HUMAN PAPILLOMAVIRUSES

1. Human Papillomavirus (HPV) Infection

1.1 Evolution, structure and molecular biology

1.1.1 *Introduction*

Papillomaviruses are small, non-enveloped, epitheliotropic, double-stranded DNA viruses that infect mucosal and cutaneous epithelia in a wide variety of higher vertebrates in a species-specific manner and induce cellular proliferation. Only bovine papillomaviruses (BPVs) 1 and 2 are known to infect mesenchymal tissues and to show cross-species transmission. More than 100 types of human papillomaviruses (HPVs) have been identified and approximately half of them infect the genital tract. Many types of HPV have been found in cervical cancers, while others are found rarely or not at all in large series of cancers, which gives rise to the nomenclature of 'high-' and 'low-risk' HPVs. These other types are associated with other anogenital and oropharyngeal cancers. A number of HPVs have been found to be present in skin cancers in patients who have epidermodysplasia verruciformis (EV); these types are also found in both non-melanoma skin cancers and normal skin. The potential associations of HPVs with these and other cancers are discussed in other sections.

All papillomaviruses share a common genetic structure that is distinct from that of polyomaviruses. A double-stranded circular DNA genome encodes approximately eight open-reading frames (ORFs). Similarly, all papillomaviruses have a non-enveloped icosahedral capsid. Understanding of the biology of papillomavirus infection was hindered by the lack of tissue culture systems to propagate the viruses, the lack of animal models for HPVs and difficulties in finding animal models of natural infection. The advent of molecular cloning of HPV genomes in the early 1980s provided the first opportunity to study individual viral genes. However, only in the late 1990s did propagation of viruses in organotypic cultures make the first attempts at viral genetics possible. The availability of complete and partial genomic sequences from a wide variety of HPV types has enabled the establishment of a new taxonomic structure and has provided a window to study the co-evolution of papillomaviruses with their primate hosts. Early evidence suggests that HPV types, as defined by DNA sequencing, also remain serologically distinct.

Molecular studies now provide a coherent picture of the mechanisms that regulate viral gene expression and replication; nevertheless, gaps in the understanding of HPV biology remain. Striking progress has been made in defining the activities of viral oncoproteins from high-risk genital HPVs, in particular HPVs 16 and 18, that promote the disruption of normal cell-cycle control. The ability to target the retinoblastoma (Rb) family of proteins and p53 and to induce telomerase are some of the critical events that contribute to the development of malignancy.

1.1.2 *Structure of the viruses*

(*a*) *Viral components and physical properties*

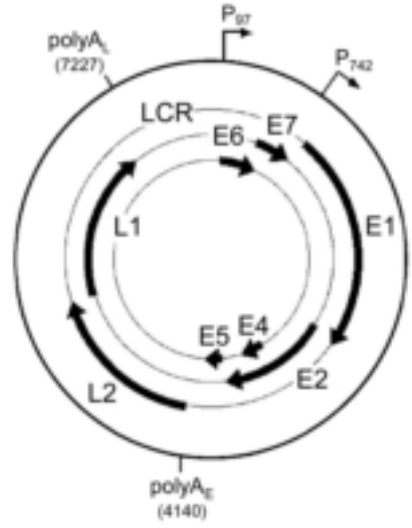
Papillomaviruses are small, non-enveloped, icosahedral DNA viruses that have a diameter of 52–55 nm. The viral particles consist of a single double-stranded DNA molecule of about 8000 base-pairs (bp) that is bound to cellular histones and contained in a protein capsid composed of 72 pentameric capsomers. The capsid contains two structural proteins — late (L)1 (55 kDa in size; 80% of total viral protein) and L2 (70 kDa) — which are both virally encoded. Virus-like particles (VLPs) can be produced by the expression of L1, alone or in combination with L2, in mammalian or non-mammalian expression systems. The intact virion has a density of 1.34 g/mL in cesium chloride and a sedimentation coefficient (S_{20}, W) of 300 (Kirnbauer *et al*., 1992; Hagensee *et al*., 1993a).

(*b*) *HPV genome, proteins and life cycle*

The genomes of all HPV types contain approximately eight ORFs that are all transcribed from a single DNA strand. The ORF can be divided into three functional parts: the early (E) region that encodes proteins (E1–E7) necessary for viral replication; the late (L) region that encodes the structural proteins (L1–L2) that are required for virion assembly; and a largely non-coding part that is referred to as the long control region (LCR), which contains *cis* elements that are necessary for the replication and transcription of viral DNA. The viral E proteins are transcribed from the early promoter (e.g. P97 in HPV 31) whereas the L proteins are transcribed principally from the late promoter (P742 in HPV 31) (see Figure 1) (Fehrmann & Laimins, 2003).

The E1 and E2 proteins of HPV act as factors that recognize the origin of replication; E2 protein is also the main regulator of viral gene transcription. E4, despite its name, is believed to be involved in the late stages of the life cycle of the virus and E5 may function during both early and late phases. The E6 and E7 proteins target a number of negative regulators of the cell cycle, primarily p105Rb and p53, respectively. During the viral life cycle, E6 and E7 facilitate stable maintenance of viral episomes and stimulate differentiating cells to re-enter the S phase. The L1 and L2 proteins assemble in capsomers, which form icosahedral capsids around the viral genome during the generation of progeny virions (Fehrmann & Laimins, 2003).

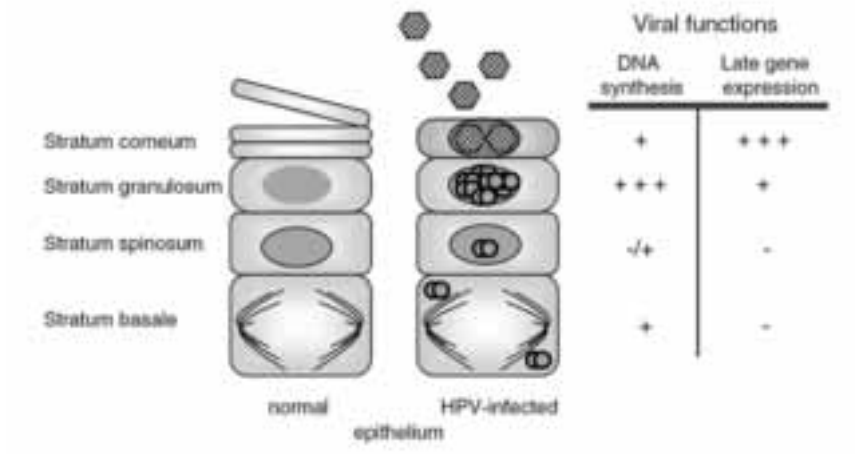
Figure 1. The genome of the high-risk HPV 31



Modified from Fehrmann & Laimins (2003)
The diagram indicates the ORFs of the early (E) and late (L) genes, the long control region (LCR), the two major promoters that drive viral expression (P97 and P742) and the two polyadenylation sites (A_E4140 and A_L7227).

Papillomaviruses are highly epitheliotropic; specifically, they establish productive infections only within stratified epithelia of the skin, the anogenital tract and the oral cavity. The viral life cycle is linked to the differentiation of the infected epithelial cell (see Figures 2 and 3). The life cycle is thought to be initiated by the infection of basal epithelial cells, presumably at sites of injury. Although several potential receptors have been reported, it is unclear which of them is of physiological importance (see Section 1.1.5(*g*)). Basal cells comprise the proliferating cellular component of stratified epithelia, in which the viral genome is established when a low copy number, nuclear plasmid and early genes are expressed preferentially although at low levels (Stoler & Broker, 1986; Schneider *et al*., 1987; Frattini *et al.*, 1996; Oguchi *et al*., 2000). The ability of HPVs to establish their genome in basal cells relies upon the *E1* (Hubert & Laimins, 2002), *E2* (Stubenrauch *et al.*, 1998), *E6* (Thomas *et al.*, 1999) and in some cases *E7* (Thomas *et al.*, 1999; Flores *et al.*, 2000) genes. Normally, when basal cells undergo cell division, the daughter cell that loses contact with the basement membrane and migrates into the suprabasal compartment withdraws from the cell cycle and initiates a programme of terminal differentiation. However, in HPV-positive human keratinocytes and cervical epithelial cells, the suprabasal cells fail to withdraw from the cell cycle and continue to support DNA synthesis and express markers for cell proliferation (Jeon *et al.*, 1995; Flores *et al.*, 1999). HPV 16 *E7* has been shown to be necessary and sufficient to induce suprabasal DNA synthesis (Flores *et al*., 2000). In addition, the E5 oncoprotein contributes quantitatively

Figure 2. Schematic representation of abnormal epithelial differentiation induced by HPV infection

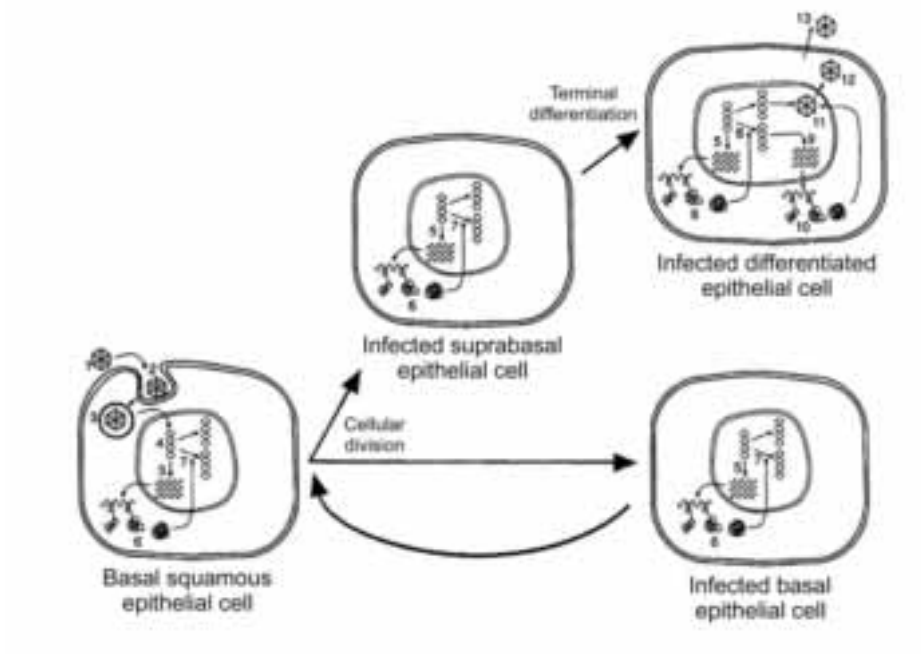


Modified from Fehrmann & Laimins (2003)
Normal and HPV-infected epithelia are compared, and differentiation-dependent viral functions are listed.

to this property both in HPV 16 (Genther *et al.*, 2003) and HPV 31 (Fehrmann *et al.*, 2003). Within this suprabasal compartment, cells support the amplification of the viral genome, expression of capsid genes and assembly of progeny virus (Peh *et al.*, 2002). The cottontail rabbit papillomavirus (CRPV) *E4* gene, which is detected preferentially in the differentiated compartment of infected tissue, is required for viral DNA amplification and expression of the L1 capsid gene (Peh *et al.*, 2004). Encapsidation of HPV DNA within capsids to generate progeny virus within the terminally differentiated cell compartment is quantitatively dependent upon L2, the minor capsid protein (Holmgren *et al.*, 2005). L2 is also required for the infectivity of HPV 16 (Yang, R. *et al.*, 2003a) and HPV 31 (Holmgren *et al.*, 2005) virions. L2 may play a role in the cell-surface binding of HPV 16 virions (Yang, R. *et al.*, 2003a), intracellular transport of the HPV 33 virion (Florin *et al.*, 2002a) and localization of viral DNA within the nucleus (Day *et al.*, 2004).

In the context of HPV-associated cervical cancer, the viral life cycle is perturbed in two fundamental ways. First, the progressive histopathological changes that arise in the cervical epithelium include the loss of terminal differentiation. This inhibition of the differentiation process leads to a cellular state that cannot support the full viral life cycle. Second, the circular viral DNA genome, which normally resides as a nuclear plasmid, often becomes integrated into the host genome and thereby becomes disrupted and its replication defective. Whether any property of the virus drives this integration event or whether it reflects random recombination events remains unclear; however, two consequences of integration can be the selective up-regulation of the viral oncogenes *E6* and *E7* and a selective growth advantage over cells that harbour the viral genome as a nuclear plasmid (Jeon & Lambert, 1995; Jeon *et al.*, 1995). Integration events that are found in

Figure 3. Replication cycle of a papillomavirus



Modified from Howley & Lowy (2001)
To establish a wart or papilloma, the virus must infect a basal epithelial cell. Knowledge of the initial steps in the replication cycle such as attachment (1), uptake (2), endocytosis (3) and transport to the nucleus and uncoating of the viral DNA (4) is limited. E-region transcription (5), translation of the E proteins (6) and steady-state viral DNA replication (7) all occur in the basal cell and in the infected suprabasal epithelial cell. Events in the viral life cycle that lead to the production of virion particles occur in the differentiated keratinocyte: vegetative viral DNA replication (8), transcription of the L region (9), production of the capsid proteins L1 and L2 (10), assembly of the virion particles (11), nuclear breakdown (12) and release of virus (13).

cervical cancer lead to the selective expression of *E6* and *E7* (Schwarz *et al.*, 1985; Yee *et al.*, 1985), which is a hallmark of cervical cancers. Whether viral integration alters cellular gene expression in any biologically relevant manner remains unclear. In a recent review, more than 190 reported integration loci were analysed with respect to changes in the viral structure and the targeted genomic locus. The results confirmed that HPV integration sites are randomly distributed over the whole genome with a clear predilection for fragile sites. There was no evidence for targeted disruption or functional alteration of critical cellular genes by the integrated viral sequences (Wentzensen *et al*., 2004). A more complete assessment of the role of HPV integration in carcinogenesis is provided in Section 4.1.4.

1.1.3 *Classification of papillomaviruses*

Papillomavirus isolates are traditionally described as 'types', and types have been detected in all carefully examined mammals and birds, with the possible exception of laboratory mice. In the only host that has been studied extensively — humans — more than 100 HPV types have been described based on the isolation of complete genomes; a yet larger number is presumed to exist based on the detection of subgenomic amplicons. Many of these HPV types have been shown to be ubiquitous and distributed globally.

Over the last 30 years, the taxonomy of papillomaviruses, which was initially based on genomic cross-hybridizations and restriction patterns, has been changed to a system based on phylogenetic algorithms that compares either whole viral genome sequences or subgenomic segments. This scientific progress has led to a refinement but never to contradictions of previous taxonomies. There is also strong evidence that papillomavirus genomes are very static, and sequence changes by mutation or recombination are very rare events. Mutational changes apparently occur at frequencies that do not differ greatly from those of the DNA genomes of the infected host organism. Papillomaviruses had originally been grouped together with polyomaviruses in one family, the *Papovaviridae*. This was based on similar, non-enveloped capsids and the common circular double-stranded DNA genomes. Because it was later recognized that the two groups of viruses have different genome sizes, completely different genome organizations and no similarities in major nucleotide or amino acid sequences, they are now officially recognized by the International Committee on the Taxonomy of Viruses (ICTV) as two separate families — *Papillomaviridae* and *Polyomaviridae*. A modified taxonomy and nomenclature has recently been proposed (de Villiers *et al.*, 2004a).

The L1 ORF is the most conserved region within the genome and has therefore been used for the identification of new papillomavirus types over the past 15 years. A new papillomavirus isolate is recognized if the complete genome has been cloned and the DNA sequence of the L1 ORF differs by more than 10% from the closest known type. Differences in homology of between 2% and 10% define a subtype and those of less than 2% define a variant. A few hundred putative new papillomavirus types have been identified since the advent of the polymerase chain reaction (PCR) and application of degenerate or consensus primers. Amplification of conserved regions, mostly within the L1 ORF, has been used. These partial fragments are usually labelled by using the initials of an individual or laboratory, followed by a laboratory number (see, e.g., Chow & Leong, 1999). A number of these short fragments constitute partial sequences of later defined HPV types (de Villiers *et al.*, 2004a).

Recently, instead of primary cloning of a complete papillomavirus genome, PCR amplification of overlapping fragments has been used to assemble a full-length genome. Such isolates are termed HPVcand(number) (de Villiers *et al.*, 2004a).

An understanding of the relationship between papillomavirus types based on a comparison of nucleotide sequences began to emerge more than 10 years ago (Chan *et al.*, 1992a,b; van Ranst *et al.*, 1992a,b). Continued research based on these principles has led

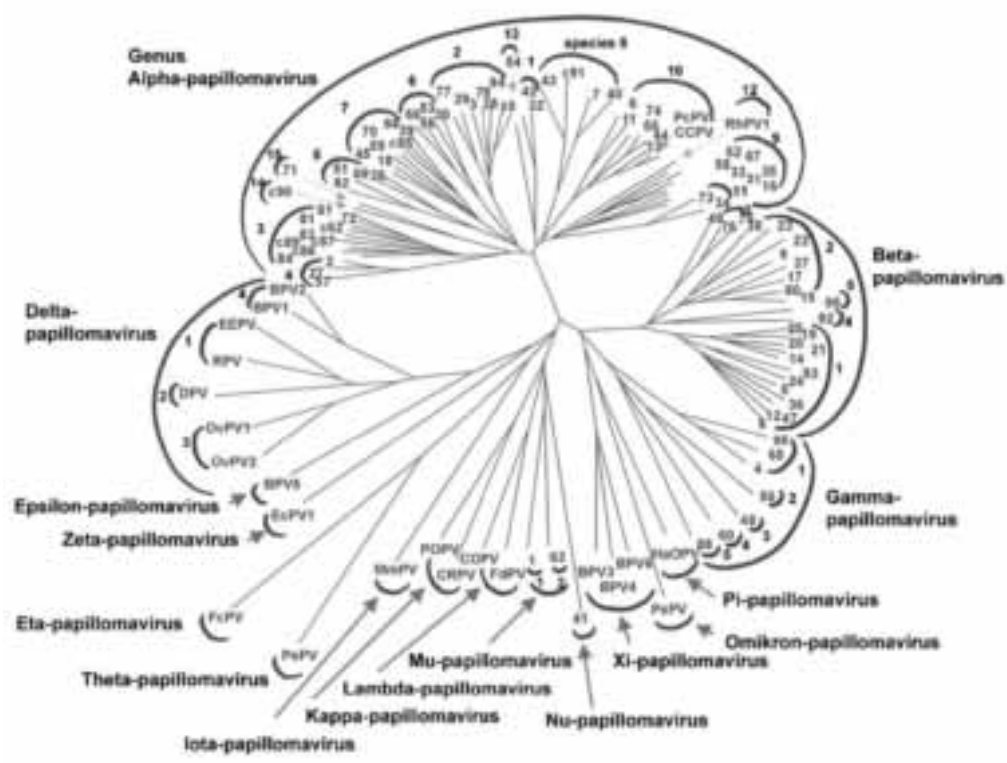
to the taxonomic groupings, which today are widely accepted. Phylogenetic assemblages occasionally coincide with biological and pathological properties, but often diverge. The closely related HPV types 2 and 27, 6 and 11, and 16 and 31, which cause common warts, genital warts and cervical cancer, respectively, are three excellent cases of the numerous consistencies between phylogeny and pathology. However, there are also some discrepancies: the phylogenetic group of genital HPV types, which incorporates all HPV types found in genital lesions, also contains some HPV types that are mostly found in cutaneous lesions, such as HPV 2. Also, highly unrelated viruses, such as HPV 2 (genus alpha) and HPV 4 (genus gamma), can cause similar cutaneous papillomas (de Villiers *et al.*, 2004a).

The evolution of papillomaviruses has often been debated (de Villiers *et al.*, 2004a). Comparative studies that used the E6, L1 or the combined E6–E7–L1 ORFs (van Ranst *et al.*, 1992a,b; Myers *et al.*, 1994; Chan *et al.*, 1995), however, have resulted in phylogenetic trees that establish similar or even identical relationships. A frequently used 291-bp amplicon, a small segment of the L1 gene, suffices as a foundation to generate highly informative phylogenetic comparisons (Bernard *et al.*, 1994). Sequence comparisons of the complete genomes of 118 papillomaviruses reveal a high diversity, but a distribution similar to that found when L1 ORF sequences were compared. A cladogram based on the complete L1 ORF of 96 HPV types and 22 animal papillomavirus types is presented in Figure 4. The frequency distribution of pairwise identity percentages from sequence comparisons of the L1 ORF demonstrates three taxonomic levels, on the basis of comparison of both complete genomes and L1 genes, namely genera, species and types (Figure 5) (de Villiers *et al.*, 2004a).

Extensive sequence comparisons using the L1 ORF of 96 HPV types and 22 animal papillomaviruses led to the establishment of the following classifications. Higher-order clusters of HPV types (e.g. the genital HPVs) had previously been called ‘supergroups’ or ‘major branches’ (Myers *et al.*, 1994; Chan *et al.*, 1995). For these taxa, the new term ‘genus’ was introduced. Different genera share less than 60% nucleotide sequence identity in the L1 ORF. Full-length sequences of complete genomes have more than 23% but less than 43% nucleotide sequence identity when compared with genera of the *Papillomaviridae*. Lower-order clusters of HPV types (e.g. HPV types 6, 11, 44 and 55) had been called ‘groups’, ‘subgroups’ or ‘minor branches’. For these taxa, the new term ‘species’ was introduced. Such species within a genus share between 60 and 70% nucleotide sequence identity. The traditional papillomavirus types within a species share between 71 and 89% nucleotide sequence identity within the complete L1 ORF (de Villiers *et al.*, 2004a).

The introduction of the term ‘genus’ is useful, as this concise term will now replace the somewhat vague expressions of ‘major branches’ or ‘supergroups’. Throughout all biology, including virology, specific genera typically unite species, which are clearly phylogenetically related but are often biologically quite diverse. The same applies to papillomavirus genera. A summary of the biological properties known for each genus is presented in Table 2, together with specific characteristics of the organization of its genome in cases where this differs from the typical pattern. The introduction of the term

Figure 4. Phylogenetic tree containing the sequences of 118 papillomavirus types



Modified from de Villiers *et al.* (2004a)

The L1 ORF sequences were used in a modified version of the Phylip version 3.572 and based on a weighted version of the neighbour-joining analysis. The tree was constructed using the Treeview programme of the University of Glasgow. The numbers at the ends of each of the branches identify an HPV type; c-numbers refer to candidate HPV types. All other abbreviations refer to animal papillomavirus types. For the meaning of each abbreviation, please refer to Table 1. The outermost semicircular symbols identify papillomavirus genera, e.g. the genus alpha-papillomavirus. The number at the inner semicircular symbol refers to papillomavirus species. To give an example taken from the upper part of the figure, the HPV types 7, 40, 43, and cand91 together form the HPV species 8 in the genus alpha-papillomavirus.

'species' is biologically useful, as these are natural taxa based on the close phylogenetic relationship of certain types and because such species typically assemble papillomavirus types that have common biological and pathological properties, a requirement of the ICTV guidelines. To give examples, all HPV types that form a species with HPV 2 are typically found in common skin warts, and all HPV types that form a species with HPV 16 are 'high-risk' HPV types that are found in cervical cancer and its precursor lesions. More detailed information about each species and papillomavirus types within a genus is presented in Table 1. The type species have been chosen either because they are the most comprehensively investigated type, because they best represent the species or because

Table 1. Characteristics of species within specific genera

Genus	Species	Type species	Other papillomavirus types	Comments
Alpha-papillomavirus	1	HPV 32 (X74475)	HPV 42 (M73236)	More frequently in benign lesions (low risk); oral or genital mucosa; third ORF in ELR
	2	HPV 10 (X74465)	HPV 3 (X74462) HPV 28 (U31783) HPV 29 (U31784) HPV 78 HPV 94 (AJ620211)	More frequently cause cutaneous than mucosal lesions; low risk; E5 biologically different
	3	HPV 61 (U31793)	HPV 72 (X94164) HPV 81 (AJ620209) HPV 83 (AF151983) HPV 84 (AF293960) candHPV 62 candHPV 86 (AF349909) candHPV 87 (AJ400628) candHPV 89 (AF436128)	Mucosal lesions; lower risk
	4	HPV 2 (X55964)	HPV 27 (X73373) HPV 57 (X55965)	Common skin warts; frequently in benign genital lesions in children; several larger un-characterized ORFs scattered throughout genome; E5 ORF biologically different
	5	HPV 26 (X74472)	HPV 51 (M62877) HPV 69 (AB027020) HPV 82 (AB027021)	High-risk mucosal lesions, also in benign lesions
	6	HPV 53 (X74482)	HPV 30 (X74474) HPV 56 (X74483) HPV 66 (U31794)	High-risk mucosal, but also in benign lesions

Table 1 (contd)

Genus	Species	Type species	Other papillomavirus types	Comments
	7	HPV 18 (X05015)	HPV 39 (M62849) HPV 45 (X74479) HPV 59 (X77858) HPV 68 (X67161) HPV 70 (U21941) candHPV85 (AF131950)	High-risk mucosal lesion
	8	HPV 7 (X74463)	HPV 40 (X74478) HPV 43 (AJ620205) candHPV 91 (AF131950)	Low-risk mucosal and cutaneous lesions; HPV 7 also known as butcher's wart virus — often in mucosal and skin lesions in HIV-infected patients
	9	HPV 16 (K02718)	HPV 31 (J04353) HPV 33 (M12732) HPV 35 (X74476) HPV 52 (X74481) HPV 58 (D90400) HPV 67 (D21208)	High-risk — malignant mucosal lesions
	10	HPV 6 (X00203)	HPV 11 (M14119) HPV 13 (X62843) HPV 44 (U31788) HPV 74 (U40822) PcPV (X62844)	Mostly associated with benign mucosal lesions; low risk; reports of HPV 6 in verrucous carcinoma
	11	HPV 34 (X74476)	HPV 73 (X94165)	Mucosal lesions — high risk
	12	RhPV 1 (M60184)	–	Mucosal genital lesions in rhesus monkeys
	13	HPV 54 (U37488)	–	Low-risk mucosal
	14	candHPV 90 (AY057438)	–	Low-risk mucosal
	15	HPV 71 (AB040456)		Low-risk mucosal

Table 1 (contd)

Genus	Species	Type species	Other papillomavirus types	Comments
Beta-papillomavirus	1	HPV 5 (M17463)	HPV 8 (M12737) HPV 12 (X74466) HPV 14 (X74467) HPV 19 (X74470) HPV 20 (U31778) HPV 21 (U31779) HPV 25 (U74471) HPV 36 (U31785) HPV 47 (M32305) HPV 93 (AY382778)	Most frequently cause cutaneous lesions, but reports of DNA in mucosa; commonly associated with lesions in EV or immunosuppressed patients; mostly benign lesions, but reported in malignant lesions, also in immunocompetent patients
	2	HPV 9 (X744464)	HPV 15 (X74468) HPV 17 (X74469) HPV 22 (U31780) HPV 23 (U31781) HPV 37 (U31786) HPV 38 (U31787) HPV 80 (Y15176)	Most frequently cause cutaneous lesions, but reports of DNA in mucosa; commonly associated with lesions in EV or immunosuppressed patients; mostly benign lesions, but reported in malignant lesions, also in immunocompetent patients
	3	HPV 49 (X74480)	HPV 75 (Y15173) HPV 76 (Y15174)	Benign cutaneous lesions
	4	HPVcand92 (AF531420)	–	Pre- and malignant cutaneous lesions
	5	HPVcand96 (AY382779)		Pre- and malignant cutaneous lesions
Gamma-papillomavirus	1	HPV 4 (X70827)	HPV 65 (X70829) HPV 95 (AJ620210)	Cutaneous lesions; histologically distinct homogenous intracytoplasmic inclusion bodies
	2	HPV 48 (U31790)	–	Cutaneous lesions
	3	HPV 50 (U31790)	–	Cutaneous lesions
	4	HPV 60 (U31792)	–	Cutaneous lesions

Table 1 (contd)

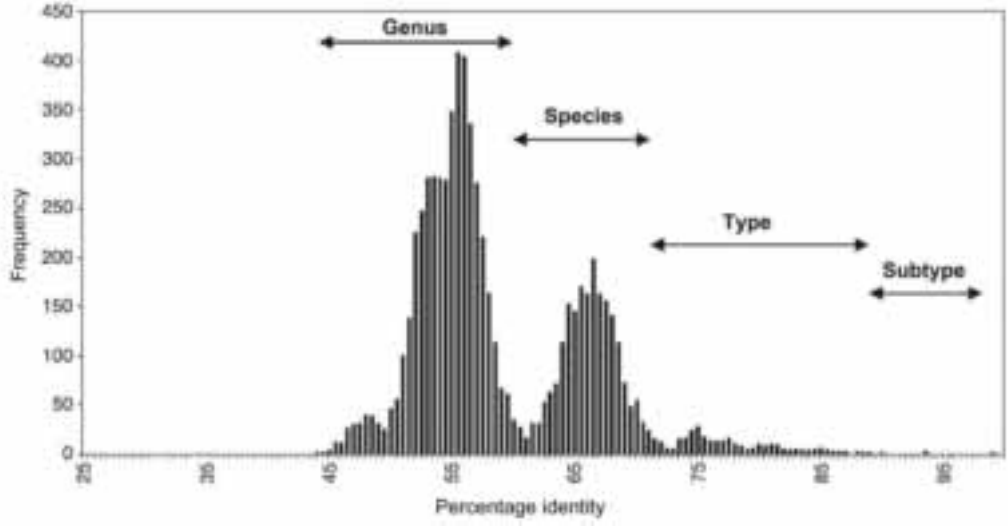
Genus	Species	Type species	Other papillomavirus types	Comments
	5	HPV 88	–	Cutaneous lesions
Delta-papillomavirus	1	EEPV (M15953)	RPV (AF443292)	E9 gene within ELR with transforming properties
	2	DPV (M11910)	–	E9 gene within ELR with transforming properties
	3	OvPV-1 (U83594)	OvPV-2 (U83585)	
	4	BPV-1 (X02346)	BPV-2 (M20219)	E5 gene in ELR with transforming properties; trans-species infection causing sarcoids in horses
Epsilon-papillomavirus	1	BPV-5 (AF457465)	–	
Zeta-papillomavirus	1	EqPV, AF498323	–	
Eta-papillomavirus	1	FcPV, AY957109	–	
Theta-papillomavirus	1	PePV, AF420235	–	
Iota-papillomavirus	1	MnPV (U01834)	–	
Kappa-papillomavirus	1	CRPV (K02708)		High divergence within the E6 and E7 ORFs described for different isolates; associated with cutaneous lesions
	2	ROPV (AF227240)		Associated with oral lesions
Lambda-papillomavirus	1	COPV (L22695)	–	ELR, 1500 bp in length
	2	FdPV (AF377865)	–	ELR, 1271 bp in length
Mu-papillomavirus	1	HPV 1 (V01116)	–	Histologically distinct heterogenous intracytoplasmic inclusion bodies; LCR, 982 bp in length

Table 1 (contd)

Genus	Species	Type species	Other papillomavirus types	Comments
	2	HPV 63 (X70828)	–	Histologically distinct filamentous intracytoplasmic inclusion bodies; LCR, 558 bp in length
Nu-papillomavirus	1	HPV 41 (X56147)	–	Several larger uncharacterized ORFs scattered throughout the genome; ELR only 17 nucleotides; all E2-BSs in LCR modified
Xi-papillomavirus	1	BPV-3 (AF486184)	BPV-4 (X05817) BPV-6 (AJ620208)	E8 gene within E6 region of BPV-4 has transforming properties similar to E5 of BPV-1
Omikron-papillomavirus	1	PsPV (AJ238373)		E7 ORF absent; several larger ORFs in L1 ORF region
Pi-papillomavirus	1	HaOPV (E15110)	–	No ELR; partial overlap between E2 and L2 ORFs

From de Villiers *et al.* (2004a)
The table shows division of the *Papillomaviridae* into genera and species, following the phylogenetic tree shown in Figure 5. For each species, the table lists a type species, other papillomavirus types that belong to these species and biological and pathological properties of each species.
bp, base pair; BS, binding site; BPV, bovine papillomavirus; candHPVs, candidate HPVs, cloned and characterized from PCR products; COPV, cannine oral papillomavirus; CRPV, cottontail rabbit papillomavirus; DPV, deer papillomavirus; DPV, deer papillomavirus; EEPV, European elk papillomavirus; ELR, region between early and late genes; EqPV, *Equus caballas* (horse) papillomavirus; EV, epidermodysplasia verruciformis; FcPV, *Fringilla coelebs* (chaffinch) papillomavirus; FdPV, *Felis domesticus* (cat) papillomavirus; HaOPV, hamster oral papillomavirus; HIV, human immunodeficiency virus; HPV, human papillomavirus; MnPV, *Mastomys natalensis* papillomavirus; ORF, open-reading frame; OvPV, ovine papillomavirus; PePV, *Psittacus erithacus timneh* (parrot); PsPV, *Phocoena spinipinnis* papillomavirus; ROPV, rabbit oral papillomavirus; RPV, reindeer papillomavirus

Figure 5. Frequency distribution of pairwise identity percentages from nucleotide sequence comparison of the L1 ORFs of 118 papillomavirus types



Modified from de Villiers *et al.* (2004a)

there is only one type in that taxon. Table 1 is an important reference that groups together (with the type species in many type-rich taxa) all those HPV types that belong to the same species and will presumably have properties similar or identical to the type species, but cannot be studied (for purposes of basic research, drug development and vaccination) as intensely as the type species. As an example, species No. 9 groups — with the type species HPV 16 — the HPV types 31, 33, 35, 52, 58 and 67, which have been studied to a lesser extent (with the exception of HPV 31) but which probably have similar biological and pathological properties as HPV 16.

Several hundred papillomavirus types have been partially identified in the form of short DNA fragments, but interest in isolating full-length genomes appears to be declining. The number of HPV types isolated and fully characterized now exceeds 100. A regulated taxonomic description of non-human papillomaviruses is particularly necessary because it is extremely probable that only a tiny fraction of all animal papillomavirus types have been identified or isolated. The present methodology used for the detection of papillomavirus types is very limiting, as it is based on the information available from known types. Hopefully, future efforts will be directed towards identifying additional types that are very distantly related to the known genera. An example of the large diversity of animal papillomaviruses are the two recently described types from birds, both of which lack traditional E6 and E7 ORFs (Tachezy *et al.*, 2002a,b; Terai *et al.*, 2002) and are less closely related to any mammalian papillomavirus type than they are to one another. Several of the papillomavirus types that presently appear as single species within a genus have in the past been identified only because of the availability of lesions that harbour

Table 2. Biological properties and characteristics of organization of genome for each genus

Genus	Biological properties	Organization of genome
Alpha-papillomavirus	Mucosal and cutaneous lesions in humans and primates High- and low-risk classification based on molecular biological data: high-risk types (pre- and malignant lesions) immortalize human keratinocytes; low-risk types (benign lesions) do not. Recent compilations of epidemiological data demonstrate more frequent association of specific species at high-risk types.	Conserved with an E5 ORF within the ELR (~300–500 bp); ORFs in ELR from different species may be divided into three groups: classical E5 ORF; closer related to the ungulate E5 ORF; putative ORF with distinct conserved motives
Beta-papillomavirus	Cutaneous lesions in humans Infections exist in latent form in general population, activated under conditions of immune suppression. Also referred to as EV–HPV types due to close association with disease EV	ELR generally < 100 nucleotides in length; E5 ORF absent
Gamma-papillomavirus	Cutaneous lesions in humans Histologically distinguishable by intracytoplasmic inclusion bodies specific for type species	ELR < 100 nucleotides in length; E5 ORF absent
Delta-papillomavirus	Lesions in ungulates Induces fibropapillomas in the respective host. Trans-species transmission occurs inducing sarcoids.	ORFs located in ELR have transforming properties.
Epsilon-papillomavirus	BPV; cutaneous papillomas in cattle	
Zeta-papillomavirus	Cutaneous lesions in horses	Undefined ORF overlapping with L2 ORF
Eta-papillomavirus	Avian papillomaviruses Cutaneous lesions in host	E4 and E5 ORFs absent; no typical E6 ORF, but an ancestral E7 ORF with partial E6 characteristics
Theta-papillomavirus	Avian papillomaviruses Cutaneous lesions in host	E4 and E5 ORFs absent; no typical E6 ORF, but an ancestral E7 ORF with partial E6 characteristics

Table 2 (contd)

Genus	Biological properties	Organization of genome
Iota-papillomavirus	Rodent papillomaviruses Cutaneous lesions	E5 ORF absent; E2 ORF considerably larger than in other genera
Kappa-papillomavirus	Isolated from rabbits Cutaneous and mucosal lesions	E6 ORF larger than in other papillomaviruses; harbours an uncharacterized E8 ORF within the E6 ORF region.
Lambda-papillomavirus	Animal papillomaviruses Benign mucosal and cutaneous lesions	ELR region exceptionally large (1500 bp and 1271 bp in two known species)
Mu-papillomavirus	Human papillomaviruses Cutaneous lesions Histologically distinguishable by intracytoplasmic inclusion bodies specific for type species	LCR relatively large (982 bp and 558 bp in two known species)
Nu-papillomavirus	Human papillomavirus Benign and malignant cutaneous lesions	Several larger uncharacterized ORFs scattered throughout genome; E2 BSs in LCR all modified
Xi-papillomavirus	Bovine papillomaviruses Induce true papillomas in host Cutaneous or mucosal lesions	Characteristic E6 ORF absent; E8 ORF (located in E6 ORF region) with properties similar to E5 ORF of BPV-1
Omikron-papillomavirus	Isolated from genital warts in cetaceans	E7 ORF absent; several larger unidentified ORFs located in L1 ORF region
Pi-papillomavirus	Isolated from hamsters Mucosal lesions	ELR absent with E2 and L2 ORFs partially overlapping

From de Villiers *et al.* (2004a)

BPV, bovine papillomavirus; BS, binding site; ELR, region between the early and late genes of the papillomavirus genome; EV, *epidermodysplasia verruciformis*; LCR, long control region; ORF, open-reading frame

many viral particles or from which substantial amounts of circular double-stranded DNA could be purified.

Subtypes of papillomaviruses are defined as being 2–10% genomically different from any papillomavirus type. This term originally had a different meaning, and was used when different isolates of the same type differed partially in their restriction enzyme cleavage patterns, such as HPV 2a, HPV 2b and HPV 2c. It later became clear that these subtypes should rather fall under the category 'variants'. Other misclassifications, which were originally based on hybridization data, include the classification of papillomaviruses as types that now fall under the subtype classification. The HPV 55 genome shares 95% homology with that of HPV 44 and therefore constitutes a subtype of HPV 44. The same classification applies to HPV 64 which is a subtype of HPV 34 and HPV 46 which is a subtype of HPV 20. The numbers HPV 46, HPV 55 and HPV 64 will remain vacant to avoid any future confusion with published data. Also, comparing published data of the L1 ORF between the pygmy chimpanzee papillomavirus and the common chimpanzee papillomavirus showed 93% similarity. The latter is therefore a subtype of the pygmy chimpanzee papillomavirus (de Villiers *et al.*, 2004a).

As the search for new papillomaviruses identified so few genomes that diverged by 2–10% from defined types, it can be concluded that papillomavirus types are clearly natural taxa. It is unclear why genomes that are intermediate to closely related papillomavirus types are so rare (de Villiers *et al.*, 2004a).

Most HPV types have been isolated repeatedly in a large number of clinical studies, and the sequences of these isolates have been compared. As may be expected, most of these isolates differ from one another. It should be stressed, however, that there is no rapid diversification as in certain RNA viruses, since most HPV types could be re-isolated in the form of only 10–100 different genomic variants that normally showed approximately 1–2% sequence diversity. The phylogenetic implications of this, namely the slow, linked evolution of host and virus, have been discussed extensively while the clinical implications, i.e. pathological diversity within individual HPV types, are still under investigation (De Villiers *et al.*, 2004a).

1.1.4 *Evolution of papillomaviruses*

Papillomaviruses are an ideal model system for the study of the evolution of DNA viruses. On several levels, phylogenetic trees of papillomaviruses reflect the relationship of their hosts. One branch of HPVs includes one ape and two monkey papillomaviruses, possibly because the diversification of the viruses predated the separation of the infected-primate taxa. This hypothesis predicts that the root of the evolution of some if not all HPV types should point to Africa, since humans evolved from non-human primates in this continent.

(*a*) *Non-human primates*

To understand the mode and time scale of the evolution of papillomaviruses, 326 genital samples from rhesus monkeys and long-tailed macaques were examined with a PCR protocol optimized to detect genital HPV types. In 28 of the samples, amplicons were found that were derived from 12 different and novel viral genomes — rhesus monkey papillomaviruses (RhPV)-a to RhPV-m, with the probable taxonomic status of 'type'. This frequency of novel RhPVs suggests that rhesus monkeys may play host to papillomaviruses with a diversity similar to that of HPVs. In phylogenetic trees, all 12 novel RhPVs and the previously described type RhPV-1 were members of the genital HPV supergroup and formed three minor branches that were distinct from the 11 branches formed by genital HPVs. It appears that the evolution of primate lineages that lead to the genus *Macaca* and to humans created transmission barriers for papillomaviruses, which resulted in a viral evolution that was closely linked to the host. Additional support for the hypothesis of linked evolution derives from the phylogenetic association of two other ape and monkey viruses with genital HPVs: the supergroup formed by at least seven ungulate papillomaviruses and the isolated phylogenetic position of the only bird papillomavirus known at that time (Chan, S.Y. *et al.*, 1997a).

Portions of the genome from two different papillomaviruses of the Abyssinian *Colobus* monkey were sequenced and analysed phylogenetically. This revealed that the major evolutionary separation between genital and EV-associated papillomaviruses, hitherto found only in humans, also exists in animals. The sequence of the LCR of *Colobus* monkey papillomavirus type 2 (CgPV-2) revealed extensive conservation of functional elements that are typical of the EV-associated viruses, which suggests that CgPV-2 could be a model to study human skin cancer in relation to EV-associated HPVs. Although isolated from the same monkey species, the other *Colobus* monkey virus, CgPV-1, is a typical genital virus as shown by comparison of E and L gene sequences. The presence of these two major phylogenetic divisions of papillomaviruses in both human and monkey hosts strongly suggests that this diversification predated the evolutionary split between monkeys and apes. This would imply that at least two different groups of papillomavirus have evolved separately in their respective primate hosts for more than 22 million years with only moderate sequence changes since their genesis (Chan, S.Y. *et al.*, 1997b).

(*b*) *Humans*

Genomic segments of 118 HPV type 16 (Chan *et al.*, 1992a) isolates from 76 cervical biopsies, 14 cervical smears, three vulval biopsies, two penile biopsies, two anal biopsies and one vaginal biopsy were amplified, cloned and sequenced. The specimens were taken from patients in Brazil, Germany, Singapore and Tanzania. The sequence of a 364-bp fragment of the LCR of the virus revealed 38 variants, most of which differed by one or several point mutations. In the phylogenetic trees that were constructed, two branches could be distinguished. Nearly all of the variants from Tanzania were assigned to one

(African) branch and all of the German and most of the Singaporean variants were assigned to the other (Eurasian) branch. While some German and Singaporean variants were identical, each group also contained variants that formed unique branches. In contrast to the internal homogeneity within the groups of the Singaporean, German and Tanzanian variants, the Brazilian variants were clearly divided between the two branches. Exceptions to this were the seven Singaporean isolates with mutational patterns typical of the Tanzanian isolates. The data suggest that HPV 16 evolved separately over a long period in Africa and Eurasia. Representatives of both branches may have been transferred to Brazil through past colonial immigration. The comparable efficiencies of transfer of the African and the Eurasian variants to South America suggest the pandemic spread of HPV 16 in past centuries. Representatives of the African branch were possibly transferred to the Far East along old Arab and Indonesian sailing routes. The data indicate that HPV 16 is a well-defined virus type, since the variants show only a maximal genomic divergence of about 5%. The small amount of divergence in any one geographical location and the lack of marked divergence between the Tanzanian and Brazilian African genome variants two centuries after their probable introduction into South America suggest a very slow rate of viral evolution. The phylogenetic tree, therefore, probably represents a minimum of several centuries of evolution, if not an age equal to that of the respective human races.

The diversity of a hypervariable 364-bp segment from the HPV 16 LCR genome was investigated in 301 virus isolates collected from 25 different ethnic groups and geographical locations. Altogether, 48 variants could be distinguished that had diversified from one another along five phylogenetic branches. Variants from two of these branches were nearly completely confined to Africa. Variants from a third branch were the only variants identified in Europeans but occurred at lower frequency in all other ethnic groups. A fourth branch was specific for Japanese and Chinese isolates. A small fraction of all isolates from Asia and from indigenous as well as immigrant populations in the Americas formed a fifth branch. Important patterns of HPV 16 phylogeny suggested co-evolution of the virus with people of the three major human races, namely, Africans, Caucasians and East Asians. However, several minor patterns are indicative of smaller bottlenecks of viral evolution and spread, which may correlate with the migration of ethnic groups in prehistoric times. The colonization of the Americas by Europeans and Africans is reflected in the composition of their HPV 16 variants. The HPV 16 genomes of today represent a degree of diversity that may have evolved over a large time span, probably exceeding 200 000 years, from a precursor genome that may have originated in Africa (Ho *et al.*, 1993a).

In a similar study, the genomic sequences of HPV type 18 isolates from four continents were compared. Diversity within HPV 18 correlates with patterns of human evolution and the spread of *Homo sapiens*: HPV 18 variants, similarly to HPV 16 variants, are specific for the major human races, with maximal diversity in Africa. African HPV 18 variants are at the root of the phylogenetic tree. The identification of an African HPV 45 isolate further reduces the evolutionary distance between HPV 18 and HPV 45. HPV 18

variants from Amazonian Indians are the closest relatives to those from Japanese and Chinese patients and suggest that a single point mutation in the phylogenetically evaluated genomic segment represents at least 12 000 years of evolution. The diversity within HPV 18, and probably within other HPV types, is estimated to have evolved over a period of more than 200 000 years and diversity between HPV types may have evolved over several million years (Ong *et al.*, 1993).

The host specificity and the benign nature of most papillomavirus infections suggest that these viruses are extremely well adapted parasites. It has been proposed that this could be indicative of host–virus co-evolution (Chan *et al*., 1992b), but it is more probable that the evolution of papillomaviruses is dominated by unilateral host selection, as adjustment to the molecular mechanism of the host cell had to be made (Shadan & Villarreal, 1993).

1.1.5 *Function of viral proteins*

The functions of the papillomavirus proteins are discussed below and summarized in Table 3. Unless otherwise stated, the description of protein functions refers to HPV proteins. When individual proteins from different papillomaviruses have a common characteristic, they are designated with the generic heading of 'papillomavirus'.

(*a*) *E1*

The 73-kDa viral protein E1 is required for viral replication; it binds to a specific DNA sequence (E1 binding site; E1BS) in the viral origin of replication and assembles into hexameric complexes with the aid of a second viral protein, E2 (Frattini & Laimins, 1994). The resultant complex has helicase activity (first predicted from similarities in amino acids to SV40 large-T antigen) and initiates DNA unwinding to provide the template for subsequent synthesis of progeny DNA (Wilson *et al.*, 2002).

The functional domains of the E1 protein have been characterized for several papillomaviruses. The carboxyl terminal half has adenosine triphosphatase (ATPase) helicase activities and is necessary and sufficient for oligomerization. A change in amino acids in the ATPase domain (Pro-479 to Ser), which is predicted to inactivate adenosine triphosphate (ATP) binding, impaired the activity of E1 (Hughes & Romanos, 1993). This domain also interacts with E2 protein and the DNA polymerase α subunit p70 (Masterson *et al.*, 1998), but is not sufficient to support replication (Amin *et al.*, 2000). A segment of approximately 160 amino acid residues immediately upstream of the ATPase/helicase domain (from approximately residue 190 to residue 350) is the DNA-binding domain (DBD; Titolo *et al.*, 2000; White *et al.*, 2001; Titolo *et al.*, 2003). A stretch of about 50 amino acids within the amino terminus of E1 acts as a localization regulatory region (LRR), that contains a dominant nuclear export sequence (NES) and a nuclear localization signal (NLS), both of which are regulated by phosphorylation (Sun *et al.*, 1998; Amin *et al.*, 2000; Deng *et al.*, 2004).

Table 3. Functions of papillomavirus proteins[a]

E1	Adenosine triphosphatase (ATPase) and DNA helicase; recognizes and binds to the viral origin of DNA replication as a hexameric complex; necessary for viral DNA replication.
E2	Main regulator of viral gene transcription; binds the viral transcriptional promoter as a dimer; involved in viral DNA replication; interacts with and recruits E1 to the origin.
E4	Acts late in the viral life cycle; interacts with the keratin cytoskeleton and intermediate filaments; localizes to nuclear domain 10; induces G2 arrest; believed to facilitate virus assembly and release.
E5	Induces unscheduled cell proliferation; interacts with 16k subunit c of vacuolar ATPase; may activate growth factor receptors and other protein kinases; inhibits apoptosis; inhibits traffic of major histocompatibility complexes to the cell surface.
E6	Induces DNA synthesis; induces telomerase; prevents cell differentiation; interacts with four classes of cellular proteins: transcriptional co-activators, proteins involved in cell polarity and motility, tumour suppressors and inducers of apoptosis, primarily p53, and DNA replication and repair factors.
E7	Induces unscheduled cell proliferation; interacts with histone acetyl transferases; interacts with negative regulators of the cell cycle and tumour suppressors, primarily p105Rb.
L1	Major viral structural protein; assembles in capsomeres and capsids; interacts with L2; interacts with cell receptor(s); encodes neutralizing epitopes.
L2	Minor viral structural protein; interacts with DNA; interacts with nuclear domain 10s; believed to facilitate virion assembly; may interact with cell receptor(s); encodes linear virus neutralizing epitopes.

[a] Some of the activities of the viral proteins have been described in cultured cells or other experimental systems; some have been observed *in vivo*.
For references, see text and Sections 4.1.2 and 4.1.3.

E1 also interacts with replication protein A (RPA), which results in the rapid stabilization of single-stranded DNA generated by E1 helicase activity (Han *et al.*, 1999; Loo & Melendy, 2004). Interaction with H1 histone may play a role in unravelling the viral chromatin by removing H1 histones before unwinding the DNA (Swindle & Engler, 1998).

(*b*) *E2*

The *E2* gene encodes a product of around 40–45 kDa, depending on the papillomavirus. The protein is tripartite. First, in the carboxyl terminus, a dimerization domain results in the formation of homodimers that recognize and bind 12-bp palindromic DNA sequences (ACCGNNNNCGGT) within the LCR, defined as E2-BSs (Desaintes & Demeret, 1996). Second, the middle region of E2 — the hinge — has a rather indeterminate function, although it is important for regulating the stability of some E2 proteins and determining their nuclear localization in others (Zou *et al.*, 2000). Third, the amino

terminal domain is essential for regulation of transcription and viral DNA replication through the interaction with E1 protein (Desaintes & Demeret, 1996).

The majority of studies have demonstrated that expression of HPV E2 protein at various levels in human cells results in the repression of transcription from the viral promoter. In one study, low levels of HPV 16 E2 were shown to activate transcription in primary human epithelial cells, but repression occurred at high levels (Bouvard *et al.*, 1994a). One of the proposed mechanisms for repression by E2 is that it binds to the E2-BS adjacent to the TATA box of the LCR and thus interferes sterically with the binding of the TATA-binding protein (TBP) to the same site as has been shown for BPV-1 E2 (Dostatni *et al.*, 1991) and HPV 18 E2 (Steger & Corbach, 1997). In support of this hypothesis, mutation of the E2-BS adjacent to TATA partially relieves repression of transcription by E2 (Dostatni *et al.*, 1991).

Low levels of E2 appear to activate transcription from the viral LCR, whereas higher levels operate solely as a transcriptional repressor. This would provide a negative feedback loop to control the levels of E6 and E7 oncoproteins. Disruption or silencing of the *E2* gene leads to the elevated levels of E6 and E7 observed in cell transformation. Conversely, overexpression of BPV-1 E2 in cell lines derived from HPV-induced cervical cancers results in suppression of HPV E6 and E7 expression and promotes the re-activation of the p53 and p105Rb pathways and the consequent senescence of cells (Goodwin *et al.*, 1998).

E2 plays an important role in the segregation of newly replicated viral DNA with mitotic chromosomes, which ensures a similar distribution of viral genomes in the daughter cells. During mitosis, E2 is associated with viral DNA and with cell centrosomes and the mitotic spindle via its carboxyl terminal domain; this association is thought to be responsible for partitioning the viral genome into daughter cells (Van Tine *et al.*, 2004).

E2 interacts with the minor viral structural protein L2, which leads to inhibition of the transactivation but not the replication function of E2 for both BPV and HPV proteins (Heino *et al.*, 2000; Okoye *et al.*, 2005). This may be a mechanism whereby, at late stages of the viral life cycle, the functions of E2 are withdrawn from transcription and directed towards the amplification of viral DNA to facilitate the production of new viral progeny.

E2 also interacts with numerous cellular proteins in cultured cells. Amongst these, three proteins are of particular interest as they are involved in the DNA damage response: topoisomerase II beta-binding protein 1 (TopBP1) (Boner *et al.*, 2002), breast cancer type 1 (BRCA1) tumour suppressor protein (Kim, J. *et al.*, 2003) and poly(ADP-ribose) polymerase 1 (PARP1) (Lee *et al.*, 2002). These interactions may be involved in the regulation of viral DNA replication and also in the protection of the viral genome when the cell is damaged. The recruitment of the viral genome to sites of DNA damage through an interaction with TopBP1 or BRCA1 may provide a quick means of repairing the viral genome and suppressing replication when the cell is exposed to DNA-damaging agents.

[The Working Group noted that it has not been proven that the interactions between E2 and cellular proteins established in cultured cells take place *in vivo*.]

(*c*) *E4*

The HPV *E4* gene is located in the E region and overlaps with *E2* but is transcribed in a different reading frame. The E4 protein is heterogeneous with the major form; it is a fusion product with a 5-amino acid sequence from the N-terminus of E1 (E1^E4). Despite its genomic location and its 'E' name, the E4 protein is expressed primarily at later stages and is the most abundant viral protein expressed during the virus life cycle. Its expression coincides almost exactly with the onset of vegetative viral DNA replication; however, although the protein is detected in cells in which viral DNA replication is ongoing and in highly differentiated cells that express the capsid genes and synthesize new progeny virions, E4 is not found in virion particles. It aggregates through sequences at its C terminus, and these aggregates are found in both the cytoplasm and the nucleus of the infected cell (Doorbar *et al*., 1991, 1997; Roberts *et al*., 1997).

The functions of the E4 protein appear to be regulated partly by post-translational modification — oligomerization, phosphorylation and proteolytic cleavage — as in the case of interference by E4 in the cell cycle. The functions of E4 have been suggested to play a role in facilitating and supporting viral genome amplification, the regulation of late gene expression, the control of virus maturation and the mediation of virus release. The HPV E4 protein plays no role in cell transformation as has been shown for BPV-1 E4 (Neary *et al*., 1987), and its expression is progressively lost from neoplastic lesions during their progression to cancer (Crum *et al*., 1990).

E4 interacts with and disrupts the organization of intermediate filaments, the cornified cell envelope (CCE), mitochondria and ND10 domains. It also interferes with the cell cycle.

(i) *E4 and intermediate filaments*

A leucine-rich motif (LLXLL) at the N-terminus of most E4 proteins is responsible for the association of E4 with the keratin cytoskeleton (Roberts *et al.*, 1994) and a hydrophobic sequence at the protein C terminus mediates disruption of the cytoskeleton (Roberts *et al*., 1997). In cultured epithelial cells, the keratin cytoskeleton is often collapsed in a perinuclear bundle (Doorbar *et al*., 1991; Roberts *et al*., 1993, 2003), but perinuclear bundles of E4 and keratins are also observed in epithelial cells *in vivo* (Wang, Q. *et al*., 2004). The ability of E4 to disrupt the cytoskeleton might compromise the structural integrity of infected cells in the upper layers of warts, and enable these cells to rupture readily and release newly synthesized virus particles into the environment (Doorbar *et al*., 1991).

(ii) *E4 and CCE*

The role of E4 in aiding virus release is supported by the association of E4 with the CCE, which is a highly resistant structure beneath the plasma membrane of differentiated keratinocytes in the stratum corneum. It comprises cross-linked proteins, including loricrin, involucrin, small proline-rich proteins, cytokeratin 10 and other proteins that are covalently linked through transglutamination. Newly synthesized papillomavirions have to pass this resistant cell envelope before release into the environment. The CCE from

HPV 11-infected genital epithelium is abnormal and more fragile than that of uninfected tissue (its thickness is ~65% that of uninfected epithelium) and its association with this compromised CCE suggests that E4 could interfere with the normal assembly of CCE and aid the release of progeny virus (Brown & Bryan, 2000).

(iii) *E4 and mitochondria*

In epithelial cell lines, E4 also binds mitochondria through its N-terminal domain and causes their redistribution from the microtubule networks to E4-containing bundles. This redistribution of mitochondria causes a reduction in their membrane potential and eventually cell apoptosis (Raj *et al.*, 2004). These observations confirm the hypothesis that E4 facilitates virus release through disruption of the cytokeratin network and the CCE and through induction of apoptosis.

(iv) *E4 and nuclear domain 10*

Nuclear domain (ND) 10s are nuclear structures that contain numerous proteins, among which promyelocytic leukaemia protein (PML) is necessary for their integrity. Studies with other viruses have shown that ND10s are associated with virus replication and transcription, and that many viral proteins induce the reorganization or disruption of ND10s (Everett *et al.*, 1999). In HPV 1-induced warts, PML is relocated from ND10s to the periphery of nuclear aggregates of full-length E4; a similar redistribution is found in keratinocytes that express E4 alone (Roberts *et al*., 2003). The E4 of HPV 16 is probably similarly responsible for the disruption of ND10s. It is still not clear why viruses need to disperse ND10s, but this process may be linked to a switch between early and later stages of replication of a virus, and would be in accordance with the role of E4 in the late stages of HPV replication. Dispersal of ND10s by E4 may also be relevant to virion assembly, as the structural proteins L1 and L2 are recruited to ND10s in both BPV and HPV (Day *et al*., 1998; Florin *et al*., 2002a,b).

(v) *E4 and the cell cycle*

The expression of several different E4 proteins, including E4 from HPV 16 and 18, induces G2 arrest in the cell cycle in keratinocytes. G2 arrest is mediated by a proline-rich sequence near to the N-terminus of E4 (Davy *et al*., 2002; Nakahara *et al*., 2002; Knight *et al*., 2004). G2 arrest is due to the sequestration and retention of activated cyclin B1 complexes to 'collapsed' E4–keratin bundles in the cytoplasm of epithelial cells (Knight *et al.*, 2004; Wang, Q. *et al*., 2004). However, E4-induced G2 arrest is not dependent on the binding of E4 to keratins (Davy *et al*., 2002).

It is not clear how relevant these activities of E4 in tissue cultures are to virus maturation and production. It has been hypothesized that suprabasal cells, driven into S phase by E7, are maintained in this phase by E4 to maximize viral genome amplification. However, continuous unscheduled replication of the host DNA would limit the availability of precursor nucleotides and replication enzymes to the virus. By inhibiting cellular DNA synthesis, E4 would make replication factors available for viral DNA replication.

Thus, the effect of E4 would be to keep the infected cell in a metabolically active state without competing with host DNA synthesis, and so boost virus genome replication.

The E5, E6 and E7 proteins are described only briefly here as they are discussed in greater detail in Sections 4.1.2 and 4.1.3.

(*d*) *E5*

Not all HPVs have an E5 ORF. The E5 ORF and the protein that it encodes vary in length among papillomaviruses. The hydrophobic nature of the protein is conserved but not the primary amino acid sequence (DiMaio & Mattoon, 2001). E5 from HPVs is considered to be a transforming protein because it transforms cultured murine fibroblasts and keratinocytes (Chen & Mounts, 1990; Leptak *et al.*, 1991), enhances the immortalization potential of E6 and E7 (Stöppler *et al.*, 1996) and, in cooperation with E7, stimulates the proliferation of mouse primary cells (Bouvard *et al.*, 1994b; Valle & Banks, 1995). When HPV 16 E5 was expressed from a heterologous promoter in cultured cells, it enhanced the activity of epidermal growth factor receptor (EGFR) in the presence of ligand (Leechanachai *et al.*, 1992; Pim *et al.*, 1992; Crusius *et al.*, 1998); co-immunoprecipitation studies have indicated that HPV 16 E5 can also form a complex with EGFR when both proteins are overexpressed (Hwang *et al.*, 1995). Through activation of EGFR, E5 can interfere with several signal transduction pathways, including the mitogen-activated protein (MAP) kinase pathway (Crusius *et al.*, 1997). However, similarly to BPV E5 (Faccini *et al.*, 1996; Ashrafi *et al.*, 2000), HPV 16 E5 inhibits gap-junction intercellular communication (Oelze *et al.*, 1995) and withdraws transformed cells from the homeostatic control of neighbouring cells. Also similarly to BPV E5 (Goldstein *et al.*, 1991; Faccini *et al.*, 1996), HPV 16 E5 binds the 16k protein subunit c of the vacuolar H^+-ATPase (v-ATPase) (Conrad *et al.*, 1993; Adam *et al.*, 2000). The interaction between BPV E5 (and HPV 16 E5) and the 16k subunit c is considered to be responsible for the lack of acidification of the cellular endomembrane compartments, including the Golgi apparatus (Schapiro *et al.*, 2000) and endosomes (Straight *et al.*, 1995), and, as a consequence, the impeded transport of proteins as is the case for major histocompatibility complexes (MHC) class I and II (Ashrafi *et al.*, 2002; Zhang *et al.*, 2003; Ashrafi *et al.*, 2005). HPV 16 E5 can also inhibit apoptosis induced by Fas-ligand and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) (Kabsch & Alonso, 2002) and by ultraviolet (UV) light (Zhang *et al.*, 2002).

(*e*) *E6*

The best known property of the E6 proteins of high-risk HPVs is the inability to bind and degrade the tumour-suppressor protein p53 through the recruitment of the protein ligase, E6-associated protein (E6-AP) (Scheffner *et al.*, 1990; Huibregtse *et al.*, 1993). This results in inhibition of the transcriptional activity of p53 (Lechner *et al.*, 1992; Mietz *et al.*, 1992) and the abrogation of p53-induced apoptosis, including apoptosis induced by E7 through the destabilization of p105Rb (Jones *et al.*, 1997a). In addition, E6 binds to numerous other cellular proteins that can be divided into four broad classes: transcrip-

tional co-activators, proteins involved in cell polarity and motility, tumour suppressors and inducers of apoptosis, and DNA replication and repair factors. Several proteins belong to more than one class.

Proteins that belong to the first class are p300 (Patel *et al.*, 1999; Zimmermann *et al.*, 1999), myc (Gross-Mesilaty *et al.*, 1998) and interferon regulatory factor 3 (Ronco *et al.*, 1998); those that belong to the second are paxillin (demonstrated for BPV 1 E6; Tong & Howley, 1997; Tong *et al.*, 1997; Vande Pol *et al.*, 1998), the mammalian homologue of *Drosophila* discs large tumour-suppressor gene product (Kiyono *et al.*, 1997; Lee *et al.*, 1997; Gardiol *et al.*, 1999), Scribble (Nakagawa & Huibregtse, 2000), membrane-associated guanylate kinase with inverted orientation (MAGI-1) (Glaunsinger *et al.*, 2000) and multiple PD2 protein 1 (MUPP1) (Lee *et al.*, 2000); those that belong primarily to the third group are p53 (Scheffner *et al.*, 1990) and Bak (Thomas & Banks, 1999); and those that belong to the fourth class are mcm7 (Kühne & Banks, 1998; Kukimoto *et al.*, 1998), XRCC1 (Iftner *et al.*, 2002) and O^6-methylguanine–DNA methyltransferase (Srivenugopal & Ali-Osman, 2002). Additional proteins that interact with E6 have been described by Mantovani and Banks (2001).

E6 induces the expression and activity of telomerase (Klingelhutz *et al.*, 1996; Gewin & Galloway, 2001; Oh *et al.*, 2001; Veldman *et al.*, 2001); this activation of telomerase has been purported to be responsible for cell immortalization by E6, although the precise mechanism by which E6 achieves this effect is still unclear (see Section 4.1.3). Through the interactions described above, E6 can affect transcriptional pathways, disrupt cell adhesion and architecture, inhibit apoptosis, abrogate DNA damage responses, induce genome instability and immortalize cells.

(*f*) *E7*

The biochemical and biological properties of the E7 protein of HPV are described in detail in Sections 4.1.2 and 4.1.3 and in Zwerschke and Jansen-Dürr (2000) and Münger *et al.* (2001). The main cellular partner of E7 is the tumour-suppressor protein p105Rb (Dyson *et al.*, 1989; Münger *et al.*, 1989a). Association of E7 with p105Rb causes its degradation (Boyer *et al.*, 1996), and leads to the loss of p105Rb control over E2F transcription factors (Phelps *et al.*, 1991; Chellappan *et al.*, 1992). In addition to binding p105Rb, E7 can bind to p107 and p130, two other members of the family of pocket proteins (Dyson *et al.*, 1992; Davies *et al.*, 1993). E7 complexes with cyclins (Dyson *et al.*, 1992; Arroyo *et al.*, 1993; Tommasino *et al.*, 1993; McIntyre *et al.*, 1996) and inactivates the cyclin-associated kinase inhibitors $p21^{cip1}$ and $p27^{kip1}$ (Funk *et al.*, 1997; Jones *et al.*, 1997b; Zerfass-Thome *et al.*, 1996). The interactions with pocket proteins underlie the ability of E7 to immortalize cells and to abrogate normal responses to DNA damage (Helt *et al.*, 2002); in addition, interaction with negative cell cycle regulators leads to unscheduled cell proliferation (Malanchi *et al.*, 2004). Other partners of E7 include the S4 subunit of the 26 S proteasome (Berezutskaya & Bagchi, 1997), Mi2beta, a component of the nuclease remodelling and deacetylase (NURD) histone complex (Brehm *et al.*, 1998, 1999), the fork head domain transcription factor, MPP2 (Lüscher-Firzlaff *et al.*, 1999), the

transcription factor, activator protein 1 (AP-1) (Antinore *et al.*, 1996), insulin-like growth factor binding protein 3 (Mannhardt *et al.*, 2000), TBP (Massimi *et al.*, 1997; Phillips & Vousden, 1997), TBP-associated factor-110 (Mazzarelli *et al.*, 1995) and a novel human DnaJ protein, hTid-1 (Schilling *et al.*, 1998).

Another important aspect of the biology of E7, independent from p105Rb binding, is its ability to destabilize centrosomes, which causes mitotic defects and genome instability (Duensing & Münger, 2001, 2003).

These interactions contribute to the interference of E7 in transcription and signal transduction pathways and in DNA repair.

(*g*) *L1*

L1 is the major structural protein of papillomaviruses. The conformation of L1 in the virion has largely been elucidated through the use of VLPs (Zhou *et al.*, 1992; Kirnbauer *et al.*, 1992; Hagensee *et al.*, 1993). VLPs are empty capsids that are assembled in tissue culture cells through the overexpression of either L1 alone or L1 plus L2. HPV 16 L1 assembles into regular 72-pentamer T=7 capsids and complex loops protrude from the surface of the capsomer structure (Chen, X.S. *et al.*, 2000).

Virions or VLPs bind to cells but dissociated capsomeres do not, which implies that interactions between capsomeres are necessary for receptor binding (Volpers *et al.*, 1995). The binding of HPV VLPs or BPV virions to a variety of cell lines of different origin from a broad range of animal species suggests that the cell surface receptor for papillomavirus is widely expressed and evolutionarily conserved (Roden *et al.*, 1994; Müller *et al.*, 1995; Volpers *et al.*, 1995). The strict host range and tissue specificity of the papillomaviruses led to the original hypothesis that an epithelium-restricted receptor existed. The promiscuity of virus binding suggests that specificity is determined by some post-binding event. However, the above results do not rule out the presence of a secondary receptor that confers specificity to a generic primary receptor, and evidence suggests that L2 may bind to a secondary viral receptor (Kawana *et al.*, 2001; Yang, R. *et al.*, 2003a).

The cell receptor is (or has) probably a protein component, because treatment of the cell surface with trypsin prevents binding to VLPs (Müller *et al.*, 1995; Roden *et al.*, 1995; Volpers *et al.*, 1995), but its nature is still elusive. Several candidate receptors have been proposed, such as integrin $\alpha 6\beta 1$, integrin $\alpha 6\beta 4$ (Evander *et al.*, 1997; McMillan *et al.*, 1999; Yoon *et al.*, 2001) or the Ig receptor, FcγRIII (CD16) (Da Silva *et al.*, 2001a). However, subsequent studies have not confirmed a prerequisite role for $\alpha 6$ integrin in papillomavirus infection (Sibbet *et al.*, 2000; Giroglou *et al.*, 2001a; Shafti-Keramat *et al.*, 2003), and the role of CD16 as a papillomavirus receptor needs confirmation. Also cell-surface glycosaminoglycans (GAGs) have been suggested to be the primary receptors of papillomaviruses (Joyce *et al.*, 1999). Sequence comparison between L1 of different papillomaviruses suggests a conserved heparin-binding domain at the C-terminus and cleavage of this domain from L1 prevents its binding to both heparin and human keratinocytes. In addition, GAGs are critical for papillomavirus infection: Chinese hamster ovary cells deficient in GAG synthesis bind VLPs very poorly, and K562 cells, which

express very little surface GAG, bind small amounts of VLPs but bind larger amounts of VLP when they express exogenous syndecan (Joyce *et al.*, 1999; Giroglou *et al.*, 2001a; Selinka *et al.*, 2002; Drobni *et al.*, 2003; Shafti-Keramat *et al.*, 2003). GAGs therefore appear to be the best candidates for the primary papillomavirus receptor.

L1 VLPs are highly immunogenic (Kirnbauer *et al.*, 1992), present conformational virus-neutralizing epitopes (Ludmerer *et al.*, 1997; White *et al.*, 1998; Carter *et al.*, 2003) and can be used to detect HPV antibodies in the sera of patients with high specificity (Kirnbauer *et al.*, 1994) (see Section 1.2.1).

(*h*) *L2*

L2 is the minor capsid protein of papillomaviruses. Despite the paucity of L2 in the virion, this protein has recently been shown to have many more functions than a purely structural role. L2 contributes to the binding of virions to the cell receptor(s), facilitates virion uptake and transport to the nucleus, delivers the viral DNA to replication centres, helps the packaging of the viral DNA into capsids and, by virtue of the presence of a neutralization epitope common to L2 proteins of many papillomaviruses, may be instrumental in conferring immunity across different types of virus.

L2 contributes to the interaction of the virion with the cell surface. Two distinct regions in the N-terminal portion of L2 interact with the cell surface; in one case, interaction takes place after binding of the capsid (Kawana *et al.*, 2001; Yang, R. *et al.*, 2003a). These results suggest that multiple cell receptors for papillomaviruses exist and that, after an initial low-specificity interaction between L1 and the cell surface, a conformational switch takes place in the capsid that allows exposure of L2 epitopes and interaction with a more specific secondary receptor. The hypothesis of a conformational change in the capsid is supported by the observation that L2 from animal and human papillomaviruses induces neutralizing antibodies as a linear protein but not when assembled in the capsid (Christensen *et al.*, 1991; Lin *et al.*, 1992; Chandrachud *et al.*, 1995; Campo *et al.*, 1997; Kawana *et al.*, 1999; Roden *et al.*, 2000). The L2 neutralizing epitope is conserved among papillomaviruses, which raises the possibility of its use in cross-protective vaccines (Kawana *et al.*, 2003).

HPV 16 L1/L2 VLPs or VLPs of L1 alone are taken up by the cell with similar kinetics. However, L1 VLPs remain widely distributed in the cytoplasm whereas L1/L2 VLPs exhibit a radial distribution across the cytoplasm and accumulate in the perinuclear region, suggesting that L2 helps the transport of the capsids across the cytoplasm. This transport was inhibited by cytochalasin B, an actin-depolymerizing agent, and an N-terminal peptide of L2 binds directly to actin, which raises the possibility that papillomavirus capsids travel along actin cables (Yang, R. *et al.*, 2003b). Other possibilities have, however, been considered; one is that the capsids infect cells via a clathrin-dependent pathway (Day *et al.*, 2003).

Input L2 deposits viral DNA at ND10, an event that is critical for the efficient transcription and replication of the viral genome (Day *et al.*, 2004) and is supported by the recruitment of E2 to ND10s (Day *et al.*, 1998). At later stages of the virus life cycle, the

binding of newly synthesized L2 to viral DNA (Zhou *et al.*, 1994) and dispersal of ND10s by E4 (Roberts *et al.*, 2003) would facilitate capsid assembly.

1.1.6 *Regulation of gene expression*

The regulation of gene expression in genital human papillomaviruses has been reviewed (Bernard, 2002).

(*a*) *Organization of the LCR*

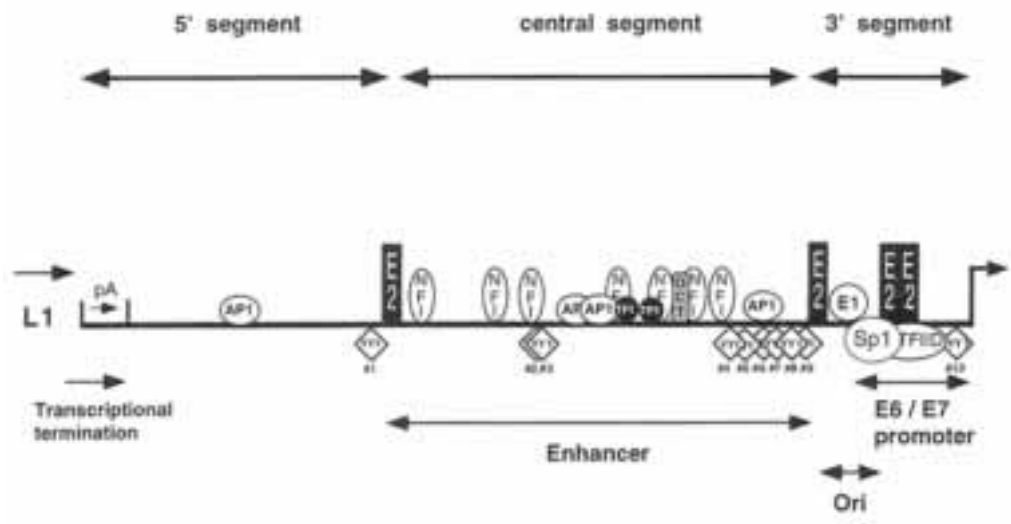
The regulation of gene expression in papillomaviruses is controlled by cellular and viral transcription factors, different promoters, differential splicing, differential transcription termination signals and the stability of different viral mRNAs. In order to be successful — from a viral point of view — the process of gene regulation should achieve: (*a*) epithelial-specific transcription; (*b*) differential expression of virus-specific genes during differentiation of squamous epithelia, in particular the switch from early to late genes; (*c*) feedback control by viral gene products, which may play an important role in the persistence of papillomavirus infection; and (*d*) response to physiological factors of the infected host on viral gene expression. Many or all of these phenomena are deregulated during malignant progression of virus-induced lesions.

Most of the regulatory events mentioned above are controlled by protein factors that are bound to *cis*-responsive elements in the LCR of the virus. The LCRs of most genital HPVs range in size from 800 to 900 bp (about 12% of the viral genome) and have a similar organization of *cis*-responsive elements (Stünkel & Bernard, 1999). Figure 6 is a schematic representation of the LCR of HPV type 16: the four E2 protein-binding sites are typical for the LCRs of all genital HPVs. The first and second E2 binding sites from the 5′ end divide the LCR into three functionally distinct segments (O'Connor *et al.*, 1996).

(i) *The 5′ segment*

The 5′ segment of the LCR is about 300 bp long and is flanked by the translation termination codon of the *L1* gene and the first E2 binding site. It contains a nuclear matrix attachment region (Stünkel *et al.*, 2000), transcription termination and polyadenylation sites for late transcripts, as well as a negative regulatory element that acts at the level of late mRNA stability (Kennedy *et al.*, 1991). The central segment functions as an epithelial-specific transcription enhancer; it fails to activate transcription from heterologous promoters in non-epithelial cell types (Gloss *et al.*, 1987; Cid *et al.*, 1993; Taniguchi *et al.*, 1993). This is probably an important mechanism for the epithelial tropism of HPVs. This enhancer is modulated by physiological factors such as steroid hormones and by intracellular signalling pathways downstream of membrane-bound receptors. A large number of cellular transcription factors have been reported to bind to about 20 different sites in this part of the LCR.

Figure 6. A schematic representation of the HPV 16 LCR, which can be considered as a model for the LCRs of all genital HPVs



Modified from O' Connor *et al.* (1996)
Four E2 binding sites serve as landmarks, and two of them divide the LCR into functionally distinct segments, which have been called the 5′, the central and the 3′ segments. The 5′ segment contains the transcription termination signal, denoted 'pA'; the central segment contains the epithelial specific enhancer that constitutes the majority of transcription factor binding sites; and the 3′ segment contains the origin of replication and the E6/E7 promoter. All the transcription factor binding sites are denoted by the abbreviation used in the text with the exception of TEF-1 which is denoted TF1.

Epithelial specificity refers to the capacity of viruses or genomic constructs to stimulate strongly homologous and heterologous promoters in cells that express epithelial markers such as certain keratin genes. This activity is similar in cells that derive from cutaneous, squamous mucosal and mucosal epithelia. The same constructs demonstrate very little activity in endothelial or hepatic cells in spite of their capacity to express keratin, and no activity in cells of other differentiation types, such as fibroblasts or lymphoid cells (Cripe *et al.*, 1987; Gloss *et al.*, 1987; Chong *et al.*, 1991). Epithelial specificity of genital HPVs is regulated by epithelial-specific transcription factors that bind to specific sites in the LCR. Non-genital HPVs have much lower enhancer activity. The activity of the enhancers is counterbalanced by silencers, which are located between the enhancer and the promoter. Their principal function appears to be repression of transcription in the basal layer of infected epithelia. The low transcriptional activity of the virus in these cells reflects the low level of gene expression required during most of its life cycle (Sailaja *et al.*, 1999).

The enhancers of many genital HPVs are activated by glucocorticoid and progesterone receptors (Gloss *et al*., 1987; Pater *et al*., 1988; Chan *et al*., 1989; Cid *et al*., 1993) which result in increased expression of the *E6* and *E7* genes. Mechanistically, gluco-

corticoid and progesterone act through the same *cis*-responsive elements. Different elements that might mediate responses to estrogen, testosterone or retinoids have not been determined to date. A repressive effect of retinoids on HPV gene expression has been observed (Bartsch *et al*., 1992).

(ii) *The 3′ segment*

The 3′ segment of the LCR is the region between the second E2 binding site and the translation start codon of the *E6* gene. It is about 140 bp long and contains a single E1 binding site, which identifies the origin of replication. The transcription start site, which is only about 5 bp upstream of the ATG codon of *E6*, is located about 90 bp downstream of the E1 binding site. A segment of about 45 bp within these 90 bp contains an Sp1 transcription factor binding site, two E2 binding sites and a TATA box (O'Connor *et al.*, 1996; Stünkel & Bernard, 1999). Together, these sites provide a complex system that can modulate the promoter activity of *E6*/*E7*.

The factor Yin Yang 1 (YY1) can both repress and stimulate a number of viral and cellular promoters (Shi *et al.*, 1997). Each of the three segments of the LCR of HPV 16 and 18, and possibly of all genital HPVs, has one or multiple YY1 binding sites (Bauknecht *et al*., 1992; May *et al*., 1994; Bauknecht *et al*., 1995; Lee *et al.*, 1998). Some of these binding sites repress *E6*/*E7* transcription. Repression is relieved by mutational change of some YY1 sites *in vivo*, which results in mutant genomes with increased carcinogenicity (May *et al*., 1994).

Regulation of expression of the late genes in genital HPVs is not well understood. The analysis of late gene expression was greatly facilitated by the availability of organotypic raft cultures that mimic differentiating epithelium. Exposure of CIN612 cells that contain episomal copies of HPV 31 to activators of protein kinase C in raft culture led to the induction of a bi-cistronic E1^E4-L1 RNA from a newly identified differentiation-dependent promoter at position 742 within the *E7* ORF (Hummel *et al.*, 1995). Time-dependent expression profiling analysis revealed a peak of late RNA expression at day 12 after exposure of the raft culture to the air–liquid interface (Ozbun & Meyers, 1997). Similarly, a differentiation-dependent late promoter has been identified at position 670 within the *E7* ORF of HPV 16 (Grassmann *et al.*, 1996). A promoter (P_{7535}) has been located in the 5′ part of the LCR of HPV 8 and has been shown to give rise to transcripts that encode late genes. Surprisingly, this promoter is stronger in transient transfections in tissue culture than the *E6*/*E7* promoter of this virus (Stubenrauch & Pfister, 1994).

1.1.7 *Methylation status of cytosine in CpG sequences in the viral genome*

Little is known about epigenetic factors that are associated with the progression of HPV infection from the subclinical stage to invasive carcinoma. In the context of the viral life cycle, there is evidence of de-novo mechanisms of methylation at cytosine residues in CpG sequences within the viral LCR in poorly differentiated cervical epithelial cells obtained from a grade 1 cervical intraepithelial neoplasia (CIN1) lesion. These cells

harbour the viral DNA as a nuclear plasmid; this methylation is lost as cells differentiate and the viral genome is amplified. The methylation pattern seen in poorly differentiated cells includes methylation of E2 binding sites, which probably suppresses E2-mediated transcriptional regulation of the viral genes (Kim, K. *et al.*, 2003b). The recognition by E2 of its cognate DNA binding site is sensitive to CpG methylation (Thain *et al.*, 1996), which could explain why expression of genes from the viral genomes in these cells is unresponsive to exogenous E2 (Bechtold *et al.*, 2003). However, in derivative cell lines that contain viral DNA in the integrated form, viral genes regain responsiveness to exogenous E2 protein (Bechtold *et al.*, 2003), which raises the possibility that the methylation pattern of the viral genome is altered upon integration. Consistent with this prediction, the LCR is hypomethylated in the single integrated copy of HPV 16 in SiHa cells. Furthermore, in an analysis of 81 patients from two different cohorts, the LCR of HPV 16 DNA was hypermethylated in 52% of asymptomatic smears, 21.7% of precursor lesions and only 6.1% of invasive carcinomas. This suggests that neoplastic transformation is inversely correlated with methylation of CpG, and that demethylation occurs before or concomitantly with neoplastic progression (Badal *et al*., 2003). A similar study with HVP 18 gave comparable results (Badal *et al*., 2004).

1.1.8 *Replication*

The replication of papillomavirus DNA has been reviewed (Lambert, 1991; Melendy *et al.*, 1995; Wilson *et al.*, 2002; Longworth & Laimins, 2004). After initiation at a single site within the LCR, replication of papillomavirus DNA proceeds bi-directionally (Yang & Botchan, 1990; Flores & Lambert, 1997). E1 is the essential origin-recognition protein for papillomavirus replication. In-vitro studies have shown that replication starts at a single E1 binding site that is located in the 3′ segment of the LCR (see Section 1.1.6). In genital HPVs, it lies approximately half way between the two E2 binding sites near the promoter and the single E2 binding site on the 5′ side of this segment; it is an A/T-rich region with only low sequence conservation (Mohr *et al*., 1990; Lu *et al*., 1993). E1 protein forms heteromers with E2 protein in solution. These heteromers stimulate initiation of replication by modulating recognition of the E1 binding site through binding of E2 to either of two flanking sites (Sverdrup & Kahn, 1995). The resultant helicase complex initiates the unwinding of DNA at the origin of replication to provide the template for subsequent synthesis of progeny DNA (Rocque *et al.*, 2000).

Due to the overlap of alternative *cis*-responsive elements involved in E1/E2 binding to DNA, replication can repress transcription from the E6 promoter (Sandler *et al*., 1993). The E1 protein–DNA complex initiates replication and requires additional cellular factors similar to those required for the replication of SV40 (Seo *et al*., 1993). These factors include replication protein A, replication factor C, proliferating-cell nuclear antigen (PCNA) and DNA polymerase alpha-primase and DNA polymerase delta. Both polymerases (also known as phosphocellulose column fraction IIA) are essential for the replication of viral DNA *in vitro* (Melendy *et al.*, 1995).

Papillomaviruses control the copy number of their genomes in infected cells, which is a prerequisite for episomal maintenance during persistent infection. This process is not under cellular control but involves the viral sequence-specific DNA-binding E2 activator and E8^E2C repressor proteins. E2 repressor proteins have been demonstrated to counteract transcriptional activation by E2 and to inhibit the E1/E2-dependent replication of papillomavirus origins (Lambert *et al.*, 1990; Bouvard *et al.*, 1994a; Stubenrauch *et al.*, 2000). All E2 repressor proteins lack the amino-terminal domain of E2 that is responsible for activation of transcription and DNA replication but retain the carboxy-terminal domain that mediates specific DNA recognition and dimerization among E2 proteins (McBride *et al.*, 1991). The E8^E2C repressor proteins consist of the peptide sequence from the small E8 ORF fused to the C-terminus of E2. E8^E2C transcripts were shown to be present throughout the entire replication cycle of HPV 31. The E8^E2C protein of HPV 31 strongly repressed the basal activity of the major viral early promoter P97 independently of E2. Mutation in the *E8* gene and disruption of the fusion protein led to a 30–40-fold increase in the transient DNA replication levels in both normal and immortalized human keratinocytes. The results suggest that the E8^E2C protein plays a role in the control of copy numbers (Zobel *et al.*, 2003).

In addition to its role in modulating viral gene expression and DNA replication, E2 also plays an important role in the efficient segregation of papillomaviral replicons to daughter cells during cell division through its capacity to bind its cognate E2 binding sites in the viral genome (Piirsoo *et al.*, 1996). E2 is thought to tether the viral genome to the host chromosomes during mitosis (Calos, 1998; Lehman & Botchan, 1998; Skiadopoulos & McBride, 1998; Ilves *et al.*, 1999; Bastien & McBride, 2000). This is probably mediated by the interaction of E2 with a cellular Brd4 bromodomain protein (You *et al.*, 2004; Brannon *et al.*, 2005).

1.2 Serological response

The study of immunity to HPV has long been hampered by the difficulty in obtaining HPV virions from cell cultures since production of the infectious virus is strictly linked to epithelial cell differentiation.

Initial studies used virions obtained from pooled material derived from warts. Experimental inoculation of BPV, CRPV or HPV (mainly HPV 1) virions into animals has indicated that denaturated virions elicit antibodies that are broadly cross-reactive among papillomavirus types, whereas intact virions induce antibodies that are largely type-specific.

Determination of the DNA sequence of several HPV types has allowed molecular biologists and immunologists to clone specific viral genes, to produce structural and regulatory viral proteins by the use of various expression vectors, and to design synthetic peptides. The experimental production of HPV 11 virions in nude mice also provided a new source of antigen. Data from these initial studies showed little sero-reactivity to denaturated virions or denaturated viral proteins, which suggests that antibodies produced

by HPV-infected patients mostly recognize conformational epitopes on the surface of the virus (Galloway, 1992, 1994).

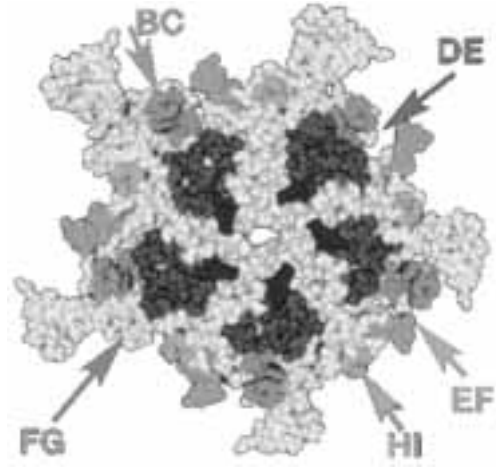
The discovery that L1 protein can assemble into VLPs that are structurally and immunochemically indistinguishable from authentic virions (Kirnbauer *et al.*, 1992; Hagensee *et al.*, 1994) has provided a valuable tool for the characterization of conformational HPV surface epitopes and strongly stimulated studies aimed at the design of prophylactic and therapeutic vaccines (for review, see Breitburd & Coursaget, 1999; Lowy & Schiller, 2006).

1.2.1 *Antigenic properties of HPV virion proteins*

Papillomavirus capsids are non-enveloped icosahedrons that comprise a major capsid protein, L1, and a minor capsid protein, L2 (Orth & Favre, 1985). L1 can assemble on its own into pentameric structures or capsomers, 72 of which in turn assemble into capsids or VLPs that are structurally and immunochemically indistinguishable from authentic virions (Kirnbauer *et al.*, 1992; Hagensee *et al.*, 1994). The repetitive structure of the capsids is highly immunogenic. Vaccination with L1 VLPs generates high-titre antibodies that are neutralizing and can protect against infection (Breitburd *et al.*, 1995; Suzich *et al.*, 1995; Kirnbauer *et al.*, 1996; Koutsky *et al.*, 2002). Recently, the crystallographic structure of a T=1 L1 VLP was determined (Chen, C.H. *et al.*, 2000). An alignment of 49 HPV L1 gene sequences showed that residues exposed on the surface were not conserved between types and were located on hypervariable loops (see Figure 7). In contrast, highly conserved residues of L1 were located below the surface of the capsomer. Consistent with this observation, neutralizing antibodies have been shown to react with conformational epitopes of L1 that are predominantly type-specific (Hines *et al.*, 1994; Roden *et al.*, 1996a; Carter *et al.*, 2000). Conformation-dependent neutralizing epitopes are present not only on capsids or VLPs, but are also retained on individual capsomers (Rose *et al.*, 1998; Yuan *et al.*, 2001). A revised model for HPV VLPs was proposed by Modis *et al*. (2002). In this model, the C-terminal extension adopts a conformation similar to that in the T1 structure but, instead of returning to the capsomer of origin, the arm is displaced onto, and ultimately invades, a neighbouring capsomer. A consequence of the invading arm model is that residues on the C-terminal arm would be accessible on the surface. It was noted that several amino acids in this C-terminal region are divergent among HPV types and, thus, may be important for recognition by type-specific antibodies. A broadly cross-reactive epitope is also found on L1 molecules, but this is folded within the virion and is only immunoreactive when denatured L1 is used as the immunogen (Firzlaff *et al.*, 1988; Jin *et al.*, 1990). Antibodies raised against denatured L1 proteins have been useful in immunohistochemical assays to detect HPV infection.

L2 is incorporated into capsids, probably at the 12 pentavalent vertices (Trus *et al.*, 1997). While L2 is not necessary for capsid formation, it is essential for genome encapsidation and infectivity (Roden *et al.*, 1996a). Although much of it remains inside the capsid, a small segment of L2 is exposed on the surface and can induce neutralizing anti-

Figure 7. Molecular structural model of the HPV 6 major capsid protein L1



From Orozco *et al.* (2005); see cover
Surface-exposed loops are indicated by arrows.

bodies (Christensen *et al.*, 1991; Campo *et al.*, 1997b; Kawana *et al.*, 1999, Roden *et al.*, 2000). Neutralizing antibodies directed against L2 tend to be much less potent than those generated against L1 (Christensen *et al.*, 1991; Roden *et al.*, 2000).

The immunogenic epitopes along the L1 and L2 proteins have been determined in two ways: first, by generating murine monoclonal antibodies to either denatured L1 or L2 proteins or to VLP proteins and, second, by mapping immunogenic epitopes that arise as a consequence of natural infection. The generation of monoclonal antibodies to VLPs has given rise to a variety of antibody types including those that were conformation-dependent and type-specific, those that were both type-specific and cross-reactive to linear epitopes and a few that were cross-reactive with intact VLPs to varying extents (Christensen *et al.*, 1990; Sapp *et al.*, 1994; Christensen *et al.*, 1996a). [The Working Group noted that inoculation of experimental animals with large amounts of VLPs, some of which could be improperly folded, may result in antibody types that are not usually produced in natural infection.]

All conformation-dependent type-specific monoclonal antibody epitopes identified to date have been found to reside on one or more hypervariable loops on the surface of VLPs (see Figure 7 and Table 4). H16.V5 was characterized as a complex epitope composed of multiple regions, the FG and HI loops (Christensen *et al.*, 2001). It was further shown that both of these loops were necessary for the transfer of HPV 16-specific binding onto HPV 31 chimeric VLPs (Carter *et al.*, 2003). It has been proposed that the F50L point mutation disrupts the binding of H16.V5 and H16.E70 (White *et al.*, 1999) by altering the conformation of residues on the FG loop (Chen, C.H. *et al.*, 2000). An alternative hypothesis is that this mutation changes the conformation of the BC loop to which it is adjacent. To address this question, hybrid VLPs were created in which the HPV 52 BC loop

Table 4. Location of conformational L1 epitopes recognized by monoclonal antibodies raised against HPV 6, 11, 16 and 31 VLPs and/or virions

Antibody	Required for binding		Region(s) required to transfer binding	Reference
	Region[a]	Amino-acid position		
H6.B10	BC, EF	49–54, 170–179	BC, EF	Christensen *et al.* (1996a); Wang, S.S. *et al.* (2003)
H6.M48	BC, EF	49–54, 170–179	BC, EF	Christensen *et al.* (1996a)
H6.N8	BC[b] and FG[c] or DE[c]	49–54	BC	Christensen *et al.* (1996a); Wang, S.S. *et al.* (2003)
H11.A3	BC, EF	49–54, 170–179	BC, EF	Christensen *et al.* (1990a); Ludmerer *et al.* (1997)
H11.B2	DE, FG	131, 132, 246, 278	DE, FG	Christensen *et al.* (1990a); Christensen *et al.* (1996a,b); Ludmerer *et al.* (1996)
H11.H3	DE[b], FG, HI	132, 246, 346	HI	Christensen *et al.* (1990a); Ludmerer *et al.* (1996)
H16.V5	FG, HI	260–290, 345–363	FG, HI	White *et al.* (1999); Christensen *et al.* (2001)
H16.E70	DE, FG	130–143, 260–290		Christensen *et al.* (1996b); White *et al.* (1999)
H16.U4	C-terminal arm	425–445		Christensen *et al.* (2001); Carter *et al.* (2003)
H31.A4	EF	175–186	EF	Carter *et al.* (2006)

VLP, virus-like particle

[a] See Figure 7

[b] Mutations in this region resulted in a partial reduction in binding.

[c] Mutations in this region showed a reduction in binding only when combined with other mutations.

was substituted onto the HPV 16 L1 backbone and the HPV 16 BC loop onto the HPV 52 L1 backbone. HPV 16 VLPs with an F50L mutation were shown to be degraded by trypsin, which indicates a failure to fold correctly; thus F50 is probably not part of the epitope (Carter *et al.*, 2003). Residues at both ends of the FG loop were shown to be involved in the binding of H16.V5 and H16.E70. To determine which residues were important for antibody binding, a series of point mutations and smaller regional mutations along the FG loop were examined. VLPs with four intertypic substitutions between amino acids 260 and 273 (16:260–273) and VLPs with three substitutions between positions 285 and 290 (16:285–290) showed substantial loss of reactivity to H16.V5 and H16.E70 (Carter *et al.*, 2003). Previous studies had shown that residues 266 and 282 were important for H16.E70 binding but not for H16.V5 binding (Roden *et al.*, 1997a; White *et al.*, 1999). None of the point mutations tested (A266T, N270S, N285T, S288N, N290T) were found to be essential for H16.V5 binding. H16E.70 binding was more sensitive to point mutations in the FG loop; the greatest loss of binding was to VLPs with substitutions at positions 285, 288 and 266.

A polar residue at position 270 was important for both H16.V5 and H16.E70 binding because substitution of Asn270 with Ala strongly reduced antibody reactivity (Carter *et al.*, 2003). Both H16.V5 and H16.E70 showed reduced binding to 16:N270S VLPs, but binding to 16:N270A VLPs was more strongly reduced. Although Ser and Ala are amino acids of similar size (somewhat smaller than Asn), Ser has a polar side-chain that can participate in a hydrogen bond similarly to Asn. Thus, the data suggest that Asn270 participates in a hydrogen bond that is important for antibody recognition of the FG loop by both H16.V5 and H16.E70.

H16.E70 was found to be a complex epitope because both the FG and DE loops were necessary for binding. The DE loop has also been shown to be essential for binding to HPV 11 by several monoclonal antibodies (Ludmerer *et al.*, 1996, 1997). However, Christensen *et al.* (2001) found that H16.E70 binding could be transferred to HPV 11/16 hybrid VLPs that did not contain the HPV 16 DE loop but possessed the HPV 16 C-terminus from residue 172 onward. A new antibody-binding site was discovered on the C-terminal arm of L1 between positions 427 and 445 (Carter *et al.*, 2003). Recognition of these residues by the H16.U4 antibody suggests that this region is exposed on the surface and supports a recently proposed molecular model of HPV VLPs (Modis *et al.*, 2002).

1.2.2 *Immune response to papillomavirus infection*

Generally there is little evidence of cross-reactive papillomavirus antibodies in human sera.

Three lines of evidence support the notion that antibody responses to HPV infection are type-specific: first, reaction of a collection of sera against a panel of HPV 6, HPV 16 and HPV 18 capsids showed that individual sera reacted differently to the three capsids (Carter *et al.*, 2000); second, pre-adsorption experiments suggest that sera that react with multiple HPV capsids contain multiple type-specific antibodies, rather than cross-reactive antibodies; third, there is a stronger correlation between seropositivity to a specific HPV capsid and detection of that type of HPV DNA than detection of any other type of HPV DNA (Kirnbauer *et al.*, 1994; Carter *et al.*, 2000).

The most consistent result from studies that investigated the immune response to HPV infection was the finding that the presence of antibodies to HPV 16 E7 protein was associated with cervical cancer at relative risks that ranged from 2.5 to 30 (Jochmus-Kudielka *et al.*, 1989; Mann *et al.*, 1990; Mandelson *et al.*, 1992; Müller *et al.*, 1992; Hamsikova *et al.*, 1994; Sun *et al.*, 1994a), and with oral and oropharyngeal squamous-cell carcinomas (Zumbach *et al.*, 2000a; Herrero *et al.*, 2003). Antibodies to E6 protein were also found to be elevated in cervical, oral and oropharyngeal cancer patients compared with controls (Meschede *et al.*, 1998; Zumbach *et al.*, 2000a,b; Herrero, 2003), as were antibodies to HPV 18 E6 and E7 in some reports. Among cases whose tumours contained HPV 16 DNA, seropositivity ranged from 25 to 50%. There was no elevation of seropositivity among individuals with preneoplastic lesions such as carcinoma *in situ*, and some studies even observed the strongest association with late-stage cervical cancer

(Fisher *et al.*, 1996; Baay *et al.*, 1995, 1997). This has led to the hypothesis that antibodies to E6 or E7 develop as a consequence of prolonged exposure to the tumour. However, antibodies to E6 or E7 do not serve as prognostic markers for progression (Park *et al.*, 1998a; Lehtinen *et al.*, 2003); nor do they predict poor survival, irrespective of the stage (Silins *et al.*, 2002).

Antibodies to E2 or E4 have also been associated with cervical cancer and CIN in some studies (Dillner *et al.*, 1989; Jochmus-Kudielka *et al.*, 1989), but not in others (Mann *et al.*, 1990; Mandelson *et al.*, 1992). In rabbits infected with CRPV, antibodies to E2 but not E4 were found in about one-third of animals bearing either papillomas or carcinomas (Lin *et al.*, 1993).

Human serum antibodies that react with fusion proteins or synthetic peptides of HPV have been found in individuals without genital tract cancers in a number of studies (Dillner, 1990; Jenison *et al.*, 1990; Köchel *et al.*, 1991). The major antigen targets appear to be the capsid proteins, in particular 6 L1, 6 L2, 16 L2 and 18 L2. Antibodies to E2 and E7 were less frequently and those to E4 were occasionally observed. Some studies found interesting correlations between seropositivity and HPV-related disease or detection of HPV DNA (Van Doornum *et al.*, 1994; Wikstrom *et al.*, 1995). However, in other studies, the prevalence of HPV antibodies was not strongly associated with other parameters of HPV infection (Jenison *et al.*, 1990; Köchel *et al.*, 1991).

To date, only a few seroepidemiological studies have used assembled HPV 1 VLPs. Carter *et al.* (1994) examined the prevalence of HPV 1 antibodies in 91 college women of whom 60% were seropositive. Among those with a history of foot warts, 89% were seropositive. The level of reactivity to HPV 1 was higher among subjects for whom foot warts were reported recently and lower among those who reported having foot warts 5–10 years previously.

HPV 6 or 11 VLPs have been used to measure seroreactivity in several studies (Carter, J.J. *et al.*, 1995; Wikstrom *et al.*, 1995; Eisemann *et al.*, 1996; Carter *et al.*, 2000). In general, there was a strong association between the detection of HPV 6/11 antibodies in individuals in whom HPV 6 DNA or genital warts were detected. The strongest association between seropositivity and genital warts was seen among women with recurrent warts. This may suggest that repeated or prolonged exposure to HPV antigens is necessary to develop a detectable antibody response. Enzyme-linked immunosorbent assay (ELISA) seropositivity was not correlated with past or present genital warts among men, in spite of higher mean ELISA values for men with genital warts versus men without genital warts (Carter, J.J. *et al.*, 1995). Men have been shown to have lower levels of seropositivity to other sexually transmitted diseases and this may reflect a reduced expression of viral antigens or less accessibility to the immune system. More studies in men are needed to confirm these observations.

A large number of studies have examined seroreactivity using HPV 16 VLPs. A comparison of the percentage of positive results among these studies is difficult because of differences in the choice of the cut-off points. Seropositivity to HPV 16 L1 was first examined in 122 women who attended health clinics for women and students (Kirnbauer

et al., 1994). Using a cut-off point based on women with no detectable HPV DNA in the genital tract, 6% of women with no HPV DNA were seropositive compared with 59% of women with HPV 16 DNA, 31% of women with HPV 18 DNA and 38% of women with HPV 31 DNA. The strongest associations were seen in women with evidence of high levels of HPV 16. For example, women who had DNA detectable by both ViraType and PCR were twice as likely to be seropositive than women in whom HPV 16 DNA was detectable by PCR only (67% versus 33%). Dysplasia was also strongly associated with seropositivity (45–75%).

In another study, in which the cut-off point was chosen by selecting an optimum optical density on the basis of the specificity and sensitivity of the results, HPV 16 L1 seropositivity was examined in subjects who were enrolled in case–control studies of CIN3 and invasive cervical cancer in Spain and Colombia (Nonnenmacher *et al.*, 1995). All cases were selected on the basis of having detectable HPV 16 DNA in the cervix. Seropositivity among cases of CIN3 was 73% and 81% in Spain and Colombia, respectively; that among cases of cervical cancer was 59% and 51%, respectively. The fact that the percentage of seropositivity was higher among cases of CIN3 may reflect the more frequent and abundant expression of L1 in premalignant lesions, although age-associated effects were not examined. In another study that examined cases of anogenital cancers, HPV 16 seropositivity ranged from 50% in HPV 16-positive vaginal cancers to 70% in HPV 16-positive vulvar cancers *in situ* (Carter *et al.*, 2001), which also supports the hypothesis that intraepithelial neoplasias that probably express high levels of L1 elicit a measurable antibody response.

The control populations from Colombia showed higher levels of reactivity (43 and 22% for CIN3 and cancer, respectively) than those from Spain (10 and 3%, respectively), a finding that parallels the increased risk for cervical cancer found in Colombia. The high level of HPV 16 L1 seropositivity in the Colombian controls probably reflects the high level of previous HPV infections in this group (Nonnenmacher *et al.*, 1995). A similar result was observed when seropositivity to HPV 16 was compared between blood donors in the USA and those in Jamaica, where the rate of cervical cancer is three times higher. Jamaican blood donors had a 4.2-fold greater probability of having HPV 16 antibodies than blood donors in the USA (Strickler *et al.*, 1999a).

In many studies, monogamous women have been found to have low seroprevalences (between 2 and 7%) (Andersson-Ellström *et al.*, 1994; Carter *et al.*, 1996; Dillner *et al.*, 1996; Wideroff *et al.*, 1996; Viscidi *et al.*, 1997; Kjellberg *et al.*, 1999). Large-scale surveys among children under 13 years of age found seroprevalences of the order of 2% (Mund *et al.*, 1997; af Geijersstam *et al.*, 1999).

Although there is consensus that carcinogenic genital HPVs are mainly sexually transmitted, controversial data exist regarding whether non-sexual transmission occurs. The specificity of HPV capsid serology for sexually transmitted HPV infections is at least 98% and it may be even higher if some non-sexually transmitted infections occurred among control groups of sexually inexperienced subjects.

The natural history of HPV 16 serum immunoglobulin (Ig) G antibodies has been examined in several large studies (Carter *et al.*, 1996, 2000; Wang *et al.*, 2003; Ho *et al.*, 2004; Viscidi *et al.*, 2004; Wang, S.S. *et al.*, 2004). In spite of differences in the populations examined, the study designs, the methodology and the choice of serological cut-off points, an overall consistent picture has emerged. HPV 16 antibodies are type-specific as shown by the fact that women with cervico-vaginal HPV 16 DNA were 8–10-fold more likely to seroconvert than women with no or other types of HPV DNA. The antibodies recognized conformational epitopes on the HPV 16 VLPs, since sera did not react with denatured VLPs or with VLPs from animal papillomaviruses. HPV 16 antibodies were slow to develop, with a median latency of 6–12 months and titres were low. Development of antibodies did not occur in all women in whom incident HPV 16 infection could be documented. Two studies found that 73% (Carter *et al.*, 2000) and 56.7% (Ho *et al.*, 2004) of women with incident HPV 16 infections seroconverted. The acquisition of HPV antibodies was most strongly associated with persistent infection. Persistence of antibodies generally lasted a few years, but results from long-term follow-up studies are not yet available. There is no evidence that antibodies modulate the state of disease, and it has been difficult to show that antibodies protect against re-infection, perhaps because it is difficult to distinguish between first infection or re-activation of infection.

A number of cross-sectional studies have demonstrated that IgA responses, specific for HPV VLPs correlate with IgG responses or with the detection of HPV DNA of the same specific type (Heim *et al.*, 1995; Wang, Z. *et al.*, 1996; Sasagawa *et al.*, 1998). Only a few longitudinal studies have been conducted (Bontkes *et al.*, 1999; Hagensee *et al.*, 2000). In a recent study (Onda *et al.*, 2003) that examined the appearance of IgA antibodies following incident HPV 16 infection, the median time to antibody detection from the primary detection of HPV 16 DNA was 10.5 months for IgA in cervical secretions and 19.1 months for serum IgA. Serum IgA antibody conversion was observed less frequently and occurred later than IgA conversion in cervical secretions or serum IgG conversion. Loss of IgA antibodies was rapid — 12.0 months for IgA in cervical secretions and 13.6 months for serum IgA — whereas approximately 20% of women with serum IgG antibodies reverted within 36 months.

In conclusion, the development of immune responses to HPV antigens is not well understood. This is in part due to the fact that different fusion proteins or peptides have been used in various studies, which has resulted in a lack of consistency. Because several studies have not found strong associations with disease, these approaches are receiving less attention than the VLP-based ELISAs. Seropositivity to E6 and E7 is clearly a consequence of tumour development, but it is not known whether factors other than prolonged exposure to antigen influence seropositivity.

1.3 Methods for the detection of HPV infection

1.3.1 *Non-molecular techniques for the detection of genital HPV infection*

The methods described in this section — visual inspection, colposcopy, cytology and histology — do not detect the factual presence of HPV, but are indirect methods that detect the clinical sequelae of an HPV infection, i.e. the presence of a clinically and/or histologically diagnosed CIN lesion or cancer. Consequently, estimates of sensitivity and specificity address the characteristics of the clinical and not the analytical test performance. Cytology and histology are restricted to a correlation with the presence of HPV.

The use of cytology as a screening tool for cervical cancer has been reviewed (IARC, 2005).

(*a*) *Visual inspection techniques*

Direct visual inspection (DVI; also known as visual inspection with acetic acid (VIA) or with Lugol's iodine (VILI)) requires that a woman lie in the lithotomy or supine position, a speculum is passed to visualize the cervix and the cervix is then washed with a dilute solution (3–5%) of acetic acid or with Lugol's iodine. Thereafter, the cervix is examined with the naked eye or with a hand-held magnifying device (usually 4 × magnification) and an adequate light source. The acetic acid causes 'whitening' (known as 'acetowhitening') of epithelial cells with a high nuclear cytoplasmic ratio. The exact reason for the acetowhitening effect is not known. A range of epithelial changes appear acetowhite after the application of acetic acid, which include immature squamous metaplasia, infection of the cervix with HPV (both low- and high-risk types) and true precursors of cervical cancer. Iodine darkens the glycogen that is stored in cervical epithelial cells. Areas of immature metaplasia, neoplasia, atrophia and condyloma stain only partially or not at all.

DVI has been evaluated in a number of large clinical trials, either alone or in comparison with cytology and HPV DNA testing. Definitions of a positive DVI test and training techniques have varied. Most studies have been cross-sectional in nature and have been limited by verification bias, since the 'gold standard' (usually colposcopy and/or biopsy) has only been applied to women with positive tests, which makes the diagnosis of disease in women with negative screening tests impossible. Verification bias tends to overestimate the specificity of the test. Most studies have used high-grade precursors of cervical cancer and/or cancer as the outcome measure. High-grade precursors of cervical cancer are known as CIN grades 2 and 3 or high-grade squamous intraepithelial lesions (HSIL), which encompasses the diagnoses of CIN2 or -3.

In some of the larger cross-sectional studies, colposcopy and/or biopsy were used to establish the presence of high-grade precursors of cervical cancer or cancer (Ottaviano & La Torre, 1982; Cecchini *et al.*, 1993; Megevand *et al.*, 1996; Sankaranarayanan *et al.*, 1998, 1999; University of Zimbabwe/JHPIEGO Cervical Cancer Project, 1999; Denny *et al.*, 2000; Belinson *et al.*, 2001a; Denny *et al.*, 2002; Cronjé *et al.*, 2003). A relatively

wide range of estimated sensitivities and specificities have been reported; although all studies showed sensitivities of more than 60%, most reported relatively low specificities and positive predictive values. However, all of them reported high negative predictive values, which has important implications for national screening programmes. One very large (n > 50 000) study compared VILI with VIA (Sankaranarayanan *et al.*, 2004a,b; IARC, 2005) and found that VILI was more sensitive than VIA and equally specific.

For low-resource countries, DVI has several potential advantages, the most important of which are the simplicity of the test, its low cost, the fact that primary health care providers can be trained to perform the test in a relatively short period of time and that an immediate result is provided, which avoids the inevitable loss to follow-up that occurs when the results of the test or treatment of lesions is delayed (Sankaranarayanan *et al*., 1998, 1999; Denny *et al*., 2002; Sankaranarayanan *et al*., 2004a).

A disadvantage of DVI is the difficulty of standardizing quality control, which is particularly important because of the subjective nature of the test. Standardization of a positive test is hindered by its subjective nature and, unlike cytology, there is no permanent record of the appearance of the cervix to allow screeners and their trainers to review the diagnosis

(*b*) *Colposcopy*

Colposcopy is a procedure that allows illuminated stereoscopic and magnified (typically × 6–40) viewing of the cervix. The woman is placed in the lithotomy position; the cervix is exposed by insertion of a bivalve speculum and various solutions (normal saline, 3–5% dilute acetic acid and Lugol's iodine) are applied to the cervical epithelium in sequence. The aim of colposcopy is to examine the transformation zone and find areas of abnormality. The latter is defined and graded according to morphological features, namely, acetowhiteness, margins, blood vessels and iodine uptake. Terminology to describe the morphological findings in a standard fashion has evolved over the years and a grading system has been proposed (IARC, 2005).

Although colposcopy continues to be used routinely as part of a standard gynaecological examination by many clinicians in some European and Latin–American countries, in the English-speaking world, it is selectively applied for diagnosis of women who are referred because of an abnormal cytological test. For this reason, studies that assess colposcopy as a diagnostic procedure are susceptible to bias and the performance of colposcopy when used for diagnostic purposes may exceed its accuracy and reproducibility when it is used as a screening tool (see Table 5).

Two meta-analyses have been performed on the accuracy of diagnostic colposcopy applied to women referred with abnormal cytology. Mitchell *et al.* (1998) performed a systematic review of 86 articles published between 1960 and 1996, nine of which met the inclusion criteria and eight of which were eligible for meta-analysis. At the cut-off level of normal versus abnormal on colposcopy, the average weighted sensitivity, specificity and area under the receiver operating characteristic curve of histological CIN2 or more were 96%, 48% and 80%, respectively. At the cut-off level of normal and low-grade SIL

Table 5. Sensitivity and specificity of diagnostic and screening colposcopy for the detection of HPV-related neoplastic lesions (≥ CIN2 and cancer)

No. of patients	Sensitivity (%)	Specificity (%)	Reference
Diagnostic colposcopy			
Meta-analysis	96	48	Mitchell *et al.* (1998)
Meta-analysis	24–90	67–97	Olaniyan (2002)
Screening colposcopy			
196	76	96	Davison & Marty (1994)
163	90.7	NA	Hilgarth & Menton (1996)
4761	13.2	99.2	Schneider *et al.* (2000)
1997	81	77	Belinson *et al.* (2001b)

CIN, cervical intraepithelial neoplasia; NA, not available

(LSIL) versus HSIL and cancer on colposcopy, the corresponding results were 85%, 69% and 82%. This suggests that, independent of prevalence and compared with low-grade lesions, high-grade lesions and cancer are diagnosed with higher sensitivity. Olaniyan (2002) reviewed publications from 1966 to 2000 and the results of his meta-analysis, based on eight studies, seven of which were also included in the previous meta-analysis, were similar.

A few studies have assessed the performance of colposcopy as a screening tool. In a cross-sectional study, 1997 unscreened Chinese women (aged 35–45 years) were first assessed by VIA performed by one gynaecologist, after which a second gynaecologist (blinded to the VIA results) performed colposcopy and took direct biopsies from abnormal areas (Belinson *et al.*, 2001b). All women also had a biopsy taken from each of the four quadrants (and all had had an endocervical curettage [ECC]) in order to estimate the performance of colposcopy in a screening setting. Sensitivity and specificity of colposcopy and direct biopsy for high-grade CIN or cancer were 81% (95% confidence interval [CI], 72–89%) and 77% (95% CI, 75–78%) compared with the combined histological findings from the direct, four-quadrant and ECC specimens. A similar study in Germany enrolled 4761 women aged 18–70 years who had visited one of 10 gynaecologists for standard care. They were screened by conventional cytology (obtained under colposcopic vision), colposcopy and HPV testing of cervicovaginal samples by PCR with probes for 13 high-risk types (Schneider *et al.*, 2000). Biopsies and ECC were performed where appropriate and, if colposcopy was normal, two biopsies and ECC were obtained. The sensitivity and specificity of screening colposcopy for detecting at least CIN2, with histological confirmation, were 13.3% (95% CI, 7.0–20.5) and 99.3% (95% CI, 99.0–99.6), respectively.

(i) *Genital HPV infections other than HPV-associated cervical neoplasia*

Both the male and female genital tracts are sites where clinically overt HPV infection can occur. Genital condylomas (warts) are easily detected with the naked eye. Bright lighting is essential and a hand-held magnifying glass is helpful. A variation on the technique of cervical colposcopy, known as high-resolution anoscopy (HRA), has been used to assess anal intraepithelial neoplasia (AIN) in the anal canal and perianal region (Jay *et al.*, 1997) using 3% acetic acid, Lugol's solution and magnification. HRA is used to guide selection of tissues from which a biopsy should be taken for the diagnosis of AIN or anal cancer. Although most authorities agree that this test is insensitive and non-specific (Beutner *et al.*, 1998a), colposcopy with or without the application of acetic acid can be helpful for the detection of smaller lesions or subclinical disease in the vagina, vulva, penis, anus and perianal skin and can help guide biopsy, especially for lesions that are suspected of being SIL or malignant.

Few studies were able to correlate the clinical or subclinical appearance of HPV-induced lesions with the presence of the virus at the molecular level. In men, Bleeker *et al*. (2005a) correlated the prevalence and size of flat condylomata, as detected by colposcopy and washing with 3% acetic acid, with penile scrapes that were positive for PCR-detected HPV and viral load: higher loads reflected higher prevalence and larger size of penile lesions.

(ii) *Non-genital HPV infection*

One earlier study (Panici *et al.*, 1992) evaluated the ability of colposcopy to detect clinical manifestations of HPV in the oral cavity in 101 male and female patients with genital condylomata who practiced orogenital sex; most of the patients (83%) had oral condylomata that could not be seen by the naked eye. Colposcopically, the oral lesions appeared as filiform (50%), moruloid (26%) and mixed (24%). HPV DNA was detected by filter in-situ hybridization in 45% of the 20 patients sampled.

(*c*) *Cytology and histology*

Reliable detection of cytological evidence of an HPV infection is notoriously difficult.

The best evaluated sign of an HPV infection is koilocytosis or koilocytotic atypia, which is the combination of nuclear atypia and the formation of a perinuclear halo (Koss & Durfee, 1955). The link between the presence of koilocytes in cervical smears and HPV was established in the mid 1970s by histological and cytological investigations (Meisels & Fortin, 1976; Purola & Savia, 1977; Della Torre *et al.*, 1978). With the advent of molecular techniques to detect the HPV genome, it became evident that cytological and histological features are not sensitive indicators of the presence of HPV. In a majority of women who are positive for HPV DNA, no cytological or histological correlates of HPV infection can be detected (Bauer *et al.*, 1991; Rozendaal *et al.*, 2000). Other cytological signs such as atypia that are indicative of the presence of (precursors of) cervical cancer do not provide a diagnostic tool for HPV infection *per se*.

In histological sections, the presence of koilocytes may be difficult to diagnose since fixation artefacts or poor dehydration can result in the presence of cells with perinuclear halos giving the cells a 'koilocyte-like' appearance. Anal cytology may also be used to diagnose AIN similarly to the use of cervical cytology to diagnose CIN (Palefsky *et al.*, 1997a,b). Anal cytology may be classified using Bethesda criteria similar to those for cervical cytology (ASCCP guidelines, discussed in Wright *et al.*, 2002).

1.3.2 *Detection of HPV proteins in infected tissues*

Immunological detection of HPV in human cells or tissues is often hindered for two main reasons: first, the late capsid proteins are only expressed in productive infections (Shah, 1992); and second, the early proteins are usually expressed in small amounts in infected tissues; in addition, the production of specific antibodies to be used for immunochemistry has long been hampered due to the lack of a suitable in-vitro culture system to obtain HPV virions (see Section 1.2). Molecular biological methods to express individual HPV antigens from any HPV type redefined the approach to produce HPV antibodies (reviewed in Galloway, 1992). Bacterial fusion proteins had several advantages: they provided an inexpensive, plentiful and reproducible source of the early and late viral antigens from any HPV type. The main disadvantage was that most fusion proteins are insoluble and had to be used in western blot assays under denaturing conditions that provide only linear epitopes. A series of type-specific antibodies have been generated from HPV recombinant proteins expressed in different heterologous systems. These antibodies can be used to demonstrate the expression of HPV proteins in biological samples using different methodologies including direct visualization in cells or tissues (immunohistochemistry) or in protein extracts (western blots and immune precipitation assays). Recently, the expression of HPV L1 protein was assessed by immunocytochemistry, using monoclonal antibodies against L1 of HPV 16 only or L1 from a pool of high-risk HPV types, in cervical smears diagnosed with LSIL or HSIL and compared with the presence of HPV DNA: 59% of the LSIL smears contained high-risk HPV DNA (types 16, 18, 33, 39, 45, 56 and 58) and 44% stained with the antibody against high-risk HPV capsid proteins; in contrast, only 33% of the HSIL were immunostained with the same antibodies while 93% were positive for HPV DNA (Melsheimer *et al.*, 2003). This suggested that loss of L1 expression in high-grade lesions, as measured with these antibodies, could be used as a prognostic marker for cervical neoplasia.

Detection of HPV early proteins is difficult due to the low expression levels generally observed in cells or tissues derived from HPV-positive lesions. Antibodies against E5, E6 or E7 are available but their use is mostly restricted to in-vitro assays (Chang *et al.*, 2001; Fiedler *et al.*, 2004). However, a polyclonal rabbit antiserum was recently raised by immunization with highly purified native HPV 16 E7 protein. Using this serum, HPV16 E7 could be detected by immunohistochemical staining of paraffin sections of biopsies of cervical HSIL and cervical cancer tissues (Fiedler *et al.*, 2004).

Since HPV infections supersede cell cycle controls, the immune detection of cell proteins that are differentially expressed in infected cells is currently being considered for use as tumour and prognostic marker, as well as for application in different modalities of cervical cancer screening (IARC, 2005). For instance, the level of expression of the cyclin-dependent kinase inhibitor $p16^{INK4a}$ was recently evaluated. An inverse relationship was found between the expression of $p16^{INK4a}$ and the presence of the normal retinoblastoma protein (pRB) in cancer cell lines in which the $p16^{INK4a}$ protein is detectable when pRB is mutated, deleted or inactivated, and is markedly reduced or absent in cell lines that contain normal pRB (Li *et al.*, 1994). pRB was shown to act as a negative regulator of $p16^{INK4a}$ gene transcription via repression of E2F activity (Li *et al.*, 1994; Khleif *et al.*, 1996). Because the E7 protein of high-risk mucosal HPVs inactivates pRB, the resulting overexpression of $p16^{INK4a}$ may be a good marker for infection by these HPV types.

A monoclonal antibody to $p16^{INK4a}$ has been developed that can detect $p16^{INK4a}$ protein in tissue sections (Klaes *et al.*, 2001). In an immunohistological study, the antibody staining was restricted to tissues from CIN2/CIN3, from CIN1 associated with high-risk HPV or from cervical cancer. Immunostaining of $p16^{INK4a}$ allowed precise identification of even small CIN or cervical cancer lesions in biopsy sections and helped reduce inter-observer variation in the histopathological interpretation of cervical biopsy specimens. Thus, p16 immunohistochemistry may reduce false-negative and false-positive biopsy interpretation and thereby significantly improve cervical (pre)-cancer diagnosis (Klaes *et al.*, 2002). Further studies are needed, however, to assess the value of $p16^{INK4a}$ immunostaining in the diagnosis of CIN and in cervical cancer screening.

1.3.3 *Detection of HPV nucleic acids*

Direct detection of HPV genomes and their transcripts can be achieved with hybridization procedures that include southern and northern blots, dot blots, in-situ hybridization, Hybrid Capture™ and DNA sequencing. A variety of signal detection procedures are available, which can further increase the sensitivity of these assays. Viral DNA and RNA can also be detected by a series of assays based on PCR. In this case, the viral genomes are selectively amplified by a series of polymerization steps, which result in an exponential and reproducible increase in HPV nucleotide sequences present in the biological specimen. Currently, the two methodologies most widely used for the detection of genital HPV types are Hybrid Capture™ version 2 and PCR with generic primers. These assays have equivalent sensitivities and specificities and both are suitable for high-throughput testing and automated processing and reading, which are necessary steps for their use in large epidemiological studies and in clinical settings.

The only procedure that is potentially capable of recognizing all HPV types and variants present in a biological specimen is DNA sequencing of an amplimer obtained by PCR with consensus primers, either after cloning into plasmids or by direct sequencing of the PCR fragment. This methodology, however, is at present labour-intensive and requires

expensive equipment. Moreover, direct sequencing does not appear to be suitable for the identification of specimens that contain multiple HPVs, since it preferentially detects the over-represented type (Vernon *et al*., 2000). Recent results obtained with multiple primer sequencing (Rady *et al.*, 1995; Gharizadeh *et al.*, 2003) and general primer-denaturing high-performance liquid chromatography (Li, J. *et al.*, 2003) suggest that it is possible to overcome this problem. The performance of these new methodologies requires confirmation in studies with large numbers of clinical samples.

The sensitivity and specificity of the various methods available vary largely but have improved considerably over the last decade, due to better quality and stability of the reagents and the accessibility to equipment that was once considered to be sophisticated. The characteristics of these assays are summarized in Table 6. Important elements to consider are collection procedure, specimen storage and sample preparation. In general, tests that use no primary amplification step, such as Hybrid Capture™ 2, are less affected by most of these variables, whereas PCR-based procedures tolerate impurities less well because of their enzymatic nature. Therefore, it is desirable to use sampling devices that allow the collection of a large cell sample and storage/transport media that not only preserve cell morphology but also stabilize DNA as well as RNA. Although a large variety of instruments for taking cervical swabs is available, further development of devices for the self-collection of vaginal samples is ongoing (Gravitt *et al.*, 2001). Procedures and devices to collect samples from men are currently being evaluated.

(*a*) *PCR-based methods*

HPV DNA can be amplified selectively by a series of reactions that lead to an exponential and reproducible increase in viral sequences present in the biological specimen. Analysis of the amplified products is generally performed by dot-blot, line-strip hybridization or restriction-fragment length polymorphism that can ultimately be coupled with direct DNA sequencing. The commonly used PCR-based methods for HPV detection in clinical samples are presented in Tables 7 and 8. The sensitivity and specificity of PCR-based methods vary, depending mainly on the primer set, the size of the PCR product, the reaction conditions and efficacy of the DNA polymerase used in the reaction, the spectrum of HPV types amplified, the ability to detect multiple types and the availability of a type-specific assay. PCR can theoretically produce 10^9 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 cross-contaminated specimens or reagents. Several procedures are available to avoid the potential problems of using PCR protocols for HPV DNA detection.

The most widely used protocols use consensus primers that are directed at a highly conserved region of the L1 gene, since they are potentially capable of detecting all mucosal HPV types. Among these are the single pair of consensus primers GP5/6 (Van den Brule *et al.*, 1990) and its extended version GP5+/6+ (Jacobs *et al.*, 1995; de Roda Husman *et al.*, 1995) and the MY09/11 degenerate primers (Manos *et al.*, 1989) and its modified version, PGMY09/11 (Gravitt *et al.*, 1998, 2000). Identification of more than 30 types can be

Table 6. Characteristics of HPV test technologies

	Test	Analytical sensitivity/ specificity	Clinical sensitivity/specificity for CIN3/cervical cancer	Comments
Based on cell morphology	Pap smears/tissues	Not applicable	Low/high	Limited because of their low sensitivities
	Colposcopy	Not applicable	Moderate/low	
	Visual inspection	Not applicable	Low/low	
Detection of HPV proteins	Immunocyto/histochemistry[a]	Low/high	Low/low	Highly dependent on sampling and tissue preservation
	Electron microscopy[a]	Low/high	Low/low	
	Western blot[a]	Low/high	Low/moderate	Cannot type HPV
Detection of HPV genomes				
Direct methods	Southern blot[a,b]	Moderate/high	Moderate/moderate	
	In-situ hybridization[a,b]	Moderate/moderate	Moderate/moderate	
	Dot blot	Low/high	Low/high	
Signal amplification	Hybrid capture[c,d,e]	High/high	High/moderate	
Target amplification	PCR[c,d,e]	High/high	Very high–high/moderate–high	
	Real-time PCR[d,e]	Very high/high	Very high/ND	
Detection of anti-HPV antibodies	ELISA			
	Peptides	Low/low	Low/low	
	VLPs	Moderate/high	Low/low	
	Fused E6/E7	High/moderate	Low–moderate/high	

CIN, cervical intraepithelial neoplasia; ELISA, enzyme-linked immunosorbent assay; ND, No data available; PaP, Papanicolaou test; PCR, polymerase chain reaction; VLPs, virus-like particles

[a] Technically cumbersome and/or time-consuming

[b] Requires DNA and tissue preservation

[c] Less dependent on sampling; can be done in crude samples

[d] Suitable for high-throughput testing and automation

[e] Provides information on viral load

Table 7. Commonly used polymerase chain reaction (PCR)-based methods for HPV detection in clinical samples: description of the main primer sets used in PCR amplification

Primer sets	Characteristics	Amplified fragment length	Specificity	Reference
MY09/11	Amplify a highly conserved L1 region	~450 bp	Mucosal HPVs	Manos *et al.* (1989)
WD72, 76, 66, 67, 154	Amplify consensus region in the E6 gene	~240–250 bp	Mucosal HPVs (HPV 6, 11, 16, 18, 31, 33, 39, 42, 45, 52...)	Resnick *et al.* (1990)
GP5/6	Amplify a highly conserved L1 region	~150 bp	Mucosal HPVs	Van den Brule *et al.* (1990)
CPI/CPIIG	Degenerate primers in the E1 gene	~188 bp	Broad spectrum, Mucosal HPVs (HPV 16, 18, 31, 33, 45, 51...) Cutaneous HPVs (HPV 1, 2, 3, 4, 5, 7, 8, 10, 14, 19, 20, 21, 22, 23, 24, 25, 36, 37, 46, 49...)	Smits *et al.* (1992)
HMB01	Primer analogous to MY09		Specific for HPV 51	Hildesheim *et al.* (1994)
HD primers	Set of 18 different degenerate primer combinations		All known as well as unknown HPVs	Shamanin *et al.* (1994a,b, 1996); de Villiers *et al.* (1997, 1999a)
L1C1/L1C2	Amplify a highly conserved L1 region	~244–256 bp	Mucosal HPVs (HPV 6, 11, 16, 18, 31, 33, 52, 58 and more..)	Shidara *et al.* (1994)
CPI/CPIIS	Degenerate primers of the E1 gene Amplify same region than CPI/CPIIG primer set	~188 bp	Broad spectrum of mucosal and cutaneous HPVs similar to CPI/CPII G	Tieben *et al.* (1994)
CP65/CP70	Degenerate primers in EV-HPV L1 region		EV-HPVs	Berkhout *et al.* (1995)

Table 7 (contd)

Primer sets	Characteristics	Amplified fragment length	Specificity	Reference
GP5+/6+	Extended version of GP5/6	~150 bp	Mucosal HPVs	Jacobs *et al.* (1995); de Roda Husman (1995)
pU-31B/2R	Amplify a consensus region within E6 and E7 genes	~228 bp	HPV 6 and 11	Sano *et al.* (1995)
pU-1M/2R	Amplify a consensus region within E6 and E7 genes	~231–268 bp	Mucosal HPVs (HPV 16, 18, 31, 33, 52b, 58 and more..)	Sano *et al.* (1995)
IU/IUDO	Amplify a consensus region in E1 gene	~188 bp	Mucosal HPVs	Paz *et al.* (1997)
CP66/CP69	Degenerate primers in EV-HPV L1 region used for a nested amplification following PCR reaction with the CP65/CP70		EV-HPVs	de Villiers *et al.* (1997)
PGMY09/11	Modified version of MY09/11		Mucosal HPVs	Gravitt *et al.* (1998, 2000)
SPF-PCR	Amplify a smaller region of L1; several primer sets have been designed	~65 bp	Mucosal HPVs	Kleter *et al.* (1998)
FAP59/64	Degenerate primers in EV-HPV L1 region	~480 bp	Cutaneous HPVs including EV-HPVs	Forslund *et al.* (1999, 2003a,b)

bp, base-pair; EV, epidermodysplasia verruciformis
Note: It is important to stress that, although highly sensitive and specific, these primer sets may differ considerably in their abilities to amplify specific types present in multiple infections (see Table 8 and Section 1.3).

Table 8. Commonly used polymerase chain reaction (PCR)-based methods for HPV detection in clinical samples: detection of the PCR-amplified products

Method	Principle	HPV typing	Potential high-throughput setting	Reference
Southern blot	PCR products are separated by electrophoresis on agarose gels then transferred onto nylon membranes; membranes are then hybridized with type specific probes.	Yes	No	Pfister & Haneke (1984)
Type-specific PCR	Following PCR amplification with consensus or degenerate primer sets, HPV amplicons are submitted to a second PCR run using type-specific primers.	Yes	Yes	Van den Brule *et al.* (1990)
Dot-blot	PCR products are denaturated and applied to replicate nylon membranes with dot-blot apparatus; membranes are then hybridized with type-specific probes.	Yes	No	Bauer *et al.* (1991)
RFLP	An aliquot of PCR amplification products is digested with a pool of restriction enzymes and the resultant restriction pattern is analysed on gel electrophoresis.	Relative typing	No	Bernard *et al.* (1994a)
ELISA or EIA	Following PCR amplification with biotin-labelled consensus primers, HPV amplicons are captured on streptavidin-coated microwell plates and detected with a digoxigenin-labelled HPV generic probe mix.	No	Yes	Jacobs *et al.* (1997); Kornegay *et al.* (2001)
Reverse line blot or LiPA	Following PCR amplification with biotin-labelled consensus primers, PCR products are hybridized to specific HPV probes immobilized on a plastic-backed nylon membrane strip.	Yes	Yes	Gravitt *et al.* (1998); Kleter *et al.* (1999); Van den Brule *et al.* (2002)

Table 8 (contd)

Method	Principle	HPV typing	Potential high-throughput setting	Reference
SSCP	Following PCR amplification with radioactive consensus primers, PCR products are separated by electrophoresis on a non-denaturing polyacrylamide gel; typing is made by comparing the migration band patterns obtained with those observed for HPV control types.	Relative typing	No	Picconi *et al.* (2000)
Sequencing	Sequencing of the PCR products can be done either directly following PCR reaction or after cloning of the amplified fragments; this is the most accurate technique for HPV typing.	Yes	Yes	Asato *et al.* (2004)

LiPA, reverse line-blot hybridization; ELISA, enzyme-linked immunosorbent assay; EIA, enzyme immunoassay; RFLP, restriction fragment length polymorphisms; SSCP, single-strand conformational polymorphisms

achieved by hybridization with type-specific probes that can be performed in different formats and analysis of restriction-fragment length polymorphism by gel electrophoresis (Bernard *et al.*, 1994a), dot-blot hybridization (Bauer *et al.*, 1991), line-strip assays (Gravitt *et al.*, 1998) and microtitre-plate assays (Jacobs *et al.*, 1997; Kornegay *et al.*, 2001) which can be automated. Another pair of consensus primers is available that amplifies a smaller fragment of the L1 gene (65 bp compared with 150 bp for the GP primers and 450 bp for MY09/11). This short PCR fragment (SPF)-PCR is designed to discriminate between a broad spectrum of HPVs in an ELISA format (Kleter *et al.*, 1998) or in reverse line-blot hybridization (LiPA) (Kleter *et al.*, 1999; Van den Brule *et al.*, 2002). The SPF and GP5+/6+ systems are widely used in epidemiological studies and have been adapted to formats for high-throughput testing. It is important to stress that, although the analytical sensitivity and specificity of these methods have been thoroughly compared (see below), they may differ considerably in their ability to detect specific types present in multiple infections. For instance, Qu *et al.* (1997) observed a 3-log decrease in the amplification of HPV 35 by MY09/11–PCR and that of HPV types 53 and 61 by GP5+/6+–PCR. In another comparison study, van Doorn *et al.* (2002) observed that the PGMY09/11–line blot assay system detected HPV 42, 56 and 59 more frequently, whereas SPF–LiPA detected HPV types 31 and 52 more frequently. This differential ability to detect specific HPV types was observed with MY09/11 and PGMY09/11 when performed with Taq Gold DNA polymerase: infections with HPV types 6, 16, 51, 53, 58, 61 and Pap 291 were detected more frequently with MY09/11–PCR while types 40, 52, 56 and 59 were detected more frequently with PGMY09/11 (Castle *et al.*, 2002a).

The first commercially available PCR-based HPV diagnostic kit for multiple types is the Amplicor™ Human Papillomavirus test kit. This assay is based on a non-degenerate pool of primers to amplify a short fragment of the L1 gene of 13 high-risk genotypes (170 bp, compared with the 450 bp obtained with PGMY09/11; see above). The amplicon is immobilized using a pool of capture molecules bound to the wells of a microtitre plate and visualized by colorimetric detection by Roche Amplicor™ chemistry. Moreover, a new test has been developed to use TaqGold™ DNA polymerase, which minimizes the amount of non-specific amplification and increases the sensitivity of the test. Because it amplifies a shorter fragment, it is considered to have a higher analytical sensitivity and a lower clinical specificity and to be adaptable for less well-preserved specimens. This system has been licensed in Europe since 2003. A PCR-based linear array HPV product, which exploits the PGMY09/11 amplification system and is capable of identifiying 37 HPV genotypes, including all high- and low-risk genotypes in the human anogenital region, is also being developed.

A fast and reliable HPV typing method has been developed using non-radioactive reverse line blotting (RLB) of GP5+/6+ PCR-amplified HPV genotypes. In this way, 40 HPV-positive clinical samples can be typed simultaneously for 37 HPV types (14 high-risk and 23 low-risk types) (Van den Brule *et al.*, 2002).

A nested PCR approach has been developed that is capable of detecting all EV-associated HPV types (Berkhout *et al.*, 1995). This methodology has been shown to be reliable

in detecting very high frequencies of known as well as new EV-HPV types in cutaneous lesions of renal transplant recipients.

An alternative PCR approach (primers FAP59/64) that is targeted to cutaneous HPV amplifies a broad spectrum of these HPV types from clinical samples, including new types, such as HPV 92 (Forslund *et al.*, 1999, 2003a,b).

Recently, PCR protocols based on a 5′-exonuclease assay and real-time detection of the accumulation of fluorescence were developed and named real-time PCR. The release of fluorescence at each amplification cycle is directly proportional to the amount of amplicon generated and is therefore considered to be an accurate method for estimating viral load. A Taqman quantitative PCR system has been reported to assess HPV viral load, while controlling for variation in the cellular content of the sample by quantification of a nuclear gene. Several reports indicated that a higher risk for cervical neoplasia was associated with higher viral loads of high-risk HPV types, in particular HPV 16 (Swan *et al.*, 1997; Joseffson *et al.*, 1999; Ylitalo *et al.*, 2000a; van Duin *et al.*, 2002). Other studies have evaluated the viral loads of different HPV types using either real-time PCR (Tucker *et al.*, 2001; Moberg *et al.*, 2004) or a low-stringency consensus PCR method (Schlecht *et al.*, 2003a). Although they showed that the risk for cervical neoplasia is associated with higher copy numbers of different HPV types (Gravitt *et al.*, 2003; Prétet *et al.*, 2004), the variability in copy numbers is too great for viral load to be used as a predictor of CIN lesions (Sherman *et al.*, 2003a). It is preferable to conclude that low viral copy numbers are associated with a low risk for developing CIN. However, further studies are warranted.

Quantitative PCR for cutaneous HPV types 5, 8, 15, 20, 24 and 36 has been developed. Using this technique, variable but low HPV DNA copy numbers were found in HPV DNA-positive non-melanoma skin cancer and actinic keratosis tissues, with a median value of 1 HPV DNA copy per 344 cells (Weissenborn *et al.*, 2005).

An HPV oligonucleotide microarray-based detection system has been developed by immobilizing HPV type-specific oligonucleotide probes and a control (β-globin probe) on an aldehyde-derivatized glass slide. Target DNA is submitted to standard PCR in the presence of fluoresceinated nucleotides (Cy5 or Cy3) using primers for both the β-globin (PC03/04) and L1 regions (modified GP5/6 primers) of several HPV types. Randomly labelled PCR products are then hybridized onto the chip, which is then scanned by laser fluorescence. In the case of multiple infections, multiple hybridization signals can be seen (Kim, C.J. *et al.*, 2003). This HPV DNA Chip® was shown to perform well in a prevalence study of HPV DNA (Hwang *et al.*, 2003, 2004). The performance of another chip (GeneTrack® HPV DNA chip) which allows the detection of 12 low-risk and 15 high-risk HPV types was successfully evaluated in HPV-positive cell lines and a small series of normal and tumour biopsies from patients with cancer of the tonsil (Oh, T.J. *et al.*, 2004). Despite its potential for further development, the utility of this system has not yet been demonstrated.

It is now being considered whether HPV RNA is an important target for the molecular diagnosis of HPV infections. The aim of testing for viral RNA is to evaluate the expression of HPV genomes (and hence their activity in infected cells) unlike HPV DNA

assays that detect only the presence of viral genomes. This is important for the identification of clinically relevant HPV infections. HPV 16 E6 and E7 transcripts can be detected with a high degree of sensitivity in clinical specimens using PCR-based methods including reverse transcriptase-PCR (RT-PCR) (Sotlar *et al.*, 1998), quantitative RT-PCR (Culp & Christensen, 2003) and real-time PCR (Lamarcq *et al.*, 2002; Wang-Johanning *et al.*, 2002). Recent studies showed that testing for E6/E7 transcripts of HPV types 16, 18, 31, 33 and 45 was more specific for discerning individuals who developed high-grade cervical disease than the detection of HPV DNA by PCR with GP5+/6+ consensus primers (Molden *et al.*, 2005). Moreover, the detection of such transcripts identified which high-risk HPV infections persisted without having to perform repeat testing (Cuschieri *et al.*, 2004a). The latter studies were performed with the PreTect HPV-Proofer™, a commercially available RNA-based real-time nucleic acid sequence based amplification kit. This reaction generates single-stranded RNAs to which specific molecular beacon probes can hybridize simultaneously to produce a fluorescent signal. The formation of newly generated RNA molecules is determined in real-time PCR by continuous monitoring of fluorescence in a fluorescent reader.

Another important application for studies of HPV RNA has been suggested by Klaes *et al.* (1999) who developed a method to amplify papillomavirus oncogene transcripts to differentiate between episomal and integrated HPV genomes. The rationale behind this method is that HPV genomes are often integrated into the host chromosomes in cervical cancers while, in normal and premalignant tissues, viral DNA is usually kept as episome. Using this assay, a strong correlation was shown between detection of integrated high-risk HPV transcripts and the presence of high-grade cervical neoplasia (Klaes *et al.*, 1999). This assay could provide a tool to predict disease progression and to monitor the efficacy of therapy (Ziegert *et al.*, 2003). The main problem with these techniques is that RNA is more prone to degradation than DNA and is therefore less available in most biological specimens, depending on the time and type of storage conditions (Habis *et al.*, 2004). For this reason, there is great interest in collection media that can preserve both DNA and RNA molecules. It was shown that the routine collection of specimens in liquid-based cytology solutions allows both morphological and immunohistochemical evaluations, and DNA and RNA studies can be performed for at least 14 days following sampling (Tarkowski *et al.*, 2001; Cuschieri *et al.*, 2004a; Nonogaki *et al.*, 2004; Cuschieri *et al.*, 2005).

Testing for the presence of more than one HPV type in a biological specimen preferentially uses PCR-based methods, since Hybrid Capture 2 does not discriminate between HPV types. In general, it appears that PCR systems that use multiple primers such as PGMY09/11 and SPF-PCR are more effectual at detecting multiple infections than those that use single consensus primers, such as GP5+/6+. This may be especially true in cases of mixed infections where one type is present in large amounts. Since more accurate tools are being developed for identifying multiple infections, it should be established whether the presence of multiple infections/lesions would be a useful marker for persistent infection and onset or progression of disease.

(*b*) *Commercial nucleic acid hybridization methods (Hybrid Capture™)*

This is the only commercially available assay for the detection of HPV DNA that has been approved by the Food and Drug Administration in the USA. The two previous versions that had a low sensitivity have now been replaced by Hybrid Capture 2, one of the most extensively used HPV tests in both epidemiological settings and clinics.

Hybrid Capture 2 is based on hybridization in solution of long synthetic RNA probes that are 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 and that are used to prepare high- (B) and low- (A) probe cocktails, which are applied in two separate reactions. DNA present in the biological specimen is then hybridized in solution with each of the probe cocktails to allow the formation of specific HPV DNA–RNA hybrids, which are then captured by antibodies that are bound to the wells of a microtitre plate and that recognize them specifically. The immobilized hybrids are detected by a series of reactions that give rise to a luminescent product that can be measured in a luminometer. The intensity of emitted light, expressed as relative light units, is proportional to the amount of target DNA present in the specimen and provides a semiquantitative measure of the viral load. Hybrid Capture 2 is currently available in a 96-well microplate format, is easy to perform in clinical settings and can be automated. Furthermore, Hybrid Capture 2 does not require special facilities to avoid cross-contamination, because it does not rely on target amplification to achieve high sensitivity, as do PCR protocols. Often, only the high-risk cocktail is used; this reduces both the duration and cost of the test. The Food and Drug Administration has recommended a cut-off value for test-positive results of 1.0 relative light unit (equivalent to 1 pg HPV DNA per 1 mL of sampling buffer). Peyton *et al.* (1998) found that Hybrid Capture 2 with the high-risk probe at a 1.0-pg/mL cut-off detected HPV types 53, 66, 67 and 73, as well as other undefined types; raising the cut-off to 10.0 pg/mL did not eliminate the cross-reactivity to types 53 and 67, which may decrease the specificity of the test (Castle *et al.*, 2002a).

A newly modified, experimental Hybrid Capture assay named Hybrid Capture 3 uses RNA probes, as in Hybrid Capture 2, but in combination with biotinylated capture oligonucleotides that are directed to unique sequence regions within the desired target to increase test specificity (Lorincz & Anthony, 2001). The assay has been developed further to reduce cross-reactivity while maintaining sensitivity and for use either on DNA or RNA as targets. A recent comparison study concluded that, at the optimal cut-off points, Hybrid Capture 2 and 3 had similar screening performance characteristics for high-grade lesions diagnosed at the enrolment visit (Castle *et al.*, 2003a).

(*c*) *Southern and northern blot hybridization*

For the analysis of HPV genomes, hybridization procedures in solid phase, such as southern blot for DNA and northern blot for RNA molecules, are excellent and can generate high-quality information; however, they are time-consuming and require large amounts of highly purified nucleic acids. Moreover, they require well-preserved, full-size

molecules and therefore cannot be carried out on all biological specimens, particularly not those derived from fixed tissues in which degradation of DNA is often observed. They are also technically cumbersome and are not suitable for large-scale population studies.

In these techniques, high-molecular-weight, highly purified DNA is digested with different restriction endonucleases and is submitted to electrophoresis on agarose gels. After denaturation, the DNA molecules are transferred to nitrocellulose or nylon filters, fixed and submitted to hybridization with specific HPV probes. Depending on the label incorporated in the probes, different signal detection systems can be used. To increase the sensitivity of the test, radioactively labelled probes are commonly used, which limits the application of southern blot to certain laboratory conditions. Despite the stringent requirements, southern blot is considered to be the golden standard for the evaluation of HPV genomes, since it can identify HPV genomes in a specimen accurately and specifically; moreover, it determines the physical status of the genomes (episomal or integrated) and gives a semiquantitative measure of viral load.

Several studies have described the presence of HPV DNA in human tissues and cell lines by southern blot (Dürst *et al.*, 1985; Lorincz *et al.*, 1992; Matsukura & Sugase, 2001). Because of the relatively lower analytical sensitivity of this test compared with target (PCR) or signal (Hybrid Capture) amplification procedures, discrepancies in HPV DNA prevalence and type distribution in cervical tumours have been reported (Matsukura & Sugase, 2004) (see the comparison of HPV testing methods in Table 9).

(*d*) *In-situ hybridization*

In-situ hybridization is a technique by which specific nucleotide sequences are identified in cells or tissue sections with conserved morphology, which allows the precise spatial localization of target genomes in the biological specimen. One great advantage of in-situ hybridization is that it can be applied to routinely fixed and processed tissues, which overcomes the relatively low analytical sensitivity of this method. Moreover, the integration status of HPV genomes can be inferred from the signal distribution in the nuclei of infected cell (Mincheva *et al.*, 1987; Berumen *et al.*, 1995). In-situ hybridization has been used to detect messenger RNA (mRNA) as a marker of gene expression when levels of viral proteins are low (Stoler *et al.*, 1989). The sensitivity of this method can be increased by combining it with PCR, a procedure known as in-situ PCR (Nuovo *et al.*, 1991a,b,c), but this is a difficult technique that has not been used widely.

The major limitation of in-situ hybridization is the potential for errors in HPV typing because of probe cross-hybridization, but recent improvements enabled its use for the detection of HPV DNA and RNA in tissues with high sensitivities and specificities (Birner *et al.*, 2001; Kenny *et al.*, 2002). Moreover, detection of HPV 16 in cervical metastatic lymph nodes of head and neck cancer patients by in-situ hybridization was highly correlated with the localization of the primary tumour (Begum *et al.*, 2003). [The Working Group noted that this methodology can clearly provide important information on HPV-mediated pathogenesis; however, its technical complexity and the requirement for intact tissue samples make in-situ hybridization inadequate for large epidemiological investigations.]

Table 9. Inter-assay comparisons of technologies for the detection of HPV DNA in clinical samples

Reference	No. of samples	Type of specimen	Method 1	Method 2	M2+/ M1+	Kappa[a]	Comments
Qu *et al.* (1997)	208	Cervico-vaginal lavages	MY09/11[b] + dot blot	GP5+/6+[b] (dot blot)	94.6%	0.8	GP5+/6+ detected fewer multiple infections; differences in the detection systems for types 35, 53 and 61
Kleter *et al.* (1998)	534	Cervical scrapes	SPF-PCR EIA[c]	GP5+/6+ (southern blot)	70.6%	0.65	
Peyton *et al.* (1998)	208	Cervical scrapes	MY09/11 + dot blot[b]	HC2 (HR)[d] cut-off[e] 1.0 pg/mL	72%	0.58	When the analysis was restricted to HPV types detected by both assays, agreement between methods was greater than 90%.
Kleter *et al.* (1999)	766	Cervical Scrapes	SPF-PCR LiPA[f]	GP5+/6+[g]	69.0%	0.77	HPV types 34, 53, 70 and 74 not represented in the GP5+/6+ system
Gravitt *et al.* (2000)	247	Cervico-vaginal lavages	PGMY09/11 + line blot assay[h]	MY09/11 + line blot assay	87.7%	0.83	
Castle *et al.* (2003a)	4345[i]	Cervico-vaginal lavages	HC3 (HR)[d] (prototype) cut-off 0.6 g/mL	HC2 (HR) cut-off 1.0 pg/mL	89%	0.53	HC3 was slightly more sensitive to detect CIN3+ than HC2; HC3 results were more concordant with MY09/11 PCR results than HC2 (1247 specimens).
Hesselink *et al.* (2004)	76	Cervical smears	GP5+/6+ EIA + rev. line blot[j]	ISH (HR)[k]	62%		Increased viral loads measured by both methods were associated with high-grade CIN, but the sensitivity of ISH to detect these lesions was too low.
Kulmala *et al.* (2004)	1511	Cervical smears	GP5+/6+ (dot blot)	HC2 (HR) cut-off 1.0 pg/mL	92%	0.67	Slightly higher sensitivities for detection of HSIL by HC2

Table 9 (contd)

Reference	No. of samples	Type of specimen	Method 1	Method 2	M2+/ M1+	Kappa[a]	Comments
Remmerbach *et al.* (2004)	106	Oral scrapes	GP5+/6+	MY09/11	7%	0.48	Negative samples were re-amplified in a nested-PCR with GP5+/6+; positivity increased further in oral but not in cervical samples.
	56	Cervical scrapes	GP5+/6+	MY09/11	73%	0.7	

See Table 7 for a description of the primers used.
CIN, cervical intraepithelial neoplasia; EIA, enzyme immunoassay; HC, hybrid capture; HR, high-risk mucosal HPV types; HSIL, high-grade squamous intraepithelial lesion; ISH, in-situ hybridization; LiPA, reverse hybridization line probe assay; SPF, short PCR fragment
[a] Agreement between positives
[b] 39 HPV types detected
[c] 43 HPV types detected
[d] 13 HPV types detected
[e] Relative light units/positive control
[f] 20 HPV types detected
[g] 14 HPV types detected
[h] 27 HPV types detected
[i] From a cohort of more than 20 000 women
[j] 37 HPV types detected
[k] BenchMark ISH View Blue Detection Kit for HPV (Ventana Med.Systems; AZ, USA)

(*e*) *Comparison of HPV testing methods*

Table 9 presents a comparison of HPV detection assays in clinical samples. In general, there are good to excellent rates of agreement between tests performed with Hybrid Capture 2 and those with generic PCR systems that employ MY09/11 and GP5+/6+, which emphasizes the availability of several viable HPV tests. An analysis of the intra- and inter-laboratory variability of these two PCR protocols (Jacobs *et al.*, 1999) showed excellent agreement between laboratories that used standardized methods. Therefore, validated protocols, reagents and reference samples assure the best test performance in different settings. It is very important to stress, however, that the analytical sensitivities and specificities of HPV tests vary largely, depending on assay characteristics, the type and quality of the biological specimen and the type and quality of the reagents used, including the use of different DNA polymerases that can affect test performance (Castle *et al.*, 2002a). Moreover, caution should be used to interpret such comparisons, because the assays differ in their ability to detect different HPV types (Kleter *et al.*, 1998) either as single or multiple infections.

Current commercially available tests have been developed to detect the most common high-risk HPV types, as confirmed by a large series of epidemiological studies that included people from all over the world. Adaptation of the assays to include HPV types according to their geographical distribution should be considered as a means of increasing test specificity.

Although the analytical sensitivity of some HPV detection assays can be very high, which is valuable in addressing the burden of HPV infections epidemiologically, its corresponding clinical significance is not so evident (Iftner & Villa, 2003; Snijders *et al.*, 2003). This is because several HPV infections do not persist and therefore do not lead to clinically relevant disease. Approaches to increase the clinical sensitivity of HPV assays that are being considered include: (*a*) testing only for the clinically relevant high-risk HPV types, (*b*) adding a viral load measure and (*c*) testing for high-risk HPV E6 and E7 transcripts. Several studies have evaluated these and other possibilities, some of which are presented here. Continuous assessment and validation of current and new methodologies is essential for the evaluation of the carcinogenic risk of certain HPVs to humans.

1.3.4 *Detection of HPV infections and HPV-associated cancers by serological assays*

The antibody response to papillomaviruses is a key determinant of protective immunity. HPV serology is also an important epidemiological tool for the assay of past and present HPV infections and for the prediction of HPV-associated cancers and their precursor lesions. Antibody responses to the HPV capsid are used as a marker of cumulative exposure to HPV while antibodies to E6 and E7 have been shown to be markers of malignant HPV-associated cervical or oropharyngeal disease. The antibody responses to HPV infections and in HPV-associated disease are discussed in detail in Section 1.2.

The development of serological assays was hampered initially by the lack of suitable cell culture systems to propagate papillomaviruses and to prepare infectious virions. This has been overcome by recombinant DNA technologies that have allowed the generation of VLPs that display conformational, type-specific epitopes of purified, correctly folded early proteins such as E6 and E7 and of infectious pseudovirions that are suitable for neutralization assays.

(*a*) *Detection of capsid antibody*

It has been shown by several groups that infection of cells with recombinant vaccinia viruses or baculoviruses that express the Ll with or without the L2 ORFs of HPV types 1, 6, 11 and 16 (Zhou *et al.*, 1992; Hagensee *et al.*, 1993; Kirnbauer *et al.*, 1993; Rose *et al.*, 1993) leads to accumulation in the nucleus of what appeared to be HPV capsids. HPV 1 particles analysed by cryoelectron microscopy at a resolution of 3.5 nm were found to be indistinguishable from HPV virions purified from foot warts (Hagensee *et al.*, 1994). Such empty capsids (also referred to as VLPs) were then used to develop ELISAs to detect antibodies in human sera and mucosal secretions for HPV types 1, 6, 11, 16 and 18 (Hagensee *et al.*, 1993; Rose *et al.*, 1993; Carter *et al.*, 1994; Hines *et al.*, 1994; Le Cann *et al.*, 1994). For these assays, VLPs are usually produced by baculovirus expression in insect cells, purified by one or more rounds of equilibrium density or other ultra-centrifugations, adsorbed to plastic surfaces and used as antigens to bind capsid-specific antibodies. ELISAs for VLPs have now become the most widely used and accepted method to analyse HPV capsid-specific antibodies. In addition, VLP-based ELISAs have been established for other mucosal high-risk HPV types 31, 33, 35 and 45 (Sapp *et al.*, 1994; Marais *et al.*, 2000a; Giroglou *et al.*, 2001b; Combita *et al.*, 2002) and for cutaneous HPV types 5, 8, 15, 20, 24 and 38 (Favre *et al.*, 1998a; Stark *et al.*, 1998; Wieland *et al.*, 2000; Feltkamp *et al.*, 2003).

Alternative methods for the detection of antibodies to HPV VLP have been developed. To increase the specificity of VLP-based ELISAs, competitive binding assays have been established for HPV types 6, 11, 16 and 18 (Palker *et al.*, 2001; Opalka *et al.*, 2003). In these tests, human antibodies compete for binding to VLPs that are adsorbed on plastic surfaces with a radio- or fluorescence-labelled monoclonal HPV type-specific reporter antibody directed to a dominant conformational epitope on the VLPs. However, such competitive assays usually have lower analytical sensitivity compared with direct binding assays. In other approaches, monoclonal antibodies that recognize conformational VLP epitopes (Hagensee *et al.*, 2000) or heparin-sulfate (cross-linked to bovine serum albumin) to which intact VLPs bind specifically (Wang *et al.*, 2005) are adsorbed on a plastic surface to capture selectively L1 that displays conformational epitopes. Finally, inhibition of VLP-mediated haemagglutination has been described for HPV types 6, 11, 16, 18, 33 and 45 (Roden *et al.*, 1996b).

HPV L1 expressed in bacteria as the glutathione-*S* transferase (GST) fusion protein has been shown to form capsomers spontaneously, to display most epitopes defined on VLPs and to be suitable as an antigen for the detection of HPV capsid antibody (Rose

et al., 1998, Yuan *et al.*, 2001). To circumvent the tedious procedures of production and purification and the varying yields and quality of VLPs from different HPV types, an alternative ELISA for HPV capsid antibody has been developed based on the affinity of GST–L1 fusion proteins purified on glutathione-coated plastic surfaces. It has been shown to have similar analytical sensitivity and specificity for HPV 16 and 18 as the conventional VLP-based ELISA (Sehr *et al.*, 2002). Recently, this type of assay has been adapted to fluorescent bead technology which allows the fast analysis of antibodies against many different (theoretically up to 100) proteins in parallel using only minute amounts of serum (Chen *et al.*, 2005). In view of the many papillomavirus types that potentially infect humans, this assay type could be of value in sero-epidemiological studies that analyse type-specific seroprevalences for large groups of HPV types simultaneously.

Several years of research were required to validate VLP-based ELISAs, and validation was laborious in the HPV system because: (*a*) early methods for the detection of HPV DNA were inaccurate, to the extent that misclassification seriously flawed early epidemiological studies of HPV (Franco, 1992); (*b*) many of the more than 100 different HPV types are not associated with malignancy and are not sexually transmitted, which renders serological cross-reactions difficult to predict on the basis of DNA homology; (*c*) most HPV infections are rapidly cleared spontaneously. In follow-up studies of HPV DNA-positive women, some 70% cleared their HPV DNA within 12 months (see also Section 1.2.2). Thus, many people who test negatively for HPV DNA may have had a previous infection; (*d*) seroconversions can appear many months after infection (see Section 1.2.2), and many people with a recently acquired HPV infection may not have seroconverted; and (*e*) testing for the HPV genome in samples taken from the uterine cervix will not detect infections at other body sites.

In spite of these major theoretical difficulties, serology with viral capsids has shown an amazing concordance with detection of viral DNA at the cervix for several HPV types. In the original report, serum IgG antibodies against capsids of HPV 16 of a wild-type strain were found in 59% of women who tested positively for cervical HPV 16 DNA, whereas only 6% and 9% of women who tested negatively for cervical HPV DNA or positively for the benign HPV types 6 and 11, respectively, had these antibodies (Kirnbauer *et al.*, 1994).

Human antibodies mostly recognize conformational epitopes on the capsid surface. HPV capsids can be disrupted, usually by treatment with high pH carbonate buffer, to destroy the type-specific epitopes; this results in the loss of type-specific serological reactivity, whereas cross-reactive antibody responses remain unaffected (Carter *et al.*, 1993; Dillner *et al.*, 1995a). Similar results were obtained previously using purified virions isolated directly from lesions (Steele & Gallimore, 1990; Bonnez *et al.*, 1991). It was also shown that neutralizing antibodies to HPV type 11 virions recognized conformational epitopes on synthetic HPV type 11 capsids. An alternative method for assaying type-specific antibodies is based on the fact that they are usually present at higher titres than cross-reactive antibodies. By assigning a ‘cut-off’ value that classifies low-titred reactivity as negative, specific results can be also obtained without a negative control or confirmatory

assays (Wideroff *et al.*, 1995). Human anti-capsid antibody responses were found to be directed against epitopes on the Ll protein, because addition of L2 protein did not augment the association between HPV infection and antibody reactivity (Carter *et al.*, 1993).

The sensitivity of assays is measured using panels of serum samples obtained from individuals with a documented infection with the virus in question, i.e. by detection of the viral genome. State-of-the-art detection of viral DNA is not entirely straightforward, and misclassification is most commonly due to the inability to distinguish between some of the many viral genotypes, to contamination in PCR assays and to inadequate sampling. Whereas there is good to excellent agreement between laboratories for certain assays such as the PCR–ELISA system based on the general primers GP5+/GP6+, there is poor agreement between different assays for the detection of HPV DNA (Jacobs *et al.,* 1999). In general, studies of the sensitivity of HPV capsid serology that have used state-of-the-art methodology for the detection of HPV DNA have found a sensitivity of 50% or more (Andersson-Ellström *et al.*, 1994; Kirnbauer *et al.*, 1994; Wideroff *et al.*, 1995; Carter *et al.*, 1996; Wideroff *et al.*, 1996a; Kjellberg *et al.*, 1999). In a large population-based study that used nested PCR technology, sensitivity was found to be 65–75% (Kjellberg *et al.*, 1999).

Persistence is a covariate of HPV seropositivity that may result from misclassification or may be a biological phenomenon. The clearly detectable presence of HPV DNA is more commonly associated with HPV seropositivity than its weakly detectable presence (Viscidi *et al.*, 1997). A heavy infection may produce more viral protein that may induce a more effective antibody response. Alternatively, a weakly detectable presence of HPV DNA may be more commonly misclassified and not be due to true infection. The persistent presence of HPV DNA in samples taken at two different occasions from the same woman is more commonly associated with seropositivity than a transient presence of HPV DNA that was not detectable in a second sample taken from the same woman (Wideroff *et al.*, 1995). Transient infections may not be present in the body long enough to evoke an antibody response. Alternatively, detection of HPV DNA that could not be repeated in a second sample may have been misclassified or may have reflected the presence of viral genomes that never resulted in an infection. The HPV virion is stable and resistant to desiccation and remains extracellularly viable for at least 1 week (Roden *et al.*, 1997b).

Specificity is assayed by comparing serum samples taken from women infected with the same HPV type, women infected with other HPV types and women not exposed to HPV. Comparisons with women infected with other types of HPV are confounded by the fact that different carcinogenic genital types are transmitted similarly and that women in the high-risk group currently infected with a certain HPV type may have had previous infections with other HPV types. All serological studies of type specificity of the HPV capsid have found a strong type-restricted component, and, in a large population-based study performed in a population with a modest number of lifetime sexual partners, no co-variation with the presence of other HPV types was found, which indicated type specificity (Kjellberg *et al.*, 1999). Type specificity of HPV capsid-based assays is also supported by

a very large number of experimental studies on immunological cross-reactivity of monoclonal antibodies against HPV capsids. Whereas disrupted or partially disrupted viruses expose epitopes that are broadly cross-reactive or even group specific (Jenson *et al.*, 1980; Dillner *et al.*, 1991), conformationally dependent epitopes on intact capsids have generally been HPV type-specific (Christensen *et al.*, 1996b). The exceptions are HPV 6 and 11 that have been shown to contain shared epitopes and type-specific epitopes on intact capsids (Christensen *et al.*, 1994, 1996b).

The specificity of HPV capsid serology is also indicated by the fact that panels of serum samples taken from subjects with no or little sexual experience have very low seroprevalences (see Section 1.2.2).

Seroprevalence from different studies and laboratories must be compared with caution due to interlaboratory variation in assays and different definitions of cut-off. Interlaboratory agreement between three laboratories has been assessed in one study that determined seropositivity for HPV 16 by VLP-based ELISA. Variation coefficients of 0.61 to 0.8 were found (Strickler *et al.*, 1997). Especially important factors include the use of different groups of sera as a basis for determination of cut-off and different mathematical definitions of cut-off. WHO is currently developing serological reference reagents for the major HPV types.

(*b*) *Neutralization assays*

Neutralization assays are thought to be more type-specific than antibody-binding assays. Many neutralization assays are based on infectious pseudovirions (Table 10). While initial assays were technically complex and tedious, and were therefore restricted to the analysis of only small numbers of sera, they allowed the definition of neutralizing epitopes by monoclonal antibodies (see also Section 1.2.1). Recent developments suggest that the high-throughput analysis that is needed for large epidemiological and vaccination studies may be feasible.

(*c*) *Detection of antibodies to E6 and E7*

Antibodies to E6 and E7 proteins of HPV types 16 and 18 are markers of HPV-associated malignant disease but, since not all patients with tumours show such antibodies, they cannot be used as diagnostic markers.

The association of E6 and E7 antibodies with cervical cancer was already apparent in initial studies that analysed only linear epitopes by either peptide ELISA or western blot analysis, despite the low sensitivity and specificity of these assays. Methods that apply full-length E6 or E7 proteins that present conformational epitopes, i.e. immunoprecipitation assays with in-vitro transcribed and translated HPV 16 E6 or E7 proteins (Stacey *et al.*, 1992, 1993; Viscidi *et al.*, 1993; Nindl *et al.*, 1994; Sun *et al.*, 1994b; Chee *et al.*, 1995; Nindl *et al.*, 1996), showed higher sensitivity and specificity.

ELISAs that use yeast-expressed biochemically purified and renatured full-length HPV 16 and 18 E6 and E7 proteins have been shown to be more specific and equally sensitive compared with radioimmunoprecipitation assays (Meschede *et al.*, 1998). These

Table 10. HPV neutralization assays

Type and source of infectious particles	Read-out	Reference
HPV 11; virions from athymic mouse xenograft	Xenografted human foreskin transformation	Christensen & Kreider (1990)
HPV 11; virions from athymic mouse xenograft	RT-PCR of HPV 11 mRNA in xenografted human foreskin	Bonnez *et al.* (1992)
BPV1; virions from lesions	C127 mouse fibroblast focus formation	Christensen *et al.* (1995)
CRPV; virions from lesions	Abortive rabbit cell infection	
HPV 16; pseudovirions generated from HPV 16 L1 and L2 expressed from Semliki Forest viruses vector and carrying BPV1 genome, in cultured hamster cells harbouring autonomously replicating BPV-1 genome (BPHE-1 cells)	C127 mouse fibroblast focus formation	Roden *et al.* (1996a)
HPV 33; pseudovirions carrying β-galactosidase marker plasmid and generated form L1 and L2 expressed by vaccinia-virus in COS-7 cells	β-Galactosidase expression in COS-7 cells	Unckell *et al.* (1997)
HPV 11; virions from athymic mouse xenograft	RT-PCR of viral mRNA in infected cultured neonatal human foreskin keratinocytes or immortalized human adult skin cell line HaCaT	Leiserowitz *et al.* (1997)
HPV 16; virions from SCID mouse xenograft	RT-PCR of viral mRNA in infected immortalized human adult skin cell line HaCaT	White *et al.* (1998)
HPV 16 and 6; pseudovirions assembled in vitro from L1/L2 VLPs produced in insect cells and β-galactosidase marker plasmid	β-Galactosidase expression in infected COS-7 cells	Kawana *et al.* (1998); Matsumoto *et al.* (2000)
HPV 6, 11, 16 and 18; pseudovirions generated by coupling of β-lactamase marker plasmid to L1/L2 VLPs produced in yeast	β-Lactamase activity in infected C33A cervical carcinoma cell line	Yeager *et al.* (2000)
HPV 6, 16 and 31b; virions from cultured trophoblast cell line 3A	HPV DNA replication and/or gene expression in infected 3A cells	Liu *et al.* (2001a); You *et al.* (2003)
HPV 16 and 31; pseudovirions generated by coupling of luciferase marker plasmid to L1 VLPs produced in insect cells	Luciferase activity in infected COS-7 cells	Bousarghin *et al.* (2002)
HPV 16 and 45; virions generated in raft cultures	RT-PCR of viral mRNA in infected immortalized human adult skin cell line HaCaT	McLaughlin-Drubin *et al.* (2003, 2004)

Table 10 (contd)

Type and source of infectious particles	Read-out	Reference
HPV 16 and 18; pseudovirions carrying secreted alkaline phosphatase marker plasmid and generated from expression of codon modified L1 and L2 genes in 293T cells	Quantification of secreted alkaline phosphatase activity	Pastrana *et al.* (2004)

BPV, bovine papillomavirus; CRPV, cottontail rabbit papillomavirus; RT-PCR, reverse transcriptase-polymerase chain reaction; VLP, virus-like particle

ELISAs have been used to demonstrate the association of antibodies for HPV 16 and 18 E6 and E7 proteins with cervical cancer (Meschede *et al.*, 1998; Zumbach *et al.*, 2000b) and also oral cancer (Zumbach *et al.*, 2000a; Herrero *et al.*, 2003).

Recently, ELISAs based on the expression of affinity-purified HPV 16 and 18 E6 and E7 in bacteria as GST fusion proteins have been developed, which appear to be of greater sensitivity (Sehr *et al.*, 2001). Epidemiological studies using these assays have not yet been published.

(*d*) *Detection of antibodies to E1, E2, E4 and E5*

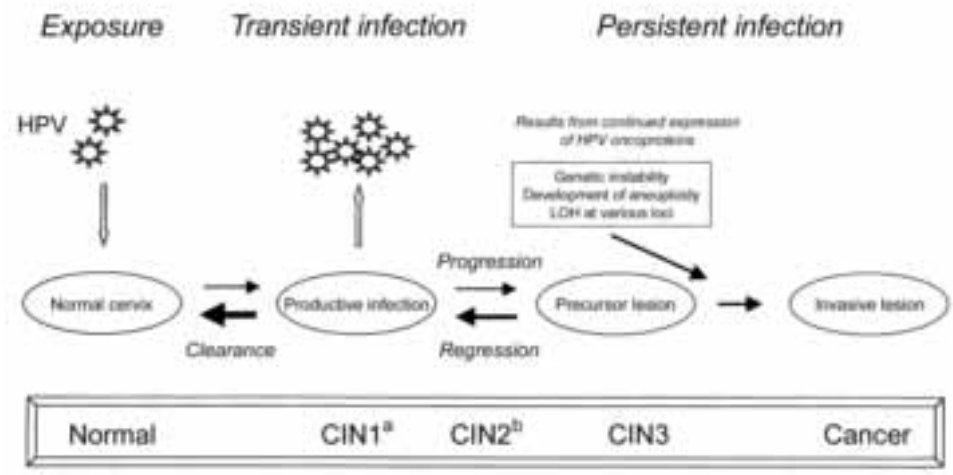
From studies that used linear epitopes as antigens in either peptide ELISA or western blot analysis, there is some indication that antibodies to E2 and E4 or some specific linear sequences of these proteins are associated with cervical cancer, but no consistent picture has emerged. As seen for antibodies to L1 and also to E6 and E7 proteins, assays that use proteins that also present conformational epitopes need to be developed before this question can be analysed appropriately.

1.4 Natural history and epidemiology of HPV infection

1.4.1 *Introduction*

HPV is a prevalent pathogen, the epidemiology of which has mostly been studied in the uterine cervix and the vagina. This section is therefore restricted to the natural history of genital HPV types. The cervical transformation zone can be considered as a ring of tissue that is susceptible to the carcinogenicity of HPV. Cervical HPV infection can be assessed visually, microscopically (via cytology or histology) and by molecular detection methods. The basic steps that lead from the normal cervix to cancer are well established (see Figure 8). To a large extent, these are probably also valid for the natural history of HPV in lesions at other anogenital sites; however, the molecular epidemiology of HPV infection at these sites is not as well characterized as that in the uterine cervix.

Figure 8. Natural history of preclinical abnormalities of the cervix



From IARC (2005)

[a] Classical histological features of CIN1 are uncommon among women who have transient infections.

[b] This entity is not as well defined as CIN3.

The major steps known to be necessary for cervical carcinogenesis include HPV infection, persistence of that infection, progression to precancerous lesions and eventually invasion. Provided that the latter step has not taken place, this process is reversible by the clearance of HPV infection and regression of precancer, which happen in many women who have ever experienced HPV infection. As discussed below, HPV infection might usefully be separated into low-viral load infections that engender no microscopically evident abnormalities and higher-viral load infections that do.

As described in Section 1.1, over 100 types of HPV exist, of which more than 40 are mucosotropic viruses that infect the anogenital and upper aerodigestive tracts (de Villiers *et al.*, 2004a). Among the latter, approximately 15 are considered to be high-risk types. The various HPV types do not all occur in different populations at the same rate; therefore, although much is known about the epidemiology and natural history of HPV infections, little is known about the long-term characteristics of infections at the type-specific level, e.g. the assessment of viral persistence. Most knowledge refers to HPV 16, which is the type most frequently found in tumours in the general population, and is discussed separately below.

1.4.2 *Transmission and acquisition*

(*a*) *Horizontal transmission*

The most common mode of horizontal transmission of anogenital HPV is by sexual activity through contact with infected cervical, vaginal, vulvar, penile or anal epithelium. In the early 1950s, Barrett *et al.* (1954) reported that genital warts developed within 4–6

weeks in wives of servicemen who had returned from overseas and who had had genital warts. Oriel (1971) reported that 64% of sexual partners of individuals who had genital warts developed genital warts themselves after a mean interval of 2–3 months. Similar results have been reported by others (Teokharov, 1969; Barrasso *et al.*, 1987). There is now overwhelming epidemiological evidence for the role of sexual activity in the transmission of anogenital HPV (Franco *et al.*, 1995; Bosch *et al.*, 1996; Dillner *et al.*, 1999; Bleeker *et al.*, 2002; Castellsagué *et al.*, 2003; Sellors *et al.*, 2003). Studies among initially virginal women strongly confirm the sexually transmitted nature of HPV infection (Rylander *et al.*, 1994; Kjaer *et al.*, 2001).

Sexual contact with an infected partner is necessary for transmission, presumably through microscopic abrasions in the mucosa or skin, and HPV infections are easily transmitted; however, on the basis of data on lesbians, it appears that intromissive intercourse in which an infected penis enters the vagina is not strictly necessary (Marrazzo *et al.*, 2001). Moreover, transmission may take place in one anogenital site, such as the introitus, and the infection may be spread by self-inoculation to another site (Winer *et al.*, 2003). As a group, anogenital HPVs are the most common sexually transmitted infections but there is some evidence that the degree of sexual transmissibility may vary among types and across populations (Franco *et al.*, 1995; Kjaer *et al.*, 1997; Rousseau *et al.*, 2000).

In addition to the sexual behaviour of women, epidemiological studies suggest that age, both of women and their partners, genetic and environmental susceptibility factors, use of barrier contraceptives, co-infections, male sexual behaviour and male circumcision are related to the prevalence of HPV (reviewed by Schiffman & Kjaer, 2003). A series of studies has also established that the sexual behaviour of and HPV infection in the male partner significantly increase the risk whereas circumcision of the male partner was associated with a significant reduction in risk for invasive cervical cancer among women (Castellsagué *et al.*, 2002).

Although fewer studies have been conducted on the prevalence of HPV infection among men than among women, HPV infections also appear to be common in men (Baldwin *et al.*, 2004; Shin *et al.*, 2004; Weaver *et al.*, 2004). In the few studies that have evaluated factors associated with infection in men, sexual history, age and possibly condom use are associated with the prevalence of HPV (Baldwin *et al.*, 2004; Shin *et al.*, 2004; Weaver *et al.*, 2004). Published data on the natural history of HPV in men are scarce; however, several large prospective studies of HPV infection in men are currently being carried out. As with any other sexually transmitted infection, prevention of HPV infection would greatly benefit from a better understanding of the determinants of transmission and infection among men.

HPV infections can be transmitted not only by peno-vaginal intercourse, but also by other sexual practices, e.g. oral sex, peno-anal intercourse, digital–vaginal sex and use of insertive sex toys (Edwards & Carne, 1998; Sonnex *et al.*, 1999; Gervaz *et al.*, 2003). Marrazzo *et al.* (2000) reviewed genital HPV infection in women who had sex with women. This review suggested that sexual practices between female sexual partners could result in transmission of HPV. Hand carriage of genital HPV types in patients with genital

warts was identified by Sonnex *et al.* (1999); their findings supported the possibility of HPV transmission by digital–genital contact.

The non-sexual mode of transmission of genital HPV remains a controversial issue. Most studies among sexually inexperienced young women (Andersson-Ellström *et al.*, 1994; Dillner *et al.*, 1999) demonstrated that non-sexual transmission of HPV is uncommon. However, a number of studies (Pao *et al.*, 1992; Cason *et al.*, 1995; Winer *et al.*, 2003) reported that HPV might occasionally be transmitted through modes other than sexual activity. The possible non-sexual routes include vertical transmission, fomites and skin contact (Mindel & Tideman, 1999; Frega *et al.*, 2003).

(*b*) *Vertical transmission*

Vertical transmission occurs when a parent conveys an infection to its unborn offspring, including a special form of vertical transmission — perinatal infection. Vertical transmission of HPV from mother to child was first suggested in the 1950s (Hajek, 1956) and was subsequently supported by several other studies (Cason *et al.*, 1995; Puranen *et al.*, 1997; Tseng *et al.*, 1998). Rare cases of anogenital warts in newborns have been reported (Tang *et al.*, 1978) and HPV DNA has been detected in mucosal scrapes and washes obtained from infants (Roman & Fife, 1986; Jenison *et al.*, 1990; Fredericks *et al.*, 1993; St Louis *et al.*, 1993). HPV DNA was rarely detected even among babies born to HPV-infected mothers (Watts *et al.*, 1998). Results from studies of transmission in infants are not consistent, and do not provide a clear indication of the rate of infection among neonates who are exposed perinatally. Differences in samples and techniques may be the reasons for the variability and inconsistency in these results.

Tenti *et al.* (1999) investigated HPV type-specific concordance between mother–infant pairs and found that HPV-positive newborns carried HPV types identical to those found in their mothers. However, discordant mother–newborn pairs have been reported in several studies, as well as HPV-positive babies born to HPV-negative mothers and transmission of HPV by the transplacental route before delivery (Puranen *et al.*, 1996).

Perinatal transmission of HPV has been demonstrated unequivocally for the rare disease juvenile respiratory papillomatosis (Dillner *et al.*, 1999). Earlier studies of juvenile-onset recurrent respiratory papillomatosis in infants and young children indicated that HPV infections may be transmitted from mother to infant, probably at the time of delivery. Age of the mother, birth order of the infant and mode of delivery are considered to be important determinants of transmission. Most infants who develop juvenile-onset recurrent respiratory papillomatosis are the first-born single or twin infant of women who tend to be younger than other mothers who gave birth at the same institutions (Kashima *et al.*, 1992a), and many are delivered vaginally rather than by caesarean section (Shah *et al.*, 1986). Cesarean delivery is generally thought to protect against perinatal transmission of HPV (Tseng *et al.*, 1998) but, as shown by other studies among children delivered by cesarean section, some of them can be HPV-positive (Chatterjee *et al.*, 1998). Kosko and Derkay (1996) and Summersgill *et al.* (2001) postulated a very limited role for cesarean section in the prevention of transmission of HPV.

Despite the evidence for vertical transmission, its overall importance in terms of public health may not be as great as that suspected by patients and health care providers (Winer & Koutsky, 2004). It would be particularly valuable to confirm the prevalence of established HPV infections in babies after vaginal birth in the absence of convincing seroconversions (using assays that provide specific although insensitive biomarkers of infection) (Dillner *et al.*, 1999). Even if anogenital infections with high viral load are rare in babies, exposure at birth could influence immune response later in life at the time of sexual exposure (Mant *et al.*, 2000), but rigorous assessment of such a theoretical effect will require very complex study designs. There seems to be consensus, however, that perinatal transmission is generally a rare event (Winer & Koutsky, 2004).

(*c*) *Issues in assessing transmission*

Assessment of type-specific concordance between genital HPV infections in heterosexual couples has been addressed in several studies as further proof of the principle of sexual transmissibility of HPVs. Although some studies (Ho *et al.*, 1993b; Baken *et al.*, 1995) found good agreement among the couples studied, most demonstrated a relatively poor correlation between HPV-positivity and types in cervical and penile samples (Strand *et al.*, 1995; Castellsagué *et al.*, 1997), even among couples where both the wife and husband reported only one lifetime sexual partner (Franceschi *et al.*, 2002). The possible explanations of HPV discordance include problems related to the sensitivity of the detection method, inadequate sampling techniques, the timing of the sampling of penile and cervical specimens, multiple partners of men or women in some couples and different rates of spontaneous regression of HPV infection in men and in women.

New epidemiological studies have begun to focus on the dynamics of HPV infection in men and on the actual characteristics of transmission in heterosexual couples. Because the basic tenet of analytical epidemiology is the observation of individual subjects, several methodological challenges need to be overcome in studies of couples or of infection that begins with an index subject and is eventually transmitted to partners and spread from that point. These studies are very important because they can estimate the probabilities of infective contact per sexual act and partner. These estimates are fundamental for models of transmission of infection that are used to assess the potential impact of HPV vaccination and the cost-effectiveness of different preventive strategies, because, to date, such models have had to make simplified assumptions concerning the parameters of sexual transmission (Hughes *et al.*, 2002).

Measurements of HPV infection in men and women are prone to error, which emphasizes the difficulties of ascertaining infection in the context of multiple types and even molecular variants and makes the distinction between persistence, recurrence and acquisition very difficult. Studies that could detect incident HPV infections among virgins who were being initiated in sexual intercourse would be useful, because the earliest aspects of transmission and immune response have not been clarified adequately by long-term cohort studies. It is uncertain whether sexual intercourse near menarche is uniquely prone to establishing infection (or persistence and progression). The proximity of first intercourse to

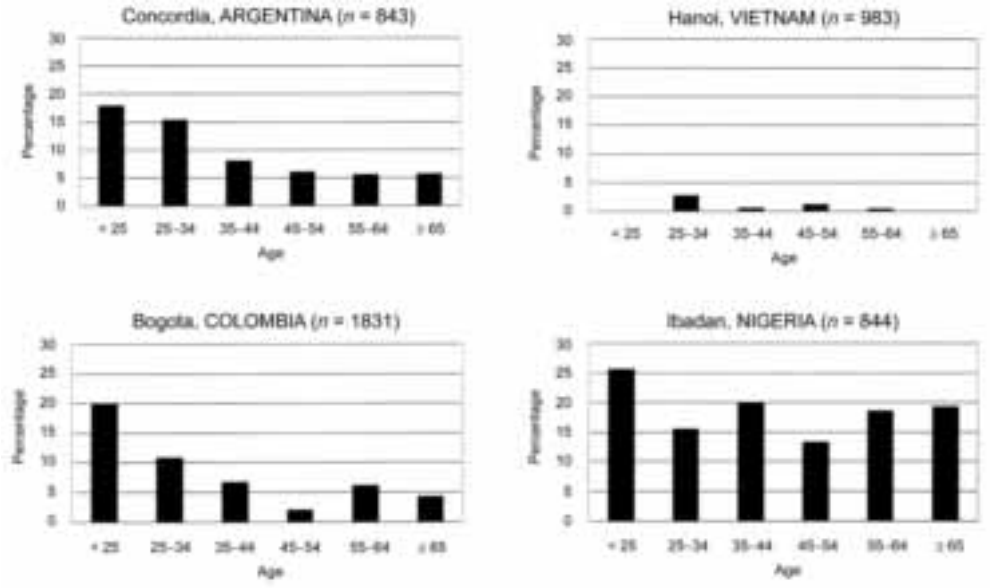
menarche does not appear to increase the risk for HPV infection (Collins *et al.*, 2005). The apparently limited protective role of condoms should be better estimated to guide the debate on this issue, and the possible role of susceptibility in the acquisition of multiple HPV types has not been assessed adequately. The currently available, limited data suggest that HPV types, although probably sexually co-transmitted, influence the transmission of each other to a minimal extent if at all (Thomas *et al.*, 2000; Liaw *et al.*, 2001; Rousseau *et al.*, 2001). The type specificity of serological responses supports this conclusion (Wideroff *et al.*, 1996a; Carter *et al.*, 2000). Recently, studies of sexual couples revealed a beneficial effect of condoms on the regression of flat penile lesions (Bleeker *et al.*, 2003). This effect was only demonstrable in couples who showed a concordance of HPV type and was associated with the maintenance of flat penile lesions or the development of new penile lesions in the areas surrounding existing penile lesions (Bleeker *et al.*, 2005b). This suggests re-infection and the development of new penile lesions in men who are susceptible to the same HPV type as that harboured by the female partner. However, further studies of multiple infections could be important to guide strategies on vaccines. For instance, it would be useful to know whether the prevention of HPV 16 infection would affect the epidemiological niche occupied by other HPV types in various populations.

In summary, improvement in our knowledge of the transmission of HPV has a significant implication for the prevention of HPV infection and also for reducing the incidence of precancerous lesions. Sexual transmission of genital HPV has been demonstrated unequivocally. However, further epidemiological studies are required to enhance the understanding of HPV transmission by non-sexual routes and to provide empirically valid parameters of sexual transmissibility to address health promotion, the (cost-)effectiveness of which will have to be evaluated. Detection of HPV mRNA may provide confirmatory evidence of infection rather than evidence of contamination or whether viral DNA is being transcribed. Large prospective cohort studies with repeated measurements of viral endpoints would be informative on the long-term persistence of HPV infection in children, since current data are usually obtained from cross-sectional studies.

1.4.3 *Prevalence of HPV infection*

The age-specific prevalence curve of cervical (and vaginal) HPV infection, as measured by HPV DNA, has a large peak that follows typical population norms of sexual initiation, which confirms sexual transmission (Burk *et al.*, 1996). In some populations, age-specific prevalences decline sharply and reach very low levels at older ages, which is consistent with viral transience as well as lower incidence at older ages (see Figure 9). However, in populations in India (Franceschi *et al.*, 2005) and sub-Saharian Africa (Thomas *et al.*, 2004), the prevalence of HPV never falls substantially. The age curve of HPV infection tends to rise again in middle age in some populations, notably in Latin America (Lazcano-Ponce *et al.*, 2001; Herrero *et al.*, 2005). The incidence rates of invasive cervical cancer tend to peak about 20–25 years after the peak age for HPV infection prevalence, and the incidence of CIN peaks in between.

Figure 9. Prevalence of high-risk types of human papillomavirus (HPV)[a] among sexually active and cytologically normal women aged ≥ 15 years, in different countries. IARC multi-centre HPV prevalence surveys



Modified from Anh *et al.* (2003), Matos *et al.* (2003), Molano *et al.* (2003), Thomas *et al.* (2004)
[a] Includes HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73 and 82.

Table 11 lists the most relevant studies of the prevalence of HPV in cytologically negative women (also excluding atypical squamous cells of undetermined significance [ASCUS]; see the footnote for exceptions) for several populations worldwide with various age ranges. The restriction of surveys on the prevalence of type-specific HPV DNA to cytologically negative women was intended to minimize any influence of longer duration of lesions related to specific types. The selected studies were population–surveillance-based, the study population consisted of about 350 women or more and the test used was type-specific PCR and HPV genotyping. Different primers were included and varied to some extent in type-specific sensitivity.

The population-wide prevalence of HPV in women varies from 1.5% in Spain to 39% in Honduras and Kenya, although careful attention needs to be given to the age distribution of the population being studied, as the prevalence of HPV is strongly age-related. In general, the prevalence is highest in Africa and South America, lowest in Europe and intermediate in Asia. However, observed rates vary within the regions (7.8% in Italy versus 1.5% in Spain; 2.0% in Hanoi versus 10.9% in Ho Chi Minh, Viet Nam). The high variability might also reflect differences in the selection of the women, although prevalence varies remarkably even across the study centres coordinated by IARC.

Table 11. Rates of detection of HPV DNA by polymerase chain reaction (PCR) amplification among women with cytologically negative Papanicolaou smears

Reference, study area	Primer system	Age range (years)	No.	Overall HPV	Specific HPV type (%)																
					6	11	16	18	31	33	35	39	45	51	52	56	58	59	68	73	82
Cuzick *et al.* (1995), United Kingdom	TS[a] 16, 18, 31, 35	20–45	1818	3.5	–	–	1.3	0.7	0.9	0.7	–	–	–	–	–	–	–	–	–	–	–
Ferrera *et al.* (1999), Honduras	MY09-11	20–65	438	39.0	0.2	1.8	10.9	4.1	3.4	0.7	0.2	0.0	0.0	0.0	0.2	0.0	1.8	0.0	0.0	0.0	0.0
Franco *et al.* (1999), Brazil	MY09-11	18–60	1425[b]	13.8	0.5[c]	0.5[c]	2.7	0.8	1.1	0.4	0.2	0.1	0.5	0.7	0.6	0.6	1.2	0.1	0.4	0.2	0.1
Liaw *et al.* (1999), USA	MY09-11	> 16	991	15.8	0.6[c]	0.6[c]	2.5	0.8	0.9	0.6	0.3	0.7	0.9	1.8	0.8	0.5	0.9	0.7	0.2	0.0	0.0
Herrero *et al.* (2000), Costa Rica	MY09-11	18–94	305	11.0	0.7	0.0	1.0	1.0	0.3	0.7	0.3	0.7	0.0	0.3	1.0	0.0	1.6	0.0	0.3	0.7	0.3
Lazcano-Ponce *et al.* (2001), Mexico	BGH 20, BPCO4	15–69	1248	13.5	0.5	1.0	1.8	1.1	1.5	1.0	0.3	1.0	0.6	0.8	0.8	0.3	1.0	0.2	0.3	0.1	0.3
Sasagawa *et al.* (2001), Japan	LCR-E7	16–72	1562	9.7	0.0	0.1	1.2	0.8	0.5	0.4	0.2	0.1	0.1	0.4	1.0	0.3	0.4	0.1	0.1	0.1	0.0
Forslund *et al.* (2002), Sweden	MY09-11	32–38	6123[b]	6.8	–	–	2.1	0.6	1.1	0.4	0.3	0.2	0.8	0.4	0.3	0.5	0.3	0.1	–	–	–
Maehama *et al.* (2002), Japan	L1C1/C2	30–85	3963[b]	10	–	–	0.3	0.1	0.3	0.3	0.6	–	–	–	–	–	0.2	–	–	–	–
Anh *et al.* (2003), Ho Chi Minh,	GP5+/6+	15–69	922[b]	10.9	0.0	0.0	3.3	1.2	0.8	1.1	0.3	0.9	0.7	0.8	1.1	1.1	1.5	0.0	0.7	0.1	0.0
De Vuyst *et al.* (2003), Kenya	SPF10	25/55	369	38.8	0.5	0.5	3.5	2.2	3.3	1.9	2.7	1.4	1.6	1.1	6.2	1.4	2.7	0.3	1.6	–	–

Table 11 (contd)

Reference, study area	Primer system	Age range (years)	No.	Overall HPV	Specific HPV type (%)															
					6	11	16	18	31	33	35	39	45	51	52	56	58	59	68	73	82
Matos *et al.* (2003), Argentina	*GP5+/6+*	15–69	987[b]	16.7	0.1	0.3	4.0	1.9	1.8	1.4	1.9	1.0	1.1	0.4	1.2	0.9	1.3	0.8	0.8	0.2	0.1
de Sanjosé *et al.* (2003), Spain	*GP5+/6+*	15–69	909[b]	1.5	0.1	0.0	1.0	0.0	0.4	0.0	0.5	0.1	0.0	0.4	0.0	0.2	0.1	0.2	0.2	0.0	0.0
Hanoi, Vietnam	GP5+/6+	15–69	994[b]	2.0	0.0	0.0	0.2	0.2	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.2	0.2	0.2	0.1	0.0	0.0
Shin *et al.* (2003), Republic of Korea	GP5+/6+	20–74	821	8.5	0.4	0.0	0.7	0.4	0.0	1.1	0.1	0.5	0.2	0.1	0.5	0.6	0.5	0.2	0.1	0.0	0.0
Sukvirach *et al.* (2003), Lampang and Songkla, Thailand	GP5+/6+	15–69	1673	4.8	0.0	0.0	0.7	0.3	0.3	0.5	0.2	0.3	0.1	0.2	0.3	0.2	0.4	0.1	0.2	0.0	0.0
Xi *et al.* (2003), Senegal	MY09-11	> 35	1639	12.5	0.2	0.0	1.0	0.9	0.4	0.7	0.0	0.1	0.2	0.3	0.5	0.3	0.7	0.4	0.1	0.3	0.1
Asato *et al.* (2004), Japan	L1C1/C2	21–93	3049	10.2	0.1	0.0	0.5	0.2	0.3	0.4	0.8	0.1	0.0	0.9	1.2	0.6	0.2	0.2	0.5	0.0	0.0
Cuschieri *et al.* (2004b), Scotland, United Kingdom	GP5+/6+	17–78	3089	12.7	–	–	3.4	1.4	0.7	0.5	0.3	0.4	0.9	0.9	0.8	0.6	0.7	0.7	0.2	0.8	0.1
Ferreccio *et al.* (2004), Chile	GP5+/6+	15–69	921	11.2	0.2	0.4	2.2	0.4	0.5	0.1	0.3	0.7	0.7	0.7	0.8	1.3	1.0	0.9	0.0	0.2	0.0
Shin *et al.* (2004), Republic of Korea	SPF10	16–29	672[b,d]	15.2	0.7	0.3	1.3	1.2	0.7	0.4	0.3	0.9	0.1	1.8	1.3	1.5	0.7	0.4	0.5	0.5	0.0
Thomas *et al.* (2004), Nigeria	GP5+/6+	> 15	844	24.8	0.4	0.4	3.0	1.7	2.6	0.6	3.0	0.4	2.1	1.1	1.5	2.1	2.5	0.6	0.2	0.5	0.4

Table 11 (contd)

Reference, study area	Primer system	Age range (years)	No.	Overall HPV	Specific HPV type (%)																
					6	11	16	18	31	33	35	39	45	51	52	56	58	59	68	73	82
Franceschi *et al.* (2005), South India	GP5+/6+	16–59	1799	14.0	0.2	0.0	2.8	0.8	0.8	0.8	0.8	0.6	0.3	0.4	0.7	1.1	0.2	0.7	0.0	0.2	0.2
Herrero *et al.* (2005), Costa Rica	MY09-11	> 17	7459	22.4	0.4	0.2	2.2	1.1	1.1	0.5	0.2	0.4	0.5	1.5	1.1	0.5	1.3	0.3	0.2	0.3	0.3
Ronco *et al.* (2005), Italy	GP5+/6+	25–64	997	7.8	0.1	0.2	2.7	0.1	0.3	0.1	0.1	0.3	0.6	0.1	0.3	0.4	0.4	0.1	0.2	0.0	0.0

See Table 7 for a description of the primers used.
[a] TS, type specific
[b] A small number of women with abnormal cytology included
[c] Estimated
[d] 75% virgins

HPV type 16 had the highest prevalence in all European studies (Cuzick *et al.*, 1995; Forslund *et al.*, 2002; de Sanjosé *et al.*, 2003; Cuschieri *et al.*, 2004b; Ronco *et al.*, 2005) and also in most of the other studies. Examples of exceptions are a study from Kenya (6.2% HPV 52 versus 3.5% HPV 16) and one from Nigeria (Thomas *et al.*, 2004) (3% HPV 35 versus 3% HPV 16). In all but one study (Asato *et al.*, 2004), HPV 16 was either first or second in rank, and no other type consistently dominated. However, among the HPV-positive women, the percentage with HPV 16 varied from 8 to 66%. Types 6, 11, 59, 68, 73 and 82 were consistently rare in all of the studies.

Table 12 summarizes the prevalence of HPV in cervical specimens among commercial sex workers. Using PCR-based methods for the detection of HPV DNA, overall prevalence of all HPV types that were tested varied by region and ranged from 14.4% in Singapore to 77.4% in Belgium. Infection with a high-risk HPV type was more common: HPV 16 had the highest prevalence that ranged from 4.3 to 13.9% . Commercial sex workers had a higher prevalence of HPV infection compared with women who were not involved in such occupations (Juárez-Figueroa *et al.*, 2001; Thomas *et al.*, 2001a; Mak *et al.*, 2005).

A study of the determinants of regional variation in age-specific HPV prevalence will help an understanding of viral persistence, clearance and possibly latency. Some studies of highly exposed women such as prostitutes (Kjaer *et al.*, 2000) have shown a significant decrease in the prevalence of HPV with age, despite continuously high sexual activity, and indicate loss of viral detection and type-specific immunity to re-infection. In contrast, a study of sexually active human immunodeficiency virus (HIV)-negative men who had sex with men (Chin-Hong *et al.*, 2004) showed that the prevalence of anal HPV infection was high among men under 30 years of age (approximately 60%) but remained high in all age groups studied. These data suggest that repeated exposures may contribute to high prevalence over a wide age range, at least in the anal canal. Studies that focus on older women and their male partners are also needed, particularly cohort studies with repeated measurements that assess male and female sexual practices and immunity.

The changes in sexual mores that began in the mid-1960s would have been expected to lead to an increase in the prevalence of HPV infection over time in most western populations. The extremely high prevalence of HPV in young women in North America (Winer *et al.*, 2003) and the United Kingdom (Peto *et al.*, 2004) supports the existence of a strong cohort effect. Confirmation of this hypothesis, however, would require that preserved specimens of representative samples from different eras be tested at the same time with the same sensitive testing technology, a proposition that could not be easily implemented. There is, however, limited evidence from seroepidemiological studies that the prevalence of antibodies against certain HPV types may have increased. For instance, in Finland, seropositivity for HPV 16 among women aged 23–31 years increased from 17% in 1983–85 to 24% in 1995–97 (Laukkanen *et al.*, 2003). In contrast, the prevalence of HPV 16 and HPV 11 was stable between the two periods at 9–12%.

Table 12. Prevalence of cervical HPV detected by polymerase chain reaction (PCR) among commercial sex workers

Reference, study area	No. at risk	Age range (years)	Method of detection	Prevalence (%)			Prevalence of specific high-risk types (%)													Prevalence of specific low-risk types (%)								
				Overall	High risk	Low risk	16	18	31	33	35	39	45	51	52	56	58	59	66	6	11	34	40	42	43	53	54	73
Kjaer *et al.* (2000), Denmark	182	20–45	GP5+/6+ primers	32.4			9.9																					
Chan *et al.* (2001), Singapore	187	19–71	PVCOU/PVCOD concensus primers with probing for 11/16/18	14.4	12.3	2.7	4.3	2.7	1.1		0.53		1.6			0.53	1.6			2.7		0.53						
Juárez-Figueroa *et al.* (2001), Mexico	495	18–62	MYBO9/MYB11/ HMBB01 L1 concensus primers	48.9	43	24.6	11.1	3.6	11.1	3.2	0.8	5.7	4.7	5.5	4.4	4.9	7.9	3.6	4	6.3	3.4		0	0		9.5	5.3	5.9
Thomas *et al.* (2001a), Thailand	251	15–35	MY09/MY11 primers with probing for 6/11/ 16/18/31/33/35/39/45	47	36.3	10.8	13.9	6		13.9[a]			2.4								10.8[b]						
Choi *et al.* (2003), Republic of Korea	417	15–51	Hybrid Capture 2 detection; genotyping by DNA oligonucleo-tide microarray with MY09/11 primers	47	64	9	11.5	3.6	1.4	1.9	4.6	2.9	3	4	4	3.1	4	2.4	1	1.2	0.2	3.1	6.7	2.4	1.4			
Ford *et al.* (2003), Indonesia	614	14–47	Oligoprobes specific for 16, 18, 31, 33, 35, 45, 52, 6, 11 and a probe with a mixture of 16, 18, 31, 52	38.4	14.5	3.5	6.6[c]			5.5[d]				2.4[e]						3.5[b]								
Tideman *et al.* (2003), Australia	288	16–36	MY09/MY11 primers	31.6	12.2	17																						
Mak *et al.* (2005), Belgium	93	17–58	SPF10	77.4	55.9		12.9[f]	3.2	–[f]	6.5	3.2	7.5	9.7	1.1	–[f]	6.5	2.2	4.3	3	5.4	2.2	1.1	3.2	1.1	1.1	6.5	3.2	10.8[g]

See Table 7 for a description of the primers used.
[a] HPV 31/33/35/39
[b] HPV 6 /11
[c] HPV 16/18
[d] HPV 31/33/35
[e] HPV 45/52
[f] HPV 16/31/52
[g] May show cross reactivity with HPV 68

1.4.4 *Incidence, persistence and clearance*

Many prospective epidemiological studies published since the last evaluation (IARC, 1995) provide data on incident infection (although such events may represent latent infections that for some reason become detectable again) and duration of infections by different types. Tables 13 and 14 show the main characteristics of these studies and illustrate the estimates of incidence and duration by type, respectively.

Table 13 summarizes the incidence of type-specific HPV infection (infection per 100 person–years). Based on these data, approximately 5–15% of HPV-negative women are infected each year with any of the high-risk types of HPV (Franco *et al.*, 1999; Moscicki *et al.*, 2001; Richardson *et al.*, 2003; Sellors *et al.*, 2003; Muñoz *et al.*, 2004). The incidence of infection with high-risk HPV types tends to be higher than that with low-risk types (Moscicki *et al.*, 2001; Richardson *et al.*, 2003; Muñoz *et al.*, 2004). The most common types of incident infection tend to include HPV 16, 18, 31, 33 and 51; one of the highest type-specific infections among the studies is HPV 16 (Ho *et al.*, 1998a; Franco *et al.*, 1999; Woodman *et al.*, 2001; Giuliano *et al.*, 2002a; Richardson *et al.*, 2003; Winer *et al.*, 2003; Harper *et al.*, 2004; Muñoz *et al.*, 2004). In addition, rates of high-risk HPV infection tend to be greater among younger than older women (Franco *et al.*, 1999; Muñoz *et al.*, 2004), although median duration of infection appears to be comparable by age (Muñoz *et al.*, 2004). Only limited prospective data are available on the duration of HPV infection with age, as determined by related longitudinal measurements of type-specific HPV DNA. One of two studies (Muñoz *et al.*, 2004) suggested that duration of HPV infection increases with age (Castle *et al.*, 2005).

It is widely accepted that persistence of HPV infection is essential for the development of cervical precancerous lesions and cancer. Fortunately, most HPV infections are transient and become undetectable within 1–2 years even by sensitive PCR assays (Ho *et al.*, 1998a; Franco *et al.*, 1999; Molano *et al.*, 2003a; Richardson *et al.*, 2003; Muñoz *et al.*, 2004). Consequently, anogenital HPV infections tend to resolve spontaneously, as do warts anywhere on the body. Presumably, they are cleared completely by the cell-mediated immune system, are self-limited or are suppressed into long-term latency. Knowledge of how often HPV transience in the short term represents successful immune clearance versus a self-limited infection would be useful. However, this question cannot easily be answered by the measurement technologies currently available to epidemiologists.

A major unresolved question regarding the natural history of HPV is the extent to which viral infections are cleared. Even when no HPV DNA is detectable by conventional molecular tests, small foci of cells that maintain infection at low DNA copy numbers could exist, and they may explain the results of studies in immunosuppressed individuals. However, it is not known how frequently this occurs in immunocompetent individuals, how long it lasts, what causes re-emergence into a detectable state or what fraction of cancers arises after a period of latency. Answers to these questions will greatly affect prevention strategies that rely on the detection of HPV DNA.

Table 13. Incident cervical HPV infection as detected by HPV DNA among women who were HPV-negative at baseline

Reference, study area	Setting	No. at risk	Mean/ median follow-up (years)	Test method	Age in years (range; mean)	Incidence rate by age	Type-specific incidence rate of HPV (per 100 person–years)																		
							16	18	31	33	35	39	45	51	52	56	58	59	53	66	HR	6	11	LR	Any
Ho *et al.* (1998a)[a], USA	University students	608	2.2	PCR and southern blot	25–49	20	3.4	1.9	1.0	0.8	0.8	1.1	0.9	3.7	1.3		1.1	1.6	1.8	3.3		2.5			19.9
Franco *et al.* (1999), Brazil	Low-income maternal and child health programme	1425	0.8	MY09/11 PCR	26–39; 33.3	33.3	1.6	0.3	1.1					1.3	1.4		1.1		1.9		8.1[b]	0.8[c]		10.9[d]	16.1
Moscicki *et al.* (2001)[a], USA	Family planning clinic	105	1.9	PCR with dot blot	13–21; 20.0	20															15.8[e]			5.9[f]	26.8
Woodman *et al.* (2001)[a], United Kingdom	Family planning clinic	1075	2.4	GP5+/6+ PCR	15–19, 18[g]	15–19	4.2	2.5	1.1	1.4					0.4		1.0					1.7[c]			15.7
Giuliano *et al.* (2002), USA	Family planning clinic	331	0.8	MY09/11 PCR	18–35; 24.2	24.2	7.1	1.0	3.0	1.5	1.5	5.5	0	4.1	3.5	1.0	1.5	4.0	4.0	2.0		1.0	1.4		35.2
Koutsky *et al.* (2002), USA	Placebo arm of HPV vaccine trial	765	1.5	Type-specific PCR	16–23; 20.1	16–23	3.8																		
Richardson *et al.* (2003), Canada	University health clinics	621	1.8	MY09/11 PCR	17–42; 23	17–42	6.2	2.3	2.0			2.2		4.1		1.8			3.0		16.8	2.7		14.9	22.8
Sellors *et al.* (2003)[a], Canada	Medical practices	253	1.2	HC2[h]	15–49; 32.7	32.7															9.5				

Table 13 (contd)

Reference, study area	Setting	No. at risk	Mean/ median follow-up (years)	Test method	Age in years (range; mean)	Incidence rate by age	Type-specific incidence rate of HPV (per 100 person–years)																	
							16	18	31	33	35	39	45	51	52	56	58	59	53	66	HR	6	11	LR	Any
Winer *et al.* (2003), USA	University students	444	3.4	MY09/11 PCR	18–20; 19.2	18–20	5.5	2.1	2.4	6.0[i]			0.7	4.5[j]		4.2						3.9	0.5	4.1[k]	
Harper *et al.* (2004), North America, Brazil	Placebo arm of HPV vaccine trial	553	2.3	SPF10 PCR	15–25	15–25	2.4	1.4													3.4[l]				
Muñoz *et al.* (2004), Colombia	Cervical cancer screening center and family-planning clinics	1610	4.1	GP5+/6+ PCR	15–85; 32.3[g]	15–85	1.0	0.7	0.7	0.4			0.5		0.5	0.5	0.7				5.0	0.2	0.2	2.0	6.2
						15–19[m]	3.7	1.3	3.0	1.2			2.5		1.8	1.2	3.0				17.4	1.2	0.6	2.6	17.2
						20–24[m]	2.3	2.2	1.1	0.0			0.5		0.0	0.0	1.1				9.5	0.6	0.5	3.4	11.3
						25–29[m]	2.0	1.3	0.7	0.7			1.0		0.3	0.0	0.0				6.9	0.0	0.0	3.7	9.5
						30–44[m]	1.3	0.9	0.4	0.2			0.5		0.6	0.2	0.6				4.1	0.1	0.3	1.8	5.4
						≥ 45[m]	0.0	0.0	0.0	0.0			0.0		0.0	0.0	1.3				0.7	0.0	0.0	0.7	1.4
						15–19															21.4				
						20–24															7.6				
						25–29															7.1				
						30–44															9.3				
						≥ 45															6.6				
						< 35	1.9	0.6	1.6					2.0	1.7		1.4		2.2		10.6[b]	1.2[c]		10.8[d]	17.9
						≥ 35	1.2	0.0	0.4					0.2	0.8		0.6		1.4		4.8[b]	0.4[c]		11.0[d]	13.8

See Table 7 for a description of the primers used.
HR, high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 and others); HC, Hybrid Capture; LR, low-risk HPV types (6, 11, 26, 40, 42, 53, 54, 55, 57, 66, 73, 82, 83, 84, 73 and others)
[a] Calculated estimate of incidence rate from reported data: incidence rate per 100 person–years = number of events/(no. at risk × mean follow-up) × 100
[b] 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 68
[c] 6, 11
[d] 6/11, 26, 32, 34, 40, 42, 44, 53, 54, 55, 57 59, 62, 64, 66, 67, 69, 70, 72, 73
[e] 16, 18, 31/33/35, 39, 45, 51, 52, 56, and 58
[f] 6/11/42/44
[g] Median age reported
[h] Hybrid Capture includes HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68.
[i] 33, 35, 39
[j] 51, 52, 55, 58
[k] 40, 42, 53, 54
[l] Infection with either HPV-16 or -18
[m] Reported cumulative risk at 1 year

Table 14. Duration of cervical HPV infection as detected by HPV DNA among women who were HPV-negative at baseline

Reference, study area	Setting	No. at risk	Mean/ median follow-up (years)	Testing method	Age at baseline in years (range; mean)	Median duration (months) of infection by specific HPV type																
						16	18	31	33	35	39	45	51	52	56	58	59	53	66	HR	6	LR	Any
Ho *et al.* (1998a), USA	University students	608	2.2	PCR and southern blot	20	11	12	6	7	6	6	6	7	7		6	6	8	6		6		8
Franco *et al.* (1999), Brazil	Low-income maternal and child health programme	1425[a]	0.8	MY09/11 PCR	26–39; 33.3															13.5[b]		8.2[c]	
Woodman *et al.* (2001), United Kingdom	Family planning clinic	1075	2.4	GP5+/6+ PCR	15–19; 18[d]	10.3	7.8	8.6	9.0					13.0		11.0					9.4[e]		13.7
Giuliano *et al.* (2002), USA	Family planning clinic	331[a]	0.8	MY09/11 PCR	24.2	8.5														9.8		4.3	
Richardson *et al.* (2003), Canada	University health clinics	621	1.8	MY09/11 PCR	17–42; 23	19.4	9.4	20.0			8.0		9.0		8.4			13.9		13.2	6.4	12.3	
Muñoz *et al.* (2004), Colombia	Cervical cancer screening centre and family-planning clinics	1610	4.1	GP5+/6+ PCR	15–85; 32.3[d]	13.7	11.9	16.5	13.4			12.2		9.7	14.6	14.8				14.8		11.1	

See Table 7 for a description of the primers used.
HR, high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 and others); LR, low-risk HPV types (6, 11, 26, 40, 42, 53, 54, 55, 57, 66, 73, 82, 83, 84, 73 and others); PCR, polymerase chain reaction
[a] Duration calculated for prevalent cases of infection
[b] 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 68
[c] 6/11, 26, 32, 34, 40, 42, 44, 53, 54, 55, 57 59, 62, 64, 66, 67, 69, 70, 72, 73
[d] Median age reported
[e] 6, 11

Persistence (i.e. long-duration of detectable HPV infection) is uncommon compared with clearance. From a practical point of view, persistence can be defined as the detection of the same HPV type (or, with a higher degree of certainty, the same intratypic variant) two or more times over a certain period. There is no consensus as to the length of time that implies persistence, but at least 6 months to 1 year is the time frame that is usually chosen. Although commonly adopted, this definition of convenience does not correspond to the understanding of the natural history of HPV. The median duration of type-specific HPV infection in several prospective studies is summarized in Table 14. Duration tends to be longer for high-risk HPV types compared with low-risk types (Franco *et al.*, 1999; Giuliano *et al.*, 2002a; Muñoz *et al.*, 2004). This approach is complemented by a study of longer-term infection with a median follow-up of 5.1 years (Schiffman *et al.*, 2005) which showed particularly pronounced persistence of HPV 16 compared with any other HPV type. In contrast to other short-term studies, the persistence of high-risk types other than HPV 16 was not much longer than that for many low-risk types (Schiffman *et al.*, 2005). The longer duration of infection with high-risk types of HPV may have implications for the pathogenesis of CIN3 and cancer.

It is of practical importance that epidemiologists agree on a uniform definition of HPV persistence, for example, taking into account whether analysis of viral variant is required as an extra level of taxonomic detail to ascertain this phenomenon (Franco *et al.*, 1994). Proof that this safeguard is essential for studies of low-risk populations has yet to be obtained; however, it seems that persistent infections tend to maintain the same original variant, at least in the case of HPV 16 or 18 (Villa *et al.*, 2000). There is considerable uncertainty concerning the importance of measurements of viral load or the presence of associated, microscopically evident abnormalities with respect to the duration of persistence. More data are needed, particularly to clarify whether infections with different types of HPV act independently on the cervix, with regard to both immunology and direct interaction. The sparse data are conflicting as to whether the presence or the absence of any one type alters the duration of any other type-specific infection (analogous to whether types influence the acquisition of each other as mentioned above) (Thomas *et al.*, 2000; Liaw *et al.*, 2001; Rousseau *et al.*, 2001). Persistence of high-risk HPV DNA after local ablation or excision of high-grade CIN is predictive of failure of treatment, whereas clearance of HPV predicts success of treatment (reviewed in Arbyn *et al.*, 2004a,b; Zielenski *et al.*, 2004).

1.4.5 *Microscopic abnormalities*

Microscopic abnormalities are diagnosed in only a minority of women who have HPV that is detectable by DNA assays. The fraction depends on the thresholds of the molecular and microscopic tests and clinical specimens examined, and can range widely from 5 to 30% (Schiffman & Kjaer, 2003). Microscopic diagnoses are prone to subjectivity and lack of interobserver reproducibility, particularly when mild or equivocal changes are involved. Therefore, misclassification is always a major concern when epidemiologists contemplate

how best to consider HPV infection as a transition state in multistage models such as that shown in Figure 8.

It is important to develop a rational classification for HPV infections that arise in a prospective epidemiological study. The persistence of at least one carcinogenic HPV type is the necessary state for the emergence of precancer. However, other aspects of infection may contribute to the likelihood that an infection will progress, such as type, load and concurrent abnormalities. Even among the carcinogenic types, HPV 16 is uniquely associated with risk for cancer and, even for HPV 16 (and other carcinogenic types), variants are relevant to the natural history. Low viral loads detectable only by PCR (not the commercially available Hybrid Capture) are associated with microscopic normalcy and with low risk of subsequent precancer and/or cancer. Viral load is clearly associated with concurrent disease (Cuzick *et al.*, 2003), but the value of increasing loads with respect to subsequent prediction of lesions has not been established (Lorincz *et al.*, 2002; Schlecht *et al.*, 2003a,b).

It is still not known whether microscopically evident abnormalities represent a stage in the natural history that is separate from HPV detected by DNA testing alone (Castle *et al.*, 2002b). In a recent 24-month prospective follow-up of women with carcinogenic HPV DNA, the presence or absence of mild histological abnormalities did not materially affect the risk for subsequent precancer (Cox *et al.*, 2003). Observations suggest that a fraction of precancers arise from HPV infections in the absence of mild or even equivocal microscopically evident abnormalities (Koutsky *et al.*, 1992; Cuzick *et al.*, 1995). This might also represent a misclassification of cytology or histology or rapid transit through the mildly abnormal phase. It has been proposed that precancers develop in HPV-infected mucosa independent from and adjacent (internal) to CIN1 rather than being an internal subclonal event (Kiviat *et al.*, 1992). These hypotheses can be addressed only through very intensive longitudinal studies that combine visual, microscopic and molecular measurements.

1.4.6 *Progression to precancer*

HPV infections (even with carcinogenic types) are so common that becoming infected is not the limiting factor in cervical carcinogenesis. The critical step for most women might be whether a precancerous lesion develops as an uncommon outcome of infection (Figure 8).

The first difficult task is to define ‘precancer’ on the basis of histology, i.e. that an intraepithelial lesion is destined to progress, although latency may be very long. There is substantial heterogeneity in the microscopic diagnosis and biological meaning of CIN2 lesions in particular. Some certainly represent acute HPV infections of particularly bad microscopic appearance that are destined, however, to regress, whereas others are incipient precancers that are destined to persist with a high risk of invasion. Some non-carcinogenic HPV infections can produce lesions that are diagnosed as CIN2, which shows that this level of abnormality is not a sufficient surrogate for cancer risk. CIN3 should be used as a surrogate for precancer and CIN2 as a buffer zone of equivocal diagnosis, similarly to

ASCUS or more minor cytological abnormalities. [The Working Group generally agreed that a combination of CIN2 and CIN3 as high-grade CIN is a sub-optimal end-point for intervention studies due to the potential for misclassification of CIN2.]

In studying the transition from HPV infection to precancer, attention should be restricted to women with carcinogenic types of HPV (unless a particular controlled comparison is being made). Within this group, viral characteristics, host factors and behavioural co-factors that increase the risk of progression or decrease the probability of viral clearance need to be determined. Persistence of HPV (defined at the type-specific level) is by far the most important determinant of progression (Nobbenhuis *et al.*, 1999) but there has been considerable heterogeneity in the way in which epidemiological studies have determined persistence and the time to ascertainment of lesion outcomes (reviewed in Schiffman & Kjaer, 2003).

The time between the occurrence of HPV infection in the late teens or early twenties and the peak of precancer at around 30 years of age is about 7–10 years. More rapid progression does occur and should be studied, but it may not be possible to study the full extent of the latency process prospectively. Using cytological end-points, it is clear that presence of carcinogenic HPVs in a specimen carries a prognostic value. The mean time to progression from ASCUS to LSIL or worse and from LSIL to HSIL or worse is significantly shorter in women who have carcinogenic HPV types than in women who have no HPV infection (e.g. mean times for ASCUS progression are 67.0 and 88.0 months, respectively, in women with carcinogenic HPV and no HPV; difference, 21.0 months; 95% CI, 11.3–30.7 months). In general, cervical abnormalities persist longer and progress more quickly in women who have carcinogenic HPV infections than in women who have non-carcinogenic infections or no HPV (Schlecht *et al.*, 2003b).

1.4.7 *Progression of lesions*

Several natural history studies have analysed the risks for progression beginning at different points in the continuum of pre-invasive lesions. For a balanced interpretation of these data, the following caveats must be considered for most of these studies: the small sample size, the highly selected study population, the insufficient follow-up time, the reporting of crude rates of progression and regression without a precise actuarial analysis of cumulative risk over time and the variability of methods to detect the development of lesions during follow-up. In particular, detection methods that use cytology cannot provide reliable estimates of rates of lesions and those that use histology may have altered the course of the natural history of the disease because frequent cervical biopsies may remove the entire lesion. Overall, these problems tend to affect the comparability of results across studies. These drawbacks notwithstanding, the following conclusions can be drawn from natural history studies: (*a*) the vast majority of CIN2 are transient and regress to normal within relatively short periods, although some may progress to CIN3 or to cancer over variable periods of time; and (*b*) in contrast, CIN3 carries a much greater probability of progression to invasion, although many such lesions may eventually regress.

Östör (1993) conducted a pooled analysis of studies published from 1950 to 1992 to derive average estimates of regression and progression by grade of CIN. The average probabilities of regression were 57% for CIN1, 43% for CIN2 and 32% for CIN3. The equivalent probabilities of progression to carcinoma *in situ* were 11% for CIN1 and 22% for CIN2, and those of progression to invasion were 1% for CIN1, 5% for CIN 2 and 12% for CIN3. A substantial proportion of lesions were biopsied, including cone biopsies, and were classified as persistent without further qualification as to the duration of the sojourn time within each grade, i.e. 32%, 35% and 56% for grades 1, 2 and 3, respectively.

Mitchell *et al.* (1994) conducted a similar meta-analysis but modified the method for ascertaining lesions during follow-up in order to stratify the estimates. By considering only studies with cytological follow-up and all grades of CIN combined, the probabilities of regression, persistence and progression to any higher-grade lesion were 34%, 41% and 25%, respectively. Regarding the latter progression figure, 10% of the lesions progressed to carcinoma *in situ* and 1% to invasive cancer. The equivalent cumulative probabilities for all grades of CIN that had been followed by both cytology and biopsy were 45%, 31% and 23% for regression, persistence and progression, respectively. Within the latter probability, progression to carcinoma *in situ* was 14% and that to invasive cancer was 1.4%. Progression rates to invasive cancer for studies that followed up only patients with carcinoma *in situ* by biopsy ranged from 29 to 36%.

In a meta-analysis of studies published since 1970 that included more than 27 000 patients who were followed without treatment, Melnikow *et al.* (1998) calculated the following weighted average rates of progression to HSIL at 24 months according to baseline cytological abnormality: ASCUS, 7.1%, LSIL, 20.8%, and HSIL (persistence), 23.4%. Cumulative progression rates to invasive cancer at 24 months by cytological abnormality were 0.3% for ASCUS, 0.2% for LSIL, and 1.4% for HSIL. The following average rates of regression to a normal Pap smear were estimated: 68.2% for ASCUS, 47.4% for LSIL, and 35.0% for HSIL. [The Working Group noted that none of the CINs was tested for HPV DNA in the above three studies.]

CIN3 lesions tend not to regress over short-term follow-up; however, risk for and timing of invasion versus eventual regression follow stochastic processes that are mediated by biological variables. The median age at diagnosis of women with precancer (CIN3) in many countries that carry out screening is approximately 30 years, whereas the median age of women with invasive cancers is skewed towards much older ages. The age of women who have screen-detected invasive cancer tends to be more than 10 years older on average than women with CIN3, which suggests a long average sojourn time in the precancer state. The size of the precancerous lesion can be used as a proxy for risk of invasion but prospective proof cannot be obtained for obvious ethical reasons. Epidemiological studies have not been able to suggest risk factors for invasion. The frequently-discussed phenomenon of HPV DNA integration is associated with invasion, but it is difficult to prove that it is causal.

1.4.8 *Accuracy and reliability of measurements*

Advances in the understanding of the natural history of HPV have followed intensive methodological efforts to standardize accurate and reliable measurements of HPV DNA. In most cases, the incoherent results from the late 1980s and early 1990s were caused by unsuspected misclassification of HPV status in the first large-scale molecular epidemiological studies of HPV and cervical cancer (reviewed by Franco, 1991, 1992; Schiffman & Schatzkin, 1994). Improvements in cytology and serology have been less extensive but very important. In future cohort studies that multiply the number of measurements taken over time, the importance of optimized methods will be even greater if observation and interpretation of the patterns of viral clearance, persistence, possible recurrence and progression are to be anticipated.

1.4.9 *Serology*

Serology of VLPs by ELISA methods is a very useful epidemiological tool for defining past and cumulative exposure to HPV infection. The assays are reasonably type-specific and are usually negative in individuals who have never been infected (Dillner, 1999; Kjaer *et al.*, 2001). This specificity is useful for the definition of HPV-infected cohorts, in whom etiological co-factors can be studied. For example, serology can be used to define HPV-exposed individuals among control subjects in case–control studies that emphasize analyses only among the exposed. However, only about half of the women with currently detectable infections of the same type (with the use of DNA and microscopy) are seropositive, which suggests that the current techniques to measure the serological response are still not sufficiently sensitive. Therefore, HPV seronegativity does not exclude exposure, partly because current assays for seropositivity do not cover more than a few types of HPV. To date, serological assays have not proved to be useful in defining immunological responses related to the natural history of HPV infection.

Two important caveats must be recognized for the interpretation of sero-epidemiological studies. The first is the cross-reactivity and relatively low sensitivity in terms of types and the second is the fact that infections in other mucosal sites of the body (e.g. the mouth) also elicit antibody responses that cannot be distinguished from those arising in the anogenital area.

1.4.10 *Other sites* (see Table 15)

A few studies have addressed the prevalence of HPV in smears from the vagina, vulva, foreskin, anus and urethra from the general population. From these results, it has been suggested that the prevalence of any HPV-type infection in the vagina and vulva is in the same range as that of the cervix. The prevalence of HPV among men (penis and urethra) varied from less than 10% to about 50%. Prevalence in neonates and primary school children (anal smear or foreskin) showed very low percentages (< 1%).

Table 15. Prevalence of HPV infection at sites other than the cervix

Reference, location	Site	No.	Age (years)	Setting	Primer	Overall HPV (%)	Specific HPV type (%)															
							6	11	16	18	31	33	35	39	45	51	52	56	58	59	73	82
Kataoka *et al.* (1991), Sweden	Urethra	105	18–23	Army conscripts (men)	Type-specific primers	17.1	2.9	8.6	1.9			5.7										
Jalal *et al.* (1992), United Kingdom	Oral epithelial scrapes	62	Not reported	Healthy young adult volunteers	Type 16-specific	44.0			44.0													
Lawton *et al.* (1992), Australia	Oral mucosa	60	Not reported		PCR	60.0																
Chen *et al.* (1993), Australia	Foreskin	92	–	Neonates	L1 consensus primers	0																
Eike *et al.* (1995), Denmark	Oral smear	61	20–79	Normal individuals	Consensus primer	0																
	Nasal smear	48				2.1																
Koch *et al.* (1997), Denmark	Anal smear	706	0–17	Nurseries, kindergarten, primary school	PCR + dot-blot	0.5																
	Oral smear	482				0.2																
Bowden *et al.* (1999), Australia	Vagina	1090	12–73	Two community-based studies using self-administered tampon	PCR	42.0																
Morin *et al.* (2000), Canada	Vulva	322	22.5 ± 4.0	Gynaecological clinic	PCR	23.9																

Table 15 (contd)

Reference, location	Site	No.	Age (years)	Setting	Primer	Overall HPV (%)	Specific HPV type (%)															
							6	11	16	18	31	33	35	39	45	51	52	56	58	59	73	82
Peixoto Guimaraes (2001), China	Oesophageal cells	57	40.5 (29–65)	Population-based oesophageal cancer screening programme	GP5+/6+	7.0			1.8[a]													
Summersgill *et al.* (2001), USA	Oral cavity	268	≤ 20	Infants, children, adolescents	MY09/11	6.0	0.7		4.0	0.4												
Kojima *et al.* (2003), Japan	Oral cavity	77	3–5	Nursery school	L1 C1/C2 1003/1004	48.1			29.7											2.7		
de Lima Soares *et al.* (2003), Brazil	Vagina	341	≥ 15	Rural area	HC	26.6																
Kreimer *et al.* (2004), USA	Oral rinse	590	40.6 (18–85)	Oral cancer screening programme	PGMY09/11	12.2			1.7	0.7			0.7	0.3	0.2		0.2	0.5	0.3	0.7	0.3	0.8
	Tonsil brush biopsy	583				3.1			1.0	0.2			0.2	0.0	0.2		0.0	0.0	0.0	0.2	0.0	0.0
Kurose *et al.* (2004), Japan	Oral mucosa	668	0–60+	Healthy volunteers	MY09/11	0.6																
Lambropoulos *et al.* (1997), Greece	Oral cavity	169	14–85	Asymptomatic subjects	PCR + southern blot	9.5	4.1	0.6	2.4	0.0												

Table 15 (contd)

Reference, location	Site	No.	Age (years)	Setting	Primer	Overall HPV (%)	Specific HPV type (%)															
							6	11	16	18	31	33	35	39	45	51	52	56	58	59	73	82
Rosenblatt *et al.* (2004), Brazil	Penis	90	Not reported	Partners of women joining cervical cancer screening programme	HC	51.1																
Shin *et al.* (2004), Republic of Korea	Penis	381	16–23 (21.3 ± 2.2)	University students	SPF_{10} primer	8.7	0.5	0.3	0.5	0.5		0.3		0.8	0.3	0.8	0.8		0.3			
Smith, E.M. *et al.* (2004a,b), USA	Oral rinse	333	≥ 18	Routine screening visits in hospital	MY09/11	18.3			10.0	< 1.0	< 1.0								< 1.0			

See Table 7 for a description of the primers used.
HC, Hybrid Capture; PCR, polymerase chain reaction
[a] HPV 16/18

Prevalence studies that address the oral mucosa in adults showed very diverse results that ranged from 0 to 60%. This was also true for the few studies among children. It has been suggested that HPV type 16 is by far the most prevalent type, and that HPV 6 and 11 are much less prevalent. The reason for the diversity between the studies needs to be explored. One study on the oesophagus showed a prevalence of HPV infection of 7.0% (type 16 or 18 had a prevalence of 1.8%) (Peixoto Guimaraes *et al.*, 2001).

1.5 Pathology of HPV infection of the genital tract and evidence therefrom for progression to malignancy

1.5.1 *Evolution of concepts and terminology*

(*a*) *Dysplasia and carcinoma* in situ

By the late 1800s, the histological changes that occurred at the margins of invasive squamous-cell cancers of the cervix had been recognized and described by Williams (1888). Their significance was not appreciated at the time, but these changes were later called carcinoma *in situ* and described precursors of cervical cancer. Reagan and Hamonic (1956) introduced the term 'dysplasia' to designate cervical epithelia that contained cytologically atypical cells but lacked the full-thickness of differentiation. Dysplasias were further divided into mild, moderate and severe, depending on their degree of differentiation. From this terminology, it was implicit that the higher the grade, the closer the lesion was in aggregate to invasion. This assumption was based upon the observation that higher-grade dysplasias resembled carcinoma *in situ* and invasive cancer more closely than those of a lower grade. However, carcinoma *in situ* remained in the minds of clinicians as the only true precursor of cancer. Patients with this disease were generally treated by total hysterectomy and those with lesser degrees of epithelial change — dysplasias — were either treated by cervical conization or followed prospectively without treatment (reviewed in Younge, 1965).

(*b*) *Cervical intraepithelial neoplasia (CIN)*

With continuing clinical experience, it became obvious both to pathologists and clinicians that there was extremely poor inter- and intra-observer reproducibility in the differentiation of carcinoma *in situ* from dysplasia. It was particularly difficult for pathologists to distinguish between severe dysplasia and carcinoma *in situ*, and clinicians became increasingly sceptical of the rationale for therapy that was dictated by the classification system for dysplasia–carcinoma *in situ*.

In view of this, and after the completion of a number of laboratory and clinical studies that were begun in the 1960s, it became apparent that severe dysplasia and carcinoma *in situ* could not be distinguished reproducibly at any level and that the lesser degrees of atypia — particularly mild and moderate dysplasia — merged imperceptibly in objective measurements with the higher-grade lesions (Richart, 1987).

These observations led to the introduction of the term 'cervical intraepithelial neoplasia' (CIN) to designate the spectrum of cervical diseases that were thought to play a role

in cervical carcinogenesis. The reasoning behind the terminology of CIN was that a continuum of change began with mild dysplasia and ended with invasive cancer after passing progressively through the intermediate stages of intraepithelial disease. The clinical impact of this new terminology was that presumed precursor lesions should be treated based on their size and location. In CIN1 (mild dysplasia), neoplastic basaloid cells occupy the lower third of the epithelium; in CIN2 (moderate dysplasia), neoplastic basaloid cells and mitotic figures occupy the lower two-thirds of the epithelium; and in CIN3, mitotic figures and basaloid cells can be found throughout the whole thickness of the epithelium. In the grading of CIN lesions, CIN3 included severe dysplasia and carcinoma *in situ* and, in terms of treatment, less emphasis was placed on hysterectomy in favour of outpatient-directed methods and conservation of the uterus (Richart, 1987).

As molecular data accumulated, it became apparent that the spectrum of atypical epithelial changes that occurred in the female lower genital tract and that were etiologically related to HPV could best be described as a two-tiered, rather than a three-tiered disease process, and the CIN classification was modified accordingly (Richart, 1990). Those lesions commonly referred to as mild dysplasia, flat condyloma or CIN1, which were thought to be the result of a productive HPV infection, were designated low-grade CIN. Those lesions that contained more severe cytological atypia (CIN2 and CIN3), which were thought to be true potential precursors of cancer and to require treatment, were designated high-grade CIN. The distinction between low-grade CIN and high-grade CIN was based upon an assessment of cytological atypia and the presence or absence of abnormal mitotic figures. However, it was emphasized that the diagnostic decision should be taken at an operational level as well as at a morphological level so that the clinician could infer accurately from the diagnosis whether the pathologist believed that the lesion being diagnosed was a true precursor of cancer or not.

Several publications have questioned whether high-grade CIN develops from existing low-grade CIN or develops *de novo* (Koutsky *et al.*, 1992, Kiviat & Koutsky, 1993). The current commonly held opinion is that CIN3 can develop either via the sequence of CIN1 and CIN2 into CIN3 or directly from a high-risk HPV infection with no demonstrable stages of CIN1 or CIN2 (Kiviat *et al.*, 1992; Park *et al.*, 1998b; Nobbenhuis *et al.*, 1999; Winer *et al.*, 2005).

(*c*) *Squamous intraepithelial lesions (SILs)*

Because of the problems caused by an extremely low degree of intra- and inter-observer reproducibility in cytological diagnoses, a group was convened in Bethesda, MD (USA), to devise a uniform cytological terminology (National Cancer Institute Workshop, 1989; Luff, 1992). This meeting concluded that molecular data are more consistent with a two-tiered, rather than a three-tiered system. This new nomenclature known as 'The Bethesda System' introduced the terms 'low-grade squamous intraepithelial lesion' (LSIL) and 'high-grade squamous intraepithelial lesion' (HSIL) (see Table 16). LSIL includes CIN1 or mild dysplasia, koilocytosis, koilocytotic atypia and flat condyloma. HSIL includes CIN2 and CIN3, or moderate and severe dysplasia, and carcinoma *in situ*.

Table 16. Relationship between histological classification of CIN and SIL and cytomorphological Pap classification

Cytological equivalent	Bethesda 2001	Negative	AGC	AGC favour neoplastic				ADC
	European/Netherlands	Negative	ASCUS/ASC-H		HSIL		HSIL	SCC
			Borderline and mild dysplasia/dyskaryosis		Moderate	Severe	In situ carcinoma	Invasive
	Netherlands/CISOE-A[a]	Pap 1	Pap 2	Pap 3a1	Pap 3a2	Pap 3b	Pap 4	Pap 5
Histology	Description	Normal	Atypia	Mild dysplasia/dyskaryosis		Moderate	Severe	In situ carcinoma	Invasive
	CIN	Grade 0		Grade 1		Grade 2	Grade 3		
	SIL			LSIL		HSIL			

From Bulkmans *et al.* (2004)

ADC, adenocarcinoma; AGC, atypical glandular cells; ASC-H, atypical squamous cells that cannot exclude HSIL; ASCUS, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; Pap, Papanicolaou test; SIL, squamous intraepithelial lesion; SCC, squamous-cell carcinoma

[a] C stands for compositon of the smear, I for inflammatory changes, S for squamous epithelium, O for other and endometrium, E for endocervical columnar epithelium and A indicates whether or not the smear is adequate.

In order to evaluate and update the 1991 Bethesda classification, the 2001 Bethesda system was established. The terminology used was agreed after a review process in which more than 400 cyto-/histopathologists, gynaecologists, cytotechnologists, epidemiologists, health physicians and lawyers were involved. The dichotomous division of SIL into LSIL and HSIL was based on virological, molecular and clinical observations that LSIL is more frequently a result of a transient HPV infection whereas HSIL is more frequently associated with viral persistence and high risk for progression. LSIL includes changes that mainly reflect HPV infection, which eliminates the distinction between condylomatous atypia and CIN1, whereas HSIL includes higher-risk lesions, including precursors of cancer (Solomon *et al.*, 2002). In addition, equivocal interpretations called atypical squamous cells of undetermined significance (ASCUS) are more common than definite lesions; approximately half of these lesions are related to HPV infection.

In addition, HSIL is usually associated with high-risk HPV types and is monoclonal and aneuploid in contrast to LSIL (Fu *et al.*, 1983; Lungu *et al.*, 1992; Park *et al.*, 1998b; Hering *et al.*, 2000).

Data from the ASCUS/LSIL Triage Study confirm that (*a*) LSIL is a fairly reproducible break-point compared with HSIL; (*b*) the subdivision of cytological HSIL into moderate and severe dysplasia or CIN2 and CIN3 is not very reproducible; and (*c*) the cytopathological effects of HPV cannot be reliably separated from those of CIN1 or mild dysplasia (Bulkmans *et al.*, 2004; Schiffman & Adrianza, 2000).

However, despite the moderate reproducibility of diagnoses into three CIN grades, pathologists in several European countries still use the three-tiered designation. They noted that (*a*) separation into CIN1, CIN2 and CIN3 correlates to a general extent with rates of progression and/or regression of the lesions (Mitchell *et al.*, 1996); (*b*) the use of MIB-1, an antibody directed against cell proliferation-associated Ki-67 antigen that stains cells in the G2M phase, increases the reproducibility of the CIN classification (Bulten *et al.*, 1996; Kruse *et al.*, 2001); and (*c*) with regard to microscopic morphological interpretation, poor reproducibility does not denigrate clinical value (Renshaw *et al.*, 2003).

The terminology of CIN is especially helpful to correlate cytopathological and histopathological findings and to manage individual patients based on the finding that moderate dysplasia (CIN2) has characteristics more similar to mild dysplasia (CIN1) than to severe dysplasia/carcinoma *in situ* (CIN3) (Östör, 1993; Nobbenhuis *et al.*, 1999). CIN or dysplasia can be substituted for SIL or used as an additional descriptor (Table 16; Solomon *et al.*, 2002; Bulkmans *et al.*, 2004). A good example of such an approach that allows easy translation to the Bethesda 2001 system is the CISOE-A classification that is used in The Netherlands (Bulk *et al.*, 2004), in which C stands for composition of the smear, I for inflammatory changes, S for squamous epithelium, O for other and endometrium and E for endocervical columnar epithelium; A determines whether or not the smear is adequate.

(*d*) *Adenocarcinoma* in situ

Adenocarcinoma *in situ* is characterized by a complex gland formation that arises within the normal endocervical glands, cytological atypia, an increased mitotic rate and a

gland-within-gland pattern. High-risk HPVs are found in nearly all adenocarcinomas *in situ* and in adenocarcinomas of the cervix. HPV 18 is more frequent in this disease than in squamous-cell carcinoma (Zielinski *et al.*, 2003).

(*e*) *Intraepithelial neoplasms of other organs in the male and female anogenital tract*

Intraepithelial lesions of the vagina, penis and anus are generally diagnosed using a modification of the CIN terminology system and are also graded into three groups. The presumed precursor lesions for these organs are referred to as vaginal intraepithelial neoplasia (VAIN), vulvar intraepithelial neoplasia (VIN), penile intraepithelial neoplasia (PIN) and anal intraepithelial neoplasia (AIN) (Zbar *et al.*, 2002). Similarly to CIN lesions, it is believed that these lesions progress via increasing degrees of intraepithelial involvement. However, their progression rate is rather slow. Follow-up data on PIN1 lesions indicate that transition to high-grade PIN is a rare event (Bleeker *et al.*, 2003).

1.5.2 *Temporal and spatial relationships between precursors of cervical cancer and invasive cancer*

(*a*) *Histological observations*

The original observations that suggested the existence of a precursor of cervical cancer and that led to the term carcinoma *in situ* were made by pathologists who noted that the epithelium overlying or adjacent to cervical cancers contained cytological alterations that were similar to those found in invasive cancers. This simple but important observation led to the concept that cancers were preceded by a precursor state that could be recognized histologically. The invention of the colposcope by Hinselmann (1925) allowed gynaecologists to recognize clinically alterations in the cervical epithelium that could be diagnosed by punch biopsy as carcinoma *in situ*. These alterations could then be treated to prevent the development of invasive cancer. However, it was not until Papanicolaou and Traut (1943) published their observations on exfoliated cells that it was discovered that these early histological and colposcopical observations could be used as part of mass screening programmes and be translated into schemes for cancer prevention. Subsequent observers noted that the mean age at diagnosis of mild, moderate and severe dysplasia, carcinoma *in situ* and invasive cancer increased progressively and that this increase was accompanied by an increase in the size of the lesion. This increase was in turn found to be accompanied by an increase in gland and canal involvement; in addition, the larger lesions were more likely to contain areas of invasion. These observations lent strong support to the hypothesis of the progression of CIN to cancer (Jones, 2006).

Retrospective analysis of lesions that were diagnosed as co-existing LSIL and HSIL in relation to the presence of high-risk HPV revealed which of the lesions (which span two grades: CIN1 and CIN2) most probably represented morphological progression from a single infection (Park *et al.*, 1998b). However, lesions that contain CIN1 and CIN3 may be attributed either to progression of the lesion or to two coincidental infections. Further-

more, in retrospective studies that analysed previous smears from women with cervical cancer for the presence of HPV and of abnormal cells, it was noted that (*a*) many women had smears with abnormal cells that had been overlooked by the cytopathologist/cytotechnician; and (*b*) the same high-risk HPV type was present both in the cervical carcinoma biopsy and in the previous smear (Walboomers *et al.*, 1995; Wallin *et al.*, 1999; Zielinski *et al.*, 2001a,b). This temporal relationship between cervical precursor lesions and cervical cancer in the presence of the same high-risk HPV type indicates the progression of such precursor lesions to cervical cancer.

(*b*) *Microinvasive and early invasive cervical cancers*

The most important direct pathological evidence that putative precursors are in fact precancerous lesions was the histological observation of invasion arising from such lesions. Tongues of invasion that range from only one or two cells to larger lesions are seen to arise directly from surface CIN lesions or from intraepithelial lesions that involve the endocervical glands. These tongues of microinvasive carcinoma may be single or multiple and are generally accompanied by a local inflammatory infiltrate and a desmoplastic response. In the cervix, the risk of metastasis depends upon the degree of stromal penetration. Microinvasive cancer with a stromal penetration of ≤ 3 mm and a length of ≤ 7 mm (FIGO [International Federation of Gynaecology and Obstetrics] stage Ia1) rarely metastasizes and can be treated conservatively. Invasive lesions with a depth of stromal penetration > 3 but < 5 mm and < 7 mm in length (FIGO stage Ia2) have a minimum risk of metastasis and can be treated conservatively if the woman wants to preserve functional integrity. Invasive lesions with a depth of stromal penetration > 5 mm or > 7 mm in length (FIGO stage IB1 or more advanced stage) are treated radically (Lécuru *et al.*, 1997).

(*c*) *Clinical and epidemiological observations*

Smith and Pemberton (1934) drew attention to the fact that patients who had invasive cervical cancer were commonly found to have had carcinoma *in situ* in their biopsies; when patients whose carcinoma *in situ* had been diagnosed by biopsy were followed without treatment, a significant number developed invasion. Similar observations were made by Kottmeier (1961) who followed 31 women with carcinoma *in situ* prospectively for at least 12 years; 72% of these women developed invasive cancer. In a similar study in New Zealand (McIndoe *et al.*, 1984), 131 patients with persistently abnormal Papanicolaou (Pap) smears were followed for 4–23 years; 22% developed invasive carcinoma of the cervix or vaginal vault and 69% had persistent carcinoma *in situ*, which was treated subsequently. These observations of the natural history of carcinoma *in situ* suggest that, in the majority of the patients, once this disease is established, it rarely regresses spontaneously. There is therefore a discrepancy between the cumulative incidence of carcinoma *in situ* observed in the natural history studies conducted in British Columbia (Canada), The Netherlands and Denmark, which suggested that a high proportion of carcinomas *in situ* do regress without treatment, and the cumulative incidence of invasive cancer seen in earlier observational studies (Smith & Pemberton, 1934; Kottmeier, 1961; McIndoe *et al*., 1984; Miller, 1992).

An explanation might be that, in these observational studies, carcinoma *in situ* was diagnosed at a relatively late phase and thus represented large lesions, whereas, in the nationwide screening programmes, much smaller lesions were diagnosed as having a lower viral load and a higher tendency for regression. The natural history of cervical precursor lesions of a lower histological grade than carcinoma *in situ* has been studied by Ho *et al.* (1998a) and Nobbenhuis *et al.* (1999) and has been reviewed extensively by Östör (1993).

1.5.3 *Histological changes in HPV-related lesions of the lower female genital tract*

The natural history of an HPV infection is age-dependent; a dramatic increase in the detection of cervical HPV DNA occurs after the initiation of sexual activity (Koutsky *et al.*, 1992; Melkert *et al.*, 1993; Hildesheim *et al.*, 1994; Ho *et al.*, 1998a; Kjaer *et al.*, 2001, Winer *et al.*, 2003). High-risk HPV is usually assumed to enter the cells of the basal and parabasal layers at sites of minor trauma or where the anatomical architecture provides easy access. Depending on host and cellular factors, the infection can be cleared spontaneously and quickly (transient HPV infection): this happens in about 70% of women within 1 year (Ho *et al.*, 1998a; Woodman *et al.*, 2001); the remaining 30% develop detectable CIN lesions. Subject to their stage and the immune status, CIN lesions may regress after the HPV infection has been cleared (Nobbenhuis *et al*., 2001). About 50% of low-grade lesions regress within 1 year, while a smaller proportion of high-grade lesions regress. The persistence of high-risk HPV infection is prerequisite for progressive CIN.

(*a*) *Latent HPV infection*

HPV genomes are present in the basal layers of infected epithelia and differentiation is required for the production of virions. The latency of HPV can be defined as a state in which viral DNA is maintained in the absence of virion production.

Latent HPV infection is operationally defined as an infection in which the replication of viral DNA is synchronized with the cell cycle but in which none of the cytopathogenic effects of HPV can be detected. Although no direct evidence for a solely latent HPV infection has been found, a number of clinical observations suggest that it may occur.

(i) HPV DNA can be detected in apparently normal cervical epithelium, and several studies have shown that the risk for women with a high-risk HPV-positive, cytomorphologically normal smear to develop an abnormal smear within 2 years or CIN3 within 4 years is substantially increased (Koutsky *et al.*, 1992; Hildesheim *et al.*, 1994; Liaw *et al.*, 1999; Rozendaal *et al.*, 2000).

(ii) A common observation is that women who have no clinical or cytological evidence of HPV while in the interpartum state may develop HPV-related lesions during the relatively immunocompromised state of pregnancy and that such lesions regress without treatment post partum (Nobbenhuis *et al.*, 2002).

(iii) Women who take immunosuppressive therapy for renal transplantation and those with HIV infection have a higher incidence of CIN and cervical cancer (Klein

et al., 1994; Williams *et al.*, 1994; Wright *et al.*, 1994; Cappiello *et al.*, 1997; Sun *et al.*, 1997; Cu-Uvin *et al.*, 1999; Ellerbrock *et al.*, 2000).

(iv) Patients in whom HPV-related lesions have been treated may have detectable HPV DNA despite normal cytological, colposcopical and histological findings. Such patients are at increased risk for recurrence compared with HPV DNA-negative controls (Koutsky *et al.*, 1992; Nobbenhuis *et al.*, 2001).

(*b*) *Low-grade CIN*

A number of studies reported that it was possible to distinguish between virus-containing flat condyloma and a true 'virus-free' CIN lesion (Meisels & Fortin, 1976). However, subsequent studies found that the distribution of HPV types in those lesions designated as flat condyloma and CIN was indistinguishable (Kadish *et al*., 1986; Willet *et al*., 1989) and that, due to this lack of consistent morphological features, the ability to make such distinctions has extremely low inter- and intra-observer reproducibility. In addition, no differences in nuclear DNA content was observed, as both have diploid/polypoid DNA distribution patterns (Fu *et al*., 1983; Fujii *et al*., 1984). It is therefore not thought to be possible to separate flat condylomas from low-grade CIN or SIL lesions.

Low-grade CIN is, by definition, a lesion that is well differentiated but abnormal and contains alterations that are characteristic of the cytopathogenic effects of a replicative HPV infection. Operationally, it is a lesion that is thought by pathologists to be the result of a productive viral infection and not to represent a true precursor of cancer. Low-grade CIN lesions can arise through infection by any of the anogenital HPV types. It supposedly arises from HPV-infected basal cells, which may gain the capacity to multiply the virus to very high copy numbers. However, this productive stage is restricted to postmitotic, differentiated cells in the suprabasal layers of the epithelium that are withdrawn from the cell cycle. Detailed in-situ hybridization and immunohistochemical studies have shown that a high expression level of viral genes, multiplication of the viral genome, synthesis of early (E6, E7, E2 and E4) and late gene products (L1 and L2), encapsulation of the HPV genome and release of virion particles together with the exfoliation of upper epithelial layers are strictly linked to terminal differentiation of the infected epithelia. The cytopathogenic effects of one HPV type compared with those of another are generally reported to be indistinguishable under light microscopy; however, some investigators have reported that HPV 16-induced lesions are more pleiomorphic than those induced by other HPV types (Crum *et al*., 1991).

Most low-grade CIN lesions have a thickened epithelium due to the acanthosis that accompanies epithelial hyperplasia and many also have papillomatosis. The basal and parabasal layers characteristically have little cytological atypia, are arranged in a uniform fashion on the basal lamina and are not highly disorganized. As viral replication begins in the upper parabasal and lower intermediate layers of the epithelium, it is accompanied by the characteristic cytopathogenic effects of HPV infection that include cytological and organizational binucleation, perinuclear cytoplasmic cavitation with a thickened cytoplasmic membrane and, most importantly, nuclear atypia. The expression of E4-encoded

proteins in squamous epithelial cells causes the cytokeratin matrix to collapse due to a specific binding to cytokeratin proteins (Doorbar *et al*., 1991) and possibly leads to the typical perinuclear cavitation, which is a feature of productive HPV infection. The combination of nuclear atypia and perinuclear halo formation is referred to as koilocytosis or koilocytotic atypia (Koss & Durfee, 1955). These koilocytotic cells are the principal hallmark of productive HPV infection of the cervical, vaginal or vulvar mucous membrane. It should be emphasized that perinuclear halos may be produced as a result of other cervical or vaginal infections or may accompany repair or metaplastic processes.

The most characteristic histological feature of anogenital HPV infection, and that which is most useful diagnostically, is nuclear atypia. HPV-related nuclear atypia is due to heteroploidy (Fu *et al*., 1981), which appears to result from mitotic spindle abnormalities and leads to DNA replication without cytokinesis. The result of this interference with the mitotic process is the formation of bi- and multinucleated cells and enlarged atypical nuclei, accompanied by heteroploidization.

In low-grade lesions, the nuclei are principally diploid and polypoid. Mitotic figures are generally increased in low-grade lesions but are mainly confined to the lower third of the epithelium, as are undifferentiated or basal-type cells, and are characteristically absent from the upper layers of the epithelium. Most of the mitotic figures have a normal appearance, but cells with tripolar mitosis or tetraploid-dispersed metaphases may also be seen (Winkler *et al*., 1984). These two types of abnormal mitotic figure are also commonly found in polyploid lesions in other organs.

(*c*) *High-grade CIN*

High-grade CIN lesions (CIN2 and -3) are substantially more atypical cytologically than low-grade CIN, have a higher degree of disorganization and have undifferentiated cells that extend beyond the lower third of the epithelium. This is reflected in the spectrum of HPV types found in low-grade CIN, which differs substantially from that found in high-grade CIN lesions (Matsukura & Sugase, 1995). In high-grade CIN, nuclear crowding, substantial pleomorphism, loss of both tissue organization and cellular polarity occur, and mitotic figures are characteristically found in the middle and upper thirds of the epithelium in addition to those in the lower third. The cytological atypia that is found in high-grade CIN lesions differs substantially from that seen in the low-grade lesions. The nuclei in high-grade CIN are generally larger, their nuclear membranes are more prominent and tend to be convoluted and distorted, and the nuclear chromatin pattern is characteristically clumped, coarsely granular and contains prominent chromo-centres. As the nuclei enlarge, the nuclear cytoplasmic ratio is altered in favour of the nucleus and the cell borders, which commonly contain visible desmosomes in low-grade lesions, become indistinct and difficult to define. In contrast to low-grade lesions, expression of the viral oncogenes *E6* and *E7* in high-grade lesions also occurs in the dividing, immature, metaplastic basal stem cells. It has been reported that the E6 protein in particular but also the E7 protein of HPV 16 induce chromosomal aberrations (White *et al*., 1994; Duensing

& Munger, 2002). The characteristic koilocyte of low-grade CIN is generally absent or markedly attenuated in high-grade lesions.

One of the most important features that distinguishes high-grade CIN from low-grade CIN is the presence of abnormal mitotic figures (Winkler *et al*., 1984). Although many different types of abnormal mitotic figure are found in high-grade CINs, the most characteristic is the three-group metaphase (i.e. chromosomal material on either side of the equatorial chromosomes in the metaphase) (Claas *et al*., 1992). Other abnormal mitotic figures that are commonly seen include the two-group metaphase, multipolar mitoses in excess of three, lagging metaphase chromosomes, coarsely clumped chromosomes and highly abnormal, bizarre mitotic figures. Abnormal mitotic figures are found in aneuploid lesions (aneuploidy is a marker for cancer or precancer) and have been reported to be the histological marker that best predicts the biological behaviour of CIN (Fu *et al*., 1981). As they are an excellent surrogate marker for aneuploidy (Bergeron *et al*., 1987a,b; Fu *et al*., 1988), these mitotic abnormalities serve as a useful objective marker to distinguish between low-grade and high-grade CIN. In the presence of an abnormal mitotic figure, a lesion is consistently aneuploid and is a true precursor of cancer. In the absence of abnormal mitotic figures, other histological features commonly used to classify these lesions should be taken into account.

(*d*) *Microinvasive and invasive squamous-cell cancer of the cervix*

Microinvasive squamous-cell cancer of the cervix consists of a single (or multiple) irregular tongue(s) of neoplastic squamous epithelium that breaks through the plane of the basal lamina and invades the cervical stroma or epithelial lamina propria. Characteristically, areas of microinvasion are better differentiated than those of high-grade CIN from which they most commonly arise. They lack the smooth contour and crisp demarcation from the subjacent stroma that is found in both surface high-grade CIN and high-grade CIN with glandular involvement. Areas of microinvasion infiltrate in an irregular fashion and split collagen bundles. Microinvasive foci are commonly accompanied by an inflammatory and desmoplastic response. Microinvasion is defined as a lesion that invades the cervical stroma to a depth of no more than 5 mm; frank invasive cancer has a histological appearance similar to that of microinvasive cancer but has invaded more than 5 mm into the cervical stroma. No convincing evidence has been found that the histological appearance of invasive cancer or the prognosis of the patient can be predicted from the HPV type that has produced the lesion (van Bommel *et al.*, 1993; Pilch *et al.*, 2001).

(*e*) *Adenocarcinoma* in situ *and adenocarcinoma of the cervix*

Adenocarcinoma *in situ* is mainly localized in the endocervical canal; representative cells are therefore rare or absent in cytological specimens, and cytology rarely results in diagnosis. Moreover, because of the incomplete overview of the endocervical canal and the poorer prognosis of adenocarcinoma of the cervix compared with squamous-cell carcinoma, clinicians always remove intraepithelial lesions of the glandular cells and data on the natural history of these lesions are therefore lacking (Boon *et al.*, 1981; Ruba *et al.*,

2004). Whereas SIL occurs on the squamous side of the cervical squamo-columnar junction, adenocarcinomas *in situ* and adenocarcinomas occur on the columnar side. They are commonly associated with CIN lesions, particularly those that are high grade (Luesley *et al*., 1987). The endocervical epithelium does not appear to sustain productive HPV infections, and low-risk HPV types have not been found in endocervical neoplasia (Higgins *et al*., 1992a).

Adenocarcinoma *in situ* is characterized by a complex gland formation in the distribution of the normal endocervical glands, cytological atypia, an increased mitotic rate and a gland-within-gland pattern. Cytological alterations similar to those seen in other aneuploid cell populations are present and abnormal mitotic figures are common. Adenocarcinoma *in situ* is distinguished from invasive adenocarcinoma by virtue of its pattern and lack of demonstrable invasion. Similarly to CIN3 and squamous-cell carcinomas, high-risk HPV is found in nearly all adenocarcinomas *in situ* and adenocarcinomas of the cervix (Zielinski *et al.*, 2003). HPV 18 is found more commonly in these adenocarcinomas and some studies have described a poorer prognosis for these tumours (Walker *et al.*, 1989; Schwartz *et al.*, 2001).

(*f*) *Condyloma acuminatum, intraepithelial neoplasia and cancer of the vagina*

The histological changes in the vaginal mucous membrane that are associated with HPV infection and HPV-induced neoplasia are similar to those that are seen in the cervical mucous membrane. Thus, condylomata acuminata and VAIN may be present. Similarly to CIN, VAIN can be separated into three histological grades. The progression to vaginal cancer appears to be slow and the tumours have the morphology of a squamous-cell carcinoma. HPV 16 is the most prevalent type described in these lesions.

(*g*) *Condyloma acuminatum, intraepithelial lesions and cancer of the vulva*

The most characteristic HPV-related lesions found on the vulva are acuminate warts. Condyloma acuminatum, which is nearly always caused by HPV 6 or 11 (Gissmann *et al*., 1982a; Nuovo *et al*., 1990; Matsukura & Sugase, 1995), is an exophytic lesion. It has cytological and histological features and organizational alterations similar to those seen in the cervical and vaginal mucous membranes, except for the presence of substantial acanthosis and papillomatosis. Condylomata acuminata that occur on the mucous membranes characteristically have the full constellation of HPV-related cytopathogenic effects, including koilocytosis. Warts that occur in the keratinizing epithelium, however, commonly contain minimal cytological atypia, and koilocytes may be difficult to identify, particularly in clinically older lesions.

The intraepithelial lesions of the vulvar skin (VIN) have a much more complicated histological pattern than those of the mucous membranes of the cervix and vagina. It is common to distinguish three different types of VIN histologically — basaloid, warty and

well-differentiated. High-risk HPV types are found principally in the warty and basaloid types of VIN and are uncommon in the well-differentiated type (van Beurden *et al.*, 1995).

The basaloid type is composed generally of small, fairly uniform cells that are hyperchromatic and contain alterations in the distribution pattern of nuclear chromatin. These cell types tend to have low mitotic activity, and abnormal mitotic figures are seldom encountered. Warty-type VIN is generally a highly pleomorphic lesion with multinucleated cells, cytological atypia, coarse chromatin clumping, large numbers of mitoses and abnormal mitotic figures. It is commonly associated with koilocytosis, and adjacent condylomatous-type changes are frequently seen. The well-differentiated type of VIN is characteristically composed of a complex proliferative lesion that is only minimally altered in pattern and contains minimal nuclear atypia. Dyskeratosis is a common feature.

VIN can be present as either a solitary patch or as multifocal lesions. Irrespective of this presentation, progression of VIN3 to vulvar carcinoma is rarer than was previously assumed (van Beurden *et al.*, 1995) and radical vulvectomy has been replaced by more conservative treatments (van Seters *et al.*, 2002). Carcinomas of the vulva are also of the basaloid, warty and well-differentiated types and have the same association with high-risk HPV as VIN. About 40% of vulvar carcinomas are high-risk HPV-positive; they occur in younger women and tend to have a more benign behaviour pattern than HPV-negative tumours (Al-Ghamdi *et al.*, 2002; Gualco *et al.*, 2003).

Recently, a modified terminology based on morphological criteria only and not on HPV type or clinical appearance has been proposed for squamous VIN (Sideri *et al.*, 2005).

(*h*) *Condyloma acuminatum, intraepithelial lesions and carcinomas of the anus and penis*

Condylomata acuminata of the anus and penis have the same histological appearance and contain the same HPV types as those in the cervix. Squamous neoplasms of the anus are similar morphologically to those that arise in other keratinizing epithelia, including HPV-related lesions of the vulva. The anus has a squamo-columnar junction and a transformation zone similar to that seen in the cervix. Squamous-cell cancers and their precursors develop at the squamo-columnar junction and in the transformation zone of the anus, as in the cervix. Anal canal tumours are histologically more similar to squamous carcinomas of the cervix, whereas perianal tumours more closely resemble those in the vulva and are of the basaloid, warty and more highly differentiated type. The association with high-risk HPV is strong (Frisch *et al.*, 1997): more than 90% of tumours in the anal canal contain high-risk HPV, mostly type 16, whereas those in the perianal canal region contain slightly lower levels of high-risk HPV and again HPV 16 is the most dominant type. Receptive anal intercourse, especially starting at a younger age, is an important risk factor (Frisch *et al.*, 1997; Gervaz *et al.*, 2003).

Squamous neoplasms of the penis are similar to those of the vulva with respect to the diversity of histological types and association with HPV (Ferreux *et al.*, 2003). Most penile cancers are basaloid, warty, verrucous or keratinizing squamous-cell cancers. As in the

vulva, basaloid and warty cancers are more strongly associated with HPV (primarily HPV 16) than squamous-cell cancers.

The histology of PIN resembles intraepithelial neoplasia at other genital sites and ranges from grade 1 to grade 3. The appearance of PIN varies considerably depending on the circumcised status of the patient and location of the lesion. PIN1 lesions have been shown to have high copy numbers of HPV DNA (Bleeker *et al.*, 2003) and form the main reservoir of HPV in men.

1.5.4 *Pathology of cutaneous HPV infection and non-melanoma skin cancer*

(*a*) *Cutaneous HPV infection*

Skin warts differ in clinical morphology and histological pattern depending on the HPV type by which they are induced. Cutaneous warts include common warts (verruca vulgaris; mainly associated with HPV 2, 4, 7 and 57), deep plantar and palmar, myrmecial warts (HPV 1), plane warts (verruca planar; HPV 3, 10 and 41), intermediate warts (mixtures of common and flat warts; HPV 26, 27, 28 and 29) and cystic or punctate, mainly plantar warts (HPV 60, 63 and 65) (reviewed by Jablonska *et al*., 1997). Skin warts are benign, show limited growth and often regress spontaneously. Common histological features comprise papillomatosis, acanthosis and parakeratosis to varying degrees. Virus-specific cytopathogenic effects are most prominent in the granular layer of the epithelium, where mature virus particles appear and spread throughout the nuclei or in paracrystalline arrays.

HPV of the beta genus induce red-brown plaque-like lesions and achromic, scaly, pityriasis versicolor-like lesions only in EV patients (see Section 2.7.1) and exceptionally in immunosuppressed patients (Orth, 1986; Majewski *et al*., 1997). These HPV types are therefore referred to as EV-HPV (Orth *et al*., 2001). They are also highly prevalent in the general population (Boxman *et al*., 1997; Astori *et al*., 1998; Boxman *et al*., 1999; Antonsson *et al*., 2000; Forslund *et al*., 2003c), but do not induce the characteristic pathology. The histology of pathognomonic EV lesions reveals large cells with pale-stained cytoplasm in the spinous and granular layers. This specific cytopathic effect is linked to high levels of viral replication in differentiating keratinocytes. Common warts, plantar warts and genital warts are rare in EV patients. However, such patients are not infrequently infected by HPV 3 and 10 that induce flat warts, as in the general population, and occasionally confluent, elevated brownish plaques mainly on the extremities and the face (Majewski *et al*., 1997). In some EV patients, the lesions are highly proliferative, with features of papilloma or seborrheic keratoses (Jacyk *et al*., 1993a; Tomasini *et al*., 1993). This cytopathic effect depends on the association of these lesions with EV-HPV or HPV 3 (Majewski *et al*., 1997).

Cutaneous warts develop in up to 90% of transplant recipients who survive the onset of immunosuppression by more than 5 years (Leigh *et al*., 1999). Two or more distinct HPV types were co-detected in most of these warts, and, in addition to the HPV types responsible for warts in the general population, EV-HPV and genital HPV DNA were also

detected. However, no EV phenotype was expressed in most of these cases (Obalek *et al*., 1992; Harwood *et al*., 1999).

A causative role of HPV in seborrheic keratoses has been speculated because of their histological similarity to warts, in that they display papillomatosis, acanthosis and hyperkeratosis. Mucosal HPV was detected in 20% of non-genital seborrheic keratoses in one study (Tsambaos *et al*., 1995) but not in others (Lee, E.S. *et al*., 2001). EV-HPV DNA was detected in small copy numbers in 76% of non-genital seborrheic keratoses (Li *et al*., 2004). EV-HPV DNA and HPV 16 DNA were also detectable by PCR in lesions of a case of stucco keratosis (Stockfleth *et al*., 2000), a skin disorder with multiple warty lesions that show papillomatous acanthokeratosis on histopathology. In view of the small copy numbers and not infrequently multiple genotypes in one specimen, it remains doubtful that HPVs are causative factors (Li *et al*., 2004).

In two cases of Darier disease, which is characterized by crusted papules, plaques and verrucous lesions on nearly all parts of the body and histologically shows suprabasal lacunae and dyskeratosis, papillomatous proliferation and vacuolated keratinocytes in the upper stratum malpighii, HPV 5, 8, 36 and 38 from genus beta were detected by nested PCR whereas PCRs for mucosotropic HPV were negative (Li, Y.H. *et al*., 2002).

In psoriasis, low levels of EV-HPV DNA can be detected in up to 90% of lesions and skin scrapings (Favre *et al*., 1998; Weissenborn *et al*., 1999; Mahé *et al*., 2003). The significantly higher prevalence of antibodies against capsid proteins of HPV 5 and 8 in patients with psoriasis compared with healthy donors (Favre *et al*., 1998; Stark *et al*., 1998) points to increased levels of productive infection in this extensive epidermal proliferation that is mediated by T-cell activation. It has been speculated that EV-HPV may contribute to the pathogenesis of psoriasis through enhancement of epidermal proliferation by early proteins and stimulation of T lymphocytes with the late, structural proteins (Majewski & Jablonska, 2003).

(*b*) *Non-melanoma skin cancer*

Non-melanoma skin cancer refers to basal-cell and squamous-cell carcinoma and includes the precancerous lesions, actinic keratoses and Bowen disease. Actinic keratosis is in essence a cutaneous counterpart of SIL in the genital mucosa (Fu & Cockerell, 2003). Keratoacanthoma, a common cutaneous lesion that broadly resembles a squamous-cell carcinoma, displays benign biological behaviour.

In about half of the patients with EV, premalignant actinic keratosis and squamous-cell carcinoma arise in the lesions of this disease, mainly on parts of the body that are exposed to the sun, more than 25–30 years after its onset. The carcinomas are locally destructive but their invasive and metastatic potential is very low (Majewski *et al*., 1997). The cytopathic effect of EV-HPV is already absent by the onset of actinic keratosis. Some carcinomas in EV patients are typical basaliomas.

In immunosuppressed transplant patients, both the clinical and histopathological features of non-melanoma skin cancer differ. Such patients have an up to 100-fold increased risk for squamous-cell carcinoma and a 10-fold increased risk for basal-cell carcinoma. It is

not possible to distinguish reliably between keratoacanthoma and squamous-cell carcinoma in transplant recipients and, for management and classification purposes, they are referred to collectively as squamous-cell carcinomas. Similarly, actinic keratoses, intraepidermal carcinoma and Bowen disease in transplant recipients are not distinct entities and, since they are all thought to be dysplastic precancerous lesions, are referred to collectively as verrucous keratoses (Blessing *et al*., 1989). Squamous-cell carcinomas appear to arise from these verrucous lesions, which contain multinucleated cells and large numbers of atypical mitoses, koilocytes and parakeratotic peaks (Price *et al*., 1988; Blessing *et al*., 1989; Glover *et al*., 1995). These histopathological features have been cited to support a putative role of HPV in these lesions.

Non-melanoma skin cancers of EV patients were consistently found to harbour large numbers of copies of extrachromosomal HPV DNA (EV-HPV types 5, 8, 17, 20 or 47) (Orth, 1987). In non-EV patients, highly sensitive detection techniques, such as nested PCR, are necessary to identify mostly EV-HPV DNA in up to 85% of actinic keratoses (Pfister *et al*., 2003), in 25–55% of basal- and squamous-cell carcinomas of immunocompetent individuals and in up to 90% of squamous-cell carcinomas in organ transplant recipients (reviewed in Harwood & Proby, 2002; Pfister, 2003; Harwood *et al.*, 2004). A diverse spectrum of HPV types was detected and no single type predominated. Infections with several types were frequently noted in immunosuppressed patients. The small amounts of HPV DNA in skin cancers of non-EV patients suggest that only a minority of the tumour cells contain HPV DNA. In quantitative PCR studies, copy numbers varied from 50 HPV DNA copies per cell to 1 copy per 14 000 cells, with a median of 1 copy per 324 cells. In-situ hybridization identified only a few HPV DNA-positive nuclei per section (Weissenborn *et al*., 2005). An exception to this picture is skin carcinomas on the fingers, which appear to be strongly associated with genital HPV types (mostly HPV 16) (Alam *et al*., 2003). HPV 16 transcripts have also been detected in these carcinomas (Sanchez-Lanier *et al*., 1994). The rate of recurrence of HPV-associated digital squamous-cell carcinomas after surgical treatment greatly exceeds that for cutaneous cancer in general (Alam *et al*., 2003).

1.6 Non-malignant clinical lesions (other than precursors of cancer) of established HPV etiology

Genital HPVs cause condylomata, laryngeal papillomas and some papillomas at other mucosal sites, e.g. the oral or sinonasal cavity and conjunctiva. Cutaneous HPV types and EV HPV types cause skin lesions. HPVs have been reported to be associated with many other conditions, but the significance of these observations is as yet unclear (Shah & Howley, 1996). This section addresses only benign conditions that are clearly associated with HPV.

1.6.1 *Anogenital area*

The terms condyloma acuminatum and genital wart are synonyms. For many years, exophytic warts were the only recognized HPV-associated manifestations of HPV infection in the genital tract. Increasing attention to the lower female genital tract with the extensive use of acetic acid, colposcopy, histology and molecular analysis revealed the presence of a spectrum of manifestations of anogenital HPV infection. Flat lesions, also called flat warts, are the most commonly reported manifestation of HPV infection that is not clinically overt. Flat warts are not easily seen by the naked eye, but application of acetic acid opacifies the thickened epithelium in contrast to the surrounding normal skin or mucosa and makes them visible, particularly under a magnifying glass or through the colposcope. Flat lesions can be found in most areas that exhibit exophytic warts. It has been estimated that flat lesions are at least twice as common as exophytic warts in the anogenital region (Koutsky *et al.*, 1988; Beutner *et al.*, 1998a; Wiley *et al.*, 2002). Flat lesions frequently cluster in multiple lesions that are often confluent. Most probably, there is a continuum between normal skin or mucosa with detectable HPV DNA (i.e. latent infection) and overt anogenital warts that are clinically evident.

Estimates of the prevalence of condylomata vary from 0.24 to 13% depending mainly on the risk of sexually transmitted diseases and age distribution in the population examined (Kjaer & Lynge, 1989). The prevalence in patients at clinics for sexually transmitted disease was 11% compared with 2% in college students and was highest in the group aged 16–24 years (Kiviat *et al*., 1989). Positivity for HPV DNA, which may reflect subclinical disease, was more than twice as common as clinical disease in 377 first attendees at such a clinic; 15% had genital warts, compared with 35% who were positive for HPV by ViraPap/ViraType™ (Borg *et al*., 1993).

In women, the vulva, vestibule, vagina, perineum and perianal region are the most common sites for condylomata acuminata. HPV 6 and 11 were detected by southern blot hybridization in up to 95% of condylomata acuminata (Gissmann *et al*., 1982a; Johnson *et al*., 1991; Nuovo *et al*., 1991b).

Several studies have investigated the relationship between vulvar vestibulitis, vestibular papillomatosis and HPV infection (Growdon *et al.*, 1985; Moyal-Baracco *et al.*, 1990; Costa *et al.*, 1991; Umpierre *et al.*, 1991; Wilkinson *et al.*, 1993; Bornstein *et al.*, 1996, 1997; Origoni *et al.*, 1999; Morin *et al.*, 2000). However, conflicting results were reported, probably because of the different populations studied and the different techniques used to reveal vulvar HPV infection. The most recent reports seem to exclude a direct role for HPV in the genesis of vulvar pain syndromes, even if a co-causal role cannot be excluded. Studies that included healthy subjects for comparison with cases found that a high percentage of asymptomatic women harbour HPV DNA in the vulvo-vestibular area (Handsfield, 1997).

Condylomata acuminata are rarely detected on the uterine cervix. HPV 6 and 11 were identified in 65% and HPV 16 and 18 in 8% of these lesions by southern blot hybridization (Mitrani-Rosenbaum *et al*., 1988). Cervical condylomata may be hyperkeratotic

and are sometimes confused with cancer owing to a bizarre pattern of vessels (Coppleson, 1991). The major capsid protein, L1, is detected more frequently and in greater quantities in condylomata acuminata of the uterine cervix than in similar lesions of the penis or the vulva (35% compared with 12% in a total of 95 cases), which indicates a higher content of virus particles (Wools *et al*., 1994).

Genital warts are rarely observed in children. In addition to HPV 6 and 11, HPV 2 has also been detected in children and the route of transmission is through either the hands or auto-inoculation since all children with HPV 2-positive condylomata also had common cutaneous warts (Obalek *et al.*, 1993).

In men, penile and urethral condylomata show a distribution of HPV types similar to that of genital warts in women. In a series of 108 male patients, condylomata were located on the penile shaft in 51%, on the shaft and perianal region in 14%, on the shaft and scrotum in 2%, on the shaft and urethral meatus in 15% and on the urethral meatus alone in 18% (Rosemberg, 1991). Several authors have described the papular and macular aspects of the lesions (Barrasso *et al*., 1987; Del Mistro *et al*., 1987; Labropoulou *et al*., 1994).

Recently, Bleeker *et al.* (2003) classified penile lesions into condylomata acuminata, papular lesions and flat lesions. Flat lesions are associated with mainly high-risk types of HPV and high viral loads, and form the reservoir of HPV in men (Bleeker *et al*., 2003, 2005a). While this information is very helpful to study viral transmission and spread between individuals, from a clinical viewpoint, routine use of 3% acetic acid, HPV typing or histology are unnecessary because these lesions do not necessitate cytodestructive treatment.

Anal condyloma is one of the most common diseases of the anal canal and perianal region (for a review, see Vukasin, 2002). Together with AIN, anal condyloma is one of the primary clinical manifestations of HPV infection in the anal canal and on the perianal skin. It is usually found in conjunction with HPV 6 or 11, but HPV types known to be associated with anal cancer, such as HPV 16 or 18 (Syrjanen *et al.*, 1987a; Bradshaw *et al.*, 1992; Soler *et al.*, 1992; Caruso & Valentini, 1999), or very rarely cutaneous HPV types may also be found (Soler *et al.*, 1992; Strand *et al.*, 1999).

Typical perianal condylomata have a papillary appearance and may be highly keratotic, may be single or multiple and may be discrete or become confluent. Lesions may be asymptomatic or may be associated with burning or itching. Condylomata in the perianal region may also be flat and hyperpigmented, although a biopsy should be obtained in the latter case to exclude high-grade AIN. Bushke-Löwenstein tumours, also known as giant condylomata, may also occur in the perianal region. These usually contain HPV 6 or 11 but may also harbour carcinogenic HPV types such as HPV 16 (Kibrité *et al.*, 1997; for a review, see Trombetta & Place, 2001). Anal condylomata are often seen inside the anal canal, where they may be associated with spontaneous bleeding or bleeding with bowel movements or anal intercourse. Inside the anal canal, the lesions may be papillary or flat.

The manifestations and natural history of anal warts may differ between HIV-positive and HIV-negative patients. In HIV-negative patients, anal condylomata, typically associated with HPV 6 or 11, rarely progress to cancer although this has been documented in

a few cases (Metcalf & Dean, 1995). However, the proportion of patients with anal condyloma who also have high-grade AIN is greater in HIV-positive patients than in HIV-negative patients (Anderson *et al.*, 2004), and progression from low-grade lesions is more common in HIV-positive than in HIV-negative patients (Palefsky *et al.*, 1998a,b; Anderson *et al.*, 2004). Progression from anal condyloma to invasive anal cancer, particularly in immunosuppressed patients, has also been reported (Byars *et al.*, 2001).

1.6.2 *Upper respiratory tract*

Recurrent respiratory papillomatosis is a relatively rare disease caused by members of the HPV family (Gissmann *et al.*, 1982b; Mounts *et al*., 1982; Mounts & Kashima, 1984). HPV 11 is the most prevalent type (50–84%) found in laryngeal papillomas (Gissmann *et al*., 1983; Ushikai *et al*., 1994). When analysis is restricted to adult papillomas, HPV 16 is found most commonly (Corbitt *et al*., 1988). Although recurrent respiratory papillomatosis can be found anywhere in the aerodigestive tract, there appears to be a predilection for areas where there is a junction of squamous and ciliary epithelium. This includes the limen vestibuli (junction of the nasal vestibule and the nasal cavity proper), nasopharyngeal surface of the soft palate, mid-zone of the laryngeal surface of the epiglottis, upper and lower margins of the ventricle, undersurface of the vocal folds and the carina and bronchial spurs (Mounts & Kashima, 1984; Kashima *et al.*, 1992a,b). HPV is also detected in the normal mucosa adjacent to lesions. Recurrent respiratory papillomatosis has a worldwide distribution, although it is more prevalent in some countries and areas than in others (Shykhon *et al.*, 2002). It is a disease of both children and adults and exhibits a bimodal age distribution. The first peak occurs at less than 5 years of age and the second between the ages of 20 and 30 years (Kashima & Shah, 1982; Gissmann *et al.,* 1983; Irwin *et al.*, 1986), with incidences in the USA of 4.3 and 1.8 per 100 000, respectively (Shykhon *et al.*, 2002). Boys and girls appear to be nearly equally affected by juvenile-onset recurrent respiratory papillomatosis in contrast with adult-onset recurrent respiratory papillomatosis, which preferentially affects men over women at a ratio of approximately 3:2 (Kashima *et al.*, 1992b; Padyachee & Prescott, 1993; Doyle *et al.*, 1994). This difference reflects the different mode of acquisition: by vertical transmission for the juvenile form and by sexual contact for the adult form. Vertical transmission of juvenile-onset recurrent respiratory papillomatosis from an active or latent maternal anogenital HPV infection was first recognized in 1956; a later prospective study showed that 50% of infants born to mothers with cervical HPV during pregnancy carried HPV in their nasopharynx (Sedlacek *et al.*, 1989). It has been estimated that 10–25% of women of child-bearing age have evidence of latent or active HPV in cervical swabs and HPV DNA has been found in one-third to one-half of aerodigestive tract swabs of children born to affected mothers. However, only one in 400 infants delivered to these women is estimated to be at risk for subsequent recurrent respiratory papillomatosis (Bauman & Smith, 1996). In adults with recurrent respiratory papillomatosis, biopsies of normal mucosa adjacent to the papillomatosis were HPV DNA-positive in a majority of patients (Steinberg *et al*., 1983; Rihkanen *et al*., 1993, 1994).

Distal disease can develop and portends a poorer prognosis owing to its inaccessibility. HPV 11 is believed to have a greater propensity for distal pulmonary spread and a poorer prognosis for ultimate remission (Bauman & Smith, 1996). Distal bronchial obstruction can also result in post-obstructive pneumonia. Tracheal involvement occurs in 2–17% of patients without tracheostomies and appears as cobblestoning of the mucosa coupled with the presence of papillomas; more distal bronchopulmonary involvement is reported in 4–11% of children with long-standing disease (Shykhon *et al.*, 2002). Although recurrent respiratory papillomatosis is considered to be a benign condition, the disease may undergo malignant degeneration.

1.6.3 *Oral cavity*

Numerous HPV types (including subtypes 1, 2, 4, 6, 7, 11 and 13) have been detected in benign lesions of the oral cavity (Garlick & Taichman, 1991; Flaitz & Hicks, 1998).

Oral HPV-related benign verrucal-papillary lesions are clinically subdivided into verruca vulgaris, condyloma acuminatum, multiple and single papillomas and focal epithelial hyperpasia (Scully *et al*., 1985). Verruca vulgaris is induced by HPV 2 and 4. All 10 verrucae vulgares from the lip in one series were positive for HPV 2 DNA (Eversole *et al*., 1987a).

Condyloma acuminatum and oral squamous papillomas are associated with HPV 6 and 11. Studies have detected the HPV capsid immunohistochemically in 10 and 22% of oral condylomatous and hyperkeratotic papillomas, respectively (Madinier & Monteil, 1987). More sensitive techniques such as southern blotting, however, have detected HPV 6 and 11 DNA in up to 85% of cases (Eversole *et al*., 1987b). Patients with genital condyloma have a high incidence of HPV-induced oral lesions; up to 50% of individuals with widespread genital condyloma have oral condyloma acuminatum (Eversole *et al*., 1987b).

Of 202 cases of benign oral leukoplakia, 2.5% was positive for HPV 6 and 11 and 3.5% for HPV-16 by in-situ hybridization (Gassenmaier & Hornstein, 1988). One study on a gingival subset of oral proliferative verrucous leukoplakia, an oral lesion characterized as a solitary, recurring, progressive white patch that develops a verruciform architecture, showed no association with HPV (Fettig *et al.*, 2000).

HPV 13 (Pfister *et al.*, 1983a) and HPV 32 (Beaudenon *et al.*, 1987) are associated with focal epithelial hyperplasia of the oral mucosa (Heck disease), which is very rare in Europe and appears to be linked to certain ethnic groups, such as Inuits, native Americans, South African blacks (Cape coloureds) and individuals of Turkish or North African extraction. Clinically, the lesions are mostly flat and of the same colour as the surrounding mucosa, have a smooth surface and do not undergo malignant conversion. In 22 Mexican patients, human leukocyte antigen (HLA) DR4 (DRB1*0404) was significantly increased (odds ratio, 3.9; 95% CI, 1.86–8.03; $p < 0.001$); 17 of 20 patients (85%) were infected with HPV 13 (Garcia-Corona *et al.*, 2004).

1.6.4 *Conjunctiva*

Conjunctival papilloma is a benign and common tumour of the stratified squamous epithelium of the conjunctiva (Santos & Gómez-Leal, 1994). Conjunctival papillomas are known to occur in both children and adults, but they are most common among people aged 20–39 years (Sjö *et al.*, 2000) with a slight preponderance among men (60%). Conjunctival papillomas are positive for genital HPV types 6, 11 and 16, which have been identified by in-situ hybridization or PCR (Naghashfar *et al.*, 1986; Mäntyjärvi *et al.*, 1989; Saegusa *et al.*, 1995). The largest PCR-based study found 92% HPV DNA positivity; most of the 52 cases examined were HPV 6- or 11-positive and only one showed a multiple infection that included HPV 16 (Sjö *et al.*, 2001). Only one report investigated normal conjunctival tissue and found HPV 16 and 18 at a frequency of 32% (Karcioglu & Issa, 1997).

The access of HPV to the conjunctiva is still under investigation. Transmission to the conjunctiva may occur as a result of fetal passage through an infected birth canal or by ocular contact with contaminated hands or objects (Bailey & Guethlein, 1990). The presence of HPV 6 and 11 in adult conjunctival papillomas may reflect either activation of a latent HPV infection acquired at birth or an infection acquired later in life by transmission from other mucosal sites through either of the latter mechanisms (Naghashfar *et al.*, 1986; McDonnell *et al.*, 1987).

1.6.5 *Skin*

The skin of both healthy populations and immunosuppressed patients harbours a very large spectrum of HPV genotypes that includes EV-HPVs (Antonsson *et al.*, 2000).

Skin warts are clearly associated with HPV and are classified according to macroscopic and microscopic morphological criteria. Infection with specific HPV types can be broadly correlated with these lesions (Gross *et al.*, 1982; Jablonska *et al.*, 1997).

Typical common or mosaic warts, i.e. rough keratotic papules or nodules, on the hands, knuckles or periungual areas contain HPV 2, 4, 7, 26, 27, 28 or 29. Using PCR on specimens obtained from 111 immunocompetent patients, HPV 2a was found in 15% of the warts, HPV 2c in 24% (now known to be HPV 27; Chan *et al.*, 1994), HPV 57 in 12%, a variant of HPV 57 in 13% and HPV 4 only in one endophytic common hand wart (Rübben *et al.*, 1993). Mucosal HPV 35 was found once in a periungueal wart of a patient with HPV 35-positive Bowenoid papulosis of the anogenital area (Rüdlinger *et al.*, 1989).

Butchers warts have the clinical appearance of common warts but occur on the hands of those who work with raw meat, fish and poultry. Using southern blot hybridization among 60 butchers, HPV 1 was found in 6.7% of warts examined, HPV 2 in 45%, HPV 3 in 15%, HPV 4 in 10% and HPV 7 in 23% (Orth *et al.*, 1981). A similar distribution of HPV types was seen with PCR analysis; 23/26 lesions were positive for HPV DNA: 7.5% for HPV 2, 11.5% for HPV 4, 27% for HPV 7 and 42% for unidentified HPV types (possibly containing HPV 1 or 3) (Melchers *et al.*, 1993). In another series, HPV 7 was

found by PCR in 74/112 (66%) warts of men who worked in meat-processing plants (abattoir workers and butchers) (Keefe *et al*., 1994).

Filiform or papillomatous common warts that are found most frequently on the face, lips, eyelids or nares contain HPV 1, 2 or 7 (Jablonska *et al*., 1985; Egawa *et al*., 1993a). HPV 7 was found in two individuals with generalized or extensive facial warts with filiform appearance (de Villiers *et al*., 1986a).

Flat or plane warts, which can appear at different locations on the body and can form a linear arrangement (i.e. Koebner warts), are associated with HPV 2, 3, 10, 26, 27, 28, 29 or 41 (Melton & Rasmussen, 1991).

Deep plantar warts, i.e. hyperkeratotic plaques or nodules on the plantar surface of the foot, are usually positive for HPV 1 or 4 (Rübben *et al*., 1993). HPV-associated epidermal cysts of the sole of the feet from 32 Japanese patients contained HPV 60 (Kato & Ueno, 1992; Egawa *et al*., 1994). HPV 1 and 63 were present in the same nucleus of one plantar wart (Egawa *et al*., 1993b).

The morphological and virological findings of skin lesions in immunocompromised patients after transplants or in patients with EV are discussed in Section 2.7.

EV-HPVs such as HPV 5 or 36 are often detected in patients with burns, cutaneous auto-immune bullous diseases or psoriatic lesions in which epidermal repair processes are very active (Favre *et al.*, 1998a, 2000). Recently, it was suggested that these viruses are commensal in healthy individuals (Antonsson *et al.*, 2000).

1.7 Therapy and vaccination

1.7.1 *Therapy of benign disease*

(*a*) *Mucosal and cutaneous warts*

Warts are the clinical manifestation of a benign productive HPV infection that can be cleared spontaneously. However, cytoreductive treatment is generally indicated to help the immune system to clear the infection more quickly and is aimed at the removal of all visible clinical lesions. This can be accomplished by medical or surgical methods, none of which is capable of removing the virus. Since this is the causative agent of the disease, the possibility of transmission and recurrence is not eliminated.

Surgical methods for the treatment of genital and cutaneous warts include cryotherapy, electrodesiccation, surgical excision and laser–ablation techniques. Current therapies for HPV-related warts and neoplasia are summarized in Table 17 (modified from Zanotti & Belinson, 2002). An overview of the efficacy of different HPV treatment regimens is given in Table 18 (modified from Rivera & Tyring, 2004; for further reviews, see Jablonska, 1998; Gibbs *et al*., 2002; Torrelo, 2002; Gunter, 2003; Stanley, 2003; Bernard, 2004; Kodner & Nasraty, 2004; Fox & Tung, 2005).

Table 17. Current therapies for HPV-related warts and neoplasia

Cytotoxic agents	
Trichloroacetic acid	**For the destruction of genital warts** An 80–90% solution is applied directly to the wart in the clinic, and causes chemical destruction of wart epithelium. Treatment is repeated weekly. It is not absorbed systemically and can be used in pregnancy. It may cause burning of the surrounding skin.
Podophyllin	**For the destruction of genital warts** A plant compound that works by arresting cells in mitosis, it is applied weekly to warts at a concentration of 10–25% in a compound of tincture of benzoin in the clinic and should be washed off after 1–4 h. Applications should be less than 0.5 mL. This compound is absorbed in the systemic circulation and should not be used in pregnancy. Excessive exposure can cause bone-marrow depression.
Podofilox	**For the destruction of genital warts** A 0.5% solution is applied twice a day for 3 days, followed by 4 days without treatment. It is designed for self-application to reduce the number of clinic visits. Not to be used in pregnancy
5-Fluorouracil	**For the treatment of multifocal or extensive VIN or VAIN** An antimetabolite, it is applied as a 5% cream. A thin layer of cream is usually spread over lesions one to three times per week, but regimens may vary. It is designed for self-application. It causes tissue destruction by interfering with DNA and RNA synthesis and may cause significant local irritation. Not to be used in pregnancy.
Physical ablation	
Laser ablation	**For the destruction of extensive genital warts or treatment of multifocal or extensive VIN or VAIN** Carbon dioxide laser uses intense focal heat to vaporize tissue. This is a destructive method that does not permit pathological assessment of involved tissue. Usually, general anaesthesia is required. Postprocedural discomfort may be significant.
Excision	
Surgical excision	**For large exophytic condylomata or confluent VIN or VAIN** Surgical excision with re-approximation and closure using absorbable suture enables the pathological assessment of diseased tissue. Multifocal disease may not be amenable to this form of therapy. General anaesthesia is usually required. Postprocedural discomfort is generally less than that with laser treatment.
Loop electrode excision procedure	**Primarily used to excise CIN** It may also be used to excise genital warts or VIN or VAIN. The depth of excision may be difficult to control for vulvar and vaginal excision. It uses a radiofrequency alternating current passed along a thin wire loop to excise lesions with minimal thermal artefact.

Table 17 (contd)

Immuno-modulation	
Imiquimod	**For the treatment of genital warts** Recent evidence in small case series also suggests efficacy in VAIN and anal dysplasia (Davis *et al.*, 2000; Pehoushek & Smith, 2001). It modifies the immune response, is a potent inducer of IFN-α and enhances cell-mediated cytological activity against viral targets. Applied topically, it induces local production of IFN and other cytokines that can be important mediators of viral clearance. It is designed for self-application as primary or adjuvant therapy of genital warts and is not recommended for mucosal surfaces, such as the vagina. A 5% cream is applied to warts overnight three times per week for up to 16 weeks; this regimen has led to complete clearance of genital warts in more than 30–60% of patients (Beutner *et al.*, 1998b; Gollnick *et al.*, 2001). Mild to moderate local inflammation is the most common side-effect, but the drug is well tolerated; no systemic side-effects have been reported.
Interferons (IFNs)	These have both immunomodulatory and direct antiviral activity. Routes of administration include intralesional injection, topical and systemic; for recombinant IFN-α or -β, intralesional injections are given at the base of each wart three times a week for 3 weeks; topical creams have little reported success; intramuscular or subcutaneous administration of IFV-γ is associated with a 30–50% clearance rate (Kirby *et al.*, 1988; Bornstein *et al.*, 1997). Systemic adverse effects, such as flu-like symptoms and leukopenia, are substantial, even with intralesional use. Despite its marked promise, IFN has never been widely used for primary therapy of genital warts because it has to be given via injection and produces systemic side-effects.

From Zanotti & Belinson (2002)
CIN, cervical intraepithelial neoplasia; IFN, interferon; VAIN, vaginal intraepithelial neoplasia; VIN, vulvar intraepithelial neoplasia

(i) *Pharmacological therapies*

Pharmacologically induced cytodestruction of virus-infected tissue has been achieved by the application of a wide variety of chemicals: podophyllin resin, podophyllotoxin, organic acids, such as salicylic acid, trichloroacetic acid and bichloroacetic acid, and cytostatic agents, such as bleomycin, cidofovir and 5-fluorouracil. More recently, immunomodulating compounds with antiviral properties, such as interferon (IFN)-α and imiquimod, have demonstrated potential efficacy.

Cytodestructive drugs

Podophyllin resin and its purified derivative podophyllotoxin belong to the lignan family of natural products that have important antineoplastic and antiviral properties. These compounds destroy virus-associated lesions by inducing tissue necrosis. The mechanism by which podophyllotoxin blocks cell division is related to its inhibition of microtubule assembly in the mitotic apparatus that results in cell-cycle arrest at metaphase (Manso-Martinez, 1982).

Table 18. Efficacy of treatment regimens for HPV-related warts and neoplasia

Therapy (reference)	Type of application	Regimen	Maximum duration	Clearance	Recurrence[a]
Podophyllin resin (Edwards *et al*., 1988; Lacey *et al.*, 2003)	P	Once or twice weekly	6 weeks	30–60%	30–70%
Podophyllotoxin (Lacey *et al.*, 2003)	S	Three consecutive days alternating with 4 days of rest	6 weeks	45–75%	30–70%
Salicylic acid (Gibbs *et al.*, 2002; Rivera & Tyring, 2004; Fox & Tung, 2005)	S	Soak in water for 5 min and dry; file wart; the solution and gel are applied two to three times daily and allowed to dry; discs are applied and covered for 48 h before removal.	20 weeks	48–87%	Insufficient data
Tri- and bichloroacetic acid (Godley *et al.*, 1987; Menendez-Velazquez *et al.*, 1993; Fox & Tung, 2005)	P	Once weekly	'Several' weeks	60–81%	36%
5-Fluorouracil, topical (Pride, 1990)	S	Apply a thin layer one to three times each week and wash with soap and water after 3–10 h	6–8 weeks	47–68%	10–70%
5-Fluorouracil, intralesional (Swinehart *et al*., 1997a,b)	P	Injection once weekly	6 weeks	39–77%	58–70%
Bleomycin (Munn *et al*., 1996)	P	Intralesional injection; a variety of techniques available	4 injections	33–92%	Insufficient data
Cryotherapy (Jablonska, 1998; Rivera & Tyring, 2004; Fox & Tung, 2005)	P	Anaesthetic followed by freezing of the lesion and 1–2 mm of surrounding healthy tissue for 20–30 sec	6 weeks	50–96%	20–70%

Table 18 (contd)

Therapy (reference)	Type of application	Regimen	Maximum duration	Clearance	Recurrence[a]
Electrosurgery or laser (Bergman & Nalick, 1991; Ferenczy, 1991; Jablonska, 1998; von Krogh, 2001; Maw, 2004; Fox & Tung, 2005)	P	Carbon dioxide or Nd:YAG; exact regimen varies according to lesion.	3 weeks	≥ 90%	6–51%
IFN, intralesional (Friedman-Kien, 1995; Syed *et al*., 1995; Monsonego *et al*., 1996; Bornstein *et al*., 1997; Cox *et al.*, 2004)	P	Two to three times weekly	8 weeks	36–75%	0–32%
Imiquimod (Beutner *et al*., 1998b,c; Edwards *et al*., 1998; Moore *et al.*, 2001; Hengge & Cusini, 2003)	S	Three times weekly for 6–10 h	16 weeks	37–50%	13–19%

IFN, interferon; Nd:YAG, neodymium/yttrium/aluminium garnet laser; P, applied by physician; S, self-applied by patient
[a] Variable follow-up

A 0.5% solution of podophyllotoxin (podophilox) applied topically reduced the mean number of anogenital warts from 6.3 to 1.1, destroyed about 70% of all warts and totally cleared warts in 29–50% of patients (Bonnez *et al*., 1994). In a comparative study, a 0.5% podophyllotoxin lotion totally cleared 81% of warts compared with a 61% clearance by 25% podophyllin ($p < 0.001$) (Kinghorn *et al*., 1993). In spite of this potency, the use of these products is no longer recommended because they engender a large variety of adverse effects and recurrence rates of up to 65% (Wiley *et al*., 2002). In addition, podophyllin and its derivatives are teratogens and should not be used in pregnant patients (von Krogh & Longstaff, 2001).

Salicylic acid, in the form of a solution, a gel or a disc soaked with solution, is commonly used for the treatment of non-genital warts in adults and children with clearance rates of up to 75% (Rivera & Tyring, 2004). Other keratolytic compounds, such as glycolic acid, pyruvic acid, formic acid and glutaraldehyde, have also been used, particularly for the treatment of viral warts in children (reviewed by Torrelo, 2002). Pooled data from six placebo-controlled trials, in which 15–60% salicylic acid was used to treat cutaneous warts, showed a cure rate of 75% (144/191) in cases and 48% (89/185) in controls (odds ratio, 3.9; 95% CI, 2.4–6.4) (reviewed by Gibbs *et al*., 2002).

Trichloroacetic acid and bichloroacetic acid have been used as an alternative to podophyllin. These compounds induce a massive coagulation of proteins, which results in destruction of the wart. They are applied topically as 50–85% solutions and can be self-administered (Godley *et al*., 1987). In a more recent study, the clinical cure rate of an 85% solution of trichloroacetic acid in pregnant patients with cervical condylomata was 83% (Menendez Velazquez *et al.*, 1993). However, trichloroacetic acid must be applied with extreme care in order to prevent acid burn to the surrounding skin (Fox & Tung, 2005).

Bleomycin is a chemotherapeutic drug that interferes with DNA synthesis and causes necrosis of lesions. It is usually given by subdermal injection, but lateral injection, topical application and pricking with a bifurcated needle have also been used. Clearance rates of 33–92% have been reported; in particular, the multipuncture method has resulted in clearance rates of over 90% (Munn *et al*., 1996).

5-Fluorouracil is not known to have a specific molecular target in the HPV life cycle, but has been reported to be effective against genital HPV precursor lesions (Krebs, 1991; Syed *et al*., 2000). Reported clearance rates are 39–77%, but recurrence rates can be as high as 58% at 3 months and 70% at 6 months after treatment (Swinehart *et al*., 1997a,b). 5-Fluorouracil is contra-indicated in pregnant women.

Immunomodulating agents

In contrast to surgical and cytodestructive therapies of cutaneous and genital warts, the goal of recently developed treatments with antiviral and immunomodulating agents is not simply to remove the lesion, but also to reduce the amount of latent and subclinical viral infection sufficiently in order to diminish the rate of recurrence. This is achieved by mobilizing the so-called ‘innate immunity’, which recognizes stress signals and activates adaptive immunity in a targeted, appropriate and effective response. Pharmacological

agents that modulate the function of dendritic cells and macrophages could play a role in this process and, therefore, could have important therapeutic value.

All IFNs have anti-HPV activity, although the specific interferon response-mediator, double-stranded RNA, is not known to occur in the HPV life cycle. Partial and total remission of laryngeal papillomas as well as cutaneous and anogenital warts have been achieved with topical, intralesional and systemic administration of IFN. Combined therapies, such as surgery in combination with IFN or podophyllin in conjunction with IFN α-n1, were proposed as the most efficacious therapies (Weck *et al*., 1986). The antiviral effects of IFN on infected cells within the lesion do not cause damage to the surrounding tissue. In general, treatment with intralesional IFN-α appears to be equally as effective as traditional therapies, and it may be particularly useful in the treatment of lesions that have failed to respond to other modalities (Browder *et al*., 1992). Although IFN-α has been approved by the Federal Drug Administration for clinical treatment of genital warts in the USA, it is not generally recommended due to dose-limiting side-effects (Wiley *et al*., 2002).

Imidazoquinolines induce immunomodulating cytokines, partly through the activation of Toll-like receptors (TLRs)[1]. The imidazoquinoline, imiquimod, and its homologues activate macrophages and other cells and thus induce secretion of pro-inflammatory cytokines — predominantly IFN-α in plasmacytoid dendritic cells, and tumour necrosis factor (TNF) α and interleukin (IL)-12 in myeloid dendritic cells. These locally generated cytokines induce a Th1 cell-mediated immune response and the production of cytotoxic effectors (Stanley, 2002). Imiquimod directly enhances the immune response to HPV and thereby reduces the viral load. The compound was the first imidazoquinoline to be used for the treatment of anogenital warts and approved by the Federal Drug Administration in the USA: application of imiquimod cream (5%) three times a week overnight for up to 16 weeks is effective and safe, and the recurrence rate is low (Cox *et al*., 2004).

(ii) *Surgical treatments*

The most frequently used surgical therapies for the treatment of HPV-related mucocutaneous lesions include cryotherapy, laser surgery, electrodesiccation/fulguration and surgical excision. These treatments are generally equivalent in terms of clearance rates of the warts but are associated with high rates of recurrence (Maw, 2004). In early studies, cryotherapy or carbon dioxide laser therapy led to the complete cure of genital warts after several sessions in the majority of patients (Rosemberg, 1991).

Cryotherapy

Cryotherapy destroys warts by freezing the tissue. Results show that cryosurgery of HPV lesions is only moderately traumatic and gives good aesthetic and functional results

[1] Named after the Toll pathway in *Drosophila melanogaster*, which controls resistance to fungal and gram-positive bacterial infections (Hoffmann & Reichart, 2002; Janssens & Beyaert, 2003)

(Kourounis *et al*., 1999). In addition, large lesions may be treated and the depth of cryonecrosis is more suitably adapted (Scala *et al*., 2002).

Laser surgery

The carbon dioxide laser is a high-precision, non-blood-letting light scalpel used for the incision and excision of tissues and to seal small blood vessels. Healing occurs by granulation and the post-operative period is relatively painless for the patient. The risk for post-operative morbidity and complications is low (Bar-Am *et al*., 1993). Hyperthermia induced by a neodymium:yttrium–aluminium garnet (Nd:YAG) laser or a 585-nm pulsed dye laser has been used for the treatment of condylomata (Volz *et al*., 1994; El-Tonsy *et al.*, 1999; Kenton-Smith & Tan, 1999).

Photodynamic therapy

Photodynamic therapy with topical application of amino-laevulinic acid followed by irradiation with light of different wavelengths has been used for some time for the treatment of superficial premalignant and malignant skin tumours (reviewed in Roberts & Cairnduff, 1995). This therapy was later shown to be effective against recalcitrant warts (Stender *et al*., 1999).

Surgical excision

Scissors or a scalpel can be used to excise genital warts. Superficial scissor excision is useful when only a few lesions are present. Extensive intra-anal warts are most conveniently removed under general anaesthesia by a proctologist. General anaesthesia may also be preferred for surgical procedures for children and sensitive patients with extensive warts on the vulvo-anal area (von Krogh, 2001).

(*b*) *Recurrent respiratory papillomatosis*

HPV infections of the mother can be transmitted to the respiratory tract of the newborn child, which may result in juvenile-onset recurrent respiratory papillomatosis, the most common benign neoplasm of the larynx in children (Kimberlin, 2004; see Section 1.6.2). The risk factors for this vertical transmission have not been well identified. The role of caesarean section in preventing the transmission of HPV-associated disease from mother to child may be limited, as infection via amniotic fluid has also been reported to occur (Kosko & Derkay, 1996; Bandyopadhyay *et al*., 2003).

Although their histology is benign, the epithelial proliferations observed in respiratory papillomatosis may result in progressive hoarseness, stridor (the sound produced by turbulent flow of air through a narrowed segment of the respiratory tract, which is a sign of airway obstruction in a child), obstruction of the airways and respiratory distress. In addition, the papillomas are characterized by multiple recurrences despite surgical removal. Additional treatments to contain the virus and growth of the papillomas include cidofovir, indole-3-carbinol, di-indolylmethane, IFN and photodynamic therapy. However, no single modality of treatment seems to be effective in eradicating this disease (Auborn, 2002).

Radiotherapy is not recommended because it can cause malignant transformation of laryngeal warts.

(i) *Pharmacological therapies*

Cidofovir

Cidofovir is an acyclic nucleoside phosphonate that has been identified as an antiviral drug that specifically inhibits viral DNA polymerases, but does not affect cellular enzymes. The strong activity of cidofovir against HPV lesions (Stragier *et al*., 2002) is unexpected, because the virus does not encode polymerase and the anti-HPV function of the drug apparently depends on other activities. A phase II trial revealed a clearance rate for HPV of 47% with minimal adverse reactions (Snoeck *et al*., 2001). Cidofovir is approved for intralesional application in laryngeal papillomas (Coulombeau *et al*., 2002). Nephrotoxicity is the dose-limiting side-effect for cidofovir when it is used intravenously (5 mg/kg) (De Clercq, 2003).

(ii) *Surgical treatments*

Surgery remains the first choice for the treatment of recurrent respiratory papillomatosis. The main goals of surgical resection are to assure an adequate airway, to improve the voice and to facilitate remission of disease while reducing morbidity. Traditionally, cryosurgery, suction diathermy and ultrasonography have been used. At present, surgical procedures that use cold steel, carbon dioxide laser and a laryngeal shaver blade are the most common (Shykhon *et al*., 2002).

Cold-steel surgery

The use of traditional surgical tools ('cold steel') to remove papillomas from the vocal cords is still preferred over the laser technique in some cases, because the latter burns healthy tissue and creates a vapour plume that may cause viral infection in the trachea or lungs. In contrast, cold-steel surgery causes loss of blood and infected tissue, which may contaminate the lower airways (Shykhon *et al*., 2002). A relatively novel device used in the surgical removal of papillomas is the powered laryngeal shaver blade, which is claimed to be safer and more accurate than traditional tools, and only causes injury to the superficial mucosa (Shykhon *et al.*, 2002).

Carbon dioxide laser vaporization

Carbon dioxide laser vaporization is widely used to treat recurrent respiratory papillomatosis. Care must be taken to avoid airway fire (Varcoe *et al*., 2004) and to protect medical personnel, as viral particles are released in the laser plume (Ferenczy *et al*., 1990; Calero & Brusis, 2003).

Nd:YAG laser

Besides surgical resection and the established carbon dioxide laser treatment, laser surgery by the use of a fibre-guided Nd:YAG laser light promises to be an effective and only minimally traumatic treatment for recurrent respiratory papillomatosis. A novel

fibre-guidance instrument was developed for endolaryngeal laser surgery of this disease. Five patients (aged 4–8 years) were treated with fibre-guided Nd:YAG continuous-wave laser light (wavelength, 1064 nm; power, 10 W; irradiance, 3.5 kW/cm^2). By 12 months after treatment, all patients showed regression of the disease. Nd:YAG laser surgery seems to prevent a rapid recurrence of juvenile respiratory papillomatosis (Janda *et al.*, 2004)

Photodynamic therapy

Photodynamic therapy of recurrent respiratory papillomatosis involves administration to the patient of a photosensitizing agent that concentrates in rapidly growing tissues. The lesions are then excised with a tuneable laser, which preferentially destroys the cells that accumulated the dye. The technique does not eradicate the virus, but may reduce the growth rate of the papillomas by 50% and may be particularly useful for the treatment of endobronchial lesions. The main side effect is photosensitivity, which lasts for weeks to months, and has sometimes led to hospitalization for cutaneous burns (Shykhon *et al.*, 2002).

1.7.2 *Therapy of precancerous lesions*

(*a*) *Therapy of CIN*

Treatment of pre-invasive disease of the cervix is based on local control and prevention of progression. When abnormal cells are detected in a cervical smear, a thorough evaluation includes colposcopy to detect the lesions, direct biopsy and removal of the lesion, where appropriate, with minimal associated morbidity. However, since cervical precancer is an HPV-induced disease, spontaneous regression is also possible.

(i) *Surgical techniques*

Two categories of treatment are available: destructive and excision techniques. The success rates for ablative or excisional techniques is > 90%. While precancer is cured in most of the treated patients, eradication of HPV from the genito-urinary tract is not always possible with currently available techniques (Cirisano, 1999); thus the possibility of persistence of the virus and recurrence of the disease remains.

Destructive techniques

Techniques that involve destruction of the whole atypical transformation zone can be applied only if strict criteria are employed to ensure that no evidence of an invasive cervical cancer lesion is present; a pretreatment biopsy is therefore mandatory. These techniques, which include carbon dioxide vaporization, cryotherapy, electrocauterization and cold (thermo) coagulation, all have success rates of approximately 90%. A meta-analysis found that there is very little difference between these techniques with regard to the success of treatment or the occurrence of complications (Cirisano, 1999).

Excision techniques

Excision techniques that involve surgical removal (followed by histological analysis) range from carbon dioxide laser excision to the cold-steel technique to the rare application of hysterectomy. However, the loop electrosurgical excision procedure (LEEP) or large loop electrosurgical excision of the transformation zone (LLETZ) using an electrosurgical unit are now the most common techniques. They must be performed after a comprehensive colposcopic examination and the intention is to remove the entire lesion (LEEP) or the whole transformation zone (LLETZ) with an adequate margin of normal squamous epithelium surrounding the abnormal area and with minimal artefactual damage (Prendiville, 2005).

(ii) *Pharmacological treatments*

Imiquimod, a non-specific modulator of immune response, has been used in limited trials to treat low-grade lesions. Results suggest a variable clinical response but with associated systemic side-effects (Diaz-Arrastia *et al.*, 2001).

HPV vaccines have been used to treat low-grade lesions in limited trials (see Section 1.7.4).

(iii) *Follow-up after treatment of CIN*

There is a well-recognized risk of recurrence of CIN and rarely of invasive cancer following both its ablative and excision treatment. Follow-up can be carried out by colposcopy, cytology or HPV DNA testing, or by a combination of any of these. Two large meta-analyses showed that the combination of cytology and HPV testing increased the sensitivity to detect persistent or recurrent CIN and the negative predictive value to identify women at little or no risk for persistence or recurrence. Cytology and colposcopy may still be needed in order to rule out false-positive and false-negative results (Paraskevaidis *et al*., 2004; Zielinski *et al.*, 2004).

(*b*) *Therapy of VIN*

Therapy of VIN is aimed at the removal of a cancer precursor lesion; however, treated patients are still at increased risk for developing invasive vulvar cancer and require long-term follow-up. Treatment modalities can be surgical or pharmacological; however, the real possibility of preventing invasive disease in patients affected by VIN by the use of extensive vulvar surgery is questioned, because relapses frequently occur and treatment-related sequelae associated with wide excisional therapy have a high psychological impact on the body image of the treated patients. However, surgery is still the preferred option in the therapy of VIN.

(i) *Surgical techniques*

The aims of the surgical approach are full histological assessment of the affected tissue combined with complete elimination of the precancerous lesion. Surgical therapies include excisional and destructive methods; excisional methods are preferred, since occult

invasion has been reported in more than 10% of cases with a pre-operative biopsy that showed VIN3. Cold-steel surgery, laser excision and laser evaporation are effective modes of treatment. The treatment can be frequently completed without hospitalization and only under local infiltration of anaesthetics. No substantial difference in the various techniques has been reported.

(ii) *Pharmacological treatments*

Topical treatment is attractive, since it can be applied directly by the patient and is easily monitored for efficacy. Unfortunately, study results have been disappointing, with only few responses and high rates of complication and recurrence. In addition, with this therapy, diagnosis has to rely on the biopsy only, with the risk that an early invasive lesion may be overlooked. Reported pharmacological treatments include 5-fluorouracil, topical bleomycin, IFN-α, cidofovir, photodynamic therapy and imiquimod.

Results on the treatment of VIN with imiquimod were first published in a report of four cases (Davis *et al.*, 2000). Several small series of patients with high response rates to imiquimod have been described since that time (Diaz-Arrastia *et al.*, 2001; Jayne & Kaufman, 2002; van Seters *et al.*, 2002). Another series of patients demonstrated a clinical improvement in only 27%. Local side-effects limited the frequency of application, which might explain this low response rate (Todd *et al.*, 2002).

(*c*) *Therapy of VAIN*

As the vagina connects the cervix and the vulva, treatment of VAIN is affected mainly by the presence of associated cervical or vulval lesions. VAIN can have different clinical presentations and treatment is tailored to the individual patient. The aim of the treatment is to remove the lesion; this can be accomplished by either pharmacological or surgical therapy, depending on the site and the size of the disease, the presence or absence of the cervix, and the age and clinical history of the patient. Pharmacological treatment includes cytostatic drugs, such as bleomycin and 5-fluorouracil, and immunomodulants, such as imiquimod. Surgical treatments include cold-steel surgery and carbon dioxide laser therapy; the latter is associated with minimal morbidity but has a low success rate with up to 50% of recurrences (Murta *et al.*, 2005). Endovaginal brachyradiotherapy is also used for VAIN3 lesions (Fine *et al.*, 1996).

1.7.3 *Therapy of invasive cancer*

(*a*) *Cervical cancer*

Although cervical cancer is preventable, once an invasive lesion occurs, it carries a substantial risk of death. The clinical stage of the disease at presentation is the single most important predictor of long-term survival (see FIGO Staging Classification for Cervical Cancer in Table 19). Recurrences more than 5 years after treatment are extremely rare. Hence, 5-year survival is a good indicator of a cure. When treated appropriately, 5-year survival exceeds 80% for patients with stage I disease, exceeds 70% for patients with

Table 19. FIGO staging classification for cervical cancer

Stage I

Stage I is carcinoma that is strictly confined to the cervix; extension to the uterine corpus should be disregarded. The diagnosis of both stages IA1 and IA2 should be based on microscopic examination of removed tissue, preferably a cone, which must include the entire lesion.

Stage IA: Invasive cancer identified only microscopically. Invasion is limited to measured stromal invasion with a maximum depth of 5 mm and no wider than 7 mm.

Stage IA1: Measured invasion of the stroma no greater than 3 mm in depth and no wider than 7 mm in diameter

Stage IA2: Measured invasion of stroma greater than 3 mm but no greater than 5 mm in depth and no wider than 7 mm in diameter

Stage IB: Clinical lesions confined to the cervix or preclinical lesions greater than stage IA. All gross lesions, even with superficial invasion, are stage IB cancers.

Stage IB1: Clinical lesions no greater than 4 cm in size

Stage IB2: Clinical lesions greater than 4 cm in size

Stage II

Stage II is carcinoma that extends beyond the cervix, but does not extend to the pelvic wall. The carcinoma involves the vagina, but not as far as the lower third.

Stage IIA: No obvious parametrial involvement; involvement of up to the upper two-thirds of the vagina.

Stage IIB: Obvious parametrial involvement, but not to the pelvic sidewall

Stage III

Stage III is carcinoma that has extended to the pelvic sidewall. On rectal examination, there is no cancer-free space between the tumour and the pelvic sidewall. The tumour involves the lower third of the vagina. All cases with hydronephrosis or a non-functioning kidney are Stage III cancers.

Stage IIIA: No extension to the pelvic sidewall, but involvement of the lower third of the vagina

Stage IIIB: Extension to the pelvic sidewall or hydronephrosis or non-functioning kidney

Stage IV

Stage IV is carcinoma that has extended beyond the true pelvis or has clinically involved the mucosa of the bladder and/or rectum.

Stage IVA: Spread of the tumour into adjacent pelvic organs

Stage IVB: Spread to distant organs

FIGO, International Federation of Gynaecology and Obstetrics
From Alliance for Cervical Cancer Prevention (2004)

stage IIA disease, is approximately 40–50% for patients with stage IIB and stage III disease and is less than 10% in patients with stage IV disease (Sankaranarayanan *et al.*, 1995; Yeole *et al.*, 1998; Alliance for Cervical Cancer Prevention, 2004).

Treatment of cervical cancer is mainly through radiotherapy; five recent studies have demonstrated that chemoradiation improves survival compared with radiotherapy alone; surgery alone or in association with radiotherapy can also be used in early-stage disease. Chemotherapy with platin compounds is used in combination with radiotherapy or surgery, or is used alone as palliation in advanced or recurrent disease (Ryu, 2002). Treatment options depending on the stage of cancer are described below and summarized in Table 20. The strengths and limitations of these treatment methods are listed in Table 21.

(i) *FIGO stage IA1*

Stage IA1 disease (depth of invasion, < 3 mm; width, < 7 mm) has a risk of metastasis to regional lymph nodes of 1.2% and with a death rate of less than 1% (Benedet & Anderson, 1996). When preservation of fertility is important, a cone biopsy may be considered as a therapeutic procedure provided that (*a*) the woman is available for long-term follow-up, (*b*) the cervix is amenable to cytological and colposcopic evaluation, (*c*) the margins of the cone biopsy are free of both intraepithelial and invasive changes and (*d*) there is no evidence of lymphatic or vascular invasion.

(ii) *FIGO stage IA2*

Stage IA2 (depth of invasion, 3–5 mm; width, < 7 mm) has a risk of metastasis to regional lymph nodes of nearly 8% and a mortality rate of 2.4% (Benedet & Anderson, 1996). The recommended treatment is modified radical hysterectomy and bilateral pelvic lymphadenectomy. If preservation of fertility is important, a large cone biopsy with nodal dissection or trachelectomy with nodal dissection (extraperitoneal or laparoscopic) may be considered (Dargent *et al.*, 2000; Shepherd *et al.*, 2001).

(iii) *FIGO stage IB*

Treatment strategies for stage IB invasive cancer include primary radiation therapy with external beam radiation and either high- or low-dose rate brachytherapy or primary surgery with radical hysterectomy and pelvic lymphadenectomy. Published observational data indicate a 5-year survival rate of 87–92% for either approach (Waggoner, 2003).

Stage IB1

The treatment of stage IB1 cervical cancer (tumour diameter of < 4 cm confined to the cervix) depends on the resources and type of oncology services available and on the age and general health of the woman. Dual treatments (surgery and radiotherapy) are more harmful, more expensive and associated with a higher rate of complications. Therefore, primary therapy should aim to use only one radical treatment — either surgery or radiation with or without concurrent chemotherapy; concurrent chemotherapy usually comprises treatment with cisplatin during external beam therapy. Five-year survival rates of 80–90%

Table 20. Options for the treatment of cervical cancer

Features	Radical surgery	Radiotherapy		Chemotherapy
		Intracavitary (brachytherapy)	External beam (teletherapy)	
Description	Major surgical procedure performed under general anesthesia that involves removal of cervix, uterus (with or without ovaries), parametrial tissue, upper part of the vagina, and lymph nodes in the pelvis. Requires careful dissection of both ureters.	Involves delivery of radiation using radioactive sources in special applicators placed in the cervical canal and vaginal fornices. Two types: low dose-rate, e.g. cesium-137 (treatment takes 1–3 days) and high dose-rate, e.g. iridium-192 (treatment takes a few minutes)	Involves delivery of a radiation beam to the cancer from an external source, i.e. the teletherapy machine. Telecobalt machines or linear accelerators can be used to deliver external beam radiotherapy.	The most common agents are cisplatin or carboplatin given as intravenous infusions.
Indication	Early stages (stage I and selected cases of stage IIA)	All stages, including palliative care	All stages, including palliative care	Advanced stages (in combination with radiotherapy) Palliative care Recurrent disease
Level of facility	Treatment for cancer is centralized and provided in tertiary-level facilities. Radical surgery is possible in some secondary-level hospitals.			

From Alliance for Cervical Cancer Prevention (2002)

Table 21. Strengths and limitations of methods of treatment of cervical cancer

Features	Radical surgery	Radiotherapy	Chemotherapy
Strengths	Surgery performed by skilled and experienced surgeons is effective in the treatment of early stage (stage I and selected stage IIA) disease. Allows preservation of ovaries in young women and avoids vaginal stenosis (narrowing). Limited capital investment is required for development of surgical services compared with radiotherapy services.	Used in the treatment of all stages of cervical cancer as well as other kinds of cancer (e.g. breast, head and neck). Effectiveness varies with the stage of the disease. Radiotherapy is the only realistic treatment once the disease has spread beyond stage IIA, when surgery is neither feasible nor effective. It is commonly used for less extensive tumours when surgical expertise is not available. Survival rates are equal to those of surgery in early-stage cancers. Suitable alternative option for women with early disease but at high risk for surgery. Mainly provided as an outpatient/ambulatory service.	Can be combined with radiotherapy for the management of locally advanced cancer. Can be used in the management of very advanced cervical cancer.
Limitations	The role of curative surgery diminishes in patients with cervical cancer that has spread beyond the cervix into the surrounding tissues. Requires skilled and experienced gynaecologists. Requires a stay in hospital (10–14 days). Complications include pelvic sepsis, pelvic thrombosis and post-operative pneumonia. Ureterovaginal or vesicovaginal fistula can occur as a post-operative complication in < 1% of patients.	Requires trained and skilled radiation oncologists, medical physicists and radiotherapy technicians to provide the treatment and to operate and maintain the equipment. Requires expensive equipment and supply of radioactive sources. Service contracts and spare parts are also necessary. If utilization is low, the cost per patient increases since the machine must be maintained and the radioactive source changed periodically, regardless of how many patients are treated. Requires a reliable power supply. Acute side-effects include radiation-induced inflammation of the rectum (proctitis) and urinary bladder (cystitis). Late complications, such as bowel obstruction and rectovaginal and vesicovaginal fistula formation, may occasionally occur. Low dose-rate brachytherapy requires an operating room and anaesthesia services to place the intrauterine catheter and vaginal ovoids. However, this machine can only be used to treat gynaecological cancers.	Requires trained and experienced medical oncologists. Chemotherapeutic agents are expensive, making them inaccessible and not widely available in many countries. Not effective as first-line treatment.

From Alliance for Cervical Cancer Prevention (2002)

following either radical surgery or radical radiation as primary therapy have generally been reported (Hopkins & Morley, 1991; Landoni *et al.*, 1997; Waggoner, 2003).

Stage IB2

For stage IB2 disease (tumour diameter of > 4 cm confined to the cervix), 5-year survival rates are reduced to approximately 65–75% (Hopkins & Morley, 1991; Sankaranarayanan *et al.*, 1995). Para-aortic nodes are commonly involved in this stage, as well as an increase in central and distant features associated with recurrence. Options for treatment include (*a*) primary chemoradiation therapy alone (Rose *et al.*, 1999), (*b*) primary radical hysterectomy with bilateral regional lymph node dissection, usually followed by radical adjuvant radiation (with or without concurrent chemotherapy) which is determined by pathological criteria such as disease-free margins, lymph–vascular space involvement and metastases to lymph nodes (Keys *et al.*, 1999) and (*c*) neo-adjuvant chemotherapy, followed by radical surgery as described above and the possible use of post-operative radiation (Sardi *et al.*, 1993).

(iv) *Advanced disease (FIGO stages II, III and IV)*

The standard treatment of advanced cervical cancer is primary radical radiation with a combination of external beam and intracavitary brachytherapy and concurrent chemo-radiation therapy (Keys *et al.*, 1999; Morris *et al.*, 1999; Rose *et al.*, 1999; Whitney *et al.*, 1999).

(v) *Recurrent disease*

Recurrent cervical cancer may be in the pelvis, at distant sites or both. The majority of recurrences occur within 2 years of diagnosis; the prognosis is poor and most patients die from the disease. Management of women with distant metastases and advanced recurrent cervical cancer requires the efforts of a multidisciplinary team, and includes palliative use of anticancer therapies (chemotherapy, radiation therapy for treatment of symptoms and surgery such as colostomy for relief of symptoms related to recto-vaginal fistulae), control of symptoms (pain, bleeding, discharge and symptoms related to specific metastases) and emotional, psychological and spiritual support of the patient and her family (Alliance for Cervical Cancer Prevention, 2004).

(*b*) *Vulvar cancer*

Invasive vulvar cancer has been treated surgically for many years. The standard radical operation consisted of radical vulvectomy with bilateral inguinofemoral lymphadenectomy. Over the last 20 years, treatment of this cancer has changed dramatically, with a progressive decrease in surgical aggressiveness and the introduction of more conservative and personalized surgery. The treatment has evolved from a single type of operation to a philosophy of individualization, conservation and restoration. Changes from the standard approach include limited resection of the primary tumour and inguinofemoral lymphadenectomy that is carried out by a separate groin incision to decrease the associated morbidity of more extensive surgery.

Vulvar surgery inevitably results in mutilation of the female genitalia and thus has a considerable psychological impact on the patient. Plastic surgery of the vulvar area is therefore more frequently used to cope with the problem of vulvar reconstruction and female body image.

The status of the lymph nodes is the most important prognostic factor in squamous-cell vulvar cancer and recurrence in an undissected groin invariably has a fatal outcome for the patient. Complete inguinofemoral lymphadenectomy is required in lesions with more than 1 mm depth of invasion (FIGO stage IB and higher). In primary tumours < 2 cm in diameter and with a depth of invasion ≤ 1 mm (FIGO stage A), dissection of groin nodes can be omitted.

Patients with negative nodes and lesions of < 8 cm in diameter have a good prognosis, with a 5-year survival rate of more than 80%. Conversely, metastasis to groin nodes carries a substantial risk for recurrence and death from the disease, and requires additional radiation treatment of the inguinopelvic areas.

Recently, a technique to determine the pathological status of early-stage vulvar cancer was introduced that limits lymphadenectomy to the sentinel nodes (De Cicco *et al.*, 2000; de Hullu *et al.*, 2000). The results of an ongoing multicentric observational study on the safety of this new surgical technique are awaited before the introduction of this conservative treatment into clinical practice.

Verrucous carcinoma of the vulva is an unusual variant of squamous-cell carcinoma that shows local malignancy. Treatment is based on wide local excision; since it rarely metastasizes to regional lymph nodes, the surgical step of inguinofemoral lymphadenectomy can be omitted. Radiation therapy is contra-indicated because it has been reported to render the tumour more aggressive and lead to the development of distant metastasis.

(*c*) *Vaginal cancer*

Primary vaginal squamous-cell cancer is a rare occurrence that comprises 1–2% of all gynaecological cancers. Radical radiotherapy is the main form of treatment, and includes external beam radiation and endovaginal brachytherapy; supplementation with concomitant chemotherapy with cisplatin is an option based on several factors that include the extent of the disease and the clinical condition of the patient. Radical surgery can be used in early lesions located in the upper third of the vagina; adjuvant radiation treatment is indicated in the presence of pathological risk factors for recurrence, such as positive pelvic lymph nodes or surgical margins close to the tumour. Pelvic exenteration is an option in selected primary or recurrent cases that are surgically suitable for such an extensive procedure (Berek *et al.*, 2005).

1.7.4 *Therapeutic vaccination*

Therapeutic vaccination would be the most obvious strategy, since host immunity plays an important role in viral clearance. Several kinds of vaccine strategies are currently under investigation.

The aim of therapeutic vaccines is to eradicate infected cells or reduce their number. Initial strategies were targeted to eliminate residual malignant cells in patients with cervical cancer, although the prevention of progression of HSIL, LSIL or even cytologically normal HPV-infected cells are all possible end-points. Therapeutic vaccines have also been used as an approach to eradicate genital warts. Once HPV infection has been established, it is improbable that antibodies play a role in the eradication of infected cells. Cytotoxic T lymphocytes (CTL) are the primary effectors of tumour eradication. Many strategies for the generation of CTL involve the stimulation of antigen-presenting cells (to process the tumour or viral antigens, and present them in the context of the MHC receptor) and adhesion of co-stimulatory molecules to produce anti-tumour lymphocytes. In many cases, HPV-associated tumours express only the E6 and E7 oncoproteins; thus, most efforts have focused on eliciting CTLs directed against E6 or E7. These viral proteins are also expressed throughout the epithelium that is undergoing lytic viral replication. It is not entirely certain, however, that these proteins are expressed in basal cells. Since basal cells are capable of proliferation, it is possible that only E1 and E2 are expressed to maintain the viral genome. CTLs that are reactive against the capsid antigens may play a role in reducing the extent of infection but would not be effective in targeting neoplastic cells. There is a considerable amount of literature on approaches that have been used to generate HPV-specific CTL and to kill tumours in preclinical models (Da Silva *et al.*, 2001b) but this is not reviewed here. Only agents that are currently being or will shortly be used in clinical trials are discussed.

Many groups have considered the use of HPV peptides because they are relatively inexpensive and are well tolerated. Much effort has been made to map HLA class I-restricted epitopes of HPV 16 and 18 E6 and E7 (Kast *et al.*, 1993; Beverley *et al.*, 1994) and clinical trials have been carried out on patients whose HLA genotype (usually A*0201) and HPV tumour type matched the viral peptide epitopes. In one trial with 15 HPV 16-positive, A*0201-positive cancer patients, no CTLs were detected nor was there evidence of clinical benefit (Ressing *et al.*, 2000). A similar trial with 19 cervical cancer patients used two E7-A*0201 epitope peptides and a helper peptide and showed little evidence of clinical improvement (Van Driel *et al.*, 1999). However, a similar approach was used in a trial with 18 women who had HSIL of the cervix or vulva: 10 mounted CTL responses to the E7 peptide and three of the 10 had a complete clinical response (Muderspach *et al.*, 2000).

Preclinical data have suggested that longer peptides that contain a helper T-cell epitope linked to the CTL epitope are more efficient at eliciting CTLs than the minimal epitope; this effect is enhanced further by mixing the peptide with a dendritic cell-activating adjuvant (Zwaveling *et al.*, 2002). Peptide vaccines are well tolerated and immunologists are making advances to understanding the mechanisms that result in robust generation of CTLs. The data suggest, however, that vaccination of peptides may be most efficient in individuals who have pre-invasive disease and are not immunocompromised (Steller, 2002).

An additional problem with the use of peptides is that the HLA genotype of the patient and the HPV genotype of the tumour must be known. This has prompted many investi-

gators to consider full-length E6 and/or E7 proteins, or fusion products with other proteins. One on-going trial is examining the safety and immunogenicity of an E6/E7 fusion protein in a saponin-based adjuvant among women with cervical HSIL (Steller, 2002). To increase the immunogenicity of the E7 protein, it has been fused to heat-shock proteins of *Mycobacterium tuberculosis* (hsp70) (Chen, C.H. *et al.*, 2000) or to hsp65 of Calmette-Guerin bacillus (Goldstone *et al.*, 2002). This fusion product has been used in an open-label trial to immunize men with anal HSIL, some of whom also had anogenital warts. Of 14 patients with warts, three had complete resolution of warts and 10 had a 70–95% reduction in the size of the warts.

A fusion protein of HPV 6 L2/E7 was developed for the treatment of genital warts. Twenty-seven subjects with genital warts were treated in an open-label trial (Lacey, C.J.N. *et al.*, 1999; Thompson *et al.*, 1999). All 27 developed L2 and/or E7 antibodies and 19/25 subjects tested had proliferative responses. By 8 weeks after vaccination, the warts of five subjects had completely cleared and the remaining subjects were offered conventional therapy. Of the 13 whose warts eventually cleared, none showed any recurrence. Similarly, an L2/E7 fusion protein of HPV 16 was designed for the treatment of anogenital dysplasia. In a trial in women with VIN/VAIN3, immunogenicity was demonstrated but no clinical response (Smyth *et al.*, 2004).

Preclinical studies have shown that dendritic cells play a critical role in antigen presentation *in vivo*. These cells can be loaded with peptide epitopes: when mixed with proteins, they engulf the protein and process fragments through the class I antigen presentation pathway. Dendritic cells can also be transfected or transduced by nucleic acids that encode the desired antigens. Several studies have shown that peptide- or protein-pulsed dendritic cells are much more effective in eliciting anti-tumour CTLs than peptides alone (Schoell *et al.*, 1999). In the context of HPV immunotherapy, monocytes were taken from the peripheral blood of cervical cancer patients and differentiated in culture using IL-4 and granulocyte macrophage colony-stimulating factor; the dendritic cells were mixed with a HLA-A*0201 E7 epitope and used to sensitize the autologous peripheral blood mononuclear cells from the cancer patients (Steller *et al.*, 1998; Santin *et al.*, 1999). A case report of a woman who had an adenocarcinoma that contained HPV 18 and who was treated over 10 months with dentritic cells that had been pulsed with HPV 18 E7 protein suggested that metastatic disease was inhibited for a period of time (Santin *et al.*, 2002). Other small clinical studies have also used autologous dendritic cells pulsed with peptides or proteins as immunogens (Adams *et al.*, 2001; Ferrara *et al.*, 2003). The use of dendritic cells will probably play an important role in future vaccine strategies.

In addition to being potent elicitors of antibodies, VLPs can also induce T-cell responses. Vaccination of subjects with HPV 16 VLPs was shown to induce both CD4+ and CD8+ T-cell responses (Pinto *et al.*, 2003). In a trial in men with genital warts, HPV 6 VLPs induced antibodies and a delayed-type hypersensitivity response with complete regression in 25/33 patients; however, no placebo group was included (Zhang *et al.*, 2000). To enhance their immunogenicity and, in particular, to stimulate a mucosal immune response, VLPs have been engineered to encapsidate a plasmid that expresses IL-2 (Oh, Y.K. *et al.*, 2004).

As discussed in Section 1.8, chimeric VLPs that contain a linked segment of E7 have been developed, and have been shown to induce specific HLA T cells in humans after in-vitro vaccination (Kaufmann *et al.*, 2001).

The use of viral vectors to introduce genes for vaccination is an effective way to stimulate many branches of the immune system. Recombinant vaccinia viruses, which have the advantage of being able to carry large inserts and not persisting in the host, have been widely used. The disadvantage of this method is that older individuals may have a pre-existing immunity to vaccinia virus which reduces the response; in addition, vaccinia virus may pose a risk to immunosuppressed recipients. A recombinant vaccinia virus that expresses the HPV 16 and 18 E6 plus E7 genes was created. In order to circumvent the potential problem of introducing oncogenes, the E6 and E7 proteins were mutated to block their binding to key tumour suppressors (Boursnell *et al.*, 1996). In an initial study, the vaccine was found to be safe when administered to nine patients with late-stage cervical cancer; as most of the patients were immunosuppressed, only one developed CTLs but she also had clinical remission (Borysiewicz *et al.*, 1996). In a more recent trial, 29 patients with stage IB or IIA cervical cancer were vaccinated (Kaufmann *et al.*, 2002). After a single vaccination, four patients developed CTLs and eight developed serological responses to the HPV proteins. Two recent studies have tested a single dose of TA-HPV, a recombinant vaccinia virus that encodes modified HPV 16 and 18 E6 and E7, in patients with VIN (Baldwin *et al.*, 2003; Davidson *et al.*, 2003). Davidson *et al.* (2003) vaccinated 18 women who had HPV 16-positive high-grade VIN with a single dose of TA-HPV, which resulted in a reduction in the size of the lesion by at least 50% in eight patients, and a further four patients showed significant relief of symptoms. A second vaccination formulation, HPV 16 L2E6E7 fusion protein, has been tested in 10 patients with high-grade VIN who had previously been primed with TA-HPV. All but one demonstrated HPV 16-specific proliferative T-cell and/or serological responses following vaccination. However, no direct correlation between immunological and clinical responses was seen (Davidson *et al.*, 2004). This approach is promising but emphasizes the difficulty of achieving immunotherapeutic responses in immunocompromised patients. Clinical trials of other viral delivery systems, including recombinant adenoviruses (Tobery *et al.*, 2003), adeno-associated virus (Liu *et al.*, 2000) and RNA-based poliovirus (van Kuppeveld *et al.*, 2002) and alphavirus (Velders *et al.*, 2001) vaccines, which have all been constructed to express E7 or poly-epitope proteins should begin soon.

DNA has emerged as an attractive candidate for a vaccine because it is inexpensive and does not require a cold chain. DNA uptake by antigen-presenting cells results in the expression of the encoded antigen, and induction of both antibodies and CTLs. In a phase I trial, a plasmid-encoding multiple HLA A2 epitope of HPV 16 E7 was encapsulated in biodegradable polymer microparticles. Twelve HLA- and HPV-matched subjects with anal HSIL were vaccinated: 10/12 exhibited an increased immune response and three showed partial histological responses (Klencke *et al.*, 2002). Enhancement of DNA vaccines by co-expression of cytokine genes such as granulocyte macrophage colony-stimulating factor has also been tested (Leachman *et al.*, 2000).

1.8 Prophylaxis[1]

The discovery that the major capsid protein L1 can assemble into VLPs that are structurally and immunogenically indistinguishable from authentic virions and studies aimed at the characterization of HPV conformational epitopes that induce neutralizing antibodies that can block new infection have had a considerable impact on the development of prophylactic vaccines (see Section 1.2). This section highlights some important innovations in prophylaxis that have occurred since the Working Group was convened, in 2005.

To date, two prophylactic vaccines have been developed and tested in large multicentric trials (Harper *et al.*, 2004; Villa *et al.*, 2005; Harper *et al.*, 2006; FUTURE II Study Group, 2007; Garland *et al.*, 2007). Both are based on the recombinant expression and self-assembly of the viral protein L1 into VLPs. The HPV VLPs contain no DNA and hence are non-infectious. Injection of the HPV VLPs elicits a strong and sustained type-specific response. One of the vaccines, Gardasil® (Merck & Co.), protects against HPV 6, 11, 16 and 18 (quadrivalent) and the other, Cervarix® (GlaxoSmithKline), protects against HPV 16 and 18 (bivalent). The expected outcome of prophylactic vaccination is a reduction in the incidence of HPV-related genital diseases, including cervical, penile, vulvar, vaginal and anal cancer and precancerous lesions. In addition, a reduction in the incidence of the genital warts has been observed among those who received the quadrivalent vaccine and a reduction in laryngeal papillomatosis can be anticipated among their children (Arbyn & Dillner, 2007).

Since 8 June 2006, the quadrivalent vaccine has been licensed for use in females 9–26 years of age in the USA by the Food and Drug Administration which recognized the indications of safe and strong protection against cervical cancer, genital warts, cervical adenocarcinoma *in situ*, CIN grades 1, 2 and 3 and VIN grades 2 and 3 that are caused by HPV 6, 11, 16 and 18 and stated that the vaccine is effective if administrated before HPV infection (Dillner *et al.*, 2007).

The Advisory Committee of Immunization Practices and the American Cancer Society recommend routine vaccination of girls aged 11–12 years, and the vaccine may be administered to girls as young as 9 years old. Vaccination is also recommended for girls and young women aged 13–26 years who have not been vaccinated previously (Markowitz *et al.*, 2007; Saslow *et al.*, 2007).

On 20 September 2006, the European Medicine Evaluation Agency officially authorized the marketing of the quadrivalent vaccine Gardasil® in the European Union. An application has also been made to this Agency for a licence for the bivalent vaccine, Cervarix®.

Although their high efficacy has clearly been shown, it is important to recognize the limitations of currently available vaccines and available data: (*a*) these vaccines do not protect against all high-risk HPV types; (*b*) they do not treat existing HPV infections; (*c*) the long-term duration of protection and the required length of protection to prevent cancer are unknown; [It should be noted, however, that follow-up of young women did not

[1] This section was updated by the IARC Secretariat after the Working Group meeting, and the text was reviewed by three members of the Working Group.

detect evidence of waning immunity over 5 years (Harper *et al.*, 2006; Villa *et al.*, 2006) and that the quadrivalent vaccine was shown to induce immune memory (Olsson *et al.*, 2007).] and (*d*) the cost of the primary vaccination, the recommended three-dose injection schedule and the possible need for additional booster vaccinations will probably limit the use of vaccine among medically underserved and uninsured populations. In addition, it will be important to evaluate the impact of the HPV VLP vaccines on other genital and non-genital HPV-associated tumours and in other populations such as individuals at high risk for anal cancer (e.g. men who have sex with men). Further, much research is needed to develop and evaluate alternative vaccine approaches to reduce the cost and expand the coverage of vaccination. It is also crucial to ensure the introduction and success of HPV vaccination programmes in developing countries (Saslow *et al.*, 2007).

Several additional approaches to prophylactic vaccines have been considered (for a review, see Breitburd & Coursaget, 1999; Schiller & Nardelli-Haegliger, 2006).

Neutralization epitopes are not only present on VLPs; advances in purifying bacterially expressed L1 proteins have shown that they can assemble into pentameric structures, such as capsomers that contain neutralizing epitopes (Li *et al.*, 1997).Vaccination of dogs with these capsomers in the canine oral papillomavirus model was fully protective (Yuan *et al.*, 2001). Capsomers may therefore offer a simplified, economical alternative to VLPs. Other approaches to provide low-cost systems that generate conformationally correct L1 protein include expression in plants, which can potentially lead to development of edible vaccines (Biemelt *et al.*, 2003; Warzecha *et al.*, 2003).

Immunization with naked DNA has the theoretical advantage of simple production. Naked DNA vaccination with L1 expression plasmids can induce antibody responses in animal models that are increased if codon-modified genes are used (Mossadegh *et al.*, 2004). Delivery of naked DNAs can be facilitated by their incorporation into recombinant viruses. Viral vectors could not only deliver the *L1* gene more efficiently but in many cases would be compatible with needle-free mucosal delivery. HPV 16 L1 recombinants of two DNA viruses, adenovirus 5 (Berg *et al.*, 2005) and adeno-associated virus (Kuck *et al.*, 2006), have been developed as candidate prophylactic vaccines. Several other attractive RNA viral vectors, including alphavirus vectors, are also currently under investigation (Vajdy *et al.*, 2004).

Live bacteria vaccines are potentially simple and inexpensive to manufacture, and can also be relatively inexpensive to deliver if administered mucosally. Four distinct L1 recombinant bacteria vaccines have been developed and tested for immunogenicity in animal models (Schiller & Nardelli-Haegliger, 2006). Among them, L1 recombinant clones of attenuated *Salmonella enterica* serovar Typhimurium and Typhi strains were shown to induce strong neutralizing antibody responses after a single intranasal or oral application in mice (Baud *et al.*, 2004). This was the case for the attenuated Ty21 strain Vivotif that expresses L1. This strain has an excellent safety record, based on its use as an oral vaccine to prevent typhoid fever in tens of millions of individuals worldwide. Therefore, this clone could potentially serve as a combined HPV/typhoid fever vaccine (Schiller & Nardelli-Haegliger, 2006).

The minor capsid structural viral protein L2 has been shown to elicit antibodies that neutralize both homologous and heterologous HPV types (Kawana *et al.*, 1999; Roden *et al.*, 2000). VLPs that consist of L1 proteins fused to L2 epitopes appear to be promising, since the presence of L2 conveys epitopes that cross-neutralize with a broad range of HPV types and was also shown to increase the yield of VLP production compared with L1-only VLPs (Slupetsky *et al.*, 2007).

In order to obtain combined prophylactic/therapeutic vaccines, ways to stimulate the cell-mediated immune response against viral non-structural proteins and neutralizing antibody production have been explored. The most advanced candidates for this type of vaccines are chimeric VLPs that incorporate peptides of early proteins as fusions of L1 or L2. To date, two chimeric VLPs have been tested in clinical trials: an HPV L1–E7 chimeric VLP that targets HPV 16-associated high-grade cervical dysplasia (Schäfer *et al.*, 1999; Schreckenberger & Kaufmann, 2004) and an HPV 16 L2–E6–E7 chimera with a potential to induce cross-neutralizing antibodies (de Jong *et al.*, 2002).

Taken together, there is a great hope for a reduction in the morbidity and mortality associated with HPV-related anogenital diseases in populations who receive the available prophylactic vaccines. The promising outcome of prophylactic vaccines from a broad public health perspective, however, can only be attained if vaccination can be achieved for those groups of women for whom access to cervical cancer screening services is most problematic. For these reasons, the development of second-generation vaccines that are expected to be cheaper, easy to deliver and/or to provide T-cell response to cure pre-existing HPV infections is highly desirable.

2. Studies of Cancer in Humans

2.1 Methodological concerns

(*a*) *Choice of disease end-point*

To obtain epidemiological evidence of the risk for cervical cancer due to a specific type of human papillomavirus (HPV), the choice of disease end-point must be appropriate. The risk for invasive cancer is examined optimally by a case–control design or among historical cohorts in which archived specimens are tested.

Prospective studies that follow women forward in time must ethically rely on surrogate end-points, the choice of which is critical. For studies of HPV infection, invasive cancer and grade 3 cervical intraepithelial neoplasia (CIN3; which subsumes diagnoses of severe dysplasia and carcinoma *in situ*) are considered to be the primary disease end-points. The inclusion of CIN3 as a surrogate for invasive cancer permits prospective studies that would otherwise be unethical, because it is a condition that often requires medical treatment, whith thus interrupts the natural history of the disease. CIN3 is the immediate precursor of invasive cervical cancer, and the two diseases share a similar