THE AETIOLOGY OF CERVICAL CANCER

F. Xavier Bosch* & Thomas Iftner†

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*IDIBELL, Institut Català d'Oncologia, Epidemiology and Cancer Registration Unit, Av. Gran Via, s/n Km. 2,7, E-08907 L'Hospitalet de Llobregat, Barcelona, Spain. Telephone: +34 93 260 7812. E-mail: x.bosch@ico.scs.es

†Universitaetsklinikum Tuebingen, Institut fuer Medizinische Virologie, Sektion Experimentelle Virologie, Elfriede Aulhorn Str. 6, D72076 Tuebingen, Germany. Telephone: +49 (0)7071 298 0246. E-mail: tsiftner@med.uni-tuebingen.de

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Fulwood House
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S10 3TH

Tel: 0114 271 1060
Fax: 0114 271 1089
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Website: www.cancerscreening.nhs.uk

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ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
ASCUS	atypical cells of unknown significance
ChIP	chromatin immunoprecipitation
CIN	cervical intraepithelial neoplasia
CIS	carcinoma in situ
CT	*Chlamydia trachomatis*
DdRp	DNA dependent RNA polymerase
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
EV	epidermodysplasia verruciformis
FDA	Food and Drug Administration
FIGO	Fédération Internationale de Gynécologie et d'Obstétrique
HBV	hepatitis B virus
HC	hybrid capture
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HPV	human papillomavirus
HR-HPV	high risk human papillomavirus
HSIL	high grade squamous intraepithelial lesion
HSV	herpes simplex virus
IARC	International Agency for Research on Cancer
ICO	Catalan Institute of Oncology
IL-2	interleukin 2
LCR	long control region
LSIL	low grade suamous intraepithelial lesions
MHC	major histocompatibility complex
NASBA	nucleic acid sequence based amplification
NHSCSP	NHS Cervical Screening Programme
OC	oral contraceptive
OR	odds ratio
ORF	open reading frame
PCR	polymerase chain reaction
RLU	relative light unit
RNA	ribonucleic acid
RR	relative risk
RT	reverse transcriptase
RT-(Q)PCR	real-time quantative PCR
SCC	squamous cell carcinoma
SIL	squamous intraepithelial lesion
SPF-PCR	short polymerase chain reaction fragment
STD	sexually transmitted diseases
WHO	World Health Organization

FOREWORD

The realisation that a virus is primarily the cause of cervical cancer has revolutionised not only our understanding of the disease but also its management. With this in mind, it is appropriate that the NHS Cervical Screening Programme (NHSCSP) should publish an up to date and authoritative version of the aetiology of cervical cancer and at the same time an in depth description of the virus concerned, the human papillomavirus (HPV).

The two authors are experts in the field, with Professor Bosch being an epidemiologist and Professor Iftner a basic scientist. They have combined their knowledge in the preparation of this publication, which will be of value to all of those concerned with managing women in the Cervical Screening Programme. The concept that a sexually transmitted agent is the cause of cervical neoplasia is frightening for many women and also disturbing for those of us who work in the programme and who have to counsel women about the place that HPV plays in initiating and promoting this condition. Be it a young woman who is concerned about future fertility or an older woman for whom the social and emotional consequences of the diagnosis of cervical disease can be profound, all women will be helped by knowledgeable and authoritative discussion with personnel who are employed in the screening programme. All of us must be aware of the true facts concerning HPV infections. This publication will go a long way in fulfilling this aim.

Euphemia McGoogan
Senior Lecturer (retired), Pathology Department,
University of Edinburgh
Associate Medical Director (retired)
Lothian University Hospitals Trust

Albert Singer
Professor of Gynaecological Research
University of London

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1. SUMMARY

Cervical cancer has been recognised as a rare outcome of a common sexually transmitted infection. The aetiological association is restricted to a limited number of viral types of the family of the human papillomaviruses (HPVs). The association is causal in nature and, under optimal testing systems, HPV-DNA can be identified in all specimens of invasive cervical cancer. It has been claimed that HPV infection is a necessary cause of cervical cancer. The evidence is consistent worldwide and implies both the squamous cell carcinomas (SCCs), the adenocarcinomas and the vast majority (ie > 95%) of the immediate precursors, namely high grade squamous intraepithelial lesions (HSIL)/cervical intraepithelial neoplasia 3 (CIN3)/carcinoma in situ. Cofactors that modify the risk among HPV-DNA positive women include the use of oral contraceptives (OCs) for five or more years, smoking, high parity (five or more full term pregnancies) and previous exposure to other sexually transmitted diseases, such as *Chlamydia trachomatis* (CT) and herpesvirus type 2. Women exposed to the human immunodeficiency virus (HIV) are at high risk of HPV infection, HPV-DNA persistency and progression of HPV lesions to cervical cancer.

Infections with HPV take place in differentiated epithelium and are usually of transient nature. Following entry into basal epithelial cells, HPV genomes are established as autonomously replicating extrachromosomal elements and a low level of HPV gene expression occurs. Upon differentiation of infected cells, productive replication and expression of capsid genes is induced, resulting in the synthesis of progeny virions. Any event inhibiting the normal differentiation of the epithelium or the prevention of the normal sequence of viral replication may lead to the development of persistent infections, which can become clinically active owing to a compromised immune status or other hitherto unknown factors. In abortively infected cells, HPV-DNA and even viral proteins are present, but no differentiation dependent synthesis of virions occurs. The viral infection leads to the generation of immunoglobulin G1 (IgG1) and IgA antibodies directed against some viral proteins, although these do not effect regression of established cervical lesions, which is mainly achieved by cellular immunity. The development of a long term persistent infection is a decisive factor for the progressive course of the disease and the identification of factors supporting this development is of major importance. Such factors are expression levels of human leucocyte antigen (HLA) class I antigens, presence or absence of specific HLA class II haplotypes, polymorphisms in certain human genes, partly in combination with viral variants, loss of heterozygosity and epigenetic events leading to the loss of cellular protein expression. In addition, any events compromising the immune system increase the frequency of clinically apparent infections and consequently the risk for malignant progression. The diagnosis of an HPV infection is usually made based on the identification of the viral DNA by molecular techniques using either complementary probes hybridising to the DNA followed by signal amplification or by polymerase chain reaction (PCR) amplification of a

subgenomic region followed by hybridisation to specific complementary probes, which allows the identification of the genotype.

2. PAPILLOMAVIRUS

Papillomaviruses are widespread among higher vertebrates but reveal a strict species specificity, and transmission from non-primates to humans has not been reported. In general, they cause local epithelial infections, with the exception of animal fibropapilloma viruses (eg bovine papillomavirus), with which the infection can also be found in the dermis. Viral spread to distant body sites does not occur.

Papillomaviruses are small icosahedral particles with a diameter of 55 nm, belong to the family of Papovaviridae, have no envelope and consist of a capsid composed of 72 capsomeres, which accommodates the viral genome. The capsomeres are made of two structural proteins: the 57 kDa late protein L1, which accounts for 80% of the viral particle and is considered to be a group specific antigen, and the 43–53 kDa minor capsid protein L2. Because of the absence of an envelope, papillomaviruses are relatively stable and resistant to desiccation and will retain viability extracellularly for at least 1 week.[1] They are also resistant to organic solvents, and heat treatment to 56°C causes only a minor loss of infectivity.

Infections with papillomaviruses may cause local cell proliferations, which become apparent in the form of benign tumours such as common warts, condylomas and cervical intraepithelial neoplasia (CIN). The majority of the benign tumours spontaneously regress in immunocompetent patients. In patients with inherited or induced immune deficiencies, we see, however, a strongly increased tendency for long term persistent infections, which in the case of the high risk HPV types (HR-HPV) (see below) carry a high risk for progression of the primary tumours into carcinomas.[2,3]

Recent studies have shown that HPV-DNA can be found in 99.7% of all cervical carcinomas, with HPV types 16, 18, 45 and 31 being the most frequent.[2,4–7] It has now been proven beyond reasonable doubt that infection with an HR-HPV is a necessary prerequisite for the development of cervical cancer, and the World Health Organization (WHO) has declared HPV16 and HPV18 as carcinogenic agents for humans.

2.1 Classification of papillomaviruses

Earlier attempts to classify HPVs were based on the rather strict tropism of certain HPV types for cornifying squamous epithelium (cutaneous types, eg HPV1, 4, 10) or mucosal epithelium (mucosal types, eg HPV6, 16, 31), with some types strongly linked to distinctive clinical presentations. However, this classification is overly simple and is incorrect in some cases, as demonstrated by the presence of the mucosal type HPV6 in cornifying genital warts. Another attempt to group papillomaviruses is the separation into skin types causing vulgar warts (eg HPV1) and genital types affecting primarily the anogenital area (eg HPV6, 16, 18). Again, this classification is rather artificial because HPV16 can also be found in nail bed carcinomas on the hands. The modern classification into different HPV genotypes is based on DNA nucleotide sequence differences within the coding regions of the proteins E6, E7 and L1

with different genotypes distinguished by < 90% sequence homology in these regions.[8–10] By this definition, over 130 different HPV types have been described to date. Further subdivision into subtypes is based on sequence homology between 90% and 98% and variants with ≥ 98% sequence homology.

HPV intratype variants are defined as having more than 98% nucleotide sequence identity determined over the E6, E7 and L1 open reading frames (ORFs) with the reference sequence.[11,12]

HPV classification

- based on DNA sequence differences within the coding regions of the early proteins E6, E7 and the late protein L1
- genotypes have < 90% DNA sequence homology in these regions: over 130 have been described to date
  - subtypes have 90–98% homology within a genotype
  - variants have ≥ 98% homology within a subtype.

2.1.1 Structure of the human papillomavirus genome

The HPV genome consists of a double stranded 8 kbp DNA molecule, which is associated with cell derived histone proteins that produce a nucleosome-like superhelical twisted structure. The relative arrangement of the 9–10 ORFs within the genome is conserved within all papillomavirus types. One speciality of papillomaviruses is that the partly overlapping ORFs are arranged on only one DNA strand. To increase their coding capacities, HPVs make use of polycistronic transcripts and fusion proteins from different reading frames. The genome can be divided into three regions: the long control region (LCR), the region of early proteins (E1–E8) and the region of late proteins (L1 and L2). In accordance with this, two ribonucleic acid (RNA) poly-A addition sites, one for the early protein transcripts and one for the late protein transcripts, are always present.[13] An example of the genome organisation of human papillomaviruses is given in Figure 1 and the functions of the different ORFs are summarised in Table 1.

2.1.2 The long control region

The size of the LCR varies from 500 to 1000 bp between different HPVs. There are no ORFs in this area of the genome but it does contain several control elements that regulate HPV-DNA replication and gene expression.[14]

2.1.3 The proteins of papillomaviruses

Transcription of the genes E6 and E7 is a consistent feature in cervical carcinomas and was the first indication of an important role for these genes in HPV associated tumorigenesis.[15–18] The E6 and E7 genes of HPV16 and HPV18 have been confirmed as potent viral oncogenes with the transforming and immortalising abilities of their gene products having been demonstrated in numerous experiments in tissue culture and experimental animal models.[19,20]

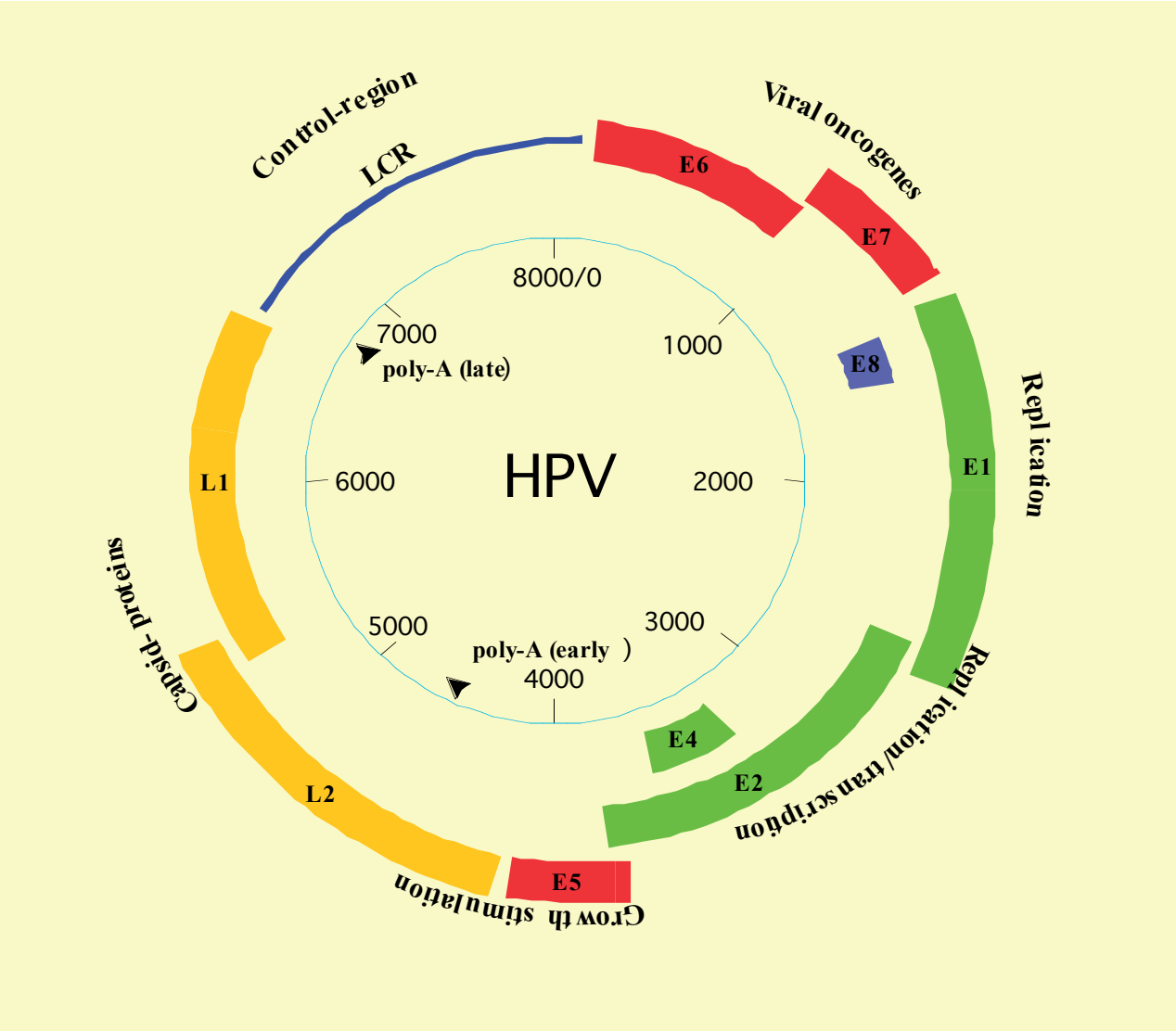


Figure 1 Genome organisation of human papillomaviruses (HPV) with the ORFs (E1–E8, L1, L2) and the long control region (LCR).

The E6 protein

The E6 ORF encodes several small proteins of approximately 150 amino acids, resulting in a molecular weight of about 16–18 kDa. Because of the presence of a splice donor and two splice acceptor sites within the E6 ORF of high risk anogenital HPV types, smaller E6 proteins (E6*I and E6*II) are produced, which may autoregulate the E6 promotor (p97) that is itself responsible for their expression. No enzymatic function of E6 proteins has been demonstrated so far; however, physical interactions with several cellular factors resulting in the deregulation of the cell cycle or interference with DNA repair have been described. The key activity mechanism of high risk E6 proteins is their ability to inhibit the function of p53. p53 is a sequence specific transcriptional transactivator with a growth arrest function and is regarded as a tumour suppressor protein that is stabilised post-translationally (increase in protein half life) in case of DNA damage.

Table 1 Size and function of papillomavirus proteins

Viral protein/genomic element	Molecular weight/size	Function
Non-coding elements		
Long control region (LCR)	500–1000 bp	Origin of replication and regulation of HPV gene expression
Early proteins		
E1	68–85 kDa	Helicase function; essential for viral replication and control of gene transcription
E2	48 kDa	Viral transcription factor; essential for viral replication and control of gene transcription; genome segregation and encapsidation
E3	Unknown	Function not known; only present in a few HPVs
E1–E4	10–44 kDa	Binding to cytoskeletal protein
E5	14 kDa	Interaction with EGF/PDGF receptors
E6	16–18 kDa	Interaction with several cellular proteins; HR-HPV type E6 causes degradation of p53 and activates telomerase
E7	~ 10 kDa	Interaction with several cellular proteins, such as with pRB and transactivation of E2F dependent promoters
E8–E2C	20 kDa	Long distance transcription and replication repressor protein
Late proteins		
L1	57 kDa	Major capsid protein
L2	43–53 kDa	Minor capsid protein

The binding of E6 to the p53 proteins leads to an enhanced ubiquitin dependent degradation of p53. This results in a shortening of its half life from 3 h to 20 min, with a corresponding loss of its biological function.[21,22] For the ubiquitination of p53, E6 needs a cellular protein called E6 associated protein (E6-AP), which acts as an E3 ubiquitin protein ligase and links ubiquitin to a lysine sidechain, forming a stable isopeptide bond.[23] In non-infected eukaryotic cells, the ubiquitin mediated proteolysis of p53 is triggered by the mdm-2 protein.[24] However, in HR-HPV infected cells the formation of the E6–p53–E6AP complex replaces the normal regulation control of p53 by mdm-2.

Independently of the E6AP dependent degradation of the p53 protein, high risk E6 proteins lead to a downregulation of p53 dependent transcription, which can be explained by the targeting of CBP/p300, a p53 coactivator,[21] and impede the efficiency of DNA repair.[25] Furthermore, E6 appears to be able to activate the cellular enzyme telomerase in differentiated cells.[21] Telomerase is an enzyme that counteracts the continuous shortening of the chromosome's telomeres, which naturally occurs during replication of the cellular genome replication. Telomere shortening correlates with cell ageing and telomerase activity correlates with an increased lifespan of the affected cell.

The E7 protein

The E7 ORF encodes for a small phosphoprotein of about 100 amino

acids (10 kDa). E7 is a proliferation inducing oncogene of human papillomaviruses; its activity is mediated through its ability to bind cellular proteins of the pRB family which, in concert with the E2F family of transcription factors, control the transition of the cell cycle from the G1 to the S phase.[26,27] Binding of E7 to the hypophosphorylated active form of pRB and its degradation leads to the activation of E2F transcription factors, permitting progression of the cell into the S phase of the cell cycle with subsequent cell replication.[28,29] Apart from this proliferative capacity that is mediated by specific sequences within the N-terminus of E7, it was shown that E7 proteins of 'low risk' types possess a 10-fold lower binding efficiency to pRB than 'high risk' E7 proteins and are very inefficient in cellular transformation assays together with activated *ras* oncogene.[27,30] Chimeric substitution assays attributed the difference in pRB binding affinity and transforming capacity between 'low risk' and 'high risk' HPV types to the exchange of a single amino acid.[27] Forced entry into the S phase is necessary for the virus to generate an environment that allows amplification of the viral DNA and induces a number of cellular responses, such as stabilisation of the p53 protein, which would lead to programmed cell death via apoptosis. To counteract these cellular responses, high risk papillomaviruses encode the E6 protein, which causes the degradation of p53.

2.1.4 Replication cycle in the infected epithelium

The initial infection by HPV probably occurs in stem cells of the basal layer of stratified epithelium or in associated hair follicles.[31–33] Following entry into the cell, HPV genomes are established as extrachromosomal elements in the nucleus. Upon cell division, one of the daughter cells migrates away from the basal layer and initiates a programme of differentiation. This leads to amplification of the viral DNA, expression of capsid proteins and finally to the production of progeny virus. The other daughter cell remains in the basal layer and provides a reservoir of viral DNA. As HPVs rely on cellular enzymes to replicate their genomes, one major consequence of an HPV infection is blockage of the cell cycle exit. HPV infected cells undergo an incomplete S phase in differentiated suprabasal cells to replicate HPV genomes to high levels.[34] In the HR-HPV types, blockage of the cell cycle exit and induction of the S phase in differentiated suprabasal cells is mediated by the E6 and E7 proteins.[35–37]

HPVs maintain their genomes at 10–100 virus copies per infected cell over long periods of time in vitro, and this is thought to reflect viral DNA replication in basal cells in vivo. Disturbances of the replication control of HR-HPV may have implications for the progression of HR-HPV induced lesions in vivo, as the viral DNA is extrachromosomal in precursor lesions but is frequently found integrated into the host chromosomes in invasive cancers.[38] As no common integration site(s) has been identified, integration does not generally target proto-oncogenes or tumour suppressor genes of the host cell. On the other hand, it has been observed that deletions and rearrangements of the integrated viral DNA occur. A model was proposed which suggests that the inactivation of the E2 gene releases E6/E7 oncogene expression from negative control.[3] However, no evidence has been presented so far that increased E6/E7 expression is necessary for the progression of HPV induced lesions. Viral DNA

integration could simply be a consequence of an environment that does not support HPV-DNA replication. This is supported by observations that long term extrachromosomal replication of HR-HPV-DNA has not been achieved in established HPV positive or negative tumour cell lines, but occurs almost exclusively in normal human keratinocytes.[39,40]

Conclusions

- HPV genotypes are distinguished by DNA sequence differences within the coding regions of the early proteins E6 and E7 and the late protein L1
- the main HPV oncoproteins are E6 and E7
- the ability of anogenital E6 and E7 proteins to degrade cellular tumour suppressor proteins correlates with oncogenic potential
- a persistently HR-HPV infected cell undergoes continuous cell division and does not halt proliferation in response to DNA damage.

2.2 Human papillomavirus detection systems

2.2.1 Hybridisation techniques

The hybrid capture 2 (HC2) assay is based on hybridisation in solution of long synthetic RNA probes complementary to the genomic sequence of 13 high risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and five low risk (6, 11, 42, 43 and 44) HPV types that are used to prepare high (B) and low (A) probe cocktails that are used in two separate reactions. Denatured DNA present in the biological specimen is then hybridised in solution with each of the probe cocktails, allowing the formation of specific HPV-DNA–RNA hybrids. These hybrids are then captured by antibodies bound to the wells of a microtitre plate that recognise specifically RNA–DNA hybrids. The immobilised hybrids are detected by alkaline phosphatase labelled anti-DNA–RNA monoclonal antibodies and the plate is washed. This is followed by an incubation of the bound enzyme conjugated antibodies with the chemiluminescent compound CDP-Star® (Tropix PE, Bedford, MA). Dephosphorylation of this substrate produces light in a glow reaction that is measured by a luminometer. Readings are transferred directly into a software programme with which the results are analysed. The intensity of emitted light, expressed as relative light units (RLUs), is proportional to the amount of target DNA present in the specimen, providing a semiquantitative measure of the viral load. The HC2 is currently available in a 96 well microplate format, is easy to perform in clinical settings and is suitable for automation. Furthermore, HC2 does not require special facilities to avoid crosscontamination as it does not rely on target amplification to achieve high sensitivity, as do PCR protocols. For screening purposes only, the high risk cocktail is used; this reduces time and cost of the test. The Food and Drug Administration (FDA) recommended cut off value for positive test results is 1.0 RLU (equivalent to 1 pg HPV-DNA per 1 ml of sampling buffer). Several studies have noted that the high risk probe cocktail of HC2 crossreacts

with HPV types that are not represented in the probe mix.[41–43] Peyton and colleagues[41] found that using the high risk HC2 probe at a 1.0 pg/ml cut off detected HPV types 53, 66, 67, 73 as well as other undefined types, and raising the cut off to 10.0 pg/ml did not eliminate the crossreactivity to types 53 and 67. Crossreactivity of the high risk HC2 probe to HPV types that have a significant risk for cervical cancer may be considered beneficial, but crossreaction with low risk types causes false positive results and may decrease the specificity of the test.[44]

The newly developed HC3 assay[45] uses RNA probes as in HC2, but in combination with biotinylated capture oligonucleotides that are directed to unique sequence regions within the desired target. These oligonucleotides are only used for capturing the desired target sequence into streptavidine coated wells of a microtitre plate. Signals are generated by RNA probes that hybridise to other regions of the captured sequences as the DNA capture oligonucleotides. Moreover, the assay has been further developed to reduce unspecific hybridisation using 'blocker oligonucleotides' (unlabelled DNA molecules that are complementary to the biotinylated capture oligonucleotides) aiming to eliminate cross-reactivity while retaining specificity. The HC3 assay can also be applied for genotyping of HPV and is capable of discriminating highly related HPV types, such as HPV18, 45 or HPV16, 31 and 35. The same assay can be used, in principle, for either DNA or RNA targets. Using this technique, it will also be possible to test for molecular variants of certain HPV types because even targets with single nucleotide exchanges can be detected specifically.

For high volume laboratory testing, Digene has developed a fully automated device for hybrid capture testing called the Rapid Capture System, which allows robotic handling of 96 well microplates. This robot station performs specimen transfer, all pipetting operations, incubations, shakings and washings. However, the denaturation of specimens in the sample device tubes still has to be performed by hand. This automatic station increases the accuracy of the test and allows a single user to test 352 specimens within 6 h.

2.2.2 *Polymerase chain reaction based assays*

Besides HC2/3, a number of companies offer in situ hybridisation HPV-DNA detection systems that allow detection of certain HPV types within the context of morphology in tissues or smears. These tests suffer from low sensitivity and from the fact that either only a single type or a group of types without further differentiation can be detected per sample.

HPV-DNA can be selectively amplified by a series of reactions that lead to an exponential and reproducible increase in the viral sequences present in the biological specimen. Analysis of the amplified products can be carried out in different ways, including gel electrophoresis, dot blot or line strip hybridisation, and, ultimately, can be coupled to direct DNA sequencing. The sensitivity and specificity of PCR based methods can vary depending mainly on the primer sets, the size of the PCR product, reaction conditions and performance of the DNA polymerase used in the reaction, the spectrum of HPV-DNA amplified and ability to detect multiple types. PCR can theoretically produce one million copies from

a single double stranded DNA molecule after 30 cycles of amplification. Therefore, care must be taken to avoid false positive results derived from crosscontaminated specimens or reagents. Several procedures are available to avoid this potential problem in using PCR protocols for HPV-DNA detection. Because of their versatility and very high sensitivity, many PCR systems are available, but care must be taken for the lack of validation and comparison with established protocols, such as those described below.

The most widely used protocols employ consensus primers that are directed to a highly conserved region of the L1 gene, and are potentially capable of detecting all mucosal HPV types.[46] Among these are the single pair of consensus primers GP5/6[47,48] and its extended version GP5+/6+[49] and the MY09/11 degenerate primers[50] and its modified version PGMY09/11.[51] Full distinction of roughly 40 types can be achieved by hybridisation with type specific probes[47,49,51–54] that can be performed in different formats, including line strip assays and microtitre plates that are amenable to automation. Another pair of consensus primers is available which amplifies a smaller fragment (65 bp compared with 150 bp for the GP primers and 450 bp for MY09/11) of the L1 gene, therefore potentially increasing the sensitivity of the assay. This short PCR fragment (SPF-PCR)[54,55] is designed to discriminate a broad spectrum of HPVs by reverse line blot hybridisation, which allows the discrimination of 43, or 24 in the new LiPAv2 format, different HPV genotypes.[54] Very recently, a reverse line blot typing assay for the GP5+/6+ system, capable of typing 37 mucosotropic HPVs, has been developed, allowing for high throughput testing both in epidemiological and clinical research.[49]

The newly developed Roche prototype microwell plate assay employs an oligonucleotide set that amplifies a short fragment of the *L1* gene of HR-HPV types (170 bp compared with 450 bp obtained with PGMY09/11). This amplicon is immobilised using a pool of capture molecules bound to the wells of a microtitre well plate and visualised by colorimetric detection with Roche Amplicor chemistry. Moreover, the new test was developed to employ TaqGold DNA polymerase, which minimises the amount of non-specific amplification and increases the sensitivity of the test. As it amplifies a shorter fragment, it is considered to be more sensitive than PGMY09/11 PCR and also amenable for less preserved specimens, and it was reported that these primers detect about 13% more HPV in cervical smears than the PGMY primers.[56] Because these primers were designed for high risk types only (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68), this test is not truly generic, but rather similar to the HC2 test. However, it has to be noted that the usage of PCR assays aiming at maximum sensitivity for detection of HPV irrespective of concomitant disease seems inappropriate in a screening setting with regard to clinical usefulness. A good screening test needs a threshold defined by sufficiently large clinical trials for detection of clinically relevant disease because higher sensitivity and less clinical specificity will result in more follow-up of women who will not have CIN3. Therefore, tests with higher analytical sensitivity are unlikely to contribute to improvements in screening.

There are extensive data describing the reproducibility and agreement of HPV testing results among the abovementioned three popular PCR protocols, as well as between them and the FDA-approved HC2 for overall HPV detection. Although agreement at the overall positivity level may be considered adequate in clinical settings, concordance at the level of type detection leaves much to be desired.[41,44,52,54,55,57–60]

Biomedlab (Korea) has developed an HPV oligonucleotide microarray based detection system of HPV types that currently allows the detection of 22 HPV types by immobilising HPV type specific oligonucleotide probes and a control (beta globin probe) on an aldehyde derivatised glass slide. The target DNA is submitted to a standard PCR in the presence of fluoresceinated nucleotides (Cy5 or Cy3) employing primers for both the beta globin (PC03/04) and the L1 region (modified Gp5/6 primers) of several HPV types. Randomly labelled PCR products are then hybridised to specific oligonucleotides on the chip, which is scanned afterwards by laser fluorescence. In the case of multiple infections, multiple hybridisation signals can be seen. Because signal detection in microarrays is subject to variation, additional levels of control would be desirable. These should include quality control of the efficiency of the PCR reaction and the hybridisation conditions and a measurement of the homogeneity of the printed probe and allow some sort of quantification. In addition, the readout requires expensive equipment for signal detection and would need to use specialised software that allows threshold settings.

2.2.3 NASBA technology

The use of novel biomarkers is proposed as a way to stratify HPV positive women into risk groups that can be managed by alternative algorithms. An approach to combine HPV testing capability limited to the detection of five types with the quantification of the transcript levels for the viral oncogenes is made by the Norwegian company Norchip, using NASBA technology. NASBA is a sensitive transcription based amplification system for the specific replication of nucleic acids in vitro. The main advantage of this technique is that the complete amplification reaction is performed at 41°C. Three enzymes are involved in this homogeneous isothermal reaction: avian myeloblastosis virus, reverse transcriptase (RT), and RNase H and T7 DNA dependent RNA polymerase (DdRp). Because of the integration of RT into the amplification process, the method is especially suited to mRNA analyses.

The target RNA is denatured at 65°C and reverse transcribed with a primer that not only possesses a sequence complementary to the target RNA but also includes a 5′ sequence corresponding to the promoter of the T7 DdRp. The RNA strand of the RNA–cDNA hybrid formed will subsequently be degraded by RNase H. With the help of a reverse primer complementary to the cDNA, double stranded DNA encompassing a T7 promoter sequence has been formed, which can be used by the T7 DdRp to synthesise new RNA molecules complementary to the target RNA. After this initial reaction, NASBA now enters the amplification (cyclic) phase and new RNA will be produced by the activity of the RT and T7 DdRp enzymes. The reaction continues in a self-sustained manner under

isothermal conditions, thus achieving large amplification of the target (106- to 109-fold).

The amplified RNA is detected by the use of molecular beacon probes, which are single stranded oligonucleotides having a stem–loop structure. One arm of the stem is labelled with a fluorescent dye and the other arm is labelled with a non-fluorescent quencher, which inhibits fluorescence by energy transfer from the fluorescence dye to the quencher. After hybridisation of the molecular beacon to its specific target and unfolding of the stem structure, the energy transfer is interrupted and fluorescence takes place, which is related in intensity to the amplicon concentration if the molecular beacon is provided in large excess. Two oligonucleotide primers that are specific for the RNA target of interest determine the specificity of the reaction.

This technique is used by the Norchip PreTect HPV-Proofer test, which is an assay for the detection of E6/E7 mRNA from the HR-HPV types 16, 18, 31, 33 and 45. It has been suggested that, on the basis of E6/E7 expression levels, it could be possible to achieve a risk stratification of HPV positive women, although direct clinical data in support of this strategy are scarce.[61,62]

Table 2 summarises the salient characteristics of the most common HPV detection methods available.

2.2.4 *Diagnosis by immunohistochemistry*

As an adjunct to Pap screening, it would be highly desirable to have viral and/or host proteins available that would allow the stratification of women

Table 2 Summary of selected diagnostic nucleic acid analyses available

				Usable for			
	Primary detection method	Secondary detection method	Brand/test name	Screening	Genotyping	Published trials	FDA approved
DNA	Probe hybridisation	Luminescence	HC2	+++	–	++++	+
	PCR amplification	ELISA	GP5+/6+	+	++	+++	–
		Reverse hybridisation	Roche prototype LBA	–	+++	++	–
			Innogenetics LiPA	+	+++		
			GP5+/6+RLB	+	+++		
		DNA ChIP	BioMedLab HPV DNA ChIP		+++	+	–
		RFLP*			+	+	–
		Direct sequencing	E1-PCR		+	+	–
RNA	NASBA	Fluorescence	Norchip PreTect HPV Proofer	+	(+)†	+/–	–
	RT-(Q)PCR			+	+/–	+	–

*Restriction fragment length polymorphisms.
†Only five types.

into different risk categories in terms of HPV persistence and malignant progression. Techniques such as immunohistochemistry provide additional information from fixed, deparaffinised or unfixed tissue specimens and from cervical samples. With this technique, cells are stained using antibodies against either viral proteins or cellular proteins that serve as surrogate markers of HPV infection or, ideally, as independent progression markers. Multiple factors affect the specificity and sensitivity of immunohistochemistry, such as the affinity and specificity of the primary and secondary antibody and the application of the respective detection systems as well as the fixation procedure of the clinical material used.

Although some candidates are already emerging, it is necessary to determine the benefits and risks of a novel marker implementation within a population before it can be included in cervical cancer screening. At present, few markers warrant such efforts, such as p16INK4a, which serves as a surrogate marker of oncogenic HPV expression and has been shown to be associated with HPV infected high grade lesions. However, marker test sensitivities, especially in HPV positive women with normal cytology, have to be extensively clarified before such markers can be used as a replacement for an HPV test.

Viral protein candidates

Because viral replication with concomitant expression of the major structural protein L1 takes place only in low grade squamous epithelial lesions, it has been postulated that the lack of L1 expression in cervical samples, as defined by immunohistochemistry, might identify those lesions with enhanced risk of progression[63] and diagnostic kits are now available. It has to be noted, however, that considering a negative test result as a prognostic indication for disease progression is questionable if the assay set up does not control for false negative results, especially in the case of a technique such as immunocytochemistry. Based on the fact that the two viral oncogenes E6 and E7 are required for the transformation of epithelial cells and have been found to be constantly expressed in all HSIL and cervical cancers, investigations are in progress to use the identification of these proteins as indicators for an 'oncogenic activity', which is thought to be superior to the identification of viral DNA.

Cellular proteins

Infections with HR-HPV types override the cell cycle control and lead to increased proliferation of the cells. Therefore, it was thought that the detection of proliferating cells in cervical samples using proliferation markers could be an adjunctive method in cervical cancer screening. However, established markers such as Ki-67 and proliferating cell nuclear antigen have not proved to be highly useful as stand alone markers,[64] and proteins regulating DNA replication, such as minichromosome maintenance proteins and cdc6, showed limited clinical usefulness because they are unable to differentiate precisely between proliferating dysplastic cells and normal proliferating cells.[65,66]

One promising candidate is the p16INK4a protein, which has been found to be overexpressed in HSIL, SCCs and adenocarcinomas of the cervix.[64,67–69] P16INK4a is a tumour suppressor protein that inhibits the

kinases cdk4 and cdk6, which are responsible for phosphorylating the Rb protein. Accumulation of p16INK4a mRNA and protein has been reported in response to inactivation of the retinoblastoma gene (Rb).[70,71] However, the link between p16INK4a and Rb is more complicated than was initially thought; p16INK4a expression does not simply reflect the status of Rb, as shown by the finding that the induction of p16INK4a expression occurs only after prolonged cell proliferation in the absence of functional Rb. This is further complicated by findings that inactivation of p53 can also result in upregulation of p16 expression.[70]

Hallmarks of immunohistochemistry with antibodies for p16INK4a are the detection of dysplastic squamous and glandular cells of the cervix with a sensitivity of 99.9% and a specificity of 100%.[68] All cases that were positive for HPV expressed the p16INK4a protein, although not all cases found to be positive for p16INK4a were HPV positive.[68] Although weak cytoplasmic staining was observed in 12% of non-neoplastic epithelium and strong focal signals in metaplasia, findings of strong diffuse staining for p16 were significantly associated with HR-HPV associated high grade squamous intraepithelial lesions (HSILs), which were diffusely positive in 70.2% versus 37.5% of low grade squamous intraepithelial lesions (LSILs) ($P = 0.02$).[64] A number of cross-sectional studies revealed a high sensitivity and specificity of p16INK4a for CIN2/3 also in liquid based cytology specimens;[72,73] however, the positive predictive value and sensitivity in prospective follow-up studies (eg prognostic value for CIN2 and 3) remains to be determined.

P16INK4a could become especially important in the diagnosis of endocervical glandular atypia and adenocarcinoma in situ (AIS); because of the difficulties of using colposcopy to recognise endocervical lesions, diagnosis of cervical adenocarcinomas must almost always be carried out by cytology. A recent study showed p16INK4a staining in all cases of invasive adenocarcinoma and AIS,[74] which is in contrast to earlier negative findings that were possibly related to the usage of different p16INK4a antibodies. Therefore, P16INK4a has the potential to be a clinically relevant surrogate marker because it is a measure of HPV gene expression and thus activity, rather than solely a detector of viral DNA presence. In summary, p16INK4A immunohistochemical analysis could provide a useful adjunct to conventional screening programmes and would help to reduce false positive and false negative results.

Other potential biomarkers are cyclin E, which correlates strongly in expression with p16INK4a ($P < 0.001$) and Ki-67, the latter being a strong indicator for HSIL only when highly expressed in the upper two thirds of the epithelium. Ki-67 was less reliable, however, for the discrimination of LSIL and immature epithelium.[64] In addition, frequency of Ki-67 positivity is influenced by menstrual cycle. Noteworthy Ki-67 staining seems to be useful for atrophic epithelium, which can have histopathological characteristics of squamous intraepithelial lesions (SIL) but should have virtually no staining for Ki-67. Because of the complementary nature of these biomarkers, it would be appropriate to combine cyclin E, Ki-67 and p16 together to distinguish reactive from neoplastic epithelium.

Other recently defined molecules with potential future diagnostic usefulness are GATA-3 mRNA (a transcription factor that activates the T cell receptor gene enhancer)[75] and TSLC1 (tumour suppressor in lung cancer; a member of the IgG super family), which show reduced expression in cervical cancer cells. In contrast, using immunohistochemical analyses, the expression of C4.8, a member of the tetraspanin protein family, was found to be increased in neoplastic cells.[76]

2.3 Immunity to HPV

Numerous studies have found a positive association between the detection of HPV antibodies and the risk of cervical neoplasia, in line with the thinking that HPV antibody detection is a marker of cumulative exposure to HPV. Although these antibodies might be effective at preventing infection, particularly those directed against the virion capsid proteins L1 and L2, it is commonly accepted that antibodies are not important effectors of the regression of established HPV infections and related cervical lesions. Neutralising antibodies are generated by a type specific conformational epitope in the viral particle. In contrast, disrupted or partially disrupted viruses expose epitopes that are broadly crossreactive or even group specific.[77,78] One exception is HPV6/11, which have been shown to contain shared epitopes and type specific epitopes on intact capsids.[77,79] Seroconversions against the HPV16 capsids are seen concomitantly with or within a few months following acquisition of HPV16 DNA,[80–84] but can be delayed many months after the detection of viral DNA in a subset of patients. In Sweden, the risk for seroconversion to the major oncogenic HPV type HPV16 increases linearly by about 4% for each lifetime sexual partner, up to a plateau of about 32% among women with on average eight lifetime sexual partners.[85] Monogamous women have, in many studies, been found to have low seroprevalences (between 2% and 7%).[80–82,85–88] Adult virginal women have so far not been found to have any HPV seropositivity, albeit the total number of virginal women tested is not very large.[80,81] Large scale surveys of seroprevalences among children younger than 13 years of age found seroprevalences in the order of 2% for HPV.[89,90]

The major isotypes of serum antibodies against HPV capsids are IgG1 and IgA.[91] The serum IgA response is also HPV type specific, as demonstrated by its correlation with presence of type-specific HPV-DNA.[91] Secretory IgA antibodies to HPV capsids are detectable in cervical mucus. In contrast with serum IgG, however, serum IgA correlates with the recent number of sexual partners and with the lifetime number of partners, mostly among young women,[91] suggesting that the IgA response is not as biologically stable over time as is the IgG response. However, it is less clear whether antibodies against one HPV type protect against subsequent reinfection with the same or another closely related type and, if so, whether this protection is related to specific IgG and IgA subclasses. After the HPV infection has been cleared, serum antibody levels remain stable over time, even after 15 years of follow-up.[92]

The cellular immune response is an important effector mechanism for the clearance of established HPV infections. The first line of defence is the immune response with natural killer (NK) cells inducing apoptosis in virus infected cells and in tumour cells. The specific activity of NK

cells requires so-called killer immunoglobulin like receptors, which enables them to distinguish normal from virally infected or tumour cells. A direct antiviral cellular immune response is mediated by cytotoxic T cells that recognise and kill infected cells that present viral peptides with the help of HLA class I molecules on their surface. Although non-infected keratinocytes in normal epithelium reveal a steady state level of expression, in HSIL HLA class I antigens tend to be downregulated or even missing,[93,94] as indicated by the fact that 30–75% of cervical cancers have downregulated the expression of at least one HLA class I antigen.[95,96] In one study, about one quarter of cervical cancers was found to retain the normal level of the HLA-A and -B antigens on the cell surface and about one sixth did not express any of these antigens.[97] HLA antigens found to be most frequently downregulated are A2, A3, A9 group, B5 group, B7, B8 and B44.

HPV infected keratinocytes are induced to express HLA (class II)-DR, but not DP or DQ, antigens on the cell surface. Even after the malignant transformation of the keratinocytes, the expression of HLA-DR antigens significantly correlates with the extent of lymphocyte infiltration, whereas that of the DP or DQ antigens is less frequent and is unrelated to the inflammatory component. The HLA-DR upregulation is inversely associated with the clinical FIGO stage of incident cervical cancer cases.[98] In summary, HLA-DR is the major histocompatibility complex (MHC) class II antigen that is most likely to be involved in antipapillomaviral immunity, although one cannot exclude the possibility that professional antigen presenting cells could activate CD4+ T lymphocytes through DP or DQ restriction.

The mediators of regression associated histological changes are known to be mainly Th1 type cytokines. Increased level of serum interleukin 2 (IL-2) was found to predict a favourable outcome for HPV16 or HPV18 associated genital lesions.[99] Also, a pathogenic role for Th2 responses is indicated by the finding that the peripheral mononuclear cells of high grade CIN patients have a decreased IL-2, gamma interferon (IFN-γ) and elevated IL-4 and IL-10 production in response to mitogenic stimuli.[100]

3. EPIDEMIOLOGY OF HUMAN PAPILLOMAVIRUS AND CERVICAL CANCER

3.1 Human papillomavirus and sexual behaviour

Several groups of studies have clearly shown that HPV is predominantly transmitted through sexual intercourse. Other forms of transmission will be outlined briefly, but their implication in cervical cancer is likely to be marginal, if any.

3.1.1 Behavioural determinants of human papillomavirus infection

Epidemiological studies investigating risk factors for HPV infection have shown clearly and consistently that the key determinants among women are the number of sexual partners, the age at which sexual intercourse was initiated and the likelihood that at least one of her sexual partners was an HPV carrier as measured by his sexual behaviour traits.[101–109] The role of males as possible vectors of HPV was measured in the early epidemiological studies by questionnaires that addressed the sexual behaviour of the husbands or sexual partners of patients with cervical cancer and control subjects. More recent studies also had the ability to measure HPV-DNA in exfoliated cells from the penile shaft, the coronal sulcus and the distal urethra.[110–114]

These and other studies have established that the risk of cervical cancer for a given woman is predictable by the sexual behaviour of her husband as much as by her own sexual behaviour. In populations in which female monogamy is dominant, the population of female sex workers plays an important role in the maintenance and transmission of HPV infections. Moreover, the probability that a woman is an HPV carrier and her risk of developing cervical cancer have been shown to be related to the presence of HPV-DNA in the penis or the urethra of her husband or sexual partner.[115–118] More recently, it has been possible to show that male circumcision protected males from being HPV carriers and their wives from developing cervical cancer.[119] In terms of HPV infections, these observations confirmed century old observations[120] and the scientific hypothesis formulated almost 30 years ago that male sexual behaviour is a central determinant in the incidence of cervical cancer.[121,122]

3.1.2 Follow-up studies in virgins initiating sexual intercourse

Studies on virgins offer a unique opportunity to demonstrate the predominantly sexual nature of HPV transmission, as one would expect women who have not experienced sexual intercourse not to harbour HPV in the cervix, although some HPV positive specimens can be isolated from the external genitalia.

Early prevalence estimates of HR-HPV-DNA infection in virgins range from 0% to 31% (reviewed in reference 123). The wide range of these estimates indicates the influence of methodological issues, including the HPV-DNA detection method used (scrapes vs. lavage), the subsite from which the samples were collected (cervix, vagina or vulva), as well as the true virginal state of the women included in these studies. As HPV testing variability was better understood, several studies in which identical sampling methods were used for all women in a closed cohort and in which

HPV-DNA was reliably measured concluded that HPV-DNA in cervical specimens is detected only in sexually experienced women.[105,124–126]

Two cross-sectional studies found a total absence of high risk genital HPV-DNA in women who had not experienced sexual intercourse.[125,126] However, 2 out of 154 samples from virginal women tested positive for HPV6, suggesting that transmission of low risk HPVs by non-sexual routes, although possible, is also extremely rare in virginal women, even in those participating in other forms of sexual activity such as digital penetration. Consistent with these results, a prospective cohort study of female Swedish students documented that only sexually experienced women harboured HPV-DNA in the cervix and that there was a positive correlation between the presence of HPV and the number of coital partners.[124]

The strongest evidence, however, that genital HPVs are predominantly sexually transmitted is provided by longitudinal studies of virgins who started sexual activity during the study period. Evidence from such a design comes from a Danish population based cohort study of 100 virgins and 105 monogamous women. As summarised in Table 3, the study showed that all women who stayed virginal throughout follow-up were negative for both HPV-DNA and HPV16 serum antibodies at enrolment and at each follow-up visit. Only a fraction of those virgins who initiated sexual activity during the study period became positive for HPV-DNA or HPV16 serum antibodies. The most important determinant of HPV-DNA acquisition in this study was the number of sexual partners the woman had had between enrolment and the follow-up visit, both among initially virginal women and among initially monogamous women. In this study, detection of HPV16 serum antibodies and development of cervical lesions occurred only after HPV transmission, suggesting that sexual intercourse is a necessary step in the acquisition of genital HPVs and in the development of cervical neoplasia.

These findings confirm results from a previous prospective cohort study of teenage girls, which found that none of the 36 girls without coital experience was positive for HPV16 or 33 antibodies or DNA whereas a majority of the girls who acquired HPV-DNA of the corresponding HPV type did seroconvert.[80,81]

A recent prospective study including 105 HPV negative women carried out in the San Francisco Bay area found that sexual behaviour, specifically exposure to new partners, represented the strongest risk factor for incident HPV infection. The association between sexual behaviour and incident HPV was quite notable, as the risk increased nearly 10-fold for each new partner per month reported.[128]

Taken together, these prospective studies clearly demonstrate (by both HPV-DNA detection and HPV serology) that the number of sexual partners is the key risk factor for acquiring HPV infection and that non-sexually transmitted HPV infections are rare or non-existent among adolescent girls.

Table 3 Prevalence of cervical human papillomavirus DNA and human papillomavirus 16 virus-like particle seropositivity in cohorts of virginal women and monogamous women by number of sexual partners at follow-up

Number of coital partners at enrolment	Number of coital partners during follow-up	HPV-DNA				HPV 16 antibodies			
		Enrolment		Follow-up		Enrolment		Follow-up	
		No positive/ no tested	Positive (%)	No positive/ no tested	Positive (%)	No positive/no tested	Positive (%)	No positive/ no tested	Positive (%)
Virgins	0*	0/30	0	0/30	0	0/28	0	0/28	0
	1	1/67	1.5	23/65	35.4	1/67	1.5	10/67	14.9
Monogamous women	1†	2/78	2.6	4/78	5.1	7/78	9.0	7/77	9.1
	2	4/27	14.8	9/26	34.6	4/27	14.8	6/26	23.1

HPV, human papillomavirus.
*Virgins that remained virgins at follow-up.
†Monogamous women that remained monogamous at follow-up.
Data from Kjaer et al.[105]
Adapted from Castellsagué and de Sanjosé.[127]

3.1.3 *Studies relating human papillomavirus DNA prevalence in the genital tract with number of sexual partners in both sexes: studies in women practising prostitution*

Genital HPV infections, as measured by the presence of HPV-DNA in the genital tract, are the most prevalent sexually transmitted viral pathogens. Genital HPV infections are considered to occur predominantly, although not exclusively, by sexual transmission. Many epidemiological studies have consistently shown that sexual behaviour related characteristics of the individual and his or her partners are the most important risk factors for acquisition of genital HPV types.[81,102,106,114,124,129–132] Specifically, the three most consistently identified sexually related determinants of HPV infection in the genital tract are: the number of sexual partners the subject has had, the age at which sexual intercourse was initiated and recent partner change.

The relationship between two of these parameters, namely number of partners and age at first intercourse, and cervical and penile HPV-DNA detection in adult women and men is shown in Figure 2 (adapted from reference 127). These graphs are derived from the controls (2225 women and 1140 men) of a series of 12 case–control studies of cervical cancer carried out by the International Agency for Research on Cancer (IARC, Lyon, France) and the Catalan Institute of Oncology (ICO, Barcelona, Spain) in 10 countries: Colombia, Brazil, Paraguay, Peru, Spain, the Philippines, Thailand, India, Morocco and Algeria.[106,112,113,115,119,133–141] Figure 2 shows that in both sexes genital HPV-DNA detection increased with increasing lifetime number of sexual partners and with decreasing age at first sexual intercourse.

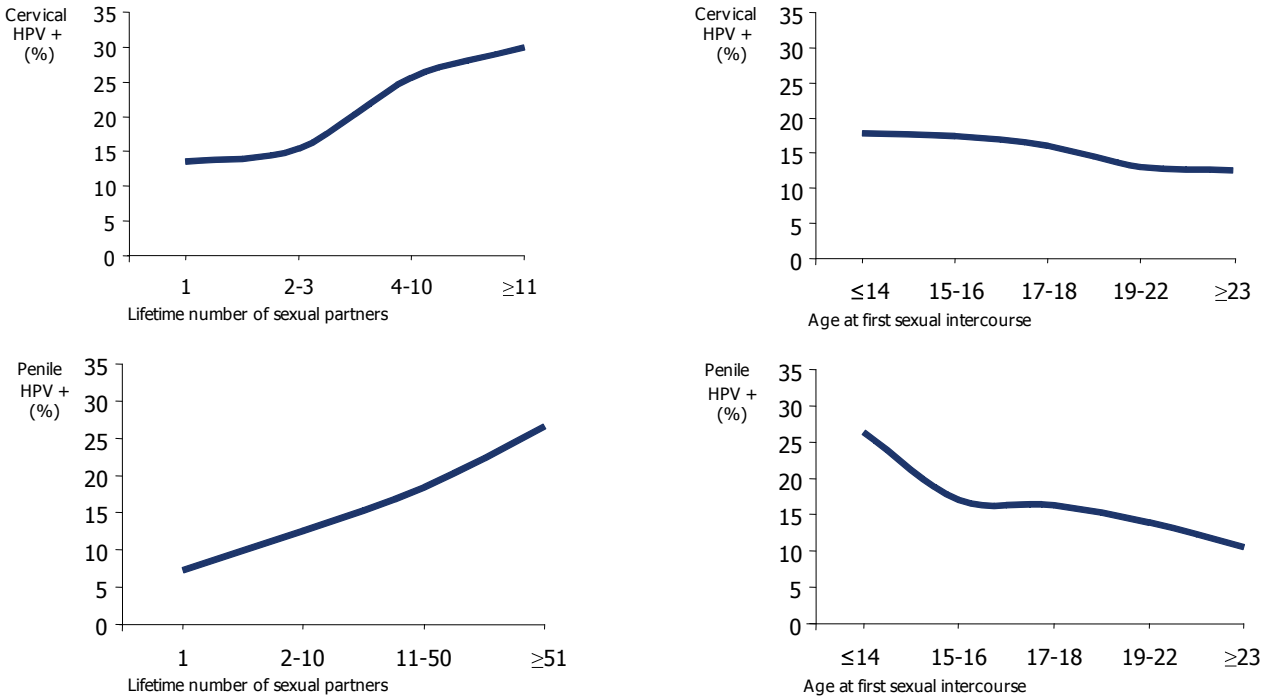


Figure 2 Prevalence of cervical and penile human papillomavirus DNA by lifetime number of sexual partners and age at first sexual intercourse (IARC studies).
HPV, human papillomavirus.
Women (n = 2225) and men (n = 1140) without genital neoplasia.
Adapted from Castellsegue and de Sanjosé, 2003.[127]

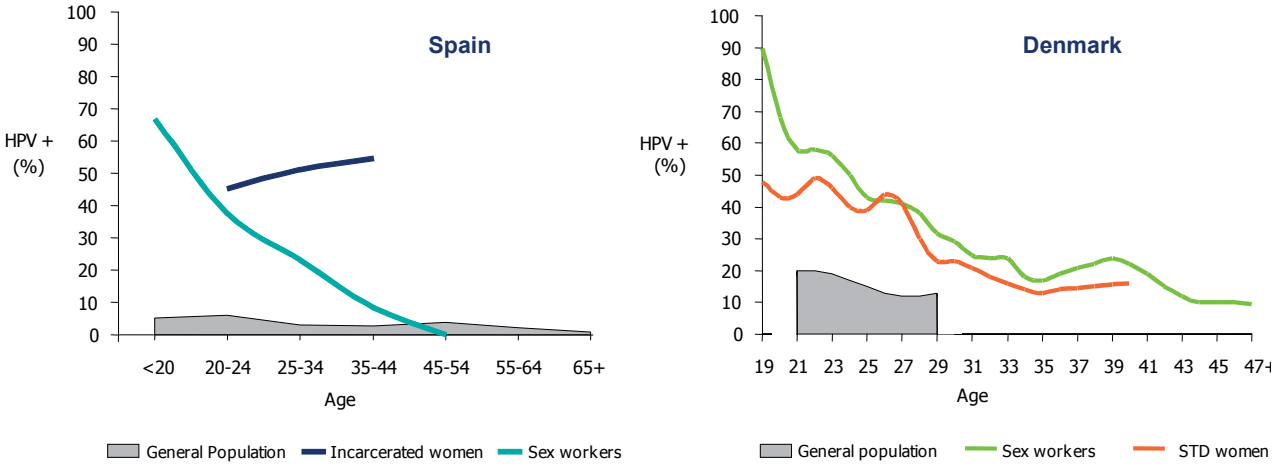
HPV data on high risk groups known to have or have had a high risk sexual behaviour, such as female sex workers and sexually transmitted diseases (STDs) clinic attendees, have provided further evidence that HPVs are predominantly sexually transmitted. Two studies conducted in Spain and Denmark compared HPV-DNA prevalence among women from the general population and women belonging to high risk groups.[142–145] As shown in Figure 3, in all age groups, HPV prevalence was highest among female sex workers, followed by women attending STD clinics or who were in prison. Women from the general population had much lower age specific HPV prevalence rates.

Many serological studies have also found a very strong correlation between the presence of HPV serum antibodies and the lifetime number of sexual partners of women in the 35–40 age range. Some authors point out that the correlation between HPV antibodies and sexual behaviour is actually stronger than that observed with herpes simplex virus-2 (HSV-2) and CT serologies.[146] HPV antibody levels are usually stable on long term follow-up, indicating that HPV seropositivity is a proxy marker of lifetime cumulative HPV exposure, as is the case for the seroepidemiology of most other STDs.[147]

3.1.4 *Studies relating the risk of cervical cancer to the number of sexual partners in both sexes*

Male sexual behaviour and cervical neoplasia in their female sexual partners

Although the implication of a sexually transmitted agent in the aetiology of cervical cancer was suggested as early as the 1940s,[120] most early studies focused on the analysis of female sexual behaviour and on the testing of cervical biological samples. However, as with any other STD, studies



Source of data: Data from Denmark includes 182 female sex workers, 187 female STD clinic attendees, and 1,000 women from the general population. Data from Spain includes 187 female sex workers, 153 incarcerated women, and 1,101 women from the general population.

Figure 3 Prevalence of cervical human papillomavirus DNA in different risk groups in Denmark and Spain.
HPV, human papillomavirus.
Data from de Sanjosé et al, 2000;[142] de Sanjosé et al, 2003;[143] Kjaer et al, 2000;[144] Touze et al, 2001.[145]
Adapted from Castellsegue and de Sanjosé, 2003.[127]

in couples have provided consistent evidence of the venereal nature of HPVs, and one would expect higher rates of HPV infection and HPV related diseases in women who had sexual contacts with promiscuous men than in women who had contacts with non-promiscuous men. Pridan and Lilienfield[148] showed for the first time an association between the number of sexual partners of the husband and the risk of cervical cancer among mostly monogamous Jewish women.

More recently, Buckley and colleagues[149] found that the risk of cervical cancer among monogamous women increased with the number of sexual partners their husbands had had. Other male factors increasing risk in this study included a husband's early age at first intercourse, reporting of extramarital affairs and history of STDs.

The potential importance of the male role in the risk of cervical cancer was also suggested in early studies of marital clusters. One study reported that subsequent wives of husbands whose previous wife developed cervical cancer had an increased risk of cervical neoplasia,[150] and other studies have shown that wives of men with cancer of the penis have a high incidence and mortality rates of cervical cancer.[151–153]

Studies showing geographic clustering of cervical and penile cancers,[154–156] and studies showing geographical correlations between incidence rates of male and female genital cancers,[153,157] provided further ecological support to the importance of men in the natural history of cervical cancer.

More recently, data from the Swedish Family Cancer Database showed that husbands of women with in situ or invasive cervical cancer had an increased risk of anal cancer, another recognised HPV-related cancer.[158] Women with cervical neoplasia also had an increased risk of anal cancer as a second primary cancer.[159] Of special note is the increased risk of husbands of cervical cancer patients developing both tonsillar cancer and cancer of the tongue,[160] supporting emerging evidence that HPV may be aetiologically involved in some of these tumours.[161]

Quantitative evidence that the risk of cervical cancer is linked to male sexual behaviour has been provided by formal case–control studies comparing either direct histories of sexual behaviour or clinical evidence of HPV-related lesions in male partners of women with and without cervical cancer.[112,114,140,162,163] For instance Zunzunegui and colleagues[163] showed that, compared with unaffected women, women with cervical neoplasia were five times more likely to be married to a man who had had more than 20 sexual partners. In another study, women who were the sole sexual partners of men with pre-existing penile condyloma showed an increased risk of cervical neoplasia.[164] Barrasso and colleagues[110] also reported a high prevalence of HPV related penile neoplasia in the sexual partners of women with cervical neoplasia.

Penile human papillomavirus DNA and cervical cancer

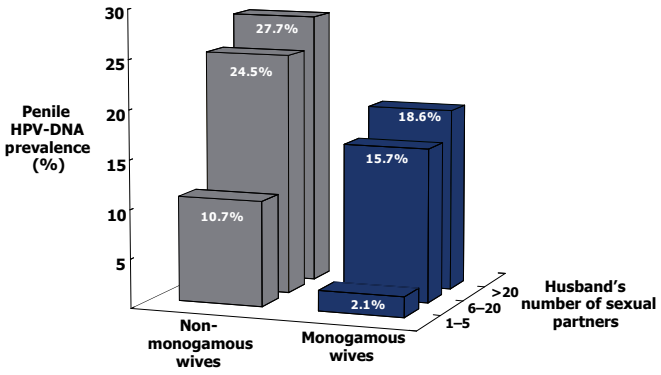
Following the identification of HPVs as the sexually transmitted agents that are aetiologically linked to cervical cancer, firm evidence for the role

of men as carriers and vectors of oncogenic HPVs resulted from studies that introduced HPV-DNA detection in penile samples.

The largest study to date exploring the male role in cervical carcinogenesis using PCR technology for the detection of penile HPV is the multicentric case–control study coordinated by the IARC and the ICO. This large study involved over 1900 couples who were enrolled in one of seven case–control studies of cervical carcinoma in situ and cervical cancer carried out in Spain, Colombia, Brazil, Thailand and the Philippines.[112,115,119,134–136,138,139,140,165] Participating men answered a detailed risk factor questionnaire and provided a specimen of exfoliated cells from the distal urethra, the glans and the coronal sulcus for HPV-DNA detection.

Figure 4 shows the correlation between penile HPV-DNA and sexual behaviour for couples enrolled in the studies conducted in Spain[112] and Colombia.[140] Penile HPV prevalence clearly increased with increasing numbers of sexual partners reported by the men. The increase was observed in male partners of both monogamous and non-monogamous women, and penile HPV prevalence was systematically higher in husbands of non-monogamous women than in husbands of monogamous women.[113]

Findings from the IARC studies conducted in low to intermediate risk countries such as Spain, Thailand and the Philippines indicate that the men's lifetime number of sexual partners and reporting of prostitutes as sexual partners are key determinants of cervical cancer risk in their wives. In Spain, the presence of HPV-DNA in the husband's penis resulted in a fivefold increased risk of cervical cancer in their wives. The risk of cervical cancer among monogamous women increased up to 9- to 10-fold in relation to the presence of HR-HPV types in the penis of their husbands. The increased risk associated with HPV type 16 was six- to ninefold. Furthermore, the prevalence of penile HPV showed a positive



Data includes 595 men that were husbands or stable coital partners of women with and without cervical cancer.

Figure 4 Penile human papillomavirus DNA prevalence by number of sexual partners of husbands of monogamous and non-monogamous women in Spain and Colombia.
HPV, human papillomavirus.
Adapted from Castellsagué et al, 1997.[113]

trend with increasing number of sexual partners and with the number of sexual partners who were prostitutes.[112] In contrast, in high risk countries such as Colombia and Brazil, no associations with cervical cancer risk were found with penile HPV-DNA or with any other indicators of male sexual behaviour.[140]

The lack of association between most male related variables and cervical cancer risk found in high risk countries could be explained by the hypothesis that, in these populations, HPV is such a widespread infection that it reduces the ability of case–control studies to discriminate subjects at a higher risk. Cross-sectional HPV-DNA detection in the penis of adult men, even if it is high, is still a poor reflection of lifetime exposure to HPV and reverse causality cannot be excluded. Other biological markers of lifetime sexual promiscuity in men, such as seropositivity to CT, are consistent in discriminating men's partners at a high risk of cervical cancer, in populations at low and high risk of cervical cancer.[112,140]

Human papillomavirus concordance in couples

Several studies have addressed concordance of genital HPVs in heterosexual couples. Most, but not all,[166] of these studies found a relatively poor correlation of HPV positivity and HPV type in cervical and penile samples.[113,137,167–169] This is particularly important in case–control studies in which the wife has cervical neoplasia, and is therefore a long-term consistent carrier of type specific HPV-DNA, and the husband is, or has been, a transient HPV-DNA carrier.[170] Moreover, in some couples, the current partner may not be the relevant one in determining the woman's risk of HPV persistence and progression to cervical neoplasia. Agreement in HPV findings was modest in couples in which both the wife and husband reported only one lifetime sexual partner.[137] Among women with cervical neoplasia, the relevant infection may have occurred years earlier, and the relatively low prevalence of penile HPV infection in their husbands suggests that viral shedding of advanced cervical lesions is limited. Also, cross-sectional detection of penile HPV may measure relatively recent exposure to HPVs that may be unrelated to the initiation of cervical neoplasia in the wife. Finally, low agreement may be partly due to technical reasons, as a smaller amount of penile exfoliated cells may be obtained in men relative to the cellular yield obtained from the cervix.

3.1.5 *Male circumcision, penile human papillomavirus and cervical cancer*

The IARC multicentric study on male circumcision and its association with cervical cancer compared penile HPV-DNA prevalence in circumcised and uncircumcised men and estimated the woman's risk of cervical cancer according to the husband's circumcision status. The authors found that circumcised men were about three times less likely to harbour HPV in their penis than uncircumcised men. Male circumcision also reduced the risk of both genital HPV-DNA prevalence and cervical cancer in the female partner, particularly and most strongly in women whose male consorts had had a promiscuous sexual history.[119]

3.1.6 *Other sources of transmission of human papillomavirus*

Despite the overwhelming evidence that genital HPVs are predominantly sexually transmitted, some clinical and epidemiological observations have documented that genital HPVs can also be transmitted in other ways, especially from mother to child. This is consistent with other microbial

and viral infections that are predominantly or exclusively sexually transmitted in adults (eg HIV, HBV, HSV-2, CT, *Treponema pallidum* and *Neisseria gonorrhoeae*) and that may be transmitted to newborns if present in a woman during pregnancy or at the time of delivery.

The evidence for the non-sexual transmission of HPVs has been reviewed by several authors[123,171] concluding that:

- genital HPV infections, including genital warts, may occur in sexually naive populations, such as virgins, infants and children
- there is some evidence of horizontal transmission of low risk HPVs
- vertical and perinatal transmission of HPVs from mother to child does exist, although rates vary widely
- high risk genital HPVs have been detected in non-genital mucosa, such as the mouth, oropharynx and conjunctiva, and they have been associated with a fraction of cancers of the oral cavity and oropharynx and with conjunctival squamous cell carcinoma
- there is low concordance of HPV types and HPV16 genomic variants between heterosexual partners.

Vertical and perinatal transmission of human papillomavirus: laryngeal papillomatosis

Non-sexual transmission of HPVs was first suggested in 1956 in a case report of a male child who was born to a mother with condyloma; the child developed symptoms of laryngeal papillomatosis and penile warts at 3 and 6 months after birth.[172] Since then, a large body of epidemiological data on perinatal transmission of HPVs has been accumulating.[123,171] However, studies evaluating transmission of HPV from mother to infant are conflicting.

Several studies have tested infants for HPV-DNA.[173–181] Detection rates in the first 1–2 days of life have ranged from 4% to 72% among infants born to women with genital HPV detected during pregnancy, and from 0.6% to 20% among infants delivered by women with no detectable HPV during pregnancy. Rates of detection at 6 weeks also vary widely, and they are not always significantly different for infants born to mothers with positive or negative assays for genital HPVs.[173,175,178] A problem that is common to these early studies is that specimens were collected only at, or very soon after, delivery, and some used HPV detection assays known to have low sensitivity and specificity.

In contrast, a study that used DNA sequence analyses to determine the source of HPV infection in infants born to HPV16 DNA positive mothers identified concordant HPV variants or prototypic sequences in 9 out of the 13 mother–infant samples analysed. This study indicated that a substantial proportion (up to 69%) of HPV16 positive infants acquire the virus from their mothers.[176]

In a carefully conducted large study, 151 infants born to mothers with known HPV status were evaluated regularly until 3 years of age for the detection of HPV-DNA in samples from the mouth, external genitalia

and anus.[182] PCR was performed with HPV L1 consensus primers and hybridisation to HPV types 6, 11, 16, 18, 31, 33, 35, 39 and 45 and to a generic HPV probe. During pregnancy, 74% (112 out of 151) of women had historic, clinical or DNA evidence of genital HPV infection. At 479 infant visits, HPV-DNA was detected in 1.5%, 1.2% and 0% of genital, anal and nasopharyngeal specimens respectively. HPV prevalence among infants born to HPV positive women was 4% (3 out of 80), and among infants born to HPV negative women was 8% (5 out of 63). All positive results in the infants were positive for unclassified HPV types and all of them were preceded or followed by HPV negative specimens. This study suggests that the few HPV infections detected in infants probably represent low level genital or non-genital HPVs, horizontal transmission or HPV contamination. The study also indicates that the risk of perinatal transmission of HPVs, although present, is probably very low (< 3%).[182]

Perinatal HPV transmission is unequivocally demonstrated for recurrent laryngeal papillomatosis, a rare, potentially life threatening condition associated with HPV types 6 and 11, the HPV types most commonly detected in genital warts. The disease can disseminate through the tracheobronchial tree and progress to pulmonary papillomatosis and subsequent fatal chest infection.[183] As the disease has a bimodal age distribution with the first peak occurring in infancy, it has been postulated that juvenile papillomatosis may be related to HPV infection acquired from a mother with genital warts or subclinical HPV infection. A study showed that the risk of the juvenile form of laryngeal papillomatosis appeared to be highest in first born infants delivered vaginally to an adolescent mother. In contrast, the risk factors identified for adult onset of the disease included lifetime number of sex partners and high frequency of oral sex, suggesting orogenital transmission.[184]

Transplacental transmission

Some studies have shown some evidence of intrauterine infection of HPVs.[185–187] In one study, 24 out of 37 samples of amniotic fluid from women with HPV-DNA or abnormal cytology were HPV positive by PCR.[185] Another study detected HPV16 DNA in cord blood specimens from neonates born to HPV16 positive mothers.[187] Detection of HPV16 DNA in infants born by caesarean section further suggests that prenatal HPV infection may occasionally occur, probably through ascending infection.[181] Finally, a case report describing the detection of epidermodysplasia verruciformis (EV)-related HPV types in amniotic fluid, placenta and cervical scrapes from an EV patient demonstrated that prenatal transmission of EV related HPVs is a plausible option.[186]

Horizontal transmission

Since the original case report on a 5 year old boy with HPV2 positive warts on the anus and hand,[188] a number of other case series have confirmed the possible horizontal transmission of HPVs, particularly of the low risk HPV types.[189] HPV-DNA was detected in the finger brush samples of three out of eight women (one with the same types) with cervical HPV, and in 9 out of 13 men (five with the same types) with penile HPV. In total, 27% of patients had the same HPV types detected in both genital

and hand samples. These findings raise the possibility that patients with genital warts may transfer not only genital HPVs to their sexual partners by finger–genital contact but also horizontally to their children.[190]

Finger–conjunctiva HPV transmission has been suggested by studies reporting presence of HPV-DNA, predominantly type 16, in human ocular surface squamous neoplasias, including conjunctival carcinomas.[191] A recent study in Uganda, a high risk area for this tumour, found a statistically significant association between high titres of HPV16 antibodies and conjunctival squamous cell carcinoma.[192]

Indirect transmission via HPV contaminated fomites (clothing, sheets, towels, objects and instruments) has also been suggested by several studies,[1,193–195] but its impact in passing and inducing active infections is most probably small, if it exists at all.

Blood, breast milk and sperm

It is very unlikely that HPVs may be transmitted via blood, as HPVs do not have a known viraemic phase, and no case reports of HPV detection in blood have been documented. Several studies have detected HPV in a significant proportion of breast cancer patients, particularly among patients with preinvasive cervical lesions and in women with benign tumours of the mammary nipple.[196,197] However, results are not consistent in the literature, and in some cases HPV lesions in mammary nipples have been associated with condyloma of the skin around the nipple.[198]

Transmission of HPV to infants via the process of breastfeeding has not been documented.

The possible role of sperm as a vector for HPVs has been explored in several studies.[168,199–203] One study detected HPV-DNA sequences in 64% of sperm specimens using PCR primers targeting small gene regions.[199] Another study found a correlation between HR-HPVs in the cervix and semen of sexual partners. The source of HPV in semen was urethral epithelial cells, isolated on a Percoll gradient, which were sloughed from the urethral epithelium during ejaculation.[168] A recent study using DNA amplification by nested PCR detected viral sequences in the sperm cells of 53% of subjects with past or current HPV infections and in 8% of healthy subjects.[202]

Evidence from these studies suggests that semen may be a transmitter of cell associated HPV during the process of ejaculation.

3.2 Human papillomavirus and cervical neoplastic lesions

The epidemiological evidence relating HPV-DNA to cervical cancer and its precursors includes a large and consistent body of studies indicating, beyond any reasonable doubt, strong and specific associations relating HPV infections to cervical cancer. The observations have been reported from all countries where investigations have taken place. Natural history and follow up studies have clearly shown that HPV infection preceded the development of cervical cancer by a number of years and confirmed that sexual transmission is the predominant mode of HPV acquisition. These studies satisfied in biological terms the long known clinical and

epidemiological observations that cervical cancer displayed the profile of an STD. Case–control studies, case series and prevalence surveys have unequivocally shown that HPV-DNA can be detected in adequate specimens of cervical cancer in 95–100% of the cases compared with a prevalence of some 5–20% from cervical specimens of women identified as suitable epidemiological control subjects.

The association has been recognised as causal in nature by a number of international review parties since the early 1990s, and the claim has been made that this is the first necessary cause of a human cancer ever identified.

The implications of the recognition that, in the absence of viral DNA, cervical cancer does not develop are of considerable practical importance. On the one hand, the concept of risk groups comes into focus. High risk women can now be redefined as the group of persistent HPV-DNA carriers. Operatively, this represents substantial progress from previous versions of the high risk group that identified women by their exposure to a number of ill defined factors (low socioeconomic status, high number of sexual partners, smoking, use of OCs, history of STDs and any combination of the above). Most of these factors are now viewed either as surrogates of HPV exposure or as relevant cofactors in the presence of HPV-DNA. On the other hand, if indeed HPV is a necessary cause of cervical cancer, the implication is that specific preventative practices targeting some putative non-HPV related cervical cancer cases are no longer justified. Finally, technology is now available to screen the HPV-DNA positive women from the general population. Therefore, the final consideration on the nature of the association between HPV and cervical cancer is of considerable public health relevance.

3.2.1 Key groups of studies and causality criteria

Epidemiological criteria to evaluate the causality of any given association have been developed and largely adopted over time. In addition to the classic Bradford Hill criteria, the IARC has developed special rules to interpret specifically the associations between viral agents (and other biological agents) and human cancer. Table 4 presents the key criteria to be examined and a qualitative appreciation of their fulfilment by the available epidemiological evidence of the association between HPV and cervical cancer published in 2002.[2] A brief selection of key studies is presented, outlining their contribution to the fulfilment of the currently accepted key causality criteria: temporality, strength and consistency of the association; biological plausibility; and exclusion of alternative explanations.

3.2.2 Temporality

Of the criteria outlined by Bradford Hill and repeatedly endorsed by the IARC, and other bodies, the demonstration that exposure has occurred before the diagnosis is considered a sine qua non condition for a risk factor and for establishing causality. Four groups of studies have contributed data relevant to the temporality criterion.

Descriptive data

Cross-sectional studies have repeatedly reported that subclinical HPV infections are highly prevalent in young individuals, whereas invasive

Table 4 Causality criteria and fulfilment evaluation of the association of human papillomavirus DNA and cervical cancer

		HPV and cervical cancer	
Criteria	**Concept**	**Type of evidence**	**Evaluation**
Time sequence	Exposure must precede disease	Cohort studies to CIN2/3	+++
Experimental (prevention)	Reduction of disease following reductions in exposure	Early vaccination trials to CIN	+
Strength and consistency	High OR/RR; robust association in different settings	Case–control studies	+++
Biological plausibility and coherence	Mechanisms: consistent with previous knowledge	Experimental	+++
Dose–response	Risk of disease is related to level of exposure	Studies on number of partners Studies on viral load	+
Qualification of causality			
Necessary	Exposure is present in all cases	Detailed investigation on 'HPV negative' cervical cancer specimens; exclusion of alternative explanations	++
Sufficient	Exposure always leads to disease	Natural history of transient infections	–

CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; OR, odds ratio; RR, relative risk.
Adapted from Bosch et al, 2002.[2]

cervical cancer typically develops in the third decade and above (Figure 5). The cross-sectional prevalence of HPV-DNA decreases spontaneously to a background level of 2–8% in most populations in groups that are 35 years old and above. In countries where intensive screening of young women takes place, part of the HPV prevalence reduction could be attributable to aggressive treatment of HPV related cervical lesions. Women who remain chronic HPV carriers are described at present as the true high risk group for cervical cancer. In some populations, a second mode of HPV-DNA prevalence has been observed for older women (ie 50 and above), and a second mode in the incidence of CIN3 lesions and invasive cervical cancer has also been reported.[161,206] In all settings investigated, the point prevalence of HPV-DNA in the young age groups is strongly related to the sexual behaviour patterns that are dominant in each population.[101,105,115,204,207,208]

These population studies provide support for the concept that HPV infections precede the development of cervical cancer by some decades. In fact, from most cancer registries, including the US based registries, it is well established that the age specific incidence of cervical cancer has a rising trend in the age interval 20–40, and shows a plateau or continues to increase smoothly after that age. Only occasionally do cases of invasive disease occur at an earlier age. Figure 5 shows the age specific cross-sectional prevalence of HR-HPV-DNA in a screening programme in the Netherlands, and the corresponding age specific incidence rates of cervical cancer in that country. The distributions shown in Figure 5 are highly reproducible in studies in other settings in high and low risk countries.[134,139,205,206]

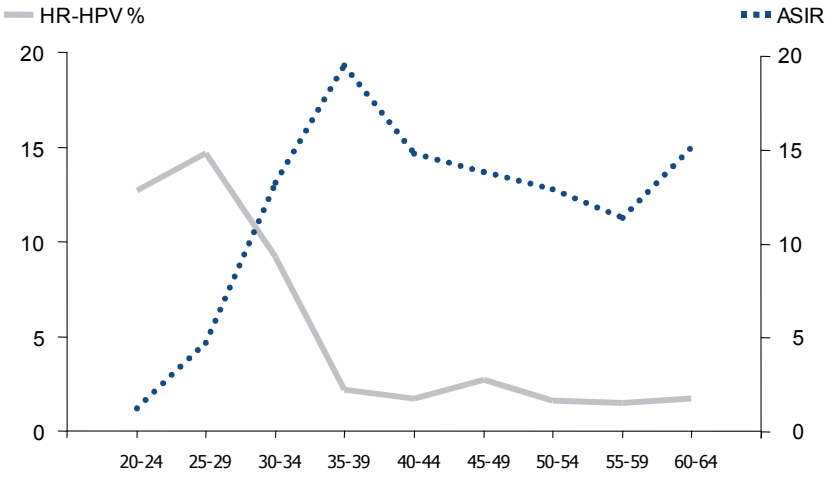


Figure 5 Age specific prevalence of high risk human papillomavirus DNA in 3700 women entering a screening programme and age specific incidence rate (ASIR) of cervical cancer in the Netherlands.
Data from Jacobs et al, 2000;[204] Parkin et al, 1997.[205]
Adapted from Bosch et al, 2002.[2]

Follow up studies

For cervical cancer and HPV, compliance with temporality criteria has been established by numerous cohort studies that monitored women from cytological normality to the stage of high grade cervical intraepithelial neoplasia (HSIL or CIN 2/3). Monitoring of women to invasive disease is not acceptable on ethical grounds, and thus that information is not available.

Repeated sampling of women being followed for viral persistence and cervical abnormalities has shown that the median duration of the infections is around 8 months for HR-HPV types compared with 4.8 months for the low risk HPV types. In two unrelated studies, the time estimates were fairly consistent. In one study in a high risk population in Brazil, the mean duration of HPV detection was 13.5 months for HR-HPV types and 8.2 months for the non-oncogenic types. HPV16 tended to persist longer than the average for high risk types other than HPV16.[209] The results were remarkably similar in student populations in the USA and in the UK.[210,211] The self-limiting course of most HPV infections is consistent with the cross-sectional profile displayed in Figure 5. However, the currently observed time intervals may still suffer from imprecision in the estimates of time at first exposure, from the variability in the endpoint definition and from censoring because of treatment of the early lesions.

Follow up studies of women with and without cervical abnormalities have indicated that the continuous presence of HR-HPV is necessary for the development, maintenance and progression of progressive CIN disease.[210,212–215] A substantial fraction (ie 15–30%) of women with HR-HPV-DNA who are cytomorphologically normal at recruitment will develop CIN2 or CIN3 within the subsequent 4 year interval.[213,216,217] Conversely, among women found to be HR-HPV-DNA negative and

cytologically identified as having either atypical squamous cells of undetermined significance (ASCUS) or borderline or mild dysplasia, CIN2/3 is unlikely to develop during a follow up of two years and the cytology is likely to return to normal.[218,219] Women found to be positive for low risk HPVs rarely become persistent carriers and their probability of progression to CIN2/3 is extremely low.[219,220]

As ongoing cohorts expand their follow up time, more precise estimates are being provided on the predictive value of viral persistence as defined by repeated measurements of viral types and variants. One such cohort in Sao Paulo has shown that the incidence of cervical lesions in women who were tested twice and found to be HPV negative was 0.73 per 1000 women–months. The corresponding incidence among women with repeated HPV16 or HPV18 positive results was increased 8- to 12-fold. The odds ratio (OR) among women who were tested twice and found to be HPV positive for the same oncogenic types, a more stringent definition of persistency, was 41.2 (95% confidence interval (CI) 10.7–158.3).[221] Retrospective assessment of HPV status using archival smears from cases of cervical cancer and control subjects has provided evidence that HPV-DNA preceded the development of invasive disease, highlighting its value in signalling false negative smears.[219] An interesting observation of the same follow up study suggests that clearance of HR-HPV in otherwise established cytological lesions is a marker associated with regression of CIN lesions.[218,222] Finally, persistence of HPV-DNA detection after treatment for CIN2/3 is an accurate predictor of relapse that is at least as sensitive as repeated vaginal cytology.[223]

These results are useful in defining the clinical role of HPV testing in screening and follow up of women following resection of CIN. However, most observations on preinvasive disease have limitations for determining cervical cancer causality because, even in controlled settings, observations are not allowed to continue beyond the stage of HSIL/CIN3 or carcinoma in situ. The role of HPV in cervical cancer must thus be measured in conventional case–control studies.

Retrospective cohorts

A particularly interesting approach to conducting follow up studies of invasive cancer (as opposed to studies of CIN3) without ethical and time constraints is provided by nested case–control studies. These are follow up studies initiated several years in the past that assembled and stored large banks of biological specimens from healthy individuals. Linkage studies with cancer registries can then identify cases of cervical cancer (or any other condition) that have occurred in the interval, and the original specimens can be analysed for the presence of HPV biomarkers. HPV-DNA prevalence can be compared with the corresponding prevalence in specimens of epidemiologically sound control subjects (individuals from the same cohort who did not develop the condition under otherwise equivalent exposures). These studies documented the existence of HPV exposure years before the development of the disease, thus reproducing the conditions of a longitudinal study. With this approach, a relative risk (RR) estimate of 16.4 (95% CI 4.4–75.1) was observed for invasive cervical cancer in Sweden using DNA extracted from stored Pap smears[224]

and of 32 (95% CI 6.8–153) in the Netherlands.[219] In a similar study design, an OR of 2.4 (95% CI 1.6–3.7) was obtained using serological markers of HPV exposure.[225]

Preventative interventions

Since the late 1980s, multiple studies have evaluated HPV testing as an adjunct to cytology in screening programmes. These have considered HPV testing either as a triage test in cases of mild abnormalities[226–228] or as a primary screening test.[229–231] This literature has been reviewed extensively.[232] Triage studies have shown that HPV testing is more sensitive that repeated cytology in identifying underlying high grade lesions in women with ASCUS.[214,220,222,233,234] Studies that reflect primary screening conditions (in the absence of fully randomised trials) have shown that the sensitivity of HPV tests is higher than standard cytology in detecting high grade lesions, whereas the specificity is age dependent. HPV tests show lower specificity than cytology in younger women, accounting for the bulk of transient infections, whereas in older women (ages 30–35 and above) specificities tend to be similar for both tests.[208,235,236]

In terms of causality assessment, these studies showed that it is possible to predict the concurrent presence of neoplastic disease (usually HSIL, CIN2/3 or severe dyskaryosis) or the risk of developing it by means of HPV-DNA detection. This property of the HPV test offers an indirect measurement of the strength of the association and of the temporal sequence of the events.

In summary, it has been established that HPV infections precede cervical precancerous lesions by a substantial number of years. The epidemiology and the dynamics of HPV infection in populations satisfy previous observations that related cervical cancer to a sexually transmitted disease.

3.2.3 *Strength of the association*

This criterion is usually discussed by examining the magnitude of the RR or the OR, which is the estimate of the RR in case–control studies. We shall use as the primary example the results of the IARC's multicentric case–control study on invasive cervical cancer, extensively published during 1992–2004. In brief, this project included nine case–control studies in different parts of the world, mostly in high risk countries. HPV-DNA testing was carried out in two central research laboratories using the MYO9/11[237] and General Primer (GP) GP5+/6+ [47,48] PCR testing systems. The published results have reported ORs for cervical cancer in the range of 50- to 100-fold for HPV-DNA. ORs for specific associations (ie HPV16 and squamous cell cancer and HPV18 and cervical adenocarcinomas) range from 100 to 900. These estimates lead to calculations of attributable fractions for the entire study that are greater than 95%.[238]

Table 5 shows the size of the multicentric case–control study, the prevalence of HPV-DNA in each relevant group, and the estimates of the OR. Figure 6 shows the HPV-DNA prevalence in cervical cancer cases and controls in eight countries. It is noteworthy that the first two studies conducted in Spain and Colombia used early versions of the MYO9/11 PCR system; HPV-DNA was identified in some 75% of the cases. The rest of the studies were analysed using the GP5+/6+ PCR system and its

Table 5 Size of the IARC's multicentric case–control study and human papillomavirus prevalence

			Cervical cancer					
	Controls		Adenocarcinoma and mixed		Squamous		Adenocarcinoma and mixed	Squamous
Number of countries	*n*	HPV-DNA (% +)	*n*	HPV-DNA (% +)	*n*	HPV-DNA (% +)	OR (95% CI)	
9*	2491	(13.4)	–	–	2365	(90.7)	–	83.3 (54.9–105.3)
6†	1466	(15.6)	141	(91.9)	2280	(96.6)	68.7 (36.2–130.5)	–

CI, confidence interval; HPV, human papillomavirus; OR, odds ratio.
*Brazil, Morocco, Paraguay, the Philippines, Thailand, Peru, Mali, Spain and Colombia.
†Brazil, Morocco, Paraguay, the Philippines, Thailand and Peru.

modifications, which resulted in an almost 20% increase in HPV-DNA detection rate in cervical cancer cases.

Given the case–control design of the study, these very high ORs reflect the risk in relation to existing HPV-DNA in cervical cells (HPV-DNA point prevalence), not in relation to 'ever' being infected with HPV (cumulative lifetime exposure). Furthermore, if HPV-DNA shedding was intermittent among control subjects, their corresponding HPV prevalence would have been underestimated, resulting in an increase in the ORs. The HPV-DNA point prevalence at advanced age (ie over 40 years of age) is usually thought to reflect viral persistency. However, much research is still devoted to properly define viral persistency and its prognosis, a crucial definition for the clarification of the uses of HPV testing in screening and patient management.[211]

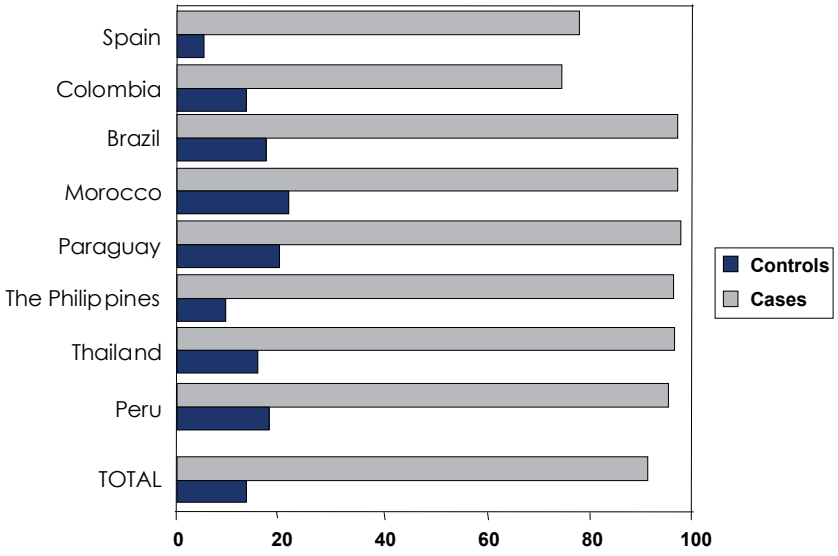


Figure 6 Prevalence of human papillomavirus DNA in cases and control subjects in the IARC's multicentric case–control study.
Data from Muñoz et al, 1992 (Spain and Colombia);[138] Eluf-Neto et al, 1994 (Brazil);[136] Chaouki et al, 1998 (Morocco);[239] Rolón et al, 2000 (Paraguay);[240] Ngelangel et al, 1998 (The Philippines);[141] Chichareon et al, 1998 (Thailand);[135] Santos et al, 2001 (Peru).[241]
Adapted from Bosch et al, 2002.[2].

Type specific risk estimates

The pool of IARC studies was large enough to provide type specific risk estimates for 18 types. Restricting the analyses to the studies that used the GP5+/6+ HPV detection system and to squamous cell carcinomas, the adjusted ORs for HPV-DNA detection (the factor by which the reference risk of cervical cancer is multiplied if HPV-DNA is detected) of any single type was 158.2 (95% CI 113.2–220.6).

Type specific risk estimates were as follows: HPV16, OR = 435; HPV18, OR = 248; HPV45, OR = 198; HPV31, OR = 124; HPV52, OR = 200; HPV33, OR = 374; HPV58, OR = 115; HPV35, OR = 74; HPV59, OR = 419; HPV51, OR = 67; HPV56, OR = 45; HPV39, infinity; HPV68, OR = 54. ORs and confidence limits are displayed in Figure 7. These studies concluded that, in addition to HPV16 and 18, the evidence is now sufficient to consider as high risk carcinogenic types HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68. Some evidence is also reported on a significant risk for HPV73 and 82.

A second group of HPV types is rarely found in cases and has been classified as low risk; this group includes HPV6, 11, 40, 42, 43, 44, 54, 61, 72 and 81, and CP6108.

From the IARC's and other studies, a small group of HPV types remains in the category of uncertain risk. The reasons are multiple: epidemiologically, the group includes the types for which the imprecision of OR is related to very small number of observations (despite very large studies). Further, it is now recognised that the type-specific sensitivity of some

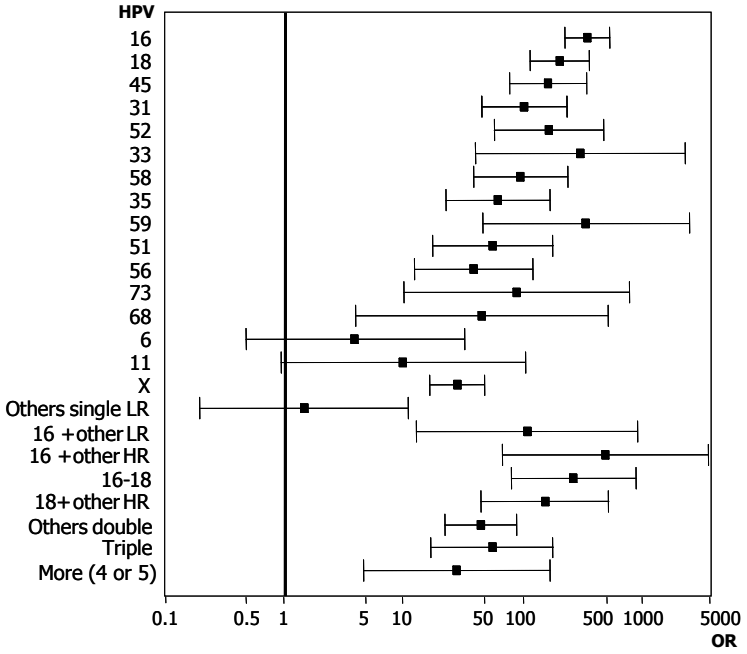


Figure 7 Human papillomavirus type specific ORs and 95% CIs for cervical cancer.
HPV, human papillomavirus; HR, high risk; LR, low risk; OR, odds ratio.
Data from Muñoz et al, 2003.[6]

of the assays used in the different studies varies for some rarer types or in the presence of multiple infections. Moreover, some HPV types (ie HPV 73 or HPV70) are classified discordantly according to whether sequence homology and experimental evidence is used or epidemiological observations are given priority. Finally, studies that are conducted in different populations reflect the underlying geographical variability, and the rarer HPV types may be uninformative in some settings while being clearly associated with disease status in other populations. The types of uncertain nature currently include HPV26, 53, 73, 82 and 70, and perhaps others.

Figure 7 shows that the risk for any given high risk type was not statistically different from the risk reported for HPV16. Likewise, the risk related to the presence of multiple HPV types in the specimen is no different from the risk linked to a single HPV type. Multiple HPV types were detected in the multicentric study in on average 9.4% of the cases and 2.2% of the control subjects, and did not show a statistically significantly increased risk (OR 114.9; 68.8–191.7) for women positive for only one HPV type (OR 172.6; 122.2–243.7).The standard estimates of the attributable fraction (the proportion of disease that is related to HPV-DNA) derived from these and most other studies ranged from 90% to 98%.

Under extremely rare circumstances, HPVs of the low risk group (ie HPV6 or 11) are found as the only type in specimens of invasive cervical cancer. Although statistically these are largely non-significant increases in risk, it should be remembered that the ability of the oncoproteins E6 and E7 of the low risk types also show transforming capacity at a very low level. It is plausible that a minute fraction of the population harbours a special susceptibility to HPV, and even the presence of a low risk type is capable of initiating a neoplastic process.

The results of the multicentric study are consistent with findings from other countries that have generated recent data on invasive cervical cancer and preinvasive disease in Costa Rica,[161] Thailand,[242] Norway,[243] Denmark[244] and virtually all other countries in which these studies have been conducted.

The proportion of multiple types in a given specimen varies across studies and particularly in relation to the HPV detection method used. Table 6 provides an indication of the proportion of specimens from cases and from the general population that showed multiple types. This table suggests that populations at high risk of cervical cancer and high rates of HIV positivity tend to show higher proportions of multiple types than populations not belonging to these risk groups. Longitudinal studies have suggested that the one time, cross-sectional detection of type specific HPV may underestimate the cumulative lifetime diversity of exposures to HPV.[211]

In summary, the association of HPV-DNA in cervical specimens and cervical cancer is one of the strongest ever observed for a human cancer. HPV16 accounts for close to 50% of the types identified in cervical cancer. The cancer risk for any one of at least 10 HPV types or for any

Table 6 Prevalence of multiple human papillomavirus types in women with and without cervical cancer

		Cases CX	Controls	
Reference	**Study**	**Percentage of all specimens**	**Percentage of the HPV+**	**Percentage of all**
1	IARC's multicentric	4–20	10	1–3
2	Rural Costa Rica	32	38	4
3	Rural Mozambique	–	41	15
4	Imprisoned women, Spain	–	71	20
5	HIV+, USA	–	42	–
	HIV–, USA	–	16	–

HIV, human immunodeficiency virus; HPV, human papillomavirus.
References: 1, Muñoz et al;[165] 2, Herrero et al;[161] 3, Castellsagué et al;[245] 4, de Sanjosé et al;[246] 5, Palefsky et al.[247]
Adapted from Bosch et al, 2002.[2]

combination of HPV types does not differ significantly. The practical conclusions from these analyses indicates that, under current evidence, group testing of clinical specimens for a cocktail of high risk types should be sufficient for screening and patient management.

3.2.4 Consistency

There is a striking consistency between the results of the multicentric case–control study and over 50 other studies conducted in other countries under different protocols and HPV-DNA testing systems. Figure 8 summarises the results of recent studies that compared the prevalence of HPV-DNA in cervical cancer cases and control subjects. The literature is consistent, despite the variability in study designs, HPV testing method or case definition. Some of the studies used the prevalence of HPV16 DNA to calculate ORs and some reported results for HPV-DNA (all types combined). Some studies focused on invasive cervical cancer, whereas others used preinvasive lesions as the definition of cases. When indicated, separate analyses are presented for squamous cell carcinomas and adenocarcinomas. Studies that have compared risk factors for CIN3 and invasive cancer have not reported any significant differences in their associations with HPV or with their epidemiological profile.[242,252]

Figure 8 also demonstrates the consistency of results between squamous cell carcinomas and adenocarcinomas, the consistency of findings between preinvasive disease and invasive cancer, and the consistency of findings between risk estimates for HPV-DNA (all types considered) and risk estimates restricted to high risk types.

In summary, the association of HPV-DNA in cervical specimens and cervical cancer is consistent in a large number of investigations in different countries and populations. There are no published studies with observations challenging the central hypothesis on causality.

3.2.5 Exclusion of alternative explanations

In the early studies of HPV and cervical cancer, and currently in the majority of real life studies, a fraction of cases are labelled as HPV negative and investigated under the hypothesis that HPV negative cases were a

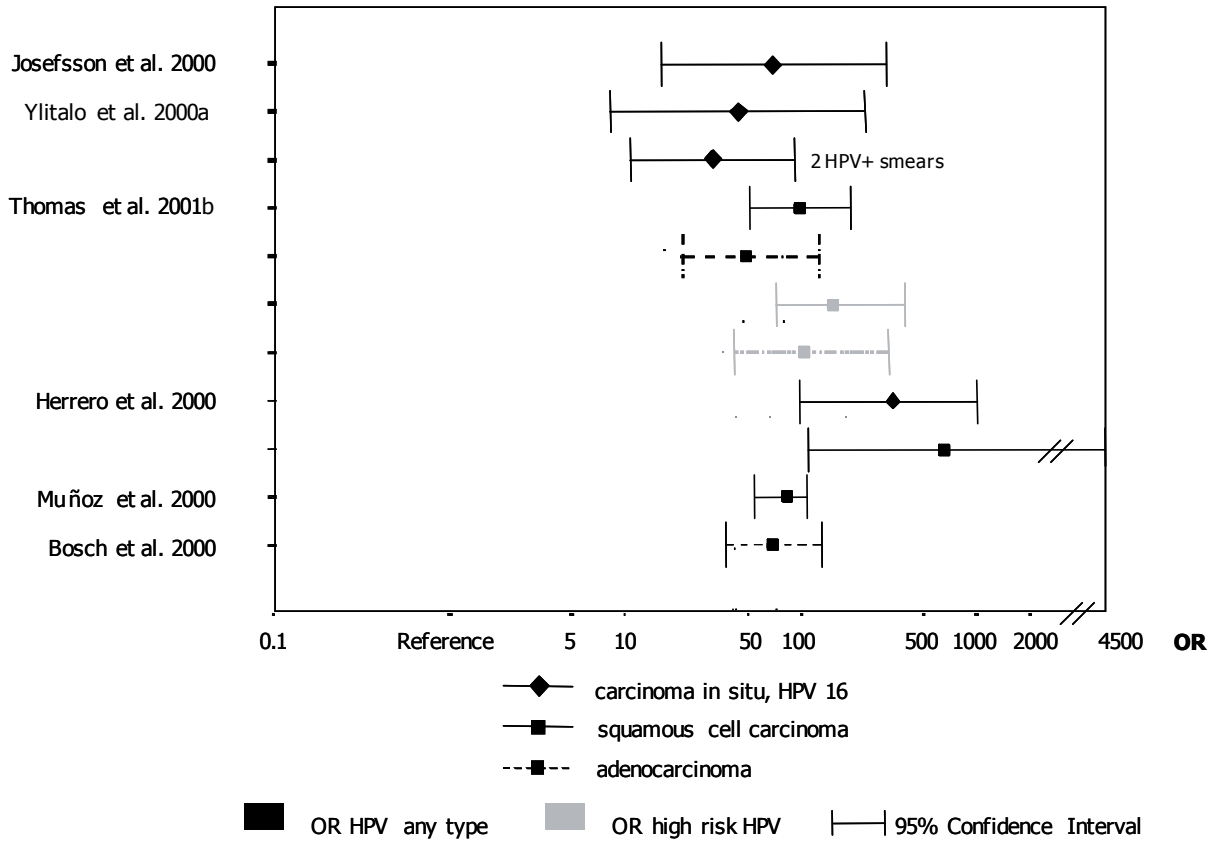


Figure 8 ORs and 95% CIs for associations found in case–control studies after 2000.
HPV, human papillomavirus; CI, confidence interval; OR, odds ratio.
Data from Josefsson et al, 2000;[249] Ylitalo et al, 2000;[251] Thomas et al 2001;[250] Herrero et al, 2000; [161] Muñoz et al, 2000;[165] Bosch et al, 2000.[2]
Adapted from Bosch et al, 2002.[2].

true biological entity.[253,254] The proportion of such cases tends to be more important in studies of preinvasive neoplasia.[255,256] As a consequence, for some time there was some uncertainty in the interpretation of the results. In the IARC's studies, HPV negative cases were compared with HPV positive cases in relation to their epidemiological profile (Table 7). In broad terms, it was clear that 'HPV negative' cases retained the same traits as the rest of the cases (ie similar age and association to high number of sexual partners, young age at first sexual intercourse, long term use of contraceptives and high parity). These results strongly suggested that the apparently HPV negative cases were also related to an STD pattern; however, none of the known sexually transmitted agents that had occasionally been associated with cervical cancer was able to satisfy the causality criteria outlined in Table 1. In the evaluation of putative HPV negative cases, it has been repeatedly demonstrated that lack of identification of HPV-DNA could be attributed primarily to the poor quality of the specimen (tumour necrosis, lack of cancer cells in the specimen, poor preservation) and to the quality of the amplification system used. Walboomers et al [7] showed that, using histological verification of the specimen and the GP5+/6+ testing system, HPV-DNA could be recovered from 99.6% of cases of cervical cancer worldwide, thus claiming that HPV was indeed a necessary cause of the disease.

Table 7 Impact of different strategies of human papillomavirus adjustment on associations between cofactors and risk of cervical cancer (from the IARC case–control studies)

	All women			HPV positive women	
Cofactor	**Cases/controls**	**Not HPV adjusted OR (95% CI)**	**HPV adjusted OR (95% CI)**	**Cases/controls**	**OR (95% CI)**
Full term pregnancies (status and number)					
Never	95/164	1	1	57/24	1
Ever	2183/2209	1.08 (0.80–1.47)	1.32 (0.88–1.98)	1616/229	2.45 (1.33–4.51)
1 or 2	444/747	0.82 (0.60–1.12)	0.99 (0.65–1.50)	279/59	1.79 (0.94–3.40)
3 or 4	644/677	1.33 (0.97–1.83)	1.68 (1.09–2.57)	450/70	2.61 (1.37–5.00)
5	1095/785	1.73 (1.24–2.41)	2.03 (1.30–3.16)	887/100	3.88 (1.99–7.55)
Oral contraceptive use (status and years)					
Never	1419/1508	1	1	1071/163	1
Ever	864/886	1.09 (0.94–1.27)	0.97 (0.79–1.19)	605/92	1.13 (0.80–1.59)
1–4 years	351/445	0.90 (0.75–1.09)	0.75 (0.58–0.97)	274/64	0.66 (0.45–0.98)
5 years	510/427	1.33 (1.11–1.59)	1.17 (0.92–1.49)	331/28	2.35 (1.44–3.85)
Smoking (status and amount)					
Never	1645/1905	1	1	1265/218	1
Ever	636/488	1.30 (1.11–1.52)	1.68 (1.36–2.08)	409/36	1.99 (1.29–3.07)
1–5 cigarettes/day	251/200	1.21 (0.98–1.51)	1.46 (1.09–1.97)	181/17	1.72 (0.98–3.01)
6 cigarettes/day	350/216	1.79 (1.45–2.22)	2.07 (1.56–2.75)	211/18	2.16 (1.18–3.97)

CI, confidence interval; HPV, human papillomavirus; OR, odds ratio.
ORs adjusted for centre, age (< 37, 37–45, 46–55, 56), educational level (none, primary, secondary or higher), smoking (never, 1–5 cigarettes per day, 6 cigarettes per day), age at first sexual intercourse (< 17, 17–18, 19–22, 23), lifetime number of sexual partners (1, 2 or 3, 4), oral contraceptive use (never, 1–4 years, 5–9 years, 10 years), lifetime number of Pap smears (0, 1–5, 6), and parity (0, 1 or 2, 3 or 4, 5 or 6, 7).

In the last decade there has not been any sound epidemiological or biological study indicating that the aetiology of cervical cancer could be independent of HPV. The hypothesis should, however, be retained as a scientific and research option. The grounds for such consideration lie in that: (1) epithelial cells are capable of developing into cancer cells and cancer growth in all human tissues regardless of a known, viral or non-viral cause; thus, cells in the human cervix might too; (2) cell genes that intervene in HPV related carcinogenesis should be able to generate spontaneous or induced mutations leading to cancer in the absence of HPV; available evidence suggests that this event is rare within the lifespan expectation of the human population; (3) relatively few cases of cervical cancer in the very old woman have been investigated; it is likely that the non-HPV related cancers currently occur very rarely and probably cluster in very old women; and (4) non-epithelial cancers do occur in the cervix at a low frequency.

In conclusion, alternative (non-HPV related) hypotheses to explain a fraction of cervical cancer are not being proposed. The hypothesis that a small fraction of cases may occur in the absence of HPV should be retained for research purposes. However, public health recommendations targeting a putative proportion of HPV negative cervical cancer cases are not supported by current results and are not justified.

Systematic reviews of causality criteria strongly indicate that the association of HPV and cervical cancer is causal in nature. The association is very strong, consistent, specific and universal. HPV infection precedes preinvasive disease, and the evidence for biological plausibility of the association is persuasive beyond reasonable doubt.

4. OTHER ENVIRONMENTAL RISK FACTORS FOR CERVICAL CANCER

Historically, several factors have been associated with cervical cancer. In light of the HPV centred model of carcinogenesis, these associations have been re-evaluated to resolve factors which are surrogate estimates of HPV exposure (such as number of sexual partners) and to identify those that may act as modulators or promoters of the HPV induced neoplastic process. Putative cofactors of interest include biological agents, smoking, hormonal exposures and dietary factors.

4.1 Analytical strategies

Using the IARC series of case–control studies, it was possible to estimate the impact of different strategies of HPV adjustment to evaluate associations between environmental cofactors and cervical cancer risk. Table 8 shows the results of these different strategies for three cofactors of interest: one model included all case patients and all control subjects but ignored adjustment for HPV; a second model included all subjects but statistically adjusting for HPV-DNA status; and a third model restricted the analysis to case patients and control subjects who tested positive for HPV-DNA. As shown in Table 8, of the three strategies, models restricted to HPV-DNA positive subjects yielded higher associations, which were between 1.1- and 2.0-fold higher than those derived from HPV adjusted models.

4.2 Biological risk factors: herpes simplex virus 2, *Chlamydia trachomatis* and human immunodeficiency virus

The IARC multicentric case–control study investigated the presence of antibodies against the common STDs to assess their role in cervical cancer risk in the presence of HPV-DNA. Relevant results have shown that among HPV positive cases and control subjects there is a residual 1.5- to 2-fold increased risk, suggesting an interaction with the oncogenic capacity of HPV.

4.2.1 Herpes simplex virus 2

The pooled analyses of seven case–control studies included 1262 cases of invasive cancer and 1117 matched control subjects. Western blot analyses were used to detect type specific antibodies to HSV-1/2. As expected, seroprevalence was higher in cases than in control subjects, and the risk of cervical cancer was significantly higher in analyses restricted to HPV-DNA positive cases and control subjects and adjusted for other possible confounders (OR 2.19; 95% CI 1.41–3.40). The association was consistent in squamous cell cancers and adenocarcinomas.[258]

4.2.2 Chlamydia trachomatis

The prevalence of *C. trachomatis* antibodies varies greatly by country, and *C. trachomatis* serum antibodies were associated with a 1.8-fold increased risk of squamous cell invasive cervical carcinoma in all countries considered except Spain. The risk was higher in women with elevated *C. trachomatis* antibody titres and in women under 55 years of age. *C. trachomatis* and *Chlamydia pneumoniae* species specific serum antibodies were differentiated using microimmunofluorescence assay. The increased risk of squamous cell invasive cell carcinoma was found in women with *C. trachomatis* but not in those with *C. pneumoniae* antibodies. The study thus supports the possibility that *C. trachomatis*

Table 8 Summary results of studies assessing the role of smoking in cervical carcinogenesis among HPV-DNA positive women

Exposure measures	Study (study outcome)						
	Denmark (HSIL)	**Norway (CIN2–3)**	**USA, Eastern (CIS and CC)**	**Manchester (CIN3)**	**Costa Rica (HSIL and CC)**	**USA, Portland (CIN3 and CC)**	**IARC (CIS and CC)**
Smoking status							
Ever vs. never	NR	**4.6 (0.9–22.9)**	1.5 (0.7–3.0)	**2.2 (1.4–3.4)**	NR	NR	**2.2 (1.5–3.2)**
Former vs. never	**3.2 (0.9–11.4)**	4.2 (0.5–37.9)	1.2 (0.5–3.1)	1.7 (0.8–3.7)	1.7 (0.8–4.0)	**2.1 (1.1–3.9)**	**1.8 (0.9–3.4)**
Current vs. never	**1.9 (1.0–3.8)**	NR	1.6 (0.7–3.5)	NR	**2.3 (1.2–4.3)**	NR	**2.3 (1.3–4.0)**
Smoking amount							
[cigarettes/day] vs. never	NR	[≤10] 3.3 (0.5–21)	[≤19] 1.6 (0.6–3.9)	[≤10] 1.4 (0.7–2.5)	[≤5] **1.8 (1.0–3.3)**	[≤19] **2.2 (1.2– 4.2)**	[≤5] **1.9 (1.1–3.4)**
		[≥11] **5.9 (1.0–36)**	[≥20] 1.3 (0.6–3.0)	[≤16] **2.2 (1.2–3.9)** [≥17] **3.1 (1.8–5.3)**	[≥6] **3.1 (1.2–7.9)**	[≥20] **2.9 (1.5– 5.6)**	[≥6] **2.2 (1.2–4.2)**
P for trend	NR	NR	*0.49*	***<0.0001***	***0.003***	NR	NS
Smoking duration							
[years] vs. never	NR	[≤9] 2.1 (0.3–12.3)	[≤10] 1.2 (0.5–3.0)	[≤9] **1.8 (0.9–3.6)**	[≤9] **2.2 (1.0–4.8)**	NR	[≤19] **2.6 (1.4–4.7)**
		[≥10] **7.5 (1.2–46)**	[≤20] 3.2 (1.0–9.7)	[≤19] **2.0 (1.2–3.3)**	[≥10] **2.0 (1.0–3.8)**		[≥20] **1.9 (1.0–3.5)**
			[>20] 0.8 (0.3–2.7)	[≥20] **3.1 (1.6–6.2)**			
P for trend	NR	NR	*0.57*	***<0.0005***	NR		NS
Comments		Stronger associations found among HPV16 seropositive subjects	For squamous cell carcinoma	Univariate OR; adjusted OR also statistically significant			

CC, cervical cancer; CIN, cervical intraepithelial neoplasia; CIS, carcinoma in situ; HSIL, high grade squamous intraepithelial lesion; NR, not reported; NS, not significant; OR, odds ratio.
Bold figures denote statistical significance.
Adapted from Castellsagué and Muñoz, 2003.[257]

increases squamous cell invasive cervical carcinoma risk, after accounting for cervical HPV infection.[259]

4.2.3 *Human immunodeficiency virus, human papillomavirus and cervical cancer*

The evidence of a putative interaction between HPV and HIV in the origin of cervical cancer was formally recognised when cervical cancer was included as one of the criteria of acquired immune deficiency syndrome (AIDS) among HIV positive women. The subsequent literature largely confirmed the evidence, although some major confounders of the epidemiological association tend to obscure the results. In brief, these refer to the powerful impact of screening in some populations, the medical surveillance of HIV carriers in developed countries and the short survival time of HIV/AIDS patients in many populations at high risk of cervical cancer in relation to the time intervals between HPV infection and cervical cancer.[260–263]

Cross-sectional studies on the prevalence of human papillomavirus and cervical intraepithelial neoplasia lesions in HIV carriers and non-carriers

Massad and colleagues[264] reported on the baseline cervical cytology from 1713 HIV positive women and 482 HIV negative control women. Cervical cytology was abnormal in 38.3% of HIV positive women compared with 16.2% of the HIV negative women. High grade lesions, low grade lesions and ASCUS were all significantly more common among HIV infected women. The RRs for any abnormal cytology were a CD4 cell count lower than 200/mm^3 (OR 2.13; 95% CI 1.45–3.13), the presence of HPV-DNA and a previous history of abnormal cytology.

Ahdieh et al[265] identified a higher baseline prevalence of cervical abnormalities among HIV infected women (13.4%) than among HIV negative women (2.4%). In this follow up study, 11 women were identified with CIN in subsequent visits; all of them were HIV positive and had median CD4 cell counts of 253/mm^3. The risk for CIN was related to HPV persistence in all cases.

Risk of acquisition of human papillomavirus DNA among HIV carriers

Thomas and colleagues[118] studied a group of 251 sex workers in Thailand. The HPV-DNA prevalence was similar to HIV status. However, the risk of high grade lesions was twofold higher in women infected with both HPV and HIV than in HIV negative/HPV positive women and 20 times higher than in HIV negative/HPV negative women.

Risk of cervical intraepithelial neoplasia among human papillomavirus carriers with and without HIV

Mandelblatt and colleagues[266] provided a pooled estimate of 15 studies on the association between HIV and CIN. HIV infected women had an eightfold increased risk of CIN (OR 8.8; 95% CI 6.3–12.5). Sun and colleagues[267] reported that, compared with HIV negative and HPV positive women, women co-infected with HPV and HIV had a decreased regression rate of low grade lesions and higher rates of progression from infection to CIN.

Ellerbrock and colleagues[268] reported that HIV positive women were 4.5-fold more likely than HIV negative women to have or develop CIN

within a 54 month interval of follow up. In the group of HIV carriers, transient HPV infections (RR 7.4; 95% CI 1.0–57.4), persistent infections with types other than 16 or 18 (RR 8.9; 95% CI 1.2–66.2) and persistent infections with HPV type 16 or 18 (RR 11.0; 95% CI 1.4–88.7) were all significantly associated with CIN.

Risk of invasive cervical cancer in HIV carriers

In 2000, the International Collaboration on HIV and Cancer group[269] published cancer data from 23 prospective studies that included 47 936 HIV infected subjects from North America, Europe and Australia for the period 1992–1999. The study concluded that there had not been a significant change in the incidence of invasive cancer (RR 1.87; 99% CI 0.77–4.56) during this period.

An overview of early African studies concluded that invasive cervical cancer was not related to exposure to HIV with a summary odds ratio of 0.8 (95% CI 0.5–1.4) from studies carried out in Rwanda, South Africa and Uganda.[270] However, recent data from a hospital based case–control study in South Africa identified an increased risk for cervical cancer (OR 1.6; 95% CI 1.1–2.3) and for vulvar cancer (OR 4.8; 95% CI 1.9–12.2) among HIV infected patients.[271]

In the USA or in Europe, reports are generally consistent in detecting an increased risk for cervical cancer among HIV infected women. Selik and Rabkin[272] from the USA reported a RR for cervical cancer of 5.5 among HIV positive women. Frisch and colleagues[273] used data from the US Cancer Match Registry for the period 1978–1996 and showed a RR of 5.4 for invasive cervical cancer among HIV positive women compared with the general population in the USA. Similar increases in magnitude were observed for in situ cervical cancer (OR 4.6), cancer of the vagina and vulva (OR 5.8) and anal cancer (OR 6.8). Rates were evaluated at the time prior to AIDS diagnosis, around the period of diagnosis and up to 60 months after the AIDS diagnosis. The authors identified no major changes in risk before or after AIDS diagnosis. Data are available from the population of New York with similar results.[274]

In southern Europe, a strong association between invasive cervical carcinoma and AIDS has consistently been found. In Italy, the linkage of the National AIDS Registry and the population cancer registries showed a 15-fold increased risk of invasive cervical carcinoma for women with AIDS.[275] The joint Italian–French follow up study of HIV positive women also showed a 13-fold increased rate of cervical cancer for HIV positive women.[276] In Spain, the Catalonian AIDS surveillance system detected 58 cases of invasive cervical carcinoma among 823 HIV positive women, an 18-fold increased risk compared with the general population.[277]

In summary, HPV and HIV share some behavioural traits that define a particularly vulnerable high risk group. Progression of HPV infections to CIN lesions and cervical cancer in the context of limited/absent screening seems to be increased among HIV carriers and AIDS patients. Furthermore, there is growing evidence to suggest that progression is related to the severity of immunosuppression, as indicated by CD4+

counts. In 2003, it was shown that, as immunosupression progresses, the relative impact of the less frequent HR-HPV types in cervical neoplasia increases, thus providing some new evidence of the biological advantage of HPV16 in escaping the immunomechanisms of response operating in immunocompetent women.[278]

4.3 Smoking

The effects of smoking have been investigated extensively in many case–control studies, and they show a moderate and statistically significant association with cervical carcinoma, even after adjusting for the strong effects of HPV. These findings are strikingly consistent with those obtained in studies restricted to HPV positive women. As shown in Table 8, all such studies report some evidence that tobacco smoking increases the risk of developing HSIL and cervical carcinoma. The ORs for ever smoking among HPV positive women are in the range of 2–5. Furthermore, most studies reporting risk estimates according to intensity, duration or pack–years show an increased risk of cervical cancer with increasing exposure to tobacco smoking. Because of the prospective nature of the study in the USA, the positive association found with smoking status and smoking intensity is particularly relevant.[279]

Despite the consistency of these findings, the possibility remains that smoking or smoking duration is a proxy for time since HPV exposure, because long duration smokers may also have had an HPV infection for a long time. Thus, residual confounding by time since HPV infection cannot be ruled out as a possible explanation for the observed effects of smoking.

The carcinogenic potential of cigarette derivatives in cervical tissue also has experimental plausibility. Almost 60 years ago, Rous and Friedwald[280] reported the carcinogenic effect of tar on virus induced rabbit papillomas. More recently, malignant transformation of HPV16 immortalised human endocervical cells by cigarette smoke condensate has been proven.[281] The fact that nicotine and tobacco specific carcinogens have been detected in the cervical mucus of smokers[282] further strengthens the hypothesis of a synergistic action between cigarette smoking and HPV for the development of HSIL/cervical carcinoma. Chemical tobacco related carcinogens may exert a direct mitogenic effect, causing DNA damage. Some authors[283] hypothesise that exposure to tobacco may affect the ability of the host to mount an effective local immune response against viral infections, as it has been shown that smoking may reduce the number of Langerhans cells and other markers of immune function. A recent prospective study[284] presented convincing evidence that smokers maintain cervical HPV infections significantly longer and have a lower probability of clearing an oncogenic infection than women who never smoked. The significant association found between the extent of smoking reduction and the reduction in lesion size in an intervention study of smoking cessation among women with minor grade lesions further strengthens the plausible role of tobacco smoking in HPV carcinogenesis.[285]

4.4 Oral contraceptives, parity

Tables 9 and 10 summarise selected studies on the effects of the long term use of OCs among HPV positive women. It is remarkable that the findings

Table 9 Summary results of studies assessing the role of oral contraceptives use as a cofactor in cervical carcinogenesis among human papillomavirus DNA positive women

	Study (study outcome)					
	Denmark	**USA, Eastern**	**Manchester**	**Costa Rica**	**USA, Portland**	**IARC**
Exposure measures	**(HSIL)**	**(CIS and CC)**	**(CIN3)**	**(HSIL and CC)**	**(CIN3 and CC)**	**(CIS andCC)**
OC use status						
Ever vs. never	NR	5.4 (0.7–43.4)	NR	NR	NR	**1.4 (1.0–2.0)**
Former vs. never	NR	3.1 (0.4–27.5)	1.2 (0.6–2.1)	0.9 (0.6–1.6)	NR	NR
Current vs. never	NR	**17.1 (1.5–188.2)**	1.3 (0.7–2.5)	1.5 (0.8–2.8)	0.8 (0.5–1.5)	NR
OC use duration						
[Years] vs. never	Decreasing risk with increasing duration	[≤2] 4.0 (0.4–44.3) [≤6] 4.8 (0.4–51.9) [≥7] 6.2 (0.7–52.7)	[≤3] 1.2 (0.6–2.4) [≤8] 0.8 (0.4–1.5) [>8] 1.5 (0.8–2.9)	[≤4] 1.8 (0.6–4.9) [≥5] **3.1 (1.1–9.1)**	NR	[1] 0.7 (0.4–1.1) [≤4] 0.8 (0.5–1.2) [≤9] **2.8 (1.5–5.4)** [≥10] **4.0 (2.1–7.8)**
P for trend	NR	*0.12*	NR	NR	NR	***< 0.001***
Comments	ORs not reported	For adenocarcinoma in situ		Duration estimates computed among women with less than three pregnancies	Relative risk refers to current vs. not current OC use at enrolment	For both squamous cell and adeno carcinoma

OC, oral contraceptive; HSIL, high grade squamous intraepithelial lesion; CIS, carcinoma in situ; CC, cervical carcinoma; CIN3, cervical intraepithelial neoplasia 3; NR, not reported. Adapted from Castellsagué and Muñoz, 2003.[257]

Table 10 Summary results of studies assessing the role of parity/pregnancy in cervical carcinogenesis among human papillomavirus DNA positive women

	Study (study outcome)				
	Denmark	**Manchester**	**Costa Rica**	**USA, Portland**	**IARC**
Exposure measures	**(HSIL)**	**(CIN3)**	**(HSIL and CC)**	**(CIN3 and CC)**	**(CIS and CC)**
Ever vs. never	NR	NR	4.6 (1.1–20)†	NR	NR
Number of live births or pregnancies					
[Number] vs. never	[0]* 0.8 (0.4–1.7)	[1] 1.6 (0.9–2.8)	[2]1.0 (0.5–2.2)‡	[1–2] 1.1 (0.6–1.7)	[1–2] **1.8 (1.0–3.5)**
	[≥1] 1.8 (0.3–2.3)	[2] 1.1 (0.6–2.0)	[3]1.5 (0.7–3.2) ‡	[≥3] 0.7 (0.3–1.6)	[3–4] **2.6 (1.3–4.9)**
		[≥3] **1.9 (0.9–3.8)**	[4–5] **3.5 (1.7–7.2)** ‡		[5–6] **2.9 (1.4–5.6)**
			[6–8] **2.2 (1.0–5.0)** ‡		[≥7] **3.9 (1.9–7.9)**
			[≥9] 1.4 (0.6–3.4) ‡		
P for trend	NR	NS	***0.04***	NR	***< 0.0001***
Comments		Unadjusted			For both CC and CIS

CC, cervical cancer; CIN, cervical intraepithelial neoplasia; CIS, carcinoma in situ; HSIL, high grade squamous intraepithelial lesion; NR, not reported; NS, not significant.
Bold figures denote statistical significance.
*Ever pregnant but 0 live births.
†OR for ever vs never pregnant.
‡Reference includes women with 0 or 1 live births.
Adapted from Castellsagué and Muñoz, 2003.[257]

are strongly dependent on background prevalence of the exposures of interest and of the practice of screening. Table 9 shows that the impact of OCs on cervical cancer (in some countries on cervical preinvasive lesions) is largely observed in countries without proper screening programmes, thus confirming the association between attendance to screening programmes and OC use in many populations (ie women who receive OC prescriptions are offered Pap smears more often). Many of these studies are uninformative for invasive cancer. A recent meta-analysis on the association between hormonal contraceptives and cervical cancer concludes that there is a linear dose–response relationship between years of use and risk of cervical neoplasia. The duration of the risk effect with time after OC cessation remains to de determined.[286]

Table 10 shows that the effect of parity is only visible in countries where a substantial number of women are in the groups reporting five or more lifetime pregnancies.

In most case–control studies high parity has consistently been found to be associated with both cervical cancer and in situ carcinoma (CIS). Most of the major studies restricting the analysis to HPV positive women also report an increased risk of HSIL/cervical carcinoma with an increasing number of pregnancies (Table 10). In the IARC pooled analysis, the OR for cervical cancer in women with seven or more full term pregnancies was fourfold higher than that in nulliparous women, and the risk increased linearly with an increasing number of full term pregnancies.[287] Risk of HSIL/cervical carcinoma significantly increased with an increasing number of live births in the Costa Rica study.[288] In contrast, a borderline association with CIN3 was found in the Manchester study,[289] and prospective studies in Denmark[290] and the USA[279] did not find an association between parity and the risk of HSIL and CIN3/cervical carcinoma respectively. These contrasting results could be explained by the low parity of the women in the last two study populations. In addition, in the USA cohort, information on parity was recruited only at enrolment, thus ignoring the impact of pregnancies occurring during the 10 year follow up period.

In conclusion, among women persistently exposed to HPV infection, some additional exposures further increased their risk of progression to advanced preinvasive lesions or invasive cancer. In the IARC studies,these cofactors were exposure to tobacco smoke, parity above five full term pregnancies and use of OCs for five or more years. Presence of antibodies to CT or to HV2 also modified significantly the risk of progression. The increase in risk for any of the cofactors was in general within the range of 2.5- to 4-fold for the extreme categories of exposure.

In our series of over 2000 cervical cancer cases, exposures to these factors at the level that generated a significant risk could be classified, and the distribution of the cases indicated that HPV-DNA was found to be the sole risk factor in close to 25% of the cases. In this group, one could speculate that the presence of HPV-DNA may be not only a necessary but also sufficient cause. HPV-DNA in association with any of the addi-

tional risk factors accounted for close to the 75% remaining cases, and less than 4 in 1000 remained as HPV negative.

4.5 Dietary factors

A systematic review of recent epidemiological evidence about the role of diet and nutrition on the risk of HPV persistence, SIL and invasive squamous carcinoma, taking into account HPV, has been completed.[291] The review included all published observational studies that controlled for HPV infection and all randomised clinical trials that were published between March 1995 and November 2003.

Twenty-nine studies were eligible for this review: six clinical trials, eight observational prospective studies and 15 case–control studies. Although there is some epidemiological support for the role of diet and nutritional status in cervical carcinogenesis, the available evidence taking HPV infection into account is insufficient for some nutrients and especially for foods. Evidence from strong study designs such as chemoprevention trials and prospective studies is discouraging. In a critical review of the chemoprevention trials available in 2001, it was pointed out that none of them had enough power to detect clinically significant differences in response rates.[292] In total, 15 out of the 21 observational studies included in the review were case–control studies, of which 11 were hospital based. Conclusions from case–control studies have to be cautious, as potential for selection and recall bias need to be considered. Also, the possible influence of the disease process on nutrient blood concentration has to be evaluated.

Overall, conclusions for food groups are consistent with a protective effect of fruits and vegetables in reducing HPV-DNA persistence.[293,294] In relation to nutrients, evidence for a protective effect is consistent for lycopene[294–296] and vitamin E,[295–301] and moderately consistent for vitamin B_{12}[302–305] and vitamin C.[293,295,298–301] Conclusions for nutrients from studies taking HPV infection into account do not differ substantially from those that did not control for it. With respect to blood levels of homocysteine, the available evidence is consistent with an increased risk.[302,303,305] Overall, the associations of nutritional c-factors were consistent for low and high grade cervical lesions and for retrospective and prospective study designs.

The protective effects found in studies using HPV persistence as the endpoint are especially relevant, as a putative protective effect of diet and specific nutrients on the neoplastic process is more plausible for intermediate endpoints than for more advanced stages of the disease process. At present, there are no published cohort studies on SIL and only a few on HPV persistence that comprehensively assess suspected nutritional or dietary factors controlling for HPV status. Thus, further studies are needed, in particular prospective studies with long follow up periods and enough power to detect small effects. It would be important to perform prospective studies in populations with a range of food and nutrient intake. The following study designs would be valuable: (1) prospective cohort studies using HSIL as the endpoint; (2) prospective studies using HPV persistence as the endpoint, with multiple HPV measures at different points in time; and (3) clinical trials looking at the effect

of supplementation with fruits and vegetables on HPV persistence and evolution of LSIL. These trials would be most efficient if conducted in populations with low fruit and vegetable intake and in studies that allow adequate statistical control of other cofactors, such as tobacco consumption, parity, screening practices and OC use.

The use of serological markers of HPV infection as a means of HPV adjustment may prove valid with the introduction of newer assays and the detection of antibodies against the most frequent HPV types. Interactions of dietary factors with genetic polymorphisms could be of interest when looking at certain nutrients. Finally, further studies should assess both food and nutrient intake through detailed questionnaires and biochemical markers of nutrient intake.

5. VIRAL AND HOST RISK FACTORS

5.1 Human leucocyte antigen haplotypes

The factors that determine whether an HPV infection is cleared or persists and that increase the risk for cervical cancer are not very well defined, but cellular immunity plays a major role. Altered HLA class I allele findings in cervical cancer have long been recognised and the presence of specific HLA class II alleles may be decisive for the risk of cervical cancer. In the case of HLA class I A2,[306] B44[307] and HLA-B7 negative associations[308] have been described. The most likely underlying mechanism is the allele specific downregulation of these antigens during cervical carcinogenesis. Downregulation of HLA-B7 on cervical cancer cells is associated with worse survival than normal expression of this antigen.[308] In addition, the existence of HPV16 variants with E6 mutations affecting HLA-A2 and -B7 binding motifs suggests that lack of CD8 restricted epitopes may enable the virus to escape the immune response.[309,310]

A large number of human studies have focused on the association of HLA class II with SIL or cervical cancer, and several HLA class II haplotypes were found to be associated with disease, eg DQw3 increases and DR13 (DRB1*1301[311]) decreases the risk for cervical cancer in general. Some associations were found to be type specific, eg DR15 increases the risk for HPV16 carrying cancer and DR7 may be either protective or increase the risk.

5.2 Cellular gene polymorphism

In addition to the functional assessment of differences in the immune response to HPV infections, natural polymorphisms or genetic variations between individuals in important cellular genes seem to constitute separate risk factors. For example, the wild type p53 protein exhibits a common polymorphism at amino acid 72, resulting in either a proline residue (p53Pro) or an arginine residue (p53Arg) at this position.

This codon 72 polymorphism of the *p53* gene has recently been proposed to increase the risk of cervical cancer by Storey and colleagues,[312] who reported that individuals with an Arg/Arg genotype at codon 72 of the *p53* gene had a sevenfold increased risk for cervical cancer. However, in a large number of preceding studies substantial concern was raised about a significant role of this polymorphism in the susceptibility to HPV associated carcinogenesis. Whereas few studies were confirmative,[313] a large body of other studies contradicted this finding.[314–322] A possible reason for the conflicting data could be large differences in the frequency of Arg72 homozygosity in the different populations studied, which made it difficult to define suitable control groups. Another pitfall is the source of DNA used to assess the p53 polymorphism, which differs greatly between the individual reports using peripheral blood lymphocytes, cervical epithelial cells or tumour biopsies. The use of tumour biopsies is a cause of erroneous results because loss of one allele at the p53 locus is a frequent phenomenon of tumour cells but not of the normal cells within the same patient. Interestingly, a very recent study in patients with the hereditary disease Fanconi's anaemia, which is an autosomal recessive disorder characterised by congenital malformations, bone marrow failure and the development of squamous cell carcinomas (SCCs), adds further

support to the role of the p53 codon 72 polymorphism as a risk factor for HPV associated SCC development.[323] It was shown that patients with Fanconi's anaemia with homozygosity for codon 72 p53Arg had a 5.6-fold increased risk of developing HPV associated cancers compared with patients with Fanconi's anaemia who did not have Arg72 homozygosity. Unfortunately, however, although vulvar cancer cases were included, no cases of cervical cancer could have been studied in this analysis.

A possible solution to the still unclear role of p53 polymorphisms could be results of more recent analyses showing a trend for an increased frequency of p53 arginine homozygotes among cervical carcinoma patients carrying HPV16 types with a specific mutation in the *E6* gene (a T→G transition at base pair 350, resulting in an amino acid change at position 83 from a leucine to a valine[324]). A significant over-representation of HPV16 350G/T variants was also evident in p53 Arg/Arg Dutch women with cervical cancer, which points to an increased carcinogenic effect of HPV16 350T variants in the context of specific p53 genotypes.

Several other groups have attempted to identify further specific genetic polymorphisms associated with cervical cancer, eg MTHFR,[325] WAFI[326] and IL-10.[327] Given the large effort and sample size required, however, more data will have to be collected in the future in order to reach conclusive results.

5.3 Loss of heterozygosity

There is a substantial body of literature regarding chromosomal abnormalities in cervical cancer. Although chromosomal aberrations have been consistently identified,[328–330] such as loss of heterozygosity at chromosomes 3p, 6, 11, 13, 16, 17 and 19 and chromosomal gains at 3q, identifying the target genes (oncogenes/tumour suppressor genes) affected in these areas will be the next major goal.

5.4 Viral variants

Although more than 100 HPV have been types identified, studies on variants in viral genes mainly relate to the *E6* gene of HPV type 16 (HPV16).[331–334] It was reported that HPV16 variants with nucleotide alterations within the *E6* gene, referred to as non-prototype like variants, are more frequently associated with high grade CIN and cervical cancer as wild type genomes,[335,336] although this phenomenon could be population dependent.[337,338] Based on regional differences, HPV16 variants have been termed European (E), Asian American (AA), African (Af1 and Af2) and North American (NA).[339]

Interestingly, a significant over-representation of HPV16 350G/T variants was detected in cervical cancers of women with a p53 Arg/Arg polymorphism, and a possible differentially oncogenic effect of HPV16 350G/T variants, which is influenced by the p53 genotype, was therefore suggested.[340] Another E6 variant described has been the 131G variant which was found to be present in 9.6% of cervical carcinoma patients (n = 94), of whom 78% had the HLA-B7 allele, already identified as a possible risk factor. Most of the studies performed did not consider other variations that may occur in the E6/E7 region or in other regions of the HPV genome. Therefore, the current risk observed, which is associated with viral variants in general, might be an underestimation. Furthermore, this

risk might be influenced by other genomic alterations, and future studies have to be performed to decipher the underlying mechanisms.

5.5 Viral load

A number of cross-sectional epidemiological studies using the semi-quantitative HC2 technique[56] have demonstrated an association between increasing viral load with HR-HPV types and the risk of cervical cancer. However, estimates of viral copy numbers depend directly on the total input of cells, and adjustment for cellular load is an absolute requirement that is frequently not fulfilled, as in the case for HC2. Using type specific real time quantitative PCR, other studies[59,341] have reported a specific association of high viral load with HPV16 infections, which was consistently associated with an increased risk for progression. Already high copy numbers of 10^7 copies/µg of cellular DNA in patients with normal cytology were found to be increased with the severity of the lesions by a factor of 100 in CIN2/3 patients.[59] Interestingly, these associations have not been found for other HPV genotypes.[342,343] Only few longitudinal data are available[341,344] and need to be extended. Recently, van Duin and colleagues[345] reported that viral load for HPV16 in women with normal and abnormal smears is an indicator of incident CIN2/3, and is predictive when falling below a certain threshold viral clearance. Unfortunately, little is known about the relationship of viral load to types other than HPV16 and cervical neoplasia.

5.6 Viral DNA integration

HPV-DNA is maintained as an episome in benign infections, whereas integrated HPV genomes are frequently detected in CIN3, cervical cancer and derived cell lines. It has been proposed that this integration event confers a certain growth advantage to the infected cells by activating the expression of the viral oncogenes.[346] The current model suggests that the inactivation of the *E2* gene as a consequence of integration releases E6/E7 oncogene expression from E2 mediated negative control. However, no evidence has been presented so far that indeed increased E6/E7 expression is necessary for the progression of HPV induced lesions. Viral DNA integration could simply be a consequence of an environment that does not support HPV-DNA replication. This is underlined by observations that long term extrachromosomal replication of HR-HPV-DNA has not been achieved in established HPV positive or HPV negative tumour cell lines, but occurs almost exclusively in normal human keratinocytes.[347] Furthermore, a number of studies reported exclusively episomal HPV16 DNA in 20–70% of cervical cancers[38,348–350] and in high percentages (75–97%) of CIN3. Therefore it remains unclear whether HPV integration is simply a consequence of loss of normal epithelial cell differentiation capacity and biologically conveys no further risk downstream or whether the integration event indeed contributes to progression.

5.7 Epigenetic events

Epigenetic events are those that alter gene expression (eg phenotype) without a change in the DNA sequence and include hypermethylation or hypomethylation of genes (eg the addition or the removal of a methyl group). For example, recent studies[351,352] have identified the silencing of tumour suppressor genes via promoter hypermethylation in HPV infected host cells as a frequent human epigenetic event. Because of the potential implications on the activity of viral oncogenes or cellular tumour suppressor genes, such as on TSLC1 (tumour suppressor in lung

cancer), which reveal reduced expression in cervical cancer because of promoter methylation,[353] continued investigation of epigenetic events in HPV infected lesions is warranted.

6. EPILOGUE

The recognition of the viral aetiology of cervical cancer is having profound implications in the paradigm of cervical cancer prevention. In public health terms, HPV technology is offering new opportunities to simplify screening programmes by using a more efficient approach to clarify the ambiguity of a sizeable fraction of the reports from conventional cytology. HPV testing can also be considered as the primary screening test in countries where cytology based programmes have not been fully implemented. In developed countries, protocols including combinations of HPV testing and cytology, probably liquid based cytology, should result again in a less demanding programme of cervical cancer screening in terms of number of visits, number of additional examinations and probably a significant reduction in overdiagnosis and overtreatment of lesions that are in fact morphological expressions of self limiting HPV infections. A full review of these options was undertaken by the IARC in March 2004, and it was concluded that there is sufficient evidence that HPV testing as standalone screening will reduce cervical cancer incidence and mortality at least as efficiently as conventional cytology.[232]

In the near future, HPV vaccines may further change the cervical cancer prevention scenario, and combined protocols of HPV vaccination and screening are expected to be adopted. HPV vaccines are viewed at present as the most important option for cervical cancer prevention in developing countries where the burden of prevention in cervical cancer mortality has not been modified despite decades of efforts based on cytology based screening protocols.

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