A STUDY OF VIRAL AND IMMUNOLOGICAL MARKERS OF HUMAN PAPILLOMAVIRUS
(HPV) RELATED PROGRESSION OF
CERVICAL NEOPLASIA

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1. Introduction

Cervical cancer is the second most common cancer among women worldwide with 493,000 new cases reported and 274,000 deaths in 2002.\(^1\) Eighty three percent of cervical cancer cases occur in the developing countries.\(^1\) In India, every year 132,082 new cases of cervical cancer cases are diagnosed and 74,118 women die from the disease.\(^2\) Thus, India has one-fourth of the global burden of cervical cancer.

Human papillomavirus (HPV) infection is an identified cause for the development of cervical cancer.\(^3\) Epidemiological studies have shown that infections with certain HPV types (genotypes) are responsible for most cases of cervical cancer. Based on available data, HPV is classified as established high risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59), probable high risk types (26, 53, 66, 68, 73, 82) and established low risk types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, CP6108).\(^4\) Globally, the most common high risk types in decreasing order of occurrence are HPV 16, 18, 45, 31, 33, 58, 52, 35, 59, 61, 82, 73 and 66.\(^5\) HPV 16 and 18 are the major types which account for 70% of the total HPV infections.\(^6\) India has a HPV prevalence rate of 96% in women with squamous cell carcinoma (SCC) and 100% in women with adenocarcinoma (ADC).\(^4\) HPV 16 and 18 are the major types reported in India which accounts for more than 70% of total infections, followed by 33, 58, 35 and 45.\(^7,8\)

The E6 and E7 proteins are the most important viral proteins needed for the transformation and immortalization of the infected cells. The E6 protein binds to the tumour suppressor protein p53 using a cellular ubiquitin ligase, E6 associated protein (E6AP). This leads to accelerated degradation of p53.\(^9\) The E6 protein also increases
telomerase activity by binding to the catalytic subunit of the telomerase enzyme, human telomerase reverse transcriptase (hTERT), thereby facilitating immortalization.\textsuperscript{10}

E7 protein binds to retinoblastoma family of proteins, sequesters it from the E2F/DP1 complex and degrades it by the ubiquitin-proteosome pathway. E7 proteins of the high risk types also bind to histone deacetylases (HDACs).\textsuperscript{11} This results in an increased expression of E2F responsive genes, thereby facilitating cell cycle progression.\textsuperscript{12,13}

E1 and E2 proteins are necessary for the regulation of viral replication and transcription. The E5 protein expression was found in both basal cells as well as differentiated cells, implying that these proteins play a role in the viral life cycle.\textsuperscript{14} L1 and L2 are the late proteins, also called as the major and minor capsid proteins respectively and form the viral capsid. They are expressed in the differentiated cells of the epithelium.

The fact that only certain women progress to cervical cancer shows that there are other risk factors that aid in the process. Several viral factors like HPV viral load in cervical tissue, HPV variants, integration into host chromosome, expression of oncogene transcripts, co-infection with HIV, HLA type of the host and host immune response are known to play an important role in deciding progression or regression of cervical disease. Epidemiological data has suggested that smoking, number of sex partners, use of oral contraceptives, and parity are also associated with development of cervical cancer.\textsuperscript{15-17}

HPVs are transmitted through skin to skin contact. High risk HPVs associated with the development of cervical cancer are transmitted through sexual contact.
Transmission of HPV through other means like blood transfusion has also been postulated.\textsuperscript{18}

The vast genetic diversity of HPV and its clinical implications makes detection and genotyping a necessary tool in management of cervical cancer. There are a various techniques used for detection of HPV. The most widely used method is target amplification by PCR with MY09/11 primers and genotyping with the restriction fragment length polymorphism (PCR-RFLP) technique\textsuperscript{19} or amplification using PGMY09/11 primers or SPF10 primers followed by reverse line blot hybridisation.\textsuperscript{20-22} Recently, a new Linear Array (LA) (Roche Molecular Systems, Inc, NJ) based on PGMY09/11 primers has been introduced that can detect 37 HPV genotypes.\textsuperscript{23} Detection of high risk HPV types is also done by signal amplification using Hybrid Capture 2 (HC2; Digene Corp, Gaithersburg, USA) which is the only U.S Food and Drug Administration licensed assay commercially available for clinical use.\textsuperscript{24}

The fact that HPV infection is a pre-requisite for development of cervical cancer has triggered research on the development of vaccines. Research in this area has been boosted by the fact that the HPV capsid proteins can self-assemble into virus like particles (VLPs) that retain the conformational epitopes. Prophylactic vaccines for the two major HPV types, 16 and 18 have been introduced and found to elicit good protective immune responses in vaccinees. Therapeutic vaccines based on the oncogenes E6 and E7 have also been tried out though with less success.

Infection with high risk HPV is considered a necessary but not a sufficient cause of cervical cancer by itself. Several studies have looked at the association of high risk HPV viral loads with viral persistence. HPV viral loads have been associated with an
increased risk of development of high grade lesions.\textsuperscript{25} Some studies have shown that HPV 16 viral load increases linearly with advancing disease stage\textsuperscript{26, 27}, whereas other studies have not found a significant association between viral load and severity of lesion.\textsuperscript{28} There is no data on HPV viral loads in Indian women at different stages of cervical cancer.

Evidence for the presence of HPV DNA in bloodstream of patients with cervical cancer is growing. The presence of HPV DNA in blood is now regarded not as a diagnostic marker, but a prognostic marker for recurrence of disease and metastasis.\textsuperscript{29, 30} Circulating HPV DNA has been detected in advanced stages of cervical cancer but not in pre-invasive lesions.\textsuperscript{29} Detection rates of HPV viremia differs between studies from 18\%\textsuperscript{31} to 24\%\textsuperscript{30} depending on the techniques used. There is only one study reported from India on HPV DNA presence in plasma and it showed that 11.8\% of women with cervical disease had HPV DNA in plasma.\textsuperscript{32}

Persistent infection of high risk HPV type is necessary for the progression of cervical cancer. The risk of developing high grade lesions is higher in patients who have persistent expression of E6/E7 transcripts.\textsuperscript{33} The expression of E6/E7 mRNA transcripts is seen to be significantly higher with advancing severity of cervical disease.\textsuperscript{34} Recent studies have also shown that E6/E7 mRNA expression is an independent prognostic marker of cervical cancer.\textsuperscript{35} There is one study in India that has looked at the prevalence of these transcripts in women with cervical cancer.\textsuperscript{36} But there are no studies from India that has looked at the load of transcripts expressed in different stages of cervical cancer.

Presence of HPV in blood as a marker of poor prognosis and metastasis has been studied extensively. Though viral DNA has been detected in blood in various studies, it
may be just reflective of tumour necrosis. However, the presence of transcripts of E6/E7 oncogenes in PBMCs might indicate the presence of active replicating tumor cells and therefore provide a basis for metastasis. There are only a few studies investigating the presence of E6/E7 transcripts in peripheral blood. HPV E6/E7 mRNA transcripts in peripheral blood mononuclear cells (PBMCs) has been found to serve as an early marker of metastasis and poor prognosis. These studies have been done in India.

The protective role of a good immune response to HPV infection can be deduced from the fact that HPV infections tend to persist longer in immunosuppressed transplant patients and HIV infected individuals. Published reports on cell mediated immune responses to HPV 16 peptides as measured by lymphoproliferation assay in patients with cervical cancer show conflicting results. Studies have shown that cell mediated immune responses to HPV 16 E7 peptide (aa 70-98) is associated with recurrence of cervical disease while responses to another E7 peptide (aa 35-74) is associated with regression from disease. One study showed that cervical cancer patients failed to mount a detectable immune response to HPV 16 peptides. Studies have also demonstrated the presence of HPV 16 E6 and E2 specific memory T helper response in healthy women. This response might be helpful in clearing persistent infection. Most of these studies have been performed in western countries, and there is a paucity of similar studies in the Indian population.

Studies have looked at the prevalence of serum antibodies to HPV 16 E6, E7 and E2 proteins. Antibody response to E2 and E7 peptides are shown to be more prevalent in cervical cancer cases compared to age matched community controls. Another study showed that antibodies to E6 and E7 proteins may be associated with advancing tumour stage whereas yet another study showed that antibodies to E7
peptides are not associated with disease stage. A study from India has shown that of these three peptides, responses to E2 peptide is significantly higher in cases of CIN than in cases with invasive cancer and women with chronic cervicitis (disease controls).

Antibody response to HPV virus like particles (VLPs) directed against the major capsid protein (L1) has been studied extensively. A study on a cohort of women in the sexually active age group has shown that women who seroconvert are 5.7 times more likely to have high grade lesions when compared to the women who did not seroconvert. The same study also showed that the median time for seroconversion is 8.3 months. The seroprevalence to HPV 16 VLPs was 46% in women who had HPV 16 in their genital tract. The presence of HPV 16 L1 antibodies has shown to be significantly associated with increased risk of cervical cancer. Thus far there are no reported studies on the presence of anti-VLP antibodies in Indian women with cervical neoplasia.

To summarize, this study aimed to look at certain viral and immunological markers of HPV in Indian women with cervical neoplasia with particular emphasis on the prognostic role of some of these markers.
Hypotheses

This thesis embodies the testing of the following hypotheses:

1. HPV DNA virus loads in cervical tissue increases with advancing HPV associated cervical disease stage.

2. HPV viremia is an useful non-invasive marker to assess cervical disease progression.

3. The presence of HPV mRNA transcripts in peripheral blood mononuclear cells (PBMCs) is an early marker of cervical disease progression.

4. Levels of expression of HPV oncogenes E6 and E7 increases with advancing disease stage.

5. T cell response to HPV as measured by an ELISPOT assay for interferon gamma is a good marker of disease progression.

6. Certain antibody responses to HPV are good markers of disease progression.
1. Aims and objectives:

2.1 Overall aim:

The overall aim of this study was to look at certain HPV related viral and immunological markers and study their association with progression of cervical neoplasia in Indian women.

2.2 Specific Objectives:

1. To amplify HPV DNA by PCR and genotype the strains present in cervical tissue of patients with cervical cancer by RFLP and line blot assay (LBA).

2. To quantitate HPV 16 and 18 DNA viral loads in cervical tissue using real time PCR and compare the findings with disease staging.

3. To detect HPV 16 and 18 plasma viremia using real time PCR and compare it with conventional MY 09/11 primer amplification.

4. To quantify HPV viral load in plasma by real time PCR and correlate the findings with disease stage.

5. To detect HPV 16 and 18 mRNA transcripts in cervical tissue using a sensitive and specific nested PCR and correlate the findings with disease stage.

6. To detect HPV 16 and 18 mRNA transcripts in PBMCs using a sensitive and specific nested PCR and correlate it with disease stage.

7. To quantitate HPV 16 and 18 mRNA transcripts in cervical tissue by real time PCR and correlate it with disease stage.
8. To detect T cell responses to HPV 16 peptides by measuring interferon gamma release using an ELISPOT assay in women with different stages of cervical neoplasia.

9. To detect the prevalence of antibodies to HPV 16 early proteins i.e., E2, E6, E7 and HPV 16 and 18 virus like particles (VLPs; capsid proteins) in Indian women with and without cervical neoplasia.
3. Review of literature:

3.1 History of Papillomavirus research:

Papillomaviruses research started in the 1930’s. The earliest evidence of work with papillomaviruses (PVs) started with the discovery that the cottontail rabbit papillomavirus (CRPV) could cause not only benign lesions in rabbits, but that some of these lesions also progressed to squamous cell cancers. The second papillomavirus that was identified was rabbit oral papillomavirus (ROPV), which was a non-oncogenic virus and caused spontaneously regressing papillomas of the mouth in domestic rabbits. These studies established the fact that papillomaviruses are host specific. Other papillomaviruses like the canine oral papillomavirus (COPV) and bovine oral papillomavirus (BOPV) were also identified during this period. Studies on papillomaviruses has since been limited because they were not cultivable in monolayer cultures.

The advent of molecular biology opened up new facets in papillomavirus research. Several medically important human papillomavirus (HPV) types were identified in the 1970’s. HPV 1 and 2 were identified from patients with cutaneous warts. Distinctly different HPV types were identified in lesions in patients with epidermodysplasia verruciformis (EV). Some lesions in patients with EV were seen to progress to squamous cell carcinoma. This was the first evidence that HPV was associated with human cancer.

The first genital HPVs that were identified were HPV 6 and 11. These HPV types were found in women with genital warts. The role of HPVs in cervical cancer was
established by the identification of two important types i.e., HPV 16 and HPV 18.\textsuperscript{58, 59} Though both these types were detected in women with cervical cancer, the real impact of the role of HPV in cervical cancer changed with the development of PCR primers for amplification of HPV DNA from cervical tissue.\textsuperscript{60} This resulted in large scale epidemiological studies that identified HPV infection as an important risk factor for the development of cervical cancer.\textsuperscript{61}

3.2 Papillomavirus structure:

Papillomaviruses are spherical, non-enveloped viruses. They are 55-60nm in diameter. The virus has a capsid made of the major capsid protein L1 and minor capsid protein L2. The capsid is made of 72 pentamers consisting of the major capsid protein L1 also called capsomers arranged in a T=7 icosahedral lattice. The exact number of the L2 monomer in the capsid is unknown. The viral genome is packaged inside the capsid. The structure of human papillomavirus virus like particles (VLPs) is shown in Figure 1.\textsuperscript{62}

3.3 Viral entry and receptors:

Studies on papillomaviruses have been hampered by the fact that they are difficult to cultivate in vitro. \(\alpha 6\) integrin has been proposed as a receptor for some papillomaviruses.\textsuperscript{63} CD16 seen in some immune cells and epithelium has also been proposed as potential accessory molecules that aid in viral attachment.\textsuperscript{64} The most studied of the receptors for papillomaviruses are the heparan sulphate proteoglycans (HSPGs). HSPGs are expressed ubiquitously at the surface of all mammalian cells and
are involved in various biological processes like cell adhesion. A study done on HPV 11 has shown that VLPs bind to heparin and cell surface glycosaminoglycans (GAGs) that resemble heparin. The same study also showed that removal of heparan sulphate by heparinise resulted in a 90% reduction in VLP attachment. This led to the belief that heparan sulphate in a receptor for the binding of papillomaviruses. Recently, another study has shown that HPV transiently binds to laminin5 (LN5), a component of the extra cellular matrix (ECM) complex. The minor capsid protein L2 is thought to have an important role after the initial binding of the virions to the receptors in the cell. This was confirmed by the fact that virions containing L2 mutants were found to be non-infectious.

After initial attachment of the virions, internalization occurs slowly through the endosomal pathway using actin at the early stages and microtubules at the late stages. A study shows that HPV L2 protein is cleaved by furin and this step is necessary for viral infection. A 40 amino acid residue in the C-terminal end of the L2 protein attaches to the microtubule network through dyenin, a motor protein resulting in intracytoplasmic transport of the virions. A mutation in this region results in reduced infectivity of the virions. The L2 protein with the encapsidated DNA then reaches the nucleus and accumulates at distinct nuclear domains called the nuclear domain 10 (ND10) which express promyelocytic leukemia protein (PML). PML expression also increases viral infectivity.
3.4 Taxonomy of Human Papillomaviruses:

Traditionally, HPVs were grouped along with polyomaviruses in the family *Papovaviridae*. This was based on electron microscopy studies that showed similar capsids and double stranded circular genomes.

Molecular characterization of the virus in the recent years has resulted in PVs being assigned a separate family *Papillomaviridae* by the International Committee on Taxonomy of Viruses. The HPV types that formed a major group are grouped as a “genus”. Genera are represented by Greek alphabets. For example, all the genital PVs are grouped under one genus named alpha papillomaviruses. The types that were closely related are grouped as “species”. The species in papillomaviruses are denoted by numbers. Each species contained types that not only share molecular similarity but also biological and pathogenic characteristics. In every species, one type is studied thoroughly and is called the “type species”. For example, in species 9, HPV 16 is the type species, as it is the type that has been mostly studied in that species.

Isolates are classified as separate “types” when the sequences in their L1 gene differ by more than 10% from other strains of the same species. Up to now, 118 types has been formally described. Papillomavirus types are usually host specific. The types causing cervical cancer are the most studied papillomavirus types. Isolates are classified as “sub-types” when the sequences in the L1 gene differ by 2-10% from other types. There are limited isolates that fall into this category. HPV 67 is a subtype of HPV 34, HPV 46 is a subtype of HPV 20 and HPV 55 is a subtype of HPV 44. The phylogenetic tree comprising 118 HPV types has been adapted from deVilliers and shown in Figure 2.
Isolates belonging to the same type with slight variations in their genome of up to 2% from the prototype are classified as “variants”. Numerous studies have been done on variants of common HPV types in humans.\textsuperscript{73, 74} Based on sequence variation of the HPV genome, HPV 16 variants are classified into six phylogenetic groups: Asian (As), European (E), African-American (AA), African1 (Af1), African 2(Af2) and North American (NA). HPV 18 variants are grouped based on sequence variation into four groups as Asian, Amerindian and African. Studies were done to look at the biological and pathogenic properties of these variants. Non-European variants of HPV 16 are shown to be more associated with higher prevalence and incidence of cervical neoplasia.\textsuperscript{75} Another study that looked at only European variants, has found a single variant of the E6 gene (L83V) to be more associated with cervical cancer risk.\textsuperscript{76}

### 3.5 Life cycle of HPVs:

The life cycle of HPV is linked to the differentiation of the host epithelium. The cellular receptors used by HPV are not clear; however heparin sulphate has been shown to attach virions in some HPV types.\textsuperscript{65} HPVs are thought to infect the basal epithelial cells through microaberations in the mucosa. After entry into the cell, the virus reaches the nucleus and establishes as extra-chromosomal plasmids (episomal forms). The virions are maintained at a copy number of 50 to 100 copies per cell.\textsuperscript{77}

The viral replication begins during the S phase of the cell cycle of the host cell with the help of the viral early proteins E1 and E2. In normal epithelium, the cells then undergo differentiation and cell cycle ceases. In HPV infected epithelium, the cells re-enter S phase of the cell cycle, resulting in high productive replication of the virus.\textsuperscript{77} This
process is mediated by the viral oncoprotein E7.\textsuperscript{78} This results in the synthesis of other viral proteins: the early proteins E1\textsuperscript{E}E4 and E5 and the capsid proteins L1 and L2.\textsuperscript{79} The viral progeny is then assembled in the differentiated cells and released into the environment.

### 3.6 HPV genome and gene products:

#### 3.6.1 HPV genome:

HPVs have a circular genome approximately 8kb in size. The genome comprises of early and late coding regions and the major regulatory region called the upstream regulatory region (URR) or the long control region (LCR). A schematic representation of the HPV genome adapted from Munoz\textsuperscript{4} is shown in Figure 3.

#### 3.6.2 HPV gene products:

The E6 and E7 proteins are the most important proteins needed for the transformation and immortalization of the infected cells. The E6 protein binds to the tumor suppressor protein p53 and induces its degradation.\textsuperscript{9} E7 protein binds to retinoblastoma family of proteins, thereby facilitating cell cycle progression.\textsuperscript{12, 13}

E1 and E2 proteins are necessary for the regulation of viral replication and transcription. Both these proteins bind to the recognition sequences in the URR and aid in the recruitment of DNA polymerases and other proteins to the origin of replication.\textsuperscript{80} E1 protein helps in unwinding of the supercoiled DNA by its ATPase and helicase
activity. The E1 protein binds with the full length E2 protein and recruits other E1 proteins to bind with higher affinity to the recognition sequences in the URR.

The E2 protein plays an important role in viral gene expression as well as viral replication. The E2 protein binds to the recognition sequences seen in the URR and thereby regulates transcription of viral promoter seen upstream of the E6 open reading frame. This leads to copy number control of the E6 oncoprotein.

L1 and L2 are the late proteins, also called as the major and minor capsid proteins respectively. They are encoded in the differentiated cells of the epithelium. These proteins form the icosahedral capsid around a single copy of the genome, before the virion is released into the environment.

The most expressed protein during the life cycle of the virus is the E1 E4 fusion protein that is expressed during the late stages of replication. The fusion protein is made from the fusion of the first five amino acids of the E1 protein and the E4 protein. The synthesis of the E1 E4 precedes that of the late proteins. The E1 E4 protein undergoes post translational cleavage, and is seen in the cytoplasm attached to cytokeratins. The E4 protein has been shown to play an important role in the productive stage of the life cycle. Expression of this fusion protein has also been shown to result in collapse of cytokeratin network. The E1 E4 protein has also been shown to arrest the cell cycle in the G2 phase.

The E5 proteins are small membrane bound proteins. The E5 protein expression is found in both basal cells as well as differentiated cells, implying that these proteins play a role in the viral life cycle. These proteins are shown to bind to and activate platelet derived growth factor (PDGF) and epidermal growth factor (EGF) receptors.
3.6.3 HPV transcription control:

Transcription and translation of papillomavirus is controlled by two major promoters and many minor promoters. The early viral promoter is called p97 in HPV 31 and HPV 16 and p105 in HPV 18 and is expressed throughout the life cycle of the virus. These promoters control the expression of the early genes. In high-risk HPV infection, a single promoter controls the expression of both the E6 and E7 transcripts, whereas in low-risk HPV infections, separate promoters are involved for E6 and E7 transcription. HPV E1 and E2 mRNAs are the first transcripts to be found after initiation of gene expression in organotypic cultures infected with HPV 31. Most early transcripts terminate after transcription of the E5 gene.

The second major viral promoter is the late viral promoter, and is called p742 in HPV 31 and p670 in HPV 16. It is seen in the E7 gene and directs the expression of two sets of transcripts. The first transcripts encoded are the E1 E4, E5 and E1/E2 proteins. The second set of transcripts that are encoded are the late viral genes, L1 and L2.

The activity of the early promoter is controlled by various cellular and viral transcriptional factors. These cellular factors bind to specific sequences in the URR, thereby regulating the expression of the promoter. The transcriptional control is found to be dependent on the stage of the viral life cycle. Transcription sites for various cellular factors like Ap-1, Sp-1, TFIID, YY1, KRF and AP2 are present in all HPV types. The cellular factors that control the late viral promoter transcription are currently unknown. However, chromatin remodelling after differentiation of cells and histone
deacetylation around the late promoter region has been found to contribute to late viral promoter transcription.\textsuperscript{104}

The early viral transcripts are polycistrionic in nature. E6 and E7 proteins are synthesised by alternate splicing of the E6 ORF. The unspliced transcript is translated as the E6 protein, and the spliced transcript E6*I is translated as the E7 protein. Splicing and poly adenylation helps in maintaining the levels of various HPV transcripts.

3.6.4 Regulation of HPV DNA replication:

HPV DNA replication during the productive life cycle of the virus occurs in three phases: establishment, maintenance and amplification. During the establishment stage, one HPV genome enters the nucleus of the host cell and directs the synthesis of 20 to 50 copies of viral DNA per cell.\textsuperscript{105} The viral genome then replicates once every cell cycle and maintains a constant copy number using the expression of the E2 protein.\textsuperscript{106} The levels of E2 protein controls early viral transcription. At low level, the E2 protein triggers the expression of E1 and E2 proteins whereas at high level, the E2 protein acts as a transcriptional repressor.\textsuperscript{106} Thus, the viral copy number is maintained at a steady level in undifferentiated cells. As differentiation occurs, the promoter usage shifts from the early promoter to a late promoter in the located in the E7 gene.\textsuperscript{107} This results in an increase in the viral amplification to thousand genome copies per cell.

3.6.5 Role of oncoproteins E6 and E7 in the viral life cycle:

The E6 and E7 proteins are encoded by all papillomaviruses, and play an important role in viral life cycle. In addition to this, they also play a major role as oncoproteins in high risk HPVs.
The E7 protein binds to retinoblastoma family of proteins and inactivates them.\textsuperscript{108} The retinoblastoma protein (pRb) is normally seen as a complex with elongation factor (E2F). The E7 protein binds to the pRb and sequesters it from the pRb-E2F complex. This results in the increased expression of E2F responsive genes. Analysis has shown that the E7 protein of the high risk HPVs bind to the pRb with a higher affinity that those seen in low risk types.\textsuperscript{109} E7 proteins of the high risk types also bind to histone deacetylases (HDACs).\textsuperscript{11} This binding results in the activation of E2F inducible genes. E2F proteins activate expression of various genes necessary for cell cycle progression. The E7 protein binds directly to Mi2beta, a component of the nucleosome remodelling-deacetylase (NURD deacetylase) complex and mediates cell cycle progression.\textsuperscript{11} This binding has been also shown to be important for the normal life cycle of the virus.\textsuperscript{110} The mechanism of action of E7 oncoprotein has been adapted from Gnamamony\textsuperscript{111} showed in Figure 4.

E6 protein also plays a very important role in the immortalization of the infected cells. The E6 protein forms a complex with the tumor suppressor protein p53 and the E6 associated protein (E6AP) and this leads to the degradation of the p53 by ubiquitination.\textsuperscript{9} \textsuperscript{112-114} The E6 protein also activates telomerase by increasing the expression of the catalytic subunit of the telomerase enzyme, human telomerase reverse transcriptase (hTERT).\textsuperscript{10} The presence of E7 protein was found to augment this expression.\textsuperscript{10} The E6 protein also binds to the PDZ domain (PSD-95/disc large/ZO-1) of a set of E6 binding cellular proteins.\textsuperscript{115} However, the role of these activities in the normal life cycle of the virus remain unclear as the low risk types effectively undergo viral replication without increasing telomerase activity, degrading p53 and binding to PDZ domain-containing cellular proteins.
The E6 protein binds to other cellular proteins that may aid in the normal life cycle of the virus. The E6 protein has been shown to bind to the c-terminal end of the tumor necrosis factor R1, blocking apoptotic signal transduction, thereby preventing the cell from tumor necrosis factor mediated apoptosis.\textsuperscript{116} However, recent studies also show that the E6 protein can act on cells in a dose dependent manner. High level of E6 expression has been shown to sensitize tumor necrosis factor (TNF) whereas low level of E6 expression has been shown to render the cells resistant to TNF.\textsuperscript{117}

The E6 protein has been shown to bind to protein kinase PKN leading to phosphorylation of the E6 protein, which may play an important role in immortalization of the cells.\textsuperscript{118} The E6 protein interferes with DNA repair by binding directly to X-ray repair complementing defective repair in Chinese hamster cells 1(XRCC1), an important protein needed for DNA repair and genomic stability in normal cells.\textsuperscript{119} The E6 protein also binds to human transcriptional adaptor 3 (hADA3), a co-activator of p53 thereby resulting in a loss of p53 function.\textsuperscript{120} This is an alternate mechanism to direct p53 degradation by which the E6 protein helps in cellular immortalization. The mechanism of action of E6 oncoprotein in bringing about loss of cell cycle control is adapted from Gnanamony\textsuperscript{111} showed in Figure 5.

### 3.7 Epidemiology of HPV associated cervical neoplasia:

Cervical cancer is the second most common cancer among women worldwide with 493,000 new cases reported and 274,000 deaths in 2002.\textsuperscript{1} About 80% of cervical cancer cases occur in the developing countries.\textsuperscript{1} In India, every year 132,082 new cases
of cervical cancer cases are diagnosed and 74,118 women die from the disease. Thus, India has one-fourth of the global burden of cervical cancer.

Epidemiological studies have shown that infections with certain HPV types (genotypes) are responsible for most cases of cervical cancer. Based on epidemiological data, HPV is classified as established high risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59), probable high risk types (26, 53, 66, 68, 73, 82) and established low risk types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, CP6108). Globally, the most common high risk types in decreasing order of occurrence are HPV 16, 18, 45, 31, 33, 58, 52, 35, 59, 56, 51, 68, 39, 82, 73 and 66. HPV 16 and 18 are the major types which account for 70% of the total HPV infections.

3.8 Natural history of cervical cancer:

Most cervical cancer occurs in the transformation zone in the cervix. The transformation zone is the junction between the columnar cells of the endocervix and the squamous cells of the ectocervix. Squamous cell cancer accounts for nearly 85% of cervical cancer followed by adenocarcinoma.

In HPV infected women, the cervical cells undergo lot of changes over a long period of time. As time progresses, the squamous cells are slowly displaced by the basoloid cells. As the stage advances, the entire zone is composed of basoloid cells. Cervical neoplasia has been classified based on the dysplastic cells as cervical intraepithelial neoplasia (CIN) grade 1, grade 2 and grade 3 followed by invasive
The role of HPV in the natural history of cervical cancer is shown in Figure 6.  

3.9 Factors associated with progression of HPV associated cervical neoplasia:

HPV infection is a pre-requisite for development of cervical cancer. However, the fact that only certain women progress to cervical cancer shows that there are other risk factors that aid in the process. Epidemiological studies have identified certain factors that show increased risk. An overview of the risk factors involved in cervical cancer is given in Figure 7.  

3.9.1 Viral and host factors:

1. Virus loads: The role of HPV viral load in the biology of progression of cervical cancer remains unclear. Variation in results of these studies has restricted the clinical utility of viral load testing. High HPV viral load is a marker of persistence of infection. Numerous studies have looked at the association of high risk HPV DNA viral load with advancing cervical disease. Several studies have argued that HPV 16 viral load is dependent on the grade of cervical disease. Median HPV viral load did not increase with advancing severity of disease. However, there are other studies that show that HPV viral load does not increase significantly between CIN grades in a study done on paraffin embedded tissues. A recent study done on liquid based cytology samples show that HPV 16 viral load in fact decreases with advancing disease stage. A study done by Gravitt shows that though high viral load of high risk types are associated with
prevalent infection, only high HPV 16 viral load is associated with incident infection.\textsuperscript{126} High HPV16 viral load has been reported in some studies as a predictive factor for development of high grade cervical lesions.\textsuperscript{25,127}

2. **Plasma viremia:** The role of HPV viremia as a non-invasive marker of cervical disease prognosis has been long debated. The reason for this is the use of different methodologies by different scientists resulting in variable results. One study that used type specific PCR detected HPV 16 or 18 DNA in plasma of 6.9% women with invasive cancer of which 18.1% had the same type in their genital tract.\textsuperscript{31} Another study using PCR based hybridisation detected HPV plasma viremia in 12% of women with invasive cancer in their genital tract.\textsuperscript{128} A sensitive nested PCR approach using consensus primers MY09/11 has shown an increase in sensitivity, and was able to detect HPV DNA in plasma in 65% of women with invasive cervical cancer.\textsuperscript{129}

3. **HPV Integration:** Integration of HPV genome into the host chromosome is associated with oncogenesis.\textsuperscript{130} Integration results in the disruption of the E2 gene, thereby removing the transcriptional control on the expression of oncoproteins E6 and E7 leading to carcinogenesis.\textsuperscript{130} Studies have reported a new assay called the amplification of oncogene transcript (APOT) that can measure integrated forms as compared to the episomal ones. The rate of integration of HPV into host genome has been shown to increase with advancing cervical disease stage.\textsuperscript{131}

4. **Viral variants:** Studies have looked at the role of HPV variants as a risk factor for the development of cervical neoplasia. The non-European variants of HPV 16
have been found to be more associated with increased risk of high grade cervical neoplasia.

5. **HLA polymorphism:** Numerous studies from different populations have shown a protective role of HLA DRB1*1301 in cervical cancer.

6. **Co-infection with HIV:** The role of HIV co-infection in the pathogenesis of HPV infection remains unclear. Studies have shown an increased prevalence of HPV in HIV seropositive individuals. A study has shown that persistent infection with high risk HPV is significantly more in HIV seropositive women compared to seronegative women. Another study showed that a CD4+ level of less than 200/mm$^3$ and not HIV RNA load is associated with increased prevalence of HPV.

3.9.2 Other co-factors:

1. **Use of oral contraceptives (OC):** The use of oral contraceptives has been shown to increase the risk of cervical cancer. However, the risk has been shown to be dose dependent. There was no risk reported for usage of OC up to 4 years. But long term usage more than 5 years have shown to increase the risk of cervical neoplasia. Though the exact mechanism of action is not known, a study has shown a eight fold increase in transcription of E6/E7 oncogenes in cell culture in the presence of oestrogen.

2. **Parity:** High parity has been associated with increased risk of developing cervical neoplasia. The odds ratio of cervical cancer was increased fourfold when women
had seven or more full term pregnancies compared to those who did not have any.\textsuperscript{16}

3. **Tobacco smoking:** Studies have shown that tobacco smoking is a risk factor in the development of cervical cancer. Various mechanisms have been postulated for this effect. One study has suggested that tobacco may cause immunosuppression in the cervix by reducing the number of Langerhans cells thereby interfering with the normal immune response to the virus.\textsuperscript{136} Another study has shown that smokers tend to have HPV infection for a longer time in their cervix than non-smokers.\textsuperscript{137}

3.9.3 **Other prognostic markers:**

1. **Squamous cell carcinoma (SCC) antigen:** SCC antigen has been shown to be elevated in women with cervical cancer. Elevated levels of SCC antigen in serum have been shown to be a risk factor for lymph node metastasis. However the role of pre-treatment SCC antigen levels in predicting treatment outcome is still debated.\textsuperscript{138}

2. **CYFRA 21-1:** Cytokeratins are usually components of epithelial cells, but soluble forms are also seen in the circulation. The EIA format to detect cytokeratin 19 is called the CYFRA 21-1. The level of the cytokeratins in serum is elevated (42-63\%) in women with cervical neoplasia. Pre-operative detection of CYFRA 21-1 is shown to be a good marker of overall survival of patients with cervical cancer.
3. **CA-125:** CA-125 is a component of a glycoprotein and has been elevated in women with cervical adenocarcinoma. Levels of pre treatment and post treatment serum CA-125 has been showed to be a marker of disease survival.\(^{138}\)

4. **Vascular endothelial growth factor (VEGF):** VEGFs are dimorphic glycoproteins that bind to tyrosine kinase receptors (VEGFR) and play an important role in the formation of vascular system during embryogenesis. They are frequently detected in serum of women with cervical cancer can be used as a marker of treatment efficacy.\(^{138}\)

### 3.10 Transmission of HPV:

HPVs are transmitted by skin to skin contact. Sexual mode of transmission is the most widely accepted mode of transmission of genital HPVs. It has also been reported from a study done in heterosexual couples that the ratio of transmission from females to males is higher than that from males to females.\(^{139}\) A recent study has suggested a possibility of transmission of HPV through other modes like blood transfusion.\(^{18}\) This study detected HPV in PBMC of paediatric patients with known history of transfusion acquired HIV. The study postulates that PBMCs may be carriers of HPV.
3.11 Lab diagnosis of HPV infection:

The fact that HPV cannot be cultured successfully has hampered the development of assays for the detection of HPV infections in cervical cancer. So, the main screening tool for cervical cancer is still cytology and histology. Newer assays like HPV DNA detection and HPV mRNA detection have become available and are discussed below.

3.11.1 Conventional cytology:

The Papanicolou (Pap) smear, introduced by Papanicolou and Traut in 1943, identifies changes in cells of the transformation zone of cervix caused by HPV infection. Abnormal cells are vacuolated with a pyknotic nucleus surrounded by a halo and are termed as “koilocytes”. The current interpretation of Pap smear is based on the Bethesda system.

However, cytology has its limitations. Inadequate sampling, poor sensitivity with false negative results, contaminants in the sample have been reported. Automated cytologic tests using PapNet (Neuromedical systems, Suffern, NY) and Autopap 300 QC (Neopath, Redmond, Wash) have been approved by the Food and Drug Administration, USA for screening smears to identify false negative smears.

3.11.2 Liquid cytology:

Liquid cytology has been used as an alternate for conventional cytology. The specimen is collected in a preservative solution; debris is removed thereby aiding clear visualization of the cells. Fluid based technology also reduces false negative smear results. The detection rate increased by 71% for low grade squamous intraepithelial lesions and 102% for high grade squamous intraepithelial lesions.
Colposcopy and colposcopy directed biopsies are done in patients with abnormal Pap smears. Visual inspection is done with naked eye after application of 3% acetic acid solution (VIA). Dysplastic cells appear as acetowhite lesions. Visual inspection with Lugol’s iodine (VILI) is another approach where the cervix is viewed with the naked eye after application of iodine solution. Normal squamous epithelial cells appear brown or almost black in colour whereas abnormal cells appear colourless, pale or mustard yellow in colour. Cervical biopsy is then done to confirm malignancy. Cervical biopsy is considered the gold standard for the detection of cervical neoplasia and HPV infection.

3.11.3 In-situ hybridisation:

In-situ hybridisation is a technique used to detect HPV directly in cervical tissue. However the sensitivity of this assay is lower than other hybridisation techniques like southern blot hybridisation.

3.11.4 HPV DNA detection:

Conventional cytology has been the modality for screening for cervical cancer. However studies have shown that women who are Pap smear negative but HPV DNA positive are at a risk of progression of cervical disease. This shows that HPV DNA testing can be used as an adjunct to conventional cytology for screening of cervical cancer.
3.11.4.1 Signal amplification assays:

3.11.4.1.1 Hybrid Capture (HC2) assay:

Hybrid Capture 2 (HC2, Digene, USA) is the only assay system that is approved by the Food and Drug Administration (FDA, USA) for detection of HPV in exfoliated cervical cells. Cells are collected in a cytobrush and suspended in specimen transport medium (STM) for preservation of virus. The samples are then treated with sodium hydroxide, and incubated with two pools containing single stranded RNA probes. Pool A contains a mixture of probes of 5 low risk types (6, 11, 42, 43, and 44) and pool B contains a mixture of probes of 13 high risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). The hybridised mixture is then added to microwell plates coated with antibodies to DNA/RNA hybrids. Alkaline phosphatase labelled monoclonal antibody to the DNA/RNA hybrids is added followed by a chemiluminescent substrate. The luminescence is captured in a luminometer. Results are interpreted as relative light units (RLU). The principle of hybrid capture is shown in Figure 8.

The HC2 is easy to perform and has shown to be highly reproducible with an intra-assay coefficient of variation of 5%. HC2 has a lower detection limit of 5000 genome copies (1 pg/ml). However, a disadvantage of this method is the presence of false positivity. The high risk cocktail was shown to cross react with some low risk HPV types.

3.11.4.2 Target amplification methods:

Amplification of target DNA is done by polymerase chain reaction (PCR). PCR is now considered the gold standard for the detection of HPV infection. PCR relies on
amplification of a specific region of the genome using specific primers. Detection of amplified products can be done by agarose gel electrophoresis, EIA, reverse line blot hybridisation and DNA sequencing. There are different approaches for PCR and are described below.

3.11.4.2.1 Consensus primer amplification:

Amplification of a broad spectrum of HPV types can be achieved by using the consensus primer approach. This technique is based on the fact that there are conserved sequences that are shared between different HPV types. This can be used as a general screening technique. Different studies have come up with different sets of primers for PCR. Since the L1 region of the HPV genome is highly conserved, consensus primers have been constructed from this region.

The most widely used consensus primers are the MY09/11 primers, the modified PGMY09/11 primers, GP5+/6(+) primers and the SPF10 primers. The MY09/11 primers amplify a 450 base pair region in the L1 genome. An earlier published study even suggests an improvement in detection of HPV by using a nested PCR approach with MY09/11 primers as outer primers and GP5+/6(+) primers as inner primers.

The modified GP5+/6+ primers were able to amplify 14 high risk HPV types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) and 6 low risk types (HPV 6, 11, 40, 42, 43, and 44). These primers amplified a smaller region of approximately 150 base pairs. Detection is based on enzyme immunoassay (EIA) and the method has a detection limit of 10 to 200 copies depending on the HPV type.
The PGMY09/11 primers are the modified version of the widely used MY09/11 primers to increase amplification efficiency.\textsuperscript{21} The primer consists of PGMY11 (pool of 5 oligonucleotides) and PGMY09 (pool of 13 oligonucleotides). This primer system was shown to increase detection rate of HPV DNA and also amplify certain types for which the MY primers were not effective.\textsuperscript{21}

Another set of primer based on the L1 gene are the SPF10 primers. These primers were designed to detect 43 different HPV types. The primers amplify a 65 base pair product and the genotypes can be detected by reverse line blot hybridisation.\textsuperscript{22} Because of the small amplicon length, these primers can amplify HPV DNA in archived formalin-fixed tissues as well.

\textbf{3.11.4.2.2 Type specific PCR:}

Unlike the consensus primers, some studies also use type specific PCRs. These studies use primers that are specific for that type. One study looked at the prevalence of HPV using primers designed from the E7 region of 14 high risk types.\textsuperscript{3} The advantage of this method is that a separate genotyping method is not necessary after amplification. However, the assay is time consuming and expensive in the case of less prevalent HPV types as many PCRs has to be done to assign the genotypes.

\textbf{3.11.5 HPV Genotyping methods:}

After amplification of HPV DNA, there are many methods available for genotyping.
3.11.5.1 Restriction fragment length polymorphism (RFLP):

RFLP is a reliable and cost effective technique for genotyping HPV. A common method is digestion of the amplified HPV DNA with a set of seven restriction enzymes. Based on the restriction sites in the sequence, different types give different digestion patterns. Comparison of the pattern obtained with already published patterns helps to ascertain the genotypes.\(^{19}\) This method can identify 44 HPV types including the 15 oncogenic types.

Another study has used digestion with a single restriction enzyme \(RsaI\) to detect five common types (HPV 6, 11, 16, 18, and 33).\(^{149}\) Another study uses digestion with a single restriction enzyme \(HpyCH4V\) to detect 41 HPV types including the most prevalent 15 oncogenic types.\(^{150}\)

3.11.5.2 Reverse line blot hybridisation:

Genotyping by reverse blot hybridisation has been commercialised by two manufacturers: the first is based on amplification by PGMY09/11 primers called Roche Linear Array HPV genotyping kit (Roche, Alameda, CA) and the second is based on the amplification by SPF10 primers and is called the Inno LiPA HPV genotyping kit (Innogenetics, Belgium). The Linear array can detect 37 genotypes whereas the Inno LiPA can detect up to 43 HPV types. Both these types are based on hybridisation of type specific probes on to strips and compared well in assigning types.\(^{23}\)

3.11.5.3 PCR based Enzyme immunoassay:

Genotyping of PCR products generated by amplification using GP5+/6(+) consensus primers has been done by enzyme immunoassay (EIA). In this method, PCR
products were generated using biotinylated primers. The amplified products were
denatured and added to streptavidin coated plates. Digoxigenin (DIG) labelled probes
were then added to the wells along with alkaline phosphatase conjugated with anti-DIG
antibodies. The endpoint was detected by measurement of color. This method showed
good agreement with hybrid capture for HPV genotyping.\textsuperscript{151}

Another recent method using PCR based EIA is the Roche AMPLICOR HPV
genotyping kit (Roche, Alameda, CA, USA). This method can detect the same 13 high
risk types that are detected by HC2. This method compared well with HC2 in detecting
HPV in women with abnormal Pap results.\textsuperscript{152}

3.11.6 Newer markers in the diagnosis of cervical disease:

3.11.6.1 p16\textsuperscript{INK4a} detection:

Cyclin-dependent kinase 4 inhibitor A (p16\textsuperscript{INK4a}) overexpression has been
observed in high grade cervical lesions and thus has been proposed to be used as a
surrogate marker of high risk HPV infection and cervical neoplasia. A recent study has
reported that p16\textsuperscript{INK4a} immunocytochemical staining has a sensitivity of 96\% in detecting
women with atypical squamous cells (ASC) and can be used as an adjunct in routing
cytological screening.\textsuperscript{153}

3.11.6.2 Pre-tect HPV Proofer:

Pre-tect HPV proofer is based on multiplex nucleic acid sequence based
amplification (NASBA) with molecular beacons. It can detect mRNA transcripts of high
risk types 16, 18, 31, 33, and 45 specifically even in the presence of background DNA
and thus have been proposed as a marker of persistent HPV infection.\textsuperscript{154}
3.12 Immune response to HPV infections:

3.12.1 Innate immunity and immune evasion of HPV:

More than 95% of women who have HPV infection clear the virus. This suggests that a good immune response is present in most women. A good innate and adaptive immune response is needed for the effective clearance of the virus.

The innate immune response is the first line of defence against viral infection. Since HPV does not have a viremic phase, the mucosal immune response plays an important role in protection from infection. Epithelial cells are the first line of defence offering a physical barrier. Several factors mediate protection of the host from HPV infection. They are

1. **Interferons**: Interferons alpha and beta (type I IFNs) are secreted by epithelial cells and contribute in the innate immune response whereas interferon gamma (type II IFN) is secreted by activated T cells and NK cells and modulate immune function. Both type I and II IFNs are known to reduce the expression of HPV E6/E7 in cell lines immortalised with HPV 16. Studies have shown that HPV 16 E6 and E7 proteins inhibits IFN expression and signalling by two mechanisms. HPV E6 protein bind to the TYK2, thereby preventing it’s binding with interferon receptors. This leads to inactivation of the JAK-STAT pathway, that is necessary for IFN-alpha action. It has also been noticed that HPV E6 protein binds to IFN regulatory factor -3 (IRF-3) and inactivates it. This has been shown to result in reduced expression of IFN-beta. The E6 protein of the high risk types bind strongly, where as the E6 protein of low risk types bind weakly to IRF-3. The HPV E7 protein has been shown to bind to p48, a
component of interferon stimulated gene factor 3 (ISGF3) transcription complex, thereby inhibiting the function of IFN alpha.\textsuperscript{159} The E7 protein also binds to interferon regulatory factor-1 (IRF-1) inhibiting its function, which is thought to be an important immune evasion mechanism.\textsuperscript{160}

2. \textbf{Inflammation:} Inflammatory response, an important component of the innate immunity is stimulated by pro-inflammatory cytokines like interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-alpha). These pro-inflammatory cytokines have been shown to down regulate E6/E7 gene expression.\textsuperscript{161} However in HPV infection, there is a lack of good pro-inflammatory response. This may be because of the low levels of expression of early viral proteins, absence of cell lysis after infection. On the other hand, it has been shown that HPV 16 E6 protein down regulates the expression of a pro-inflammatory cytokine IL-18.\textsuperscript{162} HPV has also been shown to attach to the TNF receptor and protects cells by blocking apoptosis mediated by TNF-alpha.\textsuperscript{116}

3. \textbf{NF-kB:} NF-kB plays an important role in regulating cellular responses to stress. It is a transcription factor that activates genes responsible for inflammatory and immune responses. The E5 protein of bovine papillomavirus (BPV) has been shown to activate NF-kB by induction of superoxide radicals in a mouse cell line.\textsuperscript{163} However, the role of the oncoproteins E6 and E7 in the regulation of NF-kB is controversial. Some studies show that E6 protein has been shown to stimulate expression of NF-kB responsive genes where as others show that there is a down regulation of NF-kB in cell lines.\textsuperscript{164}
4. **Cytokines and adaptive immunity:** Cytokines that are secreted during innate immune responses play an important role in the regulation of adaptive immune responses. Studies have shown that immunosuppressive cytokines such as TGF-beta and IL-10 are increased during cervical neoplasia.\(^{165}\)

5. **Macrophages:** Macrophages are an important part of the innate immune response to an infection. Increased infiltration of macrophages are seen in the epithelium and stroma in patients with CIN and cervical carcinoma.\(^{166}\) Cell lines transfected with HPV16 E7 oncogene has been found to be susceptible for cytolysis by activated macrophages.\(^{167}\) The same study also says that expression of E6 oncogene does not result in such lysis.

6. **NK cells:** NK cells are a subset of lymphocytes that destroy infected or damaged cells by releasing cytotoxic granules. Thus they play an important role in the non-specific innate immune response. Studies have shown that HPV 16 immortalised epithelial cells are resistant to the action of NK cells. NK cells activity is absent in women with pre-cancerous and cancerous lesions.\(^{168}\)

**3.12.2 Humoral immune response to HPV:**

**3.12.2.1 Early work on humoral responses:**

Studies were hampered by the lack of proper antigens. Early studies on humoral responses looked at antibody response in human sera to bovine papillomavirus antigens (BPV) and cottontail rabbit papillomavirus (CRPV) antigens.\(^{169,170}\) Also because of the asymptomatic nature of HPV infection, it was difficult to ascertain the status of infectivity of the individual.
3.12.2.2 Serological assays using synthetic peptides or proteins:

3.12.2.2.1 Early proteins as antigens:

The other main proteins studied are the E6 and E7 oncoproteins. Initial studies used either synthetic peptides or translated proteins to detect antibody response to these oncoproteins. Studies have tried to correlate antibody response to these oncoproteins and clinical disease stage of cervical cancer. Fisher\textsuperscript{44} reported that antibody response to E7 but not E6 was found to increase with advancing severity of cervical cancer.\textsuperscript{44} Other studies have shown that the antibody responses to E2 and E7 proteins are higher in women with cervical cancer compared to controls.\textsuperscript{46} Another study has shown that the presence of antibody response to E7 protein is associated with early stage cervical cancer.\textsuperscript{171} Another study looked at systemic IgG and local IgA antibody response to recombinant E7 oncoprotein in women with cervical cancer who underwent hysterectomy.\textsuperscript{172} The study found that local IgA response was lower in women with cervical cancer compared to those who did not have cervical cancer. A study showed that there was no correlation between antibody response to E6 and E7 oncoproteins and antibody response to virus like particles (VLPs) in the same women.\textsuperscript{173}

However, a study did not find a significant correlation between antibody response to E6 and E7 oncoproteins and cervical disease prognosis.\textsuperscript{174} Another study that followed up women for 20 years in whom squamous cell carcinoma (SCC) was seen found that antibodies to E6 and E7 oncoproteins were not markers of cervical disease progression.\textsuperscript{175}
3.12.2.2 Capsid antigens:

Antibody response to HPV has been tested using a variety of HPV proteins and peptides. The main proteins targeted for serological assays are the capsid proteins L1 and L2. Early studies did not use conformational epitopes of the capsid. A study used peptides spanning the entire L1 and L2 region. Another study used a single nanopeptide from the L1 region and tested IgG and IgA response in women with low-risk and high-risk HPV associated low grade squamous intraepithelial lesions (LSIL). IgG was seen in 90% of the women with high-risk HPVs but not in women with low-risk HPVs and control women. Another study using two synthetic peptides from the L1 protein has found antibody response in 91-96% of women with cervical intraepithelial neoplasia/cervical cancer compared to 3.6% of control women. These peptides had a sensitivity of 92-97% and specificity of 89-95% for detecting precancerous lesions and cervical cancer in women.

3.12.2.3 Virus like particles (VLPs):

For years, development of serological assays to detect antibody response to HPV has been hampered by the fact that HPV cannot be grown in bulk cultures. The knowledge that the HPV capsid protein L1 self-assembles when expressed in a system has lead to the development of serological assays. These self-assembled particles are called virus like particles (VLPs) and have been used successfully as they retain the conformational epitopes seen in the virus and because they are non-infectious. These VLPs have been used both in the development of serological assays as well as prophylactic vaccines.
3.12.2.4 Serological assays using VLPs:

3.12.2.4.1 Enzyme linked immunosorbent assay (ELISA):

Numerous forms of ELISA have been developed using VLPs as the antigen source. The simplest form is the direct ELISA, where the ELISA plates are coated with VLPs. Antibodies in positive sera bind to the VLPs and are detected by secondary antibodies. There are a number of studies that has used this format of ELISA to detect HPV antibody response in women with cervical cancer.\(^{180, 181}\)

VLPs have also been used as antigen source in capture ELISA.\(^{50}\) In this type of ELISA, the wells are coated with monoclonal antibody that captures the VLPs. Assays have also used capsomers expressed in bacteria as antigen source.\(^{182}\) The capsomers are pentameric structures formed by expressing the HPV L1 gene in \textit{E.coli}. The same study reports that the capsomers showed the same reactivity as VLPs. Another study has been done where ELISA was developed using capsomers as antigen source and used to detect IgA response to HPV 16 in women with incident HPV 16 infection.\(^{183}\)

The sensitivity and specificity of the VLP ELISA has always been a problem in its standardisation. Cross reactivity to epitopes may occur because of improperly folded VLPs. This problem was recently circumvented by one study that used heparin coated wells in the ELISA assay.\(^{184}\) As heparin binds to only confirmationally correct epitopes, type specific antibody response can be detected. Other ways of improving sensitivity by using polymers has also been suggested by some studies.\(^{185}\)

Sensitivity of serological assays has often been reported in comparison with the detection of HPV DNA in the cervical tract. Most studies report the presence of antibody
response in at least 50% for women who were HPV 16 DNA positive. Several studies have reported that the HPV VLP ELISA is type specific.

### 3.12.2.4.2 Neutralisation assays:

Neutralisation assay is based on the ability of antibodies in patient’s sera to prevent infection of cells by neutralising pseudovirions. Cells are transfected with plasmid which has HPV L1 and L2 genes and also the origin of replication of SV40 T antigen and expresses a reporter such as secreted alkaline phosphatase (SEAP). The pseudovirions are purified by density gradient centrifugation and used for the assay. If neutralising antibodies are present in the test sera, they bind to the epitopes in the pseudovirions thereby resulting in a reduction of signal obtained. Neutralisation assays are found to be less cross-reactive than VLP based ELISA.

### 3.12.2.4.3 Luminex bead assay:

Testing for antibody response to multiple HPV types is cumbersome using techniques like ELISA, neutralisation and complementary radioimmunoassay (cRIA) as antibody response is type specific. Recently, a study reported the multiplex detection of antibodies against HPV 16, 18, 6 and 11 by using a luminex laboratory multianalyte profiling (LabMAP3) assay system. This assay system showed a good sensitivity and compared well with already used cRIA.

Another study has developed an assay to detect antibody response to 100 distinct types in a serum sample. HPV proteins were fused with glutathione S transferase that is bound to beads. The fusion protein was mixed with the patient’s sera and the beads can
be separated using a flow cytometer like analyzer. This method showed good correlation with ELISA and can be used for large scale epidemiological studies as well as vaccine response studies.

3.12.2.3 Natural history of antibody response to capsid proteins:

Most HPV infections are transient and are clinically asymptomatic. So, presence of HPV DNA in the genital tract could be an incident infection, persistent infection or latent infection depending on when and in what interval the genital tract was examined. This causes a hindrance in serological studies that study seroconversion.

A high percentage of HPV infection is cleared within one year of infection. A study reported that 92% of women cleared HPV infection in 5 years.\(^{190}\) Most women clear the infection before antibody response can be detected. Different studies show different seroconversion rates. One study shows a seroconversion rate of 93.7% in women with prevalent infection and 67.1% in women with incident infection.\(^{50}\) The same study also shows that women who seroconvert are 5.7 times more likely to have squamous intraepithelial lesions. Another study showed a seroconversion rate of 88%.\(^{191}\) The median time for seroconversion has been shown to be 8.3 months after incident detection of HPV DNA in the genital tract.\(^{50}\) Seroconversion has been shown to be dependent on a high virus load or persistence of HPV16 in the genital tract.\(^{192}\) The same study also showed that antibody response to VLPs persisted for 36 months following incident infection.

The different classes of antibodies produced in response to HPV infection have been studied. Normally, IgA response comes first followed by IgG response. In HPV infection, it has been shown that IgA and IgG antibodies are produced around the same
time. A study has reported that IgA is first detected in the cervical secretions in 10.5 months whereas in serum it is detected only after 19.1 months. IgG response has been shown to be a marker of life time viral exposure and IgA response has been shown to be a marker of recent or ongoing viral infections.

3.12.2.3.1 HPV 16 seroreactivity studies:

HPV16 is the most common type associated with cervical cancer. There are numerous studies that have looked at seroreactivity to HPV16 capsids. The overall seroprevalence of HPV16 IgG is 13% in women between ages 12 and 59 in the United States of America (USA). Another study reports a seroprevalence of 12% for IgG and 6% for IgA in college women. A high overall seropositivity rate of 24.5% is seen in women attending a sexually transmitted disease clinic. The same study also reports that the seroprevalence of HPV16 antibodies in men is 18.7%. In a study that looked at women with incident infection, 56.7% had IgG in 8.3 months where as 37% had IgA in 14 months. Another study has shown that secretary IgA is seen in 13% of women without any cervical abnormality compared to 27% of women with cervical abnormality. Similarly cervical IgG is seen in 6% of women without cervical abnormality compared to 27.5% of women with cervical abnormality.

Another study has shown that systemic IgA response is associated with clearance of HPV infection whereas systemic IgG response is correlated with persistent infection. A study looking at different isotypes of HPV antibodies in women with cervical neoplasia has found that IgG1 and IgA are the most predominant ones.
3.12.2.3.2 Seroreactivity to multiple HPV types:

There is contradictory evidence as to whether seroreactivity to one HPV type will protect against re-infection by the same type or other high risk types. A study that looked at seroreactivity to HPV 16, 18, 31 and 45 found that though seroreactivity was type specific, reactivity to one type increased the likelihood for seroreactivity to another type.\textsuperscript{199}

3.12.2.3.2 Co-infection with HIV:

Numerous studies have been done to find the effect of co-infection with HIV on the seroprevalence of HPV. A study shows HPV seropositivity in HIV positive women is associated with an increased risk of infection with a HPV type from related species.\textsuperscript{200} However another study did not show a clear evidence of increased seroreactivity to HPV capsids in HIV positive women.\textsuperscript{201}

3.12.3 Cell mediated immune response to human papillomavirus (HPV):

HPV infection is one of the most common sexually transmitted diseases. Nearly 95% of women infected with HPV clear the infection in 5 years and around 0.1 percent of women progress to cervical cancer. This suggests a good immune response is necessary for control of HPV infection. The fact that prevalence of HPV infection increases in women with HIV infection points to the need of a good cell mediated immune response in protection from disease.\textsuperscript{202} The HPV oncoproteins E6 and E7 are constitutively expressed in cells with cervical cancer making them ideal targets for cell mediated immune response. A study showed that HPV specific CTLs were found in the peripheral blood and cervical tumour of women with cervical cancer but not in healthy
women. Another study has shown that lack of CTL response to E6 protein was well correlated with persistence of infection. This further suggested the role of cell mediated immune response in the control of cervical infection. Memory response to HPV 16 E6 peptide has been shown in healthy women, suggesting that the T cell response to E6 peptide plays an important role in protection from persistent infection.

A study has shown that women who had a detectable cell mediated immune response to an E7 peptide (aa 37-54) show regression from cervical disease. Another study has shown that lymphoproliferative response to an E7 peptide (aa70-98) is associated with recurrence of cervical disease after treatment.

3.13 Papillomavirus vaccines:

Extensive research is being carried out in the area of papillomavirus vaccines. HPV vaccines are either prophylactic (to prevent infection) or therapeutic (treat established infection). Prophylactic vaccines aim to induce protective neutralising antibodies whereas therapeutic vaccines aim to induce T cell mediated responses to clear established lesions.

3.13.1 Therapeutic vaccines:

There are several reasons why HPV associated cervical cancer is an ideal disease for therapeutic vaccination. First, development of cervical cancer is a multistep process involving a series of well defined pathological changes. Second, the time frame that leads to cervical cancer from the point of HPV infection is long. This allows for proper
intervention at many stages. Last, the E6 and E7 oncoproteins are expressed throughout the life cycle of the virus, so are excellent targets for therapeutic interventions.

Several therapeutic vaccines have been used for human trials. The vaccination strategies include either peptide/protein based or genetic immunization based vaccines. Peptide based vaccines are easy to manufacture but peptides are less immunogenic and need the presence of an adjuvant. Peptides are also HLA restricted, which limits their application. One of the first therapeutic vaccines to be tested in women with cervical cancer was the HLA-A0201*restricted lipopeptide from the HPV 16 E7 protein.\textsuperscript{205} Cellular immune responses were seen in these women but no clinical responses were achievable. A different study used another HLA A2 restricted HPV 16 E7 peptide with incomplete Freund’s adjuvant to vaccinate women with CIN. Cellular immune response was detected in the vaccinees and the dysplasia regressed in 3/18 women.\textsuperscript{206}

Whole protein based vaccines have also been used. One of the first whole protein vaccine was TA-CIN, which is a single fusion protein comprising of L2, E6 and E7 proteins.\textsuperscript{207} The vaccine was administered to healthy volunteers without an adjuvant and found to elicit both IgG and proliferative immune response.

Genetic immunisation have been done both by transfer of naked DNA as well as by vector mediated gene transfer. Transfer of naked DNA has been seen to be less effective in humans. So studies have looked at vector mediated gene transfer of viral DNA. A safety trial was done in women with cervical intraepithelial neoplasia using plasmid DNA encoding the HPV 16 E6 and E7 genes encapsidated in biodegradable microparticles.\textsuperscript{208} The women tolerated the vaccine well and significant regression was seen in women less than 25 years of age. Phase II trials are going on for a recombinant
vaccinia virus based vaccine called TA-HPV. This vaccine is a live recombinant vaccinia virus vaccine that encodes HPV 16 E6 and E7 genes.\textsuperscript{209} The vaccine was found to be safe in women with vulval intraepithelial neoplasia (VIN). All women showed an increase in the IgG titre and T cell responses and clinical regression of disease was also seen in them.

3.13.2 Prophylactic vaccines:

The fact that infection with a high-risk HPV type is a pre-requisite for the development of cervical cancer has lead to the belief that a vaccine could prevent this disease. However, the inability to grow the virus in culture has hindered research in this area for a long time. Recent revelation that the major capsid protein L1 of HPV can self-assemble when expressed in an expression system has promoted research on use of these capsid proteins as potential prophylactic vaccine targets.

The major capsid protein L1 self-assembles to form the virus like particles (VLPs).\textsuperscript{210} These VLPs are indistinguishable from the infective virions. Since the VLPs did not have any other gene but the L1, they were non-infective and non-oncogenic. However, they were able to induce neutralising antibodies similar to that of infective virions.\textsuperscript{210}

3.13.2.1 Pre clinical studies:

HPVs are very species specific. So, initial studies had to be done using other related papillomaviruses infecting animals. The most commonly used PVs were BPV in cattle and CRPV in rabbits.\textsuperscript{211 212} Vaccination with VLPs of these respective viruses was seen to induce neutralising antibodies and protected them from experimental challenge of
the virus. However, the efficacy of these VLPs in preventing sexual transmission of HPVs could not be ascertained in animal models. Though VLPs were seen to protect animals from infection, they did not cause regression of already established tumours and thus could not be used as therapeutic vaccines.\textsuperscript{212}

3.13.2.2 Clinical trials:

HPV VLPs were successfully expressed using two expression systems: insect cell lines infected with recombinant baculovirus and \textit{Saccharomyces cereviceae} with L1 coding plasmid. Phase I clinical trials showed that VLPs were safe and immunogenic with doses ranging 10-50\(\mu\)g.\textsuperscript{213} The same study also showed that VLPs induced a 40 fold higher antibody response when compared to natural infection. The study also points to the fact that similar neutralising antibody titre was achieved with or without the use of adjuvants (alum or MF59). Alum has been included in further vaccine development to stabilise VLPs.

HPV VLP vaccine research has been carried out by two commercial manufacturers, GlaxoSmithKline (GSK) Ltd and Merck Ltd. GSK developed a divalent vaccine that includes HPV 16 and 18 VLPs expressed in insect cells using their proprietary AS04 adjuvant.\textsuperscript{214} Merck developed a univalent HPV 16 vaccine and a quadrivalent vaccine that included oncogenic types HPV 16 and 18 and non-oncogenic types HPV 6 and 11.\textsuperscript{215, 216}

All three vaccine formulations were found to be safe in phase III trials and shown to be highly immunogenic and well tolerated. The vaccines showed 100\% efficacy in preventing incident and prevalent infections. The antibody response seen after all doses
were 50-100 more than what is seen in natural infections. However, it was observed that the vaccines were effective in preventing persistent infections but not transient ones.

The administration of the GSK vaccine was shown to confer protection against infection with other high risk types by 40% and against formation of cytological abnormalities in 70% of women. It has been suggested that the high antibody response achieved maybe the reason for the cross-protection. Alternatively, it has also been suggested that the L1 gene may have cross-reactive epitopes that are yet to be elucidated. The cross-protection observed in the GSK vaccine was also attributed to the use of AS04 as adjuvant. However, cross-protection was not reported for the Merck vaccine.

The mechanism of protection against persistent infection has been attributed to two facts.

1. Transudation of serum IgG to the cervical mucus epithelium was found to be higher than seen in other tissue like the gut.

2. Infection resulting from trauma in a natural infection could make the virus directly accessible by these serum exudates.

Both these vaccines have now been approved for commercial use. The recommendations for vaccination vary with the respective countries, but generally it has been accepted that vaccination will be most efficient in young girls at the age of 9-13 before the onset of sexual activity.
3.14 Treatment of cervical cancer:

HPV infection has been shown to be transient in a majority of women. Treatment of low grade lesions is not necessary as most of them regress spontaneously. Treatment options for high grade dysplasia and invasive cervical cancer differ based on the size, histological stage of disease and lymph node involvement.\textsuperscript{142} Treatment is usually by surgery, chemotherapy, radiotherapy or a combination of these methods.

3.14.1 Treatment of non-invasive lesions:

1. **Cryotherapy**: The abnormal tissue and 5 mm of surrounding tissue are frozen with a super cooled probe. The process is repeated more than once for complete necrosis of the abnormal tissue.\textsuperscript{142}

2. **Laser therapy**: Ablation of the abnormal tissue has also been done with using a carbon dioxide laser beam. Using this method, the tissue is less distorted and the healing time is short. However, it is an expensive procedure.\textsuperscript{142}

3. **Loop electrosurgical excision procedure (LEEP)**: LEEP has become the most recommended procedure for the removal of non-invasive lesions. In this method, the transition zone and the distal endocervical canal are excised using an electrically charged wire. It is less expensive than laser therapy and has the advantage that the excised tissue can be used for histological examination.

Risk of recurrence of disease is there when treated with any of the above methods. Recurrence has been seen in 31\% of women undergoing LEEP in a median of 11.9 months.\textsuperscript{217} Proper management of women after treatment is necessary. Recent data has suggested that HPV DNA testing is a good marker to detect residual infection. HPV
DNA positivity after 6 months of therapy has been shown to be a better predictor of recurrence than abnormal cytology (sensitivity 90% and 62% respectively). Also HPV DNA negativity has shown to have a better negative predictive value for clearance (99%).\textsuperscript{218}

3.14.2 Treatment of invasive cancer:

3.14.2.1 Early stage invasive cancer:

Micro invasive cancer with tumour size less than 3mm are excised using cone biopsy. Radical hysterectomy has been used traditionally as the treatment modality for early stage cervical cancer. External beam radiation therapy can also be used in these cases.

3.14.2.2 Locally advanced invasive cancer:

Traditionally, advanced cancers are treated with radiotherapy alone or in conjunction with high dose-rate intracavitary brachytherapy (ICBT) or extra beam radiotherapy (EBRT). Recent evidence has shown that when chemotherapy was given concurrently with radiotherapy, the overall 5 year survival rate increased from 58% to 73%.\textsuperscript{219}

Several immunomodulatory agents and antiviral agents have also been used for the treatment of HPV infections. Topical application of interferon alpha has been shown to increase success in patients with condyloma acuminatum.\textsuperscript{220} A study has used a topical application of 1% cidofovir gel in women with CIN I before surgery.\textsuperscript{221} Topical imiquimod (5%) has been shown to be effective in removing external genital warts.\textsuperscript{222} Recent research has shown that curcumin (diferuloyl methane) has antitumor properties.
Circumin inhibits expression of E6 and E7 oncogenes and also inhibits the action of NF-kappa B suggesting that they can be used in future in the management of cervical disease.\textsuperscript{223}

### 3.15 Indian studies on Human papillomaviruses:

Research on HPV in India started in the 1980’s. HPV was identified in patients with genital warts.\textsuperscript{224} Early studies in India reported the major types HPV 16 and 18 only. A study found HPV 16 and 18 in 82.5\% of women with invasive cancer in New Delhi using southern hybridisation.\textsuperscript{225} Another study has found HPV 16 DNA in 64\% and HPV 18 in 3\% of women with cervical neoplasia using a combination of southern blot hybridisation and PCR.\textsuperscript{226} There were many similar reports that looked at the prevalence of HPV 16 and 18 in Indian women with cervical cancer. Another study detected HPV 16 in 73.6\%, HPV 18 in 14.2\% and HPV 45 in 11.3\% of women with cervical cancer in North India.\textsuperscript{8} Another study looking at HPV prevalence in south Indian and East Indian women found that HPV 16 followed by 18 were the most prevalent types. However, other oncogenic types were also detected.\textsuperscript{7}

A study done in rural south India reported a HPV prevalence of 16.9\% in the community. The same study however pointed out the fact that widowhood, nulliparity and condom use are associated with an increased risk for HPV infection in India.\textsuperscript{227}

There are few studies that have looked at HPV 16 variants in Indian women. A study from our centre has reported that 92\% of women with cervical neoplasia harbouring HPV 16 were of the European lineage, of which 70\% carry the T350G mutant
in their E6 gene. Another study that looked at variations in the E6, E7, L1 and LCR regions has reported that the most common lineage in Indian women is the European lineage. The same study also reports that the T350G E6 mutant was seen in 100% of women with cervical neoplasia.

3.16 Role of HPV in other cancers:

The role of HPV in the etiology of cervical cancer has been proved beyond doubt. HPV infection has also been associated with cancers of other regions. The role of HPV in some of these cancers is discussed below.

1. **Cancer of the vulva:** HPV is known to be the cause for cancer of the vulva in a certain subset of individuals, whereas in others, there is no correlation. HPV was more prevalent in young women who had a basaloid squamous carcinoma (85.7%) but was less prevalent in women who had keratinising squamous carcinoma (6.3%). HPV 16 followed by 33 were the most common HPV types seen in women with vulvar dysplasia.

2. **Anal squamous cell carcinoma:** A large scale study in women has reported that high risk HPV was detected in 95% and 83% of women and men with anal carcinoma and in 80% and 28% of women and men with perianal skin cancer. HPV infection was seen more in young men with basoloid carcinoma where as HPV was not detected in older men with keratinising carcinoma.

3. **Oropharyngeal cancers:** HPV has been established as a casual factor in the development of head and neck squamous cell carcinomas. The prevalence of
HPV was low in these cancers. HPV was seen in 18% of cancer of the oral cavity, 8% of nasopharyngeal cancer, 25% of hyponasopharyngeal cancers and 7% of laryngeal cancers. However, a relatively high level of HPV was seen in oropharyngeal carcinomas (45%) particularly tonsillar carcinomas (58%).\textsuperscript{233} The same study also shows that all cases with tonsillar carcinomas had a high risk HPV type with HPV 16 being the most common type, detected in 93% of the cases.\textsuperscript{233}

4. **Epidermodysplasia verruciformis**: These are disseminated, flat warts and macular lesions seen in childhood. These lesions have a high risk of developing into SCC later in life. The HPV types seen in these lesions are called the EV types and the most common types seen are HPV 5, 8, 9, 12, 14, 15, 17, 19-25. Though many types can be seen in these lesions, the most prevalent types in SCC were HPV 5 and 8 followed by types 14, 17, 20 or 47.\textsuperscript{234}
4. Scope and plan of work:

As submitted to the university along with the application for the PhD programme, shown here as a university requirement in the thesis.

Numerous epidemiological studies have linked the presence of human papillomavirus (HPV) with cervical cancer. There are relatively few studies in India that have studied HPV associated with cervical neoplasia.

To the best of our knowledge there are no studies from the India on estimation and role of viral loads in cervical tissues. So in this study we have planned to study the role of viral load of the 2 predominant HPV types HPV 16 and 18 in the progression of cervical neoplasia in Indian women.

There has been one earlier study from our centre looking at HPV plasma vireamia. This study however only alluded to the qualitative detection of HPV DNA in plasma. There are no studies on the role of plasma viral load of HPV 16 and 18 from India. So in our study, we propose to do real time PCR to detect and quantitate HPV 16 and 18 in plasma of patients with cervical neoplasia.

Expression of oncogenes in cervical carcinoma has been looked at by an earlier study from our centre. But that study was qualitative. In this study, we propose to quantitate HPV 16 and 18 mRNA transcripts with absolute quantitation using real time PCR and associate it with clinical disease progression.

There are only 2 studies globally that have looked for HPV mRNA transcripts in PBMCs as markers of poor disease prognosis and there are no such studies from India. In our study, we propose to detect HPV 16 and 18 mRNA transcripts using a nested RT-
PCR and real time PCR in PBMCs of patients with cervical neoplasia and associate it with clinical disease progression.

There exist no Indian studies investigating T-cell responses to HPV 16 E7 peptides among patients with cervical neoplasia. In this study, we propose to study T cell responses to two peptides of the E7 protein of the most common high risk HPV, HPV 16.

There is only one study that has looked at antibody response to HPV early proteins (E2, E6 and E7) in North India. To the best of our knowledge there are no Indian studies that have investigated humoral immune responses to HPV16 VLPs. In this study, we propose to study humoral immune response to HPV 16 early proteins (E2, E6 and E7) by peptide ELISA as well as to virus like particles (VLPs) and associate it with disease progression. There are no Indian studies that have looked at immune response to HPV 18. We propose to detect antibodies to HPV 18 VLPs and associate it with disease progression.

This study will give us a better understanding of the role of different HPV markers in the development and progression of cancer cervix. It will also help in the identification of potential markers capable of predicting poor prognosis. Additionally this study would also provide comprehensive information on the role of T-cell responses to the different HPV 16 associated peptides and the role of humoral responses to HPV 16 peptides and virus like particles (VLPs) in predicting progression or recovery from cervical disease.
5. Materials and Methods

5.1 Study groups:

The study was explained to women of all study groups and a written consent was obtained.

Study group I (n =150)

This group consisted of women recruited from the departments of Obstetrics and Gynecology and Radiation therapy with a diagnosis of cervical cancer or pre-invasive cancer and who were treatment naive. Clinical staging was done by the examining clinician according to the guidelines of the International Federation of Gynaecology and Obstetrics (FIGO).235

This thesis is a part of a study funded by Department of Biotechnology (DBT), India (Ref no BT/PR5249/Med/14/613/2004). Ethical clearance was obtained from the Institutional Research Committee, Christian Medical college, Vellore (R.C.Min No 5392 dated 20.07.2004).

Study group II (n=33)

This group comprised of women who did not have any clinical evidence of cervical disease. These patients were recruited from the department of Obstetrics and Gynaecology who underwent hysterectomy for reasons not associated with cervical disease.
Study group III (n=60)

This group comprised 30 married and 30 unmarried women from the community. Samples collected from this group were used mainly for immunological studies.

5.2 Collection and processing of samples:

Cervical biopsy: This was collected from study groups I and II and apportioned into two. One sample was sent for histopathological staging to Norman Institute of Pathology, Christian Medical College, Vellore and the other was transported in viral transport medium (VTM) at +4°C to the virology laboratory. The tissue was immediately cut into portions of approximately 25mg and stored at -60°C until further analysis. Samples to be used for mRNA studies were stored in a RNA stabilising solution (RNA later, Ambion, Huntingdon, UK).

Blood: This was collected from study groups I, II and III in three separate tubes.

i. Blood collected in heparinised tubes was used to measure T cell responses to HPV peptides.

ii. Blood collected in EDTA was centrifuged and plasma separated and stored at -60°C for HPV DNA testing.

iii. Blood collected in vacutainer tubes (Becton Dickinson, California, US) was allowed to clot and the separated serum was used for HPV antibody detection studies.
5.3 HPV DNA detection and genotyping:

5.3.1 DNA extraction:

DNA was extracted from cervical biopsies using a commercial kit (QIAamp DNA mini kit, Qiagen, GmbH, Germany). Approximately 25 mg of tissue sample was used for extraction.

The following steps were carried out as per the manufacturer’s instructions.

1. Tissue was first digested with 20 µl of proteinase K in the presence of 180 µl of tissue lysis buffer (ATL) in a 1.5 ml microcentrifuge tube (Axygen, California, USA).

2. The tube was incubated at 56°C until the tissue was digested completely.

3. To the tube, 200 µl of lysis buffer (AL) was added; the tube mixed by vortexing and incubated at 70°C for 10 minutes.

4. The tube was briefly centrifuged to remove droplets on the inner surface of the lid.

5. To the tube, 200 µl of ethanol was added; the sample was mixed by vortexing and briefly centrifuged.

6. The mixture was then transferred to the QIAamp spin column and centrifuged at 8000 rpm for 1 minute.

7. The collection tube containing the filtrate was discarded and the spin column was placed in a new collection tube.
8. Five hundred microliters of wash buffer (AW1) was added and the tube was centrifuged at 8000 rpm for 1 minute. The collection tube was discarded as done in step 7.

9. Five hundred microliters of wash buffer (AW2) was added and the tube was centrifuged at 14,000 rpm for 3 minutes.

10. The collection tube was discarded and the spin column was transferred to a new 1.5 ml microcentrifuge tube.

11. Two hundred microliters of elution buffer (AE) was added to the tube. The tube was incubated at room temperature for 5 minutes and then centrifuged at 8000 rpm for 1 minute.

The eluted DNA thus obtained was used for PCR amplification either immediately or stored at -20°C until further testing.

5.3.2 Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP):

5.3.2.1 Detection using MY09/11 primers:

HPV DNA amplification was done in the eluted DNA using consensus primers MY09/11. An additional primer (HMB01) specific for HPV 51 was also included in the reaction mix. Concomitant amplification of beta globin gene was also performed in the same sample to ensure sample adequacy and integrity. The primer sequences used for the study are given in Table 1.
The reaction mix had 10 µl of extracted DNA, 2.5 units of Hotstar Taq DNA polymerase (Qiagen, GmbH, Germany), 200 µM dNTPs, 4mM MgCl₂, 50 pmoles each of MY09/11 primers, 5 pmole of HMB01 primer, 5 pmoles of each beta globin primer and appropriate PCR buffer. The reaction volume was made up to 100µl using sterile milliQ water. The primers used were custom synthesised by Metabion, Germany.

The thermal cycling conditions included a pre-PCR step at 95°C for 15 minutes followed by 40 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min 30 s and a post-PCR step at 72°C for 10 mins. Amplification was performed using one of the following thermal cyclers: Perkin Elmer GeneAmp PCR system (Roche diagnostic systems, California, USA), PTC-100 (MJ Research, California, USA) or Mycycler (Bio-Rad, California, USA).

The amplified products were detected by agarose gel electrophoresis. The products were mixed with loading dye (bromophenol blue in sucrose) at a concentration of 1:6, loaded on a 2% agarose gel and run in a gel tank. Hae III digest of ΦX174 (Bangalore Genei, Bangalore, India) was used as a molecular weight marker and the band was visualised using a gel documentation system (Geldoc 2000/Geldoc XR documentation system, Bio-Rad, California, USA). The size of the amplified product (band size) was determined using the software available with the gel documentation system (Quantitiy one, Bio-Rad, California, USA). A positive sample showed a HPV specific band at 450 basepairs and a beta globin band at 268 basepairs.

5.3.2.2 Amplification using MY09/11 primers for RFLP:

Amplification was done for HPV DNA positive samples as described above without the beta globin primers.
5.3.2.3 Restriction fragment length polymorphism (RFLP):

RFLP was done using a set of 7 restriction enzymes *BamH*I, *Dde*I, *Hae* III, *Hinf*I, *Pst*I, *Rsa*I and *Sau*3AI (New England Biolabs, Ipswich, US and Bangalore Genei, Bangalore, India). Seven microliters of amplified product was added to 10 units of each restriction enzyme and digested at 37°C for 4 hours. The digested products were separated in a 3% agarose gel stained with ethidium bromide and visualised in a gel documentation system (Geldoc 2000/ Geldoc XR documentation system, Bio-Rad, California, USA). The size of the amplified products were determined using the software (Quantity one, Bio-Rad, USA) available with the gel documentation system. The base pair sizes of the amplified product obtained were compared with previously published literature to identify the genotypes.19

5.3.3 Amplification using PGMY09/11 primers:

HPV DNA amplification was done using modified consensus primers PGMY09/11.21 The PCR reaction mix had 10 µl of extracted DNA, 7.5 units of *AmpliTaq* Gold *Taq* DNA polymerase (Applied Biosystems, California, USA), 4 mM MgCl₂, 200µM dNTPs, 50 pmoles of biotinylated PGMY09/11 primers and 2.5 pmoles of biotinylated betaglobin primers. The reaction volume was made up to 100µl with sterile milliQ water. All reagents for this PCR were received from Roche Diagnostics (courtesy Dr.Patti Gravitt, John Hopkins University, Baltimore, USA).

The thermal cycling conditions consisted of a pre-PCR denaturation step at 95°C for 9 minutes; followed by 40 cycles of 95°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute. A post-PCR extension was done at 72°C for 10 minutes. Amplification was performed using one of the following thermal cyclers: Perkin Elmer
GeneAmp PCR system (Roche Diagnostic Systems, California, USA), PTC-100 (MJ Research, California, USA) or Mycycler (Bio-Rad, California, USA).

The amplified products were run in an agarose gel electrophoresis as described in section 5.3.2.3. The HPV specific product was 450 base pairs. The amplified products were stored at +4°C until the line blot assay was done.

5.3.4 Reverse line blot hybridisation using Line blot assay (LBA):

Line blot assay (LBA) was a HPV genotyping kit produced by Roche Diagnostics, USA. The kit includes strips coated with single stranded probes from 38 HPV types (both high-risk and low-risk types) and 2 probes from beta globin gene as internal control (weak and strong reactive controls).

The assay was performed as per the manufacturer’s instructions. The following steps were carried out.

1. Forty microlitres of PCR product was denatured with equal volume of denaturation solution (NaOH).

2. HPV genotyping strips were placed in the typing trays and 4 ml of pre-warmed (53°C) hybridisation buffer was added to it.

3. The denatured product was then added to the typing tray and hybridised at 53°C in a shaking water bath (Precision Scientific, Chicago, USA) for 30 minutes.

4. The solution was then removed and the strips were washed with 4 ml of wash buffer at room temperature.
5. The strips were incubated with 4 ml of pre-warmed (53°C) wash buffer for 15 minutes and then removed by vacuum aspiration.

6. Four ml of streptavidin conjugated horse radish peroxidase was added to the strips and shaken at 70 rpm for 30 minutes at room temperature.

7. The conjugate solution was aspirated and the strips were washed with 4ml of wash buffer at room temperature.

8. Four ml of wash buffer at room temperature was added and the tray was shaken at 70 rpm for 10 minutes.

9. The solution in the tray was discarded and the wash step (step 8) was repeated.

10. The strips were allowed to soak in 4ml citrate buffer for 5 minutes.

11. Citrate buffer was aspirated and 4ml of color development solution was added to the wells and shaken at 70 rpm for 5 minutes.

12. Color development solution was aspirated completely and the strips were rinsed with distilled water.

13. The strips were stored in citrate buffer at 2-8°C.

14. The strips were interpreted manually using the HPV overlay provided by the manufacturer. Each sample was validated based on the presence of the beta globin bands.
5.4 HPV Quantitation in tissues:

5.4.1 HPV 16 quantitation:

5.4.1.1 HPV 16 quantitation procedure:

HPV 16 was performed on all samples typed as HPV 16 by LBA. Quantitation was done by Taqman-based real time PCR using primers and probe targeting the E6 region of the HPV 16 genome\textsuperscript{238} (Table 2). The PCR reaction mix contained 2.5µl of extracted DNA template, 5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, California, USA), 200 µM dNTPs, 4mM MgCl\textsubscript{2}, 0.2 µM of each HPV 16 specific primer, 0.1 µM of probe and appropriate buffers. The total volume of the mix was made up to 50 µl with sterile milliQ water.

The thermal cycling conditions used were as follows: an initial hold at 50°C for 2 minutes followed by pre-PCR denaturation step at 95°C for 12 minutes and then 50 cycles of 95°C for 15 seconds and 55°C for 30 seconds.

A previously known HPV16 positive sample was included as a positive control and milliQ water was included as negative control in each run. All samples were done in duplicate and the mean viral load calculated for analysis. PCR was done in Rotorgene 3000 or Rotorgene 6000 real time PCR machines (Corbett Research, Mortlake, Australia).

Serial dilution of a HPV 16 containing plasmid (courtesy, Dr.Patti Gravitt, JHU, USA) with known copy number was done in a background of 50ng/µl human placental DNA and included in each run as standards. An external standard curve was constructed from the standards by the software provided with the real time PCR machine (Corbett...
Research, Australia) using logistic regression. The threshold was also calculated automatically by the same software (Corbett Research, Mortlake, Australia). \( C_t \) (threshold cycle number) was calculated as the cycle at which the sample fluorescence crosses the threshold. The viral loads in the unknown samples were calculated from the regression equation using the individual \( C_t \) values by the same software.

### 5.4.1.2 Inter-assay and Intra-assay variation analysis for HPV 16:

Three samples with HPV 16 viral loads \(10^3, \ 10^5\), and \(10^6\) were tested in triplicate in the same run to check for intra-assay variability and on 3 separate days to check for inter-assay variability. Coefficient of variation was calculated on the mean logarithmic value of the viral loads obtained using the given formula

\[
\text{Coefficient of variation (CV)} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100
\]

### 5.4.1.3 Endogenous retrovirus 3 (ERV3) quantitation for HPV 16:

Due to variability in sample input during DNA extraction, normalisation of cellular content was performed by amplification of the human ERV3 gene in all the HPV16 positive samples. PCR was performed using primers targeting ERV3 gene by real time PCR based on Taqman principle as published earlier.\(^{239}\) (Table 3)

The PCR reaction mix consisted of 2.5\( \mu \)l of extracted DNA, 5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, California, USA), 200 \( \mu \)M dNTPs, 4mM MgCl\(_2\), 0.4 \( \mu \)M of each primer, 0.25 \( \mu \)M probe and appropriate PCR buffer. The total volume of the mix was made up to 50 \( \mu \)l using sterile milliQ water. Human placental
DNA (2.5µl) was used as a positive control and milliQ water (2.5 µl) was used as a negative control in all runs. The primers and probe were synthesised commercially from Metabion, Germany.

The thermal cycling conditions used were as follows: an initial pre-PCR denaturation step at 95°C for 10 minutes, followed by 50 cycles at 95°C for 15 seconds and 60°C for 30 seconds. PCR was done in Rotorgene 3000 or Rotorgene 6000 real time PCR machines (Corbett Research, Mortlake, Australia).

A standard curve was generated by serial dilution of DNA extract of human peripheral blood mononuclear cells (PBMCs) in a background of 50ng/µl salmon sperm DNA (Sigma USA). PBMCs were used as they have 2 copies of ERV3 gene per cell.239

An external standard curve was constructed from the standards by the software provided with the real time PCR machine (Corbett Research, Mortlake, Australia) using logistic regression. The threshold was also calculated automatically by the same software (Corbett Research, Mortlake, Australia). \( C_t \) (threshold cycle number) was calculated as the cycle at which the sample fluorescence crosses the threshold. The viral loads in the unknown samples were calculated from the regression equation using the individual \( C_t \) values by the same software.

5.4.1.4 Intra-assay and inter-assay variation for ERV3 quantitation:

Inter-assay and intra-assay variation analysis was done using 4 standards with ERV3 loads \( 4.7 \times 10^4, 4.7 \times 10^3, 4.7 \times 10^2 \) and \( 4.7 \times 10^1 \) after making serial dilutions. The standards were run in duplicate in the same run to assess intra-assay variation and in 2 separate runs to assess inter-assay variation. Coefficient of variation (CV) was
calculated from the logarithmic mean viral loads as done for HPV 16 (described in 5.4.1.2).

5.4.1.4 Calculation of HPV 16 viral loads:

HPV 16 viral loads was calculated using the following formula

\[
\text{Normalised HPV 16 viral load (copies/5000 cells)} = \frac{\text{HPV 16 viral load}}{\text{ERV3 viral load}} \times 10000
\]

5.4.1.5 Specificity:

To check the cross reactivity and specificity of the HPV 16 E6 primers used for quantitation, PCR was done on samples that were positive for HPV types other than HPV 16. The samples tested belonged to HPV DNA other than HPV 16 from species 9 and HPV DNA from other species (Table 4).

5.4.2 HPV 18 quantitation:

5.4.2.1 HPV 18 quantitation procedure:

HPV 18 quantitation was done on all samples typed as HPV 18 by LBA. Primers and probes used for quantitation were from the E7 region of the HPV 18 genome (Table 5).\(^{238}\) The reaction mix contained 2.5µl of extracted DNA, 5 units of AmpliTaq Gold DNA polymerase, 4mM MgCl\(_2\), 200 µM dNTPs, 0.2 µM of each primers, 0.1 µM probe and 1X PCR buffer. The volume was made up to 50 µl with sterile milliQ water.

The thermal cycling conditions used were as follows: an initial hold at 50°C for 2 minutes followed by pre-PCR denaturation step at 95°C for 12 minutes and then 50 cycles of 95°C for 15 seconds and 55°C for 30 seconds. All the assays were done in
Serial dilution of a HPV 18 containing plasmid (courtesy, Dr.Patti Gravitt, JHU, USA) with known copy number was done in a background of 50ng/µl human placental DNA and included in each run as standards. An external standard curve was constructed from the standards by the software provided with the real time PCR machine (Corbett Research, Mortlake, Australia) using logistic regression. The threshold was also calculated automatically by the same software (Corbett Research, Mortlake, Australia). 

\[ C_t \] (threshold cycle number) was calculated as the cycle at which the sample fluorescence crosses the threshold. The viral loads in the unknown samples were calculated from the regression equation using the individual \( C_t \) values by the same software.

5.4.2.2 Inter-assay and Intra-assay variation analysis:

Three samples with HPV 18 viral loads \( 10^2 \), \( 10^4 \), and \( 10^5 \) were tested in triplicate in the same run to check for intra-assay variability and on 3 separate days to check for inter-assay variability. Coefficient of variation was calculated on the mean logarithmic value of the viral loads obtained using the formula as described for HPV 16 in 5.4.1.2. A coefficient of variation less than 10 was considered acceptable.

5.4.2.3 Endogenous retrovirus 3 (ERV3) quantitation for HPV 18:

ERV3 PCR was done on all samples as described for HPV 16. PCR was performed using primers targeting ERV3 gene by real time PCR based on Taqman principle as published earlier (Table 3).
The PCR reaction mix consisted of 2.5µl of extracted DNA, 5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, California, USA), 200 µM dNTPs, 4mM MgCl₂, 0.4 µM of each primers, 0.250 µM probe and appropriate PCR buffer. The total volume of the mix is made up to 50 µl using sterile milliQ water. Human placental DNA (2.5µl) was used as a positive control and milliQ water (2.5 µl) was used as a negative control in all runs. The primers and probe were synthesised commercially (Metabion, Germany).

The thermal cycling conditions used were as follows: an initial pre-PCR denaturation step at 95°C for 10 minutes, followed by 50 cycles at 95°C for 15 seconds and 60°C for 30 seconds. PCR was done in Rotorgene 3000 or Rotorgene 6000 real time PCR machines (Corbett Research, Mortlake, Australia).

A standard curve was generated by serial dilution of DNA extracts of human peripheral blood mononuclear cells (PBMCs) in a background of 50ng/µl salmon sperm DNA. PBMCs were used as they have 2 copies of ERV3 gene per cell.

An external standard curve was constructed from the standards by the software provided with the real time PCR machine (Corbett Research, Mortlake, Australia) using logistic regression. The threshold was also calculated automatically by the same software (Corbett Research, Mortlake, Australia). C_t (threshold cycle number) was calculated as the cycle at which the sample fluorescence crosses the threshold. The viral loads in the unknown samples were calculated from the regression equation using the individual C_t values by the same software.
5.4.2.4 Calculation of HPV 18 viral loads:

HPV 18 viral loads was calculated using the following formula:

\[
\text{Normalised HPV 18 viral load (copies/5000 cells)} = \frac{\text{HPV 18 viral load} \times 10000}{\text{ERV3 viral load}}
\]

5.4.2.5 Specificity

To check the cross reactivity and specificity of the HPV 18 E6 primers used for quantitation, PCR was done on samples that were positive for HPV types other than HPV 18. The samples tested were HPV types other than HPV 18 belonging to species 7 and HPV types from other species (Table 6).

5.5 HPV detection and quantitation in plasma:

Plasma from women with HPV 16 or 18 in their cervical tissue were taken for analysis of presence of HPV DNA.

5.5.1 DNA extraction in plasma:

DNA was extracted from plasma using High Pure Viral Nucleic Acid kit (Roche Molecular Systems, California, USA). The steps included for extraction were as given in the manufacturer’s instructions and described below.

1. Two hundred microliters of binding buffer supplemented with 50 µl proteinate K was added to 200µl of plasma. The detergent and proteinase K in the lysis binding buffer lyse the viruses whereas, chaotropic salt (Guanidine HCl) helps in the binding of nucleic acid to the glass fiber fleece in the high pure filter tube. To ensure maximum yield, for every sample the binding step was done for an additional 200µl of plasma.
2. The tubes were mixed well and incubated at 72°C for 10 minutes.

3. After a brief centrifugation, 100 µl of binding buffer was added and the contents were transferred to a high pure filter tube.

4. The tubes were centrifuged at 8000 rpm for 1 minute.

5. The flow through was discarded; 500 µl of inhibition removal buffer was added and the tubes centrifuged at 8000 rpm for 1 minute.

6. The flow through was discarded; 450 µl of inhibition removal buffer was added and the tubes centrifuged at 8000 rpm for 1 minute.

7. Wash buffer was added as described in step 6.

8. The tubes were then centrifuged at 13000 rpm for 1 minute.

9. Fifty µl of elution buffer was added to the tube. The elution buffer is a low salt buffer that elutes the DNA from the glass fibre fleece.

   The eluted DNA is then used for amplification.

5.5.2. Amplification using consensus primers MY09/11:

   HPV DNA amplification was done using consensus primers MY09/11 as described for cervical tissue in section 5.3.2.1. The amplified products were detected using agarose gel electrophoresis.
5.5.3 Detection and quantitation using real time PCR:

5.5.3.1 Amplification of HPV 16:

Amplification of HPV 16 was done using real time PCR based on Taqman chemistry as described earlier\textsuperscript{240} (Table 7). Primers and probe were chosen to target the E6/E7 region of the HPV 16 genome. The PCR reaction mix had 5µl of extracted DNA, 200 µM dNTPs, 0.4 µM of each primer, 0.1 µM probe, 5mM MgCl\textsubscript{2} and 1 unit AmpliTaq Gold DNA polymerase (Applied Biosystems, California, USA). The total volume of the mix was made up to 20 µl with sterile milliQ water.

The thermal cycling conditions used were as follows: an initial pre-PCR denaturation step at 95°C for 15 minutes, followed by 45 cycles at 95°C for 10 seconds and 60°C for 1 minute. A standard curve was derived by serial dilution of a known copy number of HPV 16 plasmid (courtesy Dr.Patti Gravitt, JHU, USA). The results were expressed as genome copies per millilitre of plasma.

An external standard curve was constructed from the standards by the software provided with the real time PCR machine (Corbett Research, Mortlake, Australia) using logistic regression. The threshold was also calculated automatically by the same software (Corbett Research, Mortlake, Australia). C\textsubscript{t} (threshold cycle number) was calculated as the cycle at which the sample fluorescence crosses the threshold. The viral loads in the unknown samples were calculated from the regression equation using the individual C\textsubscript{t} values by the same software.
5.5.3.2 Amplification of HPV 18:

Amplification of HPV 18 was done by real time PCR using Taqman principle using conditions described for cervical tissues in section 5.4.2. Primers and probes used for quantitation were from the E7 region of the HPV 18 genome (Table 5). The reaction mix contained 2.5 µl of extracted DNA, 5 units of AmpliTaq Gold DNA polymerase, 4mM MgCl₂, 200 µM dNTPs, 0.2 µM of each primers, 0.1 µM probe and 1X PCR buffer. The volume was made up to 50 µl with sterile milliQ water.

The thermal cycling conditions used were as follows: an initial hold at 50°C for 2 minutes followed by pre-PCR denaturation step at 95°C for 12 minutes and then 50 cycles of 95°C for 15 seconds and 55°C for 30 seconds. All the assays were done in Rotorgene 3000 or Rotorgene 6000 real time PCR machines (Corbett Research, Mortlake, Australia).

The results were expressed as genome copies per millilitre of plasma.

5.5.4 HPV DNA sequencing:

Nucleotide sequencing was performed on five paired plasma and tissue samples that were positive for HPV 16.

5.5.4.1 HPV PCR:

Amplification was done on the samples using primers spanning the E6/E7 ORF of the HPV 16 genome (Table 8).

The reaction mix had 2.5 µl of extracted DNA, 1.5 units of Hotstar Taq DNA polymerase (Qiagen, GmbH, Germany), 120 µM dNTPs, 2mM MgCl₂, 15 pmoles each primer and
appropriate PCR buffer. The reaction volume was made up to 25µl using sterile milliQ water. The primers used were custom synthesised (Metabion, Germany).

The thermal cycling conditions included a pre-PCR step at 95°C for 15 minutes followed by 35 cycles of 95°C for 30 s, 58°C for 1 min and 72°C for 1 min and a post-PCR step at 72°C for 10 mins.

The amplified products were detected by agarose gel electrophoresis. The products were mixed with loading dye (bromophenol blue in sucrose) at a concentration of 1:6, loaded on a 2% agarose gel and run in a gel tank. Hae III digest of ΦX174 (Bangalore Genei, Bangalore, India) was used as a molecular weight marker and the band was visualised using a gel documentation system (Geldoc 2000/Geldoc XR documentation system, Bio-Rad, California, USA). The band size was determined using the software available with the gel documentation system (Quantitiy One, Bio-Rad, California, USA). A positive sample showed a HPV 16 specific band at 210 basepairs.

5.5.4.2 Pre-cycle sequencing clean up:

The amplified products were subjected to pre-sequencing clean up using a Millipore Vacuum system to remove unused dNTPs and primers. The following steps were followed.

1. The amplified product was made up to 100µl using sterile distilled water.

2. The solution was then transferred to the Millipore microtiter plate and attached to the Millipore Vacuum manifold.

3. Vacuum pressure was applied for approximately 10 minutes or until the wells dried.
4. One hundred microlitres of sterile distilled water was added and the same process was repeated.

5. Twenty microlitres of sterile distilled water was added to the wells, mixed 25 times and the contents transferred back to PCR tubes.

Agarose gel electrophoresis was done on 5 µl of the product and verified for the presence of a single specific band.

5.5.4.3 Sequencing PCR:

Sequencing PCR was done in a reaction volume of 10 µl containing 1 µl of the product, 1.6 pmoles of primer, 1 µl of Ready Reaction mix (ABI PRISM Big Dye Terminator cycle sequencing kit, Applied Biosystems, California, USA) and necessary buffers. Thermal cycling conditions include 25 cycles of 96°C for 12 s, 50°C for 15 s and 60°C for 4 minutes.

5.5.4.4 Post-cycle sequencing clean up:

The following steps were carried out for post cycle-sequencing clean up procedure.

1. The total volume of the PCR product was made up to 40 µl with injection solution and transferred to the Millipore microtiter plates in the vacuum manifold.

2. Vacuum pressure was applied to the plates until the plates are dry.

3. Forty microlitres of injection solution was added to the wells and the vacuum process was repeated.
4. Thirty microlitres of injection solution was added, the contents mixed well by pipetting 25 times and transferred to tubes for sequencing.

Sequencing was done in an automated capillary sequencer called the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, California, USA). The sequences obtained were checked and analysed using the sequence navigator software v 1.0.1 (Applied Biosystems, California, USA).

The sequences were subjected to a BLAST (Basic Local Alignment Search Tool) search (http://www.ncbi.nlm.nih.gov, National Center for Biotechnology Information, US National Library of Medicine, MD, USA) to identify the genotype.

The paired sequences from plasma and tissue of a sample were aligned to analyse sequence relatedness using an online multiple sequence alignment program, ClustalW version 1.83 (http://www.ebi.ac.uk/Tools/clustalw/).

5.6 Detection of E6/E7 mRNA in cervical tissue and PBMC:

5.6.1 Detection HPV 16 E6/E7 mRNA transcripts in cervical tissue:

5.6.1.1 mRNA extraction:

mRNA was extracted from cervical tissues using Oligotex Direct mRNA mini kit (Qiagen, GmbH, Germany). Extraction was done as per the kit’s instructions. The steps involved are given below.

1. Cervical tissue was first disrupted by grinding in a mortar and pestle.
2. The ground tissue was then homogenised using a needle and syringe after addition of 0.6 ml of lysis buffer (OL1).

3. To the lysate, 1.2 ml of dilution buffer (ODB) was added, the contents mixed and centrifuged for 3 minutes at 14000 rpm.

4. The supernatant was transferred to a new tube and 35µl of oligotex suspension was added. Oligotex particles selectively bind to poly A⁺ mRNA and thus aid in isolation of mRNA.

5. The contents were mixed well and placed at room temperature for 10 minutes.

6. The tubes were centrifuged at 14,000 rpm for 5 minutes and the supernatant discarded.

7. The oligotex:mRNA pellet was resuspended in 100 µl of OL1 buffer.

8. The mixture was diluted by adding 400 µl of ODB buffer and incubated at 70°C for 3 minutes and then at room temperature for 10 minutes.

9. The tube was centrifuged at 14000 rpm for 5 minutes and the supernatant was removed.

10. The pellet was resuspended in 350 µl of wash buffer (OW1), mixed well and transferred to a spin column.

11. The tube was centrifuged at 14000 rpm for 1 minute and the flow through was removed.

12. To the spin column, 350µl of wash buffer (OW2) was added and centrifuged at 14000 rpm for 1 minute.
13. The wash step with wash buffer OW2 was repeated.

14. The spin column was transferred to a new micro centrifuge tube and 100 µl of heated (70°C) elution buffer (OEB) was added and mixed well.

15. The spin column was then centrifuged at 14000 rpm for 1 minute.

The mRNA extract thus obtained was used for further analysis.

5.6.1.2 Reverse transcription:

cDNA synthesis was performed in a reaction volume of 20 µl with a sample input of 9µl. The reaction mix had 20 pmoles of random primers (Invitrogen, USA), 20 units RNase OUT inhibitor (Invitrogen, USA), 3mM MgCl₂, 0.5mM dNTPs, appropriate 1X buffer and 50 units of MuMLV reverse transcriptase (Invitrogen, USA). Reverse transcription was done at 37°C for 60 minutes and the reaction terminated at 95°C for 5 minutes.

5.6.1.3 Nested PCR for detection of mRNA transcripts:

First round PCR was done in a reaction mix containing 10 µl cDNA, 1.5mM MgCl₂, 0.2mM dNTPs, 20 pmoles of each outer primers, 1 unit of Hotstar Taq DNA polymerase (Qiagen, Gmbh, Germany) and 1X buffer. The reaction was made up to 50 µl with sterile milliQ water. The primers used targeted the E6/E7 ORF of the HPV 16 genome (Table 9). The thermal cycling conditions consisted of a pre-PCR denaturation step at 94°C for 15 minutes followed by 30 cycles at 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute with a final extension at 72°C for 10 minutes.
Nested PCR was done in a reaction mix containing 1 µl of first round PCR product, 1mM MgCl₂, 0.2mM dNTPs, 10 pmoles of inner primers, 1 unit Hotstar Taq DNA polymerase (Qiagen, Gmbh, Germany) and 1X buffer. The reaction was made up to 50 µl with sterile milliQ water. The primers target the E6/E7 ORF of HPV 16 genome (Table 9). The thermal cycling conditions were similar to that of the first round PCR. The amplified products were run in an agarose gel electrophoresis to identify the transcripts. The E6, E6*I and E6*II transcripts were seen as 395, 213 and 95 base pair products respectively.

5.6.1.4 Quantitation of HPV 16 E6/E7 transcripts in cervical tissue:

5.6.1.4.1 DNase treatment:

The extracted mRNA was first treated with DNase I to remove contaminating DNA. The following steps were carried out.

1. Ten microlitres of mRNA was treated with 2 units of DNase I (New England Biolabs, Ipswitch, USA) in a reaction volume of 30µl.
2. The reaction mixture was incubated at 37°C for 10 minutes.
3. EDTA (0.1M) was added to the mix to make a final concentration of 5 mM.
4. The reaction mixture was incubated at 75°C for 10 minutes to deactivate DNase I.

5.6.1.4.2 Reverse transcription:

Reverse transcription was done as described in 5.6.1.2. cDNA synthesis was done using DNase I treated mRNA as template.
5.6.1.4.3 Real time PCR for quantitation of transcripts:

Quantitation of cDNA was performed employing an UV spectrophotometer. Real time PCR was performed using an uniform input of 20 ng cDNA for all the samples. The protocol used for amplification was similar to amplification of HPV 16 in plasma explained in section 5.5.3.1.

5.6.1.5 Beta actin PCR:

Beta actin PCR was additionally performed on cDNA of all samples to check for sample integrity (Table 10).243 The PCR reaction mix contained 4µl cDNA, 50 µM dNTPs, 10 pmoles of each primer, 1 unit of Hotstar Taq DNA polymerase (Qiagen, Gmbh, Germany) and 1X PCR buffer without MgCl₂. The reaction volume was made upto 25 µl with sterile milliQ water. The thermal cycling conditions consisted of a pre-PCR denaturation at 95°C for 15 minutes followed by 40 cycles at 95°C for 1 minute, 48°C for 1 minute and 72°C for 1 minute, with a post-PCR extension at 72°C for 10 minutes. The amplified products were detected in agarose gel electrophoresis as described earlier.

5.6.2 Detection HPV 18 E6/E7 mRNA transcripts in cervical tissue:

mRNA extraction was done on all samples that were positive for HPV 18 using the LBA. cDNA synthesis was done as described for HPV 16 in section 5.6.1.2. Amplification was done by nested PCR using primers from the E6/E7 ORF of the HPV 18 genome (Table 11).244, 245

The thermal cycling conditions used for both the rounds were similar to that of HPV 16. The amplified products were detected by agarose gel electrophoresis stained
with ethidium bromide as described earlier. The expected size of the HPV 18 E6*mRNA transcript was 297 base pairs.

Beta actin PCR was done on all samples. The protocol followed was similar to that mentioned in 5.6.1.5.

5.6.3 Quantitation of HPV 18 mRNA transcripts in cervical tissue:

Quantitation of cDNA was performed employing an UV spectrophotometer. Real time PCR was performed using an uniform input of 20ng cDNA for all the samples. The protocol used for amplification was similar to amplification of HPV 18 in cervical tissue explained in 5.4.2.

5.7 Detection of HPV 16 or 18 mRNA transcripts in PBMCs:

5.7.1 mRNA extraction and cDNA synthesis:

mRNA was extracted from PBMCs using Oligotex direct mRNA mini kit (Qiagen, GmbH, Germany) as previously described for tissues in 5.6.1.1. cDNA synthesis was done using M-MuLV reverse transcriptase (Invitrogen, California, USA) as described in 5.6.1.2.

5.7.2 Detection of HPV 16 mRNA transcripts:

5.7.2.1 Detection of HPV 16 mRNA transcripts using nested PCR:

cDNA was amplified by nested PCR using primers that target the E6 region of the HPV genome (Table 12). For the first round PCR, the reaction mix consisted of 10μl cDNA, 1 unit Hotstar Taq DNA polymerase (Qiagen, GmbH, Germany), 0.2mM dNTPs, 1.5mM MgCl₂, 10 pmol of each primer and an appropriate PCR buffer in a total volume
of 50µl. For the nested PCR, the sample (1st round product) input was reduced to 1 µl and nested primers were included.

The thermal cycling conditions were same for both rounds of PCR and was as follows: a pre-PCR denaturation at 95°C for 15 minutes, followed by 32 cycles at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 60 seconds with a final extension at 72°C for 10 minutes. The amplified products were visualised in an agarose gel electrophoresis stained with ethidium bromide as described earlier. The length of the amplified product was 422 base pairs.

5.7.2.2 Detection of HPV 16 mRNA transcripts using real time PCR:

Amplification was performed on cDNA using primers that target the E6 region of HPV 16 genome as described earlier for amplification of HPV 16 in plasma (section 5.5.3.1)

5.7.3 Detection of HPV 18 mRNA transcripts:

5.7.3.1 Detection of HPV 18 mRNA transcripts using nested PCR:

Amplification was done by nested PCR using primers from the E6/E7 ORF of the HPV 18 genome as described earlier for tissues in 5.6.2.

The thermal cycling conditions used for both the rounds of PCR were similar to that done for cervical tissues 5.6.2. The amplified products were detected by agarose gel electrophoresis stained with ethidium bromide. The HPV 18 E6*mRNA showed the presence of a 297 base pair product.
Beta actin PCR was done on all samples. The protocol followed was similar to that mentioned in 5.6.1.5.

5.7.3.2 Detection of HPV 18 mRNA transcripts using real time PCR:

Amplification was done using primers targeting the E6 region of the HPV 18 genome as described earlier for HPV 18 quantitation in cervical tissues (5.6.3).

5.8 Measurement of T cell responses to HPV 16:

T cell responses to HPV 16 were detected by measurement of interferon gamma release by an ELISPOT assay. Blood collected in heparin was used for this assay. The following steps were carried out.

5.8.1 Separation of peripheral blood mononuclear cells (PBMCs):

PBMCs were separated from blood using Ficoll, a gradient that separates lymphocytes from other blood cell types. The steps done for purification of PBMCs were as follows:

1. Five ml of heparinised blood was diluted with equal volume of 1X PBS.

2. Five ml of the mixture was layered on equal volume of Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) gradient.

3. The tubes were centrifuged at 400g for 30 minutes at 18-20°C.

4. The cells were carefully removed and transferred to a new 15ml centrifuge tube.

5. The cells were suspended in 6 ml of 1X PBS and centrifuged at 1300 rpm at 18-20°C for 20 minutes.
6. The supernatant was removed, 6 ml of 1X PBS was added and the tubes centrifuged at 200g at 18-20°C for 10 minutes.

7. The previous wash step was repeated for 10 minutes.

8. The supernatant was discarded and the cells were suspended in 2 ml of 1X PBS and mixed well.

9. The numbers of lymphocytes were counted in Beckman’s coulter counter present in the department of Clinical Pathology, Christian Medical College, Vellore.

10. The cells were pelleted, and the pellet suspended in RPMI 1640 medium supplemented with 10% FBS. The volume of RPMI medium added was in such a way that 100µl of suspended solution would have 4x10⁵ cells.

5.8.2 Prediction of class I and class II T cell epitopes:

Two peptides from the E7 protein of HPV 16 were chosen for the study. One peptide E7 (aa 70-98) was reported to be associated with recurrence following treatment and the other peptide E7 (aa 37-54) was associated with regression of cervical disease.³⁹ ⁴⁰ The peptide sequences are given in Table 13. Both these peptides were shown to stimulate T cell responses in vitro in western populations. Subsequently, the common HLA types in South Indian population were identified from previously published literature (Table 14).²⁴⁷ ²⁴⁸ Prediction of class I MHC restricted T cell epitopes was done using EpiJen v1.0, an online prediction server from the Edward Jenner Institute of Vaccine research, UK (http://www.jenner.ac.uk/EpiJen/). Class II MHC binding peptide prediction was done using online software SVMHC, from the Eberhard Karls University, Tubingen, Germany (http://www-bs.informatik.uni-tuebingen.de/SVMHC/index.html).
The peptide sequences were entered into the servers, the appropriate MHC allele was chosen. Probable T cell epitopes were then generated by the servers. The epitopes were ranked based on the binding affinity denoted as IC$_{50}$. IC$_{50}$ less than 50 nM was considered high binders, 50-500nM were considered moderate binders and above 500 nM were considered non-binders. Probable epitopes are also given a score in some programs, with scores greater than 1 representing predicted binders.

The HPV 16 E7 peptides used for our study was found to contain immunodominant T cell epitopes.

**A*0201**: Four high binding epitopes were seen in the E7 peptides included in this study for this HLA type. The epitope regions were (aa 82-91), (aa 81-89), (aa 85-94) and (aa 75-84) (Table 10.1, Appendix).

**A*0301**: There were no high binders for this HLA type in the peptides that we used in the study. However, it had three moderate binders. The regions in the peptide were (aa 41-49), (aa 75-84) and (aa 89-98) (Table 10.2, Appendix).

**A*3101**: There was only one moderate binding epitope region in the peptides studied for this HLA type. The region was (aa 41-49) (Table 10.3, Appendix).

**B*3501**: There was only one moderate binding epitope region (aa 44-53) and no high binding region in the peptides studied for this HLA type (Table 10.4, Appendix).

**DRB1*1502**: The predicted binders with scores above 1 for this HLA class II type were (aa 76-85), (aa 87-96), (aa 83-92) and (aa 82-91) (Table 10.5, Appendix).

**DRB1*0701**: There were three predicted binders for this HLA type in the peptides used for the study. They were (aa 83-92), (aa 87-96) and (aa 76-85) (Table 10.6, Appendix).
The prediction results obtained for HPV 16 E7 protein from these online servers are attached in Appendix (Tables 10.1 to 10.6).

5.8.3 Enzyme linked immunospot (ELISPOT) assay for interferon gamma release:

ELISPOT assay was used to study the T cell responses to HPV 16 peptides. Both the peptides used were synthesised commercially (Anaspec, San Jose, USA). The peptides were synthesised at 95% purity and their molecular weights were 3168.3 and 1580 respectively. The peptides were dissolved in sterile milliQ water to get a final concentration of 1µg/µl. The peptides were then aliquoted and stored at -20°C.

The following steps were carried out as per the salient features of the protocol provided with the ELISPOT assay kit.

1. The wells were pre-coated with anti-interferon gamma antibody (Becton Dickinson, California, USA).

2. The separated PBMCs were added to the wells at a concentration of 4x10⁵ cells per well in the ELISPOT plate.

3. Both the peptides were added to the wells in duplicate at a concentration of 1µg per well. Phytohemagglutinin (PHA, Sigma, St.Louis, USA) was used as a positive control at a concentration of 2 µg per well and RPMI medium alone served as negative control. Negative control and positive control were used for every sample.

4. Recombinant IL-2 (Sigma, St.Louis, USA) after optimisation of dose by titration experiments was added to all wells at a concentration of 6.25 IU per ml.
5. The plate was then incubated at 37°C for 18 hours.

6. The plate was then washed with 200µl of distilled water twice and then thrice with 200 µl of reconstituted wash buffer.

7. The plate was dried and 100 µl of detection antibody was added.

8. The plate was incubated at room temperature for 2 hours.

9. The plate was washed thrice with 200 µl wash buffer and 100 µl of streptavidin-horse radish peroxidase (HRP) was added.

10. The plate was incubated at room temperature for 1 hour.

11. The plate was washed 4 times with 200 µl wash buffer and then twice with 200 µl of 1X PBS.

12. The plate was dried and 100 µl of AEC substrate was added.

13. The plate was incubated at room temperature for 5-60 minutes till spots appear.

14. The color production was stopped by washing the plate with 200 µl distilled water.

15. The spots were identified by the unaided eye using a magnifying lens.

5.8.2.1 Calculation of results:

The cut-off number of spots was calculated individually for every sample. The cut-off was mean+3SD of the spots in the negative control wells. All samples with spots more than mean+3SD was considered positive.
5.9 Measurement of humoral immune responses to HPV:

5.9.1 Measurement of antibody response to HPV 16 peptides/VLPs:

5.9.1.1 Peptide ELISA

Detection of antibodies to HPV 16 early proteins was standardised in our laboratory. Synthetic peptides spanning the regions E2 (aa 328-345), E6 (aa 1-23) and E7 (aa 11-19) were used as antigen source (Table 15). Peptides were synthesised commercially (Sigma-Aldrich, Bangalore, India). The E2 peptide was 95.5% pure and had a molecular weight of 2280.47. The E6 peptide was 99% pure with a molecular weight of 2839.33. The E7 peptide was 98.6% pure with molecular weight of 1109.25. The peptides were dissolved in 1X PBS (pH 7.4) to get a concentration of 1mg/ml and stored as aliquots at -20°C.

The protocol followed for ELISA is given below in section 5.9.3.

5.9.1.2 Virus-like particle (VLP) ELISA:

The presence of IgG antibodies to HPV 16 was determined by an in-house VLP ELISA. HPV 16 VLPs were obtained by expressing HPV late gene L1 coding for major capsid proteins into a baculovirus system. The purified VLPs were then used as the antigen (courtesy, Prof. Raphael Viscidi, John Hopkins University, Baltimore, US). The protocol followed for ELISA is given below in section 5.9.3.
5.9.1.3 Testing for cross reactivity:

Serum samples from women who were positive for HPV types other than HPV 16 were tested using both the peptide ELISA and VLP ELISA to determine the presence of cross-reacting antibodies. HPV types in these women are given in Table 16.

5.9.2 Measurement of antibody response to HPV 18:

5.9.2.1 Virus like particles (VLP) ELISA

Presence of antibodies to HPV 18 was measured by a VLP ELISA similar to that of HPV 16. HPV 18 VLPs were synthesised by expressing the major capsid protein L1 in a baculovirus system and then purified and used as the antigen (courtesy, Prof. Raphael Viscidi, JHU, Baltimore, US). The protocol followed for ELISA is given below in section 5.9.3

5.9.2.2 Testing for cross reactivity:

Serum samples from women who were positive for HPV types other than HPV 18 were tested using both the peptide ELISA and VLP ELISA to determine the presence of cross-reacting antibodies. HPV types in these women are given in Table 17.

5.9.3 ELISA procedure:

The following steps were carried out using the standardised reagents given in Table 18.

1. The plates were coated with 100µl of the necessary antigen diluted in sterile 1X PBS and incubated at 4°C for 16 hours. Nunc Maxisorp plates (Nunc, Rockslide, Denmark) were used for HPV16 E2 and E7 peptide ELISAs and Nunc Polysorp
plates (Nunc, Rockslide, Denmark) were used for HPV16 E6 peptide ELISA, HPV 16 VLP ELISA and HPV 18 VLP ELISA.

2. The contents of the plate were discarded, and the plate was blocked by adding 300 µl of 1X blocking buffer (Polyvinyl alcohol) and incubated at room temperature for 3 hours.

3. The plate was washed once with wash buffer (1X PBS with tween 20) containing 0.1% BSA and then five times with wash buffer without BSA in an automatic ELISA washer (ELx50, Biotek instruments, Winooski, USA).

4. One hundred microliters of sample dilution buffer was added to the plate and standardised volume of serum was added to the wells.

5. The plate was incubated at 37°C for 1 hour.

6. The plate was washed as described in step 3.

7. One hundred microliters of conjugate diluted with conjugate enhancing buffer was added to the plate. Anti-human IgG conjugated with horse radish peroxidase (Sigma-Aldrich, St.Louis, USA) was used as the conjugate.

8. The plate was incubated at 37°C for 30 minutes.

9. The plates were washed as described in step 3.

10. One hundred microliters of substrate (TMB, H₂O₂) was added to the wells and incubated in room temperature for 20 minutes.

11. The color development was stopped with addition of 1N H₂SO₄.
12. The plate was read at 450nm using a reference filter at 630nm in an ELISA plate reader (ELx800, Biotek instruments, Winooski, USA).

5.9.4 Derivation of cut-off:

Serum samples collected from children who were 1.5 years to 3 years old with no clinically obvious laryngeal papillomatosis were initially used to derive the cut-off. The calculated cut-off OD were 0.385 (E2), 0.047 (E6), 0.233 (E7), 0.120 (HPV 16 VLP) and 0.438 (HPV18 VLP). Subsequently, serum was collected from unmarried women in the community (mean age=23 yrs). Since unmarried women in India are believed to have lower sexual activity before marriage (13%)\textsuperscript{19}, the OD values derived from these women were finally used to derive the cut-off values for all antibody detection assays. The calculated cut-off were 0.431 (E2), 0.053 (E6), 0.243 (E7), 0.119 (HPV16 VLP) and 0.384 (HPV 18 VLP).

5.10 Statistical analysis:

Statistical analysis was done using Epi Info (version 6.04d) and SPSS (version 11.0). Chi square test and Chi square analysis for trend were performed. $P$ values less than 0.05 were considered statistically significant. Kappa statistics was done to check the agreement between the primers used (MY and PGMY) and also between the genotyping techniques (RFLP and LBA). Mann Whitney test was done to check if there was a statistical difference in HPV 16 and 18 viral loads between women with adenocarcinoma and squamous cell carcinoma. Crude and normalised HPV 16 and 18 viral load values were compared using Spearman’s correlation test.
6. RESULTS:

6.1 Clinical staging of women with cervical neoplasia:

Of the 150 women with cervical neoplasia, 10 (6.7%) were diagnosed with cervical intraepithelial neoplasia (CIN) and 140 (93.3%) were diagnosed with invasive cervical cancer. The detailed clinical disease staging in these women is given in Table 1. Since the frequency of women varied across groups, and some groups had very few women, the women were regrouped as given in Table 2 for statistical analyses.

6.2 Histopathological findings in tumour of women with invasive cervical carcinoma:

Histopathological staging was available for all 140 women with invasive cervical disease. Squamous cell carcinoma was the most common stage seen in 119 (85%) women followed by adenocarcinoma, which was seen in 13 (9.3%) women. The histopathological stage of tumour in women with invasive cancer is given in Table 3.

6.3 HPV DNA detection and genotyping:

6.3.1 Study group I:

6.3.1.1 Detection using MY09/11 primers:

Of the 150 women with cervical neoplasia, 137 (91.3%) women had detectable HPV DNA in their cervical tissue. Of the 137 women, 8 (5.8%) were diagnosed as CIN and 129 (94.2%) as with invasive cancer. Figure 1 shows a gel picture of PCR results using MY 09/11 primers. The clinical staging of 137 women is shown in Table 4. Of the 13 women who were negative for HPV DNA, 2 had CIN and 11 had invasive cancer (7
women with IIIB and 2 each with stages IIB and IB). Histological typing of the tumor from the HPV DNA negative women showed moderately differentiated squamous cell carcinoma in 7 women and 1 each with poorly differentiated squamous cell carcinoma, poorly differentiated adenocarcinoma, mixed epithelial and mesenchymal neoplasm, carcinosarcoma, undifferentiated carcinoma and cervical intraepithelial neoplasia. All the samples tested were positive for beta globin including those samples that tested negative for HPV DNA.

To check for primer specificity, PCR was done using MY primers on DNA extracts from cell culture supernatant containing viruses CMV, HSV1 or HSV2, DNA extract of a sample positive for EBV and plasmid containing JCV or BKV. None of the samples tested were positive for HPV.

6.3.1.2 HPV genotyping using PCR-RFLP:

Of the 137 women positive for HPV DNA by MY primers in cervical tissue, 125 (91.2%) were typed by RFLP. HPV 16 (66.4%) followed by HPV 18 (13.9%) were the major high risk types detected. The spectrum of genotypes identified by RFLP is given in Table 5. Figure 2 shows a gel picture of a typical RFLP pattern seen for 2 common HPV types, HPV 16 and 18. HPV 64 seen in a woman with stage IIB invasive cancer was the only low risk type identified by RFLP. Twelve (8.8%) samples were untypeable by RFLP. HPV 16 (64.7%) followed by HPV 18 (10.9%) were the most prevalent type seen in women with squamous cell carcinoma. HPV 18 (46.2%) followed by HPV 16 (30.7%) were the most common types seen in women with adenocarcinoma. The HPV type distribution in women with the two major histological types of neoplasia is shown in Tables 6 and 7.
6.3.1.3 HPV DNA detection using PGMY09/11 primers:

Of the 150 women tested, 140 (93.3%) women were positive for HPV DNA using PGMY primer amplification. Of the 140 HPV DNA positive women, 8 (5.7%) had CIN and 132 (94.3%) had invasive cancer. Of the 10 women negative for HPV DNA, 2 had CIN and 8 had invasive cancer (4 women with IIIB and 2 women each with stages IIB and IB). The histopathological stage of the tumour in the HPV DNA negative women showed moderately differentiated squamous cell carcinoma in 5 women and 1 woman each with poorly differentiated squamous cell carcinoma, poorly differentiated adenocarcinoma, mixed epithelial and mesenchymal neoplasm, undifferentiated carcinoma and cervical intraepithelial neoplasia.

To check for primer specificity, PCR was done using PGMY primers on DNA extracts from cell culture supernatant containing viruses CMV, HSV1 or HSV2, DNA extract of a sample positive for EBV and plasmid containing JCV or BKV. None of the samples tested were positive for HPV.

6.3.1.4 HPV genotyping using Line Blot assay (LBA) using PGMY09/11 primers:

Of the 140 women who were positive for HPV DNA by PGMY09/11 primer based PCR testing, 131 (93.6%) women had a single HPV type and 9 (6.4%) women had multiple types. The HPV types identified as single infections are shown in Table 8. All nine samples with multiple infections had 2 genotypes and are shown in Table 9. Of the 3 women who were negative by MY primers but positive by PGMY primers, 2 had low risk HPV types 42 and 61 and 1 had a high risk HPV type 52. The total HPV types
identified by LBA are shown in Table 10. The result as seen in LBA is shown in Figure 3.

All 12 samples that were untypeable by RFLP were resolved by LBA. The HPV types thus identified are shown in Table 11. A comparison of high risk HPV types detected by RFLP and LBA is shown in Figure 4. High risk HPV types 52, 51, 56, 58 and 33 were detected only by the LBA. The distribution of the 4 major HPV types across the disease stages is given in Figure 5.

MY and PGMY primers showed a good agreement for detection of HPV DNA in cervical tissue (kappa=0.859, p<0.001). PCR-RFLP and PCR-LBA did not correlate well in their ability to assign HPV genotypes (kappa=0.526, p<0.001). This discrepancy may be due to the presence of 12 samples untypeable by PCR-RFLP and also the presence of dual infections in 9 women.

6.3.2 Study group II:

6.3.2.1 Detection using MY09/11 primers:

Three (9.1%) of 33 women who did not have clinical cervical disease were HPV DNA positive in cervical tissue.

6.3.2.2 HPV genotyping using PCR-RFLP:

Of the 3 women with HPV DNA in cervical tissue, 2 were typed as HPV 18 and 1 was typed as HPV 31.

6.3.2.3 HPV DNA detection using PGMY09/11 primers:

Three (9.1%) of 33 women in this group had HPV DNA in cervical tissue.
6.3.2.4 HPV genotyping using LBA:

Of the 3 women with HPV DNA in cervical tissue, 2 were typed as HPV 18 and 1 was typed as HPV 31. Thus both PCR-RFLP and LBA were able to identify the HPV types in these ‘control’ women.

6.4 HPV quantitation in tissues:

6.4.1 HPV 16 quantitation:

Inter-assay and intra-assay variation analysis was done on 3 samples with HPV 16 viral loads of $10^3$, $10^5$ and $10^6$. The coefficients of variations calculated for the mean logarithm of the viral loads obtained are given in Table 12a and 12b. A graph with analysed fluorescence data obtained during quantitation of HPV 16 using real time PCR and the standard curve generated automatically by the software for HPV16 quantitation in cervical tissue are shown in Figures 6 and 7 respectively.

Using real time PCR, HPV 16 was amplifiable in all 94 samples typed as HPV 16 by LBA. Normalised HPV 16 viral load ranged from 28 copies/5000 cells to 40918563 copies/5000 cells (median viral load = 58342 copies/ 5000 cells). The median viral loads of women with different stages of cervical neoplasia are given in Table 13. HPV 16 viral load did not show statistically significant correlation with advancing disease stage (p=0.977). The median HPV16 viral load in women with squamous cellular carcinoma was 52631 copies/5000 cells and in women with adenocarcinoma was 90227 copies/5000 cells. There was no statistical difference in the median HPV 16 viral load between these 2 tumour groups (p=0.517). There was an excellent correlation between the crude and normalised HPV 16 viral load in individual samples (Spearman’s rho=0.908; $P<0.001$).
HPV 16 E6 real time PCR primers showed 100% specificity when checked with HPV DNA from samples positive for types other than HPV 16 belonging to species 9 and other species.

6.4.2 HPV 18 quantitation:

Inter-assay and intra-assay variation analysis was done on 3 samples with known viral loads. The coefficients of variations obtained are given in Table 14a and 14b. A graph with analysed fluorescence data obtained during quantitation of HPV 18 using real time PCR and the standard curve generated automatically by the software for HPV18 quantitation in cervical tissue are shown in Figures 8 and 9 respectively.

Using real time PCR, HPV 18 was amplifiable in all 20 samples typed as HPV 18 by LBA. Viral load ranged from 4118 copies/5000 cells to 11186194 copies/5000 cells (median viral load = 71367 copies/5000 cells). The median HPV 18 viral loads in women with different stages of cervical neoplasia are shown in Table 15. HPV 18 viral load did not show statistically significant correlation with advancing disease stage (p=0.263). The median HPV 18 viral load in women with squamous cell carcinoma was 65327 copies/5000 cells and adenocarcinoma was 111567 copies/5000 cells. There was no statistical difference in the HPV 18 viral load between these 2 tumour groups (p=0.219). There was a good correlation between crude and normalised HPV18 viral loads with respect to individual samples (Spearman’s rho =0.770; P<0.001). The HPV 18 viral load in the 2 control samples were 6426 and 7582 copies/5000 cells respectively which were both lower than the median virus load in any of the clinical stages (Table 15).
HPV 18 E6 real time primers showed 100% specificity when checked with HPV DNA from samples positive for types other than HPV 18 belonging to species 7 and other species.

6.4.3 ERV3 quantitation:

Inter-assay and intra-assay variation analysis was done using DNA extracts of human PBMCs with known ERV3 loads (4.68 copies/ml to 46800 copies/ml). The coefficient of variation (CVs) obtained for ERV3 viral load assay are given in Table 16a and 16b and are within acceptable range. Only one sample with ERV3 viral load (4.68 copies) showed a high CV of 18.62. A graph with analysed fluorescence data obtained during quantitation of ERV3 DNA using real time PCR and the standard curve generated automatically by the software for ERV3 quantitation in cervical tissue are shown in Figures 10 and 11 respectively.

6.5 HPV DNA detection in plasma:

6.5.1 Amplification using MY09/11 primers in conventional PCR:

6.5.1.1 HPV 16:

Of the 94 HPV 16 positive samples, 2 (2.1%) samples had HPV DNA in plasma using this technique. The 2 positive women had been diagnosed with stage IB1 and IIB invasive cervical carcinoma. The virus loads of these 2 samples in tissue were 13157 and 387541 copies/5000 cells respectively. Figure 12 shows a gel picture of the 2 samples positive for HPV DNA in plasma.
6.5.1.2 HPV 18:

None of the 20 HPV 18 positive samples had detectable HPV DNA in plasma.

6.5.2 Amplification and quantitation using real time PCR:

6.5.2.1 HPV 16:

Of the 94 HPV 16 positive women, 53 (56.4%) had HPV 16 in plasma. Plasma viral load ranged from 1 copy/ml to 31303 copies/ml (median viral load = 253 copies/ml). Percentage detection rate of plasma viremia and median plasma viral load across disease stages are given in Table 17. The rate of detection of plasma viremia increased with advancing disease stage (p=0.001). However, HPV 16 absolute plasma viral load was not significantly associated with advancing disease stage (p=0.281). There was no correlation between absolute plasma viral load and viral load in corresponding tissue (spearman’s rho=0.184, p=0.187). None of the women in study group II showed the presence of HPV 16 in plasma.

A graph with analysed fluorescence data obtained during quantitation of HPV 16 in plasma using real time PCR and the standard curve generated automatically by the software for HPV16 quantitation in cervical tissue are shown in Figures 13 and 14 respectively.

6.5.2.2 HPV 18:

Four (20%) of 20 women who had HPV 18 in their cervical tissue had HPV 18 in plasma. The viral load ranged from 200 copies/ml to 4820 copies/ml (median viral load =
500 copies/ml of plasma). HPV 18 plasma viremia and plasma viral load are shown in Table 18. HPV 18 plasma viral load was not associated with advancing disease stage (p=0.508). None of the women in study group II showed presence of HPV 18 in plasma.

A graph with analysed fluorescence data obtained during quantitation of HPV 18 in plasma using real time PCR and the standard curve generated automatically by the software for HPV16 quantitation in cervical tissue are shown in Figures 15 and 16 respectively.

6.5.3 HPV 16 sequencing in paired plasma and tissue:

Sequencing of the HPV 16 E6/E7 gene was done on 5 paired plasma and tissue samples. CLUSTAL W analysis has shown the presence of the same HPV sequences in all the paired samples tested. Alignment result of a sample is shown in Figure 17. Chromatograms showing results of nucleotide sequence analysis of a sample both in plasma and tissue is shown in Figure 18.

6.6 Detection of HPV 16 and 18 mRNA transcripts in cervical tissue and peripheral blood mononuclear cells (PBMCs):

6.6.1 Detection of HPV 16 and 18 mRNA transcripts in cervical tissue:

6.6.1.1 HPV 16 E6/E7 mRNA transcripts detection using nested PCR:

Of the 94 HPV 16 positive women, mRNA transcripts were detected in 84 (89.3%). Transcripts detected by nested PCR are shown in Table 19. However, there was no statistically significant association between detection of transcripts in tissue and
clinical staging of disease (p=0.450). Figure 19 shows the distribution of E6/E7 mRNA transcripts in various stages of cervical neoplasia. The E7 (E6*I, E6*II) transcript was the most common transcript detected (80.9%). A gel picture of different transcripts is shown in Figure 20. Beta actin was positive in all the samples. A gel picture of beta actin product is shown in Figure 21.

6.6.1.2 HPV 16 E6/E7 mRNA transcripts detection and quantitation using real time PCR:

Of the 94 HPV 16 positive samples, mRNA transcripts were detected using real time PCR in 81 (86%) women. The transcript copy number detected ranged from 1 to 38005726 copies (median copy number = 5964). Percentage positivity of transcripts detected and median transcript levels in different clinical stages of cervical neoplasia are given in Table 20. There was no significant association between the percentage positivity of detection of these mRNA transcripts in tissue and clinical stage of disease (p=0.643). However, median transcript levels in tissue appears to show a trend towards increase with advancing disease stage (p=0.06). There was no correlation between the HPV 16 mRNA transcript level (copies/20ng) in tissue and HPV 16 DNA level (copies/5000cells) in corresponding tissue (R = - 0.040, p= 0.722) (Figure 22).

A graph with analysed fluorescence data obtained during quantitation of HPV 16 mRNA transcripts using real time PCR and the standard curve generated automatically by the software for HPV16 quantitation in cervical tissue are shown in Figures 23 and 24 respectively.
6.6.1.3 HPV 18 E6* mRNA transcripts detection by nested PCR:

Of the 20 HPV 18 positive women, one sample that was negative for beta actin amplification was excluded from analysis. Of the 19 valid samples, HPV 18 E6* mRNA was detected in 15 (78.9%) of women. Table 21 shows the number of transcripts detected in different clinical stages of cervical neoplasia. A gel picture showing the HPV 18 E6 transcript is shown in Figure 25.

6.6.1.4 HPV 18 E6* mRNA transcripts detection and quantitation using real time PCR:

One sample was excluded from analysis as the internal control beta actin PCR was negative. Using real time PCR, mRNA transcripts were detected in 16 (84.2%) out of 19 HPV 18 positive women. HPV 18 transcript copy number detected ranged from 8 to 273479 copies (median copy number = 6158). HPV 18 transcript detection and copy number are shown in Table 22. A graph with analysed fluorescence data obtained during quantitation of HPV 18 mRNA transcripts using real time PCR and the standard curve generated automatically by the software for HPV18 quantitation in cervical tissue are shown in Figures 26 and 27 respectively.

6.6.2 HPV 16 and 18 mRNA transcripts detection in PBMCs:

All the samples were negative for mRNA transcripts using both nested PCR and real time PCR. However, all the samples were positive for beta actin.
6.7 Measurement of T cell responses to HPV 16:

In study group I, blood samples for ELISPOT assay was available for 101 women. Of these, results from 32 women could not be used for analyses because either the positive control wells did not show enough spots (less than 10) or the negative control wells had more than 50 spots, and were thus termed invalid. Thus, valid ELISPOT results were available for 69 women. Forty three of the 69 women were HPV 16 positive and were used for further analysis. ELISPOT reactivity to the HPV 16 E7 peptides studied in women with different stages of cervical neoplasia are given in Table 23. There was no significant difference in T cell response to these peptides in patients stratified by stage of clinical disease (p=0.424). In study group II, valid results were available for 10 women. In study group III, results were valid for 44 women. Percentage reactivity of women in the 3 study groups to the peptides are given in Table 24. There was no significant difference between the study groups in terms of T-cell responses to the HPV 16 peptides studied (p=0.789). The picture of an ELISPOT assay is shown in Figure 28.

6.8 Measurement of humoral immune response to HPV proteins:

6.8.1 Antibody response to HPV 16 viral peptides/ virus like particles (VLPs):

6.8.1.1 HPV 16 E2 peptide (aa 328-345) ELISA:

In study group I, 17 out of 94 (18%) individuals were positive for anti-E2 antibodies while 1 of 33 (3.03%) women in study group II and 3 (10%) of 30 married women in study group III were positive for anti-E2 antibodies (Figure 29). Reactivity to
E2 peptide was not significantly different between the 3 study groups (p=0.076). Antibody response to E2 peptide was not significantly higher in study group I compared to study group II (p=0.065) and study group III (p=0.445). In study group I, there was no correlation between antibody response to HPV 16 E2 peptide and clinical disease stage (p=0.771). Table 25 shows antibody response to E2 peptide in women with different clinical disease stage. The distribution of optical density (OD) values obtained for all the study groups are shown in a box and whiskers plot (Figure 30).

One (2.4%) of 42 women in study group I who had infection with HPV types other than HPV 16 was positive for anti-HPV 16 E2 antibodies. The E2 antibody status in different subsets of these women is given in Table 26 and the distribution of optical density (OD) values obtained in women with other HPV types are shown in a box and whiskers plot (Figure 31).

6.8.1.2 HPV 16 E6 peptide (aa 1-23) ELISA:

In study group I, 18 out of 94 (19.1%) women were positive for anti-E6 antibodies. Table 25 shows antibody response to the E6 peptide and clinical disease stage. None of the women in study group II were positive for anti-E6 antibodies. In study group III, 5 (16.7%) of 30 married women were positive for anti-E6 antibodies (Figure 29). There was a significant difference in the reactivity to E6 peptide between the 3 study groups (p=0.026). Antibody response to E6 peptide was significantly higher in study group I compared to study group II (p=0.027) but not with study group III (p=0.85). There was however no correlation between antibody response to HPV 16 E6 peptide and clinical stage of disease (p=0.266). Table 25 shows antibody response to E2 peptide in women
with different clinical disease stage. The distribution of optical density (OD) values obtained for all the study groups are shown in a box and whiskers plot (Figure 32).

Five (12%) of 42 women in study group I who had infection with HPV types other than HPV 16 were positive for anti-E6 antibodies. The E6 antibody status in different subsets of these women is given in Table 26 and the distribution of optical density (OD) values is shown in a box and whiskers plot (Figure 33). Median E6 antibody levels were higher in those harbouring HPV from species (HPV16 belongs to this species).

6.8.1.3 HPV 16 E7 peptide (aa 11-19) ELISA:

In study group I, 32 (34%) of the 94 women were positive for anti-E7 antibodies. Table 25 shows antibody response to the E7 peptide and clinical disease stage. One (3%) of 33 women in study group II and 10 (30%) of 30 married women in study group III were positive for anti-E7 antibodies (Figure 29). Antibody response to HPV 16 E7 peptide was significantly higher in study group I compared to study group II (p<0.001) and study group III (p<0.001). However, there was no statistically significant correlation between antibody response to HPV 16 E7 peptide and clinical stage of disease (p=0.224) (Table 25). The distribution of optical density (OD) values obtained for all the study groups are shown in a box and whiskers plot (Figure 34).

Eight (19%) of 42 women in study group I who had infection with HPV types other than HPV 16 was positive for anti-E7 antibodies. The E7 antibody status in different subsets of these women is given in Table 26 and the distribution of optical density (OD) values is shown in a box and whiskers plot (Figure 35).
6.8.1.4 HPV 16 VLP (L1) ELISA:

In study group I, 47 (50%) individuals were positive for anti-VLP antibodies. Table 27 shows antibody response to HPV 16 VLPs in study group I women with varying clinical disease stage. Fifteen (45%) of 33 women in study group II and 9 (30%) of 30 married women in study group III were positive for anti-VLP antibodies (Figure 29). Reactivity to VLPs was not significantly different between the 3 study groups (p=0.159). There was no significant difference in antibody response to HPV 16 VLPs between study groups I and II (p=0.653). However, antibody positivity tended to be higher in study group I compared to study group III (p=0.055). There was no statistically significant trend between antibody response to HPV VLPs and clinical disease stage (p=0.440) (Table 27). The distribution of optical density (OD) values obtained for all the study groups are shown in a box and whiskers plot (Figure 36).

Twelve (26%) of 42 women in study group I who had infection with HPV types other than HPV 16 was positive for anti-VLP antibodies. The antibody status in different subsets of these women is given in Table 28 and the distribution of optical density (OD) values is shown in a box and whiskers plot (Figure 37).

Antibody positivity in the 3 different HPV 16 peptide ELISAs (E2, E6 and E7) was compared with HPV 16 VLP ELISA. Women positive in atleast one of the 3 peptide ELISAs were grouped and compared with HPV 16 VLP ELISA. The agreement shown by statistical analysis is as follows: kappa 0.064, p=0.421 (E2 ELISA with VLP ELISA), kappa 0, p=1.000 (E6 ELISA with VLP ELISA), kappa 0.128, p=0.192 (E7 ELISA with VLP ELISA) and kappa 0.064, p=0.535 (all 3 peptide ELISAs combined with VLP ELISA). None of the 3 peptide ELISAs showed a good agreement with the VLP ELISA.
6.8.2 Antibody response to HPV 18 (L1) VLPs:

In study group I, 12 (60%) of 20 women were positive for anti-VLP antibodies. Table 29 shows antibody response to HPV 18 VLPs in study group I women with varying clinical disease stage. Ten (30 %) of 33 women in study group II and 8 (26.7%) of 30 married women in study group III were positive for anti-HPV 18 VLP antibodies (Figure 38). Reactivity to HPV 18 VLPs was significantly different between the 3 study groups (p=0.037). Antibody response to HPV 18 VLPs was significantly higher in study group I compared to study group II (p=0.003) and study group III (p=0.001). There was no significant trend in HPV 18 antibody positivity across increasing clinical disease stage (p=0.189). The distribution of optical density (OD) values obtained for all the study groups are shown in a box and whiskers plot (Figure 39). Fifteen (16%) of 94 women who had infection with HPV type other than HPV 18 was positive for anti-HPV 18 VLP antibodies. The antibody status in different subsets of these women is given in Table 30 and the distribution of optical density (OD) values is shown in a box and whiskers plot (Figure 40).
7. Discussion:

7.1 HPV DNA detection and genotyping:

Infection with human papillomavirus (HPV) is an important cause for the development of cervical cancer. Knowledge about the prevalence of HPV, particularly the oncogenic genotypes will be important in designing vaccine strategies to combat cervical cancer. The detection rate of HPV DNA in cervical cancer varies among studies and is influenced by various factors such as the collection medium used and DNA detection method. Early studies from India have used southern blot hybridisation and in-situ hybridisation for genotyping HPV. There are limited studies from India that have used sensitive methods like PCR for studying the prevalence of HPV genotypes in Indian women with cervical cancer. In our study, we have used a combination of PCR-RFLP and PCR based reverse hybridisation using line blot assay (LBA) for identification of HPV genotypes associated with cervical neoplasia in Indian women.

Using consensus primers MY09/11, HPV DNA was detected in 91.3% of women with cervical neoplasia (study group I) and in 9.1% of women who did not have cervical disease (study group II). Using the modified PGMY primers, HPV DNA was detected in 93.3% of women with cervical neoplasia and 9.1% of women with no cervical disease. Both the primer systems showed a good agreement in terms of detection of HPV types (kappa=0.859, p<0.001). This finding is in agreement with other studies that has shown the increased efficiency of the PGMY primers in amplification of HPV.

In addition, in our study we used 2 hot start Taq polymerases, Hotstar Taq polymerase (Qiagen, Germany) for amplification using MY primers and AmpliTaq Gold
DNA polymerase (Applied Biosystems, USA) for amplification using PGMY primers. The use of a hotstart enzyme has been previously shown to increase the detection rate of HPV. An interesting study was done to compare the efficiency of Taq DNA polymerase and AmpliTaq gold DNA polymerase to detect HPV DNA in a cohort of women attending cervical screening in Costa Rica. In that study, Castle reported that PCR employing AmpliTaq gold DNA polymerase identified 45% more infections with high risk types and also detected more high risk types than PCR using Taq DNA polymerase. The same study also shows that PCR using AmpliTaq gold DNA polymerase detected more multiple infections and infections with low viral load than PCR with Taq DNA polymerase.

There are several methods available for genotyping HPV. We have used two approaches based on consensus primers: restriction fragment length polymorphism (RFLP) and reverse line blot assay (LBA). Both these methods depend on the amplification of a 450bp region of the L1 gene. RFLP used in this study can identify 44 HPV types whereas the LBA can identify 27 HPV types.

A study from our centre has looked at the prevalence of HPV types in women with cervical neoplasia from India. This study found HPV DNA in 94.6% using PCR based line blot assay. Another study done in Chennai, India looked at the prevalence of HPV types in Indian women with invasive cervical cancer using GP5+/6+ consensus primer amplification followed by a second round of amplification using type specific PCR based on the E7 gene on negative samples. Using this approach, the study show that HPV DNA has been detected in 99.4% of women with squamous cell carcinoma and 100% in women with adenocarcinoma. The discordance between the studies was
probably due to the difference in primers used for amplification. Franceschi\textsuperscript{254} have used another set of consensus primers based on the L1 gene GP5+/6+, which amplify a smaller segment (~150bp) of the L1 gene, whereas both the primers used in our study (MY and PGMY) amplify a larger region (~450bp) of the L1 gene. Franceschi\textsuperscript{254} have also amplified all the negative samples with primers from E7 gene specific for 14 high risk HPV types. The false negative result seen in our study may be due to the deficiency in sample integrity resulting in the degradation of the viral DNA in samples from some women with cervical cancer. This has been alluded to earlier in another study done on a south Indian population, which detected HPV DNA in 87.8% of women with cervical cancer using PGMY primer based line blot assay.\textsuperscript{251} In our study, both the MY and PGMY primers showed 100% specificity when tested against other DNA viruses.

Generally, the most common types detected in India are 16, 18, 33, 45, and 31.\textsuperscript{254} The common HPV types detected in a previous study done in our centre were HPV 16 (60%), HPV 18 (14%), HPV 33 (6%), HPV 58 (6%), HPV 35 (5%) and HPV 45 (5%). Though the prevalence of HPV 16 (64.3%) and HPV 18 (13.6%) were similar in the present study, the next most common types detected were HPV 45 (5%), HPV 31 (2.9%) and HPV 59 (2.1%) (Table 10, Results). The reason for this shift in prevalence of the lesser high risk types is unclear but maybe related to the cross-reaction of the cross-section of women from different parts of the country. In this study, HPV 33 and 58 were detected only in mixed infection with HPV 52 and 58 respectively.

In our study, both PCR-RFLP and PCR-LBA were able to correctly identify HPV 16 and 18. But LBA was more efficient in picking up the less prevalent HPV types 59, 52, 58, 51, 84, 33, 56, 42, 61, 82, 39. The 3 samples that were negative by MY primers
but were positive by PGMY primers were typed as HPV 42, 52 and 61. The inefficiency of the MY primers in amplifying certain HPV types has been previously reported. Gravitt has compared the efficiency of MY primers and PGMY primers and has shown that MY primers are less efficient in picking up types 42, 26, 59, 45, 39, and 55. A similar study done by Coutlee comparing MY and PGMY primers for the typing of HPV concludes that MY primers are less efficient in amplifying HPV types 42, 66, 54, 84, 52, 53.

This study further reiterates the fact that HPV 16 and 18 contribute to approximately 80% of viral etiology of cervical cancer in Indian women. The results from genotyping studies such as ours suggest that implementation of the currently available vaccines, either Gardasil (Merck Inc) or Cervarix (GlaxoSmithkline Inc) will result in an efficient 80% reduction in the incidence of cervical cancer in India i.e., assuming 100% protection of the vaccine. A landmark study was done by Koutsky et al to study the efficacy of a HPV 16 virus like particle (VLP) based vaccine in vaccinated women. The study reports that the vaccine was 100% effective in preventing prevalent HPV16 infections in the vaccinated women.

7.2 HPV quantitation in tissue:

The role of HPV viral load in the pathogenesis of cervical cancer remains unclear. Some studies suggest that HPV 16 viral loads increase with disease severity and can be used as a biomarker in cervical cancer. Another study has reported that HPV 16 DNA copies per cell decreases with increase in clinical disease stage. A study has shown that high HPV viral load is a predictor for development of high grade cervical lesions whereas another study has shown that it is not a predictor.
There are several methods for quantitation of HPV in cervical tissue. Semi-quantitation has been done using Hybrid capture 2 (Digene HC2, US) which can detect 13 high risk types as well as 5 low risk types. However, HC2 has an inherent disadvantage in that it cannot distinguish individual types and the lower detection limit of this assay is 5000 copies.\textsuperscript{257} Real time PCR has been shown to be an excellent method of quantitation of HPV and has shown to have sensitivity (lower limit of detection) of 10 copies per test.\textsuperscript{238,258}

We have employed a sensitive real time PCR for the quantitation of HPV 16 and 18 in cervical tissue. The real time PCR we used for this study is based on the Taqman principle. The HPV 16 quantitation done in our study showed a dynamic range of 10 to $10^7$ copies/5000 cells and HPV 18 quantitation showed a dynamic range of $10^3$ to $10^7$ copies/5000 cells.

Both HPV 16 and 18 real time PCRs that we have used showed 100% specificity when checked with HPV types that belong to related species as well as other species. In a landmark study done on validation of real time PCR for the detection of HPV 16 and 18, Gravitt\textsuperscript{238} has shown that when the HPV 16 or 18 PCR was checked for cross-reactivity with plasmids containing high copy number of HPV types of the respective species, both the PCR systems showed 100% specificity. However, when clinical samples containing HPV from the respective species were tested, the HPV 16 primers showed 100% specificity whereas the HPV 18 PCR was positive 3/54 (5.5%) showing a specificity of 94.5%. Since we have employed the same PCR primers and probe as Gravitt\textsuperscript{238}, the reasons for the discrepancy in the results are unclear. Perhaps a larger number of samples
need to be tested. The HPV 18 primers have been known to show cross reactivity to HPV 45, another high risk type belonging to the same species.\textsuperscript{238}

We checked inter- and intra-assay variability of HPV 16, 18 and ERV3 quantitation assays. As per the results shown in tables 12, 14, 16, these assays demonstrated very good reproducibility as evidenced by the small co-efficient of variation (CV) observed for all three real time assays. The only deviation found was in in ERV3 quantitation where the CV calculated for intra-assay variation was 18.67%. This may be due to the low ERV3 load seen in the sample (4.68 copies). An interesting study done by Lefevre\textsuperscript{259} looked at inter- and intra-assay variability of HPV 16 real time PCR using known concentrations of HPV 16 plasmids. The intra-assay CVs obtained in that study are 7.5% (10\textsuperscript{2} copies of plasmid DNA input) and 3.1% (10\textsuperscript{4} copies of plasmid DNA input) and the inter-assay CVs obtained are 23.4% (10\textsuperscript{2} copies of plasmid DNA input) and 12.4% (10\textsuperscript{4} copies of plasmid DNA input).\textsuperscript{259} Our study shows a much smaller intra- and inter-assay CV for HPV 16 quantitation not exceeding 10%, suggesting that the methodology that we followed was much better.

In our study we found that HPV 16 and 18 viral loads did not increase with advancing disease stage (p=0.977, 0.263). Our results were similar to a study done on women with varying grades of CIN where Briolat\textsuperscript{260} found that HPV 16 quantitation as detected by both real time PCR and HC2 was not associated with severity of lesions.\textsuperscript{260} However, several other studies support the idea that HPV 16 viral loads increase with increasing cervical disease. A study done on women from Costa Rica with different grades of cervical lesions has shown that HPV 16 viral loads as measured by real time PCR increases linearly with increasing disease stage.\textsuperscript{26}
The study done by Briolat\textsuperscript{260} also reports that women with normal histology/CIN1 have a lower HPV 16 viral load than those with CIN2 or greater. In our study, none of the women with normal cytology had HPV16, so we could not explore the role of HPV 16 quantitation in women without cervical neoplasia. However, 2 of 33 women in study group II had HPV 18. We compared the viral loads in these 2 women with that of the women with cervical cancer and found that HPV 18 viral load was lower. This finding may not be of significant value as the number of cytologically normal women with HPV 18 was very few.

HPV 16 viral load in women with CIN was elevated than in women with invasive carcinoma in our study (Table 13, Results). This report is similar to that of Briolat\textsuperscript{260}, who reported that HPV 16 viral load was lower in women with invasive cervical cancer compared to those with CIN.\textsuperscript{260} This may be attributed to the highly productive life cycle of the virus in early stages of cervical neoplasia.

An important factor that recent studies have addressed is the variation in the amount of cervical cells used for the quantitation experiments. We have avoided this problem by normalisation of cell number by using an internal control, endogenous retrovirus 3 (ERV3). ERV3 was chosen as an internal control because it is known to have a single copy number and thereby 2 copies per diploid cell. The results for HPV quantitation was done relative to the ERV3 content in every sample.

In our study, we found that the cell number does not vary significantly with increasing disease severity. We checked this by looking at ERV3 loads in all the samples that were positive for HPV 16 and 18, and found that the ERV3 loads did not vary with increasing disease stage. A study done by Swan\textsuperscript{27} has reported an increase in sample
DNA content with increase in cervical disease severity.\textsuperscript{27} However, another study done by Gravitt\textsuperscript{26} in women with cervical neoplasia has shown that there was no difference in the cellularity as measured by median GADPH levels with increase in different grades of cervical neoplasia, suggesting that lack of adjustment for cellularity would not be a bias in HPV quantitation.

In our study, we did not find any significant difference between the crude viral load and normalised viral load for HPV 16 (Spearman’s rho=0.908; \( P < 0.001 \)) and HPV 18 (Spearman’s rho=0.770; \( P < 0.001 \)). Similar results showing no significant difference between crude and normalised HPV 16 viral load was observed by Gravitt\textsuperscript{26}.

To our knowledge, this is the first study that looks at quantitation of the 2 most common high risk types (HPV16 and 18) associated with cervical cancer in Indian women. Our study points to the fact that viral load is not a prognostic factor of the development of cervical cancer in Indian women. However, more longitudinal studies looking at the viral load of women with cervical intraepithelial neoplasia (CIN) have to be performed to study the role of HPV viral load in predicting patients who will progress to invasive cancer. More studies need to be also done on the viral load of other high risk HPV types to completely understand the role of HPV viral load in the pathogenesis of cervical cancer.

7.3 HPV detection in plasma:

Studies on detection of cell free DNA as a tumor marker has recently gained interest. Circulating DNA is a better marker than RNA as it is more stable. The presence of DNA in blood is not a marker for the presence of viable tumor cells, but a marker of tumor burden. Studies have shown that the presence of EBV DNA in blood is a non-
invasive marker of recurrence of nasopharyngeal carcinoma.\textsuperscript{261} This has triggered interest to look at HPV DNA in blood as a non-invasive marker of cervical cancer metastasis and recurrence.

In our study, we employed both conventional PCR based on consensus primers MY09/11 and real time PCR based on type specific primers for HPV 16 and 18 to detect plasma viremia. The detection rate with MY09/11 primers was very low. We detected HPV DNA in only 2 (2.1\%) women who had HPV 16 DNA in their cervical tissue and in none of the women who had HPV 18 in their cervical tract. The detection rate of plasma viremia with conventional PCR varies with different studies. There is only one study from India, from our own centre that has looked at plasma viremia with a single stage PCR using consensus primers MY 09/11.\textsuperscript{32} In that study, HPV DNA was detected in 11.8\% of plasma of women who had HPV DNA in their cervical tract. There are other studies with different plasma detection rates using conventional PCR. Kay\textsuperscript{30} has detected HPV 16/18 DNA in 24.3\% of women who had the same HPV type in their cervical tract using a conventional nested PCR with primers based on the E6 region. A recent study done by Wei\textsuperscript{129} used another conventional nested PCR with consensus primers based on the L1 gene and detected plasma viremia in 65\% of women who had HPV DNA in their cervical tract. However, the number of women in Wei\textsuperscript{129} was low, suggesting the result obtained might not be a true representation. The advantage of using a consensus primer based approach would be that many HPV types can be identified in one reaction, thereby reducing the cost.

The poor performance of the MY primers in detecting HPV in plasma may be attributed to the relatively lower efficiency of the MY primers in picking up low
concentration of HPV DNA present in plasma compared to that of tissue. An interesting study done by Karlsen\textsuperscript{262} comparing different consensus primers and type specific primers for the identification of HPV has shown that the lower limit of detection of MY primers was 4200 Siha cells (6300 HPV16 copies) whereas type specific primers based on the E6 region can detect up to 84 Siha cells.\textsuperscript{262}

Using real time PCR based on the E6 region of the genome, HPV 16 and 18 plasma viremia were detected in 56.4\% and 20\% of women with cervical cancer who had the same HPV type in their cervical tissue. Our study is in agreement with Yang\textsuperscript{240}, who reported plasma viremia in 50\% of patients with cervical cancer using real time PCR.\textsuperscript{240} The detection rate increased to 64.3\% when the same type was detected in the cervical tract.\textsuperscript{240} The similar results may be because we have used the same primers used by the previously mentioned study for HPV 16 real time PCR. Other studies on HPV plasma viremia have yielded different results. Plasma viremia detection rate varied between studies that used real time PCR approach. This difference may be due to the differences in study population, sample type and detection methods used. An interesting study done in Taiwan women with invasive cancer has shown that HPV 16, 18 or 52 DNA was detectable in 48\% of women who had HPV DNA in their cervical tract.\textsuperscript{29} This study has used a real time PCR approach with primers from the L1 region. Another study has looked at the presence of HPV 16 and 18 in the plasma of women with cervical cancer and found HPV 16 or 18 in 6.9\%.\textsuperscript{31}

The efficiency in amplification in our study increased with employing the real time PCR approach using primers from the E6 region of HPV 16 and 18. The PCR methodology followed in this study is similar to that done by Yang\textsuperscript{240} which reported a
lower limit of detection of up to 10 copies of HPV 16 plasmids. The same study also shows that the HPV 16 type specific primers used are highly specific and accurate. The failure of MY primers to detect HPV DNA in blood has been reported earlier. This may be because long DNA fragments are easily digested by nuclease and so may yield false negativity when using MY primers. The other reason for increased detection rate in plasma using real time PCR is the use of the Taqman methodology. Real time PCR is known to be more sensitive than conventional PCR. The increased sensitivity may also be due to the increase in sample volume input used in our study.

In our study, we found a good correlation between HPV 16 plasma positivity and advancing clinical disease stage (p=0.001). This is because, as the disease progresses, there may be more shedding of the virus into the circulation. Since this was a cross-sectional study, we do not have good follow up data to find out if these women with plasma viremia did develop disease metastasis and if plasma viremia can be used as a non-invasive marker of metastasis. Yang reported that HPV 16 DNA incidence did not depend on the clinical disease stage and histological stage of tumor.

In our study, we did not find a correlation between the absolute plasma viral load and increasing disease stage (p=0.281). However, Dong reported that HPV 16/18 viral load in plasma increased with advancing cervical disease stage. Another study done by Yang, has shown that HPV 16 viral load is not associated with clinical disease stage and histological stage.

HPV 16 plasma viral load did not depend on the HPV 16 viral load in the cervix (Spearman’s rho=0.184, p=0.187). Our result contrasts with that of Yang who showed that HPV plasma viral load was dependent of cervical tissue viral load.
In our study, only 1 woman with CIN III and none of the normal population had HPV 16 plasma viremia. Yang\textsuperscript{240} reported the presence of HPV 16 and 18 in 13% and 1% of apparently healthy women who had no obvious disease. Another study also reports the presence of HPV DNA in plasma of 5.6% of healthy women with no obvious disease. Though HPV infection in the cervix is common in cytologically normal women, the presence of HPV plasma viremia is intriguing. However, our study supports the theory that HPV DNA is shed from tumor cells in the cervix of women with established invasive cervical cancer due to necrosis and is not shed in women with low grade lesions and controls with no cervical disease.

Sequencing analysis has shown that the same HPV type was detected in plasma and cervical tissue. This confirms the fact that the HPV detected in plasma is in fact from the cervical tract. Though some studies have previously reported the presence of the same HPV type in plasma and corresponding tissue, others have found discrepant results.\textsuperscript{31} What remains unclear though is whether naked DNA is circulating or if it has arisen from disseminated tumor cells. Recent evidences have suggested that circulating tumor specific nucleic acids in serum or plasma is a non-invasive marker of disease prognosis and metastasis.\textsuperscript{263, 264}

The exact mechanism by which HPV DNA enters the blood stream and its role in the pathogenesis of HPV related disease remains unclear. One mechanism proposed is that the HPV plasma viremia is due to presence of disseminated tumor cells in circulation. This has been supported by two studies from Taiwan, which has shown that HPV mRNA was seen in patients with early and late stage cervical cancer.\textsuperscript{37, 246} Since naked mRNA cannot survive in bloodstream, they suggested that the presence of mRNA
indicated the presence of circulating viable tumor cells. Another study has reported the presence of HPV DNA in PBMCs suggesting that PBMCs may serve as carriers for transmission of HPV.\textsuperscript{18} Whatever the mechanism, the presence of HPV DNA in peripheral blood has been strongly linked to disease metastasis.\textsuperscript{265}

In conclusion, detection of HPV DNA in plasma is a marker of advancing cervical disease. HPV DNA was detected in women with high grade lesions and invasive cervical cancer but not in normal women. However absolute HPV 16 viral load in plasma is not related to either cervical disease stage or on HPV 16 viral load in tissue. Future longitudinal studies need to be done to elucidate the role of HPV DNA detection in plasma as a marker to measure tumor burden and metastasis.

7.4 HPV mRNA transcripts detection in cervical tissue:

Persistent infection with high risk HPVs are known to be necessary for the development of invasive cervical cancer. Expression of oncoproteins E6 and E7 play an important part in the transformation of normal cervical epithelium to malignant cells. We performed both nested RT-PCR and real time PCR for the detection of HPV 16 and HPV 18 transcripts in cervical tissue. We also hypothesised that the amount of mRNA transcripts in cervical tissue will increase with advancing disease stage.

Using nested PCR, HPV 16 mRNA transcripts were detected in 89.3\% of women who had HPV 16 DNA in their cervical tissue. We have employed a nested RT-PCR approach similar to that published earlier.\textsuperscript{36,266} One of these studies done by Sotlar\textsuperscript{266} has showed that there was an increase in detection of the E6*I and E6*II transcripts by using the nested PCR approach. In their study, HPV 16 mRNA transcripts were detected in 73\% of HPV 16 positive women who had cervical dysplasia. In another study done
earlier in our centre, Sathish\textsuperscript{36} detected HPV 16 mRNA transcripts in 94.3\% of women with cervical neoplasia who had HPV 16 DNA in their cervical tissue. In our study, though we used exactly the same protocol as used by the earlier study, the detection rate of HPV 16 mRNA transcripts was comparatively lower.

The E7 (E6*I/E6*II) were the most common transcripts detected in our study. A study done by Cornelisson\textsuperscript{267} has suggested that the detection of E6*I and E6*II transcripts are a proof of HPV 16 transcription.\textsuperscript{267} In our study, there was no association between the detection of HPV 16 mRNA transcripts in cervical tissue and advancing cervical disease stage. A similar result was shown by Rose\textsuperscript{268} who showed that the detection of HPV 16 mRNA transcripts did not depend on the histologic stage of the cancer. However, Sotlar\textsuperscript{266} reported that the frequency of detection of HPV 16 E6/E7 mRNA transcripts increased with progressing cervical disease, transcripts being uniformly distributed among various stages of cervical neoplasia.

Some women in our study lacked the presence of one of the transcripts. This can be attributed to mutations in the URR region that controls the expression of these oncoproteins or mutations in the E6/E7 splice sites as speculated earlier.\textsuperscript{268} It may also be due to the varying levels of expression of the individual transcripts, leading to reduced expression of some transcripts that may be below the lower limit of detection of the PCR.

We used a RNA stabilisation solution for the preservation of RNA in the cervical biopsy. This maintains the integrity of RNA. We have assessed the integrity of RNA by amplification of beta actin mRNA in all the samples. All the samples that were HPV 16 DNA positive showed a beta actin specific product. Only one sample that was HPV 18 DNA positive did not have beta actin and this sample was not included for analysis.
Using real time PCR, HPV 16 mRNA was detected in 86% of women who had HPV 16 DNA in their cervical tissue. Similar results were reported by an interesting study done by Scheurer\textsuperscript{269}, who showed HPV 16/18 mRNA transcripts in 87% of women with cervical neoplasia using an absolute real time quantitation method as done in our study. In our study, three samples that were positive by nested RT-PCR were negative by the real time PCR. Since the real time PCR is highly specific and sensitive, these three samples may be false positives obtained by nested RT-PCR. This finding has also been reported by another study done by Wang-Johanning\textsuperscript{34}, who reported that detection of HPV mRNA transcripts using real time PCR was found to correlate more with progression of cervical carcinoma than nested PCR.\textsuperscript{34}

For quantitation of HPV 16/18 mRNA oncogene transcripts, we used an absolute quantitation real time PCR method. The protocol employed type specific amplification by primers using Taqman chemistry. The methodology used by us had several advantages. First is that we used Oligotex direct mRNA kit (Qiagen, Germany) for mRNA extraction. This commercial kit selectively amplifies poly A+ mRNA. The extraction protocol has stringent conditions to minimise contamination of DNA and other species of RNA. However, in order to remove trace amounts of DNA that might be present, the extracted mRNA was treated with DNAase enzyme. The second advantage was the use of absolute quantitation. A fixed amount of cDNA (20ng) was given as input for all the samples. This reduces the need for relative quantitation using a reference gene. Normalisation to a reference gene has an inherent drawback in that there is no perfect candidate gene, thus requiring multiple genes to be employed.
HPV16 mRNA transcripts were detected equally in women with CIN and all stages of cervical cancer using real time PCR in our study. This was similar to the results obtained using the nested PCR approach. However, an important observation in our study is that the median HPV 16 transcript levels showed a trend towards increase with advancing disease stage (p=0.06). But, we did not find any significant correlation between HPV 16 DNA load and mRNA levels in cervical tissue. We have earlier shown that HPV DNA viral load does not increase with advancing cervical disease stage. This discrepancy between the DNA levels and mRNA levels may be because of individual control of transcription and translation of HPV by various cellular factors. An interesting study done by de Boer has performed both HPV 16 DNA quantitation and HPV 16 mRNA quantitation in women with cervical cancer. The study reports that there was no correlation between HPV DNA copy number and levels of mRNA expression in cervical tissue. The same study also showed that a high HPV mRNA level and not the DNA level was a marker of poor prognosis in cervical cancer. Another study done by Wang-Johanning looked at both HPV DNA and mRNA levels in the same thinprep cervical samples and has shown that though HPV DNA and mRNA levels increased with advancing disease, mRNA detection was a more accurate indicator of HPV 16 E6/E7 expression.

Our results show that active replication, as seen by increasing mRNA transcript level can be a marker of advancing cervical disease.

7.5 HPV mRNA transcripts in PBMCs:

Metastasis to different organs is a major problem associated with advancing cervical cancer. Detection of mRNA in blood cells suggests the presence of circulating
viable tumor cells. Several studies have looked at transcripts in blood as markers of disseminated tumor cells. Some of the markers studied are melanoma associated antigen family A (MAGE-A),\textsuperscript{270} carcinoembryonic antigen (CEA),\textsuperscript{271} beta human chorionic gonadotrophin (βhCG),\textsuperscript{272} and cytokeratin 19, 20.\textsuperscript{273,274}

HPV DNA has been detected in blood by various studies pointing to the fact that HPV infected cells are released into the circulation. However what remains unclear is whether HPV DNA is capable of infecting other cells/organs or if it is merely detectable in circulation as a result of cervical tumor necrosis and spill-over. The presence of HPV DNA in circulation may not necessarily mean that replicating virus is present. It is well known that the expression of E6/E7 oncoproteins is needed for malignant transformation of cells. We hypothesised that the detection of E6/E7 mRNA in peripheral blood would indicate the presence of replicative virus in circulating cells, as mRNA does not survive alone in circulation. So we checked for the presence of HPV 16 and 18 mRNA transcripts in PBMCs of women with cervical cancer using both a nested PCR and real time PCR based approach.

None of the PBMC samples tested in our study was positive for HPV 16 or 18 mRNA by either nested PCR or real time PCR. Only 2 previous reports have addressed the issue of mRNA transcripts in PBMCs. Both the reports are from the same group in Taiwan. In the first study, Pao\textsuperscript{246} detected HPV 16 mRNA in PBMCs of 92.3% women who HPV 16 in their cervical tissue and had clinical disease stage IV and therefore associated the presence of transcripts with disease metastasis\textsuperscript{246}. The second study done by Tseng\textsuperscript{37} looked for HPV 16 and 18 mRNA transcripts in various stages of cervical cancer\textsuperscript{37}. In this latter study, mRNA was detected in 51.4% of cases who had HPV DNA
in their cervical tissue and had invasive cervical cancer. Both these studies employ a
nested RT PCR to detect transcripts. We employed the same nested RT-PCR approach
used by these 2 studies. We also used a sensitive real time PCR to detect HPV 16 and 18
mRNA transcripts in PBMCs. But we were not able to detect transcripts in PBMCs.

Detection of mRNA is hampered by the fact that mRNA is likely to be degraded
by ribonucleases in blood. We have avoided this problem by storing PBMCs in a RNA
stabilisation solution (RNA later, Ambion, USA) prior to extraction. Our protocol was
validated by the amplification of beta actin in the same specimens. All the samples
showed the presence of beta actin product, ensuring sample integrity and adequacy.

mRNA was extracted in our study using a highly specific Oligotex direct mRNA
isolation kit (Qiagen, Gmbh, Germany). This kit employs the use of oligotex particles
that bind specifically to poly A+ mRNA, thereby selectively isolating them and further
binding with the silica membrane ensures the elimination of contaminating DNA. Both
the Taiwan studies that showed the presence of HPV mRNA in PBMC, employed
extraction of RNA using Trizol reagent, which has been shown to co-extract DNA. Both
those studies did not have a DNase treatment step to remove contaminating DNA.

The high percentage detection of transcripts in those studies maybe probably be due to
the presence of contaminating viral DNA. With the stringent criteria we have used, our
negative results are well validated.

We are thus able to clearly say that though viral DNA is seen in circulation as
seen earlier, viable circulating tumor cells containing viral transcripts are not seen in
women with cervical cancer. So, our study shows that detection of HPV 16 and 18
mRNA transcripts in PBMCs is not a good prognostic marker of cervical cancer progression.

7.6 Cell mediated immune responses to HPV 16 peptides:

HPV is one of the most common sexually transmitted infections in women. But the fact that less than 0.1 percent of women with HPV infection develop cancer suggests that the cell mediated immune response plays an important role in clearance of the virus. A lack of a good cell mediated immune response has been suggested as the reason for progression of cervical disease. We studied cell mediated immune response in women with HPV 16 infection, as it is the most common type seen in women with cervical cancer.

There are several methods of studying antigen specific T cells. Cytokine detection has recently become a common method of studying T cell responses. Interferon gamma (IFNγ) is a cytokine secreted by activated T cells and can be measured by different methods like ELISA, intra-cellular cytokine detection, real time PCR measurement of mRNA and by ELISPOT.276 We have used the ELISPOT assay, which has been shown to be a sensitive method to detect activated T cells. We also enhanced reactivity by adding recombinant interleukin2 (rIL2) to all the wells at a fixed concentration. It has been shown previously that addition of rIL2 enhances ELISPOT reactivity.277

T cell proliferative responses to 2 HPV 16 E7 peptides (aa 37-54 and aa 70-98) were studied by an ELISPOT assay. More than half of women (53.4%) who had cervical cancer with HPV type 16 failed to induce a response to either of the peptides studied. This may be either due to the lack of immune response to these particular peptides or maybe due to complete lack of immunity in these women. A study was done by deJong41
to look at T cell responses to HPV 16 early proteins E2, E6 and E7 in women with cervical cancer in Netherlands. The study reports that proliferative T cell response to HPV 16 peptides (E2, E6 and E7) was low in patients with cervical cancer compared to sexually active young women who did not have cervical disease, suggesting an impairment in cell mediated immune response in HPV infection.41

Another important study by deGruijl278 has looked at mRNA of various cytokines using RT-PCR in women with cervical cancer and has reported that expression of IFN gamma transcripts were low in women with invasive cervical cancer compared to those with pre-malignant lesions.278 However, a study done earlier by deGruijl279 has looked at T cell response to HPV 16 E7 oncoprotein in women who had persistent HPV infection, fluctuating HPV infection and who have cleared HPV infection and found more T cell reactivity in women who had persistent infection. 279

To study T cell immune response to the 2 peptides in healthy population, we have checked for T cell response in women who did not have any cervical disease (study group II) and healthy women from the community (study group III). But the response to peptides was poor in these women and similar to that seen in women with cervical cancer (Table 24, Results). An interesting study from Netherlands done by deJong280 has looked at the role of T cell responses to HPV 16 E2 peptides in healthy blood donors who did not have a recent history of cervical disease. The study found a good CD4+ T cell response to HPV 16 E2 peptide in more than half of the donors suggesting that a good immune response is necessary for the prevention of cervical cancer. 280

The HPV 16 E7 peptides used for our study were found to contain immunodominant T cell epitopes relevant to our population. However, despite this
feature, from our study we can come to a conclusion that T cell responses to HPV 16 E7 peptides are not a marker of progression or protection from disease. Perhaps in Indian women with cervical cancer, T cell responses to other HPV peptides might yield better insight into these responses and their prognostic potential.

7.7 Humoral immune response to HPV:

7.7.1 Humoral immune response to HPV 16:

In this study, we studied antibody response to various HPV 16 peptides (E2, E6 and E7) and HPV 16 virus like particles (VLPs) in women with HPV 16 DNA positive cervical cancer (Study group I), in women who did not have cervical disease (Study group II) and married women from the community (Study group III). Early protein E2 is one of the first proteins to be expressed during viral replication and helps to keep the oncoproteins E6 and E7 in check. The E2 region is believed to be disrupted during integration process, thereby increasing the expression of E6 and E7 proteins, which play an important role in cellular immortalization. We therefore chose to study antibody responses against peptides derived from these 3 regions. We had used two sets of controls for our study. The first set comprised of women who did not have cervical neoplasia and were HPV 16 DNA negative in cervical tissue (study group II) and the second set comprised of married healthy women from the community with unknown cervical HPV DNA status (study group III).
7.7.1.1 HPV 16 E2 peptide (328-345) ELISA:

We detected a poor antibody response to HPV 16 E2 peptide in study group I. Only 18% of women who were HPV 16 DNA positive (study group I) showed a positive response to E2 peptide. The prevalence of anti-E2 antibody in our study is low when compared to a study done in Mexican women with cervical cancer using the same E2 peptide, where anti-E2 antibodies were found in 66% of women.\(^4\)\(^3\) A study that looked at prevalence of anti-HPV16 E2 antibodies in an Indian population showed 43% reactivity to E2 peptide in women with invasive cervical carcinoma compared to 50% reactivity in women with chronic cervicitis (disease controls).\(^2\)\(^8\)\(^1\) Another study by the same researchers found anti-E2 antibodies in 68% of women with CIN and 43% of women with invasive cervical neoplasia. This reactivity observed in other studies was high compared to what we have reported.

However, the mean OD in the women in study group I in our study was low (0.292), whereas Rosales\(^4\)\(^3\) reported a high reactivity to E2 peptide (0.552).\(^4\)\(^3\) This variability may be due to the inherent difference in the assay system used. Only one woman who had infection with HPV type 59 had antibodies against the E2 peptide. It is highly possible that this woman had a transient infection with HPV 16, which was successfully cleared by the immune system. However, Rosales\(^4\)\(^3\) have reported that HPV antibody positivity is specific to the HPV type seen in the genital tract.\(^4\)\(^3\)

Reactivity to E2 peptide was not significantly different between the 3 study groups in our study (p=0.076). This is possible as women in all 3 study groups have the same risk factors and could have been exposed to HPV at some point in their life time. This also raises the question whether antibodies to E2 persist longer in circulation even
after the virus is cleared. Longitudinal studies might give us a clearer picture of the role of these antibodies in the development of cervical cancer. We also did not find an association between the antibody positivity to the E2 and advancing clinical disease stage (p=0.771).

### 7.7.1.2 HPV 16 E6 peptide (aa 1-23) ELISA

In our study, antibody reactivity to HPV 16 E6 peptide was low (19.1%) in women with cervical neoplasia (Study group I). A study on anti-E6 antibody response in Indian women has shown positivity rate of 42% in women with CIN and 37% in women with invasive cervical cancer. In our study, anti-E6 antibody positivity did not correlate with advancing disease stage, and did not serve as a marker of disease prognosis. In contrast to our study, a study done on Russian women with cervical cancer has shown that anti-HPV 16 E6 antibodies increase with disease stage. The above mentioned Russian study used purified and renatured E6 protein as antigen. In our study, the median OD in women with HPV 16 DNA positive cervical cancer was low (0.028) (Figure 17).

None of the women in study group II (HPV 16 DNA negative women with no cervical disease) were reactive to E6 peptide, but 5/30 (16.7%) married women in study group III were reactive to E6 peptide. Sharma reported that none of the women with chronic cervicitis (disease controls) in their study were reactive to HPV 16 E6 peptide. Since data on the prevalence of HPV DNA in study group III (married women) were not available, we are not able to explain why they showed reactivity whereas the women in study group II did not.

Cross reactivity was high (25%) in women with HPV types other than 16. This may not be a true representation as only 8 women were there in this group. It is possible that
either the women had an earlier infection with HPV 16 or the peptide used for the study had cross reactive epitopes.

**7.7.1.3 HPV 16 E7 peptide (aa 11-19) ELISA:**

In our study, 34% of women with cervical neoplasia (study group I) had antibodies to HPV 16 E7 peptide. An interesting study done in Mexican women with cervical cancer has looked at antibody response to a similar peptide as used in our study. The study has reported that 51% of women with cervical cancer reacted to E7 peptide.\(^4^3\) The median OD value in study group I was 0.134 (Figure 19). In another study done in India, Sharma\(^4^9\) has shown that 16% of women with invasive cervical cancer and 11% of women with CIN have anti-E7 antibodies.\(^4^9\)

In our study, antibody positivity to E7 peptide did not increase with advancing disease stage, and thus did not serve as a marker of disease prognosis. However, other studies have shown that antibody levels of E7 protein are associated with clinical stage and thus have been proposed as a marker of disease prognosis.\(^4^3\)

The antibody positivity to E7 peptide in study groups II and III was similar to that seen with E6 peptide with more married women (Study group III) reacting to the peptide (30%) than women without cervical neoplasia (study group II) (3%). In agreement with our results, another study from India, Sharma\(^4^9\) did not find any trace of antibodies in women with chronic cervicitis (disease controls).

The HPV 16 E7 ELISA was positive in 4 women who had HPV 18 and 4 women who had infection with other HPV types. However, no cross reactivity was seen within the same species. Studies on cross-reactivity of HPV 16 E6 and E7 peptide ELISA with
other types are less. Zumbach\textsuperscript{47} has shown that HPV 16 antibodies are highly type specific. However, in our study both E6 and E7 show reactivity to serum from women harbouring other HPV types (both same species and other species). This may be due to the presence of low level of infection with other HPV types or past infection with HPV types.

7.7.1.4 HPV 16 VLP ELISA

In our study, anti-HPV 16 VLP antibodies were seen in 50\% of women who had cervical neoplasia (Study group I). There are no studies in India looking at antibodies to HPV 16 VLPs. The only similar study has been done by Sharma\textsuperscript{49}, who has used a peptide ELISA based on L1 protein and found antibodies in 58\% of women with CIN, 11\% of women with invasive cervical carcinoma and 10\% of women with chronic cervicitis (disease controls).\textsuperscript{49} There was no trend in the antibody positivity to HPV 16 VLPs across disease stages, suggesting that anti-HPV16 VLP antibodies were not a marker of disease prognosis. The median OD value in study group I was 0.118 (Figure 21). The percentage positivity was higher in the VLP ELISA compared to all 3 peptide ELISAs. The women in study group I and II showed higher reactivity than study group III (Figure 14).

Serum from women with HPV types other than HPV 16 showed reactivity in the HPV 16 VLP ELISA (Table 28, Results). This cross-reactivity is a common phenomenon that has been previously reported in many studies. Wang\textsuperscript{282} has reported the presence of IgG in 40\% of women with HPV type other than 16 in their genital tract. In a South African study, women positive for one HPV type in their cervix showed antibody positivity in more than one HPV VLP ELISA.\textsuperscript{283} Though neutralising antibodies to the
major epitopes are type specific, it has been noted that after prolonged exposure to viral antigen, antibodies are produced against multiple epitopes, a phenomenon known as “immunodominance”. This may also be due to the presence of low level of infection with other HPV types or past infection with HPV types. Further, seroprevalence is related to high risk sexual behaviour. Our study has the disadvantage that details on sexual behaviour are not known for any of the study groups. So, further studies with these details might provide an insight into the reasons for this reactivity.

Very low agreement was seen when the 3 HPV 16 peptide ELISAs were compared individually and combined with HPV 16 VLP ELISA. The difference in the reactivity may be because of the difference in antigens used. The antigens used in the peptide ELISA are from the early proteins (E2, E6 and E7) whereas the antigen used for the VLP ELISA is virus like particles (VLPs) based on the late protein L1. This suggests that antibody response to HPV varies with different antigens. A study done on women with invasive cervical cancer from Columbia and Spain had compared reactivity between the capsid protein (L1) and the E6 and E7 oncoprotein and had found that there was no correlation. The study suggested that this may be due to the difference in expression patterns between the early antigens (E6 and E7) and late antigen (L1).

There paucity of studies that have looked at antibody responses to HPV 16 proteins from India. All these results suggest that antibody response to HPV 16 proteins can be used as markers of sexual exposure rather than of disease prognosis.

### 7.7.2 Humoral immune response to HPV 18 VLPs:

In our study, 60% of HPV 18 positive women with cervical neoplasia (study group I) had anti-HPV 18 VLP antibodies. The antibody response was high in our study.
compared to Zumbach\textsuperscript{47} who found anti-HPV 18 antibodies in 42.8\% of Russian women with cervical carcinoma.\textsuperscript{47} A study done in South African women found anti-HPV 18 VLP antibodies in 45\% of women with invasive cervical cancer.\textsuperscript{283} In our study, antibody reactivity did not increase with advancing disease stage (p=0.189) and thus did not serve as a marker of disease prognosis.

The prevalence of anti-HPV 18 VLP antibodies was 30\% in women who did not have cervical neoplasia (study group II) and 26.7\% in married women from the community (study group III). A study done in female blood donor controls in South Africa reported a slightly lower anti-HPV 18 IgG prevalence of 16\%.\textsuperscript{285} Another study in a similar South African population consisting of female blood donors has found anti-HPV 18 IgG prevalence to be 10.5\%.\textsuperscript{283} The difference in prevalence may be due to the diversity in the population studied. The high prevalence may also be due to the low number of control women screened in our study.

In our study, reactivity was seen with serum from women with other HPV types (Table 30, Results). Reactivity to different HPV types has been found in other studies too, but the exact mechanism is not known.\textsuperscript{283} Wang\textsuperscript{282} reported that women with HPV type other than HPV 18 in their genital tract showed 50\% positivity in HPV 18 VLP ELISA. It is difficult to ascertain whether the reactivity to other HPV types is due to cross-reactivity of antibodies or due to multiple infections. This can be sorted out only by means of cohort studies that look simultaneously at antibody levels to different HPV types and viral exposure at definite intervals.
8. Summary and conclusions:

This study attempted to evaluate the role of certain HPV markers with prognosis in women with cervical neoplasia. Three groups of women were studied. They were women with cervical neoplasia (Study group I), women without cervical neoplasia (Study group II) and healthy women from the community (Study group III). The major results obtained from this study are

- HPV DNA was detected in 91.3% of women with cervical neoplasia using MY09/11 primers and in 93.3% of women using PGMY09/11 primers.

- HPV genotypes could be ascertained in 91.2% of women with cervical neoplasia using PCR-RFLP. This technique was found to be a reliable and affordable tool for HPV genotyping.

- The HPV types detected by PCR-RFLP in decreasing order of frequency were HPV 16 (66.4%), HPV 18 (13.9%), HPV 45 (4.3%), HPV 31 (2.9%), HPV 73 (1.5%) and HPV 59, 68 (1% each).

- Line blot assay was able to type all samples (100%) that were amplified by PGMY09/11 primers.

- Line blot assay was able to resolve all RFLP untypeable strains. It also picked up multiple infections (2 HPV genotypes) in 6.4% of cases studied.

- The major high risk types detected in this study were HPV 16 (63.1%), 18 (13.4%), 45 (4.7%), 31 (3.3%), 52 (2.7%), 59 (2%), and 51, 58, 73, 84 (1.34% each).
• Both MY09/11 and PGMY09/11 primers showed 100% specificity in amplifying HPVs.

• Real time PCR was an efficient method of determining HPV 16 and 18 viral loads in cervical tissue. HPV viral loads (normalized against ERV3) were not found to correlate with clinical disease stage.

• Both sets of primers in the HPV 16 and HPV 18 real time PCR showed 100% specificity.

• HPV DNA was detectable in 2 (2.1%) plasma samples of women who had HPV 16 DNA in their cervical tract and none of the 20 women who had HPV 18 in their cervical tract using conventional MY09/11 primers.

• Real time PCR was able to detect HPV 16 DNA in plasma of 56.4% women who harboured HPV 16 in their cervix. HPV 16 plasma viremia detection rate increased with advancing disease stage suggesting poor disease prognosis.

• There was no correlation between plasma HPV 16 viral load and corresponding HPV 16 virus load in tissue.

• HPV 18 plasma viremia was detectable in 20% of HPV 18 positive women. However, HPV 18 plasma viral loads did not show any association with clinical disease stage.

• HPV 16 E6/E7 mRNA transcripts were detectable in 89.3% of HPV 16 positive samples using nested PCR. The E7 transcript (E6*I, E6*II) was the major transcript detected in cervical tissue samples from women with neoplasia in this study.
- There was no correlation between detection of HPV 16 E6/E7 mRNA transcripts using either conventional nested PCR or real time PCR and advancing cervical disease.

- HPV 16 mRNA transcripts were detected in 86% of HPV 16 positive women using real time PCR. The median transcript level showed a trend to increase with advancing disease stage (not statistically significant). This indicates that oncogene mRNA levels may play an important role in progression of cervical disease.

- There was no correlation between the HPV 16 mRNA transcript level (copies/20ng of cDNA) in cervical tissue and HPV 16 DNA level (copies/5000 cells) in the corresponding tissue sample.

- mRNA transcripts were not detected in PBMC by both conventional nested PCR and real time PCR, suggesting that mRNA detection in PBMC is not a good marker of disease prognosis.

- In terms of individual responses, reactivity to HPV 16 E7 peptides as studied by ELISPOT assay for interferon gamma (IFN \( \gamma \)) was low. This suggests that T cell responses are probably weak or absent in Indian patients or directed against other HPV proteins. The proportion of individuals showing T cell response as measured by IFN \( \gamma \) release to HPV 16 E7 peptides were not significantly different between the three study groups.

- Antibody responses to E2, E6 and E7 proteins were detected in 18%, 19.1% and 34% of HPV 16 DNA positive women respectively. There was however, no
correlation between antibody response to HPV 16 E2, E6 and E7 protein and advancing disease stage.

- Antibodies to HPV 16 VLPs were found in 50% of HPV 16 DNA positive women of study group I, 45% of HPV DNA negative women in study group II and 30% of married women in study group III suggesting that anti-VLP antibodies might serve as a marker of viral exposure (sexual activity) in the Indian context.

- Antibodies to HPV 18 VLPs were detected in 60%, 30% and 26.7% of HPV 18 DNA positive women in study group I, HPV DNA negative women of study group II and married women in study group III respectively. These findings suggest that anti-VLP antibody detection serves as a marker of viral exposure rather than a marker of disease prognosis.
Conclusion:

This study has looked at certain HPV specific viral and immunological markers and their possible association with cervical neoplastic disease progression in Indian patients. These facets of this study have been poorly reported from other parts of India. This study has found a lack of association between HPV 16 and 18 viral load in cervical tissue, HPV 18 plasma viremia, HPV16 and 18 mRNA transcript detection in tissue and PBMCs, T cell responses to HPV 16 E7 peptides and antibody response to HPV 16 E2, E6 and E7 peptides and progression of cervical neoplasia in Indian women. PCR-RFLP and LBA were found to be useful methods of HPV DNA detection and genotyping in cervical tissue. HPV 16 plasma viremia and HPV 16 E6/E7 mRNA transcript quantitation in cervical tissue were useful markers of cervical disease progression. T cell responses to HPV 16 peptides other than E7 need to be further elucidated to completely understand the role of immune responses in HPV infection. Antibody responses to HPV 16 and 18 VLPs were found to be markers of viral exposure but did not show association with disease progression.
9. Recommendations:

- Most of the women recruited in our study had invasive cervical cancer. Future studies including more women with different grades of CIN should be recruited in order to fully understand the role of these biomarkers in cervical carcinogenesis.

- Our study is a cross-sectional study looking at various factors influencing progression of cervical cancer in Indian women. A cohort study, following up women who do not have cervical disease, but have HPV DNA in their cervical tract will give a better insight into the role of these markers in the natural history of cervical neoplasia.

- Women with cervical cancer can be followed up after commencement of therapy at regular intervals to determine the efficiency of these markers in identifying women who have a poor prognosis and have disease metastasis.

- Viral loads of only 2 high risk types (HPV 16 and 18) were looked at in our study. Future studies including other lesser prevalent high risk types may give us a better idea of the role of virus loads in the development of cervical cancer in Indian women.

- T cell responses to 2 peptides from the E7 oncoprotein of HPV 16 were studied. Future studies should also study other peptides (E2, E6 and L1) to understand the role of cell mediated immune response in Indian women with cervical cancer.

To completely understand the role of HPV viral load, plasma viremia, mRNA transcripts, T cell response and antibody response to HPV in the pathogenesis of HPV in Indian women with cervical neoplasia, longitudinal studies performing these markers
need to be further evaluated taking in to account host susceptibility markers, environmental factors and improved assay systems.
10. Appendix:

10.1 Ethylene diamine tetraacetic acid (EDTA) solution (Blood collection for plasma separation):

- A stock solution (5%) was prepared by dissolving 0.5g of disodium EDTA (MW 372.2) in 10ml of sterile milliQ water.

- The solution was sterilised by passing through a Millipore membrane filter of size 0.22 micron. The stock solution was stored at 4°C.

- For collection of 3 ml blood, thirty microlitres of stock solution was used to get a final concentration of 1mg EDTA per ml of blood.

10.2 Ethidium bromide (Used in agarose gel electrophoresis):

- Ethidium bromide (stock) – 10 mg was dissolved in 10 ml of milliQ water.

- The stock solution was stored at room temperature in a dark brown bottle.

- The final concentration of ethidium bromide used was 0.5µg per ml of buffer.

10.3 Viral transport medium (VTM) (For transportation of cervical tissues):

- VTM is minimal essential medium (MEM) with 2% fetal bovine serum (FBS)

- **MEM**: Commercially obtained (GIBCO, USA) and dissolved in sterile milliQ water. It was supplemented with 50ml of HEPES buffer, 2.5ml of PAS
(penicillin, amphotericin and streptomycin) solution, 0.5 ml of ciprofloxacin and 10 ml of 8.8% sodium bicarbonate to a total volume of 500 ml.

- MEM was then supplemented with FBS, the medium aliquoted into sterile polypropylene screw cap tubes and stored at 4°C until use.

10.4 10X PBS (Used in ELISPOT assay):

- NaCl – 8.0g
- KCl – 0.2g
- Na₂HPO₄ – 1.15g
- KH₂PO₄ – 0.2g
- The above chemicals were dissolved in 100ml of sterile milliQ water.
- The pH was adjusted to 7.4 and the buffer was then sterilised by autoclaving.
- The buffer was stored at 4°C.

10.5 Tris-EDTA buffer pH 8.0 (For primer reconstitution):

- Trizma.HCl (MW-157.6) – 0.157g
- EDTA (MW-372.2) – 0.037g
- The above mentioned amount was added to 100ml of sterile milliQ water. The final concentration was 10mM Tris.Cl and 1mM EDTA.
• The pH was adjusted to 8.0. The buffer was then aliquoted and sterilised by autoclaving.

• The buffer was stored at 4°C until used.

10.6 Gel loading buffer 6X (Used in agarose gel electrophoresis):

• 0.25% (w/v) Bromophenol blue

• 40% sucrose in water.

• The buffer was stored at 4°C.

10.7 50X TAE buffer (Used in agarose gel electrophoresis):

• Tris base – 242g

• Glacial acetic acid – 57.1ml

• EDTA (0.5M) – 100ml

• EDTA (0.5M) was prepared by adding 18.61g of disodium EDTA to 80ml of milliQ water. The pH was then adjusted to 8.0 with NaOH. The total volume was made up to 100ml with milliQ water.

• The final 1X working solution had 40mM Tris-acetate/1mM EDTA.
11. Bibliography:


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103. Beger M, Butz K, Denk C, Williams T, Hurst HC, Hoppe-Seyler F. Expression pattern of AP-2 transcription factors in cervical cancer cells and analysis of their


164. Nees M, Geoghegan JM, Hyman T, Frank S, Miller L, Woodworth CD. Papillomavirus type 16 oncogenes downregulate expression of interferon-responsive


Figure 2: Phylogenetic tree showing 118 papillomavirus genotypes

This phylogenetic tree is based on sequence analysis of the L1 gene. Genera are marked with peripheral parentheses and arrows. Species are marked with smaller parentheses. Individual types are marked as numbers or abbreviations inside the parentheses. The two important species (7 and 9) that contain most of the high risk HPVs are shown in red parenthesis. Adapted from de Villiers.72
Figure 3: Human Papillomavirus (HPV) genome:

The figure shows the map of human papillomavirus (HPV) genome. The early (E), the late (L) genes and the upstream regulatory region (URR) are marked on the outside and the nucleotide positions are marked on the inside. Adapted from Munoz⁴.
Figure 1: Structure of HPV virus like particles (VLPs):

The figure shows a 3-dimensional structure of VLPs. Pentamers (consisting of major capsid protein L1) are marked by stars on both the structures.62
Table 1: Primer sequences used for PCR-RFLP in the study:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Primer</th>
<th>Region</th>
<th>Sequence</th>
<th>Base pair size of amplified product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MY09</td>
<td>L1</td>
<td>CGTCCMARRGGAWACTGATC</td>
<td>450 bp</td>
<td>Manos²³⁶</td>
</tr>
<tr>
<td>2</td>
<td>MY11</td>
<td>L1</td>
<td>GCMCAGGGWCATAAYAATGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Beta globin (GH20)</td>
<td></td>
<td>GAAGAGCCAAGGACAGGTAC</td>
<td>268 bp</td>
<td>Bauer²³⁷</td>
</tr>
<tr>
<td>4</td>
<td>Beta globin (PC04)</td>
<td></td>
<td>CAACTTCATCCACGTCACC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Primer sequences used for HPV 16 quantitation in cervical tissue:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name</th>
<th>Primer/Probe</th>
<th>Sequence</th>
<th>Base pair size of amplified product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HPV 16-U Primer</td>
<td>5'-ATG ACT TTG CTT TTC GGG AT-3</td>
<td>223</td>
<td>Gravitt&lt;sup&gt;238&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>HPV 16-L Primer</td>
<td>5'-CTT TGC TTT TCT TCA GGA CA-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>HPV 16-TM Probe</td>
<td>5'-ACG GTT TGT TGT ATT GCT GTT CTA A-3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Probe labelled with FAM at 5’ end (reporter dye) and BHQ-1 at 3’ end (quencher dye).

FAM – Carboxy fluorescein

BHQ1 – Black hole quencher 1
Table 3: Primer sequences used for ERV3 quantitation:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name</th>
<th>Primer/Probe</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PHP10F</td>
<td>Primer</td>
<td>CATGGGAAGCAAGGGAACTAATG</td>
<td>Yuan²³⁹</td>
</tr>
<tr>
<td>2</td>
<td>PHP10R</td>
<td>Primer</td>
<td>CCCAGCGAGCAATACAGAATTT</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PHP-P505</td>
<td>Probe*</td>
<td>TCTTCCCTCGAACCTGCACCATCAAGTCA</td>
<td></td>
</tr>
</tbody>
</table>

*Probe labelled with FAM at 5’ end (reporter dye) and TAMRA at 3’ end (quencher dye).

FAM – Carboxyfluorescein
TAMRA - Carboxytetramethyl rhodamine
Table 4: HPV types used for specificity testing of HPV 16 E6 real time primers:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Species</th>
<th>HPV types</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>31,33,52,58</td>
</tr>
<tr>
<td>2</td>
<td>Others</td>
<td>18,45,59,73,56,51</td>
</tr>
</tbody>
</table>
Table 5: Primer sequences used for HPV 18 quantitation in cervical tissue:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name</th>
<th>Primer/Probe</th>
<th>Sequence</th>
<th>Base pair size of amplified product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HPV 18-U Primer</td>
<td>5’-ATG TCA CGA GCA ATT AAG C-3’</td>
<td>137</td>
<td>Gravitt&lt;sup&gt;38&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>HPV 18-L Primer</td>
<td>5’-TTC TGG CTT CAC ACT TAC AAC A-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>HPV 18-TM Probe</td>
<td>5’-CGG GCT GGT AAA TGT TGA TG-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Probe labelled with FAM at 5' end (reporter dye) and BHQ-1 at 3' end (quencher dye).

FAM – Carboxyfluorescein
BHQ1 – Black hole quencher 1
Table 6: HPV types used for specificity testing of HPV 18 E6 real time primers:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Species</th>
<th>HPV types</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>39,68,45,59</td>
</tr>
<tr>
<td>2</td>
<td>Others</td>
<td>16,31,52,73,56,51</td>
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</table>
Table 7: Primer sequences used for HPV 16 quantitation in plasma:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name</th>
<th>Primer/Probe</th>
<th>Region</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HPV16F</td>
<td>Primer</td>
<td>HPV16 E6</td>
<td>5'-ATC ATC AAG AAC ACG TAG AG-3’</td>
<td>Yang²⁴⁰</td>
</tr>
<tr>
<td>2</td>
<td>HPV 16 R</td>
<td>Primer</td>
<td>HPV16 E6</td>
<td>5’-GAT CAG TTG TCT CTG GTT GCA AAT-3’</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>HPV16 probe</td>
<td>Probe</td>
<td>HPV16 E6</td>
<td>5’-TGC ATG GAG ATA CAC CTA CAT TGC-3’</td>
<td></td>
</tr>
</tbody>
</table>

*Probe was labelled with FAM at 5’ end (reporter dye) and TAMRA at 3’ end (quencher dye).

FAM – Carboxyfluorescein

TAMRA – Carboxytetramethyl rhodamine
Table 8: Primer sequences used for HPV 16 sequencing:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name</th>
<th>Primer/Probe</th>
<th>Region</th>
<th>Sequence</th>
<th>Base pair size of the amplified product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HPV16F</td>
<td>Forward</td>
<td>HPV 16 E6</td>
<td>5'-ATG ACT TTG CTT TTC GGG AT-3'</td>
<td>210</td>
</tr>
<tr>
<td>2</td>
<td>HPV 16 R</td>
<td>Reverse</td>
<td>HPV 16 E6</td>
<td>5'-CCT TGC TTT TCT TCA GGA CA-3'</td>
<td></td>
</tr>
</tbody>
</table>
Table 9: Primer sequences used for detection of HPV 16 E6/E7 mRNA transcripts in tissues using nested PCR:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name</th>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16E6/E7-I</td>
<td>Outer</td>
<td>5’ - ACA GTT ATG CAC AGA GCT GC - 3’</td>
<td>Sathish&lt;sup&gt;26&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>16E6/E7-II</td>
<td>Outer</td>
<td>5’- CTC CTC CTC TGA GCT GTC AT – 3’</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>16E6/E7-III</td>
<td>Inner</td>
<td>5’- GTG TGT ACT GCA AGC AAC AG – 3’</td>
<td>Falcinelli&lt;sup&gt;242&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>16E6/E7-IV</td>
<td>Inner</td>
<td>5’ - GCA ATG TAG GTG TAT CTC CA – 3’</td>
<td></td>
</tr>
</tbody>
</table>
Table 10: Primer sequences used for detection of beta actin in tissues using PCR:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name</th>
<th>Sequence</th>
<th>Base pair of amplified product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Beta actin</td>
<td>5' - CTCAGGAGGAGCAATGATCTTTG - 3'</td>
<td>213bp</td>
<td>Coombs²⁴³</td>
</tr>
<tr>
<td>2</td>
<td>Beta actin</td>
<td>5' - CTGGGCATGGACTCCTGTGG - 3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1: Primer sequences used for detection of HPV 18 E6/E7 mRNA transcripts in tissues using nested PCR:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name</th>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>18 E6/E7-I</td>
<td>Outer</td>
<td>5<code> - ACC GAA AAC GGT CGG GAC CGA AAA CGG T – 3</code></td>
<td>Nakagawa 244</td>
</tr>
<tr>
<td>6</td>
<td>18 E6/E7-II</td>
<td>Outer</td>
<td>5<code> - AGA CTC AGC GAA TTA ACG AG – 3</code></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>18 E6/E7-III</td>
<td>Inner</td>
<td>5’-CAC GGC GAC CCT ACA AGC TAC CT-3’</td>
<td>Czegledy 245</td>
</tr>
<tr>
<td>8</td>
<td>18 E6/E7-IV</td>
<td>Inner</td>
<td>5<code> - TGC CTT AGG TCC ATG CAT ACT – 3</code></td>
<td></td>
</tr>
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</table>
Table 12: Primer sequences used for detection of mRNA transcripts of HPV 16/18 in PBMCs using nested PCR:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name</th>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16e6-1</td>
<td>Outer</td>
<td>GAACTGCAATGTTCAGGACC</td>
<td>Pao²⁴⁶</td>
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<tr>
<td>2</td>
<td>16e6-2</td>
<td>Outer</td>
<td>CGTGTTTTGATGATCTGC</td>
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</tr>
<tr>
<td>3</td>
<td>16e6-3</td>
<td>Inner</td>
<td>CAATGTTTCAGGACCCACAGG</td>
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<tr>
<td>4</td>
<td>16e6-4</td>
<td>Inner</td>
<td>GCAACAAGACATACATCGACC</td>
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</table>
### Table 13: Sequences of peptides used for ELISPOT assay

<table>
<thead>
<tr>
<th>S.No</th>
<th>Region</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E7 (37-74)</td>
<td>EIDGPAGQAEPDRAHYNIVTFCCCKCDSTLRLCVQSTHV</td>
<td>Luxton&lt;sup&gt;39&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>E7 (70-98)</td>
<td>QSTHVDIRTLEDLMGTGLGIVCPIC SQKP</td>
<td>Kadish&lt;sup&gt;40&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

### Table 14: Common class I and class II MHC alleles found in South India

<table>
<thead>
<tr>
<th>S.No</th>
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<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>DRB1*0701</td>
<td>Shankarkumar&lt;sup&gt;247&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>DRB1*1502</td>
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<tr>
<td>3</td>
<td>DRB1*04</td>
<td></td>
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<tr>
<td>4</td>
<td>A*0201</td>
<td>Shankarkumar&lt;sup&gt;248&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>A*0301</td>
<td></td>
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<tr>
<td>6</td>
<td>A*24</td>
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<tr>
<td>7</td>
<td>A*3101</td>
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<td>8</td>
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Table 15: Sequences of peptides used for the peptide ELISA

<table>
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<tr>
<th>S.No</th>
<th>Region</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E2 (328-345)</td>
<td>HKSAITVTLTYDSEWQRDQC</td>
<td>Rosales⁴³</td>
</tr>
<tr>
<td>2</td>
<td>E6 (1-23)</td>
<td>MHQKRTAMFQDPQERPRKLPQLC</td>
<td>Sharma⁴⁹</td>
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<tr>
<td>3</td>
<td>E7 (11-19)</td>
<td>YMLDLQPET</td>
<td>Rosales⁴³</td>
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### Table 16: Panel of HPV types (from study subjects) tested for cross-reactivity in HPV 16 peptide ELISAs and VLP ELISA

<table>
<thead>
<tr>
<th>S.No</th>
<th>Species</th>
<th>HPV types</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9 (n=8)</td>
<td>31</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>33,52*</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>52,58*</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>HPV 18 (n=16)</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>Others (n=18)</td>
<td>45</td>
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<td>59</td>
<td>2</td>
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<td></td>
<td></td>
<td>73</td>
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<td></td>
<td></td>
<td>42</td>
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<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>51,82</td>
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</table>

* Co-infection with 2 HPV types
Table 17: Panel of HPV types (from study subjects) tested for cross-reactivity in HPV 18 VLP ELISA

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<th>HPV types</th>
<th>No of samples</th>
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<td></td>
<td></td>
<td>68</td>
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<td>2</td>
<td>HPV 16 (n=67)</td>
<td>16</td>
<td>67</td>
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<td>3</td>
<td>Others (n=16)</td>
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<td>4</td>
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<td></td>
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<td>64</td>
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<td>33,52</td>
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Table 18: Standardised concentrations of reagents used for ELISA:

<table>
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<tr>
<th>S.No</th>
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<th>Peptide ELISA (HPV 16 only)</th>
<th>VLP ELISA (HPV 16 &amp; 18)</th>
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<tr>
<td></td>
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<td>E2</td>
<td>E6</td>
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<tr>
<td>1</td>
<td>Sample input (µl)</td>
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<td>2</td>
<td>Antigen concentration (µg/well)</td>
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<tr>
<td>3</td>
<td>Conjugate dilution</td>
<td>1/3000</td>
<td>1/3000</td>
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</table>
(A) HPV E7 oncoprotein forms a complex with cyclin dependent kinase inhibitor p21 leading to increased expression of cyclin dependent kinase (CDK).

(B) HPV E7 oncoprotein binds to phosphorylated retinoblastoma protein (Rb) causing separation of Rb from elongation factor (E2F) complex. The Rb protein is then degraded through the ubiquitin proteosome pathway.

(C) HPV E7 oncoprotein associates with histone deacetylases (HDACs) resulting in an increased expression of transcription factors E2F/ dimerization protein (DP1) inducible genes.

Figure has been adapted from Gnanamony111
Figure 5: E6 dependent modulation of cell cycle

(A) HPV E6 oncoprotein associates with Myc/Mak and specificity protein-1 (Sp-1) proteins and these complexes induce expression of hTERT gene leading to an increase in the telomerase activity.

(B) HPV E6 oncoprotein binds to p53 protein using ubiquitin ligase E6AP and this complex is degraded by cellular proteases leading to a loss of cell control.

Figure has been adapted from Gnanamony111
Figure 1: Gel picture showing PCR results using MY primers and beta globin primers

Lane 1: Positive for HPV DNA (450bp) and beta globin (268bp)
Lane 2: Negative for HPV and positive for beta globin (268bp)
Lane 3: Negative control
Lane 4: Molecular weight ladder
Figure 2: Gel picture showing RFLP results of 2 most common HPV types 16 and 18

Lane 1: digestion pattern with Bam HI: 452bp
Lane 2: digestion pattern with Dde I: 452bp
Lane 3: digestion pattern with Hae III: 448bp  HPV 16
Lane 4: digestion pattern with Hinf I: 452bp
Lane 5: digestion pattern with Pst I: 216 bp
Lane 6: digestion pattern with Rsa I: 310 + 72 bp
Lane 7: digestion pattern with Dde I: 369 bp

Lane 8: Molecular weight ladder (ΦX174, Hae III digest)

Lane 9: digestion pattern with Bam HI: 452bp
Lane 10: digestion pattern with Dde I: 432bp
Lane 11: digestion pattern with Hae III: 452bp  HPV 18
Lane 12: digestion pattern with Hinf I: 452bp
Lane 13: digestion pattern with Pst I: 242+213 bp
Lane 14: digestion pattern with Rsa I: 135+125 bp
Lane 15: digestion pattern with Dde I: 372+63 bp
Figure 3: HPV genotyping results using line blot assay (LBA)

The picture shows individual line blot strips placed on the overlay. The presence of 2 control bands (high and low beta globin) is used for validation of the assay. The type corresponding to the band seen in the strip is HPV type of the sample. Strips 1-6 have infection with a single genotype and strips 7-9 show the presence of 2 genotypes each.
Figure 5: Distribution of the 4 major HPV types across disease stages:

The graph shows the distribution of the 4 major HPV types seen in this study. The number given in parenthesis refers to the total number of cases seen with a particular stage of cervical neoplasia.
This graph shows the analysed report of an assay of HPV 16 quantitation done in cervical tissues. The fluorescence is plotted on the Y axis and the number of cycles is plotted on the X axis. The horizontal line across the graph is the threshold.
Figure 7: Standard curve of an assay of HPV 16 quantitation in cervical tissue:

This graph shows the standard curve of the HPV16 quantitation assay shown in Figure 6. The \( C_t \) (threshold cycle) is shown in the Y axis and the input concentration is shown on the X axis. The standards are shown as blue dots and the samples as red dots. The blue dots are connected using logistic regression analysis by the software to give the standard curve.
This graph shows the analysed report of an assay of HPV 18 quantitation done in cervical tissues. The fluorescence is plotted on the Y axis and the number of cycles is plotted on the X axis. The horizontal line across the graph is the threshold.
Figure 9: Standard curve obtained in a HPV 18 quantitation assay in cervical tissue:

This graph shows the standard curve of the HPV18 quantitation assay shown in Figure 8. The \( C_t \) (threshold cycle) is shown in the Y axis and the input concentration is shown on the X axis. The standards are shown as blue dots and the samples as red dots. The blue dots are connected using logistic regression by the software to give the standard curve.
This graph shows the analysed report of a run of ERV3 quantitation done in cervical tissues. The fluorescence is plotted on the Y axis and the number of cycles is plotted on the X axis. The horizontal line across the graph is the threshold.
Figure 11: Standard curve obtained in an ERV3 quantitation assay in cervical tissue:

This graph shows the standard curve of the ERV3 quantitation assay shown in Figure 10. The
C\text{t} (threshold cycle) is shown in the Y axis and the input concentration is shown on the X
axis. The standards are shown as blue dots and the samples as red dots. The blue dots are
connected using logistic regression by the software to give the standard curve.
Figure 12: Gel picture showing HPV DNA in plasma:

Lane 1: Molecular weight ladder (ΦX174, Hae III digest)

Lane 4, 5: HPV DNA positive samples (450 bp)

Lane 2, 3, 6-11: HPV DNA negative samples

Lane 12, 13: HPV positive control (450 bp)
Figure 13: Graph showing analysed amplification pattern of HPV16 quantitation in plasma:

This graph shows the analysed report of a run of HPV 16 quantitation done in plasma. The fluorescence is plotted on the Y axis and the number of cycles of amplification is plotted on the X axis. The horizontal line across the graph is the threshold.
This graph shows the standard curve of the run of HPV16 quantitation shown in Figure 13. The Ct (threshold cycle) is shown in the Y axis and the input concentration is shown on the X axis. The standards are shown as blue dots and the samples as red dots. The blue dots are connected using logistic regression by the software to give the standard curve.
Figure 15: Graph showing analysed amplification pattern of HPV18 quantitation in plasma:

This graph shows the analysed report of a run of HPV 18 quantitation done in plasma. The fluorescence is plotted on the Y axis and the number of cycles is plotted on the X axis. The horizontal line across the graph is the threshold.
Figure 16: Standard curve obtained in a HPV18 quantitation assay in plasma:

This graph shows the standard curve of the run of HPV18 quantitation shown in Figure 15. The $C_t$ (threshold cycle) is shown in the Y axis and the input concentration is shown on the X axis. The standards are shown as blue dots and the samples as red dots. The blue dots are connected using logistic regression by the software to give the standard curve.
There was no statistically significant association between detection of transcripts and clinical disease stage (p=0.450).
Figure 20: Gel picture showing various HPV 16 transcripts detected by nested PCR:

Lane 1: Molecular weight ladder (ΦX174, Hae III digest)

Lane 2: Full length E6 transcript (395bp)

Lane 4: E6*I (213bp) and E6*II (95bp)

Lane 6: E6 (395bp) and E6*I (213bp)

Lane 8: E6*II (95bp)

Lane 3, 5, 7, 9: Negative controls
Figure 21: Gel picture showing beta actin RT-PCR results

Lane 1: Molecular weight ladder (ΦX174, Hae III digest)

Lane 2: beta actin specific product (213bp)

Lane 3: Negative control
There was no correlation between the HPV 16 mRNA transcript level and HPV 16 DNA viral load in tissue (correlation coefficient $R = -0.040$, $p=0.722$)
Figure 23: Graph showing analysed amplification pattern of HPV16 mRNA transcript quantitation in cervical tissue:

This graph shows the analysed report of a HPV 16 mRNA transcript quantitation assay done in cervical tissue. The fluorescence is plotted on the Y axis and the number of cycles is plotted on the X axis. The horizontal line across the graph is the threshold.
Figure 24: Standard curve obtained in a HPV16 mRNA quantitation assay in cervical tissue:

This graph shows the standard curve of the HPV16 mRNA transcript quantitation assay shown in Figure 23. The $C_t$ (threshold cycle) is shown in the Y axis and the input concentration is shown on the X axis. The standards are shown as blue dots and the samples as red dots. The blue dots are connected using logistic regression by the software to give the standard curve.
Figure 25: Gel picture showing HPV 18 E6 transcript using a nested PCR:

Lane 1: Molecular weight ladder (ΦX174)

Lane 2: HPV 18 E6 transcript (297bp)

Lane 3: Negative control
This graph shows the analysed report of a run of HPV 18 E6 mRNA transcript quantitation in cervical tissue. The fluorescence is plotted on the Y axis and the number of cycles is plotted on the X axis. The horizontal line across the graph is the threshold.
This graph shows the standard curve of the HPV18 mRNA transcript quantitation run shown in Figure 26. The $C_t$ (threshold cycle) is shown in the Y axis and the input concentration is shown on the X axis. The standards are shown as blue dots and the samples as red dots. The blue dots are connected using logistic regression by the software to give the standard curve.
Figure 28: Results of ELISPOT assay showing T cell responses to HPV 16 peptides

This picture shows ELISPOT results obtained for a single woman. Negative and positive controls and peptides 1 and 2 have been tested in duplicate. This sample is valid as the positive control (PHA) shows more than 100 spots and the negative control shows less than 10 spots. The sample shows reactivity to HPV 16 E7 peptide 1 and not to HPV 16 E7 peptide 2.
Figure 29: Percentage positivity of HPV 16 ELISAs in the 3 study groups

Study group I: women with cervical neoplasia and HPV 16 positive (n=94)
Study group II: women without cervical neoplasia and HPV 16 negative (n=33)
Study group III: Married women from the community (n=30)
Figure 30: Box and whiskers plot showing antibody reactivity in HPV 16 E2 peptide ELISA across the study groups:

The horizontal line across the graph shows the calculated cut-off (0.431). The horizontal line across each box is the median value. The box represents the middle 50% of values or the inter-quartile range (IQR) and the area covered by the whiskers indicate 1-99 percentile.
Figure 31: Box and whiskers plot showing antibody reactivity in HPV 16 E2 peptide ELISA in women with other HPV types:

The horizontal line across the graph shows the calculated cut-off (0.431). The horizontal line across each box is the median value. The box represents the middle 50% of values or the inter-quartile range (IQR) and the area covered by the whiskers indicate 1-99 percentile.

The HPV types included in the study groups are given in Materials and Methods section (Table 14).
Figure 32: Box and whiskers plot showing HPV 16 E6 peptide ELISA reactivity across the study groups:

The horizontal line across the graph shows the calculated cut-off (0.053). The horizontal line across each box is the median value. The box represents the middle 50% of values or the inter-quartile range (IQR) and the area covered by the whiskers indicate 1-99 percentile.
Figure 33: Box and whiskers plot showing HPV 16 E6 peptide ELISA reactivity in women with other HPV types:

The horizontal line across the graph shows the calculated cut-off (0.053). The horizontal line across each box is the median value. The box represents the middle 50% of values or the inter-quartile range (IQR) and the area covered by the whiskers indicate 1-99 percentile.

The HPV types included in the study groups (species 9 and other species) are given in Materials and Methods section (Table 14).
Figure 34: Box and whiskers plot showing HPV 16 E7 peptide ELISA reactivity across the study groups:

The horizontal line across the graph shows the calculated cut-off (0.243). The horizontal line across each box is the median value. The box represents the middle 50% of values or the inter-quartile range (IQR) and the area covered by the whiskers indicate 1-99 percentile.
Figure 35: Box and whiskers plot showing HPV 16 E7 peptide ELISA reactivity in women with other HPV types:

The horizontal line across the graph shows the calculated cut-off (0.243). The horizontal line across each box is the median value. The box represents the middle 50% of values or the inter-quartile range (IQR) and the area covered by the whiskers indicate 1-99 percentile.

The HPV types included in the study groups (species 9 and other species) are given in Materials and methods section.
The horizontal line across the graph shows the calculated cut-off (0.119). The horizontal line across each box is the median value. The box represents the middle 50% of values or the inter-quartile range (IQR) and the area covered by the whiskers indicate 1-99 percentile.
Figure 37: Box and whiskers plot showing HPV 16 VLP ELISA reactivity across the study groups:

The horizontal line across the graph shows the calculated cut-off (0.119). The horizontal line across each box is the median value. The box represents the middle 50% of values or the inter-quartile range (IQR) and the area covered by the whiskers indicate 1-99 percentile.

The HPV types included in the study groups (species 9 and other species) are given in Materials and methods section.
Figure 38: Percentage positivity of HPV 18 VLP ELISA across the 3 study groups:

Study group I: women with cervical neoplasia and HPV 18 positive (n=20)
Study group II: women without cervical neoplasia (n=33)
Study group III: Married women from the community (n=30)
Figure 39: Box and whiskers plot showing HPV 18 VLP ELISA reactivity across the study groups:

The horizontal line across the graph shows the calculated cut-off (0.384). The horizontal line across each box is the median value. The box represents the middle 50% of values or the inter-quartile range (IQR) and the area covered by the whiskers indicate 1-99 percentile.
Figure 40: Box and whiskers plot showing HPV 18 VLP ELISA reactivity across the study groups:

The horizontal line across the graph shows the calculated cut-off (0.384). The horizontal line across each box is the median value. The box represents the middle 50% of values or the inter-quartile range (IQR) and the area covered by the whiskers indicate 1-99 percentile.

The HPV types included in the study groups (species 7 and other species) are given in Materials and methods section (Table 15).
Infection with a high risk HPV may lead to low grade dysplasia. The actions of oncoproteins E6 and E7 lead to genetic changes in the host cells. The cells may progress from low grade squamous intraepithelial lesions (LSIL)/cervical intraepithelial neoplasia 1 (CIN1) to high grade squamous intraepithelial lesions (HSIL)/CIN2, 3 and rarely invasive carcinoma.  

\(^{120}\)
Figure 7: Risk factors that play a role in the natural history of cervical cancer:

- Younger age
- Increasing no. sex partners
- "Male factor"*
- Smoking?
- Pregnancy/multiparity?
- Increasing age
- HPV type (HPV-16)
- Multiple HPV types?
- Smoking?
- Chlamydia?
- Nutrients?
- Human leukocyte antigen?
- Male factor?

HPV NEGATIVE → HPV POSITIVE → HPV PERSISTENCE → ≥CIN-2/CIN-3 → INVASIVE CANCER

Increasing age
- HPV type (HPV-16), Multiple HPV types?
- Smoking
- Multiparity? OC use?
- Chlamydia? No use of condoms?
- HLA? Nutrients?

Smoking**
- Multiparity
- Long-term OC use

OC, oral contraceptives; HLA, human leukocyte antigen
*no circumcision, increasing number of sex partners, visits to prostitutes, no condom use
**data from case-control studies

Adapted from Moscicki122
Figure 8: Principle of detection of HPV DNA by Hybrid Capture2 (HC2):
Table 1: Clinical staging of women with cervical neoplasia as per the FIGO* guidelines (n=150)

<table>
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<th>S.No</th>
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<tr>
<td>1</td>
<td>CIN II</td>
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<tr>
<td>2</td>
<td>CIN III</td>
<td>8</td>
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<tr>
<td>3</td>
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<td>11</td>
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</tbody>
</table>

*FIGO – International Federation of Gynecology and Obstetrics
Table 2: Combined clinical staging of women with cervical neoplasia (n=150)

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<td>II</td>
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</tr>
<tr>
<td>Unclassified</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mixed epithelial/mesenchymal tumors</strong></td>
<td><strong>2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed epithelial/mesenchymal neoplasm</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcinosarcoma</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Carcinoma in-situ</strong></td>
<td><strong>2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Undifferentiated carcinoma</strong></td>
<td><strong>1</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4: Clinical staging of women with HPV DNA in cervical tissue (typed using PCR-RFLP, n=137)

<table>
<thead>
<tr>
<th>S.No</th>
<th>Clinical stage</th>
<th>No. of women</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CIN II</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>CIN III</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>IA2</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>IB1</td>
<td>18</td>
</tr>
<tr>
<td>5</td>
<td>IB2</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>IIA</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>IIB</td>
<td>45</td>
</tr>
<tr>
<td>8</td>
<td>IIIA</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>IIIB</td>
<td>45</td>
</tr>
<tr>
<td>10</td>
<td>IIIC</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>IVB</td>
<td>4</td>
</tr>
</tbody>
</table>
Table 5: PCR-RFLP results in HPV DNA positive women with cervical neoplasia 
(n=137)

<table>
<thead>
<tr>
<th>S.No</th>
<th>HPV type</th>
<th>Number of women</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>91</td>
<td>66.4</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>19</td>
<td>13.9</td>
</tr>
<tr>
<td>3</td>
<td>45</td>
<td>6</td>
<td>4.3</td>
</tr>
<tr>
<td>4</td>
<td>31</td>
<td>4</td>
<td>2.9</td>
</tr>
<tr>
<td>5</td>
<td>73</td>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>6</td>
<td>59</td>
<td>1</td>
<td>0.73</td>
</tr>
<tr>
<td>7</td>
<td>68</td>
<td>1</td>
<td>0.73</td>
</tr>
<tr>
<td>8</td>
<td>64*</td>
<td>1</td>
<td>0.73</td>
</tr>
<tr>
<td>9</td>
<td>Untypeable#</td>
<td>12</td>
<td>8.8</td>
</tr>
</tbody>
</table>

* HPV 64 – Low risk type identified by PCR-RFLP
# Untypeable – samples that were positive by PCR but gave an untypeable pattern in RFLP
Table 6: PCR-RFLP results in women with squamous cell carcinoma (n=119)

<table>
<thead>
<tr>
<th>S.No</th>
<th>HPV type</th>
<th>Number of women</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>77</td>
<td>64.7</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>13</td>
<td>10.9</td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>5</td>
<td>4.2</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
<td>4</td>
<td>3.4</td>
</tr>
<tr>
<td>5</td>
<td>73</td>
<td>2</td>
<td>1.7</td>
</tr>
<tr>
<td>6</td>
<td>59,64,68</td>
<td>1 each</td>
<td>0.84</td>
</tr>
<tr>
<td>7</td>
<td>Untypeable</td>
<td>8</td>
<td>6.7</td>
</tr>
<tr>
<td>8</td>
<td>Negative</td>
<td>7</td>
<td>5.9</td>
</tr>
</tbody>
</table>
Table 7: PCR-RFLP results in women with adenocarcinoma (n=13)

<table>
<thead>
<tr>
<th>S.No</th>
<th>HPV type</th>
<th>Number of women</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>6</td>
<td>46.2</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>4</td>
<td>30.7</td>
</tr>
<tr>
<td>3</td>
<td>59</td>
<td>2</td>
<td>15.4</td>
</tr>
<tr>
<td>4</td>
<td>Negative</td>
<td>1</td>
<td>7.7</td>
</tr>
</tbody>
</table>
Table 8: HPV genotypes detected in cervical tissue as single infection by LBA (n=131):

<table>
<thead>
<tr>
<th>S. No</th>
<th>HPV type</th>
<th>Number of women</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>90</td>
<td>64.3</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>19</td>
<td>13.6</td>
</tr>
<tr>
<td>3</td>
<td>45</td>
<td>7</td>
<td>5.0</td>
</tr>
<tr>
<td>4</td>
<td>31</td>
<td>4</td>
<td>2.9</td>
</tr>
<tr>
<td>5</td>
<td>59</td>
<td>3</td>
<td>2.1</td>
</tr>
<tr>
<td>6</td>
<td>73</td>
<td>2</td>
<td>1.4</td>
</tr>
<tr>
<td>7</td>
<td>51</td>
<td>1</td>
<td>0.71</td>
</tr>
<tr>
<td>8</td>
<td>52</td>
<td>1</td>
<td>0.71</td>
</tr>
<tr>
<td>9</td>
<td>56</td>
<td>1</td>
<td>0.71</td>
</tr>
<tr>
<td>10</td>
<td>42</td>
<td>1</td>
<td>0.71</td>
</tr>
<tr>
<td>11</td>
<td>61</td>
<td>1</td>
<td>0.71</td>
</tr>
<tr>
<td>12</td>
<td>64</td>
<td>1</td>
<td>0.71</td>
</tr>
</tbody>
</table>
Table 9: Women with multiple genotypes detected in cervical tissue by LBA (n=9)

<table>
<thead>
<tr>
<th>S. No</th>
<th>HPV types</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16, 84</td>
</tr>
<tr>
<td>2</td>
<td>52, 58</td>
</tr>
<tr>
<td>3</td>
<td>51, 82</td>
</tr>
<tr>
<td>4</td>
<td>16, 31</td>
</tr>
<tr>
<td>5</td>
<td>16, 84</td>
</tr>
<tr>
<td>6</td>
<td>16, 18</td>
</tr>
<tr>
<td>7</td>
<td>39, 68</td>
</tr>
<tr>
<td>8</td>
<td>52, 58</td>
</tr>
<tr>
<td>9</td>
<td>33, 52</td>
</tr>
</tbody>
</table>
Table 10: Spectrum of HPV types identified in cervical tissue as single or as multiple infections by LBA (n=149)

<table>
<thead>
<tr>
<th>S.No</th>
<th>HPV type</th>
<th>Number of women</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>94</td>
<td>63.1</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>20</td>
<td>13.4</td>
</tr>
<tr>
<td>3</td>
<td>45</td>
<td>7</td>
<td>4.7</td>
</tr>
<tr>
<td>4</td>
<td>31</td>
<td>5</td>
<td>3.3</td>
</tr>
<tr>
<td>5</td>
<td>52</td>
<td>4</td>
<td>2.68</td>
</tr>
<tr>
<td>6</td>
<td>59</td>
<td>3</td>
<td>2.0</td>
</tr>
<tr>
<td>7</td>
<td>51</td>
<td>2</td>
<td>1.34</td>
</tr>
<tr>
<td>8</td>
<td>58</td>
<td>2</td>
<td>1.34</td>
</tr>
<tr>
<td>9</td>
<td>73</td>
<td>2</td>
<td>1.34</td>
</tr>
<tr>
<td>10</td>
<td>84</td>
<td>2</td>
<td>1.34</td>
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<tr>
<td>11</td>
<td>33</td>
<td>1</td>
<td>0.67</td>
</tr>
<tr>
<td>12</td>
<td>56</td>
<td>1</td>
<td>0.67</td>
</tr>
<tr>
<td>13</td>
<td>68</td>
<td>1</td>
<td>0.67</td>
</tr>
<tr>
<td>14</td>
<td>42</td>
<td>1</td>
<td>0.67</td>
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<tr>
<td>15</td>
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<td>1</td>
<td>0.67</td>
</tr>
<tr>
<td>16</td>
<td>82</td>
<td>1</td>
<td>0.67</td>
</tr>
<tr>
<td>17</td>
<td>64</td>
<td>1</td>
<td>0.67</td>
</tr>
<tr>
<td>18</td>
<td>39</td>
<td>1</td>
<td>0.67</td>
</tr>
</tbody>
</table>
Table 11: HPV types identified in cervical tissue by LBA in PCR-RFLP untypeable samples (n=12)

<table>
<thead>
<tr>
<th>S. No</th>
<th>HPV type by LBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>52, 58</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>51, 82</td>
</tr>
<tr>
<td>6</td>
<td>59</td>
</tr>
<tr>
<td>7</td>
<td>16, 18</td>
</tr>
<tr>
<td>8</td>
<td>52, 58</td>
</tr>
<tr>
<td>9</td>
<td>33, 52</td>
</tr>
<tr>
<td>10</td>
<td>59</td>
</tr>
<tr>
<td>11</td>
<td>51</td>
</tr>
<tr>
<td>12</td>
<td>56</td>
</tr>
</tbody>
</table>
Table 12a: Intra-assay variation for the HPV 16 quantitation:

<table>
<thead>
<tr>
<th>Viral load (copies/ml)</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^3$</td>
<td>1.64</td>
</tr>
<tr>
<td>$10^5$</td>
<td>0.407</td>
</tr>
<tr>
<td>$10^6$</td>
<td>1.072</td>
</tr>
</tbody>
</table>

Table 12b: Inter-assay variation for the HPV 16 quantitation:

<table>
<thead>
<tr>
<th>Viral load (copies/ml)</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^3$</td>
<td>2.652</td>
</tr>
<tr>
<td>$10^5$</td>
<td>0.255</td>
</tr>
<tr>
<td>$10^6$</td>
<td>1.77</td>
</tr>
</tbody>
</table>
Table 13: Median HPV 16 viral loads in women with different stages of cervical neoplasia

<table>
<thead>
<tr>
<th>S.No</th>
<th>Clinical Stage</th>
<th>Number of women</th>
<th>Median viral load (copies/ 5000 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CIN</td>
<td>6</td>
<td>146382</td>
</tr>
<tr>
<td>2</td>
<td>I</td>
<td>20</td>
<td>48443</td>
</tr>
<tr>
<td>3</td>
<td>II</td>
<td>31</td>
<td>34000</td>
</tr>
<tr>
<td>4</td>
<td>III</td>
<td>35</td>
<td>67313</td>
</tr>
<tr>
<td>5</td>
<td>IV</td>
<td>2</td>
<td>2710</td>
</tr>
</tbody>
</table>

Median HPV 16 virus loads did not show significant correlation with advancing cervical disease (p=0.850)
### Table 14a: Intra-assay variation for the HPV 18 quantitation:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Viral load (copies/ml)</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(10^2)</td>
<td>0.4</td>
</tr>
<tr>
<td>2</td>
<td>(10^4)</td>
<td>3.0</td>
</tr>
<tr>
<td>3</td>
<td>(10^5)</td>
<td>0.4</td>
</tr>
</tbody>
</table>

### Table 14b: Inter-assay variation for the HPV 18 quantitation:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Viral load (copies/ml)</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(10^2)</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>(10^4)</td>
<td>3.0</td>
</tr>
<tr>
<td>3</td>
<td>(10^5)</td>
<td>0.9</td>
</tr>
</tbody>
</table>
Table 15: Median viral loads in HPV 18 positive women with different stages of cervical neoplasia (n=20)

<table>
<thead>
<tr>
<th>S.No</th>
<th>Clinical stage</th>
<th>Number of women</th>
<th>Median viral load (copies/ 5000 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>4</td>
<td>107912</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>10</td>
<td>65454</td>
</tr>
<tr>
<td>3</td>
<td>III</td>
<td>6</td>
<td>19292</td>
</tr>
</tbody>
</table>

Median HPV 18 virus loads did not show significant correlation with advancing cervical disease (p=0.110)
Table 16a: Intra-assay variation for the ERV3 quantitation:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Viral load (copies/ml)</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.68 x 10^4</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td>4.68 x 10^3</td>
<td>1.36</td>
</tr>
<tr>
<td>3</td>
<td>4.68 x 10^2</td>
<td>1.1</td>
</tr>
<tr>
<td>4</td>
<td>4.68 x 10^1</td>
<td>18.62</td>
</tr>
</tbody>
</table>

Table 16b: Inter-assay variation for the ERV 3 quantitation:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample No</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.68 X 10^4</td>
<td>0.21</td>
</tr>
<tr>
<td>2</td>
<td>4.68 X10^3</td>
<td>0.54</td>
</tr>
<tr>
<td>3</td>
<td>4.68 X 10^2</td>
<td>1.11</td>
</tr>
<tr>
<td>4</td>
<td>4.68 x 10^1</td>
<td>4.67</td>
</tr>
</tbody>
</table>
Table 17: Percentage plasma positivity and median viral loads in real time PCR of HPV 16 positive women with cervical neoplasia (n=94):

<table>
<thead>
<tr>
<th>S.No</th>
<th>Clinical stage</th>
<th>Positives</th>
<th>Percentage</th>
<th>Median viral load (copies /ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CIN</td>
<td>1/6</td>
<td>16.7</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>I</td>
<td>5/20</td>
<td>25</td>
<td>393</td>
</tr>
<tr>
<td>3</td>
<td>II</td>
<td>19/31</td>
<td>61.3</td>
<td>174</td>
</tr>
<tr>
<td>4</td>
<td>III</td>
<td>26/35</td>
<td>74.3</td>
<td>699</td>
</tr>
<tr>
<td>5</td>
<td>IV</td>
<td>2/2</td>
<td>100</td>
<td>275</td>
</tr>
</tbody>
</table>

The rate of detection of plasma viremia increased with advancing disease stage (p=0.001).
Table 18: Percentage plasma positivity and median viral load in real time PCR of HPV 18 positive women with cervical neoplasia (n=20):

<table>
<thead>
<tr>
<th>S. No</th>
<th>Clinical stage</th>
<th>Positives</th>
<th>Percentage</th>
<th>Median viral load (copies/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>1/4</td>
<td>25</td>
<td>650</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>1/10</td>
<td>10</td>
<td>200</td>
</tr>
<tr>
<td>3</td>
<td>III</td>
<td>2/6</td>
<td>33.3</td>
<td>2590</td>
</tr>
</tbody>
</table>
Table 19: HPV 16 mRNA transcripts detected in cervical tissue using nested PCR (n=84):

<table>
<thead>
<tr>
<th>S.No</th>
<th>Transcript type</th>
<th>Number of women</th>
<th>Percentage positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E7 (E6<em>I, E6</em>II)</td>
<td>68</td>
<td>80.9</td>
</tr>
<tr>
<td>2</td>
<td>E6</td>
<td>6</td>
<td>7.1</td>
</tr>
<tr>
<td>3</td>
<td>E6, E7 (E6<em>I, E6</em>II)</td>
<td>4</td>
<td>4.8</td>
</tr>
<tr>
<td>4</td>
<td>E6*II</td>
<td>4</td>
<td>4.8</td>
</tr>
<tr>
<td>5</td>
<td>E6*I</td>
<td>2</td>
<td>2.4</td>
</tr>
</tbody>
</table>
Table 20: HPV 16 mRNA transcript detection by real time PCR and median transcript levels in tissue of women with different stages of cervical neoplasia

<table>
<thead>
<tr>
<th>S.No</th>
<th>Clinical stage</th>
<th>Positives</th>
<th>Percentage positivity</th>
<th>Median transcript levels (copies/20ng of cDNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CIN</td>
<td>4/6</td>
<td>66.7</td>
<td>293.5</td>
</tr>
<tr>
<td>2</td>
<td>I</td>
<td>18/20</td>
<td>90</td>
<td>215</td>
</tr>
<tr>
<td>3</td>
<td>II</td>
<td>27/31</td>
<td>87</td>
<td>6151</td>
</tr>
<tr>
<td>4</td>
<td>III</td>
<td>30/35</td>
<td>85.7</td>
<td>20496</td>
</tr>
<tr>
<td>5</td>
<td>IV</td>
<td>2/2</td>
<td>100</td>
<td>2159422</td>
</tr>
</tbody>
</table>

Median transcript level appears to have an increasing though not statistically significant trend towards advancing clinical disease (p=0.07)
Table 21: HPV 18 mRNA transcript detection in tissue of women with different stages of cervical neoplasia using nested PCR

<table>
<thead>
<tr>
<th>S.No</th>
<th>Clinical stage</th>
<th>Positives</th>
<th>Percentage positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>3/4</td>
<td>75</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>8/10</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>III</td>
<td>4/5</td>
<td>80</td>
</tr>
</tbody>
</table>
Table 22: HPV 18 mRNA transcript detection and median transcript levels in tissue of women with different stages of cervical neoplasia using real time PCR

<table>
<thead>
<tr>
<th>S.No</th>
<th>Clinical stage</th>
<th>Positives</th>
<th>Percentage</th>
<th>Median transcript level (copies/20ng of cDNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>3/ 4</td>
<td>75</td>
<td>148148</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>9/10</td>
<td>90</td>
<td>5773</td>
</tr>
<tr>
<td>3</td>
<td>III</td>
<td>4/5</td>
<td>80</td>
<td>34805</td>
</tr>
</tbody>
</table>
Table 23: ELISPOT reactivity to E7 peptides in study group I (women with cervical neoplasia) with HPV 16:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Clinical stage</th>
<th>Response to 1 or more peptide(s)</th>
<th>No response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CIN (n=5)</td>
<td>2 (40%)</td>
<td>3 (60%)</td>
</tr>
<tr>
<td>2</td>
<td>I (n=6)</td>
<td>1 (17%)</td>
<td>5 (83%)</td>
</tr>
<tr>
<td>3</td>
<td>II (n=15)</td>
<td>8 (53%)</td>
<td>7 (47%)</td>
</tr>
<tr>
<td>4</td>
<td>III (n=17)</td>
<td>9 (53%)</td>
<td>8 (47%)</td>
</tr>
</tbody>
</table>

No significant difference between the study groups in terms of T-cell responses to the HPV 16 peptides studied (p=0.789)
Table 24: Reactivity to E7 peptides in the three study groups as detected by an ELISPOT assay:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Study group</th>
<th>Response to either peptide</th>
<th>Response to both peptides</th>
<th>Response to peptide 1(70-98)</th>
<th>Response to peptide 2(37-54)</th>
<th>No response to peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I (n=43)</td>
<td>20 (46.5%)</td>
<td>13 (30.2%)</td>
<td>6 (14%)</td>
<td>1 (2.3%)</td>
<td>23 (53.4%)</td>
</tr>
<tr>
<td>2</td>
<td>II (n=10)</td>
<td>4 (40%)</td>
<td>3 (30%)</td>
<td>-</td>
<td>1 (10%)</td>
<td>6 (60%)</td>
</tr>
<tr>
<td>3</td>
<td>III (married) (n=16)</td>
<td>6 (37.5%)</td>
<td>2 (13%)</td>
<td>-</td>
<td>4 (25%)</td>
<td>10 (63%)</td>
</tr>
<tr>
<td>4</td>
<td>III (single) (n=25)</td>
<td>7 (28%)</td>
<td>5 (25%)</td>
<td>1 (4%)</td>
<td>1 (4%)</td>
<td>18 (72%)</td>
</tr>
</tbody>
</table>

Study group I: women with cervical neoplasia and HPV 16 positive
Study group II: women without cervical neoplasia and HPV 16 negative
Study group III: Married and single women from the community
Table 25: Antibody reactivity to various HPV 16 peptides in women harbouring HPV 16 with different stages of cervical neoplasia:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Clinical stage</th>
<th>E2 peptide</th>
<th>E6 peptide</th>
<th>E7 peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CIN</td>
<td>1/6 (16.7%)</td>
<td>2/6 (33.3%)</td>
<td>3/6 (50%)</td>
</tr>
<tr>
<td>2</td>
<td>I</td>
<td>4/20 (20%)</td>
<td>5/20 (25%)</td>
<td>8/20 (40%)</td>
</tr>
<tr>
<td>3</td>
<td>II</td>
<td>6/31 (19.4%)</td>
<td>7/31 (22.6%)</td>
<td>10/31 (32.2%)</td>
</tr>
<tr>
<td>4</td>
<td>III</td>
<td>5/35 (14.3%)</td>
<td>3/35 (8.6%)</td>
<td>9/35 (25.7%)</td>
</tr>
<tr>
<td>5</td>
<td>IV</td>
<td>1/2 (50%)</td>
<td>1/2 (50%)</td>
<td>2/2(100%)</td>
</tr>
</tbody>
</table>

No statistically significant trend between antibody response to HPV 16 E2, E6 and E7 peptides and clinical disease stage (p=0.981, 0.160, 0.490)
Table 26: Antibody reactivity in women with HPV types other than HPV 16:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Groups</th>
<th>E2 peptide</th>
<th>E6 peptide</th>
<th>E7 peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HPV 18 positive</td>
<td>0/16 (0%)</td>
<td>2/16 (12.5%)</td>
<td>4/16 (25%)</td>
</tr>
<tr>
<td>2</td>
<td>Other types*</td>
<td>1/18 (5.5%)</td>
<td>1/18 (5.5%)</td>
<td>4/18 (22.2%)</td>
</tr>
<tr>
<td>3</td>
<td>HPV 16 negative, Species 9*</td>
<td>0/8 (0%)</td>
<td>2/8 (25%)</td>
<td>0/8 (0%)</td>
</tr>
</tbody>
</table>

* The HPV types included in this group are given in materials and methods.
Table 27: Antibody positivity to HPV 16 VLPs in study group I (women with cervical neoplasia:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Clinical stage</th>
<th>HPV 16 VLPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CIN</td>
<td>2/6 (33.3%)</td>
</tr>
<tr>
<td>2</td>
<td>I</td>
<td>8/20 (40%)</td>
</tr>
<tr>
<td>3</td>
<td>II</td>
<td>16/31 (50%)</td>
</tr>
<tr>
<td>4</td>
<td>III</td>
<td>19/35 (55.9%)</td>
</tr>
<tr>
<td>5</td>
<td>IV</td>
<td>2/2 (100%)</td>
</tr>
</tbody>
</table>

No statistically significant trend between antibody response to HPV 16 VLPs and clinical disease stage (p=0.440)
Table 28: HPV 16 antibody status in women with HPV types other than HPV 16:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Groups</th>
<th>16VLPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HPV 18 positive</td>
<td>6/16 (37.5%)</td>
</tr>
<tr>
<td>2</td>
<td>Other types*</td>
<td>5/18 (27.8%)</td>
</tr>
<tr>
<td>3</td>
<td>HPV 16 negative, Species 9*</td>
<td>1/8 (12.5%)</td>
</tr>
</tbody>
</table>
Table 29: Antibody positivity to HPV 18 VLPs in HPV 18 positive women with cervical neoplasia:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Clinical stage</th>
<th>HPV 18 VLPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>3/4 (75%)</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>6/10 (60%)</td>
</tr>
<tr>
<td>3</td>
<td>III</td>
<td>3/6 (50%)</td>
</tr>
</tbody>
</table>
Table 30: HPV 18 antibody status in women with HPV types other than HPV 18:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Groups</th>
<th>18VLP ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HPV 16 positive</td>
<td>10/67 (14.9%)</td>
</tr>
<tr>
<td>2</td>
<td>Other HPV types*</td>
<td>2/17 (11.7%)</td>
</tr>
<tr>
<td>3</td>
<td>HPV 18 negative, Species 7*</td>
<td>3/9 (33%)</td>
</tr>
</tbody>
</table>

* HPV types included in this group are given in materials and methods.