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

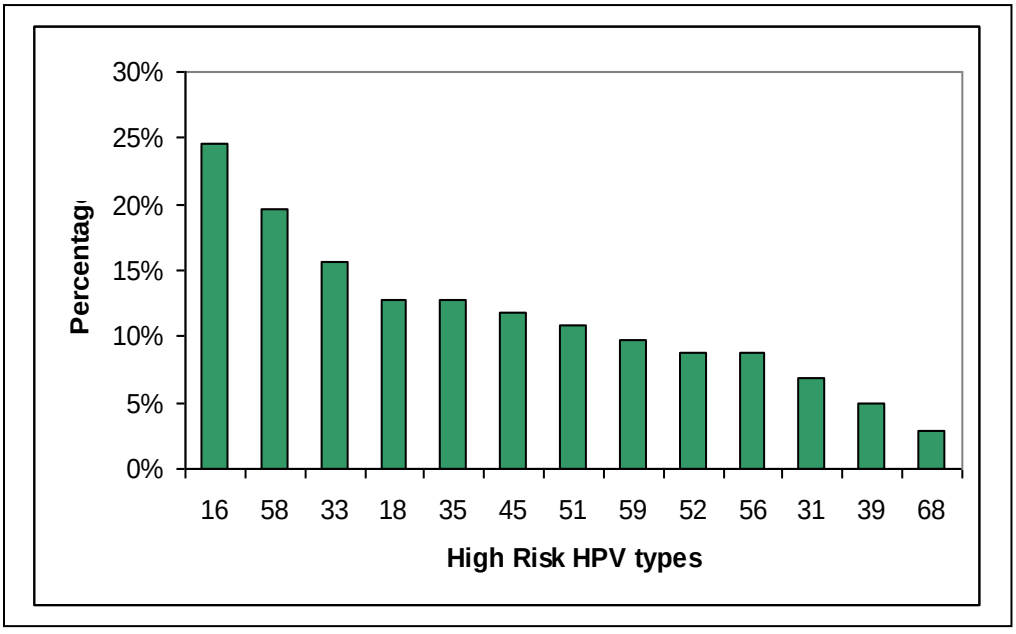
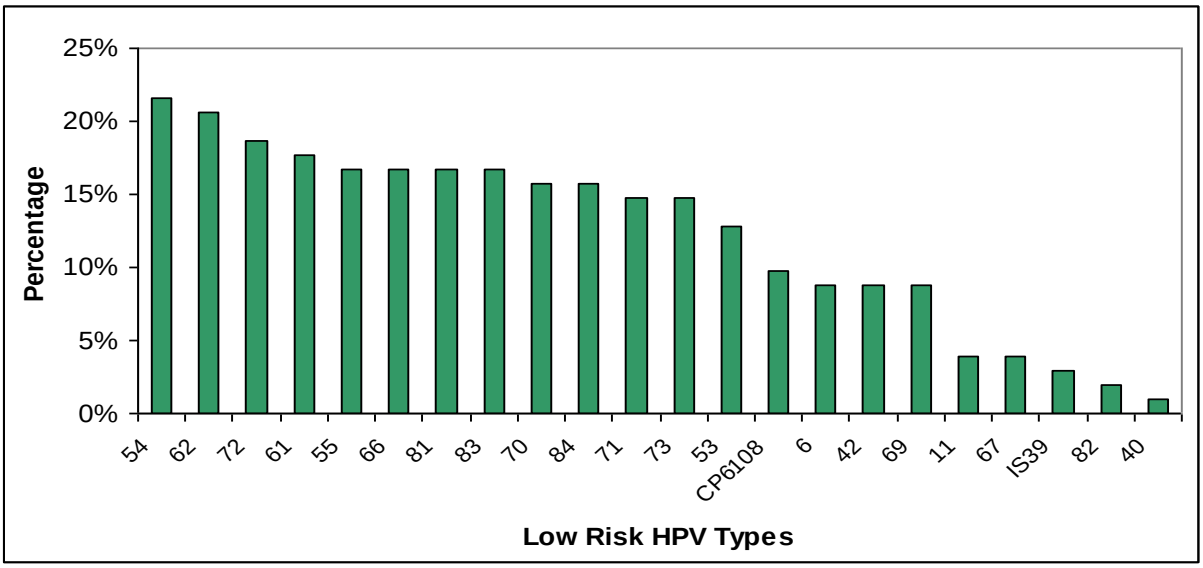


Figure 9: Low-risk HPV type distribution



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

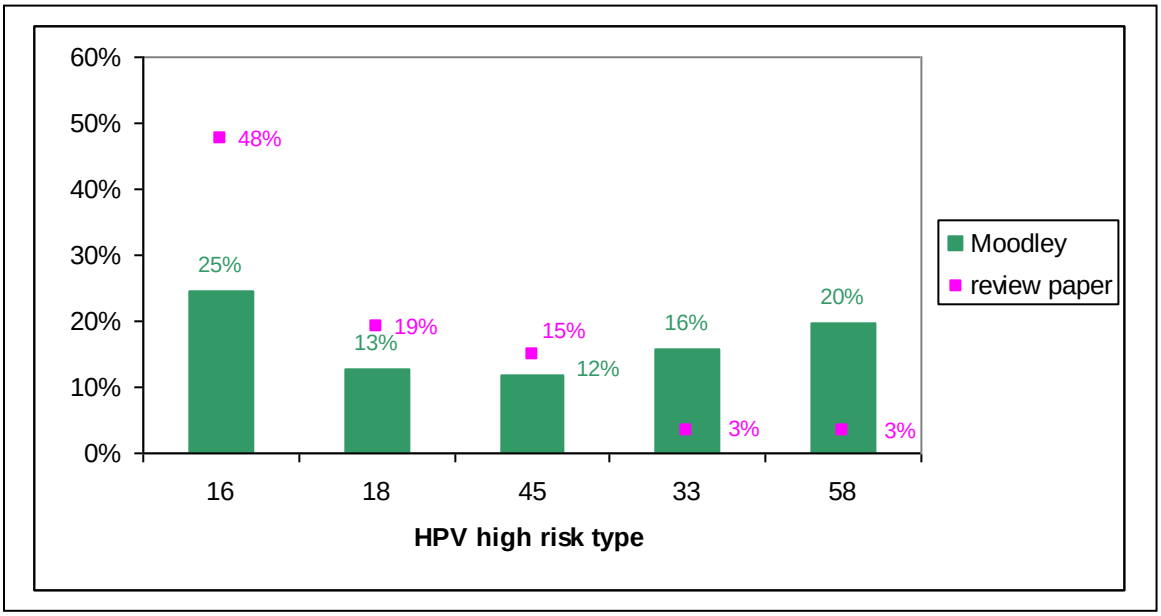


Figure 10: Comparison worldwide high-risk HPV prevalence (Clifford, 2003) and index study

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.

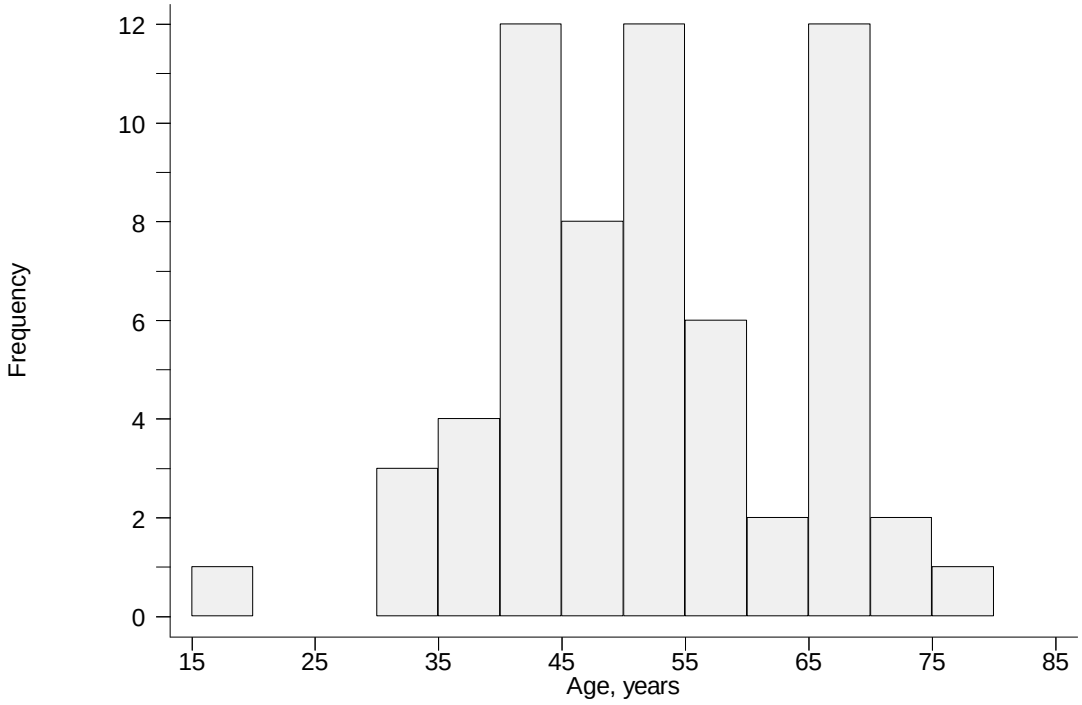


FIGURE 11: Age-distribution of patients

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)

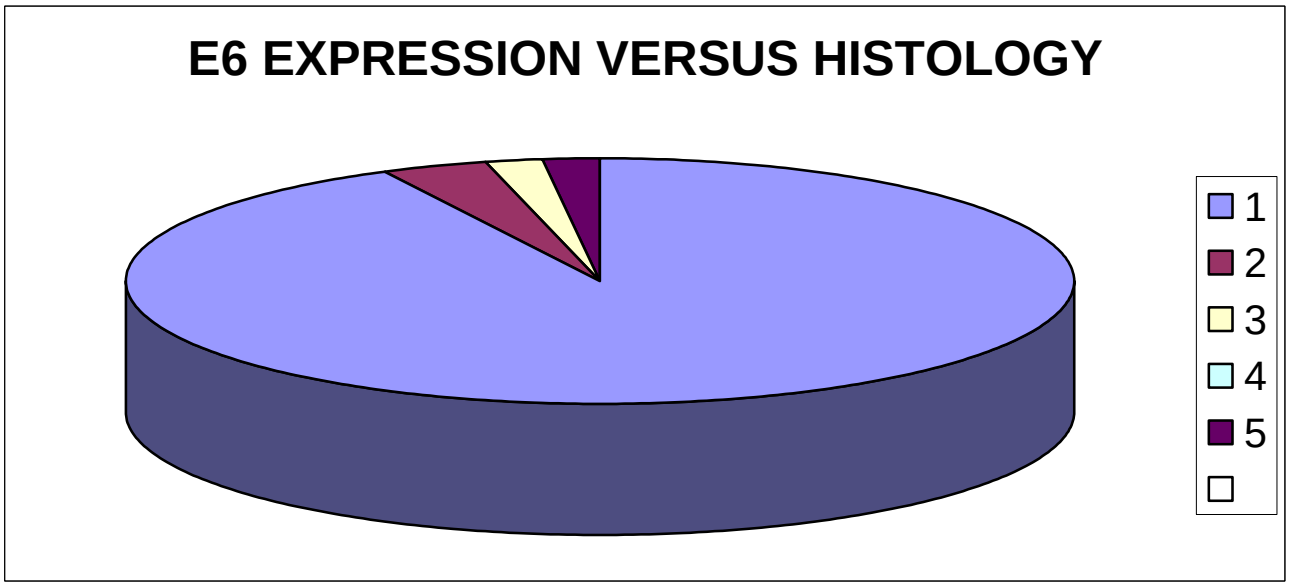
In the group of patients who used steroid contraception, the mean ages of patients **with** and **without** *E6* gene expression was 44.7 and 45.3 years, respectively (p=0.89). Of the non-contraceptive users, the mean age of patients **with** and **without** *E6* expression was 59 years (SD 12 years) 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.