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.

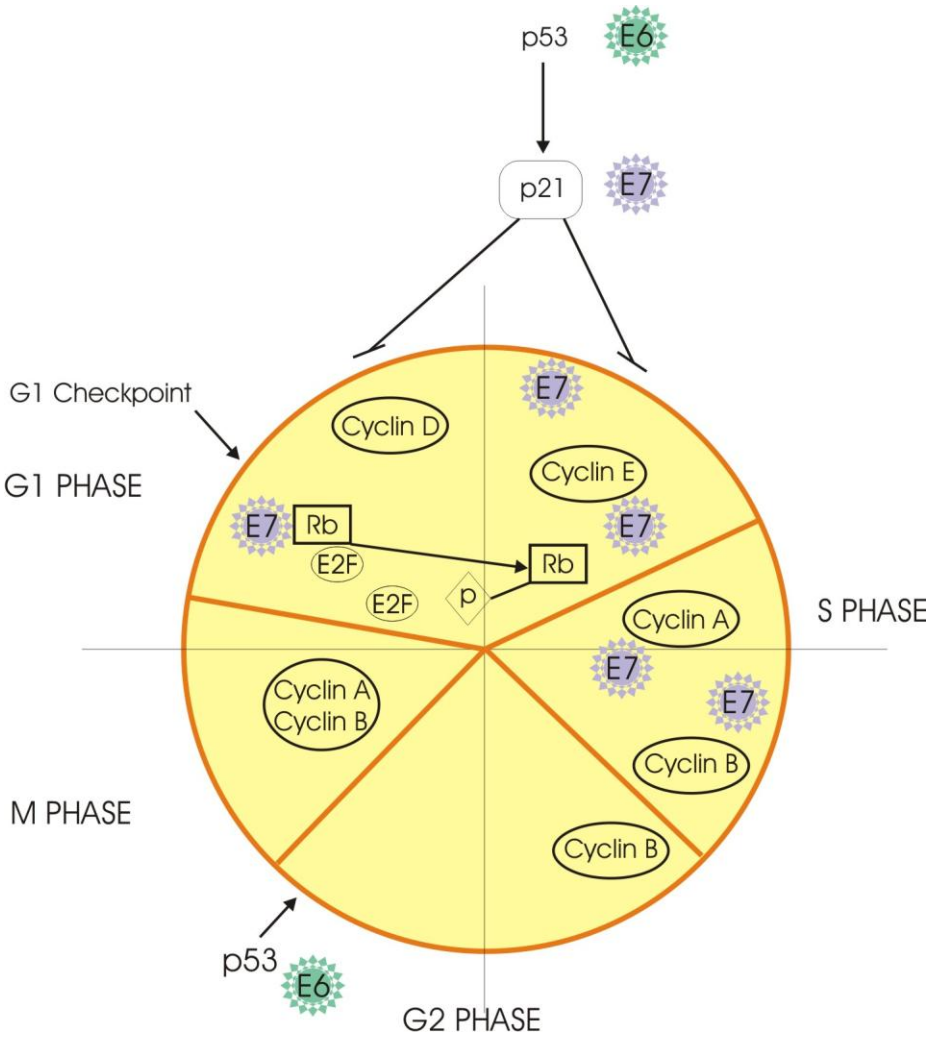

Figure 7: HPV 16 E6/E7 oncoproteins and cell-cycle interactions

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.