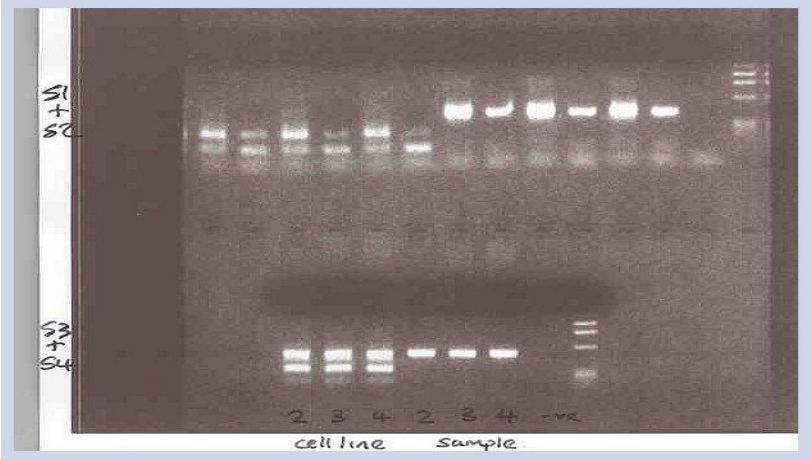
GEL IMAGE 4: RT-PCR SHOWING SPECIMENS AND GAPDH AS CONTROL



GEL IMAGE 5: NESTED PCR PRODUCTS OF SAMPLES AND CELL-LINES USING S1/2 PRIMERS DEMONSTRATING *E6/E6*I/E6* GENES



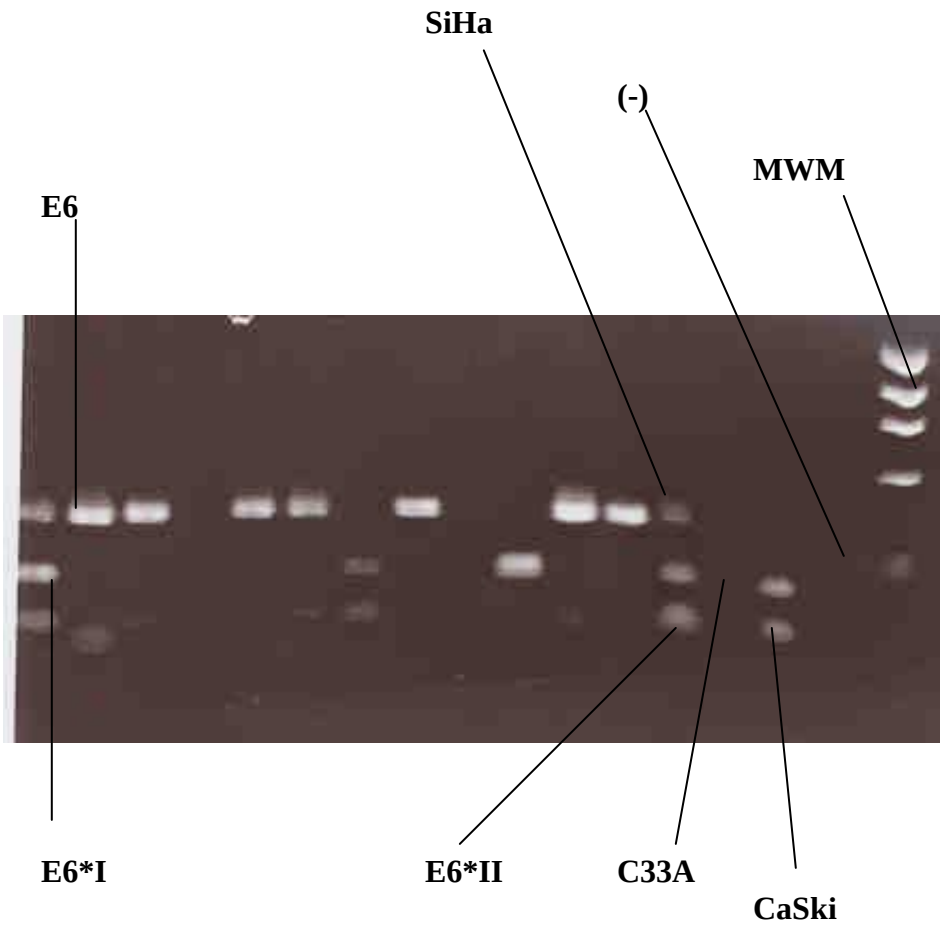
GEL IMAGE 6: RT-PCR PRODUCTS OF CELL – LINES AND CONTROLS INCLUDING NEGATIVE CONTROL



GEL IMAGE 7:

ABOVE: NESTED PCR PRODUCTS OF SAMPLES AND CELL LINES USING S1/2 PRIMERS.

BELOW: RT-PCR PRODUCTS USING S3/4 PRIMERS.



GEL IMAGE 8: NESTED RT-PCR PRODUCTS DEMONSTRATING *E6/E6*I/E6*II* GENES WITH S1/S2 PRIMERS IN SAMPLES & CELL- LINES (MWM= molecular weight marker) (-) = negative cell line

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