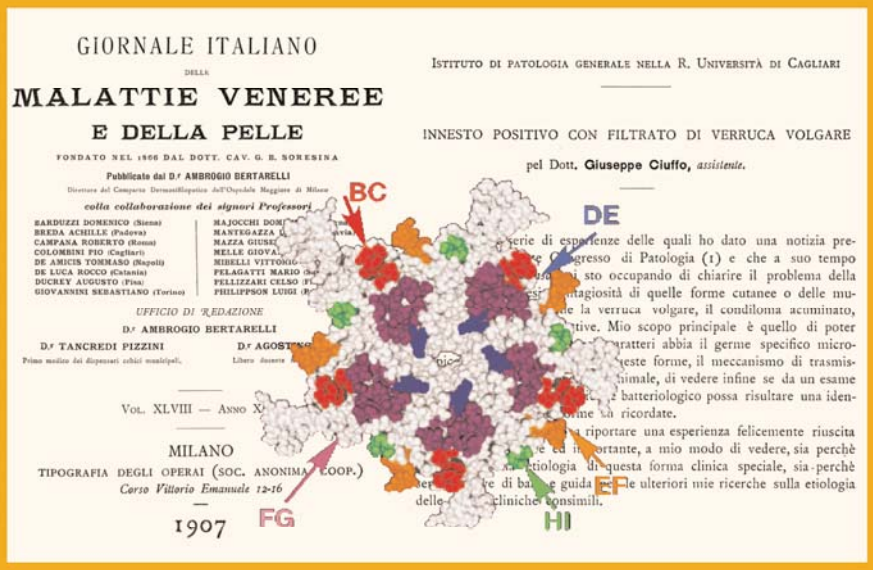
WORLD HEALTH ORGANIZATION
INTERNATIONAL AGENCY FOR RESEARCH ON CANCER

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans

VOLUME 90

Human Papillomaviruses



LYON, FRANCE
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IARC Monographs on the Evaluation of Carcinogenic Risks to Humans

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Human Papillomaviruses

This publication represents the views and expert opinions
of an IARC Working Group on the
Evaluation of Carcinogenic Risks to Humans,
which met in Lyon,

15–22 February 2005

2007

IARC MONOGRAPHS

In 1969, the International Agency for Research on Cancer (IARC) initiated a programme on the evaluation of the carcinogenic risk of chemicals to humans involving the production of critically evaluated monographs on individual chemicals. The programme was subsequently expanded to include evaluations of carcinogenic risks associated with exposures to complex mixtures, life-style factors and biological and physical agents, as well as those in specific occupations.

The objective of the programme is to elaborate and publish in the form of monographs critical reviews of data on carcinogenicity for agents to which humans are known to be exposed and on specific exposure situations; to evaluate these data in terms of human risk with the help of international working groups of experts in chemical carcinogenesis and related fields; and to indicate where additional research efforts are needed.

The lists of IARC evaluations are regularly updated and are available on the Internet at http://monographs.iarc.fr/.

This programme has been supported by Cooperative Agreement 5 UO1 CA33193 awarded since 1982 by the United States National Cancer Institute, Department of Health and Human Services. Additional support has been provided since 1986 by the European Commission, Directorate-General EMPL (Employment, and Social Affairs), Health, Safety and Hygiene at Work Unit, and since 1992 by the United States National Institute of Environmental Health Sciences.

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Cover Legend:

The cover shows the first page of a publication by Ciuffo (1907) who demonstrated — by auto-inoculation — that a cell-free extract of common warts contains an infectious agent, later to be identified as human papillomavirus (see text below).

Superimposed on this text is a molecular structural model of the HPV 6 major capsid protein L1, with surface-exposed loops that contain highly antigenic epitopes (Oroczo *et al*, 2005; reproduced with permission; see also Section 1.2.1). Virus-like particles containing these epitopes have now been successfully used to develop prophylactic vaccines against several high-risk HPVs.

[cover design: Georges Mollon]

Innesto positivo con filtrato di verruca volgare
Dr Giuseppe Ciuffo

Con una serie di esperienze delle quali ho dato una notizia preventiva nel recente Congresso di Patologia (1) e che a suo tempo pubblicherò *in extenso*, mi sto occupando di charire il problema della etiologia, patogenesi, contagiosità di quelle forme cutanee o delle mucose papillomatose, come la verruca volgare, il condiloma acuminato, che sono certamente infettive. Mio scopo principale è quello di poter trovare quale esso sia e quali caratteri abbia il germe specifico microscopico o il virus invisibile di queste forme, il meccanismo di trasmissibilità da uomo ad uomo o all'animale, di vedere infine se da un esame comparativo clinico, anatomico e batteriologico possa risultare una identità fra queste diverse forme su ricordate.

Per ora mi limiterò a riportare una esperienza felicemente riuscita sulla verruca volgare ed importante, a mio modo di vedere, sia perchè ci illumina sulla etiologia di questa forma clinica speciale, sia perchè servi e serve di base e guida per le ulteriori mie ricerche sulla etiologia delle forme cliniche consimili.

(1) Atti del IV Congresso Italiano di Patologia, Paris, Ottobre 1906

Positive graft with a filtrate of common warts
Dr Giuseppe Ciuffo

With a series of experiments, the results of which I presented at the recent Pathology Congress (1) and shall publish shortly *in extenso***,** I sought to clarify the problem of the etiology, pathogenicity and contagiousness of cutaneous or mucosal papillomatous forms, such as the common wart and condyloma acuminatum, which are assuredly infectious. My principal aim is to be able to find which is the specific microscopic germ or invisible virus that is responsible for these lesions and what are its characteristics, its mode of transmission from man to man or to animals and finally to determine whether a comparative clinical, anatomical and bacteriological examination could result in the identification of the responsible agent.

In this article, I am restricting myself to presenting an experiment on the common wart that was felicitously successful and important, the way I see it, not only because it sheds light on the etiology of the clinical form, but also because it has served and will serve as a basis and guide for my future investigations regarding the etiology of other clinically similar forms.

(1) Proceedings of the Fourth Italian Congress of Pathology, Paris, October 1906

CONTENTS

NOTE TO THE READER

The term ‘carcinogenic risk’ in the *IARC Monographs* series is taken to mean that an agent is capable of causing cancer under some circumstances. The *Monographs* evaluate cancer hazards, despite the historical presence of the word ‘risks’ in the title.

Inclusion of an agent in the *Monographs* does not imply that it is a carcinogen, only that the published data have been examined. Equally, the fact that an agent has not yet been evaluated in a monograph does not mean that it is not carcinogenic.

The evaluations of carcinogenic risk are made by international working groups of independent scientists and are qualitative in nature. No recommendation is given for regulation or legislation.

Anyone who is aware of published data that may alter the evaluation of the carcinogenic risk of an agent to humans is encouraged to make this information available to the Carcinogen Identification and Evaluation Group, International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France, in order that the agent may be considered for re-evaluation by a future Working Group.

Although every effort is made to prepare the monographs as accurately as possible, mistakes may occur. Readers are requested to communicate any errors to the Carcinogen Identification and Evaluation Group, so that corrections can be reported in future volumes.

IARC WORKING GROUP ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS: HUMAN PAPILLOMAVIRUSES

Lyon, 15–22 February 2005

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NOTE: Minor pertinent interests are not listed. These include consulting on non-regulatory matters totalling less than 2–5% of time and compensation, research grants totalling less than 5–10% of the research budget, occasional travel grants totalling less than 5–10% of time, and stock holdings valued at less than US$ 10 000 overall.

PREAMBLE

IARC MONOGRAPHS PROGRAMME ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS

PREAMBLE

1. BACKGROUND

In 1969, the International Agency for Research on Cancer (IARC) initiated a programme to evaluate the carcinogenic risk of chemicals to humans and to produce monographs on individual chemicals. The *Monographs* programme has since been expanded to include consideration of exposures to complex mixtures of chemicals (which occur, for example, in some occupations and as a result of human habits) and of exposures to other agents, such as radiation and viruses. With Supplement 6 (IARC, 1987a), the title of the series was modified from *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans* to *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, in order to reflect the widened scope of the programme.

The criteria established in 1971 to evaluate carcinogenic risk to humans were adopted by the working groups whose deliberations resulted in the first 16 volumes of the *IARC Monographs series*. Those criteria were subsequently updated by further ad-hoc working groups (IARC, 1977, 1978, 1979, 1982, 1983, 1987b, 1988, 1991a; Vainio *et al.*, 1992).

2. OBJECTIVE AND SCOPE

The objective of the programme is to prepare, with the help of international working groups of experts, and to publish in the form of monographs, critical reviews and evaluations of evidence on the carcinogenicity of a wide range of human exposures. The *Monographs* may also indicate where additional research efforts are needed.

The *Monographs* represent the first step in carcinogenic risk assessment, which involves examination of all relevant information in order to assess the strength of the available evidence that certain exposures could alter the incidence of cancer in humans. The second step is quantitative risk estimation. Detailed, quantitative evaluations of epidemiological data may be made in the *Monographs*, but without extrapolation beyond the range of the data available. Quantitative extrapolation from experimental data to the human situation is not undertaken.

The term ‘carcinogen’ is used in these monographs to denote an exposure that is capable of increasing the incidence of malignant neoplasms; the induction of benign neo-

plasms may in some circumstances (see p. 19) contribute to the judgement that the exposure is carcinogenic. The terms 'neoplasm' and 'tumour' are used interchangeably.

Some epidemiological and experimental studies indicate that different agents may act at different stages in the carcinogenic process, and several mechanisms may be involved. The aim of the *Monographs* has been, from their inception, to evaluate evidence of carcinogenicity at any stage in the carcinogenesis process, independently of the underlying mechanisms. Information on mechanisms may, however, be used in making the overall evaluation (IARC, 1991a; Vainio *et al.*, 1992; see also pp. 25–27).

The *Monographs* may assist national and international authorities in making risk assessments and in formulating decisions concerning any necessary preventive measures. The evaluations of IARC working groups are scientific, qualitative judgements about the evidence for or against carcinogenicity provided by the available data. These evaluations represent only one part of the body of information on which regulatory measures may be based. Other components of regulatory decisions vary from one situation to another and from country to country, responding to different socioeconomic and national priorities. **Therefore, no recommendation is given with regard to regulation or legislation, which are the responsibility of individual governments and/or other international organizations.**

The *IARC Monographs* are recognized as an authoritative source of information on the carcinogenicity of a wide range of human exposures. A survey of users in 1988 indicated that the *Monographs* are consulted by various agencies in 57 countries. About 2500 copies of each volume are printed, for distribution to governments, regulatory bodies and interested scientists. The Monographs are also available from IARC*Press* in Lyon and via the Marketing and Dissemination (MDI) of the World Health Organization in Geneva.

3. SELECTION OF TOPICS FOR MONOGRAPHS

Topics are selected on the basis of two main criteria: (a) there is evidence of human exposure, and (b) there is some evidence or suspicion of carcinogenicity. The term 'agent' is used to include individual chemical compounds, groups of related chemical compounds, physical agents (such as radiation) and biological factors (such as viruses). Exposures to mixtures of agents may occur in occupational exposures and as a result of personal and cultural habits (like smoking and dietary practices). Chemical analogues and compounds with biological or physical characteristics similar to those of suspected carcinogens may also be considered, even in the absence of data on a possible carcinogenic effect in humans or experimental animals.

The scientific literature is surveyed for published data relevant to an assessment of carcinogenicity. The IARC information bulletins on agents being tested for carcinogenicity (IARC, 1973–1996) and directories of on-going research in cancer epidemiology (IARC, 1976–1996) often indicate exposures that may be scheduled for future meetings. Ad-hoc working groups convened by IARC in 1984, 1989, 1991, 1993 and

1998 gave recommendations as to which agents should be evaluated in the IARC Monographs series (IARC, 1984, 1989, 1991b, 1993, 1998a,b).

As significant new data on subjects on which monographs have already been prepared become available, re-evaluations are made at subsequent meetings, and revised monographs are published.

4. DATA FOR MONOGRAPHS

The *Monographs* do not necessarily cite all the literature concerning the subject of an evaluation. Only those data considered by the Working Group to be relevant to making the evaluation are included.

With regard to biological and epidemiological data, only reports that have been published or accepted for publication in the openly available scientific literature are reviewed by the working groups. In certain instances, government agency reports that have undergone peer review and are widely available are considered. Exceptions may be made on an ad-hoc basis to include unpublished reports that are in their final form and publicly available, if their inclusion is considered pertinent to making a final evaluation (see pp. 25–27). In the sections on chemical and physical properties, on analysis, on production and use and on occurrence, unpublished sources of information may be used.

5. THE WORKING GROUP

Reviews and evaluations are formulated by a working group of experts. The tasks of the group are: (i) to ascertain that all appropriate data have been collected; (ii) to select the data relevant for the evaluation on the basis of scientific merit; (iii) to prepare accurate summaries of the data to enable the reader to follow the reasoning of the Working Group; (iv) to evaluate the results of epidemiological and experimental studies on cancer; (v) to evaluate data relevant to the understanding of mechanism of action; and (vi) to make an overall evaluation of the carcinogenicity of the exposure to humans.

Working Group participants who contributed to the considerations and evaluations within a particular volume are listed, with their addresses, at the beginning of each publication. Each participant who is a member of a working group serves as an individual scientist and not as a representative of any organization, government or industry. In addition, nominees of national and international agencies and industrial associations may be invited as observers.

6. WORKING PROCEDURES

Approximately one year in advance of a meeting of a working group, the topics of the monographs are announced and participants are selected by IARC staff in consultation with other experts. Subsequently, relevant biological and epidemiological data are

collected by the Carcinogen Identification and Evaluation Unit of IARC from recognized sources of information on carcinogenesis, including data storage and retrieval systems such as MEDLINE and TOXLINE.

For chemicals and some complex mixtures, the major collection of data and the preparation of first drafts of the sections on chemical and physical properties, on analysis, on production and use and on occurrence are carried out under a separate contract funded by the United States National Cancer Institute. Representatives from industrial associations may assist in the preparation of sections on production and use. Information on production and trade is obtained from governmental and trade publications and, in some cases, by direct contact with industries. Separate production data on some agents may not be available because their publication could disclose confidential information. Information on uses may be obtained from published sources but is often complemented by direct contact with manufacturers. Efforts are made to supplement this information with data from other national and international sources.

Six months before the meeting, the material obtained is sent to meeting participants, or is used by IARC staff, to prepare sections for the first drafts of monographs. The first drafts are compiled by IARC staff and sent before the meeting to all participants of the Working Group for review.

The Working Group meets in Lyon for seven to eight days to discuss and finalize the texts of the monographs and to formulate the evaluations. After the meeting, the master copy of each monograph is verified by consulting the original literature, edited and prepared for publication. The aim is to publish monographs within six months of the Working Group meeting.

The available studies are summarized by the Working Group, with particular regard to the qualitative aspects discussed below. In general, numerical findings are indicated as they appear in the original report; units are converted when necessary for easier comparison. The Working Group may conduct additional analyses of the published data and use them in their assessment of the evidence; the results of such supplementary analyses are given in square brackets. When an important aspect of a study, directly impinging on its interpretation, should be brought to the attention of the reader, a comment is given in square brackets.

7. EXPOSURE DATA

Sections that indicate the extent of past and present human exposure, the sources of exposure, the people most likely to be exposed and the factors that contribute to the exposure are included at the beginning of each monograph.

Most monographs on individual chemicals, groups of chemicals or complex mixtures include sections on chemical and physical data, on analysis, on production and use and on occurrence. In monographs on, for example, physical agents, occupational exposures and cultural habits, other sections may be included, such as: historical perspectives, description of an industry or habit, chemistry of the complex mixture or taxonomy. Mono-

graphs on biological agents have sections on structure and biology, methods of detection, epidemiology of infection and clinical disease other than cancer.

For chemical exposures, the Chemical Abstracts Services Registry Number, the latest Chemical Abstracts primary name and the IUPAC systematic name are recorded; other synonyms are given, but the list is not necessarily comprehensive. For biological agents, taxonomy and structure are described, and the degree of variability is given, when applicable.

Information on chemical and physical properties and, in particular, data relevant to identification, occurrence and biological activity are included. For biological agents, mode of replication, life cycle, target cells, persistence and latency and host response are given. A description of technical products of chemicals includes trade names, relevant specifications and available information on composition and impurities. Some of the trade names given may be those of mixtures in which the agent being evaluated is only one of the ingredients.

The purpose of the section on analysis or detection is to give the reader an overview of current methods, with emphasis on those widely used for regulatory purposes. Methods for monitoring human exposure are also given, when available. No critical evaluation or recommendation of any of the methods is meant or implied. The IARC published a series of volumes, *Environmental Carcinogens: Methods of Analysis and Exposure Measurement* (IARC, 1978–93), that describe validated methods for analysing a wide variety of chemicals and mixtures. For biological agents, methods of detection and exposure assessment are described, including their sensitivity, specificity and reproducibility.

The dates of first synthesis and of first commercial production of a chemical or mixture are provided; for agents which do not occur naturally, this information may allow a reasonable estimate to be made of the date before which no human exposure to the agent could have occurred. The dates of first reported occurrence of an exposure are also provided. In addition, methods of synthesis used in past and present commercial production and different methods of production which may give rise to different impurities are described.

Data on production, international trade and uses are obtained for representative regions, which usually include Europe, Japan and the United States of America. It should not, however, be inferred that those areas or nations are necessarily the sole or major sources or users of the agent. Some identified uses may not be current or major applications, and the coverage is not necessarily comprehensive. In the case of drugs, mention of their therapeutic uses does not necessarily represent current practice, nor does it imply judgement as to their therapeutic efficacy.

Information on the occurrence of an agent or mixture in the environment is obtained from data derived from the monitoring and surveillance of levels in occupational environments, air, water, soil, foods and animal and human tissues. When available, data on the generation, persistence and bioaccumulation of the agent are also included. In the case of mixtures, industries, occupations or processes, information is given about all

agents present. For processes, industries and occupations, a historical description is also given, noting variations in chemical composition, physical properties and levels of occupational exposure with time and place. For biological agents, the epidemiology of infection is described.

Statements concerning regulations and guidelines (e.g., pesticide registrations, maximal levels permitted in foods, occupational exposure limits) are included for some countries as indications of potential exposures, but they may not reflect the most recent situation, since such limits are continuously reviewed and modified. The absence of information on regulatory status for a country should not be taken to imply that that country does not have regulations with regard to the exposure. For biological agents, legislation and control, including vaccines and therapy, are described.

8. STUDIES OF CANCER IN HUMANS

(*a*) *Types of studies considered*

Three types of epidemiological studies of cancer contribute to the assessment of carcinogenicity in humans — cohort studies, case–control studies and correlation (or ecological) studies. Rarely, results from randomized trials may be available. Case series and case reports of cancer in humans may also be reviewed.

Cohort and case–control studies relate the exposures under study to the occurrence of cancer in individuals and provide an estimate of relative risk (ratio of incidence or mortality in those exposed to incidence or mortality in those not exposed) as the main measure of association.

In correlation studies, the units of investigation are usually whole populations (e.g. in particular geographical areas or at particular times), and cancer frequency is related to a summary measure of the exposure of the population to the agent, mixture or exposure circumstance under study. Because individual exposure is not documented, however, a causal relationship is less easy to infer from correlation studies than from cohort and case–control studies. Case reports generally arise from a suspicion, based on clinical experience, that the concurrence of two events — that is, a particular exposure and occurrence of a cancer — has happened rather more frequently than would be expected by chance. Case reports usually lack complete ascertainment of cases in any population, definition or enumeration of the population at risk and estimation of the expected number of cases in the absence of exposure. The uncertainties surrounding interpretation of case reports and correlation studies make them inadequate, except in rare instances, to form the sole basis for inferring a causal relationship. When taken together with case–control and cohort studies, however, relevant case reports or correlation studies may add materially to the judgement that a causal relationship is present.

Epidemiological studies of benign neoplasms, presumed preneoplastic lesions and other end-points thought to be relevant to cancer are also reviewed by working groups. They may, in some instances, strengthen inferences drawn from studies of cancer itself.

(*b*) *Quality of studies considered*

The Monographs are not intended to summarize all published studies. Those that are judged to be inadequate or irrelevant to the evaluation are generally omitted. They may be mentioned briefly, particularly when the information is considered to be a useful supplement to that in other reports or when they provide the only data available. Their inclusion does not imply acceptance of the adequacy of the study design or of the analysis and interpretation of the results, and limitations are clearly outlined in square brackets at the end of the study description.

It is necessary to take into account the possible roles of bias, confounding and chance in the interpretation of epidemiological studies. By 'bias' is meant the operation of factors in study design or execution that lead erroneously to a stronger or weaker association than in fact exists between disease and an agent, mixture or exposure circumstance. By 'confounding' is meant a situation in which the relationship with disease is made to appear stronger or weaker than it truly is as a result of an association between the apparent causal factor and another factor that is associated with either an increase or decrease in the incidence of the disease. In evaluating the extent to which these factors have been minimized in an individual study, working groups consider a number of aspects of design and analysis as described in the report of the study. Most of these considerations apply equally to case–control, cohort and correlation studies. Lack of clarity of any of these aspects in the reporting of a study can decrease its credibility and the weight given to it in the final evaluation of the exposure.

Firstly, the study population, disease (or diseases) and exposure should have been well defined by the authors. Cases of disease in the study population should have been identified in a way that was independent of the exposure of interest, and exposure should have been assessed in a way that was not related to disease status.

Secondly, the authors should have taken account in the study design and analysis of other variables that can influence the risk of disease and may have been related to the exposure of interest. Potential confounding by such variables should have been dealt with either in the design of the study, such as by matching, or in the analysis, by statistical adjustment. In cohort studies, comparisons with local rates of disease may be more appropriate than those with national rates. Internal comparisons of disease frequency among individuals at different levels of exposure should also have been made in the study.

Thirdly, the authors should have reported the basic data on which the conclusions are founded, even if sophisticated statistical analyses were employed. At the very least, they should have given the numbers of exposed and unexposed cases and controls in a case–control study and the numbers of cases observed and expected in a cohort study. Further tabulations by time since exposure began and other temporal factors are also important. In a cohort study, data on all cancer sites and all causes of death should have been given, to reveal the possibility of reporting bias. In a case–control study, the effects of investigated factors other than the exposure of interest should have been reported.

Finally, the statistical methods used to obtain estimates of relative risk, absolute rates of cancer, confidence intervals and significance tests, and to adjust for confounding should have been clearly stated by the authors. The methods used should preferably have been the generally accepted techniques that have been refined since the mid-1970s. These methods have been reviewed for case–control studies (Breslow & Day, 1980) and for cohort studies (Breslow & Day, 1987).

(*c*) *Inferences about mechanism of action*

Detailed analyses of both relative and absolute risks in relation to temporal variables, such as age at first exposure, time since first exposure, duration of exposure, cumulative exposure and time since exposure ceased, are reviewed and summarized when available. The analysis of temporal relationships can be useful in formulating models of carcinogenesis. In particular, such analyses may suggest whether a carcinogen acts early or late in the process of carcinogenesis, although at best they allow only indirect inferences about the mechanism of action. Special attention is given to measurements of biological markers of carcinogen exposure or action, such as DNA or protein adducts, as well as markers of early steps in the carcinogenic process, such as proto-oncogene mutation, when these are incorporated into epidemiological studies focused on cancer incidence or mortality. Such measurements may allow inferences to be made about putative mechanisms of action (IARC, 1991a; Vainio *et al.*, 1992).

(*d*) *Criteria for causality*

After the individual epidemiological studies of cancer have been summarized and the quality assessed, a judgement is made concerning the strength of evidence that the agent, mixture or exposure circumstance in question is carcinogenic for humans. In making its judgement, the Working Group considers several criteria for causality. A strong association (a large relative risk) is more likely to indicate causality than a weak association, although it is recognized that relative risks of small magnitude do not imply lack of causality and may be important if the disease is common. Associations that are replicated in several studies of the same design or using different epidemiological approaches or under different circumstances of exposure are more likely to represent a causal relationship than isolated observations from single studies. If there are inconsistent results among investigations, possible reasons are sought (such as differences in amount of exposure), and results of studies judged to be of high quality are given more weight than those of studies judged to be methodologically less sound. When suspicion of carcinogenicity arises largely from a single study, these data are not combined with those from later studies in any subsequent reassessment of the strength of the evidence.

If the risk of the disease in question increases with the amount of exposure, this is considered to be a strong indication of causality, although absence of a graded response is not necessarily evidence against a causal relationship. Demonstration of a decline in

risk after cessation of or reduction in exposure in individuals or in whole populations also supports a causal interpretation of the findings.

Although a carcinogen may act upon more than one target, the specificity of an association (an increased occurrence of cancer at one anatomical site or of one morphological type) adds plausibility to a causal relationship, particularly when excess cancer occurrence is limited to one morphological type within the same organ.

Although rarely available, results from randomized trials showing different rates among exposed and unexposed individuals provide particularly strong evidence for causality.

When several epidemiological studies show little or no indication of an association between an exposure and cancer, the judgement may be made that, in the aggregate, they show evidence of lack of carcinogenicity. Such a judgement requires first of all that the studies giving rise to it meet, to a sufficient degree, the standards of design and analysis described above. Specifically, the possibility that bias, confounding or misclassification of exposure or outcome could explain the observed results should be considered and excluded with reasonable certainty. In addition, all studies that are judged to be methodologically sound should be consistent with a relative risk of unity for any observed level of exposure and, when considered together, should provide a pooled estimate of relative risk which is at or near unity and has a narrow confidence interval, due to sufficient population size. Moreover, no individual study nor the pooled results of all the studies should show any consistent tendency for the relative risk of cancer to increase with increasing level of exposure. It is important to note that evidence of lack of carcinogenicity obtained in this way from several epidemiological studies can apply only to the type(s) of cancer studied and to dose levels and intervals between first exposure and observation of disease that are the same as or less than those observed in all the studies. Experience with human cancer indicates that, in some cases, the period from first exposure to the development of clinical cancer is seldom less than 20 years; studies with latent periods substantially shorter than 30 years cannot provide evidence for lack of carcinogenicity.

9. STUDIES OF CANCER IN EXPERIMENTAL ANIMALS

All known human carcinogens that have been studied adequately in experimental animals have produced positive results in one or more animal species (Wilbourn *et al.*, 1986; Tomatis *et al.*, 1989). For several agents (aflatoxins, 4-aminobiphenyl, azathioprine, betel quid with tobacco, bischloromethyl ether and chloromethyl methyl ether (technical grade), chlorambucil, chlornaphazine, ciclosporin, coal-tar pitches, coal-tars, combined oral contraceptives, cyclophosphamide, diethylstilboestrol, melphalan, 8-methoxypsoralen plus ultraviolet A radiation, mustard gas, myleran, 2-naphthylamine, nonsteroidal estrogens, estrogen replacement therapy/steroidal estrogens, solar radiation, thiotepa and vinyl chloride), carcinogenicity in experimental animals was established or highly suspected before epidemiological studies confirmed their carcinogenicity in humans (Vainio *et al.*, 1995). Although this association cannot establish that all agents

and mixtures that cause cancer in experimental animals also cause cancer in humans, nevertheless, **in the absence of adequate data on humans, it is biologically plausible and prudent to regard agents and mixtures for which there is *sufficient evidence* (see p. 24) of carcinogenicity in experimental animals as if they presented a carcinogenic risk to humans**. The possibility that a given agent may cause cancer through a species-specific mechanism which does not operate in humans (see p. 27) should also be taken into consideration.

The nature and extent of impurities or contaminants present in the chemical or mixture being evaluated are given when available. Animal strain, sex, numbers per group, age at start of treatment and survival are reported.

Other types of studies summarized include: experiments in which the agent or mixture was administered in conjunction with known carcinogens or factors that modify carcinogenic effects; studies in which the end-point was not cancer but a defined precancerous lesion; and experiments on the carcinogenicity of known metabolites and derivatives.

For experimental studies of mixtures, consideration is given to the possibility of changes in the physicochemical properties of the test substance during collection, storage, extraction, concentration and delivery. Chemical and toxicological interactions of the components of mixtures may result in nonlinear dose–response relationships.

An assessment is made as to the relevance to human exposure of samples tested in experimental animals, which may involve consideration of: (i) physical and chemical characteristics, (ii) constituent substances that indicate the presence of a class of substances, (iii) the results of tests for genetic and related effects, including studies on DNA adduct formation, proto-oncogene mutation and expression and suppressor gene inactivation. The relevance of results obtained, for example, with animal viruses analogous to the virus being evaluated in the monograph must also be considered. They may provide biological and mechanistic information relevant to the understanding of the process of carcinogenesis in humans and may strengthen the plausibility of a conclusion that the biological agent under evaluation is carcinogenic in humans.

(*a*) *Qualitative aspects*

An assessment of carcinogenicity involves several considerations of qualitative importance, including (i) the experimental conditions under which the test was performed, including route and schedule of exposure, species, strain, sex, age, duration of follow-up; (ii) the consistency of the results, for example, across species and target organ(s); (iii) the spectrum of neoplastic response, from preneoplastic lesions and benign tumours to malignant neoplasms; and (iv) the possible role of modifying factors.

As mentioned earlier (p. 11), the *Monographs* are not intended to summarize all published studies. Those studies in experimental animals that are inadequate (e.g., too short a duration, too few animals, poor survival; see below) or are judged irrelevant to

the evaluation are generally omitted. Guidelines for conducting adequate long-term carcinogenicity experiments have been outlined (e.g. Montesano *et al.*, 1986).

Considerations of importance to the Working Group in the interpretation and evaluation of a particular study include: (i) how clearly the agent was defined and, in the case of mixtures, how adequately the sample characterization was reported; (ii) whether the dose was adequately monitored, particularly in inhalation experiments; (iii) whether the doses and duration of treatment were appropriate and whether the survival of treated animals was similar to that of controls; (iv) whether there were adequate numbers of animals per group; (v) whether animals of each sex were used; (vi) whether animals were allocated randomly to groups; (vii) whether the duration of observation was adequate; and (viii) whether the data were adequately reported. If available, recent data on the incidence of specific tumours in historical controls, as well as in concurrent controls, should be taken into account in the evaluation of tumour response.

When benign tumours occur together with and originate from the same cell type in an organ or tissue as malignant tumours in a particular study and appear to represent a stage in the progression to malignancy, it may be valid to combine them in assessing tumour incidence (Huff *et al.*, 1989). The occurrence of lesions presumed to be preneoplastic may in certain instances aid in assessing the biological plausibility of any neoplastic response observed. If an agent or mixture induces only benign neoplasms that appear to be end-points that do not readily progress to malignancy, it should nevertheless be suspected of being a carcinogen and requires further investigation.

(*b*) *Quantitative aspects*

The probability that tumours will occur may depend on the species, sex, strain and age of the animal, the dose of the carcinogen and the route and length of exposure. Evidence of an increased incidence of neoplasms with increased level of exposure strengthens the inference of a causal association between the exposure and the development of neoplasms.

The form of the dose–response relationship can vary widely, depending on the particular agent under study and the target organ. Both DNA damage and increased cell division are important aspects of carcinogenesis, and cell proliferation is a strong determinant of dose–response relationships for some carcinogens (Cohen & Ellwein, 1990). Since many chemicals require metabolic activation before being converted into their reactive intermediates, both metabolic and pharmacokinetic aspects are important in determining the dose–response pattern. Saturation of steps such as absorption, activation, inactivation and elimination may produce nonlinearity in the dose–response relationship, as could saturation of processes such as DNA repair (Hoel *et al.*, 1983; Gart *et al.*, 1986).

(*c*) *Statistical analysis of long-term experiments in animals*

Factors considered by the Working Group include the adequacy of the information given for each treatment group: (i) the number of animals studied and the number examined histologically, (ii) the number of animals with a given tumour type and (iii) length of survival. The statistical methods used should be clearly stated and should be the generally accepted techniques refined for this purpose (Peto *et al.*, 1980; Gart *et al.*, 1986). When there is no difference in survival between control and treatment groups, the Working Group usually compares the proportions of animals developing each tumour type in each of the groups. Otherwise, consideration is given as to whether or not appropriate adjustments have been made for differences in survival. These adjustments can include: comparisons of the proportions of tumour-bearing animals among the effective number of animals (alive at the time the first tumour is discovered), in the case where most differences in survival occur before tumours appear; life-table methods, when tumours are visible or when they may be considered 'fatal' because mortality rapidly follows tumour development; and the Mantel-Haenszel test or logistic regression, when occult tumours do not affect the animals' risk of dying but are 'incidental' findings at autopsy.

In practice, classifying tumours as fatal or incidental may be difficult. Several survival-adjusted methods have been developed that do not require this distinction (Gart *et al.*, 1986), although they have not been fully evaluated.

10. OTHER DATA RELEVANT TO AN EVALUATION OF CARCINOGENICITY AND ITS MECHANISMS

In coming to an overall evaluation of carcinogenicity in humans (see pp. 25–27), the Working Group also considers related data. The nature of the information selected for the summary depends on the agent being considered.

For chemicals and complex mixtures of chemicals such as those in some occupational situations or involving cultural habits (e.g. tobacco smoking), the other data considered to be relevant are divided into those on absorption, distribution, metabolism and excretion; toxic effects; reproductive and developmental effects; and genetic and related effects.

Concise information is given on absorption, distribution (including placental transfer) and excretion in both humans and experimental animals. Kinetic factors that may affect the dose–response relationship, such as saturation of uptake, protein binding, metabolic activation, detoxification and DNA repair processes, are mentioned. Studies that indicate the metabolic fate of the agent in humans and in experimental animals are summarized briefly, and comparisons of data on humans and on animals are made when possible. Comparative information on the relationship between exposure and the dose that reaches the target site may be of particular importance for extrapolation between species. Data are given on acute and chronic toxic effects (other than cancer), such as

organ toxicity, increased cell proliferation, immunotoxicity and endocrine effects. The presence and toxicological significance of cellular receptors is described. Effects on reproduction, teratogenicity, fetotoxicity and embryotoxicity are also summarized briefly.

Tests of genetic and related effects are described in view of the relevance of gene mutation and chromosomal damage to carcinogenesis (Vainio *et al.*, 1992; McGregor *et al.*, 1999). The adequacy of the reporting of sample characterization is considered and, where necessary, commented upon; with regard to complex mixtures, such comments are similar to those described for animal carcinogenicity tests on p. 18. The available data are interpreted critically by phylogenetic group according to the end-points detected, which may include DNA damage, gene mutation, sister chromatid exchange, micronucleus formation, chromosomal aberrations, aneuploidy and cell transformation. The concentrations employed are given, and mention is made of whether use of an exogenous metabolic system *in vitro* affected the test result. These data are given as listings of test systems, data and references. The data on genetic and related effects presented in the *Monographs* are also available in the form of genetic activity profiles (GAP) prepared in collaboration with the United States Environmental Protection Agency (EPA) (see also Waters *et al.*, 1987) using software for personal computers that are Microsoft Windows® compatible. The EPA/IARC GAP software and database may be downloaded free of charge from *www.epa.gov/gapdb*.

Positive results in tests using prokaryotes, lower eukaryotes, plants, insects and cultured mammalian cells suggest that genetic and related effects could occur in mammals. Results from such tests may also give information about the types of genetic effect produced and about the involvement of metabolic activation. Some end-points described are clearly genetic in nature (e.g., gene mutations and chromosomal aberrations), while others are to a greater or lesser degree associated with genetic effects (e.g. unscheduled DNA synthesis). In-vitro tests for tumour-promoting activity and for cell transformation may be sensitive to changes that are not necessarily the result of genetic alterations but that may have specific relevance to the process of carcinogenesis. A critical appraisal of these tests has been published (Montesano *et al.*, 1986).

Genetic or other activity detected in experimental mammals and humans is regarded as being of greater relevance than that in other organisms. The demonstration that an agent or mixture can induce gene and chromosomal mutations in whole mammals indicates that it may have carcinogenic activity, although this activity may not be detectably expressed in any or all species. Relative potency in tests for mutagenicity and related effects is not a reliable indicator of carcinogenic potency. Negative results in tests for mutagenicity in selected tissues from animals treated *in vivo* provide less weight, partly because they do not exclude the possibility of an effect in tissues other than those examined. Moreover, negative results in short-term tests with genetic end-points cannot be considered to provide evidence to rule out carcinogenicity of agents or mixtures that act through other mechanisms (e.g. receptor-mediated effects, cellular toxicity with regenerative proliferation, peroxisome proliferation) (Vainio *et al.*, 1992). Factors that

may lead to misleading results in short-term tests have been discussed in detail elsewhere (Montesano *et al.*, 1986).

When available, data relevant to mechanisms of carcinogenesis that do not involve structural changes at the level of the gene are also described.

The adequacy of epidemiological studies of reproductive outcome and genetic and related effects in humans is evaluated by the same criteria as are applied to epidemiological studies of cancer.

Structure–activity relationships that may be relevant to an evaluation of the carcinogenicity of an agent are also described.

For biological agents — viruses, bacteria and parasites — other data relevant to carcinogenicity include descriptions of the pathology of infection, molecular biology (integration and expression of viruses, and any genetic alterations seen in human tumours) and other observations, which might include cellular and tissue responses to infection, immune response and the presence of tumour markers.

11. SUMMARY OF DATA REPORTED

In this section, the relevant epidemiological and experimental data are summarized. Only reports, other than in abstract form, that meet the criteria outlined on p. 11 are considered for evaluating carcinogenicity. Inadequate studies are generally not summarized: such studies are usually identified by a square-bracketed comment in the preceding text.

(*a*) *Exposure*

Human exposure to chemicals and complex mixtures is summarized on the basis of elements such as production, use, occurrence in the environment and determinations in human tissues and body fluids. Quantitative data are given when available. Exposure to biological agents is described in terms of transmission and prevalence of infection.

(*b*) *Carcinogenicity in humans*

Results of epidemiological studies that are considered to be pertinent to an assessment of human carcinogenicity are summarized. When relevant, case reports and correlation studies are also summarized.

(*c*) *Carcinogenicity in experimental animals*

Data relevant to an evaluation of carcinogenicity in animals are summarized. For each animal species and route of administration, it is stated whether an increased incidence of neoplasms or preneoplastic lesions was observed, and the tumour sites are indicated. If the agent or mixture produced tumours after prenatal exposure or in single-dose experiments, this is also indicated. Negative findings are also summarized. Dose–response and other quantitative data may be given when available.

(*d*) *Other data relevant to an evaluation of carcinogenicity and its mechanisms*

Data on biological effects in humans that are of particular relevance are summarized. These may include toxicological, kinetic and metabolic considerations and evidence of DNA binding, persistence of DNA lesions or genetic damage in exposed humans. Toxicological information, such as that on cytotoxicity and regeneration, receptor binding and hormonal and immunological effects, and data on kinetics and metabolism in experimental animals are given when considered relevant to the possible mechanism of the carcinogenic action of the agent. The results of tests for genetic and related effects are summarized for whole mammals, cultured mammalian cells and nonmammalian systems.

When available, comparisons of such data for humans and for animals, and particularly animals that have developed cancer, are described.

Structure–activity relationships are mentioned when relevant.

For the agent, mixture or exposure circumstance being evaluated, the available data on end-points or other phenomena relevant to mechanisms of carcinogenesis from studies in humans, experimental animals and tissue and cell test systems are summarized within one or more of the following descriptive dimensions:

(i) Evidence of genotoxicity (structural changes at the level of the gene): for example, structure–activity considerations, adduct formation, mutagenicity (effect on specific genes), chromosomal mutation/aneuploidy

(ii) Evidence of effects on the expression of relevant genes (functional changes at the intracellular level): for example, alterations to the structure or quantity of the product of a proto-oncogene or tumour-suppressor gene, alterations to metabolic activation/inactivation/DNA repair

(iii) Evidence of relevant effects on cell behaviour (morphological or behavioural changes at the cellular or tissue level): for example, induction of mitogenesis, compensatory cell proliferation, preneoplasia and hyperplasia, survival of premalignant or malignant cells (immortalization, immunosuppression), effects on metastatic potential

(iv) Evidence from dose and time relationships of carcinogenic effects and interactions between agents: for example, early/late stage, as inferred from epidemiological studies; initiation/promotion/progression/malignant conversion, as defined in animal carcinogenicity experiments; toxicokinetics

These dimensions are not mutually exclusive, and an agent may fall within more than one of them. Thus, for example, the action of an agent on the expression of relevant genes could be summarized under both the first and second dimensions, even if it were known with reasonable certainty that those effects resulted from genotoxicity.

12. EVALUATION

Evaluations of the strength of the evidence for carcinogenicity arising from human and experimental animal data are made, using standard terms.

It is recognized that the criteria for these evaluations, described below, cannot encompass all of the factors that may be relevant to an evaluation of carcinogenicity. In considering all of the relevant scientific data, the Working Group may assign the agent, mixture or exposure circumstance to a higher or lower category than a strict interpretation of these criteria would indicate.

(*a*) *Degrees of evidence for carcinogenicity in humans and in experimental animals and supporting evidence*

These categories refer only to the strength of the evidence that an exposure is carcinogenic and not to the extent of its carcinogenic activity (potency) nor to the mechanisms involved. A classification may change as new information becomes available.

An evaluation of degree of evidence, whether for a single agent or a mixture, is limited to the materials tested, as defined physically, chemically or biologically. When the agents evaluated are considered by the Working Group to be sufficiently closely related, they may be grouped together for the purpose of a single evaluation of degree of evidence.

(i) *Carcinogenicity in humans*

The applicability of an evaluation of the carcinogenicity of a mixture, process, occupation or industry on the basis of evidence from epidemiological studies depends on the variability over time and place of the mixtures, processes, occupations and industries. The Working Group seeks to identify the specific exposure, process or activity which is considered most likely to be responsible for any excess risk. The evaluation is focused as narrowly as the available data on exposure and other aspects permit.

The evidence relevant to carcinogenicity from studies in humans is classified into one of the following categories:

Sufficient evidence of carcinogenicity: The Working Group considers that a causal relationship has been established between exposure to the agent, mixture or exposure circumstance and human cancer. That is, a positive relationship has been observed between the exposure and cancer in studies in which chance, bias and confounding could be ruled out with reasonable confidence.

Limited evidence of carcinogenicity: A positive association has been observed between exposure to the agent, mixture or exposure circumstance and cancer for which a causal interpretation is considered by the Working Group to be credible, but chance, bias or confounding could not be ruled out with reasonable confidence.

Inadequate evidence of carcinogenicity: The available studies are of insufficient quality, consistency or statistical power to permit a conclusion regarding the presence or absence of a causal association between exposure and cancer, or no data on cancer in humans are available.

Evidence suggesting lack of carcinogenicity: There are several adequate studies covering the full range of levels of exposure that human beings are known to encounter, which are mutually consistent in not showing a positive association between exposure to

the agent, mixture or exposure circumstance and any studied cancer at any observed level of exposure. A conclusion of 'evidence suggesting lack of carcinogenicity' is inevitably limited to the cancer sites, conditions and levels of exposure and length of observation covered by the available studies. In addition, the possibility of a very small risk at the levels of exposure studied can never be excluded.

In some instances, the above categories may be used to classify the degree of evidence related to carcinogenicity in specific organs or tissues.

(ii) *Carcinogenicity in experimental animals*

The evidence relevant to carcinogenicity in experimental animals is classified into one of the following categories:

Sufficient evidence of carcinogenicity: The Working Group considers that a causal relationship has been established between the agent or mixture and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) in two or more independent studies in one species carried out at different times or in different laboratories or under different protocols.

Exceptionally, a single study in one species might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset.

Limited evidence of carcinogenicity: The data suggest a carcinogenic effect but are limited for making a definitive evaluation because, e.g. (a) the evidence of carcinogenicity is restricted to a single experiment; or (b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the study; or (c) the agent or mixture increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential, or of certain neoplasms which may occur spontaneously in high incidences in certain strains.

Inadequate evidence of carcinogenicity: The studies cannot be interpreted as showing either the presence or absence of a carcinogenic effect because of major qualitative or quantitative limitations, or no data on cancer in experimental animals are available.

Evidence suggesting lack of carcinogenicity: Adequate studies involving at least two species are available which show that, within the limits of the tests used, the agent or mixture is not carcinogenic. A conclusion of evidence suggesting lack of carcinogenicity is inevitably limited to the species, tumour sites and levels of exposure studied.

(*b*) *Other data relevant to the evaluation of carcinogenicity and its mechanisms*

Other evidence judged to be relevant to an evaluation of carcinogenicity and of sufficient importance to affect the overall evaluation is then described. This may include data on preneoplastic lesions, tumour pathology, genetic and related effects, structure–activity relationships, metabolism and pharmacokinetics, physicochemical parameters and analogous biological agents.

Data relevant to mechanisms of the carcinogenic action are also evaluated. The strength of the evidence that any carcinogenic effect observed is due to a particular mechanism is assessed, using terms such as weak, moderate or strong. Then, the Working Group assesses if that particular mechanism is likely to be operative in humans. The strongest indications that a particular mechanism operates in humans come from data on humans or biological specimens obtained from exposed humans. The data may be considered to be especially relevant if they show that the agent in question has caused changes in exposed humans that are on the causal pathway to carcinogenesis. Such data may, however, never become available, because it is at least conceivable that certain compounds may be kept from human use solely on the basis of evidence of their toxicity and/or carcinogenicity in experimental systems.

For complex exposures, including occupational and industrial exposures, the chemical composition and the potential contribution of carcinogens known to be present are considered by the Working Group in its overall evaluation of human carcinogenicity. The Working Group also determines the extent to which the materials tested in experimental systems are related to those to which humans are exposed.

(*c*) *Overall evaluation*

Finally, the body of evidence is considered as a whole, in order to reach an overall evaluation of the carcinogenicity to humans of an agent, mixture or circumstance of exposure.

An evaluation may be made for a group of chemical compounds that have been evaluated by the Working Group. In addition, when supporting data indicate that other, related compounds for which there is no direct evidence of capacity to induce cancer in humans or in animals may also be carcinogenic, a statement describing the rationale for this conclusion is added to the evaluation narrative; an additional evaluation may be made for this broader group of compounds if the strength of the evidence warrants it.

The agent, mixture or exposure circumstance is described according to the wording of one of the following categories, and the designated group is given. The categorization of an agent, mixture or exposure circumstance is a matter of scientific judgement, reflecting the strength of the evidence derived from studies in humans and in experimental animals and from other relevant data.

Group 1 — The agent (mixture) is carcinogenic to humans.
The exposure circumstance entails exposures that are carcinogenic to humans.

This category is used when there is *sufficient evidence* of carcinogenicity in humans. Exceptionally, an agent (mixture) may be placed in this category when evidence of carcinogenicity in humans is less than sufficient but there is *sufficient evidence* of carcinogenicity in experimental animals and strong evidence in exposed humans that the agent (mixture) acts through a relevant mechanism of carcinogenicity.

Group 2

This category includes agents, mixtures and exposure circumstances for which, at one extreme, the degree of evidence of carcinogenicity in humans is almost sufficient, as well as those for which, at the other extreme, there are no human data but for which there is evidence of carcinogenicity in experimental animals. Agents, mixtures and exposure circumstances are assigned to either group 2A (probably carcinogenic to humans) or group 2B (possibly carcinogenic to humans) on the basis of epidemiological and experimental evidence of carcinogenicity and other relevant data.

Group 2A — The agent (mixture) is probably carcinogenic to humans. The exposure circumstance entails exposures that are probably carcinogenic to humans.

This category is used when there is *limited evidence* of carcinogenicity in humans and *sufficient evidence* of carcinogenicity in experimental animals. In some cases, an agent (mixture) may be classified in this category when there is *inadequate evidence* of carcinogenicity in humans, *sufficient evidence* of carcinogenicity in experimental animals and strong evidence that the carcinogenesis is mediated by a mechanism that also operates in humans. Exceptionally, an agent, mixture or exposure circumstance may be classified in this category solely on the basis of *limited evidence* of carcinogenicity in humans.

Group 2B — The agent (mixture) is possibly carcinogenic to humans. The exposure circumstance entails exposures that are possibly carcinogenic to humans.

This category is used for agents, mixtures and exposure circumstances for which there is *limited evidence* of carcinogenicity in humans and less than *sufficient evidence* of carcinogenicity in experimental animals. It may also be used when there is *inadequate evidence* of carcinogenicity in humans but there is *sufficient evidence* of carcinogenicity in experimental animals. In some instances, an agent, mixture or exposure circumstance for which there is *inadequate evidence* of carcinogenicity in humans but *limited evidence* of carcinogenicity in experimental animals together with supporting evidence from other relevant data may be placed in this group.

Group 3 — The agent (mixture or exposure circumstance) is not classifiable as to its carcinogenicity to humans.

This category is used most commonly for agents, mixtures and exposure circumstances for which the *evidence of carcinogenicity* is *inadequate* in humans and *inadequate* or *limited* in experimental animals.

Exceptionally, agents (mixtures) for which the *evidence of carcinogenicity* is *inadequate* in humans but *sufficient* in experimental animals may be placed in this category

when there is strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans.

Agents, mixtures and exposure circumstances that do not fall into any other group are also placed in this category.

Group 4 — The agent (mixture) is probably not carcinogenic to humans.

This category is used for agents or mixtures for which there is *evidence suggesting lack of carcinogenicity* in humans and in experimental animals. In some instances, agents or mixtures for which there is *inadequate evidence* of carcinogenicity in humans but *evidence suggesting lack of carcinogenicity* in experimental animals, consistently and strongly supported by a broad range of other relevant data, may be classified in this group.

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GENERAL REMARKS

This ninetieth volume of the *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans* considers human papillomaviruses (HPVs), which were evaluated by a previous Working Group (IARC, 1995). The monograph in the present volume incorporates new data that have become available during the past decade.

HPVs represent the most common infectious agents that are transmitted sexually throughout the world; the major risk factors are behaviours associated with sexual activity. Although most infections are asymptomatic and are cleared within a period of 2 years, genital HPV infection can lead to clinical disease, including anogenital warts, cervical neoplasia, cervical cancer and other anogenital cancers. The risk for persistence of infection and progression of the more than 40 genital HPV types to grade 3 cervical intraepithelial neoplasia (CIN3) and cancer differs widely. Persistent infection with carcinogenic HPVs occurs in virtually all cases of cervical cancer.

Previous evaluations of HPVs have classified types 16 and 18 as *carcinogenic to humans (Group 1)*, types 31 and 33 as *probably carcinogenic to humans (Group 2A)* and some types other than 16, 18, 31 and 33 as *possibly carcinogenic to humans (Group 2B)*. At that time, the evaluation of types 16 and 18 was based on the strong association between infection with these HPVs and cervical cancer. For types 31 and 33, the association was less strong.

The new epidemiological data reviewed in the present volume strongly support and further confirm the previous evaluation of types 16 and 18, and provide new evidence for other HPVs. This information, which includes strong evidence of carcinogenicity at sites other than the cervix, supports new evaluations for several other HPV types in addition to those mentioned above.

A brief history of research on papillomaviruses

Research on papillomas and papillomaviruses began more than 100 years ago. Probably the earliest work was carried out in England in 1896 by McFadyean and Hobday who demonstrated cell-free transmission of canine warts (McFadyean & Hobday, 1898). This finding was followed by the more frequently quoted cell-free transmission of human warts reported by Ciuffo (1907) in Italy. However, warts were not considered to be authentic tumours at that time, and it is therefore not surprising that the subsequent reports

of cell-free transmission of chicken leukaemia by Ellermann and Bang (1908) in Copenhagen and of chicken sarcoma by Rous (1911) in New York received much more attention from the scientific community.

In spite of a limited number of studies on papillomas and their viral etiology in subsequent decades, it was almost 80 more years later when this area of research engendered broad interest, particularly in the field of medicine. This resulted from the demonstration of a relationship between specific HPV infections and cancer of the cervix, one of the most frequent cancers in women. However, the recent surge in activities in papillomavirus research basically has four (initially relatively independent) historical roots: (*a*) studies on the development of papillomas in cattle; (*b*) those on the development of papillomas in rabbits; (*c*) studies on a rare human hereditary condition (epidermodysplasia verruciformis), which is characterized by extensive verrucosis and the subsequent development of skin cancer in warts that are located at sites exposed to sunlight; and (*d*) investigations on the viral etiology of cancer of the cervix. Although they were initiated by different primary observations, all four types of study played a role in the subsequent progress and stimulated specific experimental approaches. A brief outline of the pioneer work performed in the four areas and some of the early major advances made in these fields are summarized below.

The infectious origin of bovine warts was initially demonstrated in Brazil (Magelhaes, 1920). Interest in these types of frequently giant papillomas developed from the studies of Olson and Crook (1951), who showed that transmission of these viruses to another species (horses) resulted in the induction of sarcoids. These invasive but non-metastasizing tumours are also observed in domestic horses under natural conditions. Thus, their experimental induction suggested that they originated from trans-species transmission of bovine papillomavirus (BPV), which was proven much later by molecular analyses (Lancaster & Olson, 1978). This group made another striking observation, namely the induction of bladder tumours in cattle by BPV infection (Olson *et al.*, 1959). Four years later, two additional reports by Black *et al*. (1963) and Thomas *et al*. (1963) described the transforming activity of BPV preparations in bovine and murine cells. This was the first time that tissue culture studies were used in papillomavirus research and they profoundly influenced progress in subsequent years.

The development of molecular biology and DNA-cloning techniques in the 1970s and the application of this technology to the BPV system characterized parts of the BPV genome as the elements responsible for transformation in tissue cultures (Lowy *et al*., 1980). Shortly thereafter, BPV-1 was the first type of papillomavirus to be fully sequenced (Chen *et al*., 1982).

The interest in studies of BPV continues and is mainly based on the ease with which some of the most prevalent BPV types (BPV-1 and -2) can be used in tissue culture studies to study the mechanisms of persistence of the viral genome, as well as the patterns of expression of specific viral genes. Oesophageal carcinomas that originate from BPV-4-positive papillomatosis of the oesophagus have added to this interest (Campo, 1987).

Moreover, the use of BPV DNA in shuttle vectors and the episomal persistence of this DNA greatly increased the number of studies on these types of virus.

Retrospectively, the impact of research on BPV to this field was mainly through the analysis of BPV-induced cell transformation, the dissection of the viral genome and the structural and functional characterization of individual viral genes and gene products. The data obtained particularly facilitated early studies on HPV infections.

A second root of papillomavirus research that substantially influenced cancer research in general was the identification of papillomas and their infectious origin in wild cottontail rabbits in the early 1930s (Shope, 1933). After successful transmission of this infection to domestic rabbits, Rous and Beard (1934) soon noted that the initial papillomas that developed in these animals frequently converted to squamous-cell carcinomas. Occasionally, malignant conversion also occurred in the natural host (the cottontail rabbit). In a number of ingenious studies by this group, synergistic effects of viral and chemical carcinogens were observed, and the concept of tumour initiation was developed through the analysis of this system (e.g. Rous & Kidd, 1938; Rous & Friedewald, 1944). Although Rous conceptually preceded his contemporaries by several decades, the importance of his work was only acknowledged in 1966, when he received the Nobel Prize. Ito and Evans (1961) showed that the purified DNA of the cottontail rabbit papillomavirus (CRPV) induced squamous-cell carcinomas in rabbits, and thus directly revealed the carcinogenicity of a viral genome.

The research by Peyton Rous was not specifically driven by his interest in the infectious agent of rabbit papillomas. He strove to understand the mechanisms of the induction of cancer. The frequent progression of rabbit papillomas to squamous-cell carcinomas provided a model with which to analyse the steps in cancer development and to understand the synergistic effects of different classes of carcinogen. Interestingly, the rabbit papillomavirus system drew comparatively little attention subsequently. The literature today contains comparatively few studies of CRPV in comparison with BPV, epidermodysplasia verruciformis and genital HPV infections.

The analysis of human papillomatous lesions and their relationship with viral infections and carcinogenesis began much more slowly. Because of their cell-free transmission, the infectious etiology of of human warts was clearly established. However, warts were mainly regarded as a cosmetic nuisance and were not considered to be of significant medical interest.

A gradual change from this view began with the description of a syndrome that was reported by Lewandowsky and Lutz (1922) in Basel. They described a hereditary condition that was characterized by an extensive verrucosis, and which they named epidermodysplasia verruciformis. At sites of these patients that were exposed to sunlight (the forehead, the face, the back of the hands and arms), some of these papillomatous lesions converted to squamous-cell carcinomas. Lutz (1946) and subsequently Jablonska and Millewsky (1957) proved the viral etiology of these warts in auto-inoculation experiments. Schellender and Fritsch (1970) and Ruiter and van Mullem (1970) were particularly intrigued by the restriction of the development of squamous-cell carcinomas to sites

exposed to the sun. It was largely the work of Stefania Jablonska in Warsaw, Poland, that pointed to the possible role of papillomavirus particles seen in these warts as causal factors for the subsequent development of squamous-cell cancers of the skin (Jablonska *et al.*, 1972). A collaboration between the group in Poland and the group of Gérard Orth in Paris successfully demonstrated the presence of novel types of HPV, most frequently HPV 5, within epidermodysplasia verruciformis lesions and within biopsies of squamous-cell carcinomas from these patients. (Orth *et al.*, 1977, 1978, 1979).

Although HPV 5 represents the first HPV that is regularly detected in cutaneous squamous-cell cancers of these patients, the rarity of the syndrome, the difficulties in obtaining sufficient clinical materials for extensive studies and the absence of tissue culture lines from these carcinomas were probably the reasons for the somewhat limited interest in this condition. More than 25 years after the initial discovery of HPV 5 and related viruses, most questions relating to their etiological role and to the mechanism of their interaction in infected host cells in the course of carcinogenesis still remain open. Only in more recent years has the study of cutaneous HPV infections and their relationship to non-melanoma skin cancer in immunosuppressed and immunocompetent patients found increasing attention.

A fourth root of papillomavirus research resulted in the identification of specific HPV types as causative agents for cancer of the cervix, other anogenital cancers and a subset of oropharyngeal carcinomas. These investigations were initiated to investigate a viral etiology of cancer of the cervix. Techniques that were used for the detection of Epstein-Barr viral DNA in a 'virus-free' Burkitt lymphoma cell line (zur Hausen & Schulte-Holthausen, 1970) and in biopsies from Burkitt lymphomas and nasopharyngeal cancers (zur Hausen *et al*., 1970) were applied to cervical cancer in attempts to detect herpes simplex virus type 2 (HSV 2) DNA in these biopsies. By the end of the 1960s and the during the 1970s, serological studies had suggested a role of HSV 2 in this cancer (Rawls *et al*., 1968; Naib *et al*., 1969). The failure to find traces of HSV 2 DNA in these cancer biopsies prompted the search for other potential infectious candidates in the cause of this cancer, since its epidemiology provided good reasons to suspect an infectious etiology (zur Hausen, 1976).

A number of anecdotal reports of the malignant conversion of genital warts (condylomata acuminata) had appeared in the medical literature during the preceding 100 years and resulted in speculation on a possible causal role of HPV infections in cervical cancer that led to initial attempts to characterize the viral DNA in genital warts (zur Hausen *et al*., 1974, 1975; zur Hausen 1976, 1977). These and other studies led to the early discovery of the heterogeneity of the HPV family (Gissmann & zur Hausen, 1976; Orth *et al* 1977; Gissmann *et al*., 1977), which currently numbers more than 100 fully sequenced genotypes (de Villiers *et al*., 2004).

Meisels and Fortin (1976), Meisels *et al*. (1977) and Purola and Savia (1977) interpreted the koilocytotic lesions observed in what was considered to be a flat condyloma of the cervix as being the cytopathic effect of an HPV infection. At this time, they believed that these cellular modifications could be used to differentiate between 'benign'

virus-induced and premalignant 'virus-free' lesions. Della Torre *et al*. (1978) in Italy and Laverty *et al*. (1978) in Australia first demonstrated typical HPV particles in these condylomatous lesions of the cervix. In spite of their initial interpretation as markers for non-malignant progression of the respective lesions, these observations underlined the occurrence of HPV infections at cervical sites.

Although the eventual isolation of HPV DNA from genital warts (labelled as HPV 6; Gissmann & zur Hausen, 1980) and from laryngeal papillomas (HPV 11; Gissmann *et al.*, 1982) did not yield positive data for the causality of these viruses in cervical cancer, the use of their DNA in hybridization experiments, performed under conditions of reduced stringency, permitted the subsequent cloning of HPV 16 (Dürst *et al*., 1983) and HPV 18 (Boshart *et al*., 1984), the two HPV types most frequently found to date in cervical cancer. These findings led to a burst of activity in subsequent years. Among numerous other observations, these activities resulted in (*a*) the demonstration of a specific pattern of expression of the viral *E6* and *E7* genes in carcinoma tissues (Schwarz *et al*., 1985, Yee *et al*., 1985); (*b*) the finding that human keratinocytes are immortalized by high-risk HPVs that express the *E6* and *E7* genes (Dürst *et al*., 1987; Pirisi *et al*., 1987); (*c*) the discovery that E6 and E7 proteins interact with various cellular proteins, in particular with pRb and p53 initially (Dyson *et al*., 1989; Werness *et al*., 1990); (*d*) the direct demonstration that E6 and E7 proteins are responsible for the malignant phenotype of cervical carcinoma cells (von Knebel Doeberitz *et al*., 1992, 1994); and (*e*) large-scale epidemiological studies that identified high-risk HPV types as the major risk factor for cervical cancer (Muñoz *et al.*, 1992, 2003; Bosch *et al*., 1995).

Today, the practical consequences of these studies are increasingly apparent, since an increase of an order of magnitude in the quality of diagnostic approaches to validate early precursor lesions of cervical cancer and the development of preventive vaccines that can potentially prevent one of the major cancers in women are no longer unrealistic (reviewed in zur Hausen, 2002). The recent demonstration of the efficacy of virus-like particles in the prevention of persistent infection by HPV 16 in early precursor lesions of cervical cancer (Koutsky *et al*., 2002; Harper *et al*., 2004) has had a considerable impact on the development of prophylactic vaccines. Consequently, another cancer-preventive vaccine other than that for hepatitis B virus is now available (see Section 1.8).

It is probable that research on papillomaviruses will expand in the future; the role of these viral infections in at least some subsets of other anogenital and oropharyngeal cancers has become substantially more prominent over the past few years. In addition, the potential contribution to carcinogenesis (zur Hausen, 1999) of certain types of cutaneous HPV that prevent apoptosis in cells damaged by ultraviolet light (Thomas & Banks, 1998; Jackson *et al*., 2000) and/or target tumour-suppressor genes (Accardi *et al.*, 2006) has been hypothesized. Moreover, new perspectives have emerged for the prevention of these infections by the application of HPV testing technologies and vaccines.

Public health concerns

This volume of *IARC Monographs* provides a qualitative assessment of the carcinogenicity of HPVs and groups HPV types with regard to the strength of evidence of whether or not they cause cancer. However, there are evident and critically important differences in the absolute risk posed by individual HPV types within each class of carcinogen. Among the HPV viruses that have been classified as *carcinogenic to humans* (Group 1), the absolute risk for cancer associated with HPV 16 infection is of an order of magnitude higher than that for the weaker HPV types. Similar differences in risk are evident among the HPV types that have been classified as *probably* or *possibly carcinogenic to humans*. With regard to public health, it is important to comprehend that the term 'carcinogenic' is not uniform and must be interpreted carefully for each intended intervention. In the case of vaccination, it may be prudent to include all types of HPV that can be combined in an effective and affordable manner. However, in the case of screening, such a stratagem would have a seriously negative effect on clinical specificity and may mislead many women into believing that they are at high risk for cancers (Khan *et al.*, 2005; Schiffman *et al.*, 2005). When screening tests are applied to millions of women, a high ratio of false-:true-positive results is disconcerting: false-positive results in screening may lead to unnecessary colposcopies, biopsies and ablational/excisional treatments, which increases both health-care costs and morbidity (Sadler *et al.*, 2004).

Since the association between infection with HPV and the occurrence of cervical cancer has been well established, the sections in this monograph that cover cervical cancer are focused on an evaluation of the association between specific HPV types and this cancer. In these sections, a limited number of highly stringent techniques for the detection of HPV DNA were considered to be adequate to provide evidence of an association. For cancers at sites other than the cervix, the relationship with HPV infection was not so well established. Fewer studies have been conducted on the association between HPV infection and any of these cancers, and the number of cases reported is much smaller than that for cervical cancer. To enable a preliminary assessment of the association between HPV infection and cancers other than those of the uterine cervix, a wider variety of techniques and methods were considered to be acceptable for presentation in the respective sections.

Since the Working Group was convened in 2005, important innovations in HPV prophylaxis have occurered and these needed to be included in this volume (see Section 1.8). To date, two prophylactic vaccines have been developed and used 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). One of the vaccines is Gardasil® (produced by Merck and Co.) that protects against HPV types 6, 11, 16 and 18 (quadrivalent) and another is Cervarix® (produced by GlaxoSmithKline) that protects against types 16 and 18 (bivalent). The quadrivalent vaccine was licensed in the USA by the Food and Drug Administration (US FDA, 2006) on 8 June 2006 for use in women aged 9–26 years; the European Medicines Agency (EMEA, 2006) gave official authorization for the marketing of this quadrivalent vaccine in the European Union on 20 September 2006. An application

has also been placed before this Agency to licence the bivalent vaccine. This prophylactic vaccination is expected to reduce 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 is observed among persons who receive the quadrivalent vaccine and a reduction in laryngeal papillomatosis can be anticipated among their children (Arbyn & Dillner, 2007). As a consequence, it is anticipated that a reduction in morbidity and mortality from HPV-related anogenital diseases will occur in populations who received the available prophylactic vaccines. However, the benefits of prophylactic vaccines in a broad public health perspective will be achieved only if such vaccines can be provided to those groups of women for whom access to cervical cancer screening services is most problematic. Therefore, the development of second-generation vaccines that are expected to be cheaper, easier to deliver and/or to provide T-cell response against pre-existing HPV infections is highly desirable.

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MONOGRAPH ON
HUMAN PAPILLOMAVIRUSES

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*

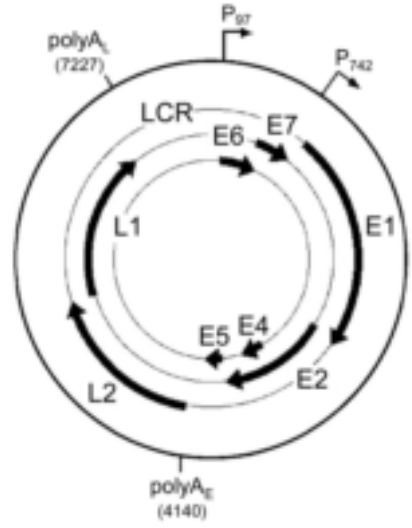
Papillomaviruses are small, non-enveloped, icosahedral DNA viruses that have a diameter of 52–55 nm. The viral particles consist of a single double-stranded DNA molecule of about 8000 base-pairs (bp) that is bound to cellular histones and contained in a protein capsid composed of 72 pentameric capsomers. 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).

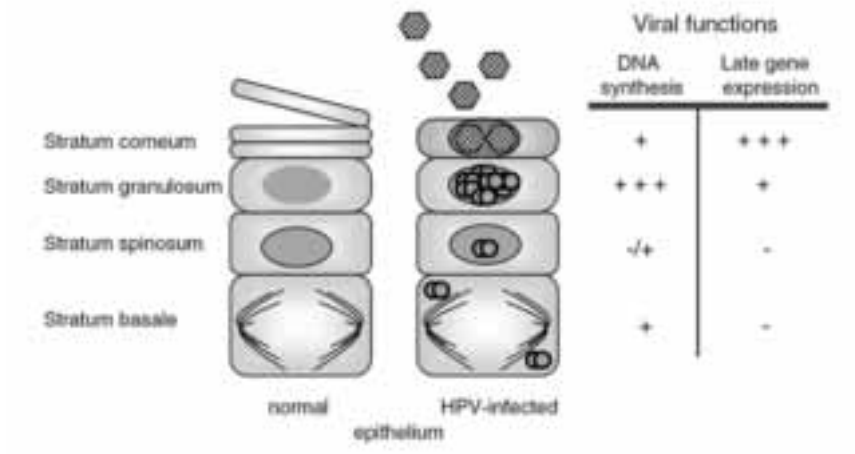
Figure 1. The genome of the high-risk HPV 31



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

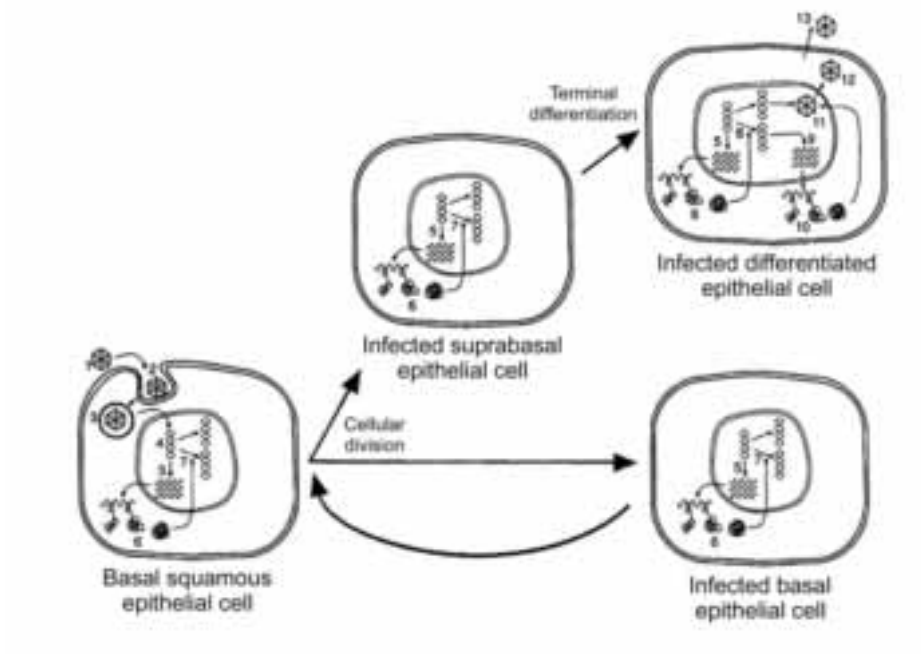


Modified from Fehrmann & Laimins (2003)
Normal and HPV-infected epithelia are compared, and differentiation-dependent viral functions are listed.

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

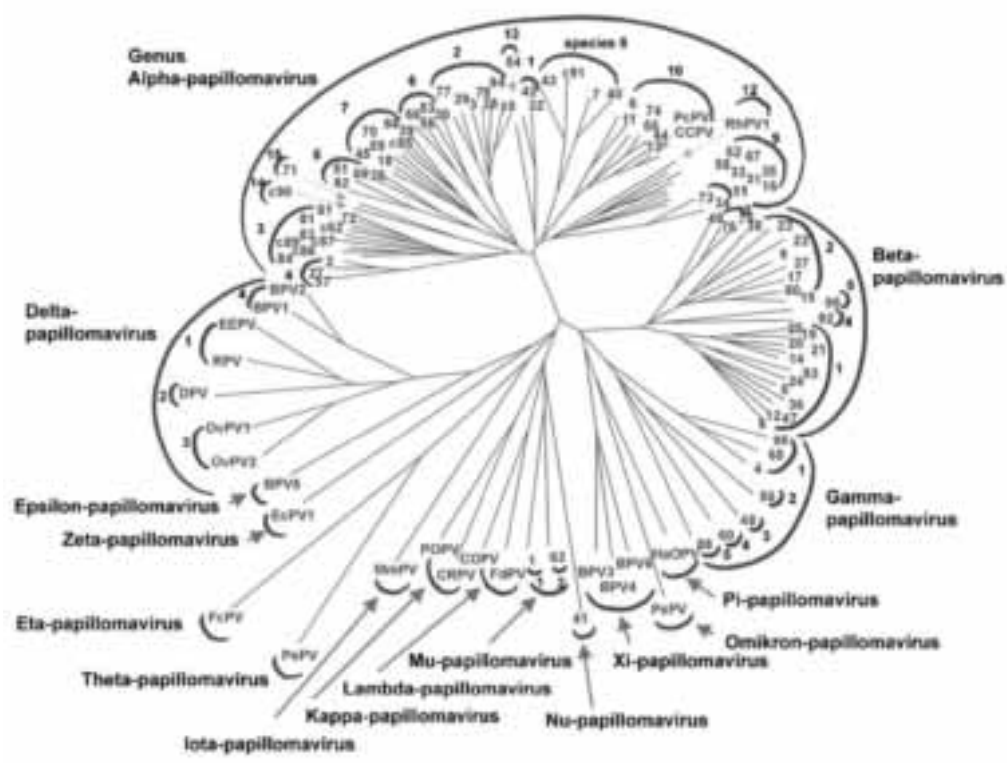
to the taxonomic groupings, which today are widely accepted. Phylogenetic assemblages occasionally coincide with biological and pathological properties, but often diverge. The closely related HPV types 2 and 27, 6 and 11, and 16 and 31, which cause common warts, genital warts and cervical cancer, respectively, 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)

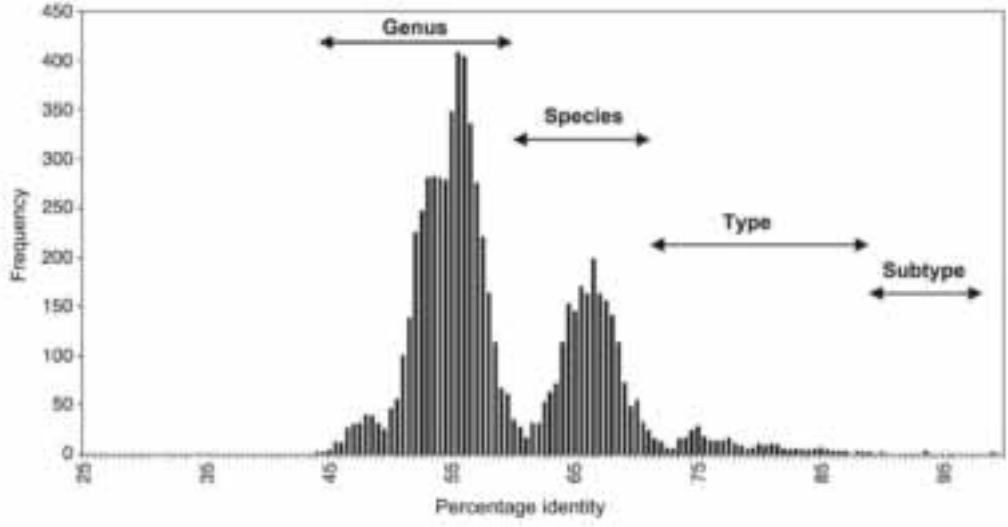
Genus	Species	Type species	Other papillomavirus types	Comments
	5	HPV 88	–	Cutaneous lesions
Delta-papillomavirus	1	EEPV (M15953)	RPV (AF443292)	E9 gene within ELR with transforming properties
	2	DPV (M11910)	–	E9 gene within ELR with transforming properties
	3	OvPV-1 (U83594)	OvPV-2 (U83585)	
	4	BPV-1 (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*

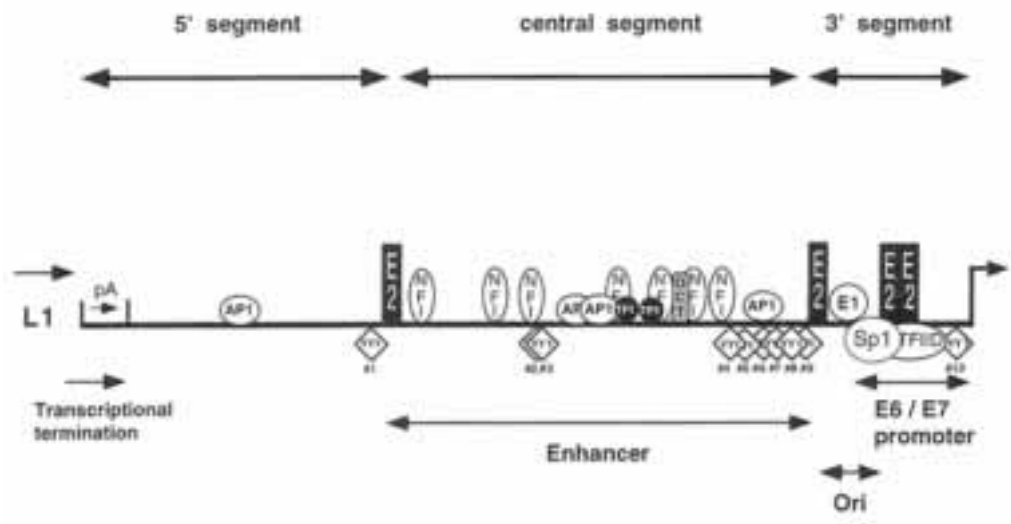
The regulation of gene expression in papillomaviruses is controlled by cellular and viral transcription factors, different promoters, differential splicing, differential transcription termination signals and the stability of different viral mRNAs. In order to be successful — from a viral point of view — 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).

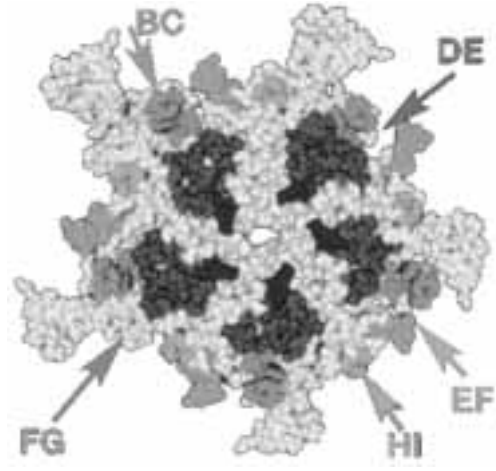
The discovery that L1 protein can assemble into VLPs that are structurally and immunochemically indistinguishable from authentic virions (Kirnbauer *et al.*, 1992; Hagensee *et al.*, 1994) has provided a valuable tool for the characterization of conformational HPV surface epitopes and strongly stimulated 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*

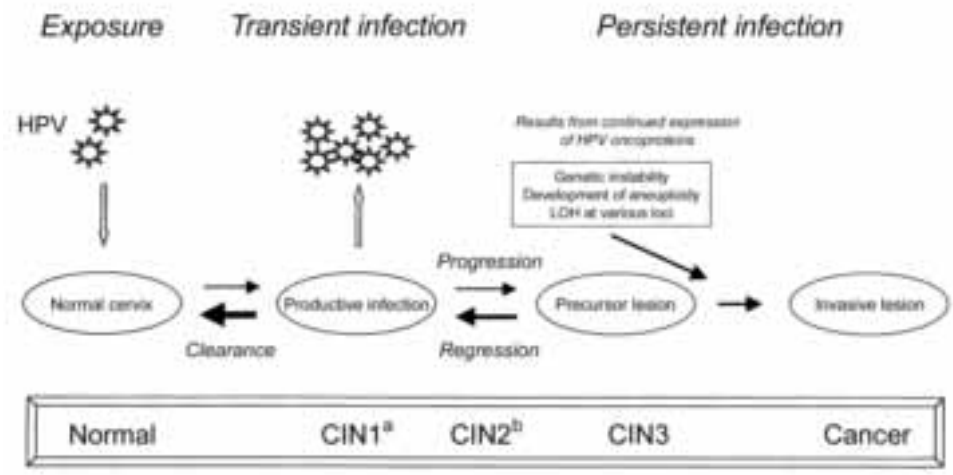
From studies that used linear epitopes as antigens in either peptide ELISA or western blot analysis, there is some indication that antibodies to E2 and E4 or some specific linear sequences of these proteins are associated with cervical cancer, but no consistent picture has emerged. As seen for antibodies 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

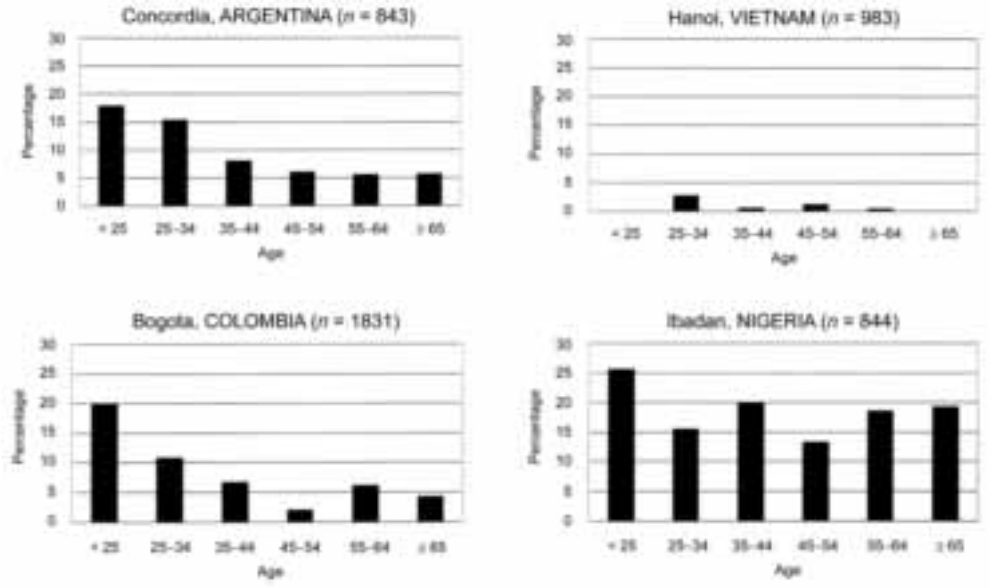
menarche does not appear to increase the risk for HPV infection (Collins *et al.*, 2005). The apparently limited protective role of condoms should be better estimated to guide the debate on this issue, and the possible role of susceptibility in the acquisition of multiple HPV types has not been assessed 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

cross-sectional virological and epidemiological profile (except for an earlier average age at diagnosis of CIN3) and demonstrate good histopathological reproducibility (Shah *et al.*, 1980; Walker *et al.*, 1983; Muñoz *et al.*, 1992, 1993). Therefore, CIN3 is a practical surrogate end-point for cervical cancer, although a proportion of cases of CIN3 regress rather than invade. However, in cohort studies, new diagnoses of small CIN3 lesions may represent diseases that were missed at the time of enrolment when HPV was assayed. This may lead to misclassification bias and spurious risk estimates.

While the choice of CIN3 is imperfect, less severe and more common grades of neoplasia, particularly CIN1, are clearly unreliable and are too closely linked to newly acquired infections with a broad range of HPV types to serve as surrogate end-points of cervical cancer. CIN2 is probably the result of a mixture of newly acquired infections and incipient CIN3; it is often treated, but can represent 'over-called' low-grade lesions.

Virologically, the persistence of HPV for several years cannot be used as an accurate surrogate of type-specific carcinogenicity because persistence is a necessary but not sufficient characteristic of carcinogenicity (Ho *et al.*, 1995; Nobbenhuis *et al.*, 1999).

(*b*) *Impact of study design*

When an etiological fraction of cervical cancers that can be attributed to a specific HPV type is small, it is more difficult to conduct prospective studies because of limitations of statistical power. At present, type-specific prospective evidence of carcinogenicity is readily available for HPV 16 and, to a lesser extent, for HPV 18. Because of the latency between average age at first HPV infection (late teens to early twenties) and average age at diagnosis of CIN3 (approximately 25–30 years of age), large longitudinal cohorts are only now attaining sufficient follow-up time to permit a reasonable assessment of a few additional HPV types. To overcome this limitation, some of the longest-term studies published to date have been based on HPV typing of archived slides from screening programmes, using a nested case–control approach. However, techniques for assaying the full spectrum of HPV types in these old specimens have not been fully validated. Since each individual HPV type is relatively uncommon, most prospective studies have combined all putative carcinogenic HPV types to assess the possible clinical utility of a pooled-probe HPV test.

As a result, epidemiologists often rely on cross-sectional designs to estimate the risk for individual HPV types. Estimation of the absolute risk, incidence rate and even the lifetime cumulative incidence rate of cervical cancer among infected compared with uninfected women and among women infected with each type of HPV alone would be ideal. The risk associated with each HPV type could then be estimated with adjustment for potential confounding due to co-infection with other HPV types. However, the correct estimation of these risks would require lifetime longitudinal follow-up of huge cohorts of women, while cross-sectional designs that use prevalence risk estimates suffer from unavoidable limitations due to the lack of a reliable measurement of lifetime exposure to HPV infection.

Case–control designs typically rely on the assessment of HPV DNA at the time of diagnosis for cases and at a similar age for controls. Since persistence of HPV DNA is a hallmark of cervical cancer/CIN3, the vast majority of cases are found to be HPV DNA-positive. In contrast, the low prevalence in controls reflects both recently acquired infections and an unknown, small fraction of infections from previous years (most of which proved to be transient after 1–2 years).

(*c*) *Problem of multiple infections*

HPV 16 and HPV 18 were classified previously as Group 1 carcinogens (IARC, 1995). In an assessment of whether additional types are also carcinogenic, possible confounding must be taken into account because different HPV types are frequently co-transmitted sexually. Multiple infections (i.e. infections with more than one HPV type) have been found in more than 25% of infected women in many surveys, but available polymerase chain reaction (PCR) assays are less sensitive and reproducible for the detection of multiple-type rather than single-type infections. When considering the possible carcinogenicity of a specific HPV type, any association with cancer due to co-infection with HPV 16 or HPV 18 must be ruled out. Possible strategies to address this type of confounding include the exclusion of all HPV 16- or HPV18-infected individuals, stratification for HPV type or group and multivariate statistical modelling. However, these strategies are constrained by the need to investigate concurrently approximately 40 relatively rare anogenital types of HPV. Thus, even very large studies typically lack statistical power to evaluate all combinations adequately. This problem is most apparent when assessing the possible carcinogenicity of uncommon types that occur mainly in combination with other HPV types.

(*d*) *Choice of method for HPV testing*

DNA testing is the reference standard for the detection of current HPV infection. There is a wealth of evidence from case–control studies that putative carcinogenic types assessed as a pooled group are associated with an increase in the risk for invasive cancer and CIN3. The collective strength of this evidence led the Food and Drug Administration in the USA to license Hybrid Capture 2, which allows the detection of 13 high-risk and five low-risk HPV types, as an adjunctive screening method. Similarly, several studies have employed PCR-based methods that still pool putative carcinogenic types, which prevents individualized assessment of the carcinogenicity of specific types. Thus, the assessment of type-specific carcinogenicity relies exclusively on studies that employ PCR with probes for individual types. However, each PCR-based system has selective differential sensitivity for individual HPV types; this could affect the risk estimates because of differential misclassification of cases and controls. Infections in cases result in lesions that are the site of viral replication, and tend to have higher viral loads than infections that do not cause obvious lesions such as those that occur in controls. Thus, a PCR system with relatively low sensitivity for a given HPV type tends to detect infections of that type

differentially in cases compared with controls, and thus overestimates the odds ratio for that specific type.

Serological data have yielded useful information for the assessment of exposure to HPV. HPV serology based on virus-like particles (VLPs) is a relatively type-specific but insensitive measure of exposure. Therefore, seropositive women appear to have been truly exposed to HPV, although the anatomical site of infection cannot be ascertained. Extremely large archives of serum specimens have permitted nested case–control studies of cervical cancer and CIN3 with an exceptional statistical power that is currently lacking in studies of DNA. Serology is included here to define HPV-exposed study populations for the consideration of etiological co-factors such as tobacco smoking or *Chlamydia trachomatis*. Finally, serology is discussed with reference to sites other than the cervix for which valid comprehensive DNA sampling is problematic.

(*e*) *Heterogeneity of definitions of initial cytomorphology*

Cohort and case–control studies have previously emphasized the distinction among HPV-infected women between those with normal versus mildly abnormal cytology. However, different types of HPV infection cause overlapping and pleiomorphic cellular changes that are sometimes pathognomonic (e.g. koiloctytotic atypia) but are often equivocal or lacking. Moreover, the interpretations of mild and equivocal HPV-related cytology differ greatly between geographical regions and assessors. A normal Papanicolaou (Pap) test in one geographical region might be called equivocal or even a low-grade squamous intraepithelial lesion (LSIL) in another region (Scott *et al.*, 2002a). Thus, for epidemiological studies to assess HPV type-specific carcinogenicity, a necessary requirement is that the study subjects are tested for a specific type of HPV and that cases have a confirmed diagnosis of CIN3 or cancer. For type-specific analyses, it is not necessary to focus excessively on the subtler, variable issues of whether control or cohort subjects had completely normal cytology.

(*f*) *HPV types, cervical cancer and cancers at other sites*

Since the association between HPV and cervical cancer is well established, the sections on cervical cancer focus on evaluating specific HPV types. In these sections, a limited number of highly stringent HPV DNA detection techniques were considered to be adequate to provide evidence of an association. For cancers at sites other than the cervix, their relationship with HPV is not well established. There are fewer studies on the association between HPV and cancers at sites other than the cervix, and the number of cases reported is much smaller. To allow for a preliminary assessment of the association between HPV and these cancers, a wider variety of techniques and methods were considered to be acceptable for presentation in their respective sections.

2.2 Cancer of the cervix

2.2.1 *Historical perspective*

Early studies on HPV and cervical cancer reported largely on HPV 16 and 18, which were the first two cancer-associated types that were isolated and used to design the initial testing systems (IARC, 1995). Developments in the technology used in large epidemiological and clinical studies evolved in two directions. Clinically designed testing systems generated cocktails of HPV probes, with the understanding that the individual risk for any HPV type of the high-risk group was clinically equivalent. Typically, these studies reported on the presence or absence of HPV DNA of the high-risk or low-risk cocktails (Peyton *et al.*, 1998; Vernon *et al.*, 2000; Castle *et al.*, 2002b). Research-oriented testing systems developed type-specific procedures and these were used to refine the understanding of type-specific risk and to make advancements in studies of HPV transmission, in the definition of HPV persistence and in investigations of HPV DNA at other organ sites (Manos *et al.*, 1989; Jacobs *et al.*, 2000).

As described in Section 1, the ability to identify multiple types of HPV in one specimen and the presence of some cross-reactivity has introduced some additional variability in the interpretation of the available data. The literature that related HPV and HPV types to cervical cancer up to 1994–95 was reviewed previously (IARC, 1995). It was concluded that there was sufficient evidence for the carcinogenicity of HPV types 16 and 18 (Group 1), HPV types 31 and 33 were classified as probably carcinogenic to humans (Group 2A) and an undefined group of other HPVs were evaluated as possibly carcinogenic to humans (Group 2B). Some evidence was suggestive that the same association existed for other HPV types, although the number of studies was limited. The only correlation observed between HPV type and clinical outcome was an increased relative frequency of HPV 18 in cervical adenocarcinoma compared with the more common squamous-cell carcinoma.

The case–control study in Spain and Colombia coordinated by IARC was instrumental in showing highly significant and high odds ratios with three different HPV DNA testing methods, namely the southern blot hybridization procedure, which was considered as the standard at the time, the first version of a testing cocktail intended for clinical use (Virapap) and the initial PCR systems based on the MY09/11 primers (Bosch *et al.*, 1992; Muñoz *et al.*, 1992). HPV type-specific risk estimates were provided for HPV 16, 18, the combination of HPV 31, 33 and 35 and for unidentified HPV types (Bosch *et al.*, 1992; Muñoz *et al.*, 1992; IARC, 1995). The high prevalence of HPV DNA among cases triggered intense research into the viral status of the apparently HPV-negative cases. Stringent laboratory analyses of case series of cervical cancer led to the conclusion that HPV is a necessary cause of cervical cancer (IARC, 1995; Walboomers *et al.*, 1999). These prompted analyses restricted to HPV-positive women for the evaluation of other risk factors. The study also showed that the risk factor profiles of the apparently HPV-negative and HPV-positive cervical cancer cases were notably similar, that the risk profile was identical for the established pre-invasive and invasive conditions and that the results

were consistent in two countries with a contrasting incidence of cervical cancer (Bosch *et al.*, 1992; Muñoz *et al.*, 1993; Moreno *et al.*, 1995).

2.2.2 *Data on pooled HPV types*

(*a*) *Cross-sectional studies and studies with short-term follow-up*

(i) *Risk for ≥ CIN2/3 from primary screening data*

Table 22 presents data from primary cervical cancer screening studies on the association between HPV positivity and the risk for ≥ CIN2/3. In some studies, verification of high-grade disease by colposcopy and histology was restricted to women who had positive cytological or HPV test results. In particular, all women screened in three studies were verified independently of screen test results and were therefore theoretically free from verification bias (Belinson *et al.*, 2001b; Blumenthal *et al.*, 2001; Sankaranarayanan *et al.*, 2004b) whereas verification bias was at least partially corrected in the statistical analyses in two studies (Schneider *et al.*, 2000; Kulasingam *et al.*, 2002).

Assessment of outcome is potentially hampered by the misclassification that is inherent to the use of an imperfect diagnostic gold standard. Nevertheless, relative risks associated with HPV status can be computed with acceptable reliability as the ratio of the risk for ≥ CIN2/3 in HPV-infected versus non-HPV-infected subjects. This relative risk is equivalent to the ratio of positive predictive value over the complement of the negative predictive value (relative risk = positive predictive value/(1-negative predictive value)).

The relative risk for ≥ CIN2 that is associated with HPV positivity varied from 5.6 (Blumenthal *et al.*, 2001) to 256 (Cuzick *et al.*, 1999). In one study in which verification bias was not taken into account, the relative risk was infinite (Clavel *et al.*, 2001) since the Hybrid Capture 2 result predicted all detected cases of ≥ CIN2. The relative risk was higher when the outcome of ≥ CIN3 was considered, and varied from 27 (Kulasingam *et al.*, 2002) to 530 (Petry *et al.*, 2003).

(ii) *Triage of women with atypical squamous cells of undetermined significance (ASCUS) or LSIL*

Using a meta-analytical approach, Arbyn *et al.* (2002, 2004a,b, 2005) documented the diagnostic performance of two management options for women with an equivocal Pap smear to detect women who need follow-up — reflexive high-risk HPV DNA testing versus repeat cytology. Two different triage groups were considered: (*a*) women with equivocal Pap smears, reported as ASCUS or borderline; and (*b*) women with LSIL or mild dyskaryosis. In these meta-analyses, diagnostic accuracy for the outcome of histologically confirmed CIN2 or CIN3 or worse was the focus of interest. The selection of studies considered was restricted to those in which ≥ CIN3 was the reported outcome and sensitive HPV DNA detection systems were used such as the Hybrid Capture 2 and PCR tests. Relative risks were computed as for the primary screening data.

The inter-study variation of the relative risks and the pooled measures are displayed graphically by the forest plots in Figures 10 for ASCUS and 11 for LSIL and are further

Table 22. Association between ≥ CIN2/3 and HPV status in women participating in cervical cancer screening

Reference, study location	Method of detection	Outcome	No. of women	Test posivity rate	Proportion of ≥ CIN2/3		Relative risk[a]	Verification bias
					HPV+	HPV–		
Cuzick *et al.* (1999), United Kingdom	HC2[b]	≥ CIN2	1703	0.073	0.160	0.001	255.84	Not corrected
Schiffman *et al.* (2000), Costa Rica	HC2	≥ CIN2	8554	0.139	0.102	0.002	45.09	Not corrected
		≥ CIN3	8554	0.075	0.096	0.001	109.05	Not corrected
Schneider *et al.* (2000), Germany	HC2[b]	≥ CIN2	4761	0.104	0.204	0.003	68.90	Partially corrected
Wright *et al.* (2000), South Africa	HC2	≥ CIN2	1365	0.208	0.137	0.007	19.36	Not corrected
Belinson *et al.* (2001b), China	HC2	≥ CIN2	1997	0.228	0.180	0.002	73.35	None
Blumenthal *et al.* (2001), Zimbabwe	HC2	≥ CIN2	2073	0.429	0.189	0.034	5.59	None
Clavel *et al.* (2001), France	HC2	≥ CIN2	5671	0.134	0.094	0.000	939.20[c]	Not corrected
Kulasingam *et al.* (2002), USA	HC2	≥ CIN2	4075	0.219	0.096	0.015	6.28	Partially corrected
		≥ CIN3	4075	0.279	0.018	0.001	27.31	Partially corrected
Salmerón *et al.* (2003), Mexico	HC2	≥ CIN2	7732	0.094	0.129	0.001	130.86	Not corrected
		≥ CIN3	7732	0.079	0.120	0.001	215.76	Not corrected
Petry *et al.* (2003), Germany	HC2	≥ CIN3	7592	0.047	0.073	0.000	530.44	None corrected

Table 22 (contd)

Reference, study location	Method of detection	Outcome	No. of women	Test posivity rate	Proportion of ≥ CIN2/3		Relative risk[a]	Verification bias
					HPV+	HPV–		
Sankaranarayan *et al.* (2004b), India	HC2	≥ CIN2	18085	0.070	0.128	0.005	28.32	None
		≥ CIN3	18085	0.070	0.089	0.002	55.27	None

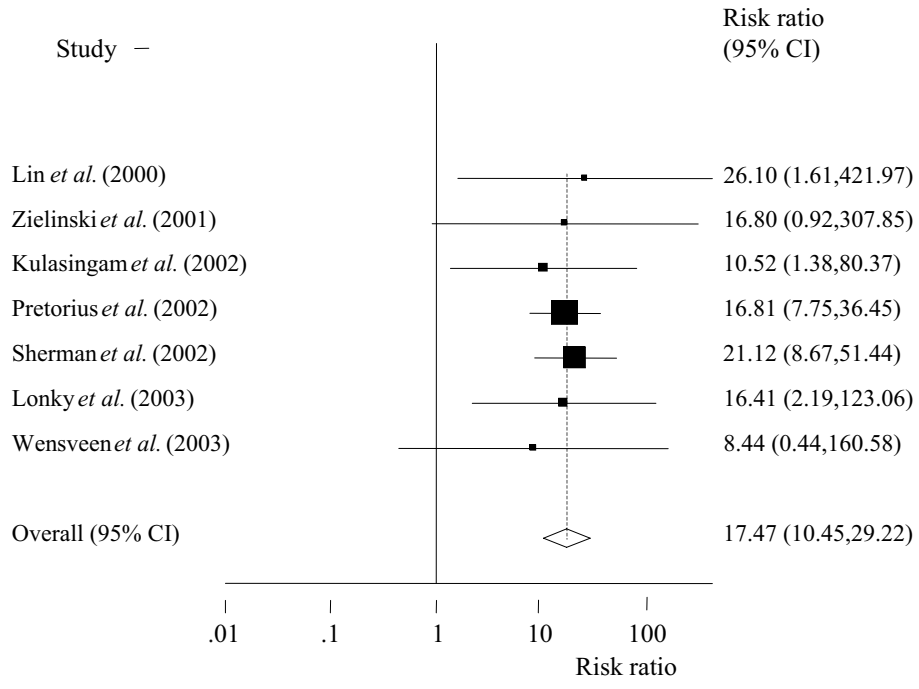
CIN, cervical intraepithelial neoplasia; HC2, Hybrid Capture 2: targets HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68; +, positive; –, negative

[a] Relative risks were calculated by the Working Group using data from Lörincz and Richart (2003), IARC (2005) and from the original publications or requested directly from the authors.

[b] PCR system that includes identification of 14 types (13 types as in HC2 + HPV 66)

[c] RR computed using Yates correction, by adding 0.5 to each cell of the 2 × 2 contingency table. This correction is required for studies where the risk for ≥ CIN2/3 in the HPV-negative group is zero. Risk for ≥ CIN2/3 if HPV positive corresponds with the positive predictive value of HPV testing for the presence of underlying ≥ CIN2/3. Risk for ≥ CIN2/3 if HPV negative corresponds with 1–negative predictive value.

Figure 10. Meta-analysis of the prediction of histologically confirmed ≥ CIN3 in women with an index Pap smear that showed ASCUS: relative risk for HPV-positive women versus HPV-negative women

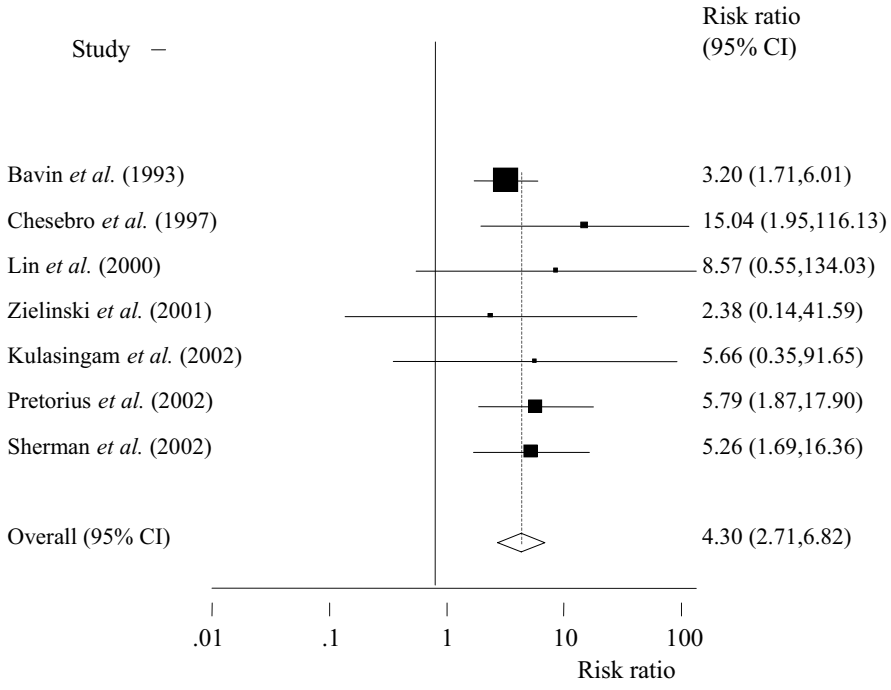


The meta-analysis is restricted to studies in which Hybrid Capture 2 or sensitive PCR was used to detect HPV DNA.

ASCUS, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; Pap, Papanicolaou test

documented in Tables 23 and 24, respectively. Overall, HPV-positive women with ASCUS from seven pooled studies had a risk for ≥ CIN3 that was 17.47 times (95% confidence interval [CI], 10.45–29.22) higher than that of HPV-negative women (Figure 10). The relative risk was substantially lower in LSIL triage settings (4.30; 95% CI, 2.71–6.82) (Figure 11). Relative risks were pooled using random-effect meta-analytical models (Dersimonian & Laird, 1986; Sutton *et al.*, 2000). The risk for ≥ CIN3 in some studies was equal to zero in the HPV-negative group, which yielded a relative risk of infinity (∞). Such studies cannot be incorporated into a meta-analysis. For these studies, 0.5 was added to the nominator and 1 to the denominator in both the HPV-negative and HPV-positive groups. This correction yielded a considerable underestimate of the relative risk, especially in small studies. Nevertheless, the underestimation in the pooled relative risk was generally smaller than when studies with a relative risk of ∞ should have been discarded.

Figure 11. Meta-analysis of the prediction of histologically confirmed ≥ CIN3 in women with an index Pap smear showing LSIL; relative risk for HPV-positive women versus HPV-negative women



CIN, cervical intraepithelial neoplasia; LSIL, low-grade squamous intraepithelial lesion; Pap, Papanicolaou test

Most triage studies were cross-sectional in design or involved a follow-up time that only lasted from the assessment of HPV status to the verification of outcome. The largest triage study (Castle *et al.*, 2005) included 5060 women who had ASCUS or LSIL. Oncogenic HPV-positive women who had ASCUS or LSIL had a 2-year absolute risk for CIN3 of approximately 15% or 17%, respectively. Women who had ASCUS or LSIL cytology who were HPV 16 DNA-positive at baseline had a 2-year cumulative absolute risk for ≥ CIN3 of 32.5% (95% CI, 28.4–36.8%) and 39.1% (95% CI, 33.8–44.7), respectively, thus the risk estimates seemed to be substantially lower than the cross-sectional relative risks. The difference in cross-sectional and longitudinal cumulative relative risks might indicate that cross-sectional studies overestimate a surplus of prevalent disease which ultimately regresses. This time-dependent effect was also observed in large cohort studies that focused on the natural history of HPV infection and precancerous cervical lesions (Liaw *et al.*, 1999; Kjaer *et al.*, 2002; Schlecht *et al.*, 2003c).

Increased risk for severe dysplasia was associated with a high risk for ≥ CIN2. The largest contribution to the relative risk derives from HPV 16 infection (Castle *et al.*, 2005)

Table 23. Triage of ASCUS: short-term outcome of ≥ CIN3 in high-risk HPV-positive versus HPV-negative women with ASCUS

Reference	Follow-up period	HPV test method	HPV types targeted	No. of women	Test posivity rate	Proportion of ≥ CIN3		Relative risk	Relative risk[a]
						HPV+	HPV–		
Lin, C.-T. *et al.* (2000)[b]	Cross-sectional	HC2	13 HR types (2)	74	0.527	0.359	0.000	∞	26.1
Zielinski *et al.* (2001a)[b]	0–4.5 years	HC2	13 HR types (2)	213	0.347	0.054	0.000	∞	16.8
Kulasingam *et al.* (2002)	Nested within primary screening setting, short follow-up	HC2	13 HR types (2)	270	0.511	0.080	0.008	10.5	
Pretorius *et al.* (2002)	Short follow-up	HC2	13 HR types (2)	949	0.322	0.183	0.011	16.8	
Sherman *et al.* (2002)	RTS, short follow-up	HC2	13 HR types (2)	2198	0.540	0.104	0.005	21.1	
Lonky *et al.* (2003)	Short follow-up	HC2	13 HR types (2)	278	0.460	0.109	0.007	16.4	
Wensveen *et al.* (2003)[b]	Cross-sectional	HC2	13 HR types (2)	148	0.453	0.045	0.000	∞	8.4

ASCUS, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; HC2, Hybrid Capture 2; HR, high-risk; RTS, randomized triage study; +, positive; –, negative

[a] Relative risk corrected by adding 0.5 to each cell that contributes to the computation of the relative risk

[b] With Yates correction (+ 0.5)

Table 24. Triage of LSIL: short-term outcome of ≥ CIN3 in high-risk HPV-positive versus high-risk HPV-negative women with ASCUS

Reference	Follow-up period	HPV test method	HPV types targeted	No. of women	Test posivity rate	Proportion of ≥ CIN3		Relative risk	Relative risk[a]
						HPV+	HPV–		
Bavin *et al.* (1993)	Cross-sectional	PCR[b]	HPV 16	179	0.374	0.343	0.107	3.2	
Chesebro *et al.* (1997)	Cross-sectional	HC2	9 HR types (1)	159	0.799	0.134	0.009	15.0	
Lin *et al.* (2000)[a]	Cross-sectional	HC2	13 HR types (2)	45	0.756	0.353	0.000	∞	8.6
Zielinski *et al.* (2001a)[a]		HC2	13 HR types (2)	65	0.800	0.077	0.000	∞	2.4
Kulasingam *et al.* (2002)[a]	Nested within primary screening setting, short follow-up	HC2	13 HR types (2)	125	0.832	0.125	0.000	∞	5.7
Pretorius *et al.* (2002)	Short follow-up	HC2	13 HR types (2)	283	0.763	0.259	0.045	5.8	
Sherman *et al.* (2002)	RTS, short follow-up	HC2	13 HR types (2)	849	0.848	0.122	0.023	5.3	

ASCUS, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; HC2, Hybrid Capture 2; HR, high-risk; LSIL, low-grade squamous intraepithelial lesion; PCR, polymerase chain reaction; RTS, randomized triage study; +, positive; –, negative

[a] With Yates correction (+ 0.5)

[b] PCR targeting HPV 16 (medium/high signal)

(see Table 25). In HPV 16-positive women with ASCUS, the risk for ≥ CIN2 within 2 years was 16.1 (95% CI, 12.0–21.7) times higher than that in high-risk HPV-negative women. Positivity for other high-risk types was associated with a relative risk of 6.1 (95% CI, 4.5–8.3), which was similar to that associated with ASCUS that was unqualified by HPV. The relative risk associated with HPV positivity was lower in LSIL patients than in ASCUS patients, but was significantly higher when women were infected with HPV 16 compared with women infected with other high-risk HPV types (Castle *et al.*, 2005).

Table 25. Two-year cumulative risk for ≥ CIN2 according to initial high-risk HPV status (positivity for HPV 16 and for other high-risk HPV types) in women with ASCUS or LSIL compared with high-risk HPV-negative women

	No.	Absolute risk (%)	Relative risk	95% CI
ASCUS HC2-negative	1559	3.0	1.0	–
All ASCUS	3488	15.3	5.1	3.8–6.8
ASCUS HPV16-positive	443	48.5	16.1	12.0–21.7
ASCUS other high-risk HPV-positive, HPV16-positive	1245	18.4	6.1	4.5–8.3
LSIL HC2-negative	237	8.4	1.0	–
All LSIL	1572	25.4	3.0	2.0–4.1
LSIL HPV 16-positive	310	51.1	6.1	3.9–8.2
LSIL other high-risk HPV-positive, HPV 16-positive	931	22.7	2.7	1.7–3.7

Adapted from Castle *et al.* (2005)
ASCUS, atypical squamous cells of undetermined significance; CI, confidence interval; CIN, cervical intraepithelial neoplasia; HC2, Hybrid Capture 2; LSIL, low-grade squamous intraepithelial lesion

(*b*) *Prospective studies*

Since the previous review (IARC, 1995), a few large prospective studies have shown that HPV infection, as assessed by DNA testing for a group of putative high-risk types, predicted an increased risk for subsequent development of CIN3 or invasive cancer. Although such studies do not add to the assessment of type-specific carcinogenicity, they are noted for completeness.

Nobbenhuis *et al.* (1999) conducted a follow-up study of 353 women aged 18–55 years who had been referred because of cervical abnormalities for a median of 33 months (range, 2–72 months) without taking any biopsies until the clinical appearance of ≥ CIN3 or until the end of study. Two hundred and ninety-seven women (87%) had mild or moderate dyskaryosis at baseline; among them, 182 (61%) were high-risk HPV-positive (defined as harbouring one or more of the following types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59,

66 or 68, using PCR with GP5+/6+ primers). Sixty-nine (31.9%) of these high-risk HPV-positive women developed ≥ CIN3 compared with only three (2.6%) of the 115 women that were high-risk HPV-negative at baseline, corresponding to a relative risk of 14.5 (95% CI, 4.7–44.8). A longer persistence of high-risk HPV-positivity was associated with an increase in the relative risk for the development of CIN3. Two of the three initially high-risk HPV-negative cases who developed CIN3 acquired HPV types during follow-up, which subsequently persisted until assessment of the outcome.

Using MY09/11 PCR with TaqGold followed by dot-blot detection of the 13 types targeted by Hybrid Capture 2, Ferreccio *et al.* (2003) examined the association of histologically confirmed CIN3 that occurred within 2 years and cancer that occurred within 7 years among 8551 women in Guanacaste, Costa Rica, who represented a mixed prevalent/incident case group. Ninety cases of CIN3 (mean age, 36.9 years) and 20 cases of invasive cancer (mean age, 43.4 years) were detected by multi-technique screening and not from symptoms. The cumulative incidence among HPV-positive women (unadjusted for loss to follow-up) was 8.6% while the comparable incidence among HPV-negative women was only 0.2%.

Sherman *et al.* (2003b) performed a 10-year follow-up of 20 810 women (mean age, 35.9 years) who were screened with a Pap smear and HPV testing from 1989 to 1999 at the Kaiser Permanente Center, Portland, USA. Among 171 women who had CIN3 or cancer diagnosed during the follow-up period, 123 (71.9%) had baseline Pap results of atypical squamous cells or worse and/or a positive HPV test, 102 (86.4%) of whom were diagnosed within the first 45 months of follow-up. During this 45-month period, the cumulative incidence of CIN3 or cancer was 4.54% among women with a Pap test result of atypical squamous cells or worse, positive HPV tests or both compared with 0.16% among women with negative Pap and HPV tests; thus, negative baseline Pap and HPV tests were associated with a lower risk for CIN3 or cancer in the subsequent 45 months.

Clavel *et al.* (2004) followed 4401 cytologically negative women for a median period of 34 months primarily to estimate the negative predictive value of Hybrid Capture 2-negativity for histologically confirmed CIN2 or CIN3 (combined). Five cases were observed and none was Hybrid Capture 2-positive at enrollment.

2.2.3 *Data on type-specific HPV*

(*a*) *Case series*

The cervix uteri of women with normal Pap smears or with mild cytological abnormalities (e.g. LSIL) harbour a broad spectrum of HPV types. Herrero *et al.* (2000) tested 3024 women in Guanacaste, Costa Rica, for 40 different HPV types and detected 34 different HPV types in women with normal cytological findings or LSIL. Franceschi *et al.* (2005) tested 1891 women in Dindigul District, India, for 44 different HPV types and detected 36 different HPV types in either single- or multiple-type infections among women with normal cytological findings or LSIL.

As the severity of cervical lesions increases, not only does the overall prevalence of HPV rise greatly, but the relative frequency of different HPV types also changes substantially. This 'enrichment' of certain HPV types, together with the depletion of others across the spectrum of cervical neoplasias, is well illustrated by the findings of three large systematic reviews carried out at the IARC on the distribution of HPV types in LSIL (8308 women from 50 studies; Clifford *et al.*, 2005), high-grade squamous intraepithelial lesions (HSIL; 4338 women from 52 studies; Clifford *et al.*, 2003a) and squamous-cell cervical carcinoma (10 058 women from 85 studies; Clifford *et al.*, 2003b).

The three IARC reviews were carried out according to the same protocol: articles that included HPV type-specific prevalence data were identified and key information (e.g. country of sample, sample size, type of cervical specimen and PCR primers used to detect HPV-positive samples) was extracted. Published findings did not generally allow the distinction of single-type from multiple-type infections and, therefore, the prevalence of each individual HPV type was evaluated independently of whether other types were detected. The three reviews were limited to studies that (*a*) included a minimum of 20 cases of LSIL, HSIL or cervical cancer and (*b*) reported type-specific prevalence of at least one HPV type other than HPV 6, 11, 16 or 18. When study methods suggested that additional type-specific data were available, these data were requested from the authors. All five continents were represented, although to varying extents. Other case series that described the distribution of HPV types in invasive cancer have been published since the IARC systematic review, including some in previously unstudied populations (Cuzick *et al.*, 2000; Bachtiary *et al.*, 2002; Dybikowska *et al.*, 2002; Mortazavi *et al.*, 2002; Nakagawa *et al.*, 2002; Pegoraro *et al.*, 2002; Alonio *et al.*, 2003; Gao *et al.*, 2003; Hwang *et al.*, 2003; Kay *et al.*, 2003; Plunkett *et al.*, 2003; Rabelo-Santos *et al.*, 2003; Stanczuk *et al.*, 2003; Tran-Thanh *et al.*, 2002; Tsuda *et al.*, 2003; Widschwendter *et al.*, 2003; Xi *et al.*, 2003; Silins *et al.*, 2004; Schellekens *et al.*, 2004).

Overall HPV prevalence was 71% among LSILs, 84% among HSILs, 88% among squamous-cell carcinomas and 77% among cervical adeno- or adenosquamous carcinomas. HPV types in Table 26 were grouped into: (*a*) HPV 16 and 18 (i.e. the types most frequently detected in cervical cancer worldwide); (*b*) other high-risk or probably high-risk types, 11 of which (i.e. HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) are currently included in the Hybrid Capture 2 DNA test approved by the US Food and Drug Administration as an adjunct to primary cytological screening and for triage of women with equivocal cytology (Wright *et al.*, 2004), and five types (i.e. HPV 26, 53, 66, 73 and 82) that have been considered for inclusion in the HPV DNA test (Muñoz *et al.*, 2003); and (*c*) the three most common low-risk HPV types (i.e. HPV 6, 11 and 70).

Figure 12 gives a graphical representation of the data of Table 26.

HPV 16 was 2.5 times more prevalent in HSIL than in LSIL. Other types showed either a similar prevalence in LSIL and HSIL or a substantially higher prevalence in LSIL than in HSIL. Most importantly, HPV 16 and 18 were found three- and two times, respectively, more frequently in squamous-cell carcinoma than in LSIL, whereas HPV 26, 39, 51, 56 and 73 were at least 10-times and HPV 53 and 66 were approximately 30-times more

Table 26. Distribution of HPV types across cervical lesions of increasing severity

HPV type	LSIL		HSIL		ADC		SCC		LSIL: SCC ratio	LSIL: ADC ratio
	No.	%	No.	%	No.	%	No.	%		
16	8308	18.7	4338	45.0	1464	31.3	8594	54.3	0.5	0.8
18	8308	6.1	4338	7.1	1455	37.7	8502	12.6	0.7	0.2
Other high-risk or possibly high-risk										
31	8155	8.2	4036	8.8	1090	1.7	7204	4.2	2.7	6.8
33	8078	5.3	4302	7.2	1331	0.9	8449	4.3	1.7	8.2
35	6395	4.3	2690	4.4	985	0.8	6223	1.0	5.7	7.1
39	4301	5.8	1841	1.1	716	0.1	3899	0.4	19.0	76.0
45	4748	3.7	2214	2.3	755	5.8	5174	4.2	1.2	0.8
51	4721	8.0	2171	2.9	693	0.1	4580	0.6	17.7	106.0
52	4380	6.7	2153	5.2	757	0.5	5304	2.5	3.6	18.0
56	4431	7.2	2110	3.0	693	0.0	4493	0.7	13.6	
58	4498	6.3	2175	6.9	811	0.5	5646	3.0	2.8	16.8
59	4281	4.6	1636	1.5	681	0.7	4488	0.8	7.6	8.7
68	4292	2.5	1763	1.0	452	0.2	4148	0.5	6.6	16.5
26[a]	3506	1.0	806	0.6	362	0.0	3728	0.1	13.0	
53[a]	3358	7.6	1589	2.3	381	0.0	3053	0.2	51.0	
66[a]	4135	6.5	1778	2.1	508	0.2	4799	0.2	43.0	43.0
73[a]	3432	2.4	1364	1.0	377	0.0	2844	0.2	16.0	
82[a]	2923	1.9	812	0.5	219	0.0	2526	0.4	6.0	
Low-risk										
6	4696	6.2	3015	1.9	1049	0.1	6569	0.6	13.3	80.0
11	4525	3.2	3015	1.3	1000	0.1	6578	0.3	13.7	41.0
70	1114	2.2	1031	1.6	493	0.0	3122	0.2	17.0	

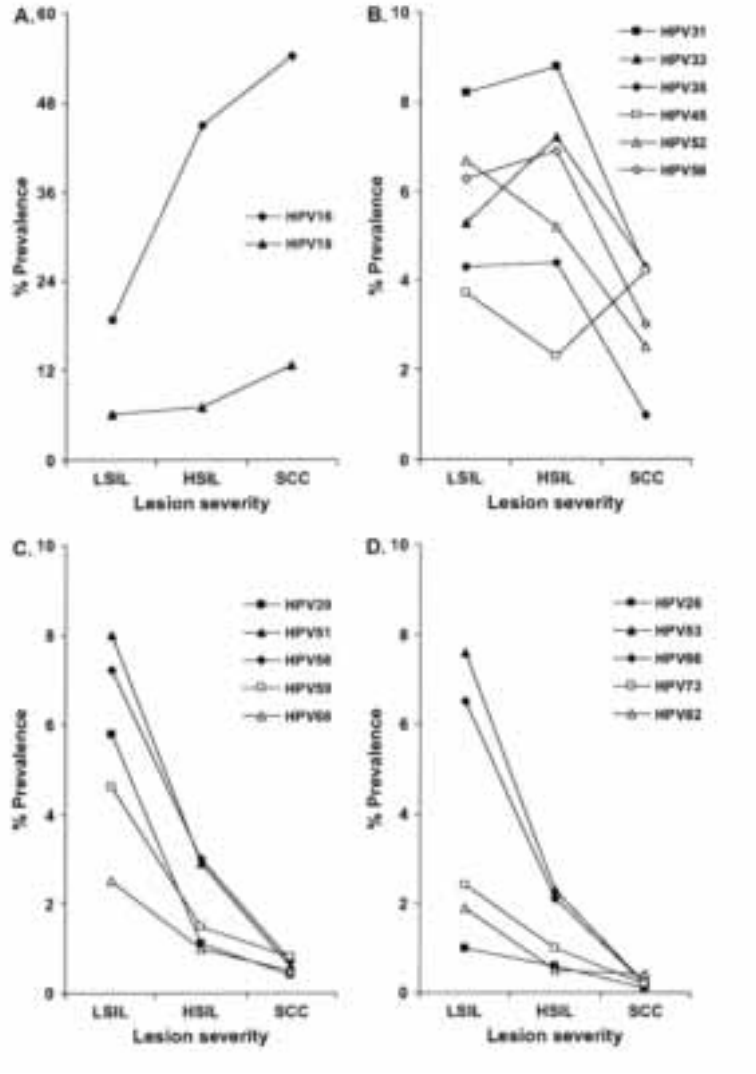
ADC, adenocarcinoma or adenosquamous carcinoma; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; SCC, squamous-cell carcinoma

[a] Not currently included in the US Food and Drug Administration-approved Hybrid Capture 2 HPV DNA test

common in LSIL than in squamous-cell carcinoma. A ratio of approximately 10 between LSIL and squamous-cell carcinoma was also found for low-risk types HPV 6, 11 and 70. The type-specific findings for adeno- or adenosquamous carcinoma were consistent with those observed for squamous-cell carcinoma except for the more marked enrichment of HPV 18 from LSIL to adeno- or adenosquamous than to squamous-cell carcinoma.

Comparisons of HPV distribution in international cross-sectional studies face several problems, including differences in the accuracy in cytological/histological classification and viral detection, as well as non-negligible heterogeneity in the distribution of HPV types across different populations.

Figure 12. Prevalence of HPV types in cervical lesions of increasing severity



Modified from Franceschi & Clifford (2005b)

Of particular note since the previous review (IARC, 1995), a high prevalence of HPV 35 has been reported in invasive cancer from previously unstudied regions in East Africa (19%) (Naucler *et al.*, 2004) and India (6%) (Castellsagué *et al.*, 2001; Franceschi & Clifford, 2005). Furthermore, a failure in the sensitivity of MY09/11 PCR primers to detect HPV 35 has also been identified, so that the prevalence of HPV 35 may have been underestimated in some of the previous case series (Iftner & Villa, 2003).

Nevertheless, the picture that emerges from the IARC systematic reviews suggests that: HPV 16 and 18 are substantially enriched in squamous-cell carcinoma compared with LSIL; some high-risk types are approximately equally represented (HPV 33 and 45) or moderately over-represented (HPV 31, 52 and 58) in LSIL than in squamous-cell carcinoma; and HPV 26, 53, 66, 73 and 82, which are not currently included in the DNA tests approved by the US Food and Drug Administration, are extremely rare in squamous-cell carcinoma, but this is also the case for some of the types that are currently included (e.g. HPV 39, 51 and 56).

In conclusion, the available evidence from cross-sectional comparisons of the distribution of HPV types in cervical lesions of increasing severity lends strong support to the notion that the risk that a woman will develop HSIL or cervical cancer varies substantially according to the specific HPV type with which she is infected.

(*b*) *Case–control studies*

Since the last review (IARC, 1995), a number of larger case–control studies have been completed that allow a more accurate evaluation of the type-specific risk of a number of additional HPV types. Only studies that reported HPV DNA results by type, as assessed by PCR, and by case and control status and included histologically confirmed end-points are reviewed and evaluated separately by disease end-point. Over the past 10 years, several specific and sensitive PCR-based methods of HPV detection have been used in epidemiological studies, and it is important to highlight that the various PCR systems differentially amplify different HPV types in disease and non-disease samples. Therefore, caution must be taken in interpreting the relative strength of the association between specific HPV types and risk for disease across studies. Due to the relative infrequency of some HPV types, smaller case–control studies have reported unstable risk estimates for certain HPV types. Greater emphasis is therefore given to larger studies and those that reported pooled data in relation to the risk associated with types other than HPV 16 and 18. As far as possible, the risk estimates presented here focus on those associated with single HPV infections only. The risk estimates published by the authors are presented where these are available by HPV type. When raw data were available from the individual publications, these were used to generate the crude odds ratio by HPV type. Finally, due to the problems of type specificity in seroepidemiological studies, those reports that only provided data on seroprevalence are not included.

In the mid-1990s, a growing interest in the risk associated with different HPV types came from examination of data from case series that indicated a relatively high prevalence of HPV types other than HPV 16 and 18 in cervical tumours (Bosch *et al.*, 1995; Huang *et al.*, 1997).

Table 27 summarizes the results of case–control studies of HPV-specific infection and pre-invasive and invasive lesions of the cervix.

In Honduras, Ferrera *et al*. (1999) conducted a population-based case–control study (149 cases of CIN3 or invasive cervical cancer and 438 controls) to investigate risk factors for cervical cancer. HPV was detected using general primer-mediated MY09/11 PCR

Table 27. Characteristics of case–control studies on HPV-specific infection and pre-invasive and invasive lesions of the cervix

Reference, study location	Study type	Methods of detection	HPV types tested	No. and type of cases	No. and type of controls
Ferrera *et al.* (1999), Honduras	Cervical screening	General primer-mediated PCR+ MY09/11 PCR sequencing	16, 18, 45, 33, 59, 31, 35, 52, 58, 56, 66, 11, 53, 70, 6, 22, 55, 62, 21	45 CIN3, 104 ICC	438 hospital-based
Hwang *et al.* (1999), Korea	Hospital-based	Consensus primer PCR; RFLP analysis	16, 18, 31, 33, 35, 52, 58	35 CIN, 41 ICC	130 healthy women
Sasagawa *et al.* (2001), Japan	Screening	LCRF1 to –4+ E7 primer; RFLP analysis	16, 18, 45, 33, 31, 35, 52, 58, 56, 51	145 LSIL, 137 HSIL, 72 SCC, 12 ADC, 16 condyloma	1562 normal cytology
Thomas *et al.* (2001b), Thailand	Hospital-based	MY09/11 PCR; generic + type-specific oligonucleotide probed for hybridization	16, 18, 31, 33, 35, 39, 45	42 ADC, 190 SCC	291 otolaryngological and general wards
Thomas *et al.* (2001c), Thailand	Hospital-based	General PCR; type-specific PCR	16, 18	190 ICC	75 CIS
Altekruse *et al.* (2003), USA	Population-based	PGMY-based 27-type reverse line blot detection	6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 66, 68, 73, 82, 83, 84	139 SCC, 124 ADC	307 population-based
Franceschi *et al.* (2003), India	Hospital-based	GP5+/6+ PCR; ETA with HPV specific oligoprobe cocktails	16, 18, 45, 33, 59, 31, 52, 58, 73, 56, 51, 66, 11, 70, 40, 42, 72, 81	193 ICC, 12 ADC	213

Table 27 (contd)

Reference, study location	Study type	Methods of detection	HPV types tested	No. and type of cases	No. and type of controls
Muñoz *et al.* (2003), Multicentre	Population- and hospital-based	E7 primer PCR for biopsies, for smears, MY0G/11 or GP5 +/6+ PCR; oligo-hybridization	16, 18, 26, 45, 33, 59, 31, 35, 52, 58, 66, 73, 39, 56, 26, 51, 68, 11, 53, 6, 81, 82	1918 prevalent SCC and ADC	1928
Asato *et al.* (2004), Japan	Population- and hospital-based	L1 consensus primer PCR; nucleotide sequencing	16, 18, 45, 33, 59, 31, 35, 52, 58, 73, 39, 56, 51, 66, 68, 82, 90, 91, 54, 53, 70, 6, 61, 71, 32, 42, 67, 72, 84, 86	356 SCC	3249 hospital-based
Hammouda *et al.* (2005), Algeria	Hospital-based	GP5+/6+ PCR; EIA with type-specific oligoprobe cocktail detecting 36 types and southern blot	16, 18, 45, 33, 31, 35, 52, 73, 39, 56, 51, 66, 42, 84	198 SCC	202
Herrero *et al.* (2005), Costa Rica	Population-based	MY09/11 PCR; dot blot with type-specific oligoprobes	16, 18, 45, 33, 59, 31, 35, 52, 58, 73, 39, 56, 26, 51, 66, 68, 82, 11, 54, 53, 70, 6, 61, 71, 22, 32, 40, 42, 55, 62, 67, 72, 81, 84, 74, 83, 85, 89, 21	73 CIN3, 35 SCC	8374 normal equivocal and low-grade dysplasia

See Table 7 for a description of the primers used.

ADC, adenocarcinoma; CIN, cervical intraepithelial neoplasia; CIS, carcinoma *in situ*; EIA, enzyme immunoassay; HSIL, high-grade squamous intraepithelial lesions; ICC, invasive cervical carcinoma; LSIL, low-grade squamous intraepithelial lesions; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SCC, squamous-cell carcinoma

followed by PCR-based sequencing that detected several different HPV types. However, due to the rarity of most HPV types, risk estimates for disease could only be generated for HPV types 16, 18, 31, 33, 52 and 58. The prevalence of any HPV type was 95% in cases of invasive cervical cancer, 87% in cases of CIN and invasive cervical cancer and 39% in controls. Compared with normal cytology, the odds ratio for invasive cervical cancer, associated with HPV 16-related types was 14.88 (95% CI, 5.12–43.25) and that for invasive disease associated with HPV 18-related types was 74.66 (95% CI, 7.77–717.62). Significantly elevated risks for invasive cervical cancer compared with normal cytology were also observed for HPV types 31 [odds ratio, 3.4], 33 [odds ratio, 34], 52 [odds ratio, 12.8] and 58 [odds ratio, 11.2]. In addition to the HPV types for which risk estimates could be generated, the authors observed HPV 45 infection among six cases and HPV 59 in one case, with none in the corresponding controls. A statistically significant association with HPV was observed for CIN2 and -3 and invasive cancer that showed an upward trend to more severe lesions and was more pronounced for HPV 16 and related types. A significantly elevated risk for CIN3 was observed with HPV 16 [odds ratio, 19], 18 [odds ratio, 8.9], 31 [odds ratio, 21.4], 33 [odds ratio, 71.5], 52 [odds ratio, 53.6] and 58 [odds ratio, 26.3]. HPV types 53, 66 and 70 were identified among controls but not among cases.

Hwang (1999) conducted a case–control study that included 130 healthy women, 35 patients with CIN and 41 patients with invasive cervical carcinoma in the Republic of Korea. HPV was detected by PCR followed by type-specific analyses by restriction fragment length polymorphism (RFLP). Significantly elevated risks for invasive cervical cancer were observed for HPV 16 [odds ratio, 146.3], 18 [odds ratio, 156], 52 [odds ratio, 39] and 58 [odds ratio, 78].

In Japan, Sasagawa *et al*. (2001) estimated the risk of HPV infection for biopsy-confirmed cervical malignancies by testing cell samples from 366 women with abnormal cytology and 1562 women with normal cytology for HPV with the long control region (LCR)-E7 PCR method that can amplify the E6–E7 DNA of more than 36 mucosal types of HPV. The prevalence of HPV infection was 9.7% in controls, 91% in HSIL and 93% in invasive cervical cancer. For HSIL and invasive squamous-cell carcinoma, the highest odds ratios were observed with HPV 16 (odds ratio, 43; 95% CI, 1.24–75 and 69; 95% CI, 36–131, respectively). For adenocarcinoma, the highest odds ratio was seen with HPV 18 (odds ratio, 94; 95% CI, 28–317). In addition to HPV types 16 and 18, elevated risks were observed for the association between HPV types 11, 31, 51, 52, 53 and 58 and squamous-cell carcinoma with magnitudes > 5.

Thomas *et al*. (2001b) studied women in Thailand who had been diagnosed with pre-invasive or invasive cervical cancer. PCR-based assays that used MY09/11 were carried out to determine HPV DNA in cervical scrapings from 232 diagnosed cases (190 women with squamous-cell carcinoma and 42 women with adenocarcinoma) and 291 hospitalized controls in Bangkok. HPV types 16, 18 and 45 were determined individually and a combined measurement of HPV types 31, 33, 35 and 39 was conducted. Only risk estimates for HPV 16 and 18 were reported separately. The prevalence of HPV types 16 and 18 was

72.4% in cases and 14.0% in controls. The 168 women with HPV 16- and 18-positive cervical cancers were compared with 250 HPV-negative controls. The odds ratio for HPV 16 was 83 (95% CI, 39–232) for squamous-cell cancer and 24 (95% CI, 8.7–76) for adenocarcinoma. In addition, HPV 45 was observed in one case but not in controls.

In another report by Thomas *et al*. (2001c) in Bangkok, Thailand, 190 women with invasive cervical cancer from the previous report were compared with 75 women with in-situ disease. HPV DNA testing of cervical scrapings showed high-risk types in 79% of invasive and 57% of intraepithelial tumours. The 291 hospital-based controls for invasive cervical cancer and 124 controls for carcinoma *in situ* had HPV prevalences of 6.9% and 10.4%, respectively. Types 16 and 18, but not types 31/33/35/39, were more common in invasive than in intraepithelial tumours, and untyped HPV DNA was more common in in-situ lesions. The odds ratio for invasive carcinoma *in situ* with HPV types 16 and 18 was reported to be 11.0 (95% CI, 3.9–33.0) and 10.0 (95% CI, 1.2–86.0), respectively.

In the northeastern USA, Altekruse *et al*. (2003) conducted a case–control study that included 124 women with cervical adenocarcinoma, 139 with cervical squamous-cell carcinoma and 307 control subjects to determine HPV genotypes and sexual and reproductive risk factors using a PCR-based reverse line blot detection system (MY09/11 L1 consensus primer system). Specimens were grouped hierarchically by HPV genotype: 18, 16, 18-related (39, 45, 59 and 68), other high-risk (26, 31, 33, 35, 51, 52, 55, 56 and 58) and low-risk (6, 11, 40, 42, 51, 53, 54, 57, 66, 73, 82, 83 and 84). HPV 18 was associated most strongly with adenocarcinoma (odds ratio, 11.9; 95% CI, 3.6–39.5) and HPV 16 was associated most strongly with squamous-cell carcinoma (odds ratio, 10.5; 95% CI, 5.2–21.2). The relative importance of HPV genotypes 16 and 18 and the differences in reproductive co-factors suggest distinctly separate causes for cervical adenocarcinoma and squamous-cell carcinoma.

Franceschi *et al*. (2003) evaluated the role of HPV and other risk factors in the etiology of invasive cervical carcinoma in a hospital-based case–control study in Chennai, southern India. A total of 205 cases of invasive cervical cancer (including 12 adenocarcinomas) and 213 frequency- and age-matched control women were included. HPV DNA in cervical cells was evaluated by a PCR assay (GP5+/6+). HPV infection was detected in all but one case of invasive cervical cancer and in 27.7% of control women. HPV 16 was the most common type in both cases and controls (60.2% and 17.4%, respectively), followed by HPV 18 and 33. Compared with women who were infected by HPV 16, those infected with HPV 18 showed an increased odds ratio of 3.9 (95% CI, 0.9–17.4). In this study, multiple HPV infections did not yield a higher odds ratio for invasive cervical cancer than single infections.

One of the largest studies that has contributed to an understanding of the association between infection by individual HPV types and cervical cancer is the analysis of data pooled from nine case–control studies of invasive cervical cancer conducted by the IARC in Brazil, Colombia, Mali, Morocco, Paraguay, Peru, the Philippines, Spain and Thailand, the results of which were reported by Muñoz *et al.* (2003). Detection of HPV DNA in cervical scrapings (exfoliated cells) and biopsy specimens was performed blindly in

central laboratories using PCR-based assays. PCR primers for the *L1* gene, MY09/11, were used in the Colombian and Spanish studies and the GP5+/6+ general primer system was used in the remaining studies. A total of 1918 cases and 1928 controls were included in the pooled analysis. Overall, the prevalence of HPV infections was 90.7% in cases and 13.4% in controls and the pooled odds ratio for any HPV type for cervical cancer was 158.2 (95% CI, 113.4–220.6). The authors concluded that, in addition to HPV types 16 (odds ratio, 435) and 18 (odds ratio, 248), HPV types 31 (odds ratio, 124), 33 (odds ratio, 374), 35 (odds ratio, 74), 39 (odds ratio, ∞), 45 (odds ratio, 198), 51 (odds ratio, 67), 52 (odds ratio, 200), 56 (odds ratio, 45), 58 (odds ratio, 115), 59 (odds ratio, 419), 68 (odds ratio, 54), 73 (odds ratio, 106) and 82 (odds ratio, ∞) should be considered as carcinogenic. In addition, HPV 53 was found in one case of invasive cervical cancer only. No significant associations were reported for HPV 6 and 11.

In Okinawa, Japan, Asato *et al*. (2004) conducted a case–control study to determine the association between HPV infections and invasive cervical cancer. The study included 356 women who had been newly diagnosed with squamous-cell carcinoma of the cervix and 3249 controls. Cervical swabs taken before any treatment was started were analysed using a consensus primer pair to amplify DNA from the L1 region of HPV by PCR. This method would, however, underestimate the prevalence of HPV in multiple infections. Direct sequencing of PCR products resulted in the identification of nucleotide sequences of 30 HPV DNA genotypes. Overall, 87.4% of cases and 10.2% of controls were HPV DNA-positive. Among cases, 84.5% were HPV-positive for types 16, 18, 31, 33, 35, 52 and 58. The odds ratio associated with being positive for HPV 16 was the highest (534.6). Significantly elevated risks for invasive cervical cancer were also associated with HPV 18 (odds ratio, 259), 31 (odds ratio, 137), 33 (odds ratio, 151), 35 (odds ratio, 31), 51 (odds ratio, 9), 52 (odds ratio, 36), 53 (odds ratio, 14), 54 (odds ratio, 22), 56 (odds ratio, 25), 58 (odds ratio, 180), 59 (odds ratio, 52), 66 (odds ratio, 65), 68 (odds ratio, 12), 70 (odds ratio, 32) and 82 (odds ratio, 65). HPV 45, 73 and 82 were each detected in a single case. This study is one of the first large case–control studies in which HPV genotyping was based completely on nucleotide sequencing which allowed the investigators to estimate the risks for cervical cancer associated with previously uncharacterized genotypes.

Hammouda *et al.* (2005) conducted a case–control study in Algiers, Algeria, that included a total of 198 cases of cervical carcinoma and 202 age-matched control women. HPV infection was detected in 97.7% of cases and 12.4% of controls (odds ratio, 635). HPV 16 was the most common type in both cases and controls, followed by HPV 18 and 45. Twelve types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 66 and 73) were found as single infections in cases. Significantly elevated risks for invasive cervical cancer were observed for HPV 16 [odds ratio, 503], 18 [odds ratio, 572], 31 [odds ratio, ∞], 33 [odds ratio, ∞], 35 [odds ratio, ∞], 39 [odds ratio, ∞], 51 [odds ratio, ∞], 56 [odds ratio, ∞], 66 [odds ratio, ∞], 73 [odds ratio, ∞] and 45 [odds ratio, 159]. In addition, HPV 52 was found in one case. Multiple HPV infections did not yield a higher odds ratio for cervical carcinoma than single infections. The distribution of HPV types in cases of cervical carcinoma

and controls in Algeria was observed to be more similar to that found in Europe than to that found in sub-Saharan Africa, where HPV 16 is less prevalent.

Herrero *et al.* (2005) presented the results of their prevalent case–control analysis of the enrolment visit of the cohort study in Guanacaste, Costa Rica. In this study, a population-based cohort of 8514 sexually active women was tested for individual HPV types and screened by cytology for CIN and cancer. An expert panel of pathologists histologically confirmed all the lesions detected. The overall prevalence of HPV was 26.5%, and HPV 16 was the type most commonly detected (3.6% of the population). High-risk HPV infection was strongly associated with risk for all grades of CIN and cancer. HPV 16, 58 and 18 were the most common types in women diagnosed with CIN3 and cancer. Significantly increased risks for CIN3 were found for HPV types 16 [odds ratio, 272], 31 [odds ratio, 83.3], 56 [odds ratio, 46.3], 58 [odds ratio, 73.5] and 68 [odds ratio, 96.1]. HPV types 16 [odds ratio, 504], 18 [odds ratio, 595], 45 [odds ratio, 390], 52 [odds ratio, 149], 39 [odds ratio, 202], 58 [odds ratio, 184] and 66 [odds ratio, 312] were significantly associated with an elevated risk for invasive cervical cancer. HPV types 59, 35, 56, 68, 53, 54, 26 and 73 were detected in controls only. Multiple-type infections were associated with an increased risk compared with single-type infections for all grades of CIN and cancer, except for HPV 16-positive CIN3 and cancer.

(*c*) *Cohort studies*

(i) *Prospective studies with data on DNA*

Table 28 summarizes the results of cohort studies of HPV type-specific infection and pre-invasive and invasive carcinoma.

Koutsky *et al*. (1992) conducted the first prominent cohort study of HPV infection with some type specificity and a disease end-point of CIN2 or CIN3. In the 24 months following study entry, the relative risk for women who were infected with HPV 16 or HPV 18 (combined) compared with HPV DNA-negative women was 11 (95% CI, 4.6–26).

Using the stored collection of cytological slides from Swedish women who participated in a multi-decade screening programme, Wallin *et al.* (1999) examined type-specific persistence of HPV DNA before the development of invasive cervical cancer. Using two different PCR techniques and DNA sequencing, HPV in cells scraped from cytological smears was typed. A total of 118 women in whom invasive cancer developed on average 5.6 years later (range, 0.5 months to 26.2 years) were compared with 118 women who remained healthy during a similar length of time. In addition to testing the cytological slides, the important issue was addressed of whether the HPV type in the pre-morbid cytological sample matched the diagnostic type among cases who had available histology blocks. There was a clear excess of HPV 16 persistence associated with the risk for developing cancer (16 cases, no controls). There was also a non-significant excess of HPV 18 persistence (four cases, no controls), and single cases with persistence of HPV 31, 33 and 73.

In a series of publications from the same project, Josefsson *et al.* (2000) and Ylitalo *et al.* (2000a,b) measured HPV 16 viral load by applying quantitative PCR to cell

Table 28. Cohort studies on HPV-specific infection and pre-invasive and invasive lesions of the cervix

Reference, study location	Method of detection (types included)	No. and type of cases	Odds ratio (95% CI)
Koutsky *et al*. (1992), USA	Dot filter hybridization, Virapap specific primers (16, 18, 6, 11, 31, 33, 35)	28 CIN	HPV 16/18 11 (4.6–26)
Wallin *et al.* (1999), Sweden	PCR MY09/MY1GP5+/6+ DNA sequencing	118 ICC	HPV 16/18/31/33/73 16.4 (4.4–75.1)
Josefsson *et al.* (2000); Ylitalo *et al.* (2000a,b), Sweden	Quantitative PCR (16)	478 CIS	High viral load 25 (12.4–31.8) Medium viral load 6.6 (1.7–11.2)
Woodman *et al.* (2001), United Kingdom	PCR GP5+/GP6+, MY09/11 and specific primers (16, 18, 31, 33, 52, 58)	23 CIN2/3	HPV 6/11 3.8 (1.5–9.8) HPV 16 8.5 (3.7–19·2) HPV 18 3.3 (1.4–8.1)
Zielinski *et al.* (2001a,b), The Netherlands	PCR GP5+/6+ using a cocktail of HPV type-specific oligoprobes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68)	57 ICC	No risk or *p*-value reported
van Duin *et al.* (2002), The Netherlands	PCR (16)	12 CIN2/3	7.7 (1.6–33)
van der Graaf *et al.* (2002), The Netherlands	Short-fragment PCR 10 general primer set (6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68/73, 70, 74)	77 CIN3, SCC	HPV 16 104.8 (29.5–372.7) HPV 18, 31, 33 10.8 (4.3–27.2)
Kjaer *et al.* (2002), Denmark	PCR GP5+/6+ (high risk 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, low risk 6, 11, 40, 42, 43, 44)	112 CIN2/3	See Table 29
Xi *et al.* (2002), USA	MY09/11, HMB01, human β-globin primers (6, 11, 16, 18, 31, 33, 35, 39, 45, 56, 40, 42, 53, 54, 51, 52, 55, 58)	6 CIN 2/3	HPV 16 non-prototype-like variants 3.5 (1.0–11.8)
Schiffman *et al.* (2005), Costa Rica	PCR MY09/11 PCR (2, 6, 11, 13, 16, 18, 26, 31–35, 39, 40, 42–45, 51–59, 61, 62, 64, 66–74v, 81–85, 82v (AE2), 89, AE9, AE10)	8 ICC 61 CIN3	No risk or *p*-value reported

See Table 7 for a description of the primers used.
CI, confidence interval; CIN, cervical intraepithelial neoplasia; CIS, carcinoma *in situ*; ICC, invasive cervical carcinoma; PCR, polymerase chain reaction; SCC, squamous-cell carcinoma

scrapings of archival cytology slides from the Swedish screening programme. A total of 2081 smears from 478 cases of carcinoma *in situ* and 1754 smears from 608 controls were tested. Elevated HPV 16 viral loads were observed among cases compared with controls starting at 13 years before diagnosis, although the smears were considered to be cytologically normal (Ylitalo *et al.*, 2000b). Thus, detection of HPV 16 DNA predicted a risk for a diagnosis of carcinoma *in situ* many years later. About 25% (95% CI, 12.4–31.8) of women infected with a high viral load before the age of 25 years developed cervical carcinoma *in situ* within 15 years. Women with a medium viral load had an absolute risk of 6.6% (95% CI, 1.7–11.2) after 15 years (Ylitalo *et al.*, 2000a). Women with low viral loads were at marginally elevated risk compared with HPV 16-negative women (Ylitalo *et al.*, 2000a). The median latency between the initial HPV 16 infection and diagnosis of carcinoma *in situ* was estimated to be between 7 and 12 years but may be up to two decades for some women (Ylitalo *et al.*, 2000b). These estimates are concordant with the time between modal ages of HPV infection and CIN3 observed in population-based cross-sectional studies.

Woodman *et al.* (2001) studied the natural history of incident cervical infection with HPV 16, 18, 31, 33, 52, 58 or 6/11 in relation to the development of CIN2 or CIN3 among 1075 British women aged 15–19 years who had recently become sexually active. The median duration of follow-up of the cohort was 29 months; thus, the few cases represented the leading edge of the incidence curve of CIN2 (14 cases) or CIN3 (14 cases). Specifically, among the 23 cases for whom HPV DNA was detected, the median time from first detection to diagnosis of CIN2 or CIN3 was 26 months (range, 0–69 months). The univariate relative risks for CIN2 or CIN3 were elevated for HPV 6/11 (3.8; 95% CI, 1.5–9.8), 16 (8.5; 95% CI, 3.7–19.2), 18 (3.3; 95% CI, 1.4–8.1), 31 (3.5; 95% CI, 1.0–11.8), 52 (2.3; 95% CI, 0.3–17.2) and 58 (2.9; 95% CI, 0.8–10.1), but not for HPV 33 (0.6; 95% CI, 0.1–4.4). The evidence of type-specific carcinogenicity afforded by these data is weakened by the short follow-up, the incomplete typing with possibility of type–type confounding and the inclusion of CIN2 in the disease group.

Zielinski *et al.* (2001b) conducted a retrospective case–control study of type-specific DNA detection (by GP5+/6+ PCR) in the last normal cervical smears archived for 57 women who developed cervical cancer approximately 8 years later compared with 114 controls matched on age and date of screening. The types found in the subsequent smears and diagnostic biopsies of the case women were the same as those detected at the baseline smear. The only statistically significant difference was for HPV 16 (29/57 cases, 2/114 controls). Other types showed non-significant excesses among cases: HPV 18 (three cases, two controls), 31 (three cases, two controls), 45 (one case, one control) and 33 (one case, no control). Most of the smears that were originally interpreted as normal were re-interpreted, blinded to other study information, as abnormal for cases but not for controls. Re-interpretation as abnormal was strongly linked to HPV DNA positivity. This study demonstrates the difficulty of determining the true cytological state at baseline in longitudinal studies.

Using quantitative PCR, van Duin *et al.* (2002) tested the viral load of archived HPV 16 DNA-positive specimens from a Dutch cohort that included 12 women who subsequently developed CIN2 or CIN3 and 47 controls who developed ≤ CIN1. All baseline smears were considered to be normal, although that interpretation does not rule out the possibility of neoplasia being missed by cytology. Over the average of almost 3 years of follow-up, an association was observed between high versus low baseline viral load of HPV 16 and risk for CIN2 or CIN3 (odds ratio, 7.7; 95% CI, 1.6–33).

van der Graaf *et al.* (2002) tested scraped cells from archived cytological smears in a nested case–control study within the Dutch (Utrecht) mass-screening programme, using a short fragment PCR 10 amplification system and typing by a reverse hybridization line probe. After exclusions for missing slides, the case group included 62 women with CIN3 and 15 with micro- or gross invasion. The 270 controls were matched to cases on age and follow-up time. During an average follow-up period of 5.6 years, 29 cases compared with three controls had slides that contained only HPV 16 (odds ratio, 104.8; 95% CI, 29.5–372.7). The presence of HPV 18, 31 and/or 33 (not distinguished individually) was associated with an odds ratio of 10.8 (95% CI, 4.3–27.2).

Kjaer *et al.* (2002) conducted a prospective cohort study among more than 10 000 women aged 20–29 years in Copenhagen, Denmark, and tested baseline specimens for type-specific DNA using GP5+/GP6+ PCR. In order to limit the study to incident cases, women with a history of cervical neoplasia, abnormal baseline cytology or abnormalities diagnosed within 9 months from baseline were excluded. A total of 112 cases of CIN2 or CIN3 were observed at the follow-up visits approximately 2 years after baseline. Because of the relatively short follow-up, some of these cases were possibly present but were missed at baseline. With this caveat, elevated univariate odds ratios were found for most putative carcinogenic HPV types, including HPV 16, 18, 31, 33, 45, 51, 52, 58 and 66 (Table 29). Increased risk estimates based on one or two cases were associated with HPV 35, 39, 56 and 59. Based on a single case each, elevated univariate risk estimates were seen for HPV 6 and 11 which are associated with condyloma acuminatum. Other identified types were rare or absent in cases and controls. However, this analysis did not take into account the possible confounding influences of multiple infections, which were found in 25% of HPV-positive cases and 12% of HPV-positive controls.

In a 5-year longitudinal cohort study among female university students in Seattle, USA, Xi *et al.* (2002) observed that incident infections with non-prototype-like HPV 16 variants conferred a 3.5 (95% CI, 1.0–11.8) increase in risk for histological CIN2 or CIN3 compared with prototype-like HPV 16 variants. Of the 48 women with incident HPV 16 prototype-like variants, six developed CIN2 or CIN3, while six of 14 women with non-prototype-like variants developed these diseases. The difference in risk was not mediated by a difference in average length of viral persistence, ethnicity or current use of oral contraceptives.

Schiffman *et al.* (2005) conducted a population-based prospective study of HPV infection and subsequent development of CIN3 and cancer in a cohort of 10 000 women in Guanacaste, Costa Rica. They tested for more than 40 types of HPV DNA using MY09/11 PCR with TaqGold polymerase, and followed more than 7000 sexually active

Table 29. Distribution of HPV types among cases and cytologically normal women who were positive for HPV at enrolment

HPV type	Cases (n = 115) of high-grade lesions (%)	Controls (n = 100) (%)	Odds ratio[a]
6	1 (0.9)	5 (0.8)	1.2
11	1 (0.9)	1 (0.2)	5.9
16	43 (37.4)	27 (4.1)	14.4
18	10 (8.9)	11 (1.7)	5.7
31	15 (13.0)	7 (1.1)	14.3
33	7 (6.1)	11(1.7)	3.9
35	1 (0.9)	1 (0.2)	5.9
39	2 (1.8)	2 (0.3)	5.9
42	0	1 (0.2)	–
44	0	1 (0.2)	–
45	5 (4.5)	1 (0.2)	30.5
51	6 (5.3)	1 (0.2)	57.0
52	5 (4.5)	2 (0.3)	15.2
56	2 (1.8)	1 (0.2)	11.9
58	5 (4.5)	5 (0.8)	6.0
59	2 (1.8)	0	–
66	4 (3.6)	1 (0.2)	24.1
X	6 (5.3)	22 (3.4)	1.6

From Kjaer *et al.* (2002)
Figures are numbers (percentages of women).
[a] Crude odds ratios calculated by the Working Group

women who had no evident prevalent CIN2, CIN3 or cancer and no hysterectomy for an average of over 5 years. Cases of incident histologically confirmed CIN3 and cancer were considered to be caused by a particular type of HPV only if that type was found both at study enrolment and at the time of diagnosis. The results confirmed the case–control literature, and were consistent with the data of Kjaer *et al.* (2002). The risks for cancer and CIN3 with HPV types were clearly associated with their phylogenetic relatedness. Compared with other types, HPV 16 was the most likely to persist for 5 years and, when persistent, to be linked to CIN3 and cancer. HPV 16-related infections also tended to lead to incident CIN3/cancer. Most of the other cancer-associated types were phylogenetically related to HPV 18. Some HPV species (clades; e.g. A3) showed virtually no association with CIN3 or cancer despite a relatively high prevalence and a tendency of some types in those clades to persist.

(ii) *Prospective studies with serological data* (Table 30)

Studies of archived sera permit the assessment of exposure to HPV before a diagnosis of CIN3 or cancer. The assays themselves tend to be type-specific, although the possibility of confounding remains because genital HPV infections are transmitted by a common sexual route and few types are assessed in any study.

Lehtinen *et al.* (1996) focused on HPV 16 serology within a cohort of Finnish women that was followed for up to 23 years. The odds ratio for HPV 16 seropositivity was 12.5 (95% CI, 2.7–57) among 72 cases (27 with cancer and 45 with carcinoma *in situ*) and 143 matched controls. The risk estimates were increased for both short and long lapses of time from sampling to diagnosis.

Dillner *et al.* (1997) compared 182 women who had developed cervical cancer with 538 controls matched on age and time of enrolment into a joint Nordic cohort. Sera were tested for antibodies to HPV 16, 18 and 33. HPV 16 seropositivity was associated prima-

Table 30. Prospective serological studies on HPV-specific infection and pre-invasive and invasive lesions of the cervix

Reference, study location	Method of detection (types included)	No. and type of cases	Odds ratio (95% CI)
Shah *et al*. (1997), USA	ELISA (16, 6, 6b)	14 ICC, 28 CIN3	HPV 16 3.9 (1.4–10.7)
Lehtinen *et al.* (1996), Finland	ELISA (16)	27 ICC, 45 CIS	12.5 (2.7–57)
Dillner *et al.* (1997) Finland, Norway, Sweden	ELISA (16, 18, 33)	182 ICC	HPV 16 3.2 (1.7–6.2)
Luostarinen *et al.* (1999), Finland, Norway, Sweden	ELISA (16, 18, 33, 6/11)	182 ICC	No signifiant association
Vonka *et al.* (1999), Czech Republic	ELISA (16, 18, 33)	43 dysplasies, 19 CIS, 5 ICC	HPV 16 3.85 (1.11–13.91)
Wallin *et al.* (2000), Sweden	ELISA (73)	41 CIN2/3	1.5 (0.35–6.65)
Hisada *et al.* (2001), USA	ELISA (16)	52 ICC, 47 CIS	2.0 (1.0–3.4)
Sigstad *et al.* (2002), Norway, Finland, Sweden	ELISA (16, 18, 33)	127 ICC	HPV 16 4.4 (2.2–8.8) HPV 18 17 (2.1–140)

CI, confidence interval; CIN, cervical intraepithelial neoplasia; CIS, carcinoma *in situ*; ELISA, enzyme-linked immunosorbent assay ; ICC, invasive cervical carcinoma

rily with an increased risk for squamous-cell carcinoma (odds ratio, 3.2; 95% CI, 1.7–6.2) while HPV 18 seropositivity tended to be associated with a higher risk for cervical adenocarcinomas (odds ratio, 3.4; 95% CI, 0.8–14.9). HPV 33 seropositivity was not significantly associated with either squamous-cell or adenocarcinoma (odds ratio, 1.6 and 1.7, respectively).

Shah *et al*. (1997) tested pre-diagnostic sera from 14 cases of invasive cancer and 28 cases of CIN3 and compared them with those from 83 matched controls. The odds ratio for antibodies to HPV 16 VLPs was 3.9 (95% CI, 1.4–10.7), but HPV 6 antibodies were not associated with the subsequent occurrence of cervical cancer or CIN3.

Luostarinen *et al.* (1999) further tested the same Nordic subjects followed by Dillner *et al.* (1997) for HPV 6/11 to assess the joint effect of simultaneous exposure with carcinogenic (HPV 16, 18 and 33) and non-carcinogenic HPV types on the risk for subsequent development of cancer. HPV 6/11 seropositivity was not strongly associated with risk for cancer. However, there was evidence of an antagonistic modification of effect for the combination of HPV 16 seropositivity and HPV 6/11 seropositivity (but not other combinations). The authors suggested that HPV 6/11 seropositivity might reflect cross-protective immunity.

Vonka *et al.* (1999) re-tested stored sera from the Prague cohort that was originally assembled in the 1970s to study herpes simplex virus (HSV) and cervical cancer (Vonka *et al.*, 1984) for HPV 16, 18 and 33 VLP antibodies. While the original study failed to find an association of HSV seropositivity with subsequent cervical cancer, the re-analysis of 67 prospective cases and 129 matched controls showed an elevated relative risk for all three HPV types. The case group included 43 moderate or severe dysplasias, 19 carcinomas *in situ* and five invasive carcinomas. The relative risks were 3.85 (95% CI, 1.11–13.91) for HPV 16, 2.70 (95% CI, 0.87–8.55) for HPV 18 and 1.51 (95% CI, 0.55–4.13) for HPV 33.

Wallin *et al.* (2000) explored the possible carcinogenicity of HPV 73 in a serological study of stored blood from 41 cases of CIN2–3 in northern Sweden and 82 matched controls. The odds ratio for HPV 73 antibodies preceding case diagnosis by an average of 2–3 years was 1.5 (95% CI, 0.35–6.65).

Hisada *et al.* (2001) tested precancer sera archived from pregnant women in a Californian cohort study and observed an age- and race-adjusted odds ratio of 2.0 (95% CI, 1.0–3.4) for the association of seropositivity to HPV 16 VLPs and subsequent risk for invasive (52 cases) or in-situ (47 cases) cervical cancer.

In a retrospective cohort study based on archived blood and cervical tissue, Sigstad *et al.* (2002) confirmed that pre-diagnostic HPV 16 and HPV 18 seropositivity was linked to a risk for subsequent cervical cancer and corresponded to the same HPV DNA types found in the tumours. A total of 127 cases of invasive cancer in the large Nordic Janus cohort and 376 controls matched on age, country and time of blood collection were studied. HPV 16-seropositive women had a relative risk of 4.4 (95% CI, 2.2–8.8) for developing invasive cancer containing HPV 16 DNA, but had no excess risk for developing other cancers. Similarly, HPV 18 seropositivity predicted a risk (odds ratio, 17; 95% CI, 2.1–140) only for cancers that contained HPV 18, of which 10/20 were

adenocarcinomas. The results for HPV 33 were not type-specific; tumours that contained HPV 33 were very rare and HPV 33 seropositivity was associated with the development of cancers that contained HPV 16.

In prospective studies of individual HPV types, the data from DNA-based and serological studies are concordant although absolute risks associated with DNA positivity are higher than those associated with seropositivity. HPV 16 is clearly carcinogenic and persists longer than other carcinogenic types. Because of its persistence, HPV 16 is apparently more closely linked to malignant transformation. There is also some prospective evidence for the carcinogenicity of HPV 18. The evidence for other types, although scant, is concordant with the more statistically powerful case–control literature.

2.3 Cancer at other anogenital sites

2.3.1 *Cancer of the vulva*

Vulvar cancer has two distinct histopathological types and sets of risk factors. Only a limited number of studies have characterized the prevalence of HPV DNA by histological type, but the results have been consistent. Keratinizing vulvar cancer is associated with a low prevalence of HPV (generally less than 10%), occurs in older women and is associated with lichen planus. In contrast, HPV DNA is found in a high proportion of basaloid and warty vulvar cancers (> 55%), which occur in younger women than keratinizing cancers and are associated with classical risk factors for the acquisition of HPV (Schiffman & Kjaer, 2003). These cancers are often associated with overlying vulvar intraepithelial neoplasia (VIN), which in turn has a strong association with HPV infection. The data suggest two distinct sets of cancer: one that is associated with HPV and may be preceded by VIN, and another that is not clearly associated with HPV and whose precancerous natural history is poorly understood.

(*a*) *Case series*

Table 31 presents series of more than 10 cases of cancer of the vulva or VIN3. Among the HPV DNA-positive vulvar cancers, HPV 16 is the most common type, followed by HPV 18 at a much smaller percentage. The proportion of HPV-positive vulvar cancers that contained HPV 31 or HPV 33 was variable in the small number of studies that specifically probed for these types. One study (Iwasawa *et al.*, 1997) found a 1.4% prevalence of HPV 33 but did investigate HPV 31. Another small study of 11 cases of vulvar carcinomas (Abdel-Hady *et al.*, 2001) found HPV 33 in two of three HPV-positive cases.

Similar to the data on vulvar cancer, case series on the prevalence of HPV DNA show that a high proportion (> 70%) of VIN3 are positive (Table 31) and that the most common type is HPV 16. Two studies (Junge *et al.*, 1995; Van Beurden *et al.*, 1998) also showed that a small proportion of lesions (< 11%) are DNA-positive for HPV 33. Two studies

Table 31. Prevalence of HPV DNA in case series of vulvar cancer (≥ 10 cases) and grade 3 vulvar intraepithelial neoplasia (VIN3) (≥ 9 cases)

Reference, study location	Method of detection[a] (types included)	No. and type of lesions	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comments
				6	11	16	18	31	33	Others (type)		
Vulvar cancer												
Hørding *et al.* (1994), Denmark	E6/E7 primers (6, 11, 16, 18, 33)	51 keratinizing 17 warty 10 basaloid	3.9 70.5 100	0 0 0	0 0 0	28.2	0 0 0	NR	3.8		1.3	Paraffin-embedded tissue; HPV DNA found in 81% of cancers with overlying VIN3, 9% of tissues with overlying VIN1–2 and 0% of tissues with adjacent lichen sclerosis
Trimble *et al.* (1996), USA	ISH (6/11/16/18/31/33/35/42/43/44/45/51/52/56)	21 basaloid-warty 48 keratinizing	85.7 6.3									Paraffin-embedded tissue; basaloid or warty carcinoma but not keratinizing squamous carcinoma associated with classical risk factors for cervical cancer
Iwasawa *et al.* (1997), Finland	MY09/11 type-specific (6, 11, 16, 18, 33)	74	36.5	0	0	25.7	12.2		1.4		4.0	Paraffin-embedded tissue; 65 of 74 cases were women 61 years of age or older; histological type not specified
Madeleine *et al.* (1997), USA	MY09/11 (6/11, 16, 18/45, 31/33/52) or RFLP	55	50.9	1.8		43.6	1.8[b]		3.6[c]	1.8[d]	1.8	Paraffin-embedded tissue; histological type not specified
Abdel-Hady *et al.* (2001), United Kingdom	GP5+/6+ (6/11, 16, 18, 31, 33)	11	27.3			27.3	9.0		18.1		18	Paraffin-embedded tissue; histological type not specified
Carter *et al.* (2001), USA	MY09/11 and RFLP; type-specific for 16, 18	38	79			55.3	2.6		13.2[e]	7.9[f]	0	Paraffin-embedded tissue; histological type not specified
VIN3												
Junge *et al.* (1995), Denmark	E6/E7 primers (6/11, 16, 18, 31, 33); ISH (6/11, 16/18, 31/33)	62 PCR [*n* = 55] ISH [*n* = 58]	89.1	0 0	0 0	[78.2] [36.2]	0	0	[5.2]	[10.9]	NR	Paraffin-embedded tissue; cases of severe dysplasia and carcinoma *in situ*

Table 31 (contd)

Reference, study location	Method of detection[a] (types included)	No. and type of lesions	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comments
				6	11	16	18	31	33	Others (type)		
Trimble *et al.* (1996), USA	ISH for 6/11/16/18/ 31/33/35/42/43/44 /45/51/52/56	54 basaloid or warty	88.9									Paraffin-embedded tissue; squamous hyperplasia (VIN2) and basaloid or warty VIN (VIN3) combined
Madeleine *et al.* (1997), USA	MY09/11 probes (6/11, 16, 18/45, 31/33/52) or RFLP	253	71.5	5.5		61.7	5.9[b]	5.9[c]		2.8[d]	9.5	Paraffin-embedded tissue; histological type not specified
Van Beurden *et al.* (1998), Netherlands	CPI and CPIIG and sequencing	27	100			92.6			3.7	3.7 (45)	0	Paraffin-embedded tissue; histological type not specified; patients with pre-existing, concomitant or subsequent cervical or vaginal neoplasm
Abdel-Hady *et al.* (2001), United Kingdom	GP5+/6+ and type-specific for 6/11, 16, 18, 31, 33	32	71.9	[18.8]		46.8					NR	Paraffin-embedded tissue; cases were VIN2 or VIN3, predominantly (26/32) warty or mixed warty-basaloid type.
Carter *et al.* (2001), USA	MY09/11 and RFLP; type-specific for 16, 18	181	91.2			74.6	6.6	8.8[e]		7.2[f]	6.1	Paraffin-embedded tissue; carcinoma *in situ*
Todd *et al.* (2004), United Kingdom	GP5/6 and type-specific for 16, 18, 31, 33	9	[88.8]			[66.7]	0	0	[22.2]		0	Paraffin-embedded tissue; 77.8% of cases had prior history of CIN.

See Table 7 for a description of the primers used.

CIN, cervical intraepithelial neoplasia; ISH, in-situ hybridization; NR, not reported; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism

[a] Unless otherwise specified, the method is PCR; ';' denotes independent methods whereas 'and' denotes subsequent steps.

[b] 18 or 45

[c] 31, 33 or 52

[d] Unknown

[e] 31, 33, 35 or 39

[f] HPV 6, 45, 52, 54, 58, 66, 72, 73 or unknown types

(Madeleine *et al.*, 1997; Carter *et al.*, 2001) showed the presence of HPV 18 or 45 in 5.9% and that of HPV 18 in 6.6% of lesions, respectively.

(*b*) *Case–control studies*

Results from case–control studies of vulvar cancer and VIN that used serological assays as detection method are consistent with the findings on DNA.

Sun *et al*. (1996) examined HPV-specific antibodies in the sera of patients in the USA who had basaloid or warty squamous-cell vulvar cancer, keratinizing vulvar cancer and VIN to determine the association between these conditions and the presence of antibodies to HPV 16. The study included a total of 54 cases (14 basaloid or warty cancers, 18 keratinizing cancers and 22 VIN) for whom serological specimens were available and 44 controls. The prevalence of antibodies to HPV 16 VLPs was significantly higher in HPV-associated VIN (59.1%) and basaloid or warty cancers (50.0%) than in keratinizing cancers (22.2%) or controls (18.2%). The odds ratios were 5.4 (95% CI, 1.7–18) for VIN and 4.5 (95% CI, 1.2–16) for basaloid and warty cancers. For keratinizing cancers, the odds ratio was not statistically significant at 1.3 (95% CI, 0.32–4.9).

A seroepidemiological nested case–control study from Finland and Norway (Bjørge *et al.*, 1997a) showed that HPV 16 seropositivity was associated with an increased risk for vulvar and vaginal cancers combined (odds ratio, 4.5; 95% CI, 1.1–22) and a strongly increased risk for pre-invasive vulvar and vaginal lesions (odds ratio, ∞; 95% CI, 3.8–∞). Seropositivity for HPV 18 was associated with an increased risk for pre-invasive lesions (odds ratio, 12; 95% CI, 1.2–590) but not for invasive cancer (odds ratio, 1.5; 95% CI, 0.3–7.5).

Hildesheim *et al.* (1997a) studied 142 histologically confirmed cases of VIN3 and invasive vulvar cancer and 126 community controls in the USA. Sera were tested for immunoglobulin G (IgG) antibodies against HPV 16 L1/L2 VLPs. Overall, 44.4% of cases and 11.9% of controls were HPV 16-seropositive. A stronger association between HPV 16 seropositivity and disease was observed for VIN3 (odds ratio, 13.4; 95% CI, 3.9–46.5) than for invasive cancer (odds ratio, 2.9; 95% CI, 0.94–8.7), although there was a suggestion that the association was stronger among women who had been diagnosed with basaloid or warty cancer (odds ratio, 3.8; 95% CI, 0.76–18.9) than among those with keratinizing cancer (odds ratio, 1.6; 95% CI, 0.35–7.4).

Madeleine *et al*. (1997) conducted a population-based case–control study in the USA to examine the association between HPV positivity, cigarette smoking, HSV-2 infection and the risk for vulvar cancer. The study included 400 in-situ and 110 invasive tumours of the vulva diagnosed among women who lived in the Seattle area from 1980 to 1994. In most analyses, cases were compared with 1043 controls. Serum samples were analysed for antibodies against HPV 6, 16 and 18. The prevalence of seropositivity to HPV 16 was 53.3% in in-situ cases, 43.8% in invasive cases and 22.2% in controls. HPV 16 seropositivity was associated with an increased risk for in-situ and invasive vulvar cancers (odds ratio, 3.6; 95% CI, 2.6–4.8; and 2.8; 95% CI, 1.7–4.7, respectively).

Overall, the data indicate that HPV 16 is the predominant HPV type in VIN3 and vulvar cancer, particularly basaloid and warty cancer. In vulvar cancers, HPV 18, 45, 31 or 33 may play a smaller role.

2.3.2 *Cancer of the vagina*

(*a*) *Case series*

The number of case series of vaginal cancer (Table 32) has remained small since the previous review (IARC, 1995). In two studies (Carter *et al.*, 2001; Daling *et al.*, 2002), the majority of vaginal cancers were positive for HPV DNA (90.7% of 54 cases and 64.0% of 25 cases, respectively). HPV 16 was the most common type and was found in at least 70% of HPV-positive tumours. HPV 6 or 11 was found in two cases of vaginal cancer in one study. HPV 18 or 45 and HPV 31, 33, 35 or 39 were also found in a small number of cases.

Similarly to the vaginal cancers, a high proportion of grade 3 vaginal intraepithelial neoplasia (VAIN3) tissues were also positive for HPV DNA and again the most common type was HPV 16 (Table 32). HPV 6 or 11, HPV 18 or 45 and HPV 31, 33 or 35 were found in a small number of cases (Van Beurden *et al.*, 1998; Daling *et al.*, 2002).

(*b*) *Case–control studies*

As with the studies of vulvar cancer, case–control studies of VAIN and vaginal cancer that used serological assays as the detection method are consistent with the findings on HPV DNA.

Hildesheim *et al*. (1997b) conducted a case–control study of VAIN and vaginal cancer in the USA. The study included 23 histologically confirmed cases of in-situ and invasive vaginal cancer and 28 community controls. Blood samples were collected from participants and tested for the presence of antibodies to HPV 16 VLPs, HSV-2 and *C. trachomatis*. Overall, 50% of cases and 25% of controls were positive for HPV 16 VLP antibodies. Women positive for HPV 16 VLP antibodies were at a 3.5-fold increased risk for vaginal neoplasia (95% CI, 0.97–13) and those with high antibody levels (high optical density) were at a 33-fold increased risk for the disease (95% CI, 2.5–430). The risk estimate was not affected by adjustment for HSV-2 or *C. trachomatis* seropositivity. The association was stronger for in-situ neoplasia than for invasive cancer, with relative risks of 5.4 (95% CI, 0.93–31) and 1.7 (95% CI, 0.22–14), respectively.

Daling *et al.* (2002) conducted a population-based case–control study that included 156 women with in-situ or invasive vaginal cancer diagnosed between January 1981 and June 1998 and 2041 control women identified through random-digit dialling in western Washington State, USA. Antibodies to HPV-16 L1 were strongly related to risk for vaginal cancer (odds ratio, 4.3; 95% CI, 3.0–6.2). Women with vaginal cancer were more likely to have had five or more lifetime sexual partners (odds ratio, 3.1; 95% CI, 1.9–4.9), to have an early age at first intercourse (< 17 years; odds ratio, 2.0; 95% CI, 1.2–3.5) and to be current smokers at diagnosis (odds ratio, 2.1; 95% CI, 1.4–3.1) than control women.

Table 32. Prevalence of HPV DNA in case series of vaginal cancer and grade 3 vaginal intraepithelial neoplasia (VAIN3 (≥ 3 cases)

Reference, study location	Method[a] of detection and types tested	No. of cases	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comments
				6	11	16	18	31	33	Others (type)		
Vaginal cancer												
Carter *et al.* (2001), USA	MY09/11 and RFLP; type-specific for 16, 18	54	90.7			63.0	5.6	3.7[b]		27.8[c]	9.3	Paraffin-embedded tissue
Daling *et al.* (2002), USA	MY09/11 and probing for 6/11, 16, 18/45, 31	25	64.0		8.0	56.0	12.0[d]	0	0	0	[12.0]	Paraffin-embedded tissue
VAIN 3												
Sugase & Matsukura (1997), Japan	Southern blot with PBM-58	3	100			66.6				33.3 (51)	0	Fresh tissue
van Beurden *et al.* (1998), Netherlands	CPI and CPIIG and sequencing	8	100			75.0			12.5			Paraffin-embedded tissue; VAIN2 and 3 with simultaneous CIN lesions or invasive cervical neoplasia
Daling *et al.* (2002), USA	MY09/11 and probing for 6/11, 16, 18/45, 31	74	82.4		8.1	54.1	8.1[d]		5.4[e]			Paraffin-embedded tissue

See Table 7 for a description of the primers used.

CIN, cervical intraepithelial neoplasia; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism

[a] Unless otherwise specified, the method is PCR; (;) denotes independent methods whereas 'and' denotes subsequent steps.

[b] 31, 33, 35 or 39

[c] 6, 45, 52, 54, 58, 66, 72, 73 or unknown HPV types

[d] 18 or 45

[e] 31, 33 or 35

Approximately 30% of cases had been treated for a prior anogenital tumour, most often of the cervix.

2.3.3 *Cancer of the penis*

Similarly to vulvar cancer, the prevalence of HPV DNA in penile cancer varies with histological type. Most case series do not specify the histological type (Table 33), but for those that do (Gregoire *et al.*, 1995; Bezerra *et al.*, 2001a,b; Rubin *et al.*, 2001; Ferreux *et al.*, 2003), warty and basaloid carcinomas of the penis in general had a higher prevalence of HPV infection than verrucous and keratinizing carcinoma. These studies mostly included a small number of cases and the range of HPV prevalence in the tissues was wide. Basaloid and warty carcinomas may be preceded by penile intraepithelial neoplasia (PIN), which is also associated with HPV infection (Aynaud *et al.*, 1994; Rubin *et al.*, 2001).

Among the HPV-positive penile cancers, HPV 16 was the most common type. However, the majority of studies included at least one case of cancer with HPV 6 or 11, which in several studies were more common than HPV 18 (Levi *et al.*, 1998; Rubin *et al.*, 2001). HPV 31 or 33 were detected only rarely.

Overall, similarly to other anogenital cancers, HPV 16 is the predominant HPV type and, similarly to vulvar cancer, warty and basaloid carcinomas tended to be those cancers with the highest proportion of HPV DNA positivity. However, the relationship between histopathology and HPV prevalence is not as clear as that in cancer of the vulva. Also, HPV 6 or 11 appear to play a more prominent role in penile cancers than in other cancers of the anogenital region.

2.3.4 *Cancer of the anus*

[For anal cancer in HIV-positive patients, see Section 2.8.3(*b*).]

(*a*) *Case series*

Case series of cancer of the anus are presented in Table 34. Anal cancer resembles cervical cancer more than the other anogenital cancers with respect to overall prevalence of HPV positivity. The prevalence of HPV DNA in anal cancer in different case series varies widely, but most studies that used MY09/MY11 or GP5+/GP6+ primers showed a prevalence of 80% or above. Basaloid cancers are similar to squamous-cell carcinomas with respect to prevalence of HPV DNA and are more probably a histological variant rather than a separate entity. HPV 16 is the most common type in squamous-cell cancers (76%) followed by HPV 18 with a much smaller percentage (9%). Most studies that included a broad range of HPV type-specific probes showed a low prevalence of HPV 31, 33 or 6 or 11.

Table 33. Prevalence of HPV DNA in case series of penile cancer (≥ 13 cases) and penile intraepithelial neoplasia (≥ 5 cases)

Reference, study location	Method of detection and types tested	No. and type of lesions	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comments
				6	11	16	18	31	33	Others		
Aynaud *et al.* (1994), France	Southern blot	4 PIN3	100	0[a]		100	0		0		0	Frozen tissue
Cupp *et al.* (1995), USA	MY09/11 and type-specific for 16, 18	42 SCC 13 carcinoma *in situ*	54.8 92.3			40.5 84.6	4.8 15.4			11.9[c] 0	7.7 2.4	
Gregoire *et al.* (1995), USA and Paraguay	Type-specific for 6, 11, 16, 18 and primer for wide range including 16, 18, 31, 33, 35, 52	45 typical 12 basaloid 10 papillary 9 warty 6 verrucous 19 mixed, warty or basaloid 8 mixed, other	11.1 75.0 0 22.2 0 47.4 0	0.9	0.9	21.1				0.9[c]	0	Paraffin-embedded tissue; HPV positivity associated with aggressive, higher-grade tumours; logistic regression showed that the only association with HPV positivity was tumour histopathology.
Levi *et al.* (1998), Brazil	Type-specific for 6/11, 16, 18	64 carcinoma	28.1		4.7	14.1	3.1			9.4[c]	1.6	Paraffin-embedded tissue
	MY09/11 and probing for 6, 11, 16, 18, 31	50 carcinoma	56.0		12.0	32.0	6.0			12.0[c]	4	Frozen tissue; no comments on histopathology
Buonaguro *et al.* (2000), Uganda	Southern blot for 16; PCR for 16 and sequencing	13 SCC	38.4			38.4						Frozen tissue
Picconi *et al.* (2000), Argentina	GP5/6 and SSCP for 6, 11, 16, 18, 31, 33	34 SCC	70.6	[5.9]	0	[23.5]	[11.8]	0	0	8.8[c]	0	Paraffin-embedded tissue; histological type not specified
Bezerra *et al.* (2001a,b), Brazil	L1 consensus primers and probing for 6/11/16/18/31/33/34/35/39/40/42/43/44/45/51/52/54/56/58	60 SCC 11 warty carcinoma	26.7 45.5		18.2	15 27.3	5		3.3[d]	3.3[e]	0 0	Paraffin-embedded tissue

Table 33 (contd)

Reference, study location	Method of detection and types tested	No. and type of lesions	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comments
				6	11	16	18	31	33	Others		
Carter *et al.* (2001), USA	MY09/11 and RFLP; type-specific for 16/18	33 SCC	81.8			69.7	3	6.0[f]		12.1[c]	9.1	Paraffin-embedded tissue; no comments on histopathology
Rubin *et al.* (2001), USA and Uruguay	SPF10 and LiPA for 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 70 and 6, 11, 34, 40, 42, 43, 44, 53, 54, 74	106 keratinizing	34.9	3.8	0	17.9	0.9	0	0	20.8[g]	NR	Paraffin-embedded tissue; no difference in prevalence between samples from Uruguay and the USA
		5 warty	100	0	0	100	0	0	0	0		
		15 basaloid	80.0	0	0	66.7	0	[6.7]	0	20.0[h]		
		30 PIN	90.0	20.0	3.3	36.7	0	30.0[i]		16.7[j]		
Ferreux *et al.* (2003), Netherlands	GP5+/6+ EIA and type-specific for 37 types; ISH	55 SCC	37.7	5.6		28.3				3.7[k]	NR	Snap-frozen samples
		48 SCC-NOS	35.4	2.1		29.2				4.2[k]		
		2 warty	100	50		50				0		
		2 sarcomatoid	0	0		0				0		
		1 verrucous	100	100		0				0		

See Table 7 for a description of the primers used.

EIA, enzyme immunoassay; ISH, in-situ hybridization; LiPA, line blot hybridization; NOS, not otherwise specified; NR, not reported; PCR, polymerase chain reaction; PIN, penile intraepithelial neoplasia; RFLP, restriction fragment length polymorphism; SCC, squamous-cell carcinoma; SSCP, single-strand conformational polymorphism

[a] 6, 11 or 42

[b] 31, 33 or 35

[c] 31, 33, 35 or 39

[d] HPV 35, 45, 52, 68, 51/70/74 and unknown types

[e] 31, 33, 39, 44, 51, 52, 58 or 66

Table 34. Prevalence of HPV DNA in case series of anal cancer (≥ 5 cases)

Reference, study location	Method of detection and types tested	No. and type of lesions	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comments
				6	11	16	18	31	33	Others		
Noffsinger *et al.* (1995a,b), Canada, China and USA	ISH for 6/11, 16/18 and 31/33/35	54 invasive and 2 in-situ	41.1	5.6		39.3		1.8[b]			7.4	Paraffin-embedded tissue; in five samples, HPV was detected with only one of the two detection methods.
	MY09/11 and type-specific for 6, 16, 18	50	46.0[c]	6.0		38.0[c]	4.0				4.0	
Shroyer *et al.* (1995), USA	MY09/11 and probing for 6/11, 16, 18, 33	11 basaloid	90.9	0		63.6	9.1	0	0		9.1	Paraffin-embedded archival tissue
		16 non-basaloid	75.0	12.5		62.5	12.5	0	0		12.5	
	ISH for 6/11, 16/18, 31/33/35	9 basaloid	66.7	0		44.4		33.3			22.2	
		11 non-basaloid	72.7	0		72.7		0			0	
Ramanujam *et al.* (1996), USA	ISH for 6, 11, 16, 18, 31, 33, 35	53 (37 women, 16 men)	34.0									Paraffin-embedded tissue
Vincent-Salomon *et al.* (1996), France	Southern blot for 6/11/42, 16/18/33, 31/35/39	15 SCC	46.7	0	0	33.3	0	0	0	13.3[d]		Frozen tissue
		9 basaloid	55.5	0	0	44.4	0	0	0	11.1[d]		
	PCR specific for 6/11, 16, 18, 33	18 SCC	66.7	0	0	66.7	0		0	0	0	
		9 basaloid	77.8	0	0	55.5	22.2		0	0	0	
Williams *et al.* (1996), United Kingdom	ISH for 6, 11, 16, 18	35 invasive	68.6	0	0	68.6	0				0	Paraffin-embedded tissue

Table 34 (contd)

Reference, study location	Method of detection and types tested	No. and type of lesions	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comments
				6	11	16	18	31	33	Others		
Frisch *et al.* (1997), Denmark and Sweden	GP5+/6+ and probes for HR types (16/18/31/33/35/39/45/51/52/56/58/59/66/68) and LR types (6/11/40/42/43/44); type-specific for 6, 11, 16, 18, 31, 33	388 (304 women, 84 men)	87.6	1.3	0	72.9	5.7	0.8	5.9	1.8[e]		Paraffin-embedded tissue
Unger *et al.* (1997), USA	ISH for 6/11, 16/18, 31/33/35	3 SCC from HIV-positive patient and 3 SCC from HIV-negative patient	100	16.7		83.3[f]	16.7[f]		16.7[f]		16.7[f]	Paraffin-embedded tissue
Cuesta *et al.* (1998), USA	ISH for 6/11, 16/18, 31/33/35	6 verrucous carcinomas from HIV-positive patients	83.3	66.7		33.3						Paraffin-embedded tissue
Lai *et al.* (1998), China	MY09/11 and probing for 6, 11, 16, 18, 33	19 SCC	5.3	0	0	5.3	0		0		0	Paraffin-embedded archival tissue
		8 cloacogenic cancers	0	0	0	0	0		0			
		23 adeno-carcinomas	0	0	0	0	0		0			
		6 adeno-squamous carcinomas	0	0	0	0	0		0			

Table 34 (contd)

Reference, study location	Method of detection and types tested	No. and type of lesions	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comments
				6	11	16	18	31	33	Others		
Poletti *et al.* (1998), Switzerland	PU-1M for 16/18/31/33/52/58 and PU-31B primers for 6/11	33	39.4	3.03				36.4[g]				Paraffin-embedded tissue
Indinnimeo *et al.* (1999), Italy	Type-specific for 6/11, 16, 18, 31/33	7 squamo-cellular	71.4			28.6			42.9			Paraffin-embedded tissue
		7 cloacogenic	57.1			57.1						
Frisch *et al.* (1999), Denmark and Sweden	GP5+/6+ and probes for HR types (16/18/31/33/35/39/45/51/52/56/58/59/66/68) and LR types (6/11/40/42/43/44); type-specific for 16, 18, 31, 33	331 (253 women, 78 men)	HR: [84] Men: 63 Women: 90 LR: [4.5] Men: 6.4 Women: 4.0			[73]	[6]	[1]	[5]	[2][h]	[2.7]	Paraffin-embedded tissue
Carter *et al.* (2001), USA	MY09/11 and RFLP; specific primers for 6, 18	64 (45 women, 38 men)	93.8			79.7	9.4		6.2[i]	10.8[j]	10.9	

Table 34 (contd)

Reference, study location	Method of detection and types tested	No. and type of lesions	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comments
				6	11	16	18	31	33	Others		
Daling *et al.* (2004), USA	MY09/11 and probing for 16, 18	179 SCC	92.2			76.0	8.9					Paraffin-embedded tissue
		41 basaloid	97.6			95.1	0					
		20 adeno-carcinoma	40.0			15.0	5.0					
		Men										
		36 in-situ	94.4			80.6	5.6					
		76 invasive	81.8			66.7	7.6					
		All	92.6									
		Women										
		34 in-situ	91.2			67.7	8.8					
		112 invasive	88.4			75.9	6.3					
		All	91.8									

See Table 7 for a description of the primers used.
HIV, human immunodeficiency virus; ISH, in-situ hybridization; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SCC, squamous-cell carcinoma
[a] Unless specified otherwise, the method is PCR; ';' denotes independent methods whereas 'and' denotes subsequent steps.
[b] 31, 33 or 35
[c] The Working Group noted some discrepancies between text and table in the number of HPV-positive tumours detected by PCR: the text reported 23/50 HPV-positive tumours (46%) whereas the table presented only 22 positive cases (44%); the text reported 21 lesions positive for HPV 16 by PCR and the table reported only 19 HPV 16-positive samples.
[d] Unknown
[e] 40/42/43/44
[f] One tissue contained 16, 18 and 33.
[g] 16, 18, 31, 33, 52 or 58
[h] Untyped high risk
[i] 31, 33, 35 or 39
[j] 6, 45, 52, 54, 58, 66, 72, 73 or unknown types

(*b*) *Case–control studies*

Frisch *et al.* (1999) studied the prevalence of HPV DNA in cancers of the anal canal and of the perianal skin. Anal cancers in women and homosexual men were more frequently HPV-positive for high-risk types ($p < 0.01$) and located in the anal canal ($p \leq 0.01$) than were cancers in heterosexual men. In both women and men, cancers of the anal canal contained high-risk HPV DNA more often than perianal skin cancers and increased high-risk HPV DNA positivity was seen with higher localization in the anal canal: 95 and 83% of cancers that involved the anal canal in women and men, respectively, were HPV-positive for high-risk types versus 80 and 28% of perianal skin cancers (p for trend < 0.001). Basaloid features, adjacent and anal intraepithelial neoplasia (AIN), poor or absent keratinization and a predominance of small or medium neoplastic cells were all strongly associated with positivity for high-risk HPV types.

The relationship between HPV infection and the subsequent risk for anal and perianal skin cancer was also studied in a case–cohort study among subjects who developed anal and perianal skin cancer during follow-up (median time, 10 years). Twenty-eight cases and 1500 controls were analysed for the presence of antibodies against HPV 16, 18, 33 or 73. An increased risk for developing anal and perianal skin cancer was observed among subjects who were seropositive for HPV 16 (odds ratio, 3.0; 95% CI, 1.1–8.2) and HPV 18 (odds ratio, 4.4; 95% CI, 1.1–17). The highest risks were seen for HPV 16-seropositive patients over the age of 45 years at serum sampling and for patients with a lag time of less than 10 years (Bjørge *et al.*, 2002).

Daling *et al.* (2004) measured antibodies to HPV 16 in cases of anal cancer and controls. HPV seropositivity was found in 51% of heterosexual male cases, 49% of not exclusively heterosexual male cases, 16% of heterosexual male controls, 42% of female cases and 15% of female controls.

In summary, cancer of the anal canal resembles cervical cancer in its high prevalence of HPV 16 and, to a lesser extent, other high-risk HPV types. Among these, HPV 18 is the next most common. HPV 6 or 11, 31 and 33 are uncommon but are found in a small proportion of tumours. In contrast, cancer of the perianal skin resembles vulvar and penile cancers, with a lower prevalence of HPV DNA positivity overall. [This may in part reflect the distance of the tumour from the anal verge, representing a mixture of true anal cancers and of skin cancers that are generally negative for infection with genital HPV types (see Section 2.5).]

2.4 Cancer of the upper aerodigestive tract

2.4.1 *Cancer of the oral cavity*

Cancer of the oral cavity (including tumours of the tongue, floor of the mouth, gum, palate and other sites of the mouth) is strongly associated with tobacco smoking (IARC, 2004) or chewing (IARC, 2007) and alcoholic beverage drinking (IARC, 1988), with attributable fractions in the order of 90%. However, some tumours occur in subjects who

are not exposed to known risk factors, and only a fraction of exposed subjects develop tumours, which suggests that other exposures may be independently involved or act as co-factors. HPV is known to infect the oral cavity of healthy individuals, and several HPV-related lesions have been characterized. However, most of the epidemiology and natural history of oral HPV infection remains to be elucidated (Herrero, 2003).

(*a*) *Case series*

Numerous studies have investigated the prevalence of HPV in tumour specimens of subjects with cancer of the oral cavity. Reported estimates have ranged from 0 to 100% (reviewed by Franceschi *et al.*, 1996; Gillison & Shah, 2001; Kreimer *et al.*, 2005). Table 35 presents series that included more than 40 cases of cancer of the oral cavity and evaluated the presence of HPV using PCR methods. The prevalence of HPV in these studies ranged from 4 to 80%. For instance, in the large IARC multicentric study, the prevalence of HPV in these cancers was 3.9% (Herrero *et al.*, 2003); in a recent systematic review that pooled HPV DNA results from oral squamous-cell cancer tissue specimens (Kreimer *et al.*, 2005), the overall prevalence in 2642 cases was 23.5% (95% CI, 21.9–25.1%). The wide variation in prevalence estimates is probably related to differences in populations, HPV testing methods, prevalence of other risk factors and the combination of specific topographical locations included in the studies. In general, studies from western countries indicate lower prevalence than those conducted in India, China or Japan.

The type most commonly reported in all studies was HPV 16, which was detected in more than 60% of HPV-positive tumours in two-thirds of the studies and in more than 80% of the tumours in half of the studies (Table 35). In the meta-analysis by Kreimer *et al.* (2005), 68.2% of the positive cancers were positive for HPV 16. The second most common type was HPV 18, with occasional reports of HPV 33, 6 and 11.

Several studies have compared the prevalence of HPV DNA in cases of oral cancer and tissues from individuals without oral cancer. Ostwald *et al.* (1994) used PCR methods to detect HPV DNA in biopsies from 26 oral squamous-cell carcinomas and exfoliated cells of the buccal mucosa from 97 healthy volunteers. HPV was detected in 61.5% of cancers and 1% of volunteers. In a study in India, frozen biopsies of 83 patients with cancer of the oral cavity were compared with exfoliated cells from 102 volunteers from a dental clinic (Koppikar *et al.*, 2005). HPV was detected by PCR in 38.6% of cancer patients and 5% of the control group. HPV 8, 16 and 18 were the most common types found in these cancers, but other types, including several from the genus beta-papillomavirus, were also detected. The latter were the only types detected in normal subjects. Zhang *et al.* (2004) reported a study of 73 cases of cancer of the oral cavity and 40 specimens from patients with benign tissue biopsies in China. PCR methods were used to detect HPV DNA for types 16 and 18, which were detectable in 74% of the cases and 55% of the non-cancer patients ($p = 0.04$).

Table 35. Prevalence of HPV DNA in case series of cancer of the oral cavity (> 40 cases) detected by the polymerase chain reaction (PCR) method[a]

Reference, study location	Method of detection and types tested	Sites included	No. of cases	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comment
					6	11	16	18	31	33	Others (type)		
Balaram *et al.* (1995), India	MY09/11; GP5/6; type-specific for 6/11, 16, 18	Buccal mucosa, tongue, floor of mouth, lower alveolus, other	91	73.6	13.2	19.8	41.7	47.3				40.7	Fresh frozen and paraffin-embedded tissue
Shindoh *et al.* (1995), Japan	Type-specific for 16, 18, 33 and dot blot hybridization	Tongue, gingiva, floor of mouth, maxillary, mouth, palate, retromolar	77	31.2			31.2	1.3				1.3	Paraffin-embedded tissue
Paz *et al.* (1997), USA	MY09/11; IU/IWDO; type-specific primers for 6, 16, 18, 31/33/35/ 44/45/56	Tongue, floor of mouth, oral cavity	64	12.5	3.1		7.8				1.6[d]		Fresh frozen tissue
Wen *et al.* (1997), China	Type-specific for 16, 18 and southern blot		45	31.1			20.0	24.4				13.3	Paraffin-embedded tissue
D'Costa *et al.* (1998), India	MY09/11 and southern blot for 6, 11, 16, 18, 33	Buccal mucosa, lower alveolus, tongue, floor of mouth, hard palate, maxilla	100	15.0	0[c]	0[c]	15.0	0		0[c]			Fresh frozen tissue
Schwartz *et al.* (1998), USA	MY09/11; type-specific primers for 6, 11, 16, 18	Tongue, gum, floor of mouth, other parts of mouth, NOS	186	20.4	6*		~11*						Paraffin-embedded tissue; *read from graph

Table 35 (contd)

Reference, study location	Method of detection and types tested	Sites included	No. of cases	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comment
					6	11	16	18	31	33	Others (type)		
Gillison *et al.* (2000), USA	MY09/MY11; type-specific primers for 33 types	Oral cavity	84	11.9			~11[e]	1.2					Snap-frozen tissue
Tsuhako *et al.* (2000), Japan	Type-specific for 6, 11, 16, 18	Tongue, mouth floor, buccal mucosa, lower gum, maxilla, lip	83	57.8	15.7	1.2	33.7	37.3			1.2[d]		Paraffin-embedded tissue
Mork *et al.* (2001), Finland, Norway and Sweden	GP5+/6+; CPI/CPIIG	Tongue, floor of mouth, oral cavity NOS	59				[7]						Paraffin-embedded tissue
Premoli-de-Percoco & Ramirez (2001), Venezuela	Type-specific for 6, 11, 16, 18	Tongue, buccal mucosa, floor of the mouth, others	50	60.0	0	0	50.0	16.0	0	0			Paraffin-embedded; women only
van Houten *et al.* (2001), Netherlands	GP5+/6+	NOS	45	4.4			4.4						Snap-frozen tissue
Ringström *et al.* (2002), USA	MY09/11	NOS	41	4.9			4.9						Snap-frozen tissue
Shin *et al.* (2002), Republic of Korea	Type-specific for 16, 18, 33	Oral cavity, salivary glands	76	14.5			5.3	10.5		2.6			Tissue collection not specified

Table 35 (contd)

Reference, study location	Method of detection and types tested	Sites included	No. of cases	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comment
					6	11	16	18	31	33	Others (type)		
Chang *et al.* (2003), Taiwan, China	MY09/11; GP5+/6+ with typing by sequencing and gene chip hybridization	Tongue, mouth, gingiva, lip, palate, floor of mouth	103	49.5			28.2	26.2		1.0			Paraffin-embedded tissue
Herrero *et al.* (2003), Multicentric, 9 countries	GP5+/6+ and southern blot for 6, 11, 16, 18, 31, 33	Base of tongue, other parts of tongue, gum, floor of mouth, palate, mouth	766	3.9			~4						Snap-frozen tissue
Kansky *et al.* (2003), Slovenia	MY09/11; GP5+/6+; multiple types of primer sets and RFLP	Tongue, floor of mouth, rectomolar trigonum, buccal mucosa	55	5.5			1.8			1.8	1.8 (58)		Paraffin-embedded tissue
Kojima *et al.* (2003), USA	PCR and DNA sequencing; ISH; immunohisto-chemistry for 38	Tongue, buccal mucosa, maxillary and mandibular gingiva, hard palate, floor of mouth	53	66.0							66.0 (38)		Paraffin-embedded tissue
Ostwald *et al.* (2003), Germany	Type-specific primers for 6/11, 16, 18	Oral cavity, lip	118	43.2		4.2	29.7	13.6					Snap-frozen tissue
Sugiyama *et al.* (2003), Japan	Type-specific for 16, 18	Gingiva, tongue, oral floor, cheek, lip, palate	86	34.9			34.9	2.3				2.3	Paraffin-embedded tissue

Table 35 (contd)

Reference, study location	Method of detection and types tested	Sites included	No. of cases	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comment
					6	11	16	18	31	33	Others (type)		
Dahlgren *et al.* (2004), Sweden	GP5+/GP6+ or CPI/CIIPG and sequencing; type-specific PCR for 16, 18, 33	All tumours were from the oral tongue.	85 mobile	2.4			2.4	0		0			Paraffin-embedded tissue
			25 base	40.0			28.0	0		4.0	4.0 (35)		
Smith, E.M. *et al.* (2004a), USA	MY09/11 and dot blot and sequencing	Tongue, floor of mouth, gingiva, hard palate, lip, major salivary glands, other oral mucosa	126	10			7.9			2.3			Paraffin-embedded tissue

See Table 7 for a description of the primers used.
EIA, enzyme immunoassay; ISH, in-situ hybridization; NOS, not otherwise specified; RFLP, restriction fragment length polymorphism
[a] Only studies that used PCR as the method of detection of HPV were included.
[b] ';' denotes independent methods whereas 'and' denotes subsequent steps.
[c] Subgroup of 70 samples examined
[d] Untyped
[e] 90% of the 253 tumour specimens analysed in the study were positive for HPV 16.

(*b*) *Case–control studies*

Table 36 presents case–control studies of cancer of the oral cavity that used exfoliated cells or immunological markers to determine the presence of HPV.

The study by Schwartz *et al.* (1998) included 284 cases of cancer of the oral cavity and oropharynx combined and 477 controls. Results for an HPV 16 capsid antibody response were available for 259 cases and 446 controls. Antibodies against HPV 16 L1 VLPs were equally prevalent in tumours of the floor of the mouth and in controls (odds ratio, 1.1; 95% CI, 0.5–2.5), but were more frequent in cancers of the tongue (odds ratio, 2.4; 95% CI, 1.5–3.8) after adjustment for age, sex, tobacco smoking and alcoholic beverage drinking.

In a joint Nordic cohort, serum samples were collected from almost 900 000 individuals who were then followed through record linkage with tumour registries. Mork *et al.* (2001) evaluated the association of serum levels of antibodies against HPV 16 capsids with the occurrence of different cancers of the head and neck in a case–control study nested within this cohort. Elevated odds ratios were detected for cancers of the tongue (2.8; 95% CI, 1.2–6.6) and of the oral cavity not otherwise specified (3.6; 95% CI, 0.5–23.6). [The Working Group noted that this study adjusted for serum levels of cotinine and is important because of its prospective nature.]

In a case–control study by Dahlstrom *et al.* (2003), antibodies against HPV 16 L1 VLPs were detected at similar levels in 36 cases of squamous-cell carcinoma of the oral cavity and 120 cancer-free controls selected from a managed care organization (8.3 versus 9.2%, respectively).

Herrero *et al.* (2003) conducted a multicentre case–control study in Australia, Canada, Cuba, India, Italy, Northern Ireland, Poland, Spain and the Sudan from April 1996 to December 1999. The study included 1670 cases (1415 with cancer of the oral cavity and 255 with cancer of the oropharynx) and 1732 controls. Oral exfoliated cells and blood were obtained from all participants and fresh biopsy specimens from cases. HPV DNA was detected by PCR followed by enzyme immunoassay or southern blot hybridization. Antibodies against HPV 16 L1, E6 and E7 proteins in plasma were detected by enzyme-linked immunosorbent assay (ELISA). Detection of HPV DNA in exfoliated cells was performed in only less than 50% of samples and did not correlate with detection of HPV DNA in biopsy specimens. HPV DNA was detected in biopsy specimens of 3.9% (95% CI, 2.5–5.3%) of 766 cancers of the oral cavity with valid PCR results. Antibodies against HPV 16 L1 were associated with increased odds ratios for cancers of the oral cavity (1.5; 95% CI 1.1–2.1), as were antibodies against either HPV 16 E6 or E7 (2.7; 95% CI, 1.6–4.7) after adjusment for country, age, sex, tobacco smoking, alcoholic beverage drinking and *paan* chewing.

Van Doornum *et al.* (2003) reported the prevalence of antibodies against HPV 16 E7 to be 5.3% among 56 cases of carcinoma of the tongue and 2% among 100 non-cancer controls.

Smith, E.M. *et al.* (2004b) reported a case–control study that included 130 cases of cancer of the oral cavity and 333 control subjects, frequency-matched for age and sex.

Table 36. Case–control studies of HPV prevalence and cancer of the oral cavity

Reference, study location	Sites included	No. of cases	No. of controls	Method of detection	HPV prevalence (%)		Odds ratio (95% CI)	Comments/adjustments
					Cases	Controls		
Schwartz *et al.* (1998), USA	Oral cavity and oropharynx combined; see Table 35 for organs included and Table 38	259	446	Antibodies against HPV 16 L1 VLPs	51.4	35.0	2.3 (1.6–3.3)	Adjusted for age, sex, tobacco smoking and alcoholic beverage drinking *Read from graph
	Floor of mouth	38	446		26*	35.0	1.1 (0.5–2.5)	
	Tongue	107	446		20*	35.0	2.4 (1.5–3.8)	
		237	435 pop. controls	PCR in exfoliated cells	9.3	9.2	0.9 (0.5–1.6)	
Mork *et al.* (2001), Finland, Norway and Sweden	Tongue, floor of mouth, oral cavity NOS	19	Cohort of ~950 000 residents	Antibodies against HPV 16 L1 VLPs	11	2	3.6 (0.5–26.3)	Seropositivity for HPV 16
	Tongue	57			16	7	2.8 (1.2–6.6)	
Herrero *et al.* (2003), Multi-centric, 9 countries	Base of tongue, other parts of tongue, gum, floor of mouth, palate, mouth	511	613	PCR of exfoliated cells	4.7	6.9	0.6 (0.3–1.1)	Adjusted for country, sex, age, tobacco smoking, alcoholic beverages, *paan* chewing
		1299	1527	Antibodies against HPV 16 L1 VLPs	8.9	6.0	1.5 (1.1–2.1)	
		1319	1581	Antibodies against E6 and E7 proteins	4.2	1.5	2.7 (1.6–4.7) 4.3 (0.8–23.2) if both positive	
Smith *et al.* (2004b), USA	Oral cavity and oropharynx combined (see Table 38)	201	333	PCR and sequencing of oral exfoliated cells	28.4	18.3	1.8 (1.1–2.7)	Adjusted for age, sex, tobacco smoking, alcoholic beverage consumption
	Oral cavity: lip vermillion and inner mucosa, tongue, gingiva, floor of mouth, hard palate, other oral mucosa, parotid gland, submandibular gland	130	333		15	18.3	NR	

CI, confidence interval; NR, not reported; PCR, polymerase chain reaction; VLP, virus-like particles

[a] Detection of HPV DNA in exfoliated cells was considered to be an inadequate indication of HPV infection in this study as it did not correlate with detection of HPV DNA in tumours.

Oral exfoliated cells and tumour tissue were analysed for HPV content by PCR and dot blot hybridization and for HPV type by DNA sequencing. HPV DNA was detected in oral cells from 15% of cases of cancer of the oral cavity and 18.3% of controls. Risk estimates restricted to cancers of the oral cavity were not presented.

Studies of non-genital sites present a special challenge for the assessment of exposure to HPV because oral exfoliated cells do not appear to demonstrate infection adequately. As an alternative, serological markers of HPV exposure or expression (e.g. antibodies against HPV L1 VLPs or against E6 or E7 proteins) have been used in some recent studies (Schwartz *et al.*, 1998; Herrero *et al.*, 2003; see above).

Moreover, it is not always feasible to define clearly the precise anatomical location of the primary tumour, and multiple locations are frequent. Misclassification of the primary tumour site can introduce distortions in prevalence estimates for individual sites. For example, the base of the tongue is not classified consistently in the different studies when sites are grouped as oral cavity or oropharynx. A recent, carefully conducted study (Dahlgren *et al.*, 2004) indicated that the base of the tongue has an HPV prevalence of 40% compared with 2.3% in the oral tongue.

2.4.2 *Cancer of the oropharynx and tonsil*

Similar to cancers of the oral cavity, cancers of the oropharynx and tonsil are strongly associated with tobacco smoking and alcoholic beverage drinking, but other etiological agents may play an independent role or act as co-factors.

(*a*) *Case series*

A series of studies have reported prevalence of HPV in cancers of the oropharynx and tonsil. Those that used PCR as the method of detection and included more than 40 cases are summarized in Table 37. The prevalence of HPV in the selected studies ranged from 14 to 57%, and the largest study to date (Herrero *et al.*, 2003) reported a prevalence of 18%. In a systematic review that pooled HPV DNA results from 969 cases of squamous-cell cancer of the oropharynx (Kreimer *et al.*, 2005), the overall prevalence of HPV was 35.6% (95% CI, 32.6–38.7%). Among HPV-positive tumours, there was a marked and very consistent predominance of HPV 16. In all studies included in Table 37, HPV 16 was present in at least 78% of HPV-positive tumours and, in the review by Kreimer *et al.* (2005), the corresponding figure was 87%. HPV 18 is almost never present and other types (HPV 6, 11, 31 and 33) are only detected sporadically. In the studies that presented separate estimates for cancer of the tonsil, this anatomical site consistently showed the highest prevalence of HPV.

Using in-situ hybridization with probes for HPV 6, 11 and 16 under high stringency, Niedobitek *et al*. (1990) tested 28 tonsillar carcinomas and 30 tonsils removed because of chronic inflammation. Six of the cases and none of the controls were HPV 16-positive [$p < 0.001$].

Table 37. Prevalence of HPV DNA in case series of oropharyngeal and tonsillar cancer (> 40 cases)

Reference, study location	Method[a] of detection and types tested	No. and type of lesions	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Comment
				6	11	16	18	31	33	Others (type)	
Schwartz *et al.* (1998), USA	MY09/11; type- specific for 6, 11, 16, 18	11 oropharynx 44 tonsil	36.4 44[b]	0 10		36.4 34.1					Paraffin-embedded tissues; *read from graph
Gillison *et al.* (2000), USA	MY09/MY11/HMB01; mixture for 16/18/51/66 and probing with 33 type-specific probes	60 oropharynx 52 tonsil 8 others	56.7 62 25		1	51	1	1	3		Fresh-frozen tissues; type distribution for combination of cases of the oral cavity and oropharynx (n = 253)
Mellin *et al.* (2000), Sweden	GP5+/6+; type-specific for 16, 33	60 tonsil carcinomas	43.3			43.3			1.7		Paraffin-embedded tissues
Lindel *et al.* (2001), Switzerland	SPF 1/2 for high-risk types and sequencing	99 oropharynx 40 tonsil	14.1 15			11.1			1.0	1.0 (35) 1.0 (45)	Paraffin-embedded tissues
Strome *et al.* (2002), USA	MY09/11, others; type-specific for 16	52 tonsil carcinoma	46.2			40.4				3.8 (12)	Paraffin-embedded tissues
Herrero *et al.* (2003), Multi-centric, 9 countries	GP5+/6+ and EIA for 6, 11, 16, 18, 31, 33	142 tonsil	18.3 26			~17				[< 1]	Snap-frozen tissues
Li, W. *et al.* (2003), Australia	GP5+/6+ and other consensus primers; type-specific for 16	67 tonsil carcinoma	46.3			44.7					Paraffin-embedded tissues
China		16 tonsil carcinoma	0								Paraffin-embedded tissues
Smith *et al.* (2004a), USA	MY09/11/HMB01; dot blot and sequencing	31 tonsil 5 oropharynx	58.1 0			51.6	3.2		3.2		Paraffin-embedded tissues

See Table 7 for a description of the primers used.
EIA, enzyme immunoassay; PCR, polymerase chain reaction;
[a] Only those studies where HPV was detected by PCR were included; ';' denotes independent methods whereas 'and' denotes subsequent steps.

Snijders *et al.* (1992a,b) used PCR and southern hybridization techniques to test 10 cases of carcinoma of the tonsil and seven control patients with tonsillitis. All cases tested positive for HPV versus none of the controls [odds ratio, ∞; 95% CI, 7.5–∞]. The presence of HPV in cancer cells was confirmed by RNA in-situ hybridization.

In Japan, Watanabe *et al.* (1993) tested 12 cases of carcinoma of the oropharynx, hypopharynx and tonsil and 28 control specimens from patients with chronic tonsillitis. Three methods for HPV testing were used — dot-filter, southern hybridization and PCR. The results from PCR showed a prevalence of HPV DNA of 25% among cases and 4% among controls.

(*b*) *Case–control studies*

Table 38 presents case–control studies of cancer of the oropharynx that used exfoliated cells or immunological markers to determine the presence of HPV.

In the study by Schwartz *et al.* (1998), the odds ratio for cancer of the tonsil associated with the detection of antibodies against HPV 16 L1 was 3.9 (95% CI, 2.0–7.8) after adjustment for age, sex, tobacco smoking and alcoholic beverage drinking.

In the nested case–control study reported by Mork *et al.* (2001), the risk for developing cancer of the oropharynx associated with pre-diagnostic detection of antibodies against HPV 16 L1 VLPs was 14.4 (95% CI, 3.6–58.1).

In the serological case–control study of Dahlstrom *et al.* (2003), HPV 16 L1 VLP seropositivity was associated with a 60-fold (95% CI, 5.7–620) increase in risk for oropharyngeal squamous-cell carcinoma (70 cases) compared with 120 cancer-free, clinic-based controls, following adjustment for reported alcoholic beverage use and serum cotinine level, which is a biomarker of recent exposure to tobacco.

In the IARC multicentre study (Herrero *et al.*, 2003), there was no difference in the prevalence of HPV DNA in exfoliated cells from cancers of the oropharynx and those from controls. However, the odds ratio for antibodies against HPV 16 L1 VLPs was 3.5 (95% CI, 2.1–5.9), and that for antibodies against HPV 16 E6 or E7 proteins was 4.5 (95% CI, 2.0–10.1). When both antibodies were detected, the odds ratio was 67.1 (95% CI, 12.9–348). These estimates were adjusted for country, tobacco smoking, alcoholic beverage drinking and *paan* chewing.

In another case–control study of 48 cases of oropharyngeal carcinoma and 100 cancer-free controls, levels of HPV 16 L1 antibodies were significantly elevated in cases compared with controls (33 versus 18%; $p = 0.04$); however, seroprevalence of HPV E7 was similar (4% versus 2% for cases and controls, respectively) (Van Doornum *et al.*, 2003).

Smith, E.M. *et al.* (2004a) reported an adjusted odds ratio for oropharyngeal cancer of 3.6 (95% CI, 1.8–7.1) associated with detection of HPV in exfoliated cells.

The oropharynx, and in particular the tonsils, are the extragenital sites where the role of HPV is most clear for a defined subset of tumours. Similarly to vulvar cancer, a dual etiology has been postulated for oropharyngeal tumours (Herrero *et al.*, 2003). One subset includes smoking-related cancers, with squamous histological features and *p53* mutations.

Table 38. Case–control studies of HPV prevalence and cancers of the oropharynx and tonsil

Reference, study location	Sites included	No. of cases	No. of controls	Method of detection	HPV prevalence (%)		Odds ratio (95% CI)	Comments/adjustments
					Cases	Controls		
Schwartz *et al.* (1998), USA	Oral cavity and oropharynx combined	259	446	Antibodies against HPV 16 L1 VLPs	51.4	35.0	2.3 (1.6–3.3)	Adjusted for age, sex, tobacco smoking, alcoholic beverage drinking
	Tonsil	49	446		44.0	35.0	3.9 (2.0–7.8)	
Mork *et al.* (2001), Finland, Norway and Sweden	Oropharynx (ICD 145)	26	Cohort of ~900 000 residents	Antibodies against HPV 16 L1 VLPs	38	10	14.4 (3.6–58.1)	Seropositivity for HPV 16
Dahlstrom *et al.* (2003), USA	Base of tongue, tonsil, other oropharynx	70	120	Antibodies against HPV 16 L1 VLPs	58.6	9.2	59.5 (5.7–620)	Adjusted for cotinine, alcoholic beverages, matching variables; prevalence in tonsil cancers, 59.4%
Herrero *et al.* (2003), Multi-centric, 9 countries	Oropharynx and tonsil	90	613	PCR in oral exfoliated cells	8.9	6.9	1.0 (0.4–2.5)[a]	Adjusted for country, sex, age, tobacco smoking, alcoholic beverages, *paan* chewing
		238	1527	Antibodies against HPV 16 L1 VLPs	13.4	6.0	3.5 (2.1–5.9)	
		243	1581	Antibodies against E6 and E7 proteins	5.3	1.5	4.5 (2.0–10.1) 67.1 (12.9–348) if both positive	
Van Doornum *et al.* (2003), Netherlands	Oropharynx	48	100	Antibodies against HPV 16 L1 VLPs and E7 proteins	L1, 33 E7, 4	L1, 18 E7, 2	L1, 2.25 (1.0–4.9)	

Table 38 (contd)

Reference, study location	Sites included	No. of cases	No. of controls	Method of detection	HPV prevalence (%)		Odds ratio (95% CI)	Comments/adjustments
					Cases	Controls		
Smith *et al.* (2004b), USA	Oral cavity and oropharynx combined (see Table 36)	201	333	PCR of oral exfoliated cells and sequencing	28.4	18.3	1.8 (1.1–2.7)	
	Oropharynx: base of tongue, soft palate, uvula, palatine tonsil fossa, pillar and overlapping regions, oropharynx, oropharynx NOS	71	333		38.0	18.3	NR	

CI, confidence interval; NOS, not otherwise specified; NR, not reported; PCR, polymerase chain reaction; VLP, virus-like particles

[a] Detection of HPV DNA in exfoliated cells was considered to be an inadequate indication of HPV infection as it did not correlate with the detection of HPV DNA in tumours.

The other includes HPV-related tumours (reviewed by Gillison & Shah, 2001), which have been shown to have basaloid histological features, to occur more frequently in nonsmokers, to be less frequently associated with *p53* mutations (Braakhuis *et al.*, 2004; Dai *et al.*, 2004), to be associated with distinct patterns of genetic alterations (Braakhuis *et al.*, 2004) and possibly to have a better prognosis (Pintos *et al.*, 1999).

2.4.3 *Cancer of the oesophagus*

The possibility that HPV infection may play a role in the etiology of squamous-cell carcinoma of the oesophagus has been proposed, but the data have not been consistent (reviewed by Gillison & Shah, 2003). The epithelium of the oesophagus is similar to that of the oral cavity, and papillomas have been described at this anatomical site although they are rare, and HPV is not consistently detected therein.

(*a*) *Case series*

Table 39 presents series of cancer of the oesophagus that studied more than 40 cases; the 19 studies show great heterogeneity with regard to the prevalence of detection of HPV DNA, which ranged from 0 to 55%. Five studies from France (Benamouzig *et al.*, 1995), Italy (Talamini *et al.*, 2000), Japan (Saegusa *et al.*, 1997), the Netherlands (Kok *et al.*, 1997) and Slovenia (Poljak *et al.*, 1998), which included more than 45 cases and used PCR methods with common consensus primers, showed 0% prevalence of HPV in oesophageal cancers. In contrast, many studies from China, an area with a high incidence of cancer of the oesophagus, reported a high overall prevalence of HPV that was generally around 50%. The largest study, also from China and which included 700 cases, showed an overall HPV prevalence of 17% using in-situ hybridization (Chang *et al.*, 2000).

In oesophageal cancers, the predominance of HPV 16 is less marked than that at other sites of the head and neck, and HPV 18, 6 and 11 are detected commonly.

A recent study by de Villiers *et al.* (2004b) among patients with other head and neck cancers detected HPV DNA in 67% of the premalignant or malignant oesophageal tissue biopsies, which pointed to a particular subgroup of cancers of the oesophagus.

In the study by Benamouzig *et al.* (1992), the presence of oesophageal HPV infection was studied in endoscopic biopsies of 12 patients with oesophageal squamous-cell carcinoma, 24 control patients exposed to similar known risk factors (alcoholic beverages and tobacco) and seven non-exposed controls. Five of 12 patients with oesophageal carcinoma had HPV infection in the normal oesophagus tissue using dot blot hybridization (three of these also had HPV-positive tumour tissue). Only one of the controls had an oesophageal HPV infection ($p < 0.01$). HPV 16 and 18 were the types most frequently detected.

(*b*) *Case–control studies*

Case–control studies that assessed HPV prevalence by serology have reported contradictory results. A carefully conducted population-based case–control study in Sweden

Table 39. Prevalence of HPV DNA in case series of oesophageal carcinomas (> 40 cases)

Reference, study location	Method[a] of detection and types tested	No. of cases	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comments
				6	11	16	18	31	33	Others (type)		
Chang *et al.* (1992), China	Type-specific for 6, 11, 16, 18	51	49.0	5.9	13.7	17.6	13.7				2.0	Paraffin-embedded tissue
Toh *et al.* (1992), Japan	Consensus PCR for 16/18/31/33/52/58, 6/11/16/18/31/33 and type-specific primers for 16, 18	45	6.7			2.2	4.4					Fresh tissue
Togawa *et al.* (1994), International	Consensus primers and RFLP for 6, 11, 16, 18	72	23.6			12.5	1.4			9.7 (unknown)		Paraffin-embedded or frozen tissue
Benamouzig *et al.* (1995), France	MY09/11 and type-specific for 6/11, 16/18, 31/33; dot blot for 6/11, 16/18	75	0									Frozen tissue
Suzuk *et al.* (1996), China	MY09/11 Type-specific for 6, 16, 18	70 70	0 4.3	1.4		2.9						Paraffin-embedded tissue
Lam *et al.* (1997), Hong Kong, SAR	Type-specific for 16, 18 and southern blot with consensus probes	70	8.6			8.6						Snap-frozen tissue
Turner *et al.* (1997), Canada, USA	MY09/11 type-specific primers for 16, 18/33	51	2.0			2.0						Paraffin-embedded tissue
Kok *et al.* (1997), Netherlands	MY09/11, GP5+/6+, CPI/CPIIG, CPI/CPIIS	63	0									Paraffin-embedded tissue

Table 39 (contd)

Reference, study location	Method[a] of detection and types tested	No. of cases	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comments
				6	11	16	18	31	33	Others (type)		
Mizobuchi *et al.* (1997), Japan	Type-specific for 16, 18 and southern blot	41	7.3				7.3					Snap-frozen tissue
Saegusa *et al.* (1997), Japan	L1C1/L1C2 for 6/11/16/18/31/33/42/52/58; pU-1M/pU-2R for 16/18/31/33/52/58	92	0									Paraffin-embedded tissue
Poljak *et al.* (1998), Slovenia	MY09/11, GP5+/6+, WD; nested PCR; type-specific PCR for 6, 16, 18	120	0									Paraffin-embedded tissue
de Villiers *et al.* (1999a), China	Degenerate primer set HD; CP; GP5+/6+	70	4.3			1.4			1.4			Fresh frozen tissue
Lavergne & de Villiers (1999), China and South Africa	Degenerate PCR and sequencing	63	30.2	9.5		0	1.6			25.3 (untyped)	6.3	Snap-frozen tissue
Chang *et al.* (2000), China	ISH with broad-spectrum probe and type-specific probes for 6/11, 16, 18, 30, 53	700	16.6		1.1	3.0	1.7			12.0 (untyped)		Paraffin-embedded tissue
Talamini *et al.* (2000), Italy	MY09/11, GP5+/6+	42	0									Paraffin-embedded tissue

Table 39 (contd)

Reference, study location	Method[a] of detection and types tested	No. of cases	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comments
				6	11	16	18	31	33	Others (type)		
Kawaguchi *et al.* (2000), China and Japan	Type-specific for 16, 18 and sequencing	75	22.7									Snap-frozen tissue
Li, L.T. *et al.* (2001, 2002), China	Type-specific for 16, 18	62	62.9									Paraffin-embedded tissue
Matsha *et al.* (2002), South Africa	MY09/11, GP5+/6+	50	46.0		22.0	4.0				14.0 (39), 2.0 (52), 4.0 (un-typed)		Paraffin-embedded tissue
Shen *et al.* (2002), China	Consensus primers PCR for 6/11, 16/18	55 44	65.5		34.1		43.2					Fresh tissue
	PCR for 16, 18	77	54.5			39.0	22.1				10.4	
Si *et al.* (2003), Hong-Kong, SAR	MY09/11 and type-specific for 16, 18	319	13.5			12.2	1.9					Paraffin-embedded and snap-frozen tissues
Lu *et al.* (2004), China	Type-specific for 16	104	52.9			52.9						Paraffin-embedded tissue
Katiyar *et al.* (2005), India	MY09/11 Type-specific for 16, 18	101 60	26.7 66.7			16.8	2.0					Snap-frozen tissue

See Table 7 for a description of the primers used.
ISH, in-situ hybridization ; PCR, polymerase chain reaction
[a] Unless otherwise specified, the method is PCR; ';' denotes independent methods whereas 'and' denotes subsequent steps.

(Lagergren *et al.*, 1999) showed no association of antibodies against HPV 16 or HPV 18 L1 VLPs with either squamous-cell or adenocarcinoma of the oesophagus after adjustment for age, sex, smoking status, alcoholic beverage intake, education and the presence of the other HPV type. Another serology-based study from the Netherlands (Van Doornum *et al.*, 2003) reported 17% HPV 16 seropositivity among cases of oesophageal carcinoma compared with 18% among cancer-free controls. No cases were seropositive for HPV 16 E7 compared with 2% of controls. In contrast, a hospital-based study in China assessed HPV16 VLPs in cases of oesophageal cancer (95% of which were squamous-cell carcinomas) and cancer-free controls and reported that higher antibody levels significantly increased the relative risk for oesophageal cancer (odds ratio, 4.5; 95% CI, 1.8–11.9), after adjustment for age and sex (Shen *et al.*, 2002a). [The Working Group noted that the study lacked the ability to adjust further for potentially important confounders, such as tobacco use, alcoholic beverage consumption, dietary patterns or sexual behaviour.]

(*c*) *Cohort studies*

Prospective seroepidemiological studies in Scandinavian cohorts point to an association between HPV and oesophageal cancer. An initial study in Finland (Dillner *et al.*, 1995b) indicated a 14-fold increase in risk for oesophageal cancer associated with prediagnostic detection of HPV 16 capsid antibodies, and another study in Norway indicated a 6.2-fold increase in risk after adjustment for cotinine levels as markers of tobacco exposure (Bjørge *et al.*, 1997b).

2.4.4 *Cancer of the larynx*

The laryngeal epithelium is known to be susceptible to HPV infection because of the well-established association of HPV types 6 and 11 with juvenile- and adult-onset laryngeal papillomatosis. A few retrospective case series have reported laryngeal squamous-cell carcinoma among patients with a history of laryngeal papillomatosis; HPV 11 DNA was most commonly detected in these cancer specimens (Shen *et al.*, 1996; Reidy *et al.*, 2004). However, laryngeal papillomatosis is not a precursor for most laryngeal cancers. Similarly to cancers of the oral cavity and pharynx, the main risk factors for laryngeal squamous-cell carcinoma are tobacco use and alcoholic beverage consumption and the attributable fraction for these exposures is large.

(*a*) *Case series*

Table 40 presents studies of at least 40 cases of squamous-cell carcinomas of the larynx that employed PCR-based detection methods. The overall HPV prevalence ranged from 7% in a study in the USA to 59% in a large study in China (Ma *et al.*, 1998). HPV 16 was the predominant type detected, and accounted for approximately all [74%] HPV-positive laryngeal squamous-cell carcinomas. HPV 18 was the second most commonly detected type; HPV 6, 11 and 33 were detected in a few cases, and no other carcinogenic HPV types were reported. [The Working Group noted that the number of specimens exa-

Table 40. Prevalence of HPV DNA in case series of laryngeal cancer (> 40 cases)

Reference, study location	Method[a] of detection and types tested	No. of cases	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comment
				6	11	16	18	31	33	Others (type)		
Pérez-Ayala *et al.* (1990), Spain	Type-specific for 11 and 16 and dot blot	48	54.2		0	54.2						Snap-frozen tissues
Shidara *et al.* (1994), Japan	L1C1/L1C2 and RFLP	45	24.4			20.0	4.4					Paraffin-embedded tissues
Suzuki *et al.* (1994), Japan	L1C1/L1C2 and RFLP	41	26.8	0	0	22.0	4.9	0	0			Paraffin-embedded tissues
Fouret *et al.* (1995), France	Primers WD 72, 76, 66, 67, 154 and southern blot for 6, 11, 16, 18, 31, 33	59	5.1									Paraffin-embedded tissues
Almadori *et al.* (1996), Italy	PCR for 6, 11, 16, 18 and southern blot	45	20.0	4.4		20.0	0					Snap-frozen tissues
Paz *et al.* (1997), USA	MY09/11, IU/IWDO, type-specific for 6, 16, 18, probing for 31, 33, 35, 44, 45, 56	49	8.2	0		6.1	0			2.0 (un-known)		Snap-frozen tissues

Table 40 (contd)

Reference, study location	Method[a] of detection and types tested	No. of cases	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comment
				6	11	16	18	31	33	Others (type)		
Cattani *et al.* (1998), Italy	MY09/11 and EIA for 6, 11, 16, 18, 31	75	29.3			12.0	10.7		1.3	5.3 (un-typed)	1.3	Snap-frozen tissues
Hoffmann *et al.* (1998), Germany	Type-specific primers for 6, 11, 16, 18, 33 and southern blot; consensus primers and southern blot for type-specific negative samples	29	20.7			6.8						Snap-frozen tissues
Ma *et al.* (1998), China	pU-1M/pU-2R for 6/11 and pU-31B/pU-2R for 16/18/31/33/52/58	102	58.8	25.5	2.0	29.4	21.6	0	1.0		19.6	Paraffin-embedded tissues
Mineta *et al.* (1998), Japan	Type-specific for 16, 18	42	31.0			26.2	4.8					Snap-frozen tissues
Gorgoulis *et al.* (1999), Greece	MY09/11 and GP5/6; type-specific for 6, 11, 16, 18, 31, 33, 35	91	20.9	3.3	0	14.3	3.3	0	3.3		3.3	Snap-frozen tissues
Pintos *et al.* (1999), Canada	GP5+/6+ and southern blot	52	15.4									Paraffin-embedded tissues
Gillison *et al.* (2000), USA	MY09/11/HMBO1, TS for 16/18/51/66, southern blot with 33 type-specific probes and sequencing; type-specific for 16 and 18	86	18.6			~17				1.2 (un-typed)		Fresh frozen tissues; type-specific data in combination with other sites of head and neck cancers

Table 40 (contd)

Reference, study location	Method[a] of detection and types tested	No. of cases	Overall HPV positivity (%)	Type-specific HPV positivity (%)							Multiple infections (%)	Comment
				6	11	16	18	31	33	Others (type)		
Jacob *et al.* (2002), India	Type-specific for 16 and 18	44	34.1			34.1	0					Paraffin-embedded tissues
Báez *et al.* (2004), Puerto Rico	Type-specific for 6	52	46.2			46.2						Snap-frozen tissues

See Table 7 for a description of the primers used.
EIA, enzyme immunoassay; PCR, polymerase chain reaction
[a] Unless otherwise specified, the method is PCR; ';' denotes independent methods whereas 'and' denotes subsequent steps.

mined in each study was small (< 105 cases), which may in part explain the large variability in the prevalence estimates.] A relatively low prevalence of overall HPV DNA, coupled with the high prevalence of HPV16 and the lack of other HPV types among HPV-positive laryngeal carcinomas, have been consistently reported in the more recent studies of HPV and laryngeal squamous-cell carcinoma.

Similar results were reported in a large systematic review of published studies that met the same inclusion criteria of adequate sample size and PCR-based HPV detection methods (Kreimer *et al.*, 2005). Of 1435 laryngeal and hypopharyngeal squamous-cell carcinomas, the pooled HPV DNA prevalence was 24% (95% CI, 22–26%). In the type-specific analysis among HPV-positive laryngeal squamous-cell carcinomas, HPV16 accounted for 66% of infections, and HPV18 and HPV6 accounted for 16 and 14% of infections, respectively.

(*b*) *Case–control studies*

Table 41 reports the results of case–control studies. One early study (Brandsma & Abramson, 1989) reported similarly low prevalence of HPV in both cases (5%) and controls with benign disease or structural abnormalities (4%), which suggested no association between HPV and laryngeal cancer.

Smith *et al.* (2000) investigated HPV DNA in 44 patients with laryngeal or hypopharyngeal squamous-cell carcinoma and 12 controls with benign laryngeal conditions. HPV DNA was collected from biopsy material from the cases and from upper respiratory tract brushings from the controls. HPV DNA was detected in 25.0% (11/44) of cases and in 16.7% (2/12) of controls. The prevalence of carcinogenic HPV types (HPV 16, 18, 31, 45 and 70 were detected) was similar in cases (18.2%) and controls (16.7%). However, after adjustment for tobacco, alcoholic beverage consumption and age, the presence of carcinogenic HPV DNA elevated the relative risk for laryngeal squamous-cell carcinoma threefold (odds ratio, 3.0) (although not significantly) compared with controls.

One prospective serological study that included laryngeal squamous-cell carcinoma (Mork *et al.*, 2001) investigated the seroprevalence of HPV16 L1 VLP in a nested case–control study of 76 cases of laryngeal cancer and 411 controls using several Nordic cancer registries linked with serum banks. HPV seropositivity increased the risk for developing laryngeal cancers 2.4-fold (95% CI, 1.0–5.6), following adjustment for serum cotinine levels, a biological marker for tobacco exposure. [The serological samples were collected on average 9.4 years before the diagnosis of cancer, and thereby provide evidence of exposure to HPV that preceded the development of disease. However, this study lacked additional information on potential confounding factors, such as alcoholic beverage use and sexual behaviour.]

One serological case–control study detected HPV16 L1 VLPs in 35.7% of 14 laryngeal squamous-cell carcinomas (Dahlstrom *et al.*, 2003), which was a higher prevalence than that detected in controls (9.2% of 120).

In contrast, another serological study of 127 cases of laryngeal cancer and 100 cancer-free controls reported prevalences of HPV 16 of 20 versus 18%, respectively (Van Doornum *et al.*, 2003).

Table 41. Case–control studies of prevalence of HPV and laryngeal cancer

Reference, study location	No. of cases	No. of controls	Method of detection and types tested	HPV prevalence (%)		Odds ratio (95% CI)	Comments/adjustments
				Cases	Controls		
Brandsma & Abramson (1989), USA	60 SCC	53 with benign disease or structural abnormalities	Southern blot	5	4	NR	HPV-positive tumours harboured DNA sequences related to HPV 11.
García-Milían *et al.* (1998), Cuba	33 SCC	25	PCR with MY09/11	48	16	$p < 0.05$	Matched for age
Nishioka *et al.* (1999), Japan	27	35	PCR for 16/18 and dot blot for 16 and 18	19	6	3.75 (0.72–19.67)	Matched for age and sex; adjusted for tobacco smoking
Smith *et al.* (2000), USA	44 SCC	12 patients with benign laryngeal conditions	PCR with MY09/11 or MY09/GP5+ and sequencing	18[a]	17[a]	3.0 (CI not given)	DNA from oral source, laryngeal source and biopsies; adjusted for age and alcoholic beverage and tobacco consumption
Mork *et al.* (2001), Norway, Finland and Sweden	76 SCC	411	Antibodies against HPV 16 L1 VLP	12	5	2.4 (1.0–5.6)	Adjusted for cotinine levels
Dahlstrom *et al.* (2003), USA	14 SCC	120	Antibodies against HPV 16 VLP	35.7	9.2	NR	
Van Doornum *et al.* (2003), Netherlands	127	100	Antibodies against HPV 16 L1 VLP and E7 protein	L1, 20 E7, 2	L1, 18 E7, 2	$p = 0.876$ NR	

See Table 7 for a description of the primers used.

CI, confidence interval; NR, not reported; PCR, polymerase chain reaction; SCC, squamous-cell carcinoma; VLP, virus-like particles

[a] The high-risk types found in the study and accounted for were 16, 18, 31, 45 and 70.

Altogether, the evidence suggests that HPV may be involved in the development of some laryngeal cancers, but the associations documented to date are not as clear nor as strong as those observed at other upper aerodigestive sites, such as the tonsils and the oropharynx (Herrero, 2003). If HPV DNA causes a subset of laryngeal cancers, it is probably a smaller subset than that documented for other sites of the head and neck. However, HPV 16 predominates over other HPV types among HPV-associated laryngeal cancers. There is some evidence against an association between HPV and laryngeal squamous-cell carcinoma: (*a*) cell lines derived from laryngeal tumours contain low viral load, which probably reflects the absence of a clonal relationship (Atula *et al.*, 1999); (*b*) the presence of HPV DNA is not limited to the tumour specimen and is also found in normal surrounding tissue (Venuti *et al.*, 2000); and (*c*) HPV DNA is not consistently detected in premalignant lesions of laryngeal squamous-cell carcinoma (Fouret *et al.*, 1995; Poljak *et al.*, 1997; Smith *et al.*, 2000), which suggests a lack of continuity of HPV infection throughout the malignant process.

In the oral region, a clear lack of concordance of HPV prevalence between biopsy tissue and oral specimens has been demonstrated (Herrero, 2003). To take samples from the the larynx of healthy controls is practically impossible; therefore, new methods to assess exposure to HPV must be considered. Immunological markers (e.g. antibodies against HPV E6 and E7) may also help distinguish cancers in which HPV has played an etiological role.

2.5 Cancer of the skin and conjunctiva

2.5.1 *Cancer of the skin*

In contrast to the papillomavirus types found in the cancers of the mucosae which are classified in the genus alpha of the papillomaviruses, the papillomavirus types found in skin cancers mainly belong to the genus beta of the family also termed as epidermodysplasia verruciformis (EV)-HPV (see Section 1.1.3). This can be explained by a different tropism of the two genera, because the skin is histologically distinct from the mucosae. The pathology of skin lesions is described in Section 1.5.4.

(*a*) *Case series*

Early studies of skin cancer that used southern blot or PCR for the detection of mucosal HPV types suggested a low prevalence of HPV in skin cancers except for those that occur at periungual and palmoplantar sites. In these rare tumours (occasionally found in patients who also have HPV 16/18-positive cervical lesions), the high prevalence of HPV 16/18 DNA suggests possible transmission of HPV infection from genital sites (IARC, 1995).

Since the development of highly sensitive, partially nested PCRs designed to detect epidermodysplasia verruciformis (EV)-related and cutaneous HPV types (Berkhout *et al.*, 1995; Shamanin *et al.*, 1996; see Section 1.3.3), a much higher prevalence of HPV DNA

has been found in non-melanoma skin cancers (Tables 42 and 43). However, HPV DNA is also frequently detected in specimens of normal skin and in plucked hairs (see Section 2.5.1(*b*)). A diverse spectrum of HPV types, including HPV 20, 38, 41 and 48, have been detected, and many new partial HPV DNA sequences (350–430 nucleotides from the L1 gene) have been identified. Most of them have been assigned to genera beta (including EV-associated HPV) and gamma (see Section 1.1.3) (Berkhout *et al.*, 1995; Shamanin *et al.*, 1996; Bens *et al.*, 1998; Forslund *et al.*, 2003a).

The need for highly sensitive detection techniques can be explained by the very small amounts of HPV DNA present in skin tumours. When HPV DNA was determined by quantitative, type-specific real-time PCR in precancerous actinic keratoses and non-melanoma skin cancers that were positive in nested PCR, viral loads ranged from 1 HPV DNA copy per 14 200 cell equivalents to 50 HPV DNA copies per 1 cell equivalent (Weissenborn *et al.*, 2005). The HPV DNA load was significantly higher in actinic keratoses than in squamous-cell carcinomas and Bowen disease. In most cases, probably not every tumour cell harbours an HPV genome, which is supported by in-situ hybridization that shows only a few HPV DNA-positive cell nuclei per section.

The genus- and species-specific PCRs used differ in sensitivity towards individual types, and thus affect the spectrum of HPV types identified in cases in which DNA levels are close to the limit of detection (Pfister, 2003). When HPV 5 and 8-specific nested PCR was added to an EV-specific nested PCR with degenerate primers, for example, these types were shown to be more prevalent than had been anticipated previously (Meyer *et al.*, 2000). Using HPV 38-specific, hot-start PCR to amplify part of the E6 open-reading frame (ORF), HPV 38-related DNA was detected in 55% of basal-cell carcinomas, 46% of squamous-cell carcinomas and 10% of healthy skin samples (Caldeira *et al.*, 2003).

(i) *Squamous-cell carcinoma and keratoacanthoma*

Nearly all studies published after 1996 revealed HPV prevalences in squamous-cell carcinoma of 27–60% (Table 42). The prevalence of EV-associated beta-HPV was generally in the range of 30–50%; probably as a result of the primers employed for the PCR, it was only 12% or lower in two studies (Shamanin *et al.*, 1996; Iftner *et al.*, 2003). HPV 16 and related high-risk mucosal HPV were occasionally detected in some case series; however, the high prevalence (28%) of HPV 16 in a French study (Cairey-Remonnay *et al.*, 2002) could not be confirmed by others even when highly sensitive mucosal HPV-specific PCR was used. HPV 4 was found in one of 26 and nine of 72 squamous-cell carcinomas in two case series (Shamanin *et al.*, 1996; Iftner *et al.*, 2003) and HPV 41 in two of 10 squamous-cell carcinomas in another (Grimmel *et al.*, 1988).

The original findings of a high prevalence of HPV 16/18 in periungual squamous-cell carcinomas (Moy *et al.*, 1989; Eliezri *et al.*, 1990) were confirmed by numerous case reports and more recent surveys of digital squamous-cell carcinomas (Forslund *et al.*, 2000; Alam *et al.*, 2003; Table 44). HPV 16 and occasionally HPV 31, 35 and 73 were detected in up to 90% of these tumours (Alam *et al.*, 2003). Of 72 cases of digital squamous-cell carcinomas, 10% had an antecedent genital dysplasia or carcinoma that contained the same HPV type as

Table 42. Prevalence of HPV DNA in case series of squamous-cell carcinoma (SCC) and keratoacanthoma (KA) of the skin

Reference, study location	Method of detection and types tested	No. and type of lesions	Overall HPV positivity (%)	Type-specific HPV positivity (%)		Other types detected (no.)	Comment
				EV-, beta-HPV	16/18, related types[a]		
Pfister *et al.* (1986), Germany	Southern blot and restriction digest	6 KA	33.3			25-related (2)	
Scheurlen *et al.* (1986), Germany	Dot blot hybridization and restriction digest	7 KA	14.3	14.3			Frozen tissue; HPV 9 and 37 in a single KA present at ~10 copies/cell
Grimmel *et al.* (1988), Germany	Southern blot for 41	6 KA 10 SCC	0 20.0			41 (2)	Frozen tissue. HPV 41 not found in any of 44 melanomas or 47 non-malignant skin lesions in other patients
Eliezri *et al.* (1990), USA	ISH for 6/11, 16/18, 31/33/35, 42/43/44, 45/56, 51/52	16 SCC	0				Tissue stored at –20 °C; see also Tables 43 and 44
Kawashima *et al.* (1990), Poland	Dot blot for 5/8/14, 17/20/23/24, 6/11, 16/18/33, 2/3, 1/4/7, 3/10/28; PCR and Southern blot for 11/16	33 KA 51 SCC (NOS) 25 SCC (lip)	0 2.0 4.0		 4.0*	Untyped (1)	Frozen tissue.; no HPV DNA found in any of 14 cutaneous horns in control patients (see also Tables 43 and 46) *HPV 16
Pierceall *et al.* (1991), USA	PCR for 6/11, 16, 18	21 SCC	19.0		19.0*		Fresh or frozen tissue; no HPV DNA found in the normal skin biopsies from the HPV 16- positive tumour patients; *all HPV 16
Shamanin *et al.* (1996), Germany	PCR with broad range degene-rate primers [not specific for EV]	26 SCC 4 KA	30.8 50.0	11.5	3.8	4, 32, 51 (1 each), 42 (2) 6 (1), 34 (1)	Frozen tissue

Table 42 (contd)

Reference, study location	Method of detection and types tested	No. and type of lesions	Overall HPV positivity (%)	Type-specific HPV positivity (%)		Other types detected (no.)	Comment
				EV-, beta-HPV	16/18, related types[a]		
Hsi *et al*. (1997), USA	Nested PCR with L1 consensus and degenerate primers and sequencing	30 KA	26.7				Paraffin-embedded tissue; in non-lesional skin samples from reduction mammoplasty specimens or uninvolved skin from melanoma patients, 1/26 was positive for HPV DNA.
Harwood *et al.* (2000), United Kingdom	PCR with 9 degenerated primers for EV, cutaneous, and mucosal HPV	22 SCC	27.2	27.2	0		Frozen tissue
Meyer *et al*. (2000), Germany	Nested PCR with mucosal-, cutaneous-, and EV-HPV-specific degenerate and type-specific (5, 8) primers, followed by RFLP analysis	10 SCC	50.0	40.0		6 (1), untyped (1)	
O'Connor *et al*. (2001a), Ireland	Nested PCR with MY09/11 and EV-HPV-specific primers and sequencing	12 SCC	83.3	58.3		Unknown (3)	Three of 20 normal skin samples from controls positive in a nested PCR with EV-HPV-specific primers
Cairey-Remonnay *et al*. (2002), France	PCR with MY09/11 and FAP-primers; hybridization for 6, 11, 16, 18, 31, 33, 35, 45, 51, 52, 58, 68	51 SCC	37.2		29.4	Untyped cutaneous (6)	Paraffin-embedded tissue
Forslund *et al.* (2003a), Norway	PCR with FAP primers	12 KA	33.3				Frozen tissue
Forslund *et al.* (2003c), Australia	PCR with FAP and HPV 38-specific primers, cloning, and sequencing	12 SCC	33.3	33.3			Tissue stored at –20°C; 92% of perilesional and 63% of buttock swabs from same patients were HPV-positive.

Table 42 (contd)

Reference, study location	Method of detection and types tested	No. and type of lesions	Overall HPV positivity (%)	Type-specific HPV positivity (%)		Other types detected (no.)	Comment
				EV-, beta-HPV	16/18, related types[a]		
Iftner *et al.* (2003), Germany, USA	PCR with broad range degenerate primers	72 SCC	59.7	5.6	12.5	4 (9)	Frozen tissue; other types reported in combination with basal-cell carcinomas
Meyer *et al.* (2003), Germany	(Nested) PCR with mucosal-cutaneous-, and EV-HPV-specific degenerate primers	15 SCC	46.7	46.7	6.7	6 (1)	Frozen tissue; 2/13 normal skin samples from patients with non-melanoma skin cancers (see Table 46) contained HPV DNA.
Pfister *et al.* (2003), Poland	Nested PCR with group-specific (mucosal, EV) and type-specific (HPV 8) primers and sequencing	20 SCC	45.0	45.0	5.0		Paraffin-embedded tissue

See Table 7 for a description of the primers used.
EV, epidermodysplasia verruciformis; ISH, in-situ hybridization; NOS, not otherwise specified; PCR, polymerase chain reaction
[a] Alpha 7- and alpha 9-HPV

Table 43. Prevalence of HPV DNA in case series of basal-cell carcinoma of the skin

Reference, study location	Method of detection and types tested	No. of lesions	Overall HPV positivity (%)	Type-specific HPV positivity (%)		Other types detected (no.)	Comment
				EV-, beta-HPV	16/18, related types[a]		
Grimmel *et al.* (1988), Germany	Southern blot for 41	13	0				Frozen tissue
Eliezri *et al.* (1990), USA	In-situ hybridization (probe specificity not reported)	26	3.8				Paraffin-embedded tissue; see also Tables 42 and 44
Kawashima *et al.* (1990), Poland	Dot blot for 5/8/14, 17/20/23/24, 6/11, 16/18/33, 2/3, 1/4/7, 3/10/28; PCR and southern blot for 11/16	53	1.9	1.9			Frozen tissue; see also Tables 42 and 46
Pierceall *et al.* (1991), USA	PCR for 6/11, 16, 18	16	18.8		18.8*		Fresh or frozen tissue. No HPV DNA was found in the normal skin biopsies from the HPV-positive tumour patients; *all HPV 16
Nahass *et al.* (1992), USA	PCR [with consensus primers] and dot blot hybridization	3 scrotal	0				Fixed tissue
Zhu *et al.* (1993a,b), USA	PCR with MY09/11 and southern blot	13	0				Fresh tissue stored at 4 °C
Shamanin *et al.* (1996), Germany	PCR with broad range degenerate primers	11	36.4	27.3		4 (1), 6 (1), 7 (1)	Frozen tissue
Biliris *et al.* (2000), Greece	Multiplex, type-specific PCR for 1, 2, 5, 8, 11, 16, 18, 33	72	30.6	26.4	13.9*		Frozen tissue; *all HPV 18
Harwood *et al.* (2000), United Kingdom	PCR with EV, cutaneous and mucosal HPV-specific primers	30	36.7	33.3		Alpha 2- (1), mucosal (1)	Frozen tissue

Table 43 (contd)

Reference, study location	Method of detection and types tested	No. of lesions	Overall HPV positivity (%)	Type-specific HPV positivity (%)		Other types detected (no.)	Comment
				EV-, beta-HPV	16/18, related types[a]		
Wieland *et al.* (2000), Germany and Poland	(Nested) PCR with genital/mucosal-, cutaneous- and EV-specific primers	69	43.4	40.6	0	6, 28, 34 (1 each)	Snap frozen; 8 of 31 (26%) perilesional skin tissues were HPV DNA-positive.
Forslund *et al.* (2003c), Australia	PCR with FAP and HPV 38-specific primers, and sequencing	19	21.1	21.1	0		Tissue stored at –20 °C; 52% of perilesional and 57% of buttock swabs from same patients were HPV-positive.
Iftner *et al.* (2003), Germany	PCR with broad range degenerate primers	18	27.8	0	16.7	27 (1), untyped (1)	Frozen tissue

See Table 7 for a description of the primers used.
EV, epidermodysplasia verruciformis; PCR, polymerase chain reaction
[a] Alpha 7- and alpha 9-HPV

Table 44. Prevalence of HPV DNA in case series of periungual and palmar squamous-cell carcinoma (SCC) of the skin

Reference, study location	Method of detection and types tested	No. and type of lesions	Overall HPV positivity (no. positive/ total[a])	Type-specific HPV positivity (no. positive/total)[a]		Other types detected (no.)	Comment
				6/11	16/18		
Moy *et al*. (1989), USA	Dot blot for 6/11, 16, 18	10 periungual	8/10		6/10		Frozen tissue; episomal HPV 16 found in 4/6 HPV 16-positive specimens
Ostrow *et al*. (1989a), USA	Southern blot for 2, 6, 16, 18, 31	1 digital	1/1		1/1		Episomal and integrated HPV 16 demonstrated in tumour tissue by two-dimensional gel electrophoresis
Eliezri *et al*. (1990), USA	ISH (NA)	12 periungual	9/12		7/12	Untyped (2)	Paraffin-embedded tissue; see also Tables 42 and 43
Guitart *et al*. (1990), USA	ISH for 6/11, 16/18, 31/33/35	9 nail bed SCC	1/9		1/9*		Paraffin-embedded tissue; *HPV 16; the patient also had HPV 16 in cervical tissue.
Ashinoff *et al*. (1991), USA	PCR for 16/18 and dot blot	2 periungual	2/2		2/2*		Paraffin-embedded tissue; *HPV 16 in both lesions
Moy & Quan (1991), USA	Dot blot for 6/11, 16, 18	1 digital	1/1		1/1*		Frozen tissue; *HPV 16
Sánchez-Lanier *et al*. (1994), USA	Southern blot for 6/11, 16, 18	4 digital	4/4		4/4*		Expression of unspliced E6 and spliced E6I transcripts in all patients; *all HPV 16
McHugh *et al*. (1996), USA	RT, in-situ PCR	1 metastatic digital	1/1			35 (1)	HPV 35 RNA in axillary lymph node metastases
Downs *et al*. (1997), United Kingdom	PCR with L1 consensus primers	1 digital	1/1			Untyped (1)	Subungual SCC in Darier disease
Forslund *et al*. (2000), Sweden	PCR with neighbour primers, type-specific for 16 and consensus primers	15 digital	67%		53%	73 (2)	Paraffin-embedded tissue; study investigating link with genital SCC
Alam *et al*. (2003), USA	NA	[~25] digital	~90%		[~65%]	31 (1), 35 (1), unknown (5)	History of cervical SCC in 2 HPV-positive tumour patients

ISH, in-situ hybridization; NA, not available; PCR, polymerase chain reaction; RT, reverse transcriptase

[a] Unless otherwise specified

the digital tumour (Alam *et al.*, 2003), which underlines the possibility of genital–digital spread. It should be emphasized that tumours at this site are extremely rare.

Whereas HPV was found in only a few keratoacanthomas when detected by southern blot hybridization (Pfister *et al.*, 1986; Scheurlen *et al.*, 1986), studies that used sensitive PCR reported a prevalence of about 30% in such lesions (Table 42).

(ii) *Basal-cell carcinoma*

In broad-spectrum PCR-based studies, the prevalence of HPV in basal-cell carcinoma was 21–44% in series of 11–72 cases (Table 43). EV-associated HPV usually predominated.

(iii) *Verrucous carcinoma (epithelioma cuniculatum)*

Individual reports have documented HPV 1, 6/11 and 16/18 in single cases of verrucous carcinoma (Garven *et al.*, 1991; Noel *et al.*, 1993; Sasaoka *et al.*, 1996), a rare, indolent (typically non-metastasizing) tumour that occurs at acral sites. Verrucous carcinomas also develop in the anogenital region, where they may be found in conjunction with Buschke-Löwenstein tumours, and are usually associated with HPV 6 or 11. Verrucous carcinomas may also be found in the anogenital region in the absence of Buschke-Löwenstein tumours (see Section 2.3.3). However, the relationship between HPV infection and verrucous carcinomas in the anogenital region is poorly understood. HPV was not identified in any of 11 tumours in one case series (Petersen *et al.*, 1994) (Table 45).

(iv) *Premalignant cutaneous disease (Bowen disease and actinic keratoses)*

As for squamous-cell carcinoma, early southern blot-based studies indicated a very low prevalence of HPV in Bowen disease and actinic keratoses (Table 46), except for the rarely occurring periungual and palmoplantar Bowen disease; HPV 16/18 was found in 57–70% of these lesions in several case series (Table 47). When broad-range, sensitive PCRs were used, HPV DNA was generally detected in 35–85% of actinic keratoses and Bowen disease (Table 46).

EV-associated HPV predominated in several cases series, whereas, in one study, they were found in only 7% of premalignant lesions in which a broad spectrum of cutaneous as well as high- and low-risk mucosal HPV were detected (Iftner *et al.*, 2003). In one comparative study, the prevalence of EV-HPV DNA was similar in both low- and high-grade actinic keratoses (Pfister *et al.*, 2003).

(v) *Malignant melanoma*

There is little evidence for an association between HPV and malignant melanoma. HPV 17 and HPV 38 were found in a superficial spreading malignant melanoma of an immunosuppressed patient but not in 35 other malignant melanomas (Scheurlen *et al.*, 1986). HPV DNA related to EV-associated HPV 5 and 20 was detected in two of 15 melanoma biopsies (13%) in one study in which HPV DNA was also found in seven of 20 normal skin samples (35%) from randomly selected patients who were undergoing cosmetic surgery (Astori *et al.*, 1998). In another case series, beta-HPV were identified in

Table 45. Prevalence of HPV DNA in case series of verrucous carcinoma (VC) or epithelioma cuniculatum (EC) of the skin

Reference, study location	Method of detection and types tested	No. and type of lesions	Overall HPV positivity (no. positive/total)	Type-specific HPV positivity (no. positive/total)		Other types detected (no.)	Comment
				6/11	16/18		
Knobler *et al.* (1989), Austria	Dot blot (6, 11, 16/18)	1 EC, lower leg	1/1	1/1			Frozen tissue; the tumour contained HPV 11.
Garven *et al.* (1991), USA	ISH for 6/11, 16, 18/31/33	1 VC, leg	1/1	1/1	1/1		Paraffin-embedded tissue; the tumour contained HPV 11 and 18.
Noel *et al.* (1993), Belgium	ISH for 1, 2, 3, 4, 11, 16, 18	1 VC, leg	1/1			1 (1)	Paraffin-embedded tissue
Petersen *et al.* (1994), Denmark	PCR with MY09/11	11 CC, site not specified	0/11				Paraffin-embedded tissue
Sasaoka *et al.* (1996), Japan	PCR with consensus primers, restriction mapping and southern blot for 16	2 VC, foot	2/2		2/2		Both tumours contained HPV 16.

CC, carcinoma cuniculatum [belongs to the group of verrucous carcinoma]; PCR, polymerase chain reaction

Table 46. Prevalence of HPV DNA in case series of Bowen disease (BD) and actinic keratoses (AK) of the skin

Reference, study location	Method of detection and types tested	No. and type of lesions	Overall HPV positivity (no. positive/ total)[a]	Type-specific HPV positivity (no. positive/total)[a]		Other types detected (no.)	Comment
				EV-, beta-HPV	16/18, related types[b]		
Ikenberg *et al*. (1983), Germany	Southern blot for 16	3 BD, thumb, lower leg, dorsal hand	1/3		1/3		Frozen tissue; thumb not further defined
Pfister & Haneke (1984), Germany	Southern blot for 1, 3, 6, 8, 11, 13	1 BD, dorsal hand	1/1			2 (1)	Tissue type not reported
Kawashima *et al.* (1986), Poland	Southern blot for 1/2/4/7, 3/10/28, 5/8/14, 17/20/23/24	12 BD	0				Frozen tissue
Stone *et al.* (1987), USA	Southern blot for 1, 4, 6, 11, 16, 18	1 BD, foot	1/1		1/1		Fresh tissue
Grimmel *et al*. (1988), Germany	Southern blot for 41	6 AK	1/6			41 (1)	Frozen tissue
Guerin-Reverchon *et al*. (1990), France	ISH for 1, 2, 5, 6/11, 16/18	11 BD, leg, face, hand	5/11		2/11*	2 (1), untyped (3)	Paraffin-embedded tissue; *1 lesion with HPV 2 and 16, 1 lesion with HPV 16 and 18; no HPV DNA in 4 samples of normal skin or 6 samples of unrelated skin disease
Kawashima *et al*. (1990), Poland	Dot blot for 5/8/14, 17/20/ 23/24, 6/11, 16/18/33, 2/3, 1/4/7, 3/10/28; PCR and southern blot for 11/16	83 non-genital BD 55 AK	2/83 3/55	2/55		34 (2) Untyped (1)	Frozen tissue; see also Tables 42 and 43
Kettler *et al*. (1990), USA	ISH for 1, 6, 11, 16, 18	25 non-genital BD	6/25		[4]/25		Paraffin-embedded tissue

Table 46 (contd)

Reference, study location	Method of detection and types tested	No. and type of lesions	Overall HPV positivity (no. positive/ total)[a]	Type-specific HPV positivity (no. positive/total)[a]		Other types detected (no.)	Comment
				EV-, beta-HPV	16/18, related types[b]		
Inaba *et al*. (1993), Japan	ISH for 1, 6, 11, 16, 18	1 BD forearm (6-year-old boy)	1/1			1 (1)	Paraffin-embedded tissue
Harwood *et al.* (2000), United Kingdom	PCR with EV-, cutaneous- and mucosal-HPV-specific degenerate primers	11 carcinoma *in situ* (BD) and AK	6/11	3/11		3/10/28/77 (2); 6/11/16/66 (1)	Frozen tissue
Forslund *et al.* (2003c), Australia	PCR with FAP and type 38-specific primers, cloning, and sequencing	10 AK	7/10	7/10			Tissue stored at –20 °C; 80% of perilesional and 60% of buttock swabs from the same patients were HPV-positive.
Iftner *et al.* (2003), Germany	PCR with broad range degenerate primers	71 AK 20 BD	58% 70%	}7%	}11%	1 (5), 6 (3), 27 (6), 2, 3, 4, 7, 34, 57, 73 (1 each)	Frozen tissue
Meyer *et al.* (2003), Germany	(Nested) PCR with mucosal-cutaneous-, and EV-HPV-specific degenerate primers	36 AK/BD	36%	22%	8%	6 (1)	Frozen tissue; 2/13 normal skin samples from patients with non-melanoma skin cancers (see Table 42) contained HPV DNA.
Pfister *et al.* (2003), Poland	Nested group-specific (mucosal, EV) and type-specific PCR for type 8	54 AK 60 AK 18 BD	85% 67% 33%	80% 40% 33%	0% 0% 11%		Frozen tissue Paraffin-embedded tissue Paraffin-embedded tissue

See Table 7 for a description of the primers used.
EV, epidermodysplasia verruciformis; ISH, in-situ hybridization; NA, not available; PCR, polymerase chain reaction
[a] Unless otherwise specified
[b] Alpha 7- and alpha 9-HPV

Table 47. Prevalence of HPV DNA in case series of periungual and palmoplantar Bowen disease of the skin

Reference, study location	Method of detection and types tested	No. and type of lesions	Overall HPV positivity (no. positive/ total)	Type-specific HPV positivity (no. positive/ total)		Other types detected (no.)	Comments
				6/11	16/18		
Kawashima *et al*. (1986), Poland	Southern blot hybridization	1 periungual	1/1			34 (1)	Frozen tissue
Rüdlinger *et al*. (1989), Switzerland	Southern blot hybridization for 1–5, 7, 10, 27, 30, 31, 33–38	1 periungual	1/1			35 (1)	Bowenoid lesion on vulva also contained HPV 35.
Kettler *et al*. (1990), USA	ISH for 1, 6, 11, 16, 18	5 palmoplantar	4/5		4/5		Paraffin-embedded tissue; 3/4 contained HPV 16, 1/4 contained HPV 16-related type.
Ashinoff *et al*. (1991), USA	PCR for 16/18 and dot-blot	5 periungual	3/5		3/5		Fixed tissue; all HPV 16
McGrae *et al.* (1993), USA	PCR and dot blot for 6, 11, 16, 18, 31, 33, 39, 45	3 periungual (1 patient)	3/3	?	3/3		All contained HPV 16; the patient had HPV 6-containing condylomata of the penis.
Nordin *et al*. (1994), Sweden	PCR for 16	1 digital	1/1		1/1		HPV 16 also found in vulvar and cervical dysplastic tissue from this patient
Sau *et al*. (1994), USA	ISH for 6/11, 16/18, 31/33/ 51	7 nail bed	4/7		4/7		Paraffin-embedded tissue

ISH, in-situ hybridization; PCR, polymerase chain reaction

four of 54 malignant melanomas (7%). Samples of normal skin of the patients were not available (Miracco *et al.*, 2001).

(*b*) *Case–control studies*

A few studies compared the prevalence of HPV DNA in skin cancer tissue with that in healthy skin samples from control subjects or in swabs from perilesional skin. Depending on the sensitivity of the detection systems, prevalence rates of 22 and 86% were found in cancer tissue from immunocompetent patients compared with 8 and 22% in uninvolved skin, respectively (Stark *et al.*, 1994; O'Connor *et al.*, 2001a). In a study that compared the prevalence of EV-HPV DNA in 91 cases of solar keratosis or Bowen disease, 72 squamous-cell carcinomas and 106 normal skin samples, adjusted odds ratios for the presence of EV-HPV DNA were 9.2 (95% CI, 1.0–80) for solar keratosis and Bowen disease and 9.6 (95% CI, 0.9–100) for squamous-cell carcinoma (Iftner *et al.*, 2003). In another study in Poland, very little difference between the positivity rates of basal-cell carcinomas and paired healthy skin (32% and 26%) was observed (Wieland *et al.*, 2000). The prevalence of HPV DNA was much higher in perilesional swabs (92% and 52%, respectively) than in squamous-cell carcinomas (33%) and basal-cell carcinomas (21%) of patients in Australia (Forslund *et al.*, 2003a). The high prevalence in swabs may reflect contamination of the skin surface by HPV rather than infection (Forslund *et al.*, 2004).

A significant association between the presence of EV-HPV DNA and skin cancer was observed in punch biopsies from the clinically normal skin of 38 immunosuppressed renal transplant recipients and 39 immunocompetent individuals (odds ratio for both groups combined, 6.41; 95% CI, 1.79–22.9) (Harwood *et al.*, 2004). Conversely, no association was found between the presence of cutaneous or mucosal HPV types and skin cancer.

A slightly positive, non-significant association was found between EV-HPV DNA in plucked eyebrow hairs and squamous-cell carcinoma in a population in subtropical Australia (odds ratio, 2.00; 95% CI, 0.50–8.0) (Boxman *et al.*, 2000). However, a strong association was observed between EV-HPV DNA and solar keratoses among men (odds ratio, 3.40; 95% CI, 1.77–6.53) but not among women (Boxman *et al.*, 2001). In a Dutch population, HPV DNA in eyebrow hairs (all EV-HPV types except for HPV 2 in one individual) was associated with a history of squamous-cell carcinoma (odds ratio, 1.7; 95% CI, 1.1–2.7) (Struijk *et al.*, 2003). Positive associations were observed for the individual EV-HPV types 5, 8, 15, 20, 24 and 38 and the adjusted odds ratios for HPV 5, 15 and 20 were statistically significant.

Two seroepidemiological case–control studies evaluated HPV infection as a risk factor for cutaneous squamous-cell carcinoma in immunocompetent individuals (Feltkamp *et al.*, 2003; Masini *et al.*, 2003). Infection with EV-related HPV types (5, 8, 15, 20, 23, 24, 36 and 38) was assessed by serology using a L1-VLP ELISA method. In Italy (Masini *et al.*, 2003), positive serology for HPV 8 was associated with an odds ratio for cutaneous squamous-cell cancer of 3.2 (95% CI, 1.3–7.9). Other variables significantly associated with this tumour were family history of non-melanoma skin cancer, high professional or recreational exposure to the sun, light eye colour, large number of solar

keratoses and seborrheic keratoses on the body surface and residence in buildings that emit radon. In the Netherlands (Feltkamp *et al.*, 2003), the estimated relative risk for squamous-cell carcinoma was significantly increased in HPV 8- and HPV 38-seropositive subjects after adjusting for age and sex (odds ratio, 14.7; 95% CI, 1.6–135; and 3.0; 95% CI, 1.1–8.4, respectively). The estimated relative risk for nodular and superficial multifocal basal-cell carcinoma was also significantly increased in HPV 8-positive subjects (odds ratio, 9.2; 95% CI, 1.1–78.2; and 17.3; 95% CI, 2.1–143, respectively) and to a lesser extent in HPV 20-seropositive subjects (odds ratio, 3.2 and 3.4, respectively). No associations were found for HPV 16. The relative risk for developing malignant melanoma was not increased among HPV-seropositive individuals.

Available studies do not allow an evaluation of whether exposure to the sun confounds or modifies the effect of HPV on skin cancer.

2.5.2 *Cancer of the eye and conjunctiva*

Early case reports and case series on the prevalence of HPV in eye lesions have been reviewed previously (IARC, 1995). HPV (mostly type 16) was found by PCR in both intraepithelial neoplasia of the conjunctiva (80–100%) and in nearly all invasive carcinomas of the conjunctiva, eyelid and lacrimal sac that were tested. In studies published since that time, the detection rate of HPV has varied widely.

In a study in subtropical Tanzania, most cases of conjunctival epithelial dysplasia and epithelial neoplasms were found to be HPV-positive by in-situ hybridization (Moubayed *et al.*, 2004).

Using PCR-RFLP and in-situ hybridization methods, Saegusa *et al.* (1995) detected HPV 16 in two of four dysplasias (50%) and one of four squamous-cell carcinomas (25%) but in no basal-cell epithelioma of the conjunctiva. No other HPV types were found. Nakamura *et al.* (1997a) detected HPV 16 in two of four dysplasias and HPV 18 in one case of severe dysplasia and in one of four carcinomas of the conjunctiva. Eng *et al.* (2002) failed to detect DNA of HPV types 6, 11, 16 or 18 in any of 20 formalin-fixed, paraffin-embedded malignant epithelial tumours of the conjunctiva.

HPV DNA was detected in about 30% of non-familial sporadic retinoblastoma in two studies (Orjuela *et al.*, 2000; Palazzi *et al.*, 2003). In spite of a similar overall prevalence of HPV, the spectrum of types differed between the two studies, e.g. for HPV 16 (10% versus 23%) and for HPV 18 (28% versus 0%).

It has been proposed that HPV infection represents an alternative carcinogenic mechanism to retinoblastoma gene inactivation but there was no significant correlation between the detection of HPV DNA and immunohistochemical detection of the retinoblastoma protein (Orjuela *et al.*, 2000).

HPV 16 DNA and E6-specific mRNA were detected by in-situ hybridization and reverse transcriptase in-situ PCR, respectively, in five of 10 conjunctival intraepithelial neoplasias; HPV 18 DNA and mRNA were present in the other five specimens (Scott *et al.*, 2002b). Neither HPV DNA nor mRNA were detected in clinically uninvolved con-

junctival specimens from the same patients or from five age-matched control subjects ($p < 0.001$). In contrast to these findings, other studies detected HPV not only in epithelial neoplasms but also in non-neoplastic lesions as well as in apparently healthy conjunctiva. In one study, HPV 16 DNA was found by PCR with consensus primers and dot blot hybridization using 28 type-specific probes, in two of 10 invasive cancers of the conjunctiva, and in the normal muscosa of one of 30 age- and sex-matched controls (Palazzi *et al.*, 2000). In another study, HPV 16 infection was found by PCR in seven of 20 samples from carcinomas (35%) and in two of six samples from conjunctivitis (Waddell *et al.*, 1996). Karcioglu and Issa (1997) identified HPV 16 and 18 DNA by PCR in eight of 14 (57%) in-situ squamous-cell carcinomas, in 17 of 31 (55%) invasive squamous-cell carcinomas, in four of 20 (20%) samples of climatic droplet keratopathy, in 11 of 31 (35%) samples of scarred corneas and in six of 19 (32%) samples of normal conjunctival tissue obtained during routine cataract extractions. HPV DNA was not detected by PCR with MY09/11 primers in any of 28 pathological specimens that ranged from intraepithelial neoplasia to invasive squamous-cell carcinoma or in 23 disease-free, age- and sex-matched patients (Tulvatana *et al.*, 2003).

The weak association between infections with genital HPV types and carcinoma of the conjunctiva is supplemented by the lack of a statistically significant association between anti-HPV 16 antibody status and the risk for conjunctival neoplasia (Newton *et al.*, 2002; Waddell *et al.*, 2003).

In a pilot study of 21 squamous-cell carcinomas of the conjunctiva and 22 conjunctival samples of control subjects from Uganda, broad-spectrum and EV-specific PCR-based assays detected EV-HPV types in 86% of the cases and in 36% of controls (odds ratio after adjustment for exposure to the sun, 22.7; 95% CI, 1.7–312) (Ateenyi-Agaba *et al.*, 2004). No mucosal HPV types were found in either cases or controls by genus alpha- and type 16-, 18-, and 45-specific PCR. As human immunodeficiency virus (HIV) serology was not available for the study patients, the strong association between EV-HPV and carcinoma of the conjunctiva could not be adjusted for a possible immunosuppression due to HIV infection, which appeared to be strongly associated with conjunctival cancer in Uganda (Newton *et al.*, 2002).

2.6 Cancer at other sites

2.6.1 *Cancer of the nose and nasal sinuses*

Inverted papillomas are rare tumours of the nasal cavity and paranasal sinuses. Although commonly benign, they frequently reveal signs of invasive growth and convert into malignant tumours in up to 13% of cases (Bernauer *et al.*, 1997). They are therefore discussed jointly with carcinomas of the nasal cavity.

The presence of HPV infection in an extensive squamous-cell papilloma of the nasal cavity was first detected by immunoperoxidase staining of group-specific antigens (Syrjänen *et al*., 1983). A further study analysed 14 patients with 13 inverted papillomas

and three squamous-cell carcinomas that extended to several sites of the nasal cavity and paranasal sinuses by indirect immunoperoxidase staining (Syrjänen *et al*., 1987b). Seven of the 25 papilloma biopsies analysed expressed HPV antigens. By in-situ hybridization with probes for HPV 6, 11 and 16, nine lesions in seven patients were shown to contain HPV 11 DNA. The three carcinomas tested were positive for HPV 16 DNA. In another study that used only in-situ hybridization to detect HPV 6 and 11, a high prevalence of both HPV 6 and 11 was noted in the 21 inverted papillomas analysed (Weber *et al*., 1988). A new virus type, HPV 57, was subsequently isolated from an invasively growing inverted papilloma of the maxillary sinus (de Villiers *et al.*, 1989). When type-specific primers were used, this virus was subsequently identified in six of eight inverted nasal papillomas, in one of three inverted papillomas with dysplasia and in two of four inverted papillomas with carcinoma (Wu *et al.*, 1993). HPV 57 was also detected in a further case of inverted papilloma (Ogura *et al.*, 1996).

Most other studies that used type-specific primers detected HPV 11 and HPV 6 in inverted papillomas and in some malignant tumours, and a limited number of cases were reported to be positive for HPV 18 and 16 DNA. Two inverted nasal papillomas contained HPV 11 DNA, one of which had a 500-base-pair insertion (Respler *et al.*, 1987). In another study, HPV 6 DNA was identified by southern blot in one of seven inverted papillomas (Ishibashi *et al*., 1990). Kashima *et al*. (1992b) found HPV 11 in five of 29 inverted papillomas; two other papillomas contained HPV 6 DNA, and one of 24 squamous carcinomas contained HPV 18 DNA. A specific search for HPV 16 and 18 sequences by PCR in nasal carcinomas found that six of 49 cases were positive for HPV 16 and one for HPV 18 DNA (Furuta *et al*., 1992). Bernauer *et al.* (1997) detected HPV DNA in seven of 21 inverted papillomas; one lesion, which was associated with a squamous-cell carcinoma, was positive for HPV 18. One of two carcinomas that occurred within papillomas of the nasal septum contained HPV 6/11 DNA and the other contained HPV 18 DNA (Buchwald *et al.*, 1997). Expression of E6/E7 genes of HPV 6 was detected in an inverted papilloma (Harris *et al.*, 1998a). HPV 11 was found by in-situ hybridization and by PCR in one inverted papilloma (Kraft *et al.*, 2001). Among 28 cases of squamous-cell carcinoma associated with inverted papillomas, four were HPV-positive: one for HPV 6/11, one for HPV 16/18, one for HPV 6/11 and 16 and one for HPV 18 (Buchwald *et al*., 2001).

In summary, it appears that inverted papillomas are frequently positive for HPV 11, 6 and 57 DNA, whereas a small percentage (~5–15%) of carcinomas arising at the same sites contain HPV 18, 16, 11, 6 and 57 DNA at decreasing frequency.

2.6.2 *Cancer of the lung*

A number of studies have investigated the prevalence of HPV DNA in lung cancer tissues in patients with juvenile-onset recurrent respiratory papillomatosis and in women with a history of CIN3. [It must be noted that almost all studies are case series and laboratory personnel were not blinded as to the nature of the specimens; other aspects of proper epidemiological design were also lacking.]

Table 48 summarizes the prevalence of HPV detected by PCR in studies of lung cancer that involved at least 20 subjects. HPV DNA prevalence in lung tumours ranged from zero (Shamanin *et al.*, 1994a; Szabó *et al.*, 1994; Welt *et al.*, 1997; Wistuba *et al.*, 1998; Gorgoulis *et al.*, 1999) to very high levels, especially in studies conducted in Asia. Many of the positive reports that come from Asia are from Taiwan, China (Cheng *et al.*, 2001, 2004; Wu *et al.*, 2005) and Okinawa, Japan (Hirayasu *et al.*, 1996; Tsuhako *et al.*, 1998; Iwamasa *et al.*, 2000; Miyagi *et al.*, 2000, 2001).

A recent study in Taiwan, China, that tested non-cancer lung specimens as controls (Cheng *et al.*, 2001) found that 77 (55%) of 141 lung cancers were positive for HPV 16/18 by nested PCR and in-situ hybridization, and that the detection of HPV 16 and HPV 18 was more common in tumour specimens from women than in those from men ($p < 0.0001$), in those from nonsmokers than in those from smokers ($p < 0.001$) and in adenocarcinomas than in squamous-cell carcinomas ($p < 0.03$). Furthermore, specimens from cases were significantly more likely to be HPV DNA-positive than lung tissue from non-cancer patients (55% versus 27%; $p < 0.001$). From these results, it was suggested that HPV infection may play a role in lung carcinogenesis among Taiwanese nonsmoking women: only 10% of lung cancer cases in women occur in patients with a history of cigarette smoking, and adenocarcinomas constitute 59% of lung cancers among women compared with 31% among men (Chen *et al.*, 2004). In two other studies that involved many of the same patients, HPV 11 was also reported in 13% of lung specimens from non-cancer patients (Cheng *et al.*, 2004) and HPV 16 was reported in 13% of peripheral blood specimens from non-cancer patients (Chiou *et al.*, 2003). [Although the high prevalence of HPV DNA in normal control specimens could reflect false-positive results, a high concordance with in-situ hybridization was demonstrated, and the prevalence of HPV was higher in specimens obtained from lung cancer patients than in those from non-cancer patients in each of these studies.]

Similarly, in tumours collected during 1993 in Okinawa, Japan, 34 (79%) of 43 squamous-cell carcinomas tested were positive for HPV DNA when type-specific E6/E7 PCR for HPV 16, 18, 6 and 11 was used (Hirayasu *et al.*, 1996). Subsequently, it was reported that the prevalence of HPV-positive squamous-cell carcinomas steadily decreased in specimens obtained after 1995 (Miyagi *et al.*, 2000): 68% were HPV DNA-positive in 1995, 35% in 1996, 23% in 1997 and 24% in 1998. The decreasing prevalence of HPV-positive specimens correlated with a marked fall in the number of squamous-cell carcinomas of the lung in Okinawa during the same time frame, and a concordance of nearly 100% between PCR and in-situ hybridization results was shown. A separate evaluation of adenocarcinomas showed that 78% of 23 case specimens contained HPV DNA (Tsuhako *et al.*, 1998). Cases of HPV DNA-positive squamous-cell carcinoma that had high infiltration with Langerhans cells were found to have a better prognosis (Miyagi *et al.*, 2001). Normal control tissue specimens were not tested.

In spite of the above results, several large case series failed to detect HPV DNA in any lung cancer specimens tested or detected it in a very small percentage among a total of 290 cases (Shamanin *et al.*, 1994a; Hiroshima *et al.*, 1999; Clavel *et al.*, 2000).

Table 48. Prevalence of HPV DNA in case series of lung cancer detected by the polymerase chain reaction (PCR) method

Reference, study location	Method of detection and types tested	No. of cases	Type of lesion	Overall HPV positivity (%)	Type-specific HPV positivity (%)					Multiple infections (%)	Comment
					6	11	16	18	Others (type)		
Ogura *et al.* (1993), Japan	PCR, southern blot for 16, 18	29	SCC	10.3			10.3	0			Snap-frozen tissue
Liu *et al.* (1994), China	PCR for 11, 16; ISH for 11, 16	49	SCC	14.3		12.2	4.1			2.0	Paraffin-embedded archival tissue
Shamanin *et al.* (1994a,b), Germany	L1 consensus primers and 4/60/65 + southern blot	85	SCC (40%), ADC (15%), others	0							Frozen samples
Szabo *et al.* (1994), Japan	PCR	47	SCC (85%), large cell carcinoma	0							Paraffin-embedded tissue
Xing *et al.* (1994), China [cited in Syrjänen (2002)]	PCR, ISH	49	SCC	14.2		8.2	8.2	2			
Al-Ghamdi *et al.* (1995), United Kingdom	E1 consensus primers and type-specific for 6, 7, 11, 16, 18	66	SCC (50%), others	9.1	1.5	4.5	1.5			1	Paraffin-embedded tissue
Kinoshita *et al.* (1995), Japan	PCR for 16, 18, 33 and southern blot for 18; ISH for 18	36	ADC (61%), SCC (28%), small cell carc.	8.3				36.1			Frozen (PCR, southern blot) or fixed (ISH) samples
Li *et al.* (1995), China	Dot blot for 16, 18; PCR for 16, 18	50	SCC (54%), ADC (32%), small cell (4%)	32.0			44.0	12.0		2.0	Paraffin-embedded (90%) or frozen tissue (10%)
Nuorva *et al.* (1995), Finland	MY09/11 and nested PCR for 6, 11, 16, 18, 31, 33; ISH for 6, 11, 16, 18, 31, 33	22	Bronchioalveolar carcinoma	36.4	9.1	13.6	4.5	4.5	22.7 (31); 22.7 (33)	31.8	Paraffin-embedded tissue
Zhang, Z.F. *et al.* (1995), China	PCR for 6/11, 16, 18	34	[SCC]	11.8							
Da *et al.* (1996), China	Consensus primer; ISH for 16/18	40	SCC (40%), ADC (30%); small-cell carcinoma (23%)	PCR, 55.0 ISH, 25.0							

Table 48 (contd)

Reference, study location	Method of detection and types tested	No. of cases	Type of lesion	Overall HPV positivity (%)	Type-specific HPV positivity (%)					Multiple infections (%)	Comment
					6	11	16	18	Others (type)		
Hirayasu *et al.* (1996), Japan	PCR for 6, 11, 16, 18 and southrn blot	73	SCC, ADC, small-cell carcinoma, large-cell carcinoma	58.9	15.1	0	37.0	43.8		30.1	Paraffin-embedded tissue
	ISH for 6/11, 16/18, 31/33/51	94		28.7	9.6		28.7			7.4	
Noutsou *et al.* (1996), Greece	Consensus and mixed type-specific primers for 11/16/18/33, and RFLP	99	SCC (41%), ADC (41%)	15.2		3.0	4.0	8.1	2.0		Paraffin-embedded tissue
Soini *et al.* (1996), Finland	MY09/11 and nested PCR for 6, 11, 16, 18, 31, 33; ISH for 6, 11, 16, 18, 31, 33	43	SCC (65%), ADC (26%), small-cell carcinoma (7%)	30.2	14.0	14.0	18.6	16.3	20.9 (31); 20.9 (33)	25.6	Paraffin-embedded tissue
Welt *et al.* (1997), Germany	ISH for 6, 11, 16, 18; PCR with MY09/11 and nested with CN3/MY09	32	SCC	0							Paraffin-embedded tissue
Bohlmeyer *et al.* (1998), USA	MY09/11, southern blot, and dot blot for 6, 11, 16, 18, 33	34	SCC	5.9				5.9			Paraffin-embedded tissue
Papadopoulou *et al.* (1998), Greece	MY09/11 and Southern blot for 6/11, 16/18	52	SCC	69.2	11.5		21.2		13.5	5.8	Paraffin-embedded tissue
Tsuhako *et al.* (1998), Japan	ISH; PCR for 6, 11, 16, 18, 31/33/51	23	Adenosquamous carcinoma	78.3	13.0	13.0	52.2	30.4		30.4	Paraffin-embedded tissue
Wistuba *et al.* (1998)	PCR for 16, 18, 31, 33	35	Small cell (40%), SCC (31%), ADC (29%)	0							Paraffin-embedded archival tissue
Gorgoulis *et al.* (1999), Greece	MY09/11, nested GP5/6 and type-specific for 6, 11, 16, 18, 31, 33, 35 and dot-blot	68	SCC (46%), ADC (47%), large cell carcinoma	0							Frozen and paraffin-embedded tissue
Hennig *et al.* (1999a), Norway	GP5+/6+ and probing for 6, 11, 16, 18; ISH for 6, 11, 16, 18	75	ADC (37%), SCC (24%), small-cell carcinoma, others	49.3	17.3	1.3	33.3	1.3		9.3	Paraffin-embedded tissue; patients with history of CIN3

Table 48 (contd)

Reference, study location	Method of detection and types tested	No. of cases	Type of lesion	Overall HPV positivity (%)	Type-specific HPV positivity (%)					Multiple infections (%)	Comment
					6	11	16	18	Others (type)		
Hiroshima *et al.* (1999), Japan	PCR for 16, 18, 33 and southern blot	285	AdC	0.4			0.4				Paraffin-embedded tissue
Clavel *et al.* (2000), France	Hybrid Capture II	185	SCC (55%), AdC (32%)	2.7					2.7*		Snap-frozen tissue; *oncogenic types in Hybrid Capture II assay
Iwamasa *et al.* (2000), Japan	PCR for 6, 11, 16, 18 and southern blot										Storage of tissue not reported; *read from graph
1993		43	SCC	80*							
1998		21	SCC	24*							
Miyagi *et al.* (2000), Japan	PCR for 6, 11, 16, 18 and southern blot; ISH for 6, 11, 16, 18	157	SCC	51.0	14.0	8.9	24.2	25.5		18.5	Paraffin-embedded tissue
Cheng *et al.* (2001), Taiwan, China	MY09/11 and type-specific for 16, 18; ISH for 16, 18	141	SCC (41%), AdC (59%)	54.6			35.5	41.1			Tissue section
		60	Normal biopsies	26.7			15.0	11.7			
Miyagi *et al.* (2001), Japan	PCR for 6, 11, 16, 18 and southern blot; ISH for 6, 11, 16, 18	59	SCC	49.2	10.2		18.6	20.3			Paraffin-embedded tissue
		62	AdC	19.4	1.6		6.4	11.3			
Cheng *et al.* (2004), Taiwan, China	MY09/11 and type-specific for 6, 11; ISH for 6, 11	141	SCC (41%), AdC (59%)	38.3	28.4	9.9					Tissue section
		60	Normal biopsies	15.0	1.7	13.3					
Zafer *et al.* (2004), Turkey	MY09/11 and RFLP for 16, 18	40	SCC (63%), ADC (33%)	5.0				5.0			Tissue stored at –20 °C
Brouchet *et al.* (2005), France	ISH; immuno-histochemistry with VP1 antibody for 6/11/16/18/31/33/42/51/52/56/58	122	SCC (33%), ADC (25%)	0							Paraffin-embedded tissue
Wu *et al.* (2005), Taiwan, China	Not reported	166	SCC (43%), ADC (57%)	54.8							Frozen tissue

See Table 7 for a description of the primers used.
ADC, adenocarcinoma; CIN, cervical intraepithelial neoplasia; ISH, in-situ hybridization; RFLP, restricted fragment length polymorphism; SCC, squamous-cell carcinoma
* Three additional cases with HPV-31/33/35
[a] ';' denotes independent methods whereas 'and' denotes subsequent steps.

At multiple locations in the respiratory tract, including the bronchial spurs, squamo-columnar junctions are found, which are types of tissue that may be particularly prone to HPV-associated tumorigenesis (Syrjänen, 2002). Juvenile-onset recurrent respiratory papillomatosis, an HPV-associated lesion that predominantly contains HPV 6 and 11, may spread to the trachea and bronchi, and solitary squamous-cell papillomas of the bronchi have been reported (Syrjänen, 2002). Some of these papillomas have been found to contain HPV, mostly types 6 and 11 (Flieder *et al.*, 1998; Syrjänen, 2002). Although HPV 6 and 11 are thought to cause primarily benign lesions in anogenital epithelium, a recent study detected HPV 11 DNA in three lung cancers in patients with juvenile-onset respiratory papillomatosis; HPV 6, 16 or 18 were not detected. In one tumour specimen for which adequate material was available for testing, the HPV 11 genome was found to be integrated into the host genome (Reidy *et al.*, 2004). Several other studies of lung cancer in patients with respiratory papillomatosis also detected HPV 11 in tumour specimens (Byrne *et al.*, 1987; Guillou *et al.*, 1991; Rady *et al.*, 1998; Cook *et al.*, 2000). In one case, HPV 6 was found (DiLorenzo *et al.*, 1992). Together, these data suggest that, within the setting of juvenile-onset recurrent respiratory papillomatosis, HPV 11 and, to a lesser extent HPV 6, may on rare occasions (estimated to occur in less than 1% of patients) be associated with the development of lung cancer (Cook *et al.*, 2000; Reidy *et al.*, 2004).

An increased risk for lung cancer has been reported in women who have been diagnosed with anogenital cancer or CIN3 (Frisch & Melbye, 1995). It was hypothesized that the relationship was due to a mutual association of lung cancer and anogenital tumours with tobacco smoking, but a possible connection with HPV infection has also been suggested (Hennig *et al.*, 1999a). There is at least one well-documented case of an HPV 16-positive anaplastic lung carcinoma in a woman who had had a cervical cancer 9 years previously, although the possibility of a late metastasis could not be fully excluded (Stremlau *et al.*, 1985). Furthermore, in 75 women with bronchopulmonary cancer following a diagnosis of CIN3 (with no radiotherapy), 37 (49%) were shown to be HPV-positive by PCR with GP5+/GP6+ primers (18 for HPV 16, 12 for HPV 6, five for HPV 16/6, one for HPV 16/11 and one for HPV 16/18), and the overall concordance between the HPV types in the lung tumours and in CIN3 specimens was greater than 60% (Hennig *et al.*, 1999a). [It was not stated whether this was more than a chance finding, in view of the high prevalence of HPV 16 in lung tumours and the predominance of HPV 16 in CIN3.] Of 22 cases of bronchopulmonary cancers who did not have a history of CIN (controls), three were HPV 6-positive and none contained HPV 16, 11 or 18. In contrast, no HPV-positive bronchopulmonary carcinomas were detected when tested by in-situ hybridization.

In two studies summarized earlier in this section (Chiou *et al.*, 2003; Cheng *et al.*, 2004), it was speculated that haematogenous spread of HPV 16 and 18 from the cervix may explain some HPV-associated cancer in the lung. In this connection, another study reported the detection of HPV DNA sequences in 52% of peripheral blood mononuclear cells from patients with genital HPV infections, but not in the 19 control subjects with no HPV infection (Pao *et al.*, 1991).

In summary, with the exception of the studies in Taiwan, China, there is a paucity of data in non-cancer lung specimens, which greatly limits the interpretation of the large number of studies that have been reported to date. In those studies that did test non-cancer lung specimens, the high prevalence of HPV DNA reported was unexpected. [Simultaneous testing of normal human tissues, for which there is broad agreement that the prevalence of HPV is very low (in addition to cancer and non-cancer lung specimens), is necessary before the specificity of the assay results reported can be accepted entirely.]

2.6.3 *Cancer of the colon and the rectum*

Cancers of the colon and the rectum are biologically distinct from cancer of the anus. Thus, data that combine anal and rectal cancers should be interpreted with caution and the term anorectal cancers should be avoided. Whereas anal cancer has a strong association with HPV (see Section 2.3), the relationship between HPV and cancers of the colon and rectum has not been established.

Most studies that analysed a possible role of HPV in cancer of the colon were carried out in the early 1990s. A study performed in former Czechoslovakia that tested 13 adenocarcinomas and 10 adenomas of the colon for HPV 2, 6, 16 and 18 by southern blot hybridization failed to find any evidence of HPV DNA (Boguszakova *et al.*, 1988). Similarly, Gilbert *et al.* (1991) failed to find HPV 16 DNA in eight adenocarcinomas of the anus, rectum or sigmoid colon in patients with Crohn disease. Shroyer *et al.* (1992) analysed 22 colon adenocarcinomas by PCR for HPV 6, 11, 16, 18 and 33 and by in-situ hybridization for HPV types 6/11, 16/18 and 31/33/35. None of the colon cancers revealed a positive reaction. Similar results were reported by Shah *et al*. (1992) who found no HPV DNA in 19 primary tumours of the colon using PCR with the MY09/MY11 consensus primers and southern blot hybridization with a generic probe for HPV 16/18. A more recent study (Audeau *et al.*, 2002) also reported negative results after testing 20 colorectal cancers by immunohistochemistry staining with a monoclonal antibody that reacts with HPV 6, 11, 16 and 18.

The number of positive reports is small and some were based mainly on immunohistochemistry. Kirgan *et al.* (1990) detected the presence of HPV antigen in 29/30 (97%) invasive carcinomas, in 18 of 30 adenomas and in seven of 30 biopsies (23%) of normal mucosa of the colon. Two years later, it was reported that 13 of 38 (32%) carcinomas, 8/21 (38%) adenomas and two of 24 (8%) normal biopsies of the colon contained the HPV L1 region as demonstrated by PCR amplification (McGregor *et al*., 1993). A study from Taiwan, China, reported the presence of both HPV 16 and HPV 18 DNA in three cell lines derived from colorectal adenocarcinomas (Cheng *et al*., 1991); subsequently, it was found that NIH3T3 cells transformed with colonic cancer cells contained HPV 16 DNA (Cheng *et al*., 1993). A study from Turkey reported HPV 18 and 33 infections in 39 and 30, respectively, of 51 colon cancers using PCR and direct sequencing (Sayhan *et al*., 2001).

In a retrospective case–control study of 55 cases of colorectal cancer, the same number of tissues adjacent to the tumour and 10 control specimens were tested by nested

PCR and in-situ PCR for the presence of HPV DNA (Bodaghi *et al.*, 2005). In this series, 23 of 55 (42%) samples of colorectal cancer tissue and 15 of 52 (29%) samples of tissues adjacent to the tumour were positive for HPV DNA; 31 had HPV 16, five were positive for HPV 18 and two contained HPV 45 DNA. Ten samples contained HPV DNA in both the tumour and adjacent tissues and five contained HPV DNA only in the tissues adjacent to the tumour. None of the control tissues was HPV-positive. The findings were confirmed by in-situ hybridization, although the HPV DNA copy number was generally low.

In view of the limitations of the techniques used in most of the studies and in the absence of larger studies that include more case–control analyses, the observed positive reports require a cautious interpretation.

2.6.4 *Cancer of the breast*

Data on HPV in malignant tumours of the breast are controversial. In a series of 15 breast cancers analysed by low-stringency filter hybridization, Ostrow *et al.* (1987) failed to find evidence of HPV DNA. In studies that used HPV 16- and 18-specific primers, Wrede *et al.* (1992) and Gopalkrishna *et al.* (1996) also failed to find HPV DNA in 80 and 30 breast cancer biopsies, respectively. Similarly, Bratthauer *et al.* (1992) were unable to detect HPV 6, 11, 16 or 18 in 15 intraductal papillomas, 15 papillary carcinomas and 13 infiltrating ductal carcinomas of the breast. The analysis of paraffin sections from 20 nipples with Paget's disease (10 central intraductal and 10 invasive carcinomas) by PCR with MY09/MY11 consensus primers and by dot blot hybridization for HPV 6/11, 16/18 or 31/33/35 provided no evidence of HPV DNA (Czerwenka *et al.*, 1996).

These negative findings contrasted with several positive reports: Di Lonardo *et al.* (1992) detected HPV 16 DNA sequences by PCR and southern blot hybridization using specific primers in ten of 40 breast carcinomas and some axillary lymph node metastases. HPV 11 and 18 were not detected in any sample. More recently, Hennig *et al.* (1999b) examined 41 breast carcinomas from 38 patients with a history of CIN3 by PCR with specific primers and detected HPV 16 DNA in 19 cases (46%). Only one of these tumours was also positive by in-situ hybridization. A study conducted in China reported the presence of HPV 33 DNA in 14/32 cases of invasive ductal carcinomas of the breast, detected by PCR and Southern blot hybridization (Yu *et al.*, 2000). Liu *et al.* (2001) examined 17 breast cancer samples using broad spectrum PCR, cloning and sequencing and by Southern blot hybridization for HPV 16, 18 and 31. Six (35%) of the biopsies were positive for HPV types 16, 18 and 31, and viral DNA was largely episomal. Another report from China found 19 of 28 (68%) breast carcinoma samples to be HPV-positive (Li, T. *et al.*, 2002).

In a study that compared cancer tissue with normal biopsies, Damin *et al.* (2004) analysed 20 specimens of reduction mammoplasty, 21 fibroadenomas and 101 breast carcinomas using specific primer sets that target the E6 region of HPV 16 or 18. Twenty-five (24.7%) of the carcinomas, but none of the other biopsies, were found to be HPV 16- (15 cases) or HPV 18- (11 cases) positive. One specimen contained sequences of both virus types. Recently, de Villiers *et al.* (2005) cloned several HPV types from 25 of 29 (86%)

breast carcinomas and 20 of 29 (69%) corresponding samples of the mamilla from the same patient. Many ductal areas of the mamillae revealed condyloma-like histological patterns. The most prevalent HPV type in carcinomas and nipples was HPV 11, followed by HPV 6. A number of additional types were found, including those commonly detected in mucosal and cutaneous lesions, such as HPV 16, 23, 27 and 57 (nipples and carcinomas), 20, 21, 32, 37, 38, 66 and GA3-1 (nipples only) and 3, 15, 24, 87 and DL473 (carcinomas only). [It is plausible that surface areas may be infected frequently by a variety of different HPV types (see Section 2.5); thus the significance of these findings is currently difficult to assess.]

2.6.5 *Cancer of the ovary*

HPV was not detected in eight case series of cancer of the ovary from North America and Europe (de Villiers *et al.*, 1986b; Ostrow *et al.*, 1987; Leake *et al.*, 1989; McLellan *et al.*, 1990; Beckmann *et al.*, 1991; Trottier *et al.*, 1995; Anttila *et al.*, 1999; Chen *et al.*, 1999). Initial results that showed the presence of HPV 6 in 10/12 samples of epithelial ovarian carcinomas using in-situ hybridization (Kaufman *et al*., 1987) could not be reproduced in later analyses by either in-situ or southern blot hybridization or by PCR of the same and additional ovarian carcinoma specimens (Kaufman *et al*., 1990).

In two studies of ovarian cancer from Taiwan, China, HPV 16 DNA was found in 50 and 8% and HPV 18 DNA in 17 and 2% of 18 and 60 cases, respectively, using PCR (Lai *et al.*, 1994; Ip *et al.*, 2002). Quantitative real-time PCR of the samples from the latter study revealed a prevalence of HPV 16 in 18 of 56 cases and between less than one and four HPV 16 DNA copies per cellular genome (Yang, H.J. *et al*., 2003). Only one ovarian cancer contained several copies per genome.

One study from China compared the prevalence of HPV 16 detected by in-situ hybridization with an E6-specific probe in 50 ovarian epithelial cancers and in 30 non-malignant ovarian tissues, most of which had been removed for uterine pathology (Wu, Q.-J. *et al.*, 2003). Twenty-six (52%) of the cancers were positive compared with two (7%) of the controls (odds ratio, 16.7; 95% CI, 3.2–71.4).

In rare cases, squamous-cell carcinoma of the ovary may originate from cervical squamous-cell carcinoma *in situ*. Pins *et al.* (1997) described a case of CIN3 with contiguous upward spread to the endometrium, fallopian tubes and ovaries, focal invasion and HPV 16 DNA in all tumours detected by PCR. Upward spread of HPV 16-positive CIN may also explain the HPV 16-positivity of primary squamous-cell carcinoma of the ovary in two further cases (Mai *et al.*, 1996; Manolitsas *et al.*, 1998).

2.6.6 *Cancer of the prostate*

A possible association of prostatic cancer with sexual behaviour and exposure to sexually transmitted infection has been reported (Hayes *et al.*, 2000; Rosenblatt *et al.*, 2001; Strickler & Goedert, 2001). Furthermore, men with anal cancer, a disease that has

been associated with HPV, have an increased risk for developing subsequent prostatic cancer (Rabkin *et al.*, 1992).

Table 49 presents case series of cancer of the prostate and benign prostatic hypertrophy in association with HPV prevalence. A few studies found an association of HPV with prostatic cancer (McNicol & Dodd, 1990a; Anwar *et al.*, 1992a; Serth *et al.*, 1999; Carozzi *et al.*, 2004). In three of these studies, specimens of non-cancerous prostate were also found to have a substantial, although lower, prevalence or copy number of HPV DNA (McNicol & Dodd, 1990a; Serth *et al.*, 1999; Carozzi *et al.*, 2004). Other studies reported that HPV DNA was equally prevalent in cancers, benign prostatic hypertrophy and normal prostatic tissue (McNicol & Dodd, 1990b, 1991; Ibrahim *et al.*, 1992; Dodd *et al.*, 1993; Wideroff *et al.*, 1996b). The majority of studies did not detect HPV in prostatic cancer specimens (Masood *et al.*, 1991; Effert *et al.*, 1992; Serfling *et al.*, 1992; Anderson *et al.*, 1997; Gherdovich *et al.*, 1997; Noda *et al.*, 1998; Strickler *et al.*, 1998a; Saad *et al.*, 1999). A few studies have reported the detection of HPV DNA in specimens of benign hypertrophic prostate tissue and prostate cancer using non-amplification methods that are less prone to false-positive test results than PCR (McNicol & Dodd, 1990a).

Seroepidemiological studies of exposure to HPV have similarly reported conflicting findings. Four cross-sectional studies failed to detect a relationship between the presence of HPV antibodies and prostatic cancer (Strickler *et al.*, 1998a,b; Hayes *et al.*, 2000; Rosenblatt *et al.*, 2003), and another cross-sectional study found a borderline association of prostatic cancer with antibodies to HPV 33 (odds ratio, 1.6; 95% CI, 1.0–2.7) but not to HPV 16 or 18 (Adami *et al.*, 2003). In contrast, two nested case–control studies reported odds ratios for the association between HPV 16 antibodies and prostatic cancer greater than 2.5 (Dillner *et al.*, 1998; Hisada *et al.*, 2000). These latter findings have been interpreted by some authors as evidence that prospective HPV serological data reflect the strong association of HPV antibodies with sexual behaviour and the relationship of sexual behaviour with the risk for prostatic cancer. Such an association is not found in cross-sectional studies because, by the time prostatic cancer occurs, men have aged sufficiently that some have lost detectable levels of HPV antibody (Strickler & Goedert, 2001).

Overall, the failure of most studies that used sensitive PCR methods to detect HPV in prostatic cancer or, when detected, to find similar or higher prevalence of HPV DNA in non-cancer than in cancer tissues does not support a role of HPV in prostate carcinogenesis.

2.6.7 *Cancer of the urinary bladder and urethra*

The majority of bladder cancers that occur in the developed world are transitional-cell carcinomas (approximately 90%), and the proportion of squamous-cell carcinomas ranges from 3 to 10%. In contrast, in countries where schistosomes are endemic, the majority of bladder cancers are squamous-cell cancers (60–80%) (Cooper *et al.*, 1997).

The prevalence of HPV DNA in case series of cancers of the urinary bladder is summarized in Table 50. [For these tumours, contamination from the lower genital tract during acquisition of tissues is a particular concern.] In studies of transitional-cell carcinomas, or

Table 49. Prevalence of HPV DNA in case series of prostate cancer and benign prostatic hypertrophy

Reference, study location	Method of detection and types tested	No. of cases	Type of lesion	Overall HPV positivity (%)	Type-specific HPV positivity (%)			Comment
					6	16	18	
McNicol & Dodd (1990a), Canada	Southern blot for 16/18	4 12	Cancer Benign hypertrophy	75.0 33.3				Mostly TURP, also SPP; frozen tissue
McNicol & Dodd (1990b), Canada	PCR for E6 HPV 16, 18	4 15 5	Cancer Benign hypertrophy Normal autopsies	100 93.3 20.0		100 93.3 20.0	 20.0	Mostly TURP, also SPP and autopsy; frozen samples
Masood *et al.* (1991), USA	ISH for 6/11, 16, 18/31, 33/35	20 20	Cancer Benign hypertrophy	0 0				Biopsies and TURP; paraffin-embedded tissue
McNicol & Dodd (1991), Canada	PCR for E6 HPV 16, 18	27 56	Cancer Benign hypertrophy	51.9 62.5		51.9 60.7	3.7 5.4	Mostly TURP, also SPP; frozen samples
Anwar *et al.* (1992a), Japan	PCR for E6 HPV 16, 18, 33	68 10 10	Cancer Benign hypertrophy Normal autopsies	41.2 0 0		16.2	25.0	TURP, SPP and autopsy; paraffin-embedded tissue
Effert *et al.* (1992), USA	'Differential' PCR for 16, 18 and southern blot	30	Cancer	0				Collection not specified; frozen tissue
Ibrahim *et al.* (1992), USA	PCR and dot-blot for 6, 11, 16, 18, 31, 33, 35, 39, 45	40 12 17	Cancer Benign hypertrophy Normal tissue	15.0 0 11.8		15.0 11.8		Biopsies/TURP/SPP; paraffin-embedded and frozen samples
Rotola *et al.* (1992), Italy	PCR for E6 HPV 6/11, 16	8 17	Cancer Benign hypertrophy	NS [> 75] NS [≥ 82]	50.0[b] 64.7[b]	75.0 82.3		Collection not specified; frozen samples
Serfling *et al.* (1992), USA	PCR with L1 consensus primer and Southern blot	30	Cancer	0				Collection not specified; frozen samples
Dodd *et al.* (1993)[c], Canada	RT-PCR for E6/E7 mRNA of HPV 16	7 10	Cancer Benign hypertrophy	42.9 50.0		42.9 50.0		Collection not specified; frozen samples

Table 49 (contd)

Reference, study location	Method of detection and types tested	No. of cases	Type of lesion	Overall HPV positivity (%)	Type-specific HPV positivity (%)			Comment
					6	16	18	
Sarkar *et al.* (1993), USA	PCR for E6/E7 of 6/11/16/18; Southern blot for 16	23	Cancer and intraepithelial neoplasia	13.0[d]		13.0		Surgical, not TURP; paraffin-embedded tissue
Tu *et al.* (1994), USA	PCR with L1 consensus primer and Southern blot for 16, 18	43 17 1	Cancer Metastases in lymph nodes Normal tissue	2.3 5.9 0		2.3	 5.9	Surgical not TURP; tumours, paraffin embedded; metastases, frozen samples
Moyret-Lalle *et al.* (1995), France	PCR for E6 of 16, 18 and hybridization	17 22	Carcinoma Adenoma	52.9 31.8		52.9 31.8	0 0	Collection not specified; frozen samples
Suzuki *et al.* (1996), Japan	PCR with L1 consensus primer and RFLP	51	Cancer	15.7		15.7		Surgery or autopsy; frozen tissue
Wideroff *et al.* (1996b), USA	PCR with L1 and E6 consensus primers and dot blot for 6, 11, 16, 18, 31, 33, 45	56 42	Cancer Benign hypertrophy	L1, 12.5 L1, 9.5	0 0	0 0	0 0	TURP, surgery and biopsy; paraffin-embedded tissue
Anderson *et al.* (1997), United Kingdom	PCR with E2- and E6-specific primers for 16, and E1 consensus primer	14 10	Cancer Benign hypertrophy	0 0				TURP; frozen tissue
Gherdovich *et al.* (1997), Italy	PCR with MY09/11; nested PCR	5 60	Cancer Benign hypertrophy	0 0				Surgery; frozen tissue

Table 49 (contd)

Reference, study location	Method of detection and types tested	No. of cases	Type of lesion	Overall HPV positivity (%)	Type-specific HPV positivity (%)			Comment
					6	16	18	
Terris & Peehl (1997), USA	PCR for E6 HPV 16 (2 probes) and for L1 HPV 6/11/16/18/33	53	Cancer	$E6_a$, 3.8 $E6_b$, 18.9 L1, 0		3.8 18.9		Radical retropubic resection of the prostate; paraffin-embedded tissues from 41 patients
		21	Benign hypertrophy	$E6_a$, 9.5 $E6_b$, 33.3 L1, 0		9.5 33.3		
		37	Normal tissue	$E6_a$, 2.7 $E6_b$, 13.5 L1, 0		2.7 13.5		
Noda *et al.* (1998), Japan	Nested PCR with consensus primer and RFLP for 16, 18, 31, 33, 35, 52, 58	38 71	Cancer Benign hypertrophy	0 4.2		 4.2		Surgical and TURP; paraffin-embedded tissue
Strickler *et al.* (1998a), Italy and USA	PCR with MY09/11 and GP5+/6+	63 61	Cancer Benign hypertrophy	0 0				Mostly TURP, also SPP; frozen samples
Saad *et al.* (1999), Canada	PCR with MY09/11 and southern blot	40	Cancer	0				Collection not specified; fresh samples
Carozzi *et al.* (2004), Italy	PCR with consensus primer and primer for 16/18/31/33/35/45/52/58, and typing for 6, 11, 16, 18, 31, 33, 35, 45, 52, 58	26 25	Cancer Benign hypertrophy	65.4 48.0				Transperineal biopsy fixed in 10% formalin

See Table 7 for a description of the primers used.
ISH, in-situ hybridization; NS, not specified; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; RT, reverse transcriptase; SPP, suprapubic resection of the prostate; TURP, transurethral resection of the prostate
[a] ';' denotes independent methods whereas 'and' denotes subsequent steps.
[b] 6 or 11
[c] All samples also reported in study by McNicol & Dodd (1991)
[d] Samples positive only after Southern blot analysis
[e] HPV positivity determined in the preservation fluid after 1–2 h of storage of the biopsies

Table 50. Prevalence of HPV DNA in case series of cancer of the urinary bladder

Reference, study location, year(s) of study	Method[a] of detection and types tested	No. and type of lesions	Overall HPV positivity (%)	Type-specific HPV positivity (%)				Other types (n)	Multiple infections (%)	Tissue collection; storage
				6	11	16	18			
Bryant *et al.* (1991), United Kingdom, NR	ISH for 6/11, 16/18	76 TCC 3 SCC	15.8 0			15.8				Transurethral resection; paraffin-embedded specimens
Kerley *et al.* (1991), USA, NR	PCR for 6, 11, 16, 18 and restriction digest	18 TCC 4 SCC 5 controls	0 25.0 0		25.0					Paraffin-embedded specimens
Anwar *et al.* (1992b), Japan, NR	PCR for 6, 11, 16, 18, 33 and dot-blot	46 TCC 2 SCC 21 controls	82.6 50.0 33.3		47.8[b] 19.0	28.3 50.0[c] 9.5	38	33 (14) 33 (2)	60 5	Paraffin-embedded archival specimens; type-specific positivity combines TCC and SCC.
Knowles (1992), United Kingdom, NR	PCR with GP5/6 and Southern blot	100 TCC	0							Cystoscopic biopsies; frozen samples
Furihata *et al.* (1993), Japan, 1981–92	ISH for 16, 18, 33	90 TCC	31.1			21.1	18.9	33 (16)	19	Cystectomy; paraffin-embedded specimens
Chang *et al.* (1994), Finland, 1966–87	PCR with MY09/11	108 TCC	0							Paraffin-embedded archival specimens
Maloney *et al.* (1994), USA, 1979–92	PCR with GP5/6 and type-specific for 16, 18	20 TCC 22 SCC	0 4.5				4.5			Cystectomy; paraffin-embedded specimens
Kamel *et al.* (1995), Finland, 1987–92	ISH for 6, 11, 16, 18, 31, 33	40 TCC 7 SCC	60.0 42.9	30.0 14.3	25.0	20.0 28.6	40.0	31 (16); 33 (12) 31 (3); 33 (1)	45 29	Paraffin-embedded archival specimens

Table 50 (contd)

Reference, study location, year(s) of study	Method[a] of detection and types tested	No. and type of lesions	Overall HPV positivity (%)	Type-specific HPV positivity (%)				Other types (n)	Multiple infections (%)	Tissue collection; storage
				6	11	16	18			
LaRue *et al.* (1995), Canada, NR	PCR and southern blot with L1 consensus primer and probe; typing for 6, 11, 16, 18, 33 by dot blot and sequencing	71 TCC 8 controls	39.4 0		1.4	38.0				Transurethral resection or cystectomy; frozen specimens
Lopez-Beltran & Muñoz (1995), Spain, NR	PCR for 6, 11, 16, 18; ISH for 6/11, 16/18, 31/33/35	76 TCC	PCR, 9.2 ISH, 5.3	1.3		9.2 5.3			1.3	Transurethral resection; paraffin-embedded specimens
Sano *et al.* (1995), Japan, 1989–93	PCR with pU-31B/2R, pU-1M/2R, L1C1/L1C2 and type-specific for 16, 18	80 TCC 11 SCC	0 0							Paraffin-embedded archival specimens
Smetana *et al.* (1995), Israel, 1986–90	ISH for 6/11, 16/18, 31/33/35; PCR with E1 consensus primer and southern blot for 6/11, 16/18	110 TCC 41 controls	25.5 4.9		16.4 2.4	9.1	2.4			Paraffin-embedded specimens and control biopsies
Boucher *et al.* (1996), United Kingdom, NR	Southern blot for 6/11, 16	54 TCC, 1 SCC	0							Radical cystectomy or transurethral resection; formalin-fixed specimens?
Mvula *et al.* (1996), Japan, NR	PCR with L1 consensus primer and type-specific for 16, 18	34 TCC 2 SCC	8.8 0			2.9				Paraffin-embedded archival specimens
Tenti *et al.* (1996), Italy, NR	PCR with MY09/11 and type-specific primers and southern blot for 6/11, 16, 18, 33	79 TCC	32.9			29.1	12.7		8.9	Transurhetral resection or cystectomy; paraffin-embedded specimens

Table 50 (contd)

Reference, study location, year(s) of study	Method[a] of detection and types tested	No. and type of lesions	Overall HPV positivity (%)	Type-specific HPV positivity (%)				Other types (n)	Multiple infections (%)	Tissue collection; storage
				6	11	16	18			
Cooper *et al.* (1997), South Africa, NR	ISH for 6, 11, 16, 18, 31, 33; PCR with 5 E6 consensus primers	25 SCC	0							Paraffin-embedded archival specimens from patients infested with *Schistosoma haematobium*
Lu *et al.* (1997), United Kingdom, 1987–94	ISH for 16, 18	22 TCC 5 SCC	0 0							Paraffin-embedded archival specimens
Aynaud *et al.* (1998), France, NR	PCR with MY09/11 and type-specific for 6, 11, 16, 18, 33	58 TCC	0							Transurethral excision; frozen specimens
Simoneau *et al.* (1999), Canada, 1990–92	PCR with MY09/11 and dot-blot for 6, 11, 16, 18, 33; PCR for 16	187 TCC	9	2.1	0.5	4.8	2.1		1.1	Transurethral resection; frozen specimens
Sur *et al.* (2001), South Africa, 1994–96	PCR with GP5+/6+	64 TCC	1.6							Paraffin-embedded archival specimens
Westenend *et al.* (2001), Netherlands, NR	ISH for 6/11, 16/18 31/33/51	16 SCC	0							Biopsy or cystectomy; paraffin-embedded archival specimens

See Table 7 for a description of the primers used.
ISH, in-situ hybridization; NR, not reported; PCR, polymerase chain reaction; SCC, squamous-cell carcinoma; TCC, transitional-cell carcinoma
[a] ';' denotes independent methods whereas 'and' denotes subsequent steps.
[b] Not clear from article if 22 or 28 HPV 6/11 positive samples (47.8 and 60.9%, respectively)
[c] 16, 18 or 33

in a few studies in which the type of bladder cancer was not specified and that were conducted in countries where schistosomes are not endemic, no HPV DNA was found in a total of 430 bladder cancers using PCR (Kerley *et al.*, 1991; Knowles, 1992; Saltzstein *et al.*, 1993; Sinclair *et al.*, 1993; Chang *et al.*, 1994; Maloney *et al.*, 1994; Sano *et al.*, 1995; Aynaud *et al.*, 1998) or in a total of 91 bladder cancers using in-situ hybridization or Southern blot technique (Ostrow *et al*., 1987; Boucher *et al.*, 1996; Lu *et al*., 1997).

In other PCR-based studies, HPV was detected in 3–80% of the samples of transitional-cell carcinomas: in about 3% in the studies by Chetsanga *et al.* (1992; 1/44), Mvula *et al.* (1996; 1/34), Tekin *et al.* (1999; 2/42), Sur *et al.* (2001; 1/91) and Fioriti *et al.* (2003; 1/32); in 9% in the studies by Lopez-Beltran and Muñoz (1995; 7/76) and Simoneau *et al.* (1999; 16/187); in 20% in the study by Gopalkrishna *et al.* (1995; 2/10); in about 30% in the studies by Smetana *et al.* (1995; 20/59), Tenti *et al.* (1996; 26/79), Chan *et al.* (1997; 6/20) and Gazzaniga *et al.* (1998; 11/35); and in 39%, 50% and 83% in the studies by LaRue *et al.* (1995; 28/71), Aglianò *et al.* (1994; 23/46) and Anwar *et al.* (1992b; 38/46), respectively.

Widely variable prevalences of HPV positivity were also determined using less sensitive methods such as in-situ or southern blot hybridization: 5% (Lopez-Beltran & Muñoz, 1995; 4/76), 16% (Bryant *et al.*, 1991; 12/76), 20% (Shibutani *et al.*, 1992; 4/20), 31% (Furihata *et al.*, 1993; 28/90), 40% (de Gaetani *et al.*, 1999; 17/43) and 60% (Kamel *et al.*, 1995; 24/40).

Only genital (low- and high-risk) HPV types have been assessed in these studies and HPV 16 and 18 were detected most frequently.

Relatively few studies addressed HPV positivity in squamous-cell carcinoma of the bladder using PCR (Kerley *et al*., 1991; Anwar *et al*., 1992b; Maloney *et al.*, 1994; Sano *et al*., 1995; Mvula *et al*., 1996) or in-situ or southern hybridization (Bryant *et al.*, 1991; Kamel *et al.*, 1995; Boucher *et al.*, 1996; Lu *et al*., 1997; Westenend *et al*., 2001) (see Table 50). From these studies, six of 73 (8%) squamous-cell carcinomas were found to be positive for HPV 6, HPV 11 or high-risk HPV. HPV 6 was reported in another squamous-cell carcinoma of the bladder and was identified by southern blot, PCR and in-situ hybridization (Wilczynski *et al*., 1993). No HPV DNA was detectable by PCR in 25 schistosomiasis-associated bladder cancers (Cooper *et al.*, 1997).

In carcinomas of the urethra, HPV 6 or 11 has been detected in two individual cases and in three of a series of four cases (Grussendorf-Conen *et al.*, 1987; Mevorach *et al.*, 1990; Alonso *et al.*, 1997). HPV 16 has been detected in eight of 17 invasive cancers (47%) and in four of six metastases in women with urethral carcinoma (Wiener & Walther, 1994), as well as in four of 14 squamous-cell cancers of the urethra (29%) in men (Wiener *et al.*, 1992). In a study that investigated the location within the male urethra, HPV 16 DNA was found in six squamous-cell carcinomas of the pendulous urethra, but in none of the primary cancers of the bulbous (six) and posterior (two) urethra (Cupp *et al.*, 1996).

2.7 Co-factors of HPV in cervical cancer

It is now clear that HPV infection is causally related to cervical cancer and its precursor lesions (IARC, 1995). However, because of the high prevalence of HPV infection in the general female population and the relatively low rate of cervical cancer, environmental and host genetic factors may influence the progression from infection to cancer (Santos *et al.*, 2001; Castellsagué & Muñoz, 2003; Wang & Hildesheim, 2003). To evaluate the co-factors of HPV, it is important to note the complex and multifactorial origin of cancer. One or more co-factors could substantially contribute to HPV-initiated cervical carcinogenesis depending on its (their) prevalence in the population studied. For example, in female populations whose use of tobacco use is low to non-existent, significant associations between tobacco smoking and disease are unlikely to be detected. Similarly, variations in the prevalence of hormonal contraceptive use in populations by region and country result in the differences in risk estimates for this factor that are observed across countries. These population differences need to be taken into account when interpreting the strength of the association observed between any putative HPV co-factor and cervical cancer.

Evidence for co-factors of HPV derives from two types of study design: prospective cohort studies in which exposure is assessed before the occurrence of disease and case–control studies in which exposure is ascertained after the diagnosis of disease. Moreover, several different analytical approaches may be used in the statistical analyses of these data. Since cervical cancer only develops in women who are infected with HPV, the most appropriate analytical approach is to restrict all statistical analyses to HPV-positive women; that is, women who are at risk for development of the disease. The ideal controls or comparison group would be women who were infected with HPV at the same time as the case women, but who did not develop the disease. Methodologically, this is difficult to achieve; therefore, one approach has been simply to restrict analyses to both cases and controls who were infected with either any HPV type or carcinogenic HPV types. It is important to note that the use of this method of restriction involves a potential for disease misclassification that would bias the risk estimate observed towards the null. Therefore, the studies summarized below may have reported underestimates of the true association between, for example, tobacco smoking and disease.

2.7.1 *Non-infectious co-factors for cervical cancer*

A complete summary of possible co-factors for cervical cancer and its precursor lesions has not been attempted here because many potential co-factors have not been evaluated rigorously in epidemiological studies, and the focus has therefore been placed on tobacco smoking, hormonal contraceptive use, parity and nutritional and genetic factors.

(*a*) *Tobacco smoking*

The role of tobacco smoking in the etiology of cervical cancer has been a topic of debate for many years (Layde, 1989). In an extensive review of the literature through to 1985, Winkelstein (1986) stated that 15 of 18 studies reported a significantly increased risk for cervical cancer in tobacco smokers. In another review of the literature that covered the years 1986–89 (Winkelstein, 1990), 11 of 15 studies reported significant positive associations of different magnitude between tobacco smoking and risk for cervical cancer. Despite this evidence, there have still been doubts as to whether tobacco smoking is truly associated with the risk for cervical cancer. The discrepancy is based on the problem of misclassification with respect to exposure to HPV. Moreover, most studies conducted before 1990 did not measure HPV status or control for sexual activity, which is known to differ according to smoking status. Since 1990, most studies have shown a significant association between tobacco smoking and cervical cancer and its precursor lesions after either adjustment for HPV infection in the analyses or restriction of the analyses to HPV-positive women. Evidence for the association between tobacco smoking and cervical cancer reported here derives from the most rigorously designed studies that restricted statistical analyses to HPV-positive women.

(i) *Case–control studies*

Over the past few decades, numerous case–control studies have been conducted worldwide to quantify the association between tobacco smoking and the risk for cervical cancer (reviewed by Haverkos *et al*., 2003). Muñoz *et al*. (1993) first reported an analysis of the association between tobacco smoking and the risk for CIN3/carcinoma *in situ* that was restricted to 218 HPV-positive women who participated in a case–control study in Colombia and Spain. No significant association was observed, but the study was hampered by low statistical power.

Studies on tobacco smoking and cervical cancer that have been published subsequently are summarized in Table 51.

In a case–control study conducted in Denmark (Kjaer *et al*., 1996) that included 141 prevalent cases, current and former smokers had a twofold increased risk for SIL or ASCUS compared with those who had never smoked. In an update of the same study, the risk appeared to be higher (Kruger-Kjaer *et al*., 1998).

In a comparison of cases of CIN3 and CIN1 in the USA, Ho *et al*. (1998b) observed a significant dose–response in the increase in risk among women who smoked 10 or more cigarettes per day and had a smoking history of more than five pack–years compared with those who had never smoked or former smokers. No association with CIN2 was observed.

Among HPV 16-positive women, Olsen *et al*. (1998a) observed an increase in risk of 4.6 for CIN2/3 among those who had ever smoked tobacco in a population-based case–control study conducted in Norway.

Ylitalo *et al*. (1999) conducted a case–control study among women who were selected from those registered in the Uppsala county cervical cytology programme in Sweden. Cases and controls were matched by HPV status and selected on the basis of cytology

Table 51. Case–control studies of tobacco smoking and pre-invasive and invasive cervical cancer restricted to HPV-positive women

Reference, study location	No. and type of cases	No. and type of controls	Smoking status[a]	Odds ratio (95% CI)	Intensity/duration of smoking	Odds ratio (95% CI)	Detection method and comments
Kjaer *et al.* (1996), Denmark	141 ASCUS and SIL combined	153 normal cytology	Former Current	2.3 (0.8–6.6) 1.9 (1.2–3.2)	–	–	PCR GP5+/6+; adjusted for age
Ho *et al.* (1998b), USA	44 CIN3	163 CIN1	Former Current	1.8 (0.64–5.22) 2.1 (1.09–5.15)	≤ 10 cigarettes/day* > 10 cigarettes/day* ≤ 5 pack–years* > 5 pack–years*	1.49 (0.61–3.67) 3.35 (1.22–9.15) *p* trend = 0.018 1.75 (0.71–4.31) 2.66 (1.15–6.15) *p*-trend = 0.019	PCR and southern blot; no association with CIN2 (n = 52); adjusted for age, education, ethnicity, no. of Pap smears in past 3 years, high-risk versus low-risk HPV infection; *reference category is never or former smokers.
Kruger-Kjaer *et al.* (1998), Denmark	82 ASCUS 86 LSIL 71 HSIL	155 normal cytology	ASCUS LSIL HSIL *Current versus former* ASCUS LSIL HSIL	4.2 (1.4–12.6) 2.5 (0.8–8.0) 3.2 (0.9–11.4) 1.9 (1.0–3.4) 1.5 (0.8–2.7) 1.9 (1.0–3.8)	–	–	PCR GP5+/6+; adjusted for age, years of sex life without barrier contraceptive, partner's education, marital status [follow-up of Kjaer *et al.* (1996)]
Olsen *et al.* (1998a), Norway	60 CIN2/3, histologically confirmed	14 with no dysplasia	Ever versus never smoker	4.6 (0.9–22.9)	Former smoker 1–10 cigarettes/day > 10 cigarettes/day	4.2 (0.5–37.9) 3.3 (0.5–20.8) 5.9 (1.0–35.6)	PCR; population-based controls; adjusted for age
Ylitalo *et al.* (1999), Sweden	178 CIS HPV 16/18-positive	178 HPV16/18-positive; no history of in-situ or ICC and no hysterectomy	Former Current	2.1 (1.0–4.3) 2.3 (1.3–4.3)	1–9 years 10–19 years ≥ 20 years 0.15–3.95 pack–years 4.00–7.95 pack–years ≥ 8.00 pack–years	2.3 (1.1–5.2) 2.5 (1.3–4.7) 1.8 (0.8–4.1) *p* trend = 0.62 2.3 (1.1–4.8) 2.4 (1.6–7.3) 1.6 (0.8–3.2) *p*-trend = 0.17	PCR; adjusted for marital status, OC use, age at sexual debut, no. of sexual partners, age at menarche, parity, years in school
Kjellberg *et al.* (2000), Sweden	122 CIN2/3 histologically confirmed	346 cytologically normal	Never and party smokers Former Current	1.0 2.3 (1.0–5.6) 2.6 (1.2–5.6)	Never and party smokers 1–4 cigarettes/day 5–14 cigarettes/day ≥ 15 cigarettes/day	1.0 0.5 (0.1–1.9) 3.2 (1.2–8.4) 5.8 (1.7–19.4) *p*-trend < 0.001	PCR; adjusted for HPV DNA, age

Table 51 (contd)

Reference, study location	No. and type of cases	No. and type of controls	Smoking status[a]	Odds ratio (95% CI)	Intensity/duration of smoking	Odds ratio (95% CI)	Detection method and comments
Hildesheim *et al.* (2001), Costa Rica	136 HSIL/cancer high-risk HPV-infected, histologically confirmed	624 high-risk HPV	Former Current	2.4 (1.2–5.1) 2.3 (1.3–4.3)	1–5 cigarettes/day ≥ 6 cigarettes/day < 10 years ≥ 10 years	2.3 (1.3–3.9) 2.7 (1.1–6.7) 2.6 (1.2–5.3) 2.2 (1.2–4.2) *p*-trend = 0.003	HC2 + PCR; no association with passive exposure; adjusted for age, no. of pregnancies, HPV types
Lacey *et al.* (2001), USA	58 ADC	49 healthy community members	Ever Former Current	0.7 (0.3–1.5) 1.0 (0.4–2.5) 0.5 (0.2–1.1)	< 1 pack/day ≥ 1 pack/day ≤ 10 years 11–20 years > 20 years	1.2 (0.5–2.9) 0.4 (0.2–1.0) *p*-trend = 0.10 0.9 (0.4–4.0) 1.3 (0.4–2.1) 0.4 (0.1–4.0) *p*-trend = 0.25	PCR MY09/11; adjusted for age, ethnicity
	70 SCC		Ever Former Current	1.5 (0.7–3.0) 1.2 (0.5–3.1) 1.6 (0.7–3.5)	< 1 pack/day ≥ 1 pack/day ≤ 10 years 11–20 years > 20 years	1.6 (0.6–3.9) 1.3 (0.6–3.0) *p*-trend = 0.49 1.2 (0.5–3.0) 3.2 (1.0–9.7) 0.8 (0.3–2.7) *p*-trend = 0.57	
Plummer *et al.* (2003), 4 continents	1463 squamous ICC 211 CIS	254 hospital- and population-based	Ever Former Current	2.08 (1.33–3.27) 1.80 (0.95–3.44) 2.30 (1.31–4.04)	≤ 5 cigarettes/day > 6 cigarettes/day < 20 years ≥ 20 years	1.89 (1.05–3.41) 2.23 (1.18–4.20) 2.36 (1.30–4.29) 1.85 (0.97–3.51)	PCR MY09/11; pooled analysis of 10 case–control studies; adjusted for age, centre, education, no. of sexual partners, age at first intercourse, OC use, parity, screening
Giuliano *et al.* (2004), Mexico border	35 ASCUS/AGUS 25 LSIL 19 HSIL cytology-based diagnosis	201 normal cytology	*ASCUS/AGUS* Former Current *LSIL* Former Current *HSIL* Former Current	 1.57 (0.52–4.76) 0.75 (0.29–1.92) 0.44 (0.05–3.78) 2.19 (0.82–5.85) 0.43 (0.05–3.63) 0.61 (0.16–2.30)	–	–	PCR MY09/11; adjusted for country, parity, *C. trachomatis* infection, Pap smear in the past 3 years, age

Table 51 (contd)

Reference, study location	No. and type of cases	No. and type of controls	Smoking status[a]	Odds ratio (95% CI)	Intensity/duration of smoking	Odds ratio (95% CI)	Detection method and comments
Harris *et al.* (2004), USA	High-risk HPV-positive 137 CIN 1 143 CIN 2/3 histologically confirmed	181 ≤ ASCUS and high-risk HPV-positive	*CIN1* Former Current *CIN2/3* Former Current	 1.7 (0.8–3.6) 1.8 (1.1–3.1) 2.0 (0.9–4.1) 1.6 (1.0–2.7)	 1–10 cigarettes/day > 10 cigarettes/day 0.1–5 pack–years > 5 pack–years 1–10 cigarettes/day > 10 cigarettes/day 0.1–5 pack–years > 5 pack–years	 1.4 (0.9–2.5) 2.5 (1.2–5.3) 1.7 (1.0–2.8) 2.1 (1.0–4.5) 1.4 (0.8–2.4) 2.6 (1.3–5.5) 1.4 (0.8–2.4) 2.6 (1.3–5.2)	PCR MY09/11; adjusted for age, no. of HPV types

ADC, adenocarcinoma; AGUS, atypical glandular cells of undetermined significance; ASCUS, atypical squamous cells of undetermined significance; CI, confidence interval; CIN, cervical intraepithelial neoplasia; CIS, carcinoma *in situ*; HC2, Hybrid Capture 2; HSIL, high-grade intraepithelial lesion; ICC, invasive cervical cancer; LSIL, low-grade squamous intraepithelial lesion; OC, oral contraceptive; Pap, Papanicolaou test; PCR, polymerase chain reaction; SCC, squamous-cell carcinoma; SIL, squamous intraepithelial cells
The reference category is never smokers, if not otherwise specified.

from the most recent Pap smear. A significant approximately twofold increase in risk for carcinoma *in situ* was observed for both former and current smokers compared with those who had never smoked.

Among women who participated in a study in Västerbotten County, Sweden, ever having used tobacco was associated with a strong dose–response in the increase in risk for CIN2/3 (Kjellberg *et al*., 2000).

Among women with high-risk HPV infection, Hildesheim *et al*. (2001) observed a significant approximately twofold increase in risk for HSIL and cervical cancer among former and current smokers.

In a multicentric case–control study among women in the USA, Lacey *et al*. (2001) did not observe a significant increase in risk for adenocarcinoma or squamous-cell carcinoma of the cervix with smoking.

Plummer *et al*. (2003) pooled data from eight IARC case–control studies of invasive cervical cancer and two studies of carcinoma *in situ*, including the study of Muñoz *et al*. (1993). More than 1600 cases were included in the pooled analysis. Ever and current smokers had a twofold higher risk for cervical cancer compared with those who had never smoked. Increasing intensity and duration of smoking did not confer substantial additional risk.

In a study conducted at the USA–Mexico border among Hispanic women, Giuliano *et al*. (2004) did not observe a significant association between tobacco smoking and the risk for LSIL or HSIL.

Harris *et al*. (2004) reported an increase in risk for ≥ CIN1 among women in the USA who were positive for high-risk HPV-type infections and who were current smokers. A dose–response in the increase in risk for ≥ CIN1 was observed for intensity of smoking.

(ii) *Prospective studies*

Several large prospective cohort studies have published risk estimates for the association between tobacco smoking and risk for cervical cancer (Table 52).

Deacon *et al*. (2000) reported a significant increase in risk among participants in a cohort study of 61 570 women in Manchester, United Kingdom. In this nested case–control study, 199 histologically confirmed incident cases of CIN3 were compared with 181 HPV-positive controls (women with normal to CIN2 lesions). In adjusted analyses, ever use of tobacco was associated with a significant more than twofold increase in risk, as was smoking one or more packs of cigarettes per day.

Among participants of the Kaiser Permanente Cohort in Portland, Oregon (USA), Castle *et al*. (2002b) reported a significant increase in risk of at least twofold for incident CIN3 among former smokers and current smokers compared with nonsmokers, regardless of the intensity of smoking and analytical method used to assess the association (Kaplan Meier estimates versus logistic regression) among women infected with high-risk HPV types.

Other prospective studies supported a role for tobacco smoking in the modulation of the natural history of HPV infections. In a prospective study in the USA, Giuliano *et al*.

Table 52. Prospective studies of tobacco use and pre-invasive and invasive cervical cancer restricted to HPV-positive women

Reference, study location	Parent cohort	No. and type of cases	No. and type of controls	Smoking status	Odds ratio (95% CI)	Intensity/ duration of smoking	Odds ratio (95% CI)	Detection method and comments
Deacon *et al.* (2000), United Kingdom	61 570	199 incident CIN3, histologically confirmed	181 normal and < CIN3	Ever Former	2.20 (1.44–3.35) 1.69 (0.76–3.75)	< 1 pack/day ≥ 1 pack/day	1.48 (0.79–2.76) 2.57 (1.49–4.45) *p* trend < 0.001	PCR MY09/11; adjusted for age at first intercourse, total no. of sex partners, years since start of last regular relationship, history of spontaneous abortion
Castle *et al.* (2002b), USA	20 759 women of whom 1812 high-risk HPV-positive	58 incident CIN3 10 incident cancers (high-risk HPV-positive only)	1790 with normal cytology	Former Former	2.1 (1.1–3.9)[a] 3.3 (1.6–6.7)[b]	< 1 pack/day ≥ 1 pack/day < 1 pack/day ≥ 1 pack/day	2.2 (1.2–4.2)[a] 2.9 (1.5–5.6)[a] 2.9 (1.4–6.1)[b] 4.3 (2.0–9.3)[b]	HC2; 10 years follow-up; matched by cytologic interpretation of baseline Pap smears, age, screening behaviour [a] Kaplan Meier analysis [b] Logistic model

See Table 7 for a description of the primers used.
CI, confidence interval; CIN, cervical intraepithelial neoplasia; HC2, Hybrid Capture 2; Pap, Papanicolaou test; PCR, polymerase chain reaction

(2002a) observed a longer duration of high-risk HPV infections and a lower probability of clearing these infections among women who had ever smoked. Similarly, in a cohort of HIV-positive and -negative women, Minkoff *et al*. (2004) observed a significant increase in the incidence of persistent HPV infections among current smokers.

(*b*) *Hormonal contraceptive use*

The use of hormonal contraceptives, most commonly combined oral contraceptive formulations of estrogen and progesterone, has been hypothesized to be associated with development of pre-invasive and invasive cervical lesions (Castellsagué & Muñoz, 2003). In addition to the limitations in design of research conducted without taking HPV status appropriately into account, the investigation of hormonal contraceptives relative to the risk for cervical cancer has been limited by confounding with Pap smear history, and formulation and dose of hormonal contraceptives.

(i) *Case–control studies* (Table 53)

Several case–control studies evaluated the risk for pre-invasive and invasive cervical cancer associated with the use of exogenous hormones, either for contraception or control of menopausal symptoms, among HPV-positive women (Kjaer *et al.*, 1996; Kruger-Kjaer *et al.*, 1998; Lacey, J.V. *et al.*, 1999; Ylitalo *et al.*, 1999; Lacey *et al.*, 2000; Hildesheim *et al.*, 2001; Moreno *et al.*, 2002; Berrington *et al.*, 2002; Smith *et al.*, 2003; Giuliano *et al.*, 2004; Shields *et al.*, 2004). Of these, four observed significant associations with invasive lesions (Ylitalo *et al.*, 1999; Berrington *et al.*, 2002; Moreno *et al.*, 2002; Smith *et al.*, 2003) and one with adenocarcinoma *in situ* (Lacey *et al.*, 1999).

In a case–control study in a Danish population reported by Kjaer *et al*. (1996) and updated by Kruger-Kjaer *et al*. (1998), no association with preneoplastic disease was observed for current users of oral contraceptives or current users with a long duration of use. In contrast, Ylitalo *et al*. (1999) reported a significantly increased cervical cancer risk of 2.65 for current oral contraceptive use in Sweden. The risk was significant after 2 years of use. Studies conducted among populations in the USA have failed to detect associations with either pre-invasive or invasive squamous-cell carcinoma despite adjustment for Pap smear screening history (Lacey, J.V. *et al*., 1999; Giuliano *et al*., 2004). However, Lacey, J.V. *et al*. (1999) detected a significant positive association between current oral contraceptive use and adenocarcinoma *in situ*. In the same study population, no association between non-contraceptive hormonal use and either adenocarcinoma or squamous-cell carcinoma of the cervix was detected (Lacey *et al.*, 2000). In a prevalent case–control study conducted by Hildesheim *et al*. (2001) in Costa Rica, a significant increase in risk for HSIL/cancer was observed only among oral contraceptive users with a duration of use of 5 years or more who had had three or fewer pregnancies. In a meta-analysis of studies restricted to HPV-positive women, Smith *et al.* (2003) observed a significantly increased risk (odds ratio, 2.5; 95% CI, 1.6–3.9) for CIN3 and invasive cervical cancer only for long-term oral contraceptive use (≥ 10 years). Shields *et al*. (2004) found no significant association between endogenous

Table 53. Case–control studies of oral contraceptive (OC) use and pre-invasive and invasive cervical cancer restricted to HPV-positive women

Reference, study location	No. and type of cases	No. and type of controls	OC use status	Odds ratio (95% CI)	Intensity/ duration of use	Odds ratio (95% CI)	Detection method and comments
Kjaer *et al.* (1996), Denmark	141 HSIL and SIL combined	153 cytologically normal	Never users ≥ 20 years 17–19 years ≤ 16 years	 0.5 (0.2–1.4) 0.6 (0.3–1.3) 0.8 (0.4–1.7)	–	–	PCR GP5+/6+; adjusted for age
Kruger-Kjaer *et al.* (1998), Denmark	82 ASCUS 86 LSIL 71 HSIL	155 cytologically normal	No association reported	NR	–	–	PCR GP5+/6+; adjusted for age, years of sex life without barrier contraceptive, partner's education, marital status; [update of the study by Kjaer *et al.* (1990)]
Lacey, J.V. (1999), USA	48 squamous CIS 91 squamous ICC 33 ADC *in situ* 91 ADC	48 healthy population-based	*ADC* in situ Ever Former Current	 5.4 (0.7–43.4) 3.1 (0.4–27.5) 17.1 (1.5–188.2)	 ≤ 2 years 2–6 years > 6 years	 4.0 (0.4–44.3) 4.8 (0.4–51.9) 6.2 (0.7–52.7) *p*-trend = 0.12	PCR MY09/11; multicentre study; adjusted for age, ethnicity, income, lifetime no. of sexual partners, no. of Pap smears; current use is defined as use 12 months before diagnosis for cases and at reference date for controls.
			Invasive ADC Ever Former Current	 1.3 (0.4–4.4) 1.3 (0.4–4.1) 2.1 (0.4–11.9)	 ≤ 2 years 2–6 years > 6 years	 1.5 (0.3–6.6) 1.1 (0.2–5.2) 1.0 (0.2–4.2) *p*-trend = 0.88	
			Squamous-cell CIS Ever Former Current	 1.7 (0.5–6.2) 1.8 (0.5–6.7) 1.6 (0.3–8.5)	 ≤ 2 years 2–6 years > 6 years	 1.4 (0.3–7.2) 3.8 (0.7–19.3) 1.1 (0.3–5.0) *p*-trend = 0.85	
			Squamous ICC Ever Former Current	 1.2 (0.4–3.8) 1.0 (0.3–3.2) 0.7(0.1–3.6)	 ≤ 2 years 2–6 years > 6 years	 1.1 (0.3–4.2) 1.9 (0.4–8.4) 0.9 (0.2–3.7) *p* trend = 0.99	

Table 53 (contd)

Reference, study location	No. and type of cases	No. and type of controls	OC use status	Odds ratio (95% CI)	Intensity/ duration of use	Odds ratio (95% CI)	Detection method and comments
Ylitalo *et al.* (1999), Sweden	178 CIS	178 HPV16/18-positive	Former Current	1.54 (0.76–3.12) 2.65 (1.06–6.67)	< 2 years 2–< 10 years ≥ 10 years	1.55 (0.65–3.70) 2.23 (1.02–4.86) 2.79 (1.14–6.87)	PCR; adjusted for marital status, smoking, age at sexual debut, no. of sexual partners, age at menarche, parity, years in school
Lacey *et al.* (2000), USA	139 SCC 124 ADC	49 healthy community members matched by age, ethnicity, residence	Ever use ADC SCC	 1.1 (0.31–3.9) 0.49 (0.13–1.9)	 < 3 months ≥ 3 months < 3 months ≥ 3 months	 1.4 (0.23–8.4) 0.9 (0.17–4.7) 0.8 (0.13–5.1) 0.3 (0.04–1.9)	PCR MY09/11; multicentric study; non-contraceptive hormone use; no association with either age at first use or whether estrogen was opposed or unopposed.
Hildesheim *et al.* (2001), Costa Rica	136 HSIL/cancer high-risk HPV-postive histo-logically confirmed	624 high-risk HPV	Former Current	0.93 (0.55–1.6) 1.5 (0.83–2.8)	< 5 years ≥ 5 years	0.99 (0.58–1.7) 1.30 (0.70–2.3)	HC2 + PCR; adjusted for age, no. of pregnancies, cigarettes/day; no statistical interaction with parity; however, significantly elevated risk observed among women with OC use ≥ 5 years and having < 3 pregnancies (odds ratio, 3.1; 95% CI, 1.1–1,9)
Berrington *et al.* (2002), United Kingdom	221 ICC	393 from general practitioners' records	–	–	0 year 1–4 years 5–9 years ≥ 10 years	1.00 (0.1–7.8) 1.74 (0.8–3.8) 0.76 (0.3–2.1) 3.92 (1.1–14.1)	Serology; odds ratio calculated as floating absoluted risk with floating CI
Moreno *et al.* (2002), 4 continents	1676 squamous ICC and CIS	255 population- or hospital-based	Ever	1.42 (0.99–2.04)	1 year 2–4 years 5–9 years ≥ 10 years	0.67 (0.41–1.08) 0.80 (0.51–1.24) 2.82 (1.46–5.42) 4.03 (2.09–7.79)	PCR MY09/11; pool of 10 case–control studies (8 ICC and 2 CIS); adjusted for age, centre, education, no. of sex partners, age at first intercourse, parity, no. of Pap smears in life

Table 53 (contd)

Reference, study location	No. and type of cases	No. and type of controls	OC use status	Odds ratio (95% CI)	Intensity/ duration of use	Odds ratio (95% CI)	Detection method and comments
Shapiro *et al.* (2003), South Africa	484 SCC 40 ADC	254	*Injectable (progesterone)* Ever	0.9 (0.6–1.5)	< 1 year 1–4 years 5–9 years 10–14 years ≥ 15 years	0.9 (0.5–1.6) 0.9 (0.6–1.6) 0.8 (0.5–1.4) 1.1 (0.6–2.2) 0.8 (0.4–1.7)	Study population had high exposure to hormonal contraceptives and high rate of disease; adjusted for age, ethnicity, age at first intercourse, lifetime no. of sex partners, education, smoking, rural/urban residence, no. of previous Pap smears
			Combined OC use Ever	0.9 (0.7–1.3)	< 1 year 1–4 years ≥ 5 years	0.8 (0.5–1.2) 0.9 (0.6–1.6) 1.3 (0.6–2.7)	
Smith *et al.* (2003), 4 continents	1279 cases CIN 3 and ICC	265	NR		< 5 years 5–9 years ≥ 10 years	0.9 (0.7–1.2) 1.3 (1.0–1.9) 2.5 (1.6–3.9)	Meta-analysis of 5 case–control studies including Lacey *et al.* (1999), Deacon *et al.* (2000), Hildesheim *et al.* (2001), Berrington *et al.* (2002) and Moreno *et al.* (2002). Each study adjusted for different potential confounding factor.
Giuliano *et al.* (2004), USA–Mexico Border	35 ASCUS/AGUS 25 LSIL 19 HSIL cytology–based diagnosis	201	HSIL *OC use* Former Current *Injectable use* Former Current ASCUS/AGUS *OC use* Former Current *Injectable use* Former Current LSIL Former Current *Injectable use* Former Current	 0.50 (0.15–1.66) 0.66 (0.17–2.59) 0.63 (0.16–2.54) 1.75 (0.40–7.65) 0.87 (0.53–1.42) 1.38 (0.81–2.36) 0.86 (0.49–1.52) 0.90 (0.41–2.00) 0.59 (0.28–1.24) 0.35 (0.11–1.11) 0.86 (0.33–2.22) 1.61 (0.54–4.82)			PCR PGMY09/11; adjusted for country, parity, *C. trachomatis* infection, Pap smears in the past 3 years, age

Table 53 (contd)

Reference, study location	No. and type of cases	No. and type of controls	OC use status	Odds ratio (95% CI)	Intensity/ duration of use	Odds ratio (95% CI)	Detection method and comments
Shields *et al*. (2004), Costa Rica	67 ≥ CIN2 pre-menopausal 43 ≥ CIN2 post-menopausal	134 pre-menopausal 86 post-menopausal		No association			No associations between sex hormone binding globulins, estradiol, free estradiol, estrone, estrone sulfate, or dehydroepiandrosterone and disease regardless of menopausal status; adjusted for menopausal status, age, days since last menses or years since menopause

See Table 7 for a description of the primers used.
ADC, adenocarcinoma; AGUS, atypical glandular cells of undetermined significance; ASCUS, atypical squamous cells of undetermined significance; CI, confidence interval; CIN, cervical intraepithelial neoplasia; CIS, carcinoma *in situ*; HC2, Hybrid Capture 2; HSIL, high-grade intraepithelial lesion; ICC, invasive cervical cancer; LSIL, low-grade squamous intraepithelial lesion; NR, not reported; OC, oral contraceptive; Pap, Papanicolaou test; PCR, polymerase chain reaction; SCC, squamous-cell carcinoma; SIL, squamous intraepithelial cells

circulating hormone concentrations and risk for HSIL among the same Costa Rican study population.

In summary, no study reported a significant increased risk for invasive cervical cancer in ever versus never users of oral contraceptives. When data were pooled across the studies, a significant elevation in risk was only observed among women who had used oral contraceptives for 5 or more years and the risk increased further among those with a duration of use of 10 or more years (Smith *et al.*, 2003). Among a South African population that has a high burden of cervical cancer, a high prevalence of hormonal contraceptive use and a low prevalence of Pap smear screening, Shapiro *et al*. (2003) observed no significant associations with hormonal contraceptive use regardless of the formulation (combined oral estrogen and progesterone or injected progesterone) or duration of use.

(ii) *Prospective studies* (Table 54)

Two prospective studies evaluated the association between hormonal contraceptive use and incidence of CIN among HPV-positive women. Deacon *et al*. (2000) reported no significant increase in risk among current users of oral contraceptive in a study conducted in the United Kingdom. Similarly, Castle *et al*. (2002b) reported no association with current oral contraceptive use among women aged 16 and older in the USA.

(*c*) *Parity*

(i) *Case–control studies* (Table 55)

For several decades, high parity has been suspected to increase the risk for in-situ carcinoma and cancer of the cervix. Unfortunately, many of the studies on cervical cancer either did not measure HPV or did not control for HPV infection or other variables in sexual history that are potential confounders of the association between parity and risk for cervical cancer. The few studies that restricted their statistical analyses to HPV-positive women are reviewed below.

In Denmark, Kjaer *et al*. (1996) observed an increased risk of borderline significance of 1.9 between one or more live births and the risk for ASCUS and SIL combined compared with women who reported no previous pregnancies. However, in a later update of the study by Kruger-Kjaer *et al*. (1998), no significant associations were observed when the cytological categories ASCUS, LSIL and HSIL were examined separately.

Hildesheim *et al*. (2001) observed a significant elevation in risk for HSIL/cancer among women who had had four to five live births among participants in Costa Rica. However, a linear increase in risk with increasing parity was not observed.

The strongest evidence for an association between parity and risk for cervical cancer is from the pooled analysis conducted at IARC on 10 case–control studies by Muñoz *et al*. (2002). Although only two of the eight individual studies of invasive cervical cancer and one of the two studies of carcinoma *in situ* observed significant associations with parity, when the studies were pooled, an odds ratio of 1.81 was observed for women who reported one to two full-term pregnancies compared with none. The risk estimate increased to 3.82 among women who had had seven or more full-term pregnancies. While no statistically

Table 54. Prospective studies of oral contraceptive (OC) use restricted to HPV-positive women

Reference, study location	Parent cohort	No. and type of cases	No. and type of controls	OC use status	Odds ratio (95% CI)	Intensity/ duration of OC use	Odds ratio (95% CI)	Detection method and comments
Deacon *et al*. (2000), United Kingdom	61 570	199 incident CIN3 histologically confirmed	181 < CIN3	Former Current	1.15 (0.63–2.10) 1.28 (0.66–2.50)	1–47 months 48–95 months ≥ 96 months	1.19 (0.58–2.43) 0.76 (0.38–1.53) 1.52 (0.80–2.88)	PCR MY09/MY11; no age restriction
Castle *et al*. (2002b), USA	20 759 women of whom 1812 high-risk HPV-positive	58 incident CIN3 10 incident cancers (high-risk HPV-positive only)	1790 with normal cytology	Current	0.84 (0.49–1.5)[a] 0.61 (0.32–1.1)[b]			HC2; 10 years follow-up; no adjustment [a] Kaplan Meier [b] Conditional logistic model

See Table 7 for a description of the primers used.
CI, confidence interval; CIN, cervical intraepithelial neoplasia; HC2, Hybrid Capture 2; PCR, polymerase chain reaction

Table 55. Case–control studies of parity and pre-invasive and invasive cervical cancer restricted to HPV-positive women

Reference, study location	No. and type of cases	No. and type of controls	No. of full term pregnancies	Odd ratio (95% CI)	Detection method and comments
Kjaer *et al*. (1996), Denmark	141 ASCUS and SIL combined	153 cytologically normal	0 ≥ 1	0.9 (0.5–1.5) 1.9 (1.0–4.4)	PCR GP5+/6+; adjusted for age
Kruger–Kajer *et al*. (1998), Denmark	82 ASCUS 86 LSIL 71 HSIL	155 cytologically normal	*HSIL* 0 ≥ 1	 0.8 (0.4–1.7) 1.8 (0.3–2.3)	PCR GP5+/6+; adjusted for age, years of sex life without barrier contraceptive, partner's education, marital status; no association with either ASCUS or LSIL; follow-up of the study by Kjaer *et al.* (1996)
Hildesheim *et al*. (2001), Costa Rica	136 HSIL/cancer high-risk HPV-positive, histologically confirmed	624 high-risk HPV	0–1 2 3 4–5 6–8 ≥ 9	1.0 1.0 (0.48–2.2) 1.5 (0.73–3.2) 3.5 (1.7–7.2) 2.2 (0.98–5.0) 1.4 (0.56–3.4) *p* trend = 0.04	HC2 + PCR; no association with passive exposure; adjusted for age, no. of pregnancies, cigarettes/day

Table 55 (contd)

Reference, study location	No. and type of cases	No. and type of controls	No. of full term pregnancies	Odd ratio (95% CI)	Detection method and comments
Muñoz *et al.* (2002), 4 continents	1676 squamous CIS and ICC 124 adeno CIS and ICC	255	*Squamous CIS/ ICC* 0 1–2 3–4 5–6 ≥ 7 *Adeno CIS/ICC* 0 1–2 3–4 5–6 ≥ 7	 1.00 (0.55–1.81) 1.81 (1.31–2.52) 2.55 (1.95–3.34) 2.83 (2.02–3.96) 3.82 (2.66–5.48) *p* trend = 0.0002 1.00 (0.21–4.86) 3.47 (1.80–6.70) 2.90 (1.77–4.75) 2.47 (1.44–4.23) 2.92 (1.55–5.50)	PCR MY09/11; pool of 10 case–control studies (8 ICC and 2 CIS); adjusted for age, centre, education, no. of sex partners, age at first intercourse, OC use, smoking, history of Pap smears; CIs were estimated by treating the relative risk as a floating absolute risk.
Giuliano *et al.* (2004), USA–Mexico Border	35 ASCUS/AGUS 25 LSIL 19 HSIL cytology-based diagnosis	201	0 1–2 3–4 ≥ 5	1.0 0.38 (0.08–1.88) 2.14 (0.60–7.64) 0.81 (0.06–10.65)	PGMY 09/11; adjusted for country, parity, *C. trachomatis* infection, Pap smears in the past 3 years, age

See Table 7 for a description of the primers used.

AGUS, atypical glandular cells of undetermined significance; ASCUS, atypical squamous cells of undetermined significance; CI, confidence interval; CIS, carcinoma *in situ* HC2, Hybrid Capture 2; HSIL, high-grade intraepithelial lesion; ICC, invasive cervical cancer; LSIL, low-grade squamous intraepithelial lesion; OC, oral contraceptive; Pap, Papanicolaou test; PCR, polymerase chain reaction; SIL, squamous intraepithelial cells

significant interaction was detected, there appeared to be a higher risk among women with high parity and young age at first full-term pregnancy, and high parity and 5 or more years of oral contraceptive use. A significant increase in risk for adenocarcinoma was also detected among women with one to two full-term pregnancies, although this did not increase linearly with increasing parity.

In a study of women residing along the USA–Mexico border by Giuliano *et al*. (2004), no significant association between SIL and parity was observed.

(ii) *Prospective studies* (Table 56)

The number of prospective studies that have evaluated the association between parity and risk for cervical cancer among HPV-positive women is limited. Deacon *et al*. (2000) observed no increase in risk with increasing parity among women participating in a cohort study in Manchester, United Kingdom. Similarly, Castle *et al*. (2002b) found no increase in risk for CIN3 or cancer among participants in the cohort study in Guanacaste, Costa Rica. However, this study is limited by the fact that parity was assessed only at baseline with no further assessment throughout the 10-year follow-up period.

(*d*) *Nutrients*

Over the past few decades, numerous studies have examined the association between risk for cervical cancer and dietary intake or serological measures of nutrient concentrations. However, most of these studies have methodological limitations that include lack of measurement or adequate consideration of HPV infection in the analyses. Of the studies that did measure HPV infection, very few restricted their analyses to HPV-positive women. Therefore, only a small proportion of all studies are reviewed here.

In addition to the problems of assessment of HPV infection, a drawback that is common to all studies that attempt to examine associations between nutrients and disease is the incomplete consideration of confounding factors, such as tobacco smoking or oral contraceptive use, which are associated with both cervical cancer and nutritional status. In addition, most studies were conducted before the availability of reliable laboratory methods for separating and quantifying the major carotenoids and their geometric isomers in serum. Finally, due to the significant disparity in the content of nutrients in foods by region and variety, the data on carotenoid, selenium and folate content in the food supply are liable to be imprecise. The net result of these limitations is a significant exposure misclassification that results in attenuation of the true association.

(i) *Case–control studies* (Table 57)

Of the studies that restricted analyses to HPV-positive women, only two assessed the association between dietary intake of nutrients and the risk for cervical cancer or CIN (Wideroff *et al.*, 1998; Rajkumar, 2003). Wideroff *et al*. (1998) examined the association between vitamins A, C and E, β-carotene, folate and zinc and the risk for incident HSIL among women resident in Portland, Oregon (USA). Although the risk appeared to be lower among those who consumed higher concentrations of β-carotene, folate and zinc, none of

Table 56. Prospective studies of parity and pre-invasive cervical cancer restricted to HPV-positive women

Reference, study location	Parent cohort	No. and type of cases	No. and type of controls	Number of full term pregnancies	Odds ratio (95% CI)	Detection method and comments
Deacon *et al*. (2000), United Kingdom	61 570	199 incident CIN3 histologically confirmed	181 (includes < CIN 3) stratified random sampling	0 1 2 ≥ 3	1.0 1.57 (0.88–2.77) 1.13 (0.64–1.99) 1.90 (0.94–3.85)	PCR MY09/MY11
Castle *et al*. (2002b), USA	20 759 women of whom 1812 high-risk HPV-positive women	58 incident CIN3 10 incident cancers (high-risk HPV-positive only)	1790 with normal cytology	0 1–2 ≥ 3 0 1–2 ≥ 3	1.0 1.1 (0.64–1.7)[a] 0.7 (0.31–1.6)[a] 1.0 1.2 (0.67–2.1)[b] 0.7 (0.24–1.9)[b]	HC2; parity assessed at enrolment only, not throughout the 10 year follow–up period [a]Kaplan Meier analysis [b]Logistic regression models

See Table 7 for a description of the primers used.
CI, confidence interval; CIN, cervical intraepithelial neoplasia; HC2, Hybrid Capture 2; PCR, polymerase chain reaction

Table 57. Case–control studies of nutrients and pre-invasive and invasive cervical cancer restricted to HPV-positive women

Reference, study location	No. and type of cases	No. and type of controls	Odds ratio for diet (95% CI)	Odds ratio for serum/plasma levels (95% CI)	Detection method and comments
Ho *et al.* (1998b), USA	44 CIN3	163 CIN1		*CIN3 versus CIN1* Vitamin C 2.86 (0.61–13.52) Log α-toc. 0.63 (0.04–9.01) Log β-car. 0.49 (0.13–1.82) Retinol 1.01 (1.00–1.03)	PCR MY09/11 ; adjusted for age, education, ethnicity, no. of Pap smears in past 3 years, high-risk versus low-risk HPV, smoking status; odds ratio for a unit increase in micronutrient level
Ho *et al.* (1998c), USA	262 histo-logically confirmed ≥ CIN1	80 normal cytology with no history of abnormal cytology		*CIN1–3 versus normal* Vitamin C 0.34 (0.13–1.00) Log α-toc 0.25 (0.04–1.66) Log β-car. 0.88 (0.33–2.33) Retinol 0.99 (0.98–1.00) Vitamin C < 0.8 mg/dL 1.00 ≥ 0.8 mg/dL 0.41 (0.19–0.89)	PCR MY09/11; adjusted for age, ethnicity, income, smoking status; odds ratio for a unit increase in micro-nutrient level
Wideroff *et al.* (1998), USA	68 high-risk HPV-positive HSIL	69 high-risk HPV-positive	**Diet + supplements** *Vitamin A quartiles* 1 1.0 2 1.9 (0.6–5.5) 3 1.0 (0.3–2.8) 4 1.4 (0.5–4.2) *β-Carotene quartiles* 1 1.0 2 0.6 (0.2–2.0) 3 0.8 (0.2–2.3) 4 0.6 (0.2–2.0)		PCR and dot blot hybri-dization; adjusted for age

Table 57 (contd)

Reference, study location	No. and type of cases	No. and type of controls	Odds ratio for diet (95% CI)	Odds ratio for serum/plasma levels (95% CI)	Detection method and comments
Wideroff *et al.* (1998) (contd)			*Vitamin C quartiles* 1 1.0 2 1.9 (0.7–5.6) 3 1.0 (0.4–2.8) 4 1.3 (0.4–3.6) *Vitamin E quartiles* 1 1.0 2 0.8 (0.2–2.2) 3 0.6 (0.2–1.7) 4 1.0 (0.4–2.6) *Folate quartiles* 1 1.0 2 0.7 (0.2–1.9) 3 0.7 (0.2–2.2) 4 0.7 (0.3–2.1) *Zinc quartiles* 1 1.0 2 1.0 (0.3–2.9) 3 1.0 (0.4–2.9) 4 0.8 (0.3–2.2)		
French *et al.* (2000), USA	208 HIV-infected SIL	673 HIV-infected normal cytology		*Retinol* ≥ 1.1 µmol/L 1.0 < 1.1 µmol/L 1.8 (1.1–1.3)	PCR MY09/11; analyses adjusted for age, race/ethnicity, CD4+ cell count, HIV type 1 RNA, body mass index, serum albumin
Goodman *et al.* (2001), USA	150 histologically confirmed SIL	179 normal cytology		*MTHFR variants* CC 1.0 CT 2.0 (1.1–3.7) TT 2.9 (1.0–8.8) *p* trend = 0.02	PCR MY09/11 and dot-blot hybridization; adjusted for age, ethnicity, tobacco, alcohol, no. of sex partners before age 20 years, HPV infection

Table 57 (contd)

Reference, study location	No. and type of cases	No. and type of controls	Odds ratio for diet (95% CI)	Odds ratio for serum/plasma levels (95% CI)	Detection method and comments
Weinstein *et al.* (2001a), USA	75 (serum)/63 (RBC folate) histologically confirmed ICC	27 (serum)/23 (RBC folate) HPV 16-seropositive		*Serum folate* High 1.0 Low 2.4 (0.8–7.4) *RBC folate* High 1.0 Low 1.4 (0.5–4.8)	Serology ELISA; adjusted for ethnicity, study site
Weinstein *et al.* (2001b) USA	183 ICC	79 HPV 16-seropositive		*Homocysteine quartiles* 1 1.00 2 2.45 (0.9-7.1) 3 3.81 (1.3-11.2) 4 1.93 (0.6-5.9) *p* trend = 0.42	Serology only; adjusted for age, ethnicity, study site, no. of sex partners, age at first intercourse, years since last Pap smear, no. of pregnancies, smoking, OC use, education, income
Rajkumar *et al.* (2003), Chennai, India	190 ICC	51 cyto-logically normal	*Vegetable and fruit intake (servings/week)* < 6 1.00 6 0.88 (0.26–3.03) ≥ 7 0.37 (0.11–1.22) *p* trend = 0.08		PCR GP5+/6+; adjusted for age, area of residence, occupation, marital status, age at first marriage, no. of pregnancies, husband's extramarital affairs, body mass index, chewing habit

See Table 7 for a description of the primers used.

β-car., β-carotene; CI, confidence interval; CIN, cervical intraepithelial neoplasia; ELISA, enzyme-linked immunosorbent assay; HSIL, high-grade intraepithelial lesion; ICC, invasive cervical cancer; MTHFR, methylene tetrahydrofolate reductase; OC, oral contraceptive; Pap, Papanicolaou test; PCR, polymerase chain reaction; RBC, red blood cell; α-toc., α-tocopherol

these associations reached statistical significance. Among women resident in Chennai, India, Rajkumar *et al*. (2003) observed a non-significant inverse association between consumption of vegetables and fruit and the risk for invasive cervical cancer.

Serum retinol has been examined in two studies that restricted their analyses to HPV-positive women. Ho *et al*. (1998b,c) did not observe significant associations between serum retinol and CIN1–3 among women in the USA. This was the only study that examined the association between serum carotenoids, α-tocopherols and vitamin C concentrations and risk for CIN1–3 combined or considered separately. Only serum vitamin C was significantly associated with a reduced risk for disease (odds ratio, 0.41 for ≥ 0.8 mg/dL versus < 0.8 mg/dL), and the association was limited to the comparison between women with CIN1–3 and those with normal cytology. Among women infected with HIV in the USA, French *et al*. (2000) observed significant associations between higher serum retinol concentrations and the risk for SIL.

Two studies examined the concentration in serum or red blood cells of nutrients that are involved in one-carbon methyl transfer reactions, such as folate and vitamin B12, or the accumulation in serum of homocysteine, a biomarker of insufficient one-carbon nutrient status (Goodman *et al.*, 2001; Weinstein *et al.*, 2001a,b). Weinstein *et al*. (2001a,b) observed elevated risks for invasive cervical cancer among women in the USA who had low serum and red blood cell concentrations of folate, although these associations did not reach statistical significance. In the same study, an increase in risk for invasive cervical cancer with elevated levels of serum homocysteine was observed, but no significant trend in risk. Goodman *et al*. (2001) observed a significant increase in risk for SIL among women with single nucleotide variants in the methylenetetrahydrofolate reductase gene, which is involved in the methylation of homocysteine to methionine. The analysis was not restricted to HPV-positive women but HPV status was controlled for in the statistical analysis.

(ii) *Prospective studies* (Table 58)

Of the prospective studies that restricted analyses to HPV-positive women, only one examined risk for invasive cervical cancer (Lehtinen *et al.*, 1999) and the others examined the risk for persistent HPV infection (Giuliano *et al.*, 1997; Sedjo *et al.*, 2002a,b; Giuliano *et al.*, 2003; Sedjo *et al.*, 2003a,b).

Lehtinen *et al*. (1999) examined the association between serum retinol and α-tocopherol and the risk for invasive cervical cancer among women who were resident in Finland and Sweden. No significant associations were observed, although retinol levels appeared to interact with HPV status.

Giuliano *et al.* (1997) observed significant associations among Hispanic women resident in the USA between serum β-carotene, β-cryptoxanthin, lutein and α-tocopherol when those who were transiently infected with HPV were compared with those who were persistently infected with high-risk HPV over a 3-month period.

The Young Women's Health Study reported a decreased risk for HPV persistence for the highest versus the lowest tertile of serum levels of vitamin B12 (odds ratio, 0.40) and *cis*-

Table 58. Prospective studies of nutrients restricted to HPV-positive women

Reference, study location	No. and type of cases	No. and type of controls	Odds ratio for diet (95% CI)	Odds ratio/*p* value for serum/ plasma levels (95% CI)	Detection method and comments
Giuliano *et al.* (1997), USA	33 persistently HPV-positive women	32 intermittently HPV-positive women		α-Carotene 0.028 β-Carotene 0.179 Lycopene 0.351 β-cryptoxanthin 0.010 Lutein 0.034 α-Tocopherol 0.001 Vitamin C 0.556	HC 1 high-risk probe; *p*-value for difference in adjusted mean (μM) value; Mexican American women; adjusted for age, age at first intercourse, no. of pregnancies, duration of OC use
Lehtinen *et al.* (1999), Finland and Sweden	34 ICC	105 matched controls		*Retinol* Low 1.0 High 1.19 (0.55–2.58) *α-Tocopherol* Low 1.0 High 1.52 (0.70–3.30)	HPV 16-, 18-, 33-seropositive
Sedjo *et al.* (2002a), USA	131 persistent HPV (diet) 109 persistent HPV (nutrient biomarkers)	70 intermittent HPV (diet) 60 intermittent HPV (biomarkers)	*Folate tertiles* 1 1.00 2 0.63 (0.29–1.38) 3 0.52 (0.23–1.18) *p* trend = 0.109 *Vitamin B12 tertiles* 1 1.00 2 1.47 (0.68–3.19) 3 0.68 (0.30–1.54) *p* trend = 0.439 *Vitamin B6 tertiles* 1 1.0 2 0.92 (0.43–2.00) 3 0.61 (0.27–1.39) *p* trend = 0.254	*Folate tertiles* 1 1.00 2 0.95 (0.39–2.29) 3 1.20 (0.51–2.78) p-trend = 0.662 *Vitamin B12 tertiles* 1 1.00 2 0.57 (0.24–1.32) 3 0.40 (0.17–0.96) *p* trend = 0.037	HC 2 Probe B; risk of persistent infection; adjusted for age, age at first intercourse, marital status, smoking, race, body mass index

Table 58 (contd)

Reference, study location	No. and type of cases	No. and type of controls	Odds ratio for diet (95% CI)	Odds ratio/*p* value for serum/ plasma levels (95% CI)	Detection method and comments
Sedjo *et al.* (2002b), USA	131 persistent HPV (diet) 101 persistent HPV (nutrient biomarkers)	69 intermittent HPV (diet) 58 intermittent HPV (bio-marker)	*Fruit tertiles* 1 (low) 1.00 2 0.89 (0.42–1.87) 3 (high) 0.59 (0.27–1.30) *p* trend = 0.206 *Vegetable tertiles* 1 (low) 1.00 2 0.38 (0.17–0.84) 3 (high) 0.46 (0.21–0.97) *p* trend = 0.033 *Lutein tertiles* 1 (low) 1.00 2 0.37 (0.13-0.82) 3 (high) 0.50 (0.24–1.07) *p* trend = 0.054	trans-*Lycopene tertiles* 1 (low) 1.00 2 0.49 (0.21–1.14) 3 (high) 0.78 (0.35–1.78) *p* trend = 0.496 cis-*Lycopene tertiles* 1 (low) 1.00 2 0.57 (0.25–1.29) 3 (high) 0.44 (0.19–1.01) *p* trend = 0.046	HC 2 Probe B; adjusted for age, race, smoking, body mass index; risk of persistent infection; no significant association detected with plasma vitamin A, α- and β-carotene, β-cryptoxanthin, lutein
Sedjo *et al.* (2003a), USA	84 high-risk HPV-positive with persistent infection			trans-*Lycopene tertiles* 1 (low) 1.00 2 3.03 (1.02–7.65) 3 (high) 2.79 (1.17–6.66) *p* trend = 0.025 cis-*Lycopene tertiles* 1 (low) 1.00 2 3.50 (1.51–8.08) 3 (high) 2.92 (1.28–6.63) *p* trend = 0.010	PCR PGMY 09/11; Cox proportional hazard model adjusted for age, ethnicity, no. of new male partners, marital status; hazard ratios were estimated for the association of HPV clearance to each nutrient tertile; no significant association detected with α- and β-carotene, β-cryptoxanthin, lutein/zeaxanthin, tocopherols, folate, vitamin B12

Table 58 (contd)

Reference, study location	No. and type of cases	No. and type of controls	Odds ratio for diet (95% CI)	Odds ratio/*p* value for serum/ plasma levels (95% CI)	Detection method and comments
Giuliano *et al.* (2003a), Brazil	185 HPV type-specific persistent infection	248 transiently HPV-infected	*β-Cryptoxanthin quartiles* 1 (low) 1.00 2 0.60 (0.33–1.09) 3 0.48 (0.27–0.87) 4 0.47 (0.26–0.85) *p* trend = 0.007 *Lutein/zeaxanthin quartiles* 1 (low) 11.00 2 20.58 (0.32–1.05) 3 30.44 (0.24–0.78) 4 40.49 (0.27–0.87) *p* trend = 0.066 Vitamin C quartiles 1 1.00 2 0.63 (0.35–1.15) 3 0.84 (0.47–1.48) 4 0.50 (0.27–0.92) *p* trend = 0.66		PCR MY09/11; risk of persistent infection; adjusted for kcal, income, education, no. of persons in household, no. of sex partners during past 5 years, total no. of pregnancies; significant inverse association also detected with papaya and orange consumption; no significant association detected with α- and β-carotene

See Table 7 for a description of the primers used.
CI, confidence interval; HC, Hybrid Capture; OC, oral contraceptive; PCR, polymerase chain reaction

lycopene (odds ratio, 0.44) (Sedjo *et al.*, 2002a,b). In the same study, the authors reported an approximately threefold higher probability of oncogenic HPV clearance among women in the highest compared tertile of both *trans*- and *cis*-lycopene concentrations (Sedjo *et al.*, 2003a).

Increasing levels of dietary vegetables decreased the risk for persistent HPV infection (Sedjo *et al.*, 2002b). Giuliano *et al*. (2003) assessed the association between dietary nutrient intake and risk for HPV persistence among women who participated in the Ludwig-McGill HPV Natural History Study in Sao Paolo, Brazil. Dietary intakes of β-cryptoxanthin, lutein/zeaxanthin and vitamin C were significantly inversely associated with risk for persistent type-specific HPV infection. In addition, consumption of papaya was inversely associated with persistent HPV infection in this population.

(iii) *Clinical trials* (Table 59)

In this section, all nutrient-based clinical trials are evaluated, although several did not measure HPV infection and none limited their analyses to HPV-positive women.

Randomization assures that the proportion of HPV-positive women is comparable between treatments and that the majority of women who had cervical preneoplastic lesions were HPV-positive. Although randomized clinical trials are considered to be the gold standard to demonstrate the efficacy of a chemopreventive agent, intervention studies of nutrients and risk for cervical cancer present unique problems. First, the widespread adoption of the use of supplemental vitamins and minerals in the USA, where most chemoprevention studies have been carried out to date, can adversely affect the feasibility of testing the efficacy of a single nutrient to prevent cervical cancer. Unlike pharmaceutical agents that proceed through phase I and II trials to determine the safe and optimal dose, a 'best-guess' estimate has been used to choose doses for phase III nutrient chemoprevention trials. Information on duration of treatment and length of follow-up that is needed to demonstrate an effect has also been lacking. Finally, the statistical power of a study to assess efficacy is based on the accumulation of an adequate number of events (e.g. regression or progression of CIN). Non-compliance to the study regimen and high rates of spontaneous regression of lesions such as CIN1 (> 80% spontaneous regression) combine to reduce the power of any study to detect differences between treatment and control groups. As a consequence of the above limitations, only one of 10 clinical trials conducted has shown significant protective effects of the nutrient that was tested, which was topical all-*trans*-retinoic acid (Meyskens *et al*., 1994).

Folate trials

Two phase II trials of folic acid for the prevention of cervical cancer have been completed (Butterworth *et al.*, 1992; Childers *et al.*, 1995). Both found no significant effect of treatment on the regression or progression of lesions. In Alabama, USA, Butterworth *et al*. (1992) found no protective effect of a daily dose of 10 mg folate on either regression or progression of cervical lesions after 6 months of treatment. However, the majority of participants entered the trial with CIN1 lesions that have a high rate of spontaneous

Table 59. Clinical trials of nutrients and pre-invasive cervical lesions

Reference, study location	No. of subjects enrolled	No. of subjects completing trial	Entry diagnosis	End-point	Detection method of primary end-point	Dose	Duration	Results
Folic acid								
Butterworth *et al.* (1992), USA	235	199	CIN1/2	Lesion regression and HPV DNA	Biopsy and PCR HPV 16	10 mg/day	6 months	No effect
Childers *et al.* (1995), USA	331	262	89% KA/CIN1 11% CIN2	Lesion regression	Colposcopy	5 mg/day	6 months	No effect
β-Carotene								
De Vet *et al.* (1991), Netherlands	278	278	28% CIN1 42% CIN2 30% CIN3	Lesion regression	Cytology, colposcopy	10 mg/day	3 months	No effect
Fairley *et al.* (1996), Australia	114	111	5% Atypia 62% HPV 23% CIN1 10% CIN2	Lesion regression and HPV DNA	Cytology and PCR and HC HPV DNA	30 mg/day	12 months	No effect on either lesion regression or HPV positivity
Romney *et al.* (1997), USA	98	69	51% CIN1 46% CIN2 39% CIN3	Lesion regression and HPV 16	Biopsy and PCR HPV 16	30 mg/day	9 months	Decreased regression in β-carotene arm
Mackerras *et al.* (1999), Australia	147	141	100% minor atypia or CIN1	Lesion regression	Cytology	30 mg/day β-carotene 500 mg/day vitamin C	24 months	No effect of either β-carotene or vitamin C
Keefe *et al.* (2001), USA	103	78	43% CIN2 57% CIN3	Lesion regression and HPV	Biopsy and PCR HPV DNA	30 mg/day	24 months	No effect on regression

Table 59 (contd)

Reference, study location	No. of subjects enrolled	No. of subjects completing trial	Entry diagnosis	End-point	Detection method of primary end-point	Dose	Duration	Results
Retinoic acid								
Meyskens *et al.* (1994), USA	301	232	50% CIN2 50% CIN3	Lesion regression	Biopsy	1 mL 0.375% topical all *trans*-retinoic cream	Treated at 1, 3, 6 months; end-point assessed at 15 months	Increased regression of CIN2 at 15 months
Follen *et al.* (2001), USA	39	36 at 6 months 30 at 12 months	33% CIN2 67% CIN3	Lesion regression and HPV DNA	Biopsy and HC	200 mg/day oral *N*-(4-hydroxyohenyl) retinamide	6 months	No effect
Alvarez *et al.* (2003), USA	114	104	39% CIN2 61% CIN3	Lesion regression	Cytology	25 or 50 mg aliretinoin or placebo daily	3 months	No effect

CIN, cervical intraepithelial neoplasia; HC, Hybrid Capture; KA, koilocytic atypia; PCR, polymerase chain reaction

regression. In Arizona, USA, Childers *et al*. (1995) similarly found that 5 mg folic acid per day had no significant effect on cervical lesions after 6 months of treatment. Again, the majority of participants entered the study with CIN1 lesions.

β-Carotene trials

Five phase II/IIII trials of β-carotene supplements have been conducted, none of which demonstrated an increase in regression or a decrease in progression of any preneoplastic lesion. De Vet *et al*. (1991) found no effect of treatment with 10 mg per day β-carotene for 3 months among women in The Netherlands. A longer duration of treatment (9–24 months) with higher doses (30 mg) was also ineffective in altering rates of regression of lesions in studies conducted by Fairley *et al*. (1996) in Australia, Romney *et al*. (1997) in the USA, Mackerras *et al*. (1999) in Australia and Keefe *et al.* (2001) in the USA.

Retinoic acid trials

Three trials tested different formulations of retinoic acid (Meyskens *et al.*, 1994; Follen *et al.*, 2001; Alvarez *et al.*, 2003). In the only placebo-controlled trial of a topical retinoid (all-*trans*-retinoic acid), Meyskens *et al*. (1994) found a significant effect of three administrations of the compound on the regression of CIN2 lesions after 15 months. In comparison, two studies of oral doses of retinoids failed to demonstrate an effect (Follen *et al.*, 2001; Alvarez *et al.*, 2003). In a small trial conducted by Follen *et al*. (2001), daily oral doses of 200 mg *N*-(4-hydroxyohenyl) retinamide had no effect on regression of CIN2/3 lesions after 6 months of treatment. Similarly, daily administration of 25 or 50 mg aliretinoin had no effect on the regression of CIN2/3-lesions after 3 months of treatment (Alvarez *et al*., 2003).

Most of the studies were small and high rates of spontaneous regression of lesions were observed. As a result, these studies had insufficient power to test adequately the efficacy of the selected agents. Since effective ablative treatments are available for CIN2/3, it is questionable whether continued efforts should be made to find chemopreventive alternatives to the current standard of care. Future efforts in this area will require multisite collaboration to ensure that an adequate sample size of women with histologically confirmed CIN2/3 lesions are enrolled and complete the treatment protocol.

(*e*) *Genetic factors*

Familial clustering of cervical cancer has been explored as a potential marker of inherited genetic susceptibility.

Population-based studies that used routinely collected data and record linkage in Sweden, Iceland and America consistently found moderately increased risk estimates for carcinoma *in situ* and invasive carcinoma among women who had a first- or a second-degree relative with cervical cancer (Amundadottir *et al.*, 2004; de Zelmanowicz *et al.*, 2005; Couto & Hemminki, 2006). However, these investigations did not fully take into account relevant covariates such as HPV status or screening practices.

Heritability estimates from the Swedish Cancer Registry suggest that genes are responsible for less than 30% of cervical tumours (Hemminki *et al.*, 1999; Magnusson *et al.*, 2000; Couto & Hemminki, 2006).

In a multicentric case–control study in Latin America that included 481 patients with invasive cervical cancer and 801 population controls, Brinton *et al.* (1987) found a familial tendency for all cell types of cervical cancer. Women who had a family history of cervical cancer had a 2.49-fold higher risk for adenocarcinoma (based on one case), a 9.93-fold higher risk for adenosquamous carcinoma (based on 2 cases) and a 3.11-fold higher risk for squamous cell carcinoma (based on 13 cases).

In the Republic of Korea, Yoo *et al.* (1997) conducted a case–control study that included 203 cases of invasive cervical cancer and reported a 2.20-fold (95% CI, 1.21–4.01) increase in risk associated with a family history of cervical cancer. Cusimano *et al.* (1989) reported an odds ratio of 2.87 (95% CI, 1.05–7.83) for a family history of cervical cancer in Sicily. In a case–control study in the USA, Hildesheim *et al.* (1999) reported a significantly increased risk for rapid onset of cervical cancer among young women whose mothers had a history of cervical cancer.

Familial studies are limited in their capacity to separate fully the effects that can be attributed to genetic susceptibility from those that are related to common environmental and behavioural traits. However, reports have consistently suggested that a familial risk exists and further studies aimed at identifying relevant biomarkers would be pertinent.

(*f*) *Human leukocyte antigen (HLA) polymorphisms and risk for cervical cancer*

Major histocompatibility gene products that are complexed with peptides derived from viral antigens can induce T-cell responses on the surface of antigen-presenting cells that clear viral infections. HLA class II genes (*DR*, *DQ* and *DP*) are expressed on B cells and macrophages, where they present antigen fragments to $CD4^+$ T cells. Although they are not usually expressed on epithelial cells, expression of HLA class II genes is increased in cervical cancer cells (Glew *et al.*, 1992). $CD4^+$ T cells have been reported to have killer activity and could thus potentially kill cervical cancer cells directly (de Jong *et al.*, 2004; Steele *et al.*, 2005); however, $CD4^+$ T cells more commonly provide helper functions that assist the maturation of $CD8^+$ T cells. $CD8^+$ T cells recognize peptides in conjunction with the more ubiquitously expressed HLA class I genes. These genes (*A*, *B* and *C*) are highly polymorphic and present antigens on most cells, including cervical epithelial cells. Although class I and II gene products were initially defined using serological reagents, it has more recently been possible to characterize the HLA genotype using PCR-based methods with specific primers. Several studies have examined the association between HLA genotypes and the risk for cervical cancer. Most of these focused on class II alleles because the methods for genotyping these genes were developed earlier than those for the detection of class I alleles.

Class II *DR* and *DQ* alleles are co-dominantly expressed. The large number of polymorphisms of these alleles lead to variations in the antigen recognition site on the cell

surface, which may confer susceptibility or resistance to HPV infection and neoplastic progression. Malignant transformation and regression of cottontail rabbit papillomavirus-induced lesions were clearly shown to be associated with class II DR and DQ genes (Han *et al.*, 1992).

The results of several selected studies are summarized in Table 60.

The first associations between HLA class II genes and cervical cancer were reported with *DR5*, *DR6* and *DQ3* (Wank & Thomssen, 1991). A second report on the same samples employed HLA typing using DNA-based methods and assigned the increase in risk to *DQB1*0301/0303* (Wank *et al.*, 1993). A number of studies that used different ethnic populations confirmed this association (Helland *et al.*, 1992; Gregoire *et al.*, 1994; Nawa *et al.*, 1995; Duggan-Keen *et al.*, 1996) but others did not find statistically significant associations for these alleles (Glew *et al.*, 1992; Apple *et al.*, 1994; Allen *et al.*, 1996; Lin *et al.*, 2001).

Other allele groups that have been reported to confer risk include *DRB1*11* (Duggan-Keen *et al.*, 1996), *DRB1*15* (Cuzick *et al.*, 2000; Maciag *et al.*, 2000; Beskow *et al.*, 2001) and *DQB1*06* (Gregoire *et al.*, 1994; Beskow *et al.*, 2001) and the related haplotypes *DRB1*0401–DQB1*0301* (Cuzick *et al.*, 2000), *DRB1*1101–DQB1*0301* (Lin *et al.*, 2001) and *DRB1*1501–DQB1*0602* (Apple *et al.*, 1994). In addition, *DRB1*13* and the *DRB1*13–DQB1*06* haplotype have been reported to confer protection against the development of cervical cancer (Apple *et al.*, 1995; Sastre-Garau *et al.*, 1996).

Class II *DRB1* and *DQB1* genes were examined in 315 women who had invasive squamous-cell cervical cancer and 381 population-based controls who were residents of the metropolitan area of Seattle, WA (USA) (Madeleine *et al.*, 2002). An increased risk for squamous-cell cancer was associated with two *DRB1* alleles (*DRB1*1001* and *DRB1*1101*) and one *DQ* allele (*DQB1*0301*). Decreased risks for squamous-cell cancer were associated with *DRB1*0301* and *DRB1*13*. The relative risks for squamous-cell cancer that contains HPV 16 were different from those for squamous-cell cancers that contain other high-risk HPV types for three alleles: *DRB1*0401*, *DRB1*07* and *DQB1*06*. The increased risks associated with the DQB1*0301 allele were specific to the *DRB1*0401–DQB1*0301* (odds ratio, 1.9; 95% CI, 1.2–3.1) and *DRB1*1101–DQB1*0301* (odds ratio, 2.9; 95% CI, 1.6–5.2) haplotypes. Similarly, the associations with disease for the *DRB1*0301* and *DRB1*13* alleles were specific to the *DRB1*0301–DQB1*02* (odds ratio, 0.7; 95% CI, 0.5–1.0) and *DRB1*13–DQB1*06* (odds ratio, 0.6; 95% CI, 0.4–0.9) haplotypes.

Fewer studies have examined the association between class I alleles and cervical cancer. In some cases, the risk was associated with the genotypes *A2* or *A*3303* (Wang *et al.*, 2002a), although the association with *A*3303* was seen only in a population of women from Portland (OR, USA) and not in two other populations that were examined. In contrast, the same study found a protective effect for *CW*0202* in all three populations, and a protective effect for *CW*0401* for both populations in the USA. The *B*07* allele has been examined frequently and some (Hildesheim *et al.*, 1998; Wang *et al.*, 2002b), but not all (Gostout *et al.*, 2003) studies found that it conferred risk. In the progression of HPV-related dysplasia, the *B*44* allele was associated with a strong risk (odds ratio, 9.0) in one

Table 60. Association of human leukocyte antigen (HLA) class II alleles with the risk for cervical cancer

Reference, study location	HLA groups/allele(s)	Total no. of cases	Type of cases	*p* Value or odds ratio (95% CI)
	DRB1*			
Madeleine *et al.* (2002), USA	DRB1*07	315	HPV 16 SCC	1.5 (1.0–2.3)
Allen *et al.* (1996), Sweden	DRB1*08			
	DRB1*0802	150	Cervical cancer	$p = 0.005$
Madeleine *et al.* (2002), USA	DRB1*10	315	SCC	5.6 (1.2–26.1)
			HPV SCC	7.3 (1.5–36.7)
	DRB1*1001		HPV SCC	7.3 (1.5–36.7)
Wank & Thomssen (1991), USA	DR5 (DRB1*11, DRB1*12)	66	Invasive cancer	$p = 0.009$
Duggan-Keen *et al.* (1996), United Kingdom	DRB1*11	150	Cervical cancer	NS
Lin *et al.* (2001), Senegal	DRB1*11	55	Invasive carcinoma	1.0 (0.4–2.2)
Madeleine *et al.* (2002), USA	DRB1*11	315	SCC	1.8 (1.2–2.9)
	DRB1*1101		SCC	2.4 (1.4–4.2)
			HPV 16 SCC	2.4 (1.3–4.6)
Wank & Thomssen (1991), USA	DR6 (DRB1*13, DRB1*14)	66		$p = 0.004$
Apple *et al.* (1994, 1995), USA	DRB1*13	98	SCC and ADC	0.3 (0.1–0.7)
Sastre-Garau *et al.* (1996), France	DRB1*1301/1302	126	Invasive cancer	$p = 0.0004$
Cuzick *et al.* (2000), United Kingdom	DRB1*1301	116	SCC	NS
Lin *et al.* (2001), Senegal	DRB1*13	55	Invasive cancer	0.5 (0.2–1.1)
Krul *et al.* (1999), Netherlands	DRB1*13	172	SCC	NS
Madeleine *et al.* (2002), USA	DRB1*13	315	SCC	0.6 (0.4–0.9)
			HPV 16 SCC	0.6 (0.3–0.9)
	DRB1*1301		SCC	0.6 (0.4–1.0)
			HPV 16 SCC	0.6 (0.3–1.1)
	DRB1*1302		SCC	0.6 (0.3–1.1)
			HPV 16 SCC	0.4 (0.2–1.0)

Table 60 (contd)

Reference, study location	HLA groups/allele(s)	Total no. of cases	Type of cases	*p* Value or odds ratio (95% CI)
Maciag *et al.* (2000), Brazil	DRB1*15	161	SCC	2.2 (1.3–3.9)
Krul *et al.* (1999), Netherlands	DRB1*15	172	SCC	NS
Madeleine *et al.* (2002), USA	DRB1*15	315	SCC	1.0 (0.7–1.4)
Beskow *et al.* (2001), Sweden	DRB1*1501	440	CIS	$p = 0.027$
Madeleine *et al.* (2002), USA	DRB1*1501	315	SCC	1.0 (0.7–1.4)
Cuzick *et al.* (2000), United Kingdom	DRB1*1501	116	HPV 16 SCC	$p = 0.05$
Gostout *et al.* (2003), USA	DR2 (1501)	127	Cervical cancer	$p = 0.023$
	DQB1*			
	DQ1 (DQB1*05, DQB1*06)			
Maciag *et al.* (2000), Brazil	DQB1*05	161	SCC	0.6 (0.4–0.9)
Madeleine *et al.* (2002), USA	DQB1*05	315	SCC	1.2 (0.9–1.7)
Beskow *et al.* (2001), Sweden	DQB1*0602		CIS	$p = 0.028$
Madeleine *et al.* (2002), USA	DQB1*0602	315	HPV 16 SCC	0.9 (0.6–1.4)
Lin *et al.* (2001), Senegal	DQB1*0602	55	Invasive carcinoma	0.6 (0.1–2.7)
Sastre-Garau *et al.* (1996), France	DQB1*0603	126	Invasive cancers	
Madeleine *et al.* (2002), USA	DQB1*0603	315	SCC	0.6 (0.4–1.0)
Gregoire *et al.* (1994), USA	DQB1*0604	66	SCC	$p = 0.02$
Helland *et al.* (1992), Norway	DQB1*0604	158	SCC	0.4 (0.1–1.1)
Madeleine *et al.* (2002), USA	DQB1*0604	315	SCC	0.6 (0.3–1.3)

Table 60 (contd)

Reference, study location	HLA groups/allele(s)	Total no. of cases	Type of cases	*p* Value or odds ratio (95% CI)
Wank & Thomssen (1991), USA	DQ3 (DQB1*07, DQB1*08, DQB1*09)	66	Invasive cancer	$p = 0.0009$
Helland *et al.* (1992), Norway	DQ3	158		2.0 (1.3–3.2)
Nawa *et al.* (1995), Japan	DQ3	23	SCC	$p = 0.0003$
Sastre-Garau *et al.* (1996), France	DQ3	126	Invasive cancer	$p = 0.03$
Allen *et al.* (1996), Sweden	DQ3	150	Cervical cancer	$p = 0.11$
Apple *et al.* (1994), USA	DQ3	28	SCC and ADC	NS
Krul *et al.* (1999), The Netherlands	DQ3	172	SCC	NS
Glew *et al.* (1992), United Kingdom	DQ3	53	SCC	NS
Madeleine *et al.* (2002), USA	DQB1*07	315	SCC HPV 16 SCC	1.6 (1.2–2.2) 1.5 (1.0–2.2)
Duggan-Keen *et al.* (1996), United Kingdom	DQB1*0301	150	Cervical cancer	$p = 0.04$
Cuzick *et al.* (2000), United Kingdom	DQB1*0301	116	SCC	$p = 0.02$
Helland *et al.* (1992), Norway	DQB1*0301	158	SCC	1.8 (1.1–3.0)
Madeleine *et al.* (2002), USA	DQB1*0301	315	SCC HPV 16 SCC	1.6 (1.2–2.2) 1.5 (1.0–2.2)
Gregoire *et al.* (1994), USA	DQB1*0301	66	SCC	NS
Wank *et al.* (1993), USA	DQB1*0301			8.71 $p = 0.0001$
Vandenvelde *et al.* (1993), Belgium	DQB1*0301/0302			NS
Lin *et al.* (2001), Senegal	DQB1*0301/0302	55	Invasive cancer	0.8 (0.4–1.9)
Madeleine *et al.* (2002), USA	DQB1*09 DQB1*0303	315	SCC SCC	1.3 (0.8–2.1) 0.9 (0.6–1.3)

Table 60 (contd)

Reference, study location	HLA groups/allele(s)	Total no. of cases	Type of cases	*p* Value or odds ratio (95% CI)
Gregoire *et al.* (1994), USA	DQB1*0303	66	SCC	2.7
Wank *et al.* (1993), USA	DQB1*0303			4.50 *p* = 0.0012
	DRB1*:DQB1*			
Cuzick *et al.* (2000), United Kingdom	DRB1*0401–DQB1*0301	116		*p* = 0.02
Madeleine *et al.* (2002), USA	DRB1*0401–DQB1*0301	315	HPV 16 SCC	1.9 (1.2–3.1)
Allen *et al.* (1996), Sweden	DRB1*0401–DQB1*0301	150		*p* = 0.01
Lin *et al.* (2001), Senegal	DRB1*1101–DQB1*0301	55	Invasive carcinoma	2.6 (1.0–7.1)
Madeleine *et al.* (2002), USA	DRB1*1101–DQB1*0301	315	HPV 16 SCC	2.9 (1.6–5.2)
Allen *et al.* (1996), Sweden	DRB1*1101–DQB1*0301	150	Cervical cancer	NS
Duggan-Keen *et al.* (1996), United Kingdom	DRB1*07–DQB1*0201	150		NS
Allen *et al.* (1996), Sweden	DRB1*0802–DQB1*0402	150	Cervical cancer	*p* = 0.001
Maciag *et al.* (2000), Brazil	DRB1*15–DQB1*0602	161	SCC	2.0 (1.2–3.6)
Cuzick *et al.* (2000), United Kingdom	DRB1*1501–QB1*0602	116	 HPV 16	NS 1.8 (1.0–3.3)
Apple *et al.* (1994, 1995), USA	DRB1*1501–DQB1*0602	98	SCC and ADC HPV 16	2.9 (1.3–6.7) 4.8 (1.9–11.8)
Madeleine *et al.* (2002), USA	DRB1*1501–DQB1*0602	315	HPV 16 SCC HPV 16	1.0 (0.7–1.4) 0.9 (0.6–1.4)
Allen *et al.* (1996), Sweden	DRB1*1501–DQB1*0602	150	Cervical cancer	NS
Madeleine *et al.*, USA	DRB1*0301–DQB1*02 DRB1*13–DQB1*06	315	HPV 16 SCC	0.7 (0.5–1.0) 0.6 (0.4–0.9)
Apple *et al.* (1994, 1995), USA	DRB1*13–DQB1*0603	98		0.3 (0.1–0.8)

ADC, adenocarcinoma; CIS, carcinoma *in* situ; NS, not significant; SCC, squamous-cell carcinoma

study (Bontkes *et al.*, 1998) and the *B*07–DQB1*0302* haplotype was associated with a strong risk (odds ratio, 8.2) in another (Wang *et al.*, 2002b).

Epidemiological studies in different populations have found various relationships between risk for cervical cancer and HLA polymorphisms. Comparisons are hampered by issues such as small sample sizes, inappropriate controls and chance findings. The probability of chance findings is increased by multiple comparisons that are often made between the extensive number of polymorphisms and the disease. Ethnic admixture within study groups of one race also may contribute to differences in the distribution of HLA polymorphisms between seemingly homogeneous populations. This heterogeneity may influence risk estimates by masking true effects.

2.7.2 *Infectious co-factors*

The central etiological role of HPV (a sexually transmitted infection) in cervical tumorigenesis has led to hypotheses that other microbial agents that also infect the cervicovaginal epithelium could act as co-factors and increase or decrease the risk for cervical cancer in the presence of a high-risk HPV infection (Lacey, 1992). The proposed mechanisms of co-factors (described in Section 4.1.5(*a*)) include direct biological interactions, such as viral co-activation of HPV replication, and indirect effects, such as damage to the epithelial barrier that protects against HPV infection. In this section, special emphasis has been placed on studies of co-factors that controlled for the effects HPV infection in the analyses.

Two sources of bias were common concerns in the studies reviewed: (*a*) the inability to assess temporality; many studies assessed exposure using serological assays that do not distinguish between current and past infections. Moreover, most studies that tested directly for infections in the cervix were cross-sectional. Therefore, it was generally not known whether the presumptive infectious co-factor was present concurrently with a high-risk HPV infection, or whether it preceded or followed the development of cervical neoplasia. This most probably caused bias towards the null. (*b*) residual confounding by HPV; detection of a sexually transmitted infection, even among women who are all currently positive for HPV DNA, may be a surrogate marker for high-risk behaviours or high-risk sexual partners and a consequently greater cumulative lifetime exposure to high-risk HPV. Because of the very strong association of HPV with cervical cancer, a small degree of residual confounding by HPV could account for moderate effects that are putatively associated with a sexually transmitted infection.

(*a*) *Herpes simplex virus (HSV)*

Genital HSV infection is one of the microbial agents that is most frequently studied as a potential co-factor for cervical cancer. Before the causal role of HPV in the development of cervical cancer was firmly established, HSV was itself regarded as a candidate etiological agent — one that, similarly to HPV, could help explain the association of cervical cancer with sexual behaviour (Brinton, 1992). Although in-vitro studies conducted during

the 1970s demonstrated the carcinogenic potential of HSV (Duff & Rapp, 1971a,b; Duff, 1975), HSV DNA was not consistently detected in cervical cancer specimens (Brinton, 1992). An increased understanding of the causal role of HPV and the publication of a large prospective study that found no association of HSV-2 seroantibodies with incident cervical cancer (Vonka *et al*., 1984) shifted the focus away from HSV-2 as an etiological agent (Brinton, 1992; Ferrera *et al*., 1997a; Lehtinen *et al*., 2002). In retrospect, the study by Vonka *et al.* (1984) may have lacked statistical power because of the small number of cases observed during follow-up, and concerns were expressed regarding over-matching (Brinton, 1992). Moreover, laboratory studies demonstrated that HSV-2 DNA does not need to persist for HSV-2 to play a role in the transformation of cervical epithelial cells (i.e. a possible 'hit and run' mechanism) (Galloway & McDougall, 1983; Jones, 1995).

HSV-2 has a much greater tropism for genital tissue and recurs with greater frequency in the genital tract than HSV-1 (Engelberg *et al*., 2003; Sacks *et al*., 2004). Therefore, seroepidemiological studies conducted during or after the 1990s, when assays that could distinguish between HSV-2 and HSV-1 infection came into use, are of most interest (Ashley & Wald, 1999).

The majority of seroepidemiological studies found a moderate or no association between HSV-2 antibodies and cervical neoplasia (Table 61). Lehtinen *et al.* (2002) pooled data and specimens from three population-based Nordic cohorts to form a collective population of more than 500 000 women. No difference was found between the baseline seroprevalence of HSV-2 among 178 incident cervical cancer cases and 525 controls after adjustment for antibodies to HPV 16/18/33 VLPs and cigarette smoking (odds ratio, 1.0; 95% CI, 0.6–1.7).

A study by the IARC (Smith *et al.*, 2002a) that pooled data from seven separate case–control investigations conducted in Brazil, Colombia, Morocco, Peru, the Philippines, Spain and Thailand found a higher seroprevalence of HSV-2 in 1158 cases of squamous-cell carcinoma and 105 cases of adeno-/adenosquamous carcinoma than in 1117 controls; after limiting the analysis to HPV DNA-positive subjects and adjusting for seropositivity to *C. trachomatis*, the associations of HSV-2 seroantibodies with squamous-cell carcinoma (odds ratio, 2.2; 95% CI, 1.4–3.4) and adeno-/adenosquamous carcinoma (odds ratio, 3.4; 95% CI, 1.5–7.7) were still significant.

Several additional investigations reported significant associations of HSV-2 seroantibodies with cervical cancer (Hildesheim *et al*., 1991; Jha *et al*., 1993; Becker *et al*., 1994; Koffa *et al*., 1995; Daling *et al*., 1996; Thomas *et al*., 2001b, c) or cervical neoplasia (Olsen *et al*., 1998b), but most of these studies either did not control statistically for HPV infection despite having tested for HPV (Becker *et al*., 1994; Thomas *et al*., 2001b,c) or observed an association only among HPV-negative women (Jha *et al*., 1993; Koffa *et al*., 1995; Daling *et al*., 1996).

Studies that tested for HSV DNA in the cervix also gave conflicting results. A high prevalence of HSV DNA was detected in neoplastic cervical specimens using sensitive PCR methods in some studies (Koffa *et al*., 1995; Han *et al*., 1997) but these data were not confirmed by others (Vecchione *et al*., 1994; Tran-Thanh *et al*., 2003). After laboratory data

Table 61. Selected epidemiological studies (that assessed exposure to HPV) of seroantibodies to herpes simplex virus-2 (HSV-2) and risk for cervical neoplasia

Reference, study location	Study design	No. and type of cases	No. and type of controls	HSV-2 sero-prevalence	%	Association or odds ratio (95% CI)	Best epidemio-logical control for HPV and method of HPV detection	Comments
Hildesheim *et al.* (1991), Costa Rica, Panama, Colombia, Mexico	Case–control	766 ICC	1532 normal cytology	*HPV 16/18-positive* Cases Controls	 57 39	*HPV 16/18* 1.6 (1.3–1.9) *HPV 16/18 and HSV-2* 8.8 (5.9–13.0)	Filter in-situ hybridization for HPV 16/18/6/11	An insensitive and non-specific assay for HPV, complicating the interpretation of these data.; results suggest interaction between HPV 16/18 and HSV-2.
Peng *et al.* (1991), China	Case–control	101 ICC	146 normal cytology	ICC Controls	42 29	*ICC* 1.3 (0.7–2.3)	Adjusted for HPV 16 and 33 DNA by PCR	HPV DNA assay detected few high-risk types.
Koutsky *et al.* (1992), USA	Prospective cohort of cytologically normal women enrolled through an STD clinic	28 CIN2/3	213 who did not develop CIN2/3	Cases Controls	45 43	*CIN2/3* 1.0 (0.5–2.3)	Adjustment for HPV by Virapap or dot-filter hybridization	HPV assays were insensitive but few HPV-negative women developed CIN2/3; no details of HSV assay given
Jha *et al.* (1993), United Kingdom	Case–control	219 ICC	387 normal cytology	Invasive cancer Controls	11 5	*HPV-adjusted* 2.2 (1.1–4.5) *HPV-positive* 1.8 (0.4–8.6) *HPV-negative* 3.0 (1.5–6.0)	Adjusted by antibodies to HPV 16/18 E7	Insensitivity of HPV 16/18 E7 assay increases change of residual confounding by HPV; HSV-2 ELISA may not have been type-specific; blood collected years after diagnosis
Becker *et al.* (1994), USA	Case–control	128 Hispanic and 73 non-Hispanic CIN2/3	216 Hispanic and 121 non-Hispanic with normal cytology	*All subjects* CIN2/3 Controls	 36 29	*All subjects* 1.3 (0.8–1.9) *Hispanic* 3.1 (1.6–6.2) *Non-Hispanic* 0.7 (0.4–1.2)	Tested for HPV by PCR	No adjustment for HPV

Table 61 (contd)

Reference, study location	Study design	No. and type of cases	No. and type of controls	HSV-2 sero-prevalence	%	Association or odds ratio (95% CI)	Best epidemio-logical control for HPV and method of HPV detection	Comments
Dillner *et al.* (1994), Finland	Case–control	94 ICC	188 normal cytology	IgA in cases IgG in cases Data for controls not reported	28 92	*IgA* 1.0 (0.6–1.8) *IgG* 1.1 (0.4–2.6)	Tested for multiple HPV 16 peptide antibodies	No adjustment for HPV
de Sanjosé *et al.* (1994), Spain and Columbia	Case–control	*Spain* 249 CIN3 223 ICC *Columbia* 276 CIN 3 150 ICC	*Spain* 242 matched to CIN3 238 matched to cancer *Columbia* 270 matched to CIN 3 149 matched to cancers	*Spain* CIN3 Controls ICC Controls *Columbia* CIN3 Controls ICC Controls	 14 11 26 12 61 50 73 60	*Spain* CIN3 1.2 (0.7–2.0) ICC 1.1 (0.6–1.7) *Columbia* CIN3 1.1 (0.7–1.7) ICC 1.1 (0.6–2.1)	Adjusted for HPV DNA by PCR	HSV assay may have had some cross-reactivity between HSV-1 and HSV-2 (Lacey, 1992).
Bosch *et al.* (1996), Spain	Case–control of patients' male partners	306 husbands of women with CIN3 or SCC	327 husbands of women with normal cytology	Data not shown		No association	Adjusted for penile HPV DNA by PCR; HPV in husbands strongly associated with case status	Husbands' HSV-2 seroprevalence data are indirect evidence for or against HSV-2 as a co-factor.
Daling *et al.* (1996), USA	Case–control	264 SCC	541 normal cytology	Squamous carcinoma Controls	37 26	*All subjects* 1.2 (0.8–1.8) *HPV sero- and DNA-negative* 3.6 (1.6–8.0)	Stratified and/or adjusted for HPV 16 serology (all subjects), HPV DNA (cases) by PCR	Only 77% of cases were HPV DNA-positive and given HPV serology insensitivity, residual HPV confounding was a concern.

Table 61 (contd)

Reference, study location	Study design	No. and type of cases	No. and type of controls	HSV-2 sero-prevalence	%	Association or odds ratio (95% CI)	Best epidemio-logical control for HPV and method of HPV detection	Comments
Lehtinen *et al.* (1996), Finland	Nested case–control	27 ICC 72 in-situ cancer	143 cancer-free women	Cancer Controls	15 26	0.6 (0.2–1.4)	Statistical adjustment for HPV 16 VLP antibodies	Analysis controlled for detection of HPV 16 antibodies
Muñoz *et al.* (1996a), Colombia	Case–control of patients' male partners	210 husbands of women with CIN3 92 husbands of women with SCC	262 husbands of women with normal cytology	Data not shown		No association	Tested for penile HPV DNA by PCR	Husbands' HSV-2 seroprevalence data are indirect evidence for or against HSV-2 as a co-factor.
Ferrera *et al.* (1997a), Honduras	Case–control	25 CIN3 48 ICC	50 normal cytology matched to CIN3, 93 matched to ICC	All subjects tested	92	No significant association	Statistical adjustment for HPV DNA by PCR	Few details of the HSV-2 assay were provided, and its specificity could not be confirmed.
Olsen *et al.* (1998), Norway	Case–control	94 CIN2/3	228 normal cytology	CIN2/3 Controls	41 25	2.6 (1.0–6.3)	Adjusted for HPV DNA by PCR and antibodies to HPV 16 VLPs	Stronger association among HPV 16 DNA-positive subgroup of cases and controls. HSV-1 antibodies also associated with CIN2/3
Yoshikawa *et al.* (1999), Japan	Cross-sectional	94 CIN1 40 CIN2 33 CIN3	130 normal cytology	Any CIN Controls	79 72	2.0 (0.7–5.9)	Adjusted for HPV DNA by PCR	Details of HSV assay not reported
Thomas *et al.* (2001c), Thailand	Case–control	190 SCC	291 normal cytology	Squamous carcinoma Controls	58 50	1.4 (1.0–2.0)	Tested for HPV DNA by PCR, but no control for HPV in analyses	

Table 61 (contd)

Reference, study location	Study design	No. and type of cases	No. and type of controls	HSV-2 sero-prevalence	%	Association or odds ratio (95% CI)	Best epidemio-logical control for HPV and method of HPV detection	Comments
Lehtinen *et al.* (2002), Finland, Norway and Sweden	Nested case–control set in three cohorts	178 cancers identified through cancer registries	527 cancer-free women	Controls Cases	12 15	1.0 (0.6–1.7)	Statistical adjust-ment for HPV 16/18/31 by VLP serology	A meta-analysis of prior longitudinal studies also reported in this paper found no HSV-2 effect; total cohort size, 550 000
Smith *et al.* (2002a), Brazil, Columbia, Morocco, Peru, Philippines and Thailand	Pooled analysis of multiple case–control studies	1158 SCC 105 adeno-carcinomas	1117 normal cytology	Controls Squamous carcinoma Adenocarcinoma	26 44 44	*Squamous carcinoma* 2.2 (1.4–3.4) *Adenocarcinoma* 3.4 (1.5–7.7)	Analysis limited to HPV DNA-positive by PCR	Based on comparison with the 164 HPV DNA-positive normal controls
Castle *et al.* (2003b), Jamaica	Cross-sectional	92 CIN3/ICC 117 CIN2	201 CIN1	CIN1 CIN2 CIN3/cancer	61 62 74	*CIN3/cancer* 1.2 (0.6–2.3) *CIN2* 0.8 (0.5–1.5)	Analysis limited to HPV DNA-positive by PCR	
Yokoyama *et al.* (2003), Japan	Cohort	41 CIN1 and 43 CIN2 that persisted or progressed	73 CIN1 and 28 CIN2 that regressed	Regressed Persisted/progressed	79 77	Regressed 1.1 (0.6–1.9)	Adjusted for HPV DNA by PCR	Details of HSV assay not reported

CI, confidence interval; CIN, cervical intraepithelial neoplasia; ELISA, enzyme-linked immunosorbent assay; ICC, invasive cervical cancer; Ig, immunoglobulin; SCC, squamous-cell carcinoma; STD, sexually transmitted disease; VLP, virus-like particles

had shown that selected HSV-2 oncogenes (e.g. *Xho-2*) may be integrated into and persist in cervical cancer cells, Tran-Thanh *et al*. (2003) tested 200 cervical cancer specimens and 244 normal specimens and failed to detect these or other HSV-2 sequences in any cervical specimens.

Molecular epidemiological data have provided only inconsistent support for the hypothesis that HSV-2 is a co-factor in cervical tumorigenesis. Null results were often observed despite the potentially positive bias related to residual confounding by HPV, especially since the chronic and recurrent nature of genital HSV-2 infection would suggest that studies of HSV-2 might be less affected than studies of transient (e.g. bacterial) infections by an inability to address temporality.

(*b*) *Other herpes viruses*

All three β-herpesviruses, cytomegalovirus (CMV), human herpes virus (HHV)-6 and HHV-7 have been detected in cervical specimens (Han *et al*., 1997; Chan *et al*., 2001). While several studies that used sensitive assays to test for viral DNA have suggested that these viruses may be by-standers rather than co-factors in cervical tumorigenesis (Thompson *et al*., 1994; Boyle & Smith, 1999; Chan *et al*., 2001; Tran-Thanh *et al*., 2002), a few small studies reported associations between CMV and cervical neoplasia (Koffa *et al*., 1995), between HHV-6 and cervical cancer (Chen *et al*., 1994; Yadav *et al*., 1996) and between HHV-7 and high-grade cervical neoplasia (Lanham *et al*., 2001). Serological studies have also given conflicting results. Yokoyama *et al*. (2003) found that higher CMV IgG seroantibody titres were significantly associated with a greater risk of persistent CIN and Koutsky *et al*. (1992) reported a higher incidence of CIN2/3 in young patients with sexually transmitted diseases who were CMV-seropositive. Other studies, however, observed no association of CMV seroantibodies with cervical neoplasia (Jha *et al*., 1993; Dillner *et al*., 1994; de Sanjosé *et al*., 1994; Ferrera *et al*., 1997a; Yoshikawa *et al*., 1999).

Epstein-Barr virus (EBV), a γ-herpesvirus, can infect epithelial cells and may play a role in nasopharyngeal carcinoma. However, the role of EBV in cervical tumorigenesis is controversial and consideration must be given to the fact that positive PCR results can occur due to the ability of EBV to infect infiltrating immune cells (Shoji *et al*., 1997; Boyle & Smith, 1999). Several studies using standard PCR (Koffa *et al*., 1995; Voog *et al*., 1997; Ammatuna *et al*., 2000; Lanham *et al*., 2001) and studies that used in-situ PCR and in-situ hybridization to identify the specific infected cells (Payne *et al*., 1995; O'Leary *et al*., 1997; Shoji *et al*., 1997; Elgui de Oliveira *et al*., 1999) found no association of EBV with the presence of cervical neoplasia, whereas positive findings were reported by others using similar methods (Landers *et al*., 1993; Se Thoe *et al*., 1993; Sasagawa *et al*., 2000). In two seroepidemiological studies, serum EBV IgG antibodies were not associated with cervical cancer (Jha *et al*., 1993; Dillner *et al*., 1994).

(*c*) Chlamydia trachomatis

C. trachomatis is the most common sexually transmitted bacterial infection; it is an obligate intracellular bacterium that has prevalence rates of 3–10% among young sexually

active women in the general community, which can rise to 24% in high-risk populations (Burstein *et al*., 1998; Stamm, 1999; Burstein *et al*., 2001; Turner *et al*., 2002). Because 85–90% of *C. trachomatis* infections are asymptomatic, many remain undiagnosed (Turner *et al*., 2002) and untreated, and can persist for several months or even years (Stamm, 1999; Peipert, 2003; Stephens, 2003). *C. trachomatis* infection may also recur, or even possibly be reactivated, similarly to viral infections (Stephens, 2003; Hogan *et al*., 2004). Infection with *C. trachomatis* is associated with squamous metaplasia and hypertrophic ectopy and, when infection is chronic, may lead to sequelae, including pelvic inflammatory disease (Paavonen *et al*., 1988; Stamm, 1999). Therefore, although *C. trachomatis* is a treatable bacterial infection, it is an important cause of chronic intracellular cervical infection and, as a result, long-term co-infection with high-risk HPV may not be uncommon.

C. trachomatis has also been studied extensively as a potential co-factor in cervical tumorigenesis. In comparison with HSV-2, many more epidemiological studies have reported a positive association between this bacterium and high-grade cervical neoplasia and/or invasive cervical cancer (Table 62).

Two large epidemiological investigations observed highly significant associations between *C. trachomatis* seroantibodies and cervical squamous-cell carcinoma (Koskela *et al.*, 2000; Smith, J.S. *et al.*, 2004). The Nordic nested case–control study found that 30% of 149 squamous-cell carcinoma cases compared with 13% of 442 controls were seropositive for *C. trachomatis* at baseline, a difference that persisted after adjustment for detection of antibodies to HPV 16, 18 and/or 31 VLPs (odds ratio, 2.2; 95% CI, 1.3–3.5) (Koskela *et al*., 2000). In the IARC study, 53% of 1139 squamous-cell carcinoma cases but only 31% of 1100 controls were seropositive for *C. trachomatis* and the strength of this association increased with increasing *C. trachomatis* antibody titre (p for trend < 0.001) (Smith, J.S. *et al*., 2004). No association with adeno-/adenosquamous carcinoma was observed in either study. The Nordic nested case–control study also reported a possible association of *C. trachomatis* serovar with risk for cervical cancer (Anttila *et al*., 2001); this association was not found in the IARC study (Smith, J.S. *et al*., 2004).

Similar associations between antibodies to *C. trachomatis* and cervical neoplasia were observed in several other seroepidemiological studies that controlled for HPV (Koutsky *et al*., 1992; de Sanjosé *et al*., 1994; Smith *et al*., 2002b; Matsumoto *et al*., 2003). Two cross-sectional studies observed a significant association between *C. trachomatis* antibodies in men and CIN3 or cancer in their wives (Bosch *et al*,. 1996; Muñoz *et al*., 1996a). Only a minority of studies did not detect a significant association between *C. trachomatis* antibodies and cervical neoplasia (Lehtinen *et al*., 1996; Ferrera *et al*., 1997a).

Furthermore, detection of *C. trachomatis* DNA in the cervix was also associated with neoplasia in several studies. A nested case–control investigation based on the Swedish contingent of the Nordic cohort found that *C. trachomatis* DNA in prediagnostic (still normal) Pap smears was strongly related to the risk for subsequent cervical cancer (odds ratio, 17; 95% CI, 2.6–∞) (Wallin *et al*., 2002). A cross-sectional study showed an association between HSIL (odds ratio, 5.8; 95% CI, 1.5–22) but not squamous cancer (odds ratio, 2.1; 95% CI, 0.36–12) and cervical *C. trachomatis* DNA in HPV DNA-positive subjects

Table 62. Selected epidemiological studies (that assessed exposure to HPV) on the association between Chlamydia trachomatis (CT) infection, cervical neoplasia and cervical cancer

Reference, study location	Study design	No. and type of cases	No. and type of controls	Prevalence of *Chlamydia*	%	Association(s) and/or odds ratio (95% CI)	Best epidemiological control for HPV and method of HPV detection	Comments
Koutsky *et al.* (1992), USA	Prospective cohort of cytologically normal women enrolled through an STD clinic	28 incident CIN2/3	213 who did not develop CIN2/3	*CT culture* Cases Controls *CT seroprevalence* Cases Controls	 21 13 75 55	*CT culture* 1.1 (0.5–2.8) *CT seroprevalence* 2.4 (1.0–5.7)	Adjustment for HPV by Virapap or dot-filter hybridization	HPV assays used were insensitive, but few HPV-negative women developed CIN2/3; no details of the CT seroassay provided
Jha *et al.* (1993), United Kingdom	Case–control	219 ICC	387 normal cytology	ICC Controls	17 7	*HPV-adjusted* 2.2 (1.2–3.9) *HPV-positive* 1.5 (0.4–5.6) *HPV-negative* 3.1 (1.7–5.5)	Adjusted or stratified by antibodies to HPV 16/18 E7	Insensitivity of HPV 16/18 E7 assay increases chance that CT could be surrogate for HPV; blood collected years after diagnosis
Becker *et al.* (1994), USA	Case–control	128 Hispanic and 73 non-Hispanic CIN2/3	216 Hispanic, and 121 non-Hispanic women with normal cytology	*CT seroprevalence* CIN2/3 Controls	 85 82	 1.1 (0.7–1.9)	Tested for HPV DNA by PCR, but no control for HPV in analyses	No adjustment for HPV
de Sanjosé *et al.* (1994), Spain and Columbia	Case–control	*Spain* 249 CIN3 223 ICC *Columbia* 276 CIN3 150 ICC	*Spain* 242 normal cytology matched to CIN3 cases 238 normal cytology matched to cancer cases *Columbia* 270 normal cytology matched to CIN3 cases 149 normal cytology matched to cancer cases	*CT seroprevalence* *Spain* CIN3 Controls ICC Controls *Columbia* CIN3 Controls ICC Controls	 29 11 28 16 48 25 53 41	*Spain* CIN3 2.2 (1.1–4.6) ICC 1.7 (0.9–3.1) *Columbia* CIN3 1.8 (1.1–2.9) ICC 0.9 (0.5–1.8)	Adjusted for HPV DNA status determined using consensus PCR	Increasing CT titre had stronger association with case status, but assay specificity was not optimal (Smith, J.S. *et al.*, 2004).
Bosch *et al.* (1996), Spain	Case–control of patients' male partners	306 husbands of women with CIN3 or SCC	327 husbands of normal cytology women	*CT seroprevalence* Case husbands Control husbands	 21 8	 2.6 (1.4–4.6)	Adjusted for penile HPV DNA by PCR	Husbands' CT seroprevalence data are indirect evidence of CT as a co-factor; HPV in husbands was strongly associated with cancer in wives.

Table 62 (contd)

Reference, study location	Study design	No. and type of cases	No. and type of controls	Prevalence of *Chlamydia*	%	Association(s) and/or odds ratio (95% CI)	Best epidemiological control for HPV and method of HPV detection	Comments
Lehtinen *et al.* (1996), Finland	Nested case–control	72 in-situ cancer or ICC	143 matched controls from same cohort	*CT seroprevalence* Cancer Controls	 10 4	3.0 (0.7–13.4)	Statistical adjustment for HPV 16 VLP antibodies	
Muñoz *et al.* (1996a), Colombia	Case–control of patients' male partners	210 husbands of women with CIN3 92 husbands of women with SCC	262 husbands of normal cytology women	*CT seroprevalence* Case husbands Control husbands	 29 15	2.4 (1.4–4.1)	Tested for penile HPV DNA by PCR, but this was not associated in wives.	Husbands' CT seroprevalence data are indirect evidence of CT as a co-factor, but null association of HPV DNA with cancer may imply residual HPV confounding.
Muñoz *et al.* (1996b), Spain, Colombia	Case–control	85 HPV DNA-negative and normal cytology	725 HPV DNA-negative and normal cytology	*CT seroprevalence* HPV-negative HPV-positive	 20 40	2.3 (1.2–4.2)	HPV DNA by PCR	Some data incorporated in Smith, J.S. *et al.* (2004)
Ferrera *et al.* (1997a), Honduras	Case–control	25 CIN3 50 ICC	50 normal cytology matched to CIN3, 95 matched to ICC	*CT seroprevalence* ICC Controls CIN 3 Controls	 70 62 80 68	*ICC* 0.95 (0.36–2.5) *CIN3* 2.0 (0.5–7.9)	Statistical adjustment for HPV DNA by PCR	
Lehmann *et al.* (1999), Germany	Cross-sectional	29 HPV DNA-positive	115 HPV DNA-negative	*CT DNA* Any HPV HPV-negative	 10 2	$p < 0.05$	HPV DNA by PCR	No control for shared sexual risk factors between CT and HPV
Koskela *et al.* (2000), Finland, Norway and Sweden	Nested case–control	149 SCC 32 ADC	442 women from same cohorts who remained cancer-free, matched to SCC and 94 to ADC	*CT seroprevalence* SCC Controls ADC Controls *CT DNA prevalence* SCC	 30 13 9 7 10	*SCC* 2.2 (1.3–3.5) *ADC* 0.4 (0.1–1.7)	Adjustment for HPV 1618/33 by VLP serology	Some data reported earlier (Dillner *et al.*, 1994, 1997); current data later re-analysed to assess effect of CT serovar (Anttila *et al.*, 2001) and possible interactions between HPV 16 and CT (Hakama *et al.*, 2000; Luostarinen *et al.*, 2004)

Table 62 (contd)

Reference, study location	Study design	No. and type of cases	No. and type of controls	Prevalence of *Chlamydia*	%	Association(s) and/or odds ratio (95% CI)	Best epidemiological control for HPV and method of HPV detection	Comments
Giuliano *et al.* (2001, 2002b), USA–Mexico border	Cross-sectional	259 high-risk HPV 65 low-risk HPV	2153 HPV-negative	*CT DNA* HPV-negative Any HPV High-risk HPV Low-risk HPV	 8 16 18 9	*Any HPV* 1.8 (1.2–2.7) *High-risk HPV* 2.1 (1.4–3.2) *Low-risk HPV* 1.2 (0.5–3.2)	HPV DNA by PCR	Adjusted for sexual behaviour, age, other risk factors
Smith *et al.* (2002b), Africa, Asia, South America, Spain	Case–control	455 SCC 44 ADC	539 normal cytology	*CT seroprevalence* Controls SCC ADC	 22 48 30	*SCC* 2.1 (1.1–4.0) *ADC* Not significant	Cancer analysis limited to HPV DNA-positive by PCR	Some data incorporated in Smith, J.S. *et al.* (2004)
Tamim *et al.* (2002), Lebanon	Cross-sectional	61 HPV DNA-positive 49 HPV DNA-positive	478 HPV DNA-negative 80 HPV DNA-negative	HPV-negative HPV-positive *CT DNA* HPV-negative HPV-positive	22 26 13 59	 1.4 (0.7–2.7) 10.2 (4.2–24.3)	HPV DNA by PCR	No control for shared sexual risk factors between CT and HPV-positive. CT associated with 'abnormal cytology' in HPV-negative strata. Updates Finan *et al.* (2002).
Wallin *et al.* (2002), Sweden	Nested case–control	118 ICC	118 cancer-free women from same cohorts	*CT DNA prevalence* Controls Invasive cancers	 0 8	 17.1 (2.6–∞)	Adjusted analysis for detection of HPV by PCR	CT DNA and HPV DNA detected in baseline specimens, obtained years before cancer
Castle *et al.* (2003b), Jamaica	Cross-sectional	117 CIN2 92 CIN3	201 CIN1	*CT DNA* CIN1 CIN2 CIN3 *CT seroprevalence* CIN1 CIN2 CIN3	 9 10 9 20 17 22	By laboratory method *CT DNA* *p* trend = 0.96 *CT serology* *p* trend = 0.61	Analysis limited to HPV DNA-positive by PCR	Assessed CT gradient by grade of neoplasia; association of CT with HPV DNA not assessed, no comparison of CIN with normal controls
Matsumoto *et al.* (2003), Japan	Cross-sectional	80 CIN1 34 CIN2 27 CIN3	109 normal cytology	*CT seroprevalence* Any CIN Control	 23 11	**Any CIN adjusted for:** *HPV DNA and age* 1.7 (0.7–4.3) *HPV serology and age* 2.7 (1.3–6.0)	Adjusted for HPV DNA by PCR, and/or HPV 16/52/58 by VLP serology	Disparity in results raises concern that false-negative HPV serology resulted in residual HPV confounding; some data reported in Yoshikawa *et al.* (1999)

Table 62 (contd)

Reference, study location	Study design	No. and type of cases	No. and type of controls	Prevalence of *Chlamydia*	%	Association(s) and/or odds ratio (95% CI)	Best epidemiological control for HPV and method of HPV detection	Comments
Molano *et al.* (2003b), Colombia	Cross-sectional	216 high-risk HPV-positive 52 low-risk HPV-positive 9 other HPV	1536 HPV-negative	*CT DNA* HPV-negative > 1 HPV Any HPV High-risk HPV	 5 10 7 7	*> 1 HPV* 2.5 (1.1–5.9) *Any HPV* 1.3 (0.8–2.4) *High-risk HPV* 1.3 (0.7–2.4)	HPV DNA by PCR	Adjusted for no. of regular and casual sex partners, age at first intercourse, condom use; no association of CT with abnormal Pap test, but analysis combined ASCUS and SIL.
Yokoyama *et al.* (2003), Japan	Cohort	41 CIN1 and 43 CIN2	73 CIN1 and 28 CIN2 that regressed	*CT IgA Seroprevalence* Regressed Persisted/progressed	24 29	0.8 (0.5–1.3)	Adjusted for HPV DNA by PCR	Tested for CT IgA (rather than IgG — as in most studies); no report of assay sensitivity or specificity
Giuliano *et al.* (2004), USA–Mexico border	Cross-sectional	30 HSIL	1876 normal cytology	*CT DNA* Control HSIL	 7 13	1.1 (0.3–4.1)	Analysis limited to HPV DNA-positive by PCR	Subjects same as those in study of Giuliano *et al.* (2001)
da Silva *et al.* (2004), Brazil	Case–control	26 HPV DNA-positive	26 HPV DNA-negative	*CT DNA* HPV-negative HPV-positive	 8 35	6.4 (1.1–55.4)	HPV DNA by PCR	No control for shared sexual risk factors
Smith, J.S. *et al.* (2004), Brazil, Colombia, Morocco, Peru, Philippines, Spain, Thailand	Pooled analysis of multiple case–control studies	1139 SCC 99 ADC	1100 normal cytology	*CT seroprevalence* Controls SCC ADC	 31 53 39	*SCC* 1.8 (1.2–2.7) *ADC* 1.0 (0.5–2.0)	Analysis limited to HPV DNA-positive by PCR	Based on comparison with 164 HPV DNA-positive normal controls; some data from Smith *et al.* (2002b) and de Sanjosé *et al.* (1994) included; association of SCC increased with CT titre.
Golijow *et al.* (2005), Argentina	Cross-sectional	75 HSIL 35 SCC	79 normal cytology	*CT DNA* Control HSIL SCC	 11 47 20	*HSIL* 5.8 (1.5–22) *SCC* 2.1 (0.36–12)	Analysis limited to HPV DNA-positive by PCR	Based on comparison with 24 HPV DNA-positive normal controls

ADC, adenocarcinoma; ASCUS, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; HSIL, high-grade squamous intraepithelial lesion; ICC, invasive cervical cancer; Ig, immunoglobulin; PCR, polymerase chain reaction; SCC, squamous-cell carcinoma; SIL, squamous intraepithelial lesion

(Golijow *et al*. 2005). Another cross-sectional study that did not find an association between *C. trachomatis* DNA and HSIL (Giuliano *et al*., 2004) reported an association between the detection of HPV and *C. trachomatis* DNA after adjustment for sexual behaviour (Giuliano *et al*., 2001, 2002b). Several other studies (Muñoz *et al*., 1996b; Lehmann *et al*., 1999; Tamim *et al*., 2002; Molano *et al*., 2003b; da Silva *et al*. 2004), but not all (Smith, J.S. *et al*., 2002b, 2004; Golijow *et al*., 2005), also observed an association between *C. trachomatis* (DNA or antibodies) and detection of HPV.

Only limited data, however, address the more specific question of the stage at which *C. trachomatis* might have its effects in the multistage process of HPV-associated cervical tumorigenesis and whether it has an effect on (*a*) the risk for HPV infection, (*b*) the persistence of HPV, (*c*) the development of neoplastic cervical lesions and/or (*d*) the persistence and progression of cervical lesions after their development. Most of the data that are available do not support a role of *C. trachomatis* infection in the progression of cervical neoplasia. *C. trachomatis* was not associated with the persistence or progression of lesions in a study of 114 prevalent CIN1 and 71 CIN2 that used *C. trachomatis* IgA seroantibodies as markers of active infection (Yokoyama *et al*., 2003) or in follow-up studies conducted during the 1980s that detected *C. trachomatis* by culture (Syrjanen, K. *et al*., 1986, 1987; Yliskoski *et al*., 1992). Similarly, a cross-sectional investigation found no differences in the detection of *C. trachomatis* DNA or *C. trachomatis* IgG seroantibodies by grade of neoplasia in colposcopy patients with CIN1 ($n = 201$), CIN2 ($n = 117$) or CIN3 ($n = 92$) (Castle *et al*., 2003), and at least one study that had detected a greater prevalence of *C. trachomatis* DNA in SIL relative to normal specimens did not find differences in prevalence between LSIL, HSIL and cancer (Golijow *et al*., 2005).

Overall, the data reported to date provide initial evidence of a possible epidemiological association between *C. trachomatis* and cervical neoplasia. Although the possibility of residual confounding by HPV can not be excluded, the frequent null results reported for HSV-2 and other sexually transmitted infections make it more difficult to attribute the association of *C. trachomatis* and cervical neoplasia entirely to their shared sexual risk factors. The exact stage(s), however, of the multistage process of HPV-associated tumorigenesis that might be affected by *C. trachomatis* has not been examined carefully and remains uncertain.

(*d*) *Other non-viral infections*

Several other non-viral infectious agents have been postulated as co-factors for cervical cancer. An association of *Trichomonas vaginalis* with cervical neoplasia was observed in several studies (Zhang & Begg, 1994), including a few large prospective cohort investigations (Gram *et al*., 1992; Zhang *et al*., 1995; Viikki *et al*. 2000). However, these studies did not control appropriately for HPV and were noted to have other limitations (Boyle & Smith, 1999; Watts *et al*., 2005). In contrast, two recent cross-sectional investigations (Becker *et al*., 1994; Schiff *et al*., 2000) and one prospective cohort study (Watts *et al*., 2005) found no relation between *T. vaginalis* and CIN2/3 or incident SIL, respectively, and neither was bacterial vaginosis found to be associated with cervical neoplasia in several

recent investigations (Peters *et al*., 1995; Frega *et al*., 1997; Castle *et al*., 2001; Boyle *et al*., 2003; Watts *et al*., 2005). Two studies that did report a significant association between bacterial vaginosis and cervical neoplasia did not control for possible confounding factors (Platz-Chrisensen *et al*., 1994; Schiff *et al*., 2000). Studies of the relationship between bacterial vaginosis and HPV infection gave conflicting results (Peters *et al*., 1995; Sikstrom *et al*., 1997; Castle *et al*., 2001; Jamieson *et al*., 2002; Mao *et al*., 2003; Watts *et al*., 2005).

A few epidemiological investigations reviewed by Boyle and Smith (1999) have assessed the possible association of *Neisseria ghonnorrhoeae* with cervical neoplasia and most reported no association (Takac, 1998; Boyle & Smith, 1999). Among the studies that reported a positive association, a large case–control study found some increase in risk for CIN3 or cancer with seroantibodies to *N. ghonnorrhoeae* in the women (de Sanjosé *et al*., 1994) but no association with seroantibodies in their husbands (Bosch *et al*., 1996; Muñoz *et al*., 1996a). A prospective study of young patients at clinics for sexually transmitted disease reported an increased risk for incident CIN2/3 among culture-positive women, but no information on other possibly correlated risk factors was available (Koutsky *et al*., 1992).

There is also little evidence to suggest that *Candida albicans* (Becker *et al*., 1994; Takac, 1998; Schiff *et al*., 2000) or *Treponema pallidum* (de Sanjosé *et al*., 1994; Bosch *et al*., 1996; Muñoz *et al*., 1996a; Ferrera *et al*., 1997a; Schiff *et al*., 2000; Thomas *et al*., 2001c) are co-factors for cervical tumorigenesis.

(*e*) *Inflammation caused by various infections*

Although only infection with *C. trachomatis* has been consistently associated with cervical neoplasia in epidemiological studies, it has been suggested that cervical inflammation in general, regardless of the specific microbial agent involved, may be a risk factor for progression of HPV infection (Castle & Giuliano, 2003). If this assumption is correct, it might help to explain some of the variable findings reviewed above. Consistent with this hypothesis, a cross-sectional study observed that the specific level of inflammation (graded by the number of invading neutrophils) was directly associated with a risk for CIN2/3 (Castle *et al*., 2001). In other studies, CIN2/3 was found to be more strongly associated with 'any' cervical co-infection than with co-infection assessed on an agent-specific basis (Schiff *et al*., 2000), and data from two independent studies suggested that the risk for cervical cancer increased with increasing numbers of possible co-infections detected (Schmauz *et al*., 1989; Dillner *et al*., 1994). Moreover, a small cohort study observed that variations in the detection of HPV DNA over time were related to the presence of any one of a number of cervicovaginal infections rather than to one specific infectious agent (McNicol *et al*., 1994).

(*f*) *Possible protective effects of adeno-associated virus*

Adeno-associated virus (AAV) is the one infectious agent that may reduce the risk for cervical neoplasia. AAV can suppress papillomavirus replication and cellular transformation *in vitro* (Hermonat, 1992, 1994a). However, although a few epidemiological studies

found that detection of AAV DNA was associated with a decreased risk for the presence and/or grade of neoplasia (Walz *et al*., 1997; Coker *et al*., 2001), most studies did not (Strickler *et al*., 1999b; Odunsi *et al*., 2000; Lanham *et al*., 2001; Ahn *et al*., 2003; Grce *et al*., 2004). Serological studies have also given conflicting results. Although several earlier seroepidemiological studies found possible inverse associations of AAV antibodies with cervical neoplasia (Sprecher-Goldberger *et al*., 1971; Mayor *et al*., 1976; Georg-Fries *et al*., 1984; Tobiasch *et al*., 1994), two subsequent serological studies gave negative results. The first found no association of AAV antibodies with cervical neoplasia or invasive cervical cancer in two separate, independent populations of patients (Strickler *et al*., 1999). The second study found a non-significant inverse association with cervical cancer (odds ratio, 0.4; 95% CI, 0.1–1.6) after controlling for HPV, and no association with CIN3 (odds ratio, 1.4; 95% CI, 0.3–6.8) (Smith *et al*., 2001).

2.8 Special populations

2.8.1 *Skin cancer in patients with epidermodysplasia verruciformis (EV) and HPV infection*

EV is a very rare, inherited condition that was first described by Lewandowsky and Lutz (1922). During the following 60 years, approximately 250 cases were reported worldwide (Lutzner & Blanchet-Bardon, 1985). The condition is usually recognized before puberty and is characterized by widespread HPV infection and the later development of multiple cutaneous squamous-cell carcinomas, predominantly at sites that are exposed to the sun. Although basal-cell carcinomas have been described in EV patients, they appear to be rare, and there are no reports of increased risk for malignant melanoma in EV patients (Orth *et al.*, 1980; Orth, 1986, 1987; Majewski *et al*., 1997; Pfister, 2003). Two cases of eccrine carcinoma and a single case of a malignant proliferating trichilemmal tumour have been described in EV patients (Motegi *et al*., 2003). No published standardized mortality ratios (SMRs) are available for skin cancer in EV patients, but a squamous-cell carcinoma:basal-cell carcinoma ratio of 16:1 was reported in one study of 66 EV patients (Tanigaki *et al.*, 1986). About half of these patients had developed warts by the age of 10 years, whilst squamous-cell carcinoma developed between the ages of 30 and 50 years. The average time lag between onset of EV-type skin warts and squamous-cell carcinoma was 24.5 years (Tanigaki *et al*., 1986). Other virus-associated cancers, including liver cancer associated with chronic hepatitis B virus infection (van Voorst Vader *et al*., 1986; see also IARC, 1994), genital carcinoma, tonsillar carcinoma and Burkitt lymphoma, have rarely been described in EV patients (Lutzner & Blanchet-Bardon, 1985; Ishiji *et al*., 2000).

The limited epidemiological data on EV have pointed to a considerable time lag between primary HPV infection and the development of HPV-associated changes. HPV infection is probably acquired during the first days after birth, particularly in EV families; even in the general population, 45% of skin swab samples were positive for HPV DNA shortly after birth (Antonsson *et al*., 2003). There is one report of possible vertical

transmission in an EV patient (Favre *et al*., 1998b). The same HPV types as those found in the skin lesions of the mother were detectable in her amniotic fluid, placenta and genital scrapes. In contrast, EV-type skin warts never start to appear before 4–5 years of age and carcinomas develop much later. Majewski and Jablonska (1997) observed EV patients with skin autografts from the uninvolved internal aspect of the arm that covered areas of the forehead that had been excised for carcinomas. Within the grafted skin, benign lesions started to develop only several years after transplantation. No carcinoma developed for up to 20 years of graft life, whereas premalignant and malignant changes appeared around the grafts. This suggests that HPV-associated skin carcinogenesis is a very slow process.

The high level of consanguinity in EV families suggests an autosomal recessive mode of inheritance (Lutzner, 1978; Tanigaki *et al*., 1986) but, in one family, the inheritance appeared to be X-linked (Androphy *et al*., 1985). Genetic linkage analyses of consanguineous EV families identified susceptibility loci on chromosomes 17q25 and 2p21-p24 (Ramoz *et al*., 1999, 2000). A more detailed analysis of three Algerian and two Colombian EV1 (17q25)-linked families revealed homozygous nonsense mutations in one of two adjacent novel genes, *EVER1* and *EVER2*, in affected family members (Ramoz *et al*., 2002). No such mutations were observed in 90 unrelated individuals. The predicted full-length EVER1 and EVER2 proteins share 28.4% of their amino acids. They have features of integral membrane proteins and are localized in the endoplasmic reticulum.

The immunogenetic background responsible for defects in immunosurveillance of EV-HPV infections and the inability to eliminate EV-HPV-infected cells remains poorly defined (Majewski *et al*., 1997). A comparison of the prevalence of interleukin (IL) 10 promoter polymorphisms in 22 Brazilian EV patients and 27 healthy individuals indicated that genotypes that determine a low IL10 production are significantly increased in EV patients (de Oliveira *et al*., 2003). This is not easy to interpret because IL10 is generally regarded as immunosuppressive, although it has been speculated that low levels of IL10 would allow higher production of tumour necrosis factor-α (TNFα), which could impede antigen-presenting Langerhans cells.

Mutations in exons 5–8 of the *TP53* gene were detected in one of three benign lesions, two of five actinic keratoses, three of nine Bowen carcinomas *in situ* and five of eight squamous-cell carcinomas of two EV patients (Padlewska *et al*., 2001). Five of nine mutations characterized by sequencing were C→T transitions at dicytidine sites that are considered to be ultraviolet (UV) signature mutations. The other four mutations could be caused by reactive oxygen species that result from exposure to the UVA component of sunlight or from oxidative metabolism.

The importance of sunlight (see also IARC, 1992) in the development of EV-associated squamous-cell carcinomas is suggested by the fact that, although skin warts are found on all body sites, the carcinomas occur almost exclusively on sites exposed to the sun (Tanigaki *et al*., 1986). Furthermore, squamous-cell carcinomas appear to develop more frequently in Caucasian EV patients who live in subtropical and tropical climates than in those who live in temperate climates, and are rare in black EV patients. Only two of 33 (6%) black South African EV patients developed squamous-cell carcinomas (van Voorst Vader *et al.*, 1987)

compared with 40–50% of Caucasian patients who lived in Europe (Orth *et al.*, 1979), 58% of Japanese patients who lived in Japan (Tanigaki *et al*., 1986) and 100% of patients who lived in South America (Rueda & Rodriguez, 1976).

HPV types in warts and skin cancers in EV patients

(*a*) *Skin warts*

EV patients develop a variety of skin warts: common warts (verruca vulgaris), consistently plane warts (verruca planar, that are usually somewhat flatter than plane warts in the general population) and frequently the so-called EV-specific lesions, namely, red plaque-like lesions and scaly, pityriasis versicolor-like lesions (Orth *et al*., 1979; Orth, 1987; Majewski *et al*., 1997).

Table 63 summarizes studies of HPV typing of EV-associated skin warts, all of which used restriction enzyme cleavage and hybridization methods without amplification. Reports are based on a limited number of specimens, often from single patients, and only one study included information on control material (Jacyk *et al*., 1993a). Some studies did not specify the type of skin wart examined and many did not specify the number of samples examined.

A large number of HPV types has been found in plane warts and EV-specific lesions. The phylogenetically related types HPV 5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 38, 47 and 50 all belong to the papillomavirus genus beta according to the new nomenclature (de Villiers *et al*., 2004a). They are widely referred to as EV-HPV because they were originally found only in EV patients. In addition to EV-HPV, HPV 3 and 10, which are found in plane warts in the general population, were frequently detected.

A very similar spectrum of HPV types has been identified in five EV patients by PCR with degenerate HPV primers (HPV 8, 19, 20, 24, 38, 5-related and 9-related from genus beta, and HPV 2 and 57 that are found in common warts in the general population) (Harris *et al*., 1997; Surentheran *et al*., 1998).

Cutaneous lesions induced by EV-HPV are highly polymorphic, and include verruca planar, red plaque-like lesions, pityriasis versicolor-like lesions and lesions similar to seborrheic keratoses. However, they share a specific cytopathic effect that is identical for various EV-HPVs, the intensity of which depends only on the viral load and the activity of the disease (Majewski *et al*., 1997). The cytopathic effect is characterized by large clear cells with clear nucleoplasma and cytoplasmic keratohyaline granules. A keratinocyte differentiation-dependent viral transcription pattern has been revealed by in-situ hybridization of benign HPV 5-induced lesions, which is characteristic of productive warts with strong E4-specific signals almost throughout the epithelium and L1/2-specific signals in the superficial layers of the stratum granulosum (Haller *et al*., 1995). HPV 3 and 10 are predominantly associated with plane warts, which may be large, pigmented and confluent. The cytopathic effect is again specific with a ‘bird’s eye-like’ appearance of cells due to vacuolization around pyknotic dark nuclei (Majewski *et al*., 1997).

Table 63. HPV types detected by southern blot in skin warts in epidermoplasia verruciformis (EV) patients with multiple lesions

Reference, study location	Types included	No. of cases	Clinical description	HPV type-specific positivity						Comments
				1–3, 10	5	8	17	20	Others[a]	
Orth *et al.* (1979)[b], Europe	1, 2, 3, 4, 5, 8, 9, 12	14	VP RP PV	+ +	 + +	 +			2, 9 9, 12 12	Frozen and paraffin-embedded tissue
Ostrow *et al.* (1982), USA	5	2	NA		+					Frozen tissue
Pfister *et al.* (1983a) [Turkish patient]	1, 2, 3, 4, 5, 6, 8, 10, 11, 20, 25, 29	1	NA		+	+		+[c]	19[c], 25[c], one type not specified	Frozen tissue
Kremsdorf *et al.* (1984), France	14a, 14b, 15, 17a, 17b, 19, 20, 21, 22, 23, 24	8	VV/VP PV				 +	+	14a, 14b, 15, 21 19, 21, 23, 24	Frozen tissue
Lutzner *et al.* (1984), France	2, 3, 5, 8, 9, 14, 15, 17, 20, 21, 22, 23, 24	11	NA	+	+	+		+	2, 14, 22, 9, 9-related	Frozen tissue
van Voorst Vader *et al.* (1986), Netherlands	5, 8, 17, 19, 20, 24	1	NA		+	+	+		19, 24	Frozen tissue
Kanda *et al.* (1989), Japan	1, 2, 3, 5, 8, 12, 14, 17, 20, 21, 38	12	VP, VV PV RP RP	+	 +		 +	 + +	14, 38 12, 14, 38 (multiple HPV types in some lesions) 14, 21	Frozen tissue

Table 63 (contd)

Reference, study location	Types included	No. of cases	Clinical description	HPV type-specific positivity						Comments
				1–3, 10	5	8	17	20	Others[a]	
Jacyk & de Villiers (1993), South Africa	20 not specified	20	VP PV	+	+ +		 +			Frozen tissue
Jacyk *et al.* (1993a), South Africa	20 not specified	5	Seborrheic keratoses PV VP	 +	+ + +		 +		No HPV found in 10 keratoses from non-EV patients	Frozen tissue; includes information on controls
Jacyk *et al.* (1993b), South Africa	20 not specified	1	PV VV						9 4, 9	Frozen tissue
Yutsudo *et al.* (1994), Japan	3, 17, 20, 38 and others not specified	1 1	PV VV, VP	 +			+	+	38	Fresh tissue
Adachi *et al.* (1996), Japan	3, 5, 8, 9, 12, 14, 17, 20, 25, 47; type-specific primers for HPV 5, 14, 21, 47	1	NA		+				14, 21, 47	Paraffin-embedded tissue

NA, not available; PV, pityriasis versicolor-like lesions; RP, red plaque-like lesions; VP, verruca planar; VV, verruca vulgaris

[a] Of those types tested

[b] EV-HPV were named HPV 4 in the original paper and further differentiated and later renamed as HPV 5, 8, 9 and 12 (Orth *et al.*, 1980; Kremsdorf *et al.*, 1984).

[c] Specified later (Gassenmaier *et al.*, 1984)

(*b*) *HPV types in squamous-cell carcinoma of EV patients*

Table 64 summarizes data on HPV types in EV-associated skin cancer, which are derived mostly from single case reports. All studies were performed using hybridization methods without amplification. In the limited number of invasive squamous-cell carcinomas analysed, HPV 5, 8, 14, 17, 20 and 47 have been identified. HPV-21 DNA was disclosed by PCR amplification and sequence analysis in a malignant proliferating trichilemmal tumour (Motegi *et al*., 2003). The dominance of HPV-5 and -8 in malignant tumours from Europe and the USA is in contrast to the presence of multiple EV-HPV types in benign lesions of the same patients (Pfister *et al*., 1983b; Van Voorst Vader *et al*., 1986; reviewed in Orth, 1987), which may point to an increased carcinogenic potential. However, due to the overall small number of patients, data on prevalence should still be regarded as preliminary. In contrast, HPV 3 or 10 were never detected in EV-associated squamous-cell carcinomas and no malignant conversion was observed in EV patients only infected by HPV 3 (Majewski *et al*., 1997).

HPV 5, 8, 17, 20 and 47 DNAs have been found in squamous-cell carcinomas as episomal oligomers and monomers, some in concatemeric form (approximately 100 copies/cell) (Pfister *et al*., 1983b; Yutsudo *et al*., 1985; Deau *et al.*, 1991; Yutsudo *et al*., 1994; Adachi *et al*., 1996). This is in contrast to the frequently observed integration of HPV DNA in cervical cancers. HPV 14 was identified only rarely in a skin carcinoma and appeared to be integrated into the cellular DNA (Orth, 1987). In another case, HPV 5 DNA was integrated in a metastasis whereas viral genomes persisted extrachromosomally in the corresponding primary tumour (Yabe *et al*., 1989, 1991, 1999). Transcripts of HPV 5, 17 and 20 have been demonstrated in squamous-cell carcinomas (Orth, 1987; Yutsudo & Hakura, 1987; Yutsudo *et al*., 1994).

Both wild-type HPV genomes and those with deletions have been found in primary and metastatic tumours (Ostrow *et al*., 1982; Yabe *et al*., 1989; Deau *et al*., 1991). Some cancers contain mostly or only viral DNA with deletions, which primarily affect the late genes but may extend into the non-coding genome region. In addition, sequence variants of the HPV 5 and 8 *E6* gene have been demonstrated in some EV-associated cancers (Deau *et al*., 1991). The significance of these findings and their role in transformation remains unclear.

2.8.2 *Studies of the incidence of HPV-associated neoplasia in transplant patients*

A consistent increase in the incidence of malignancies in organ transplant recipients has been attributed to the effect of chronic immunosuppression that is required to prevent rejection of the transplanted tissue. Immunocompromised individuals are at increased risk for HPV-associated anogenital and cutaneous cancers compared with age-matched healthy individuals. Organ transplant recipients have an almost 100-fold increased risk for squamous-cell skin carcinoma and a 10-fold increased risk for basal-cell carcinoma (Leigh *et al.*, 1999). In Australia, where exposure to UV light is high, the cumulative incidence of skin cancer increased progressively from 7% after 1 year of immunosuppression to 40%

Table 64. HPV types detected by southern blot in skin cancer in epidermoplasia verruciformis (EV) patients

Reference, study location	Types included	No. of cases	HPV type-specific positivity				Comments
			5	8	17	20	
Ostrow *et al*. (1982), USA	5	2	+				Frozen tissue; HPV 5 found in PV lesions, primary SCC and metastatic SCC in same patient; wild-type and sub-genomic HPV 5 found in primary and metastatic tumour
Pfister *et al*. (1983b) [Turkish patient]	1, 2, 3, 4, 5, 6, 8, 10, 11, 20, 25, 29	1	+				Frozen tissue; 100 copies/cell; oligomeric DNA, some persisting in concatemeric form
Lutzner *et al*. (1984), France	1, 2, 3, 5, 8, 9, 14, 15, 17, 20, 21, 22, 23, 24	5	+	+			Frozen tissue; HPV 14 found in one SCC; the seven skin cancers include three Bowen disease and four SCC.
Yutsudo *et al*., (1985), Japan	17	1			+		Fresh tissue; 100 copies/cell; episomal DNA as oligomers and monomers; HPV transcripts in SCC suggests infection.
Orth (1986, 1987), International	1, 2, 3, 4, 5, 8, 9, 12, 14a/b	14	+	+			Frozen tissue; HPV-14b (1 tumour) (approximately 100–300 copies/cell)
van Voorst Vader *et al.* (1986), Netherlands	1, 2, 3, 4, 5, 8, 9, 10, 12, 14, 15, 17, 19–24	1	+				Frozen tissue; HPV 17 and 24 found in peri-lesional tissue
Yabe *et al*. (1989, 1991, 1999), Japan	5	1	+				Tissue storage not specified; HPV 5 found in benign lesions; deleted forms of HPV 5 found in both primary and metastatic tumours from same patient
Yutsudo *et al*. (1994), Japan	3, 17, 20, 38 and others not specified	1				+	Fresh tissue; 100 copies/cell; episomal DNA as oligomers and monomers; HPV transcripts in SCC suggests infection.
Adachi *et al*. (1996), Japan	3, 5, 8, 9, 12, 14, 17, 20, 25, 47; type-specific primers for 5, 14, 21, 47	1					Paraffin-embedded tissue; HPV 47 episomal DNA as oligomers and monomers
Ishiji *et al*. (2000), Japan	3, 5, 16, 20, 57, 58, 60	1				+	Paraffin-embedded tissue; HPV 20 DNA also disclosed by in-situ hybridization in the nuclei of some cancer cells

PV, pityriasis versicolor-like lesion; SCC, squamous-cell carcinoma

after 9 years and 70% after 20 years (Bouwes Bavinck *et al.*, 1996). These data suggest the interplay of UV light and immunosuppression as risk factors in the development of skin cancer (IARC, 1992). The exact role of immunosuppression in conferring increased risk is not known. Current data suggest that it is most strongly associated with the early stages of dysplasia, and that progression to cancer *per se* is not associated with immunosuppression. Similarly, the biology of HPV infection among immunocompromised individuals is not yet known in detail. Also, questions remain about the biology of HPV infection among transplant patients compared with immunocompromised individuals who are HIV-positive (Palefsky & Holly, 2003).

The first reports of cutaneous and anogenital lesions as a result of HPV infection in immunosuppressed transplant recipients appeared in the 1980s. Lutzner *et al*. (1980) described two immunosuppressed renal allograft recipients who developed skin lesions. In both patients, structural antigens of HPV 5 were identified in these lesions by immunofluorescence. The histological and ultrastructural features observed were similar to those previously seen in patients with HPV 5-associated EV, the only condition in which this HPV type had been detected until that time. The data suggested a role of this potentially high-risk virus in skin cancers that are known to occur with increased frequency in immunosuppressed allograft recipients.

Lower genital cytopathology was evaluated in 105 immunosuppressed renal transplant recipients (Halpert *et al*., 1986). Evidence of HPV infection was found in 17.5% and lower genital neoplasia was observed in 9.5% of the patients. The rate of viral infection in the immunosuppressed patients was ninefold greater than that in a general population and 17-fold greater than that in a matched immunocompetent population. The rate of cervical neoplasia was 16-fold greater than that in the general population and nine-fold greater than that in a matched immunocompetent population. In one-third of patients with HPV lesions and one-half of patients with neoplastic lesions, multiple lower genital sites were also involved. Of the risk factors evaluated, only the number of sexual partners was associated with the development of HPV-related lower genital neoplasia.

Studies on the incidence of HPV-associated cancer in transplant patients have been reviewed previously (IARC, 1995). A number of more recent studies are reviewed below.

(*a*) *HPV infection, CIN and invasive cervical and anogenital carcinomas in transplanted patients*

Studies up to 1994 on the prevalence of anogenital and cervical lesions and/or HPV infection in immunosuppressed women following transplantation were reviewed previously (IARC, 1995) and are summarized in Table 65. At least seven studies of transplanted patients have been published subsequently.

To test the hypothesis that renal allograft recipients are at high risk for anal HPV infection and AIN, 133 renal allograft recipients and 145 control patients underwent anoscopy and biopsy (Ogunbiyi *et al.*, 1994). PCR was used to detect HPV 16 DNA in biopsy samples. A histological diagnosis of anal HPV infection or AIN was made in 32 allograft recipients: HPV infection was detected in five patients, 20 had AIN1, three had AIN2,

Table 65. Prevalence or risk of cervical HPV infection, cervical intraepithelial neoplasia (CIN) and invasive carcinoma of the cervix and anogenital carcinomas in transplanted patients

Reference, study location	Method of detection	No. and % with HPV or lesion				Relative risk (95% CI) or *p* value	Comments
		Transplanted patients		Controls			
		No.	%	No.	%		
HPV infection							
Schneider *et al.* (1983), USA	Cytology (koilocytotic atypia)	11/132	8.5	–		9[a] (3.4–20.2)	Frozen tissue
Halpert *et al.* (1986), USA	Cytology	18/81	22.8	2/81	2.5	17[b] (5.0–50.6)	Frozen tissue
MacLean *et al.* (1986), New Zealand	Cytology	5/24	21	–			Frozen tissue
Alloub *et al.* (1989), United Kingdom	DNA hybridization	22/49	45	26/69	38		Paraffin-embedded tissue
	HPV 6/11	9/49	18.4	22/69	32	$p = 0.36$	
	HPV 16/18	10/49	20.4	4/69	6	$p < 0.005$	
	Mixed 6/11 and 6/18	3/49	6.1	–			
Gentile *et al.* (1991), Italy	Cytology/histology	12/39	31	–			Frozen tissue
Gitsch *et al.* (1992), Germany	Histology (condyloma)	7/23	30	–			Frozen tissue
Fairley *et al.* (1994a), Australia	PCR (*L1* consensus primers)	15/69	22	1/22	4.5	$p = 0.05$	Frozen tissue; the *p* value was obtained using a Fisher's exact test.
Ogunbiyi *et al.* (1994), United Kingdom	Anoscopy/biopsy	5/133	3.8	0	0	–	Frozen tissue

Table 65 (contd)

Reference, study location	Method of detection	No. and % with HPV or lesion				Relative risk (95% CI) or *p* value	Comments
		Transplanted patients		Controls			
		No.	%	No.	%		
Ozsaran *et al.* (1999), Turkey	Histology	2/48	4.2	–	–	–	Frozen tissue
Roka *et al.* (2004), Austria	Hybrid Capture 2 with high-acid low-risk probes	14/60	23.3	–	–	–	Frozen tissue; kidney and liver; transplanted patients
Invasive carcinoma							
Schneider *et al.* (1983), USA	Cytology	6/132	4.5	–			Frozen tissue
MacLean *et al.* (1986), New Zealand	Cytology	0/24	0	–			Frozen tissue; mean time since transplant, 61 months
Fairley *et al.* (1994b), Australia and New Zealand	Cytology	12 cases	NA	–		3.3 (1.7–5.8)	Frozen tissue; mean follow-up, 5.8 years; SIR comparing patients on dialysis with transplanted patients
Birkeland *et al.* (1995), Denmark, Finland, Norway and Sweden	Histology	28 cases	NA	–		8.6 (5.7–13)	Frozen tissue; mean follow-up, 4.8 years; SIR; population-based cancer registry data
Ozsaran *et al.* (1999), Turkey	Histology	20/48	4.7	–	–	–	–
Brown *et al.* (2000), USA	PCR MY11/09 and specific primers for 6, 11, 16, 18	13/16	81	8/13	62	$p = 0.02$	Paraffin-embedded tissue

Table 65 (contd)

Reference, study location	Method of detection	No. and % with HPV or lesion				Relative risk (95% CI) or *p* value	Comments
		Transplanted patients		Controls			
		No.	%	No.	%		
CIN							
Porreco *et al.* (1975), USA	Cytology	3/131	2.3	–		[14 (2.8–40)]	Frozen tissue; mean follow-up, 3.6 years
Cordiner *et al.* (1980), United Kingdom	Cytology/histology	5/26	19	–			Frozen tissue; after a mean of 3.8 years of immunosuppression
Ingoldby *et al.* (1980), United Kingdom	Cytology	0/50	0	–			Paraffin-embedded tissue; 3 years of follow-up
Schneider *et al.* (1983), USA	Cytology	6/132	4.5	–			Frozen tissue; mean time to CIN since transplant, 38 months
Halpert *et al.* (1986), USA	Cytology	10/81	12	2/81	2.5	[5.6 (1.1–38)]	Frozen tissue; mean time since transplant, 47 months
Alloub *et al.* (1989), United Kingdom	Histology	24/49	49	7/69	10	[8.5 (3.0–25)]	Paraffin-embedded tissue
Gentile *et al.* (1991), Italy	Cytology/histology	1/39	2.6	–			Frozen tissue; mean time since transplant, 77 months
Gitsch *et al.* (1992), Austria	Histology	2/23	8.7	–			Frozen tissue
David *et al.* (1993), Germany	Cytology/histology	5/58	8.6	–			Frozen tissue

Table 65 (contd)

Reference, study location	Method of detection	No. and % with HPV or lesion				Relative risk (95% CI) or *p* value	Comments
		Transplanted patients		Controls			
		No.	%	No.	%		
Fairley *et al.* (1994a), Australia	Cytology	5/69	7.2	0/22	0		Frozen tissue
Ogunbiyi *et al.* (1994), United Kingdom	Anoscopy/biopsy	27/133	20.3	1/145	0.68	$p < 0.05$	Frozen tissue
Longuet *et al.* (1996), France	Southern blot hybrid	4/81	4.9	0/3000	0	–	Frozen tissue
Sasadeusz *et al.* (2001), Australia	Cytology	Before transplant 7/77	9.1	–		2.2 (1.1–4.2)	Includes four high-grade smears; the risks are for transplanted women compared with the general population.
		After transplant 11/87	12.6	–		7.0 (4.8–10.2)	
Malouf *et al.* (2004), Australia	Cytology	17/166	10.2	–			Includes CIN3; incidence of CIN3 lesions in lung transplanted patients versus women screened from the New South Wales cytology registry

[ ] Calculated by the Working Group
CI, confidence interval; CIN, cervical intraepithelial neoplasia; PCR, polymerase chain reaction; SIR, standardized incidence ratio
[a] Compared with the general population
[b] Compared with the matched immunocompetent population

three had AIN3 and one patient had anal cancer. One subject with AIN was detected in the control group. HPV 16 DNA was detected in 47% and 12.4% of anal biopsies in the transplant recipients and the controls, respectively. Renal allograft recipients were found to be at high risk for anal HPV infection and neoplasia ($p < 0.05$).

The presence of HPV and the ensuing risk for CIN were studied in 48 renal transplant patients who received immunosuppressive therapy (Özsaran *et al*., 1999). Cervical smears were analysed and colposcopy was conducted. Genital neoplasia was found in 20 of the patients. Koilocytosis developed in six of eight (75%) patients who received high-dose immunosuppressive therapy that was necessitated by rejection of the transplant. HPV was found in two of 48 patients, both of whom had koilocytosis in their cervical biopsies. The data show that renal transplant patients who receive immunosuppressive therapy are at increased risk for CIN.

The prevalence of anal HPV infection was studied in organ transplant patients before immunosuppressive therapy (Roka *et al*., 2004). Patients (40 men, 20 women) who underwent solid-organ transplantation (kidney, liver) for the first time were routinely screened for anal HPV infection. Anal swabs were obtained within 24 h after transplantation and analysed for the presence of mucosal-type HPV DNA by liquid DNA/RNA hybridization. Some type of HPV DNA was detected in 14 patients (23.3%), nine patients (15%) were positive for high-risk HPV, eight (13.4%) were positive for low-risk HPV and three (5%) were positive for both types. The prevalence of HPV infection tended to be higher in liver transplant than in kidney transplant recipients (29.4% versus 20.9%), but the difference was not significant. The prevalence of previous HPV infection (23.3%) before immunosuppressive therapy was started was higher than that found in previous studies or in a control group. In particular, the rate of infection with high-risk HPV types was 15%.

HPV types were analysed in lower genital tract neoplasms of renal transplant recipients and compared with virus types found in immunocompetent patients who had similar neoplasms and in normal immunocompetent controls (Brown *et al.*, 2000). Twenty specimens from lower genital tract neoplasms of 16 renal transplant patients, 13 specimens from 13 immunocompetent patients with similar histology and samples from 13 patients with normal lower genital-tract histology were analysed by PCR for the presence of HPV infection. Primers included the L1 region consensus primers and primers specific for the HPV E6 region for subtypes 6, 11, 16 and 18. HPV infection was detected in 21 of 46 specimens tested. Thirteen of the HPV-positive specimens were from transplant patients and eight were from immunocompetent patients (five with and three without disease). This difference was statistically significant between the transplant and immunocompetent group ($p = 0.02$). Although no difference in HPV 6 and/or 11 was detected between the two groups, the difference in HPV types 16 and/or 18 approached statistical significance ($p = 0.06$). High-risk HPV 16 and/or 18 were found at a higher rate in transplant patients than in their immunocompetent counterparts. The combination of reduced immune function and increased HPV 16 and/or 18 infection rate places these patients at increased risk for aggressive lower genital tract neoplastic progression.

The genome of a novel HPV type was cloned from an iatrogenically immunosuppressed woman with persistent low-grade vaginal AIN (Longuet *et al.*, 1996). HPV 74 was found to be phylogenetically related to the low-risk HPV types 6, 11, 44 and 55. HPV 74 or a variant of this type was found in specimens from three additional immunosuppressed women but not in about 3000 anogenital specimens from immunocompetent patients.

Immunocompromised patients, such as renal allograft recipients, have a higher rate of cytological abnormalities following infection with HPV. This is thought to be due to prolonged persistence of the virus because of impaired clearance by the immune system. A retrospective review was conducted of the cervical cytology of women who underwent bone-marrow transplantation and who had had cervical smears performed in the period 1990–98 (Sasadeusz *et al.*, 2001). The number of cytological abnormalities was significantly higher than that in the general population, both before (age-adjusted odds ratio, 2.2; $p = 0.02$) and after bone-marrow transplantation (odds ratio, 7.0; $p < 0.0001$). After transplantation, allogeneic recipients had more abnormalities than autologous patients (odds ratio, 2.6; $p = 0.02$) although only allogeneic recipients had a higher level of abnormalities after transplantation than before (allogeneic odds ratio, 6.8; $p = 0.004$). These observations suggested that pre-transplant disease and treatment factors increase the risk for cytological abnormalities and that transplant-related factors such as conditioning therapy and immunosuppression further increase this risk.

The incidence and outcomes of HPV infection and cervical abnormalities after lung transplantation were investigated in a retrospective cross-sectional study of 166 female recipients who underwent transplantation between February 1989 and June 2001 (Malouf *et al.*, 2004). The incidences of low-grade epithelial abnormality of the cervix, CIN1 and the earliest pre-cancerous changes of the cervical epithelial cells (CIN3) in the post-transplant cohort were 42.2 and 30, respectively, per 1000 women screened. In a large reference population of 20–69-year-old women, these figures were 8.3 and 6.2, respectively, per 1000 women screened. It was concluded that the incidence of cervical abnormalities in lung transplant recipients is about five times higher than that in the general population.

(*b*) *HPV DNA in transplant-associated skin lesions*

(i) *Skin warts*

Individual case reports documented the presence of multiple HPV types, including EV-associated types (Soler *et al.*, 1992; Purdie *et al.*, 1993), in skin warts of transplant recipients. HPV 27 (Ostrow *et al.*, 1989b) and HPV 49 (Favre *et al.*, 1989) were first identified in warts of transplant recipients and HPV 26 in warts of a patient with an unusual immune deficiency syndrome (Ostrow *et al.*, 1984).

Table 66 summarizes the results of larger studies (i.e. that include more than five lesions) of transplant-associated viral warts. In most studies that used in-situ hybridization or southern blot, the overall detection rate of HPV DNA ranged from 60 to 90%. Three studies that used PCR amplification with several sets of genus- and species-specific, degene-

Table 66. Prevalence of HPV DNA in skin warts of transplant recipients

Reference, study location	Method of detection (types included)	No. of cases (warts)	Overall HPV positivity (%)[a]	HPV type-specific positivity				Comments
				1–4, related types[b]	EV-, beta-HPV	6/11, 16/18	Other types (no. of lesions)	
Gassenmaier *et al*. (1986), Germany	Southern blot (1, 2, 3, 4, 5/8, 16/18)	16	8/16 (50)	6/16	1/16	1/16	–	Paraffin-embedded tissue
Rüdlinger *et al*. (1986), United Kingdom	Southern blot (1–4, 10, 5, 6/11, 16)	54	39/54 (72)	39/54	0/54	0/54	–	Frozen tissue; multiple HPV types found in single lesions; no control warts examined
van der Leest *et al.* (1987), USA	Southern blot (1–6)	32 (44)	39/44 (89)	50/44*	4/44*	0/44	–	Frozen tissue; *double infections in 15 cases
Barr *et al*. (1989), United Kingdom	Dot blot and southern blot (1, 2, 4, 5/8)	77	NA	NA	12/77	–	–	Frozen tissue; no control warts examined
Wilson *et al*. (1989), United Kingdom	Southern blot (1, 2, 3, 4, 5, 8)	18	13/18 (72)	9/18	0/18	–	4/18 not further characterized	Frozen tissue; viral genome in HPV 2 warts showed poly-morphism at PvuII and PstI sites.
Blessing *et al*. (1990), United Kingdom	ISH (4, 5, 8)	20	4/20 (20)	0/20	4/20	–	–	Frozen tissue; simple warts, dysplastic warts and EV-like lesions (3) studied; no specimen contained > 1 HPV type; no control wart samples
Obalek *et al*. (1992), France and Poland	Southern blot (1–7, 10, 16, 18, 28, 41, 50)	56 (82)	72/82 (88)	72/82	10/82	0/82	2/82 detected under non-stringent hybridization conditions	Frozen tissue; EV HPV always co-detected with HPV 3 or related types.

Table 66 (contd)

Reference, study location	Method of detection (types included)	No. of cases (warts)	Overall HPV positivity (%)[a]	HPV type-specific positivity				Comments
				1–4, related types[b]	EV-, beta-HPV	6/11, 16/18	Other types (no. of lesions)	
Euvrard *et al.* (1993), France	ISH (1a, 2a, 5, 16/18)	17	14/17 (82)	9/17	0/17	10/17	–	Frozen and paraffin-embedded tissue; multiple HPV types found in single lesions; no control warts examined
Soler *et al.* (1993), France	Southern blot, ISH and PCR (5, 6/11, 16/18, 1a, 2a)	18 transplant 3 non-transplant	11/18 (61) 0/3 (0)	1/18 0/3	1/18 0/3	4/18 0/3	–	Frozen tissue
Trenfield *et al.* (1993), Australia	Southern blot (1, 2, 3, 4, 5/8, 10, 11, 16/18, 41)	18	5/18 (28)	5/18	0/18	0/18	–	Frozen tissue
Hepburn *et al.* (1994), New Zealand	Dot blot (1–5, 6/11, 8, 41, 48, 49)	36 (44)	19 (43)	26/44	4/44	5/44	41 (1)	Multiple types found in some lesions
Pélisson *et al.* (1994), France	ISH (1a, 2a, 5, 6a, 11a, 16 and 18)	8 transplant 7 non-transplant 7 non-transplant normal skin	5/8 (63) 4/7 (57) 0/7 (0)	4/8 4/7 0/7	1/8 0/7 0/7	4/8 0/7 0/7	– – –	Frozen tissue; simple warts examined; multiple HPV types found in single lesions
Shamanin *et al.* (1994b), United Kingdom	PCR and direct sequencing (1–4, 10, 5/8, 6/11, 16/18 and others)	50	28 (60)	15/50	6/50	1/50	Uncharacterized (14)	Frozen tissue; benign warts and EV-like lesions (3) studied; no control warts examined
Stark *et al.* (1994), United Kingdom	Southern blot and PCR (1, 2, 5/8, 6/11, 16/18 and others)	18 transplant 6 non-transplant	10/18 (55) 2/6 (33)	4/18 2/6	3/18 0/6	3/18 0/6	0/18 0/6	Frozen tissue

Table 66 (contd)

Reference, study location	Method of detection (types included)	No. of cases (warts)	Overall HPV positivity (%)[a]	HPV type-specific positivity				Comments
				1–4, related types[b]	EV-, beta-HPV	6/11, 16/18	Other types (no. of lesions)	
de Villiers *et al*. (1997), United Kingdom	Nested PCR (2 sets of degenerate primers)	8 (15)	1/15 (93)	10/15	2/15	0/15	–	Frozen tissue.
Harwood *et al*. (1999), United Kingdom	Several nested PCR with degenerate, cutaneous, mucosal, EV-HPV-specific primers and direct sequencing	23 (51)	51/51 (100)	47/51	41/51	14/51	7 (4), 41 (5)	Co-detection of two or more distinct HPV types in 94% of lesions
Berkhout *et al*. (2000), Netherlands	Nested PCR with degenerate primers, EV, alpha 2/4, direct sequencing	12 VV 7 VP 16 VS	9/12 (75) 6/7 (86) 10/16 (63)	6/12 6/7 4/16	2, 6, 3*/12 0, 1, 2*/7 7, 7, 5*/16			Frozen tissue; *subgroups of beta-HPV, multiple HPV types in some lesions
O'Connor *et al*. (2001b), Ireland	Nested PCR with mucosal and EV-HPV-specific degenerate primers	11	11/11 (100)	6/11	10/11	0/11	–	Co-detection of two or more distinct HPV types in 6 warts

EV, epidermodysplasia verruciformis; ISH, in-situ hybridization; NA, not available; PCR, polymerase chain reaction; VP, verruca plana; VS, verruca seborrheica; VV, verruca vulgaris

[a] Of those types tested

[b] Alpha 2-, alpha 4-, gamma- and mu-HPV

rate primers detected HPV DNA in more than 90% of skin warts (de Villiers *et al.*, 1997; Harwood *et al.*, 1999; O'Connor *et al.*, 2001b).

Common skin-associated HPV types from the genera alpha2, alpha4, gamma and mu, including HPV 1, 2, 3, 4, 10, 27, 28, 57 and 77, were the most common types to be identified in studies in which appropriate probes were used (83–92%). Depending on the genus-specific sensitivity of the detection system, EV-HPV types (genus beta) were found in 10–90% of the warts. In earlier studies, mucosal HPV types 6/11 and 16/18 were found in 23 of 199 (11%) transplant samples and in none of 16 (0%) controls when probes that detect these HPV types were employed [$p = 0.32$]. A large PCR-based study of 51 warts identified mucosal types in 14 of these (27%). This study employed the most comprehensive set of PCR primers, which allowed co-detection of two or more distinct HPV types in 94% of the lesions (Harwood *et al.*, 1999). Mixed infections were seen predominantly with cutaneous and EV-HPV types. In contrast, in immunocompetent individuals, single HPV types only were detected with the same complex PCR system in all but one of 20 warts from 15 immunocompetent individuals (Harwood *et al.*, 1999). It should be emphasized that no EV phenotype was usually expressed in mixed infections of skin warts of transplant patients (van der Leest *et al.*, 1987; Obalek *et al.*, 1992; Harwood *et al.*, 1999). However, Morrison *et al.* (2002) described verrucae planae with the histological diagnosis of EV in eight of 17 patients who had had organ transplants (HPV 11) or acquired immunodeficiency syndrome (AIDS) (HPV 6) and identified HPV 8 and HPV 5 by in-situ hybridization.

(ii) *Verrucous keratoses (precancerous lesions)*

Table 67 summarizes the prevalence of HPV DNA in case series (of more than five lesions) of verrucous keratoses. HPV DNA detection rates in transplant-associated verrucous keratoses were approximately 20–30% in most studies that used southern blot or in-situ hybridization without amplification. In early PCR-based studies, detection rates were between 24 and 48% (Shamanin *et al.*, 1994b; Stark *et al*., 1994; Tieben *et al*., 1994), which increased to 49–88% by the use of more sophisticated and comprehensive primer systems (de Jong-Tieben *et al*., 1995; de Villiers *et al*., 1997; Berkhout *et al*., 2000; Harwood *et al*., 2000). HPV DNA was also found in 80% of precancerous lesions by a nested PCR with degenerate primers (de Jong-Tieben *et al*., 1995) but in no more than 30% by single-step PCRs that had been developed earlier (Tieben *et al*., 1994).

This underlines the importance of assay sensitivity and indicates that most HPV DNA persists at low copy levels in precancerous skin lesions and in skin cancers (see Sections (iii) and (iv)) of transplant recipients. This notion corresponds to observations of skin cancers in the general population. In contrast, the prevalence of HPV DNA in skin warts was similar when determined by southern blot (60–90%) and highly sensitive PCRs (100%).

The combination of data from studies that used probes designed to detect type-specific HPV, albeit with different methodologies, showed that overall common skin-associated HPV types were found in 27 of 219 (12%) transplant samples compared with four of 23 (17%) control samples [$p = 0.68$]. These types were found in 11 of 17 lesions (65%) by broad-spectrum PCR assays. When comparing studies with similar methodologies, common

Table 67. Prevalence of HPV DNA in verrucous keratoses of transplant recipients

Reference, study location	Method of detection (types included)	No. of cases (lesions)	Overall HPV positivity (%)[a]	HPV-type specific positivity				Comments
				1–4, related types[b]	EV-, beta-HPV	6/11, 16/18	Other types (no. of lesions)	
Rüdlinger *et al.* (1986), United Kingdom	ISH (1a, 2, 3, 4, 5/8, 6/11, 16)	11	1/11 (9)	1/11	0/11	0/11	–	Frozen tissue; no control samples examined
Barr *et al.* (1989), United Kingdom	Dot blot (1, 2, 4, 5/8)	NA	NA	NA	7/44	NA	NA	–
Blessing *et al.* (1990), United Kingdom	ISH (4, 5/8)	19	5/19 (26)	2/19	3/19	–	–	Frozen tissue; no control samples examined
Euvrard *et al.* (1991), France	ISH (1, 2, 5, 16/18)	7	0/7 (0)	0/7	0/7	0/7	–	Frozen tissue
Viac *et al.* (1992), France	ISH (multiple probes)	11	4/11 (36)	2/11	0/11	0/11	Uncharacterized (2/11)	Frozen tissue
Euvrard *et al.* (1993), France	ISH (1, 2, 5, 16/18)	21	5/21 (24)	5/21	1/19	3/21	–	Multiple HPV types identified in single lesions; no control tissue examined
Soler *et al.* (1993), France	Southern blot, ISH and PCR (1, 2, 3, 4, 5/8, 6/11, 16/18)	18	11/18 (61)	4/18	1/18	15/18	–	Frozen tissue; multiple HPV types found in single lesions
Trenfield *et al.* (1993), Australia	Southern blot (1, 2, 3, 4, 5/8, 11, 16/18)	26	4/26 (15)	3/26	1/26	0/26	–	Frozen tissue

Table 67 (contd)

Reference, study location	Method of detection (types included)	No. of cases (lesions)	Overall HPV positivity (%)[a]	HPV-type specific positivity				Comments
				1–4, related types[b]	EV-, beta-HPV	6/11, 16/18	Other types (no. of lesions)	
McGregor *et al*. (1994), United Kingdom	PCR (5/8, 6/11, 16/18)	31 transplant 13 non-transplant	0/31 (0) 0/13 (0)	– –	0/31 0/13	0/31 0/13	–	Paraffin-embedded tissue
Pélisson *et al*. (1994), France	ISH (1, 2a, 3, 4, 5, 6a/11a, 16/18)	10 transplant 2 non-transplant	4/10 (40) 0/2 (0)	2/10 0/2	1/10 0/2	4/10 0/2	–	Frozen tissue; multiple HPV types found in single lesions
Shamanin *et al*. (1994b), United Kingdom	Southern blot and PCR (1–4, 10, 5/8, 6/11, 16/18 and others)	40	19/40 (48)	6/40	6/40	0/40	Uncharacterized (7/40)	Frozen tissue; no control samples studied
Stark *et al*. (1994), United Kingdom	Southern blot and PCR (1, 2, 3, 4, 5/8, 6/11, 16/18)	46 transplant 21 non-transplant	11/46 (24) 4/21 (19)	5/46 3/21	2/46 2/21	1/46 0/21	Unknown (3/46)	Frozen tissue; no control samples examined
Tieben *et al*. (1994), Netherlands	PCR and direct sequencing (multiple probes)	10	3/10 (30)	1/10	1/10	0/10	Uncharacterized (1/10)	Frozen tissue; no control samples
de Jong-Tieben *et al*. (1995), Netherlands	PCR (degenerate nested primers, direct sequencing)	15 AK 5 BD	14/15 (93) 2/5 (40)		14/15 2/5			Frozen tissue; frequently more than one HPV type detected
de Villiers *et al*. (1997), United Kingdom	Nested PCR (2 sets of degenerate primers, sequencing)	12 (17)	11/17 (65)	1/17	6/17		7 (1)	Frozen tissue

Table 67 (contd)

Reference, study location	Method of detection (types included)	No. of cases (lesions)	Overall HPV positivity (%)[a]	HPV-type specific positivity				Comments
				1–4, related types[b]	EV-, beta-HPV	6/11, 16/18	Other types (no. of lesions)	
Harwood *et al.* (2000), United Kingdom	Degenerate PCR for EV, cutaneous, and mucosal HPV	9 (17)	15/17 (88)	11/17	12/17	2/1		Frozen tissue; 55% of 11 lesions of immunocompetent patients HPV-positive (see Table 24?)
Berkhout *et al.* (2000), Netherlands	Nested PCR with degenerate primers, EV, alpha 2/4, direct sequencing	56	38/56 (68)	NA/56	21/56			Frozen tissue
de Jong-Tieben *et al*. (2000), Netherlands	PCR (degenerate nested primers, direct sequencing)	37 AK 11 BD	18/37 (49) 8/11 (73)		18/37 8/11			Three of 28 (11%) clinically normal skin samples EV-HPV-positive
Forslund *et al.* (2003a), Australia	PCR with FAP- and HPV 38-specific primers, cloning and sequencing	6	2/6 (33)	1/6	2/6			Frozen tissue; 67% of perilesional and buttock swabs were HPV-positive; 70% of 10 lesions of immunocompetent patients HPV-positive (see Table 24?)

AK, actinic keratoses; BD, Bowen's disease; EV, epidermodysplasia verruciformis; ISH, in-situ hybridization; NA, not available; PCR, polymerase chain reaction

[a] Of those tested

[b] Alpha 2-, alpha 4-, gamma- and mu-HPV

cutaneous HPV types are clearly less prevalent in precancerous lesions than in skin warts. Mucosal HPV types were found in about 10% of transplant samples. The high prevalence of HPV now detected in precancerous lesions is due to the frequent detection of EV-HPV types in an average of 65% of transplant samples compared with 6% in earlier studies.

(iii) *Squamous-cell carcinoma*

Table 68 summarizes the prevalence of HPV DNA in case series of transplant-associated squamous-cell carcinoma (see also Table 21 in IARC, 1995 for comparison).

Rates of detection of HPV DNA in these tumours in studies that used southern blot and in-situ hybridization and in early studies that used PCR varied extremely from 0 to 100%. In one case–control study that used PCR and multiple probes, HPV DNA was found in two of nine (22%) control squamous-cell carcinomas compared with 10 of 30 (33%) transplant-associated squamous-cell carcinomas (Stark *et al*., 1994). Consistently high rates of detection of HPV DNA ranging from 54 to 91% have been found since 1995 by the use of sophisticated and comprehensive primer systems. Direct comparisons within individual studies consistently showed higher HPV DNA prevalences in squamous-cell carcinomas of immunosuppressed patients than in those of immunocompetent patients (Harwood *et al*., 2000; Meyer *et al*., 2000; O'Connor *et al*., 2001b; Forslund *et al*., 2003b).

The combination of the data from studies before 1995 showed that common skin-associated HPV types were found in 34 of 452 (7%) transplant and one of nine (11%) control samples. These types were found in 24 of 44 carcinomas (55%) by the broadest PCR spectrum employed (Harwood *et al*., 2000). Mucosal HPV types were found in about 10% of transplant-associated squamous-cell carcinomas. As in the case of precancerous lesions, the high prevalence of HPV found in squamous-cell carcinoma during the past 10 years is due to the frequent detection of a broad spectrum of beta-HPV, including EV-HPV and related types, in 70–80% of the cases.

HPV 8 was detected in a primary squamous-cell carcinoma from the arm and its lymph node metastasis (Morrison *et al.*, 2002).

(iv) *Basal-cell carcinoma*

Table 69 summarizes the prevalence of HPV DNA in case series of basal-cell carcinoma. In studies from 2000 and later, the overall rates of detection were between 33 and 80%. Combining the data from these four studies, the overall detection rate was 30 of 52 (57%); common skin-associated HPV types were found in 23%, beta-HPV in 44% and mucosal HPV in 4%.

(v) *Multiple lesions*

In patients from whom multiple lesions were analysed, certain HPV types were found to prevail in both benign, precancerous and malignant lesions located on different anatomical sites, and partially removed on different occasions up to 7 years apart (Höpfl *et al*., 1997; de Villiers *et al*., 1997; Harwood *et al*., 2000). However, overall no single HPV type seemed to prevail in cutaneous precancerous lesions or skin cancers of transplant recipients.

Table 68. Prevalence of HPV DNA in squamous-cell carcinoma (SCC) of transplant recipients

Reference, study location	Method of detection (types included)	No. of cases (lesions)	Overall HPV positivity (%)[a]	HPV type-specific positivity				Comments
				1–4, related types[b]	EV-, beta-HPV	6/11, 16/18	Other types (no. of lesions)	
Barr *et al.* (1989), United Kingdom	Dot blot (1, 2, 4, 5/8)	25	16/25 (64)	1/25	15/25	–	–	Frozen tissue
Magee *et al.* (1989), USA	ISH (1–4, 16/18, 6/11)	8	8/8 (100)	0/8	–	8/8	–	–
Blessing *et al.* (1990), United Kingdom	ISH (4, 5/8)	11	2/11 (18)	2/11	0/11	–	–	Frozen tissue
Dyall-Smith *et al.* (1991), United Kingdom	PCR amplification (1–4, 5, 7, 9, 11, 16/18, 19, 25)	188	0/188 (0)	0/188	0/188	0/188	–	Frozen tissue; no control SCC studied
Viac *et al.* (1992), France	ISH (multiple probes)	8	2/8 (25)	1/8	0/8	1/8	–	–
Euvrard *et al.* (1993), France	ISH (1, 2, 5, 16/18)	46	25/46 (54)	20/46	2/46	15/46	–	Frozen tissue; multiple HPV types found in single lesions; no control samples studied
Purdie *et al.* (1993), United Kingdom	Dot blot and Southern blot (1–4, 10, 5/8, 6/11, 16/18)	10	6/10 (60)	2/10	2/10	0/10	Unknown (4/10)	–
Smith *et al.* (1993), Australia	PCR amplification (probes not specified)	20	0/20 (0)	–	–	–	–	–
Soler *et al.* (1993), France	Southern blot, PCR and ISH (1–4, 5/8, 6/11, 16/18)	26	21/26 (81)	0/26	6/26	20/26	–	Frozen tissue; multiple HPV types found in single lesions
Trenfield *et al.* (1993), Australia	Southern blot (multiple probes)	40	2/40 (5)	1/40	1/40	0/40	–	Frozen tissue

Table 68 (contd)

Reference, study location	Method of detection (types included)	No. of cases (lesions)	Overall HPV positivity (%)[a]	HPV type-specific positivity				Comments
				1–4, related types[b]	EV-, beta-HPV	6/11, 16/18	Other types (no. of lesions)	
McGregor *et al.* (1994), United Kingdom	PCR amplification (5/8, 6/11, 16/18)	14 transplant 22 non-transplant	0/14 (0) 0/22 (0)	– –	0/14 0/22	0/14 0/22	–	Paraffin-embedded material
Pélisson *et al.* (1994), France	ISH (1a, 2a, 5, 6a/11a, 16/18)	13	8/13 (62)	3/13	1/13	7/13	–	Frozen tissue; no control SCC studied
Shamanin *et al.* (1994b), United Kingdom	Southern blot and PCR (1, 2, 3, 5, 7, 10, 37, 40)	23	13/23 (57)	4/23	0/23	0/23	41 (1); unknown (8)	Frozen tissue; no control samples examined
Stark *et al.* (1994), United Kingdom	Southern blot and PCR (1–4, 5/8, 6/11, 16/18)	30 transplant patients 9 controls	10/30 (33) 2/9 (22)	3/30 1/9	0/30 1/9	2/30 0/9	Unknown (6)	Frozen samples
Tieben *et al.* (1994), Netherlands	PCR and direct sequencing (multiple probes)	24	5/24 (21)	1/24	3/24	0/24	Unknown (2)	Frozen tissue
Berkhout *et al.* (1995), Netherlands	PCR (degenerate nested primer, direct sequencing)	53	43/53 (81)	0/53	43/53	0/53		Multiple HPV types found in some lesions
Shamanin *et al.* (1996), United Kingdom	Broad-range PCR with degenerate primers	20	13/20 (65)	7/20	2/20	1/20	41, 54, 61 (1 each), 69 (4)	Frozen tissue
de Villiers *et al.* (1997), United Kingdom	Nested PCR (2 sets of degenerate primers)	22 11 intra-epidermal carcinomas	20/22 (91) 10/11 (91)	9/28	> 70%	1*		Frozen tissue; *HPV 11 in a SCC on the thumb

Table 68 (contd)

Reference, study location	Method of detection (types included)	No. of cases (lesions)	Overall HPV positivity (%)[a]	HPV type-specific positivity				Comments
				1–4, related types[b]	EV-, beta-HPV	6/11, 16/18	Other types (no. of lesions)	
Harwood *et al.* (2000), United Kingdom	Degenerate PCR for EV, cutaneous, and mucosal HPV	18 (44)	37/44 (84)	24/44	33/44	4//44	66 (1)	Frozen tissue; multiple HPV types found in single lesions
Berkhout *et al.* (2000), Netherlands	Nested PCR with degenerate primers, EV, alpha 2/4, direct sequencing	81	63/81 (78)	18/81	32,41, 23*/81			Frozen tissue; *subgroups of beta-HPV, multiple HPV types in some lesions. 10/31 clinically normal skin samples HPV-positive
de Jong-Tieben *et al*. (2000), Netherlands	PCR (degenerate nested primers, direct sequencing)	50	34/50 (68)		34/50			Three of 24 (13%) clinically normal skin samples EV-HPV-positive
Meyer *et al*. (2000), Germany	Nested PCR with mucosa, cutaneous, and EV-HPV-specific degenerate and type-specific (5, 8) primers	9	6/9 (67)		5/9		70 (1)	Frozen tissue
O'Connor *et al*. (2001b), Ireland	Nested PCR with mucosa and EV-HPV-specific degenerate primers	9	8/9 (89)	0/9	8/9	0/9	–	
Forslund *et al.* (2003a), Norway	PCR with FAP-specific primers	60	33/60 (55)	NA	NA	NA	NA	One HPV 10 and mostly beta-HPV in 8 patients
Forslund *et al.* (2003c), Australia	PCR with FAP- and HPV38-specific primers, cloning and sequencing	11	6/11 (54)	2/11	5/11	–	–	Frozen tissue; 91% of perilesional and 73% of buttock swabs were HPV-positive.

EV, epidermoplasia verruciformis; ISH, in-situ hybridization; KA, keratoacanthoma; NA, not available; PCR, polymerase chain reaction

[a] Of those types tested

[b] Alpha 2-, alpha 4-, gamma- and mu-HPV

Table 69. Prevalence of HPV DNA in basal-cell carcinoma (BCC) of transplant recipients

Reference, study location	Method of detection (types included)	No. of cases (lesions)	Overall HPV positivity (%)[a]	HPV type-specific positivity				Comments
				1–4, related types[b]	EV-, beta-HPV	6/11, 16/18	Other types	
Rüdlinger *et al*. (1986), United Kingdom	Southern blot (1–4, 5/8, 6/11, 16)	1	0/1 (0)	0/1	0/1	0/1	–	–
Obalek *et al*. (1988), Poland	Southern blot (1, 4, 5, 10, 11, 16/38)	2	2/2 (100)	2/2	0/2	0/2	–	–
Euvrard *et al*. (1993), France	ISH (mixed probe)	2	0/2 (0)	0/2	0/2	0/2	–	–
Trenfield *et al*. (1993), Australia	Southern blot (1–4, 5/8, 11, 16/18)	11	1/11 (9)	1/11	0/11	0/11	–	–
McGregor *et al*. (1994), United Kingdom	PCR amplification (5/8, 6/11, 16/18)	11 (transplant) 15 (non-transplant)	0/11 (0) transplant 0/15 (0) non-transplant	–	0/11 0/15	0/11 0/15	–	Paraffin embedded tissue
Pélisson *et al*. (1994), France	ISH (1, 2, 5, 6/11, 16/18)	4	3/4 (75)	1/4	0/4	3/4	–	Frozen tissue; no control BCC samples
Tieben *et al*. (1994), Netherlands	PCR (four consensus primers designed to detect cutaneous HPV types)	4	0/4 (0)	0/4	0/4	0/4	0/4	Frozen tissue
Shamanin *et al*. (1996), United Kingdom	Broad-range PCR with degenerate primers	5	3/5 (60)	3/5	0/5	0/5	51, 56	Frozen tissue; multiple HPV types in two cases
Harwood *et al*. (2000), United Kingdom	Degenerate PCR for EV-, cutaneous and mucosal HPV	15 (24)	18/24 (75)	9/24	13/24	2/24		Frozen tissue

Table 69 (contd)

Reference, study location	Method of detection (types included)	No. of cases (lesions)	Overall HPV positivity (%)[a]	HPV type-specific positivity				Comments
				1–4, related types[b]	EV-, beta-HPV	6/11, 16/18	Other types	
Berkhout *et al*. (2000), Netherlands	Nested PCR with degenerate primers, EV, alpha 2/4, direct sequencing	14	5/14 (36)	2/14	0,3, 4*/14			*Subgroups of beta-HPV; multiple HPV types in some lesions
de Jong-Tieben *et al*. (2000), Netherlands	PCR (degenerate nested primers, direct sequencing)	9	3/9 (33)		3/9			Three of 24 (13%) clinically normal skin samples EV-HPV-positive
Forslund *et al.* (2003c), Australia	PCR with FAP- and HPV 38- specific primers, cloning and sequencing	5	4/5 (80)	1/5	3/5			Frozen tissue; 83% of perilesional and 60% of buttock swabs were HPV-positive.

See Table 7 for a description of the primers used.
EV, epidermodysplasia verruciformis; ISH, in-situ hybridization; PCR, polymerase chain reaction
[a] Of those types tested
[b] Alpha 2-, alpha 4-, gamma- and mu-HPV

(*c*) *HPV infection and cancer at other sites in transplant patients*

(i) *Head and neck region*

Three cases of head and neck squamous-cell carcinoma were reported in patients who were 18, 29 and 53 years of age at the time of tumour diagnosis after renal, cardiac or bone-marrow transplantation (Bradford *et al.*, 1990). Time from transplant to diagnosis of tumour ranged from 7 months to 12 years. Only the youngest patient had no history of exposure to the traditional pre-disposing factors, tobacco and alcohol use. Histopathology of all three tumours showed features of koilocytosis with hyperkeratosis and parakeratosis suggestive of HPV infection (Bradford *et al*., 1990) [the Working Group noted that this paper does not provide specific data on the presence of HPV].

A 36-year-old renal transplant recipient who took cyclosporin A presented with bilateral nasal polypoid lesions that involved the nasal septum and lateral nasal walls (Harris *et al.*, 1998b). Pathological findings from surgical excision demonstrated an inverted papilloma with focal atypia and mild dysplasia. DNA from the tissue was tested by PCR and revealed the presence of HPV type 6. Analysis of RNA showed transcription in the tissue of the HPV early proteins E6 and E7. Histologically normal nasal tissue from the same patient contained HPV DNA and transcripts similar to those described in the inverted papilloma specimen (Harris *et al*., 1998b).

HPV infection in oral cyclosporin-induced gingival overgrowth was investigated in renal transplant recipients by assessing morphological changes and by the use of in-situ hybridization with HPV-specific probes (Bustos *et al.*, 2001). Biopsies of gingival overgrowth lesions from 13 renal transplant recipients and four samples of healthy mucosa from these patients were analysed. The pathologist was not aware of the HPV result. Twelve of the 13 samples studied (92.3%) contained HPV, of which four tested positive for HPV 6/11 and one for HPV 16. In 11 of the HPV-positive cases, koilocytotic atypia was found. The four biopsies of normal mucosa from gingival overgrowth patients also contained HPV DNA. These data show that suppression of T-cell function by cyclosporin therapy can result in an increase in HPV infection and add to the growth-stimulating activity of cyclosporin in the oral mucosa.

A total of 10 paraffin-embedded biopsy specimens of epithelial tumours from six heart transplant recipients were studied for the presence of HPV (Auvinen *et al*., 2002). These cases included all epithelial cancer cases among the malignancies seen in 249 heart transplant patients at Helsinki University. HPV DNA was amplified by PCR. A specimen from one patient revealed the presence of HPV 16. In this patient, who had received a heart transplant in 1991 and subsequent chemotherapy including cyclosporin, a tonsillar tumour (epidermoid carcinoma) was discovered in 1997.

(ii) *Urinary tract*

Three cases of de-novo lower urinary tract carcinoma in renal transplant recipients were reported, which showed the potential for unusually rapid urothelial extension and invasion in chronically immunosuppressed individuals (Lemmers & Barry, 1990). Two patients had a history of perianal condylomata acuminata. Tumours from one of these

harboured the genetic sequences of HPV type 6. One patient had multiple manifestations of cyclophosphamide-related urothelial injury, including bladder carcinoma.

One report described two cases of rapidly progressive, multifocal transitional-cell carcinomas of the bladder that developed in two patients after renal and cardiac transplantation, respectively (Noel *et al*., 1994). In both cases, HPV 16 DNA was detected using the PCR amplification method. This HPV type has not been previously described in this type of tumour in transplant recipients. HPV infection may play a role in the development of rapidly progressive multifocal transitional-cell carcinoma in the bladder of immunosuppressed patients.

2.8.3 *Studies in human immunodeficiency virus (HIV)-infected persons*

(*a*) *Studies of the uterine cervix*

(i) *Prevalence of cervical HPV infection and SIL* (Table 70)

In a study from Italy that included 221 women at high risk for HIV, among the 121 HIV-positive women, 58 (47%) had HPV lesions, 23 (40%) of whom had CIN1–3. In the 100 HIV-negative women, 23 (23%) had HPV lesions; among these 23 women, six (26%) had CIN1–3. These findings suggest that HIV infection is associated with HPV lesions and that cervical cytological abnormalities develop in this situation (Branca *et al.*, 1995).

Murphy *et al*. (1995) performed a retrospective study of 136 HIV-positive women who attended an inner city ambulatory HIV clinic over a 6-year period between 1987 and 1992 in Dublin, Ireland. During this time, a total of 165 HIV-infected women attended for management of their HIV disease. The results of cervical cytological specimens (smears) were available for 136 (82.4%) women. Forty-one (30.1%) women had mild dysplasia/CIN1, 21 (15%) had CIN2 and 17 (12.5%) had CIN3. The overall prevalence of dysplasia was 58.1%. Twenty-seven (34.2%) of the women with CIN had cytological evidence of HPV infection. No association between the clinical stage of HIV disease and the presence or degree of SIL was observed (Murphy *et al.*, 1995).

Sun *et al*. (1995) compared the prevalence of HPV infection and CIN in more than 650 HIV-positive and HIV-negative women from the New York area: 60% of HIV-positive and 36% of HIV-negative women had detectable cervical HPV DNA ($p < 0.001$); 27% of HIV-positive women had HPV 16, 24% had HPV 18 and 51% had more than one type. Of the HIV-negative women, 17% had HPV 16, 9% had HPV 18 and 26% had multiple types. Latent HPV infection was defined as the presence of HPV in the absence of disease. Among 208 HIV-positive women with HPV infection, 126 (61.6%) had no evidence of CIN compared with 97.3% of HIV-negative women with HPV infection. Thus, HPV infection in HIV-positive women appears to be more probably associated with CIN than that in HIV-negative women.

Bongain *et al*. (1996) studied 111 HIV-positive women in Nice, France, 39 of whom were pregnant. Each participant underwent four cervical biopsies: 9.9% had CIN2 and 8.2% had CIN3. No significant differences in the prevalence of CIN were noted between

transmission group, Centers for Disease Control stage of disease, $CD4^+$ cell count and pregnancy.

Langley *et al*. (1996) studied 68 HIV-1-positive, 58 HIV-2-positive, 14 HIV-1-positive/HIV-2-positive and 619 HIV-negative women who attended clinics for sexually transmitted diseases in Senegal. HPV was detected in 43% of women by PCR and in 7% by southern transfer hybridization; 7.4% of all women had SIL. Both HIV-1 and HIV-2 were associated with HPV infection. HIV-2 was also associated with SIL but the association between HIV-1 and SIL did not reach statistical significance. Most lesions were LSIL. HIV-positive women who had SIL had a lower ratio of CD4:CD8 cells than HIV-positive women without SIL ($p = 0.003$).

Petry *et al*. (1996) compared the prevalence of cervical HPV infection and CIN among 62 HIV-positive women and 77 HIV-negative women who were immunosuppressed due to other causes. HIV-positive women had a higher prevalence of cervical HPV infection and CIN than HIV-negative immunosuppressed women.

Sopracordevole *et al*. (1996) assessed the relationship between HIV status, level of $CD4^+$ cells and SIL in 51 HIV-positive women in Aviano, Italy. Thirty of 51 patients (58.8%) had confirmed SIL. There was no significant difference in the $CD4^+$ cell count between women with or without SIL, which suggests that the expression of HPV-related dysplasia is a complex process.

Cappiello *et al.* (1997) assessed the association between different HPV genotypes, HIV infection and CIN in a multisite study carried out in Italy. The women were intravenous drug users or sexual partners of intravenous drug users. CIN was detected in 36% of HIV-positive women and in 9% of HIV-negative women. The prevalence of HPV did not differ significantly between HIV-positive and HIV-negative women. The most frequently detected genotypes in both groups were HPV 16 and HPV 18 and were similar between HIV-positive and HIV-negative women. HIV-positive women showed a wider spectrum of HPV genotypes, including low-risk and rare types.

Chiasson *et al*. (1997) studied the prevalence of CIN and vulvovaginal lesions in a group of HIV-positive and HIV-negative women from the New York area, USA. Vulvovaginal condylomata acuminata were found in 5.6% of HIV-positive and 0.8% of HIV-negative women. Multicentric disease was more common among HIV-positive than HIV-negative women and HIV-positive women with vulvovaginal disease were more likely to have CIN than those without (odds ratio, 2.9; 95% CI, 1.1–74). In a multivariate analysis, HIV positivity (adjusted odds ratio, 5.3; 95% CI, 1.3–35.3) and HPV infection (adjusted odds ratio, 6.1; 95% CI, 1.7–39.4) were associated with the detection of vulvovaginal condylomata.

Drapkin *et al*. (1997) performed a retrospective chart review of 89 HIV-positive and 100 HIV-negative women who attended Duke University clinics in southeastern USA. SIL was found in 49.4% of HIV-positive and in 23.0% of HIV-negative women (odds ratio, 3.3; 95% CI, 1.7–6.1).

Table 70. Prevalence of cervical HPV infection and squamous intraepithelial lesions (SIL) in HIV-positive and HIV-negative women

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p* value	%	Odds ratio (95% CI)/ *p*-value		
Branca *et al*. (1995), Italy	No HPV DNA detection	121	100			23.2 HIV+, 11.0 HIV–		Cytology, histology	HIV– women were at high risk for HPV infection.
Murphy *et al*. (1995), Ireland	No HPV DNA detection	136				58.1 SIL HIV+, 27.9 HSIL		Cytology	Retrospective chart review; 80.9% of women acquired HIV through IDU.
Sun *et al*. (1995), USA	MY09/MY11 primers with RFLP typing and E6 primers (16, 18)	344	325	60.4 HIV+, 35.7 HIV–	$p < 0.001$	*LSIL* 14.2 HIV+, 2.8 HIV– *HSIL* 4.9 HIV+, 0.6 HIV–		Cytology, histology	HPV testing performed on cervicovaginal lavage specimen
Bongain *et al*. (1996), France		111				6.1 CIN1 9.9 CIN2 8.2 CIN3		Histology	Of 111 participants 39 were pregnant at time of study; each patient had 4 cervical biopsies.
Langley *et al*. (1996), Senegal	PCR with SBH	68 HIV-1, 58 HIV-2, 14 HIV-1 and HIV-2	619	57.1 HIV-1, 50.0 HIV-2, 75.0 HIV-1 and HIV-2, 40.1 HIV–	2.9 (1.7–4.9) 1.7 (1.0–2.9) 4.9 (0.8–10.3)	*SIL* 7.5 HIV-1, 11.1 HIV-2, 16.7 HIV-1 and HIV-2, 6.8 HIV–	1.8 (0.7–4.7) 2.9 (1.2–7.2) 5.2 (1.4–19.6)	Cytology, histology	Adjusted odds ratio; women enrolled from STD clinics in Senegal; HPV testing performed on endocervical swabs
Petry *et al*. (1996), Germany	Viratype	62	77 allograft recipients, 19 immuno-suppressed	50.0 HIV+, 19.5 HIV– allograft recipients, 31.6 HIV– immuno-suppressed		46.8 HIV+, 16.9 HIV– allograft recipients, 31.6 HIV– immuno-suppressed		Cytology, histology	Cervical swab material used for HPV testing

Table 70 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p* value	%	Odds ratio (95% CI)/ *p*-value		
Sopracordevole *et al*. (1996), Italy	No HPV DNA detection	51				58.8 SIL		Cytology, histology	
Cappiello, *et al*. (1997), Italy	MY09/MY11 PCR and RFLP (specific types not specified)	135	101	40.3 HIV+, 29.6 HIV–	1.61 (0.89–1.21)	35.8 HIV+, 9.2 HIV–	5.52 (2.43–12.91)	Cytology, histology	HPV typing performed on cells scraped from glass slides; participants or their sexual partners were IDUs.
Chiasson *et al*. (1997), USA	PCR on cervicovaginal lavage specimen using L1 consensus primers	396	375	61.7 HIV+, 35.7 HIV–	$p < 0.001$	5.6 HIV+, 0.8 HIV–	$p < 0.001$	Cytology, histology	Study examined prevalent condyloma of vulva, vagina and perianal region and multicentric condyloma
Drapkin *et al*. (1997), USA	No HPV DNA detection	89	100			*SIL* 49.4 HIV+, 23.0 HIV–	3.3 (1.7–6.1)	Cytology	Retrospective chart review of women in southeastern USA
Ferrera *et al*. (1997b), Honduras	GP5/GP6 PCR with SB analysis (6, 11, 16, 18, 31, 33)	23	28	56.5 HIV+, 18.0 HIV–	6.0 (1.5–26.7)				Study of prostitutes in Tegucigalpa, Honduras; HPV testing on wooden spatula specimen
Frankel *et al*. (1997), USA		55 with adequate cytology results				23 genital condyloma, 47.3 cervical SIL on cytology, 2 vaginal SIL		Cytology	Study of women hospitalized with HIV infection at Yale-New Haven Hospital from October 1994 to April 1995
Rezza *et al*. (1997), Italy	MY09/MY11 primers with RFLP (16, 18, 31, 33, 35, 53, 58)	135	101	40.0 HIV+, 31.7 HIV–	$p = 0.19$	*LSIL* 35.6 HIV+, 8.9 HIV–	5.64 (2.5–13.2)	Cytology	HPV testing performed on cervical cytobrush sample
Calore *et al*. (1998), Brazil	No HPV DNA detection	82				LSIL, 19.5 HIV+ HSIL, 6.1 HIV+		Cytology	Women aged 13–21 years

Table 70 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p* value	%	Odds ratio (95% CI)/ *p*-value		
La Ruche *et al*. (1998), Cote d'Ivoire	MY09/ MY11 primers with RFLP analysis for typing	151 HIV+ and HIV– women with LSIL 60 HIV+ and HIV– women with HSIL	151 HIV+ and HIV– 240 HIV+ and HIV–	68.2 LSIL 30.5 LSIL controls 81.7 HSIL 20.4 HSIL controls	4.4 (2.6–7.4) 14.3 (6.6–30.9)			Cytology, histology	Cervical Viva-brush specimen used for HPV testing; controls were chosen at random among women shown not to have cervical lesions; adjusted odds ratio
Maiman *et al.* (1998), USA	MY09/MY11 primers with probing (6, 11, 32, 40, 42, 43, 44, 53, 54, 55, 61, 70, Pap 155, Pap 291, AE2, 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 68, 73)	253	220	75.1 HIV+, 46.8 HIV–	$p < 0.0001$	*LSIL* 15.4 HIV+, 3.6 HIV–, *HSIL* 7.9 HIV+, 1.6 HIV– *SIL* 50.8 HIV+, CD4 < 200; 28.3 HIV+, CD4 < 200–499; 24.6 HIV+, CD4 > 500	$p < 0.0001$	Cytology, histology	Cervicovaginal lavage specimen used for HPV testing
Rezza *et al*. (1998), Italy	MY09/MY11 primers with RFLP typing (16, 18, 31, 33, 35, 53, 58, 11, 44, 46, 54, 59, 66, CP 6108, CP 8304)	135	101	40.0 HIV+, 31.7 HIV–					HPV testing performed on cytobrush specimen

Table 70 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p* value	%	Odds ratio (95% CI)/ *p*-value		
Rugpao *et al.* (1998), Thailand	Viratype Plus with typing (16, 18, 31, 35, 45, 52, 56 and 6, 11, 42, 43, 44)	224	257	*HR type* 15.8 HIV+, 3.6 HIV– *LR type* 5.4 HIV+, 8.3 HIV–	5.0 (1.8–14.6) 1.8 (0.6–4.9)	*SIL* 10.6 HIV+, 2.5 (HIV–)	5.3 (2.0–15.2)	Cytology	Women were sexual partners of men with HIV-1 infection.
Six *et al*. (1998), France	SBH (6/11/42, 16/18/33, 31/35/39) and MY09/MY11 PCR with probing (16, 18, 33) and GP1/GP2 primers with probing (all other HPV types)	253	160			*SIL* 265 HIV+, 7.5 HIV– *Among HIV+* 14.9, CD4 > 500; 26.0, CD4 200–500; 38.7, CD4 < 200	 1.5 (0.5–4.2) 3.4 (1.4–8.3) 4.4 (1.7–11.4)	Cytology (with biopsy for HSIL)	HPV testing on cervical cytobrush sample; 278 women were followed prospectively at 6-month intervals for 1 year; reference category was HIV– women.
Uberti-Foppa *et al*. (1998), Italy	HC2 and MY09/MY11 primers with typing by RFLP	168	100	*HC* 66.1 HIV+, 15.0 HIV– *PCR* 91 HIV+, 48 HIV–	$p < 0.001$ $p < 0.001$			Cytology, histology	Collection method for HPV testing not specified; 91 HIV+ women acquired HIV through heterosexual contact and 74 through IDU.
Cu-Uvin *et al*. (1999), USA	MY09/MY11/ HMBO1 L1 PCR	851	434	64.3 HIV+, 27.6 HIV–	4.7 (3.7–6.1)	–			HERS population; HPV testing in cervicovaginal lavage specimen

Table 70 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p* value	%	Odds ratio (95% CI)/ *p*-value		
Goncalves *et al.* (1999), Brazil	MY09/MY11 PCR with RFLP typing (more than 44 types)	141		65.7 vaginal samples, 64.4 cervical samples, 47.4 perianal samples				Cytology, histology	HPV testing on cytobrush specimens from cervical, vaginal and perianal areas
Hankins *et al*. (1999), Canada	MY09/MY11 primers with probing (14 different types)	375		67.2 HIV+		10.9 confirmed SIL HIV+		Cytology	Women were participants in the Canadian Women's HIV Study.
Kapiga *et al*. (1999), Tanzania	No HPV DNA detection	691				*SIL* 2.9 HIV+; CD4 > 500, 2.1; CD4 200–500, 2.3; CD4 > 500, 8.8	*p* for trend = 0.02	Cytology	Participants were HIV+ pregnant women; cytology collected 3–6 months after delivery
Leroy *et al*. (1999), Rwanda	No HPV DNA detection	103	107			24.3 HIV+, 6.5 HIV–	4.6 (1.8–12.3)	Cytology	All women were pregnant.
Luque *et al*. (1999), USA	HC2	93		47.3 overall, 21.5 HR, 12.9 LR	2.57 (1.29–13.56)	51.3 HIV VL > 10 000 copies/mL, 24.3 HIV VL < 10 000 copies/mL	2.11* (1.12–10.19)	Cytology	Sampling from Cervex brush; *relative risk

Table 70 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p* value	%	Odds ratio (95% CI)/ *p*-value		
Massad *et al*. (1999), USA	MY09/MY11/HMB01 with probing (6, 11, 16,18, 26, 31, 32, 33, 35, 39, 40, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 66, 68, 69, 70, 73, Pap 155, Pap 291, AE2)	1713	482			*LSIL* 14.9 HIV+, 2.3 HIV– *HSIL* 2.5 HIV+, 1.2 HIV–	8.9 (4.81–16.4) 2.68 (1.13–6.34)	Cytology	Women were participants in the WIHS; HPV testing was performed on a cervicovaginal lavage specimen.
Palefsky *et al*. (1999), USA	MY09 MY11/HMB01 with probing (6, 11, 16,18, 26, 31, 32, 33, 35, 39, 40, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 66, 68, 69, 70, 73, Pap 155, Pap 291, AE2)	1778	500	63.4 HIV+, 29.8 HIV–	4.08 (3.29–5.05)			Cytology	Women were participants in the WIHS; HPV testing was performed on a cervicovaginal lavage specimen.
Stratton *et al*. (1999), USA	No HPV DNA detection	452 pregnant 126 non-pregnant				*LSIL* 17.0 pregnant, 23.8 non-pregnant *HSIL* 2.0 pregnant, 2.4 non-pregnant		Cytology	Women were participants in the WITS; 240 women had a Pap smear *post partum*.
Temmerman *et al*. (1999), Kenya	GP5+/GP6+ primers with probing (6, 11, 16, 18, 31, 33)	51	469	41.2 HIV+, 14.3 HIV–	3.91 (2.00–7.65)	17.6 HIV+, 5.1 HIV–	4.77 (1.84–12.36)	Cytology	HPV testing on endocervical brush specimens; women recruited from a family planning clinic in Nairobi

Table 70 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p* value	%	Odds ratio (95% CI)/ *p*-value		
Ahdieh *et al*. (2000), USA	MY09/MY11/HMB01 PCR primers	184	84	69.6 HIV+, 75.7 $CD4^+$ < 200, 68.5 $CD4^+$ ≥ 200, 26.2 HIV–					Women were participants in the ALIVE cohort and followed semi-annually; HPV was tested on cervicovaginal lavage specimen.
Ammatuna *et al*. (2000), Italy	HC2 and 2-step PCR combining MY09/ MY11 primers with GP5+/GP6+ PCR	110		60.9 HIV+		53.6 HIV+	3.55 (1.96–6.48)	Cytology, histology	HPV typing performed on Ayre's spatula specimen
Branca *et al*. (2000), Italy	MY09/MY11 PCR with RFLP analysis (16, 18, 31, 33, 35, 53, 58)	266	193			*LSIL* 21.8 HIV+, 6.6 HIV– *HSIL* 7.6 HIV+, 3.4 HIV–	3.9 (2.2–7.0) for SIL	Cytology	Women were participants in the DIANAIDS cohort; HIV– women were at high risk for HPV infection; an Ayre's spatula specimen was used for HPV testing.
Cubie *et al*. (2000), Scotland, United Kingdom	HC2	63		12.5 HIV VL < 500, 15.4 HIV VL 500–5000, 32.4 HIV VL 5000–50 000, 52.9 HIV VL > 50 000		25.0 HIV VL < 500, 23.1 HIV VL 500–5000, 37.8 HIV VL 5000–50 000, 52.9 HIV VL > 50 000		Cytology, histology	Sampling from Cervex brush; women followed at 6-month intervals

Table 70 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p* value	%	Odds ratio (95% CI)/ *p*-value		
French *et al*. (2000), USA	MY09/MY11/HMB01 (6, 11, 16,18, 26, 31, 32, 33, 35, 39, 40, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 66, 68, 69, 70, 73, Pap 155, Pap 291, AE2)	1314		66.2 overall, 96.2 SIL, 61.3 normal cytology	11.45 (5.51–23.82)			Cytology	Women were participants in the WIHS; HPV testing was performed on a cervicovaginal lavage specimen; women in this analysis had measurement of serum retinol; multivariate odds ratio
Heard *et al*. (2000), France	MY09/MY11 primers (16, 18, 33) plus SBH with probing (6/11/42, 16/18/33, 31/35/39, other types) and sequencing of unidentifiable types	307		49.5 by PCR 55.5 by SBH		13.7 CIN1 13.3 CIN2/3		Cytology, histology	HPV testing on cotton swab and cervical spatula specimens
Marais *et al*. (2000), South Africa	MY09/MY11 primers and an HPV 16-specific primer set	47	52	85.1 HIV+, 42.3 HIV–	$p = 0.00001$				HPV testing performed on cervicovaginal lavage specimens; participants were commercial sex workers.
Moscicki *et al*. (2000), USA	MY09/MY11 primers (6, 11, 42, 44, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58)	133	55	77.4 HIV+, 54.5 HIV– *HR* 54.9 HIV+, 29.1 HIV–	1.4 (1.1–1.8) 1.8 (1.2–2.7)	*SIL* 33.1 HIV+, 10.9 HIV–		Cytology	Young women aged 13–18 years were participants in the REACH cohort; HPV testing was performed on a cervicovaginal lavage specimen; most of the women had high $CD4^+$ levels, with 50% having $CD4^+ > 500/mm^3$.

Table 70 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p* value	%	Odds ratio (95% CI)/ *p*-value		
Torrisi *et al.* (2000), Italy	MY09/MY11 PCR with restriction endonuclease typing	104	106 previously normal (Group 2), 112 previously abnormal cytology (Group 3)	53.8 HIV+, 6.6 Group 2, 41.9 Group 3		*SIL* 50 HIV+, 5.7 Group 2, 56.3 Group 3		Cytology, histology	HPV performed on cervicovaginal lavage specimen
Womack *et al.* (2000), Zimbabwe	HC2	249	217	64.3 HIV+, 27.6 HIV–		17.3 HIV+, 5.9 HIV–		Cytology, histology	HPV typing performed on Cyto-soft cervical brush specimen; primary care setting in Harare, Zimbabwe
Duerr *et al.* (2001), USA	SBH/HC2 and MY09/MY11 primers (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 66, 68, 70, Pap 155, Pap 291, W13B)	709	341	64.5 HIV+, 29.2 HIV–	$p < 0.001$	18.8 HIV+, 5.3 HIV–	$p < 0.001$		Cervicovaginal lavage specimen used for HPV testing; women were participants in the HERS.
Hameed *et al.* (2001), USA	HC	209		48 HIV+		11 LSIL 0.1 HSIL		Cytology	Study of HPV testing and cytology among women being followed for HIV infection; HPV testing performed on cervical swab material

Table 70 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p* value	%	Odds ratio (95% CI)/ *p*-value		
Mayaud *et al*. (2001), Tanzania	MY09/MY11 primers with reverse line blot assay and specific priming (6, 11, 16, 18, 31)	100	555	*Age* *< 20 years* 41 HIV+, 34 HIV– *20–29 years* 33 HIV+, 36 HIV– *≥ 30 years* 33 HIV+, 25 HIV–	1.02 (0.6–16)			Cytology	Pregnant women in Tanzania studied; endocervical cytobrush specimen used for HPV testing
Thomas *et al*. (2001a), Thailand	MY09/MY11 PCR (6/11, 16, 18, 31, 33, 35, 39, 45)	37	214	*6/11* 27.0 HIV+, 8.4 HIV– *16* 24.3 HIV+, 12.1 HIV– *31/33/35/39* 27.0 HIV+, 11.7 HIV–	1.1 (0.4–2.9) 1.2 (0.4–3.2) 1.2 (0.5–3.3)			Cytology	Study of commercial sex workers in Bangkok; HPV testing performed on cervical samples obtained with a Teflon-coated swab
Volkow *et al*. (2001), Mexico	MY09/MY11 primers and GP5/GP6 primers, specific priming of HPV 16 E6/E7 and HPV 18 LCR	85	44 with HIV+ male partner, 55 commercial sex workers	68.7 HIV+, 28.6 HIV–		*SIL* 17.8 HIV+, 12.5 HIV– *HSIL* 8.2 HIV+, 1.8 HIV–	$p < 0.05$	Cytology, histology	HPV testing performed on cervical cytobrush
Chirenje *et al*. (2002), Zimbabwe	No HPV DNA detection	207	355			25.6 HIV+, 6.7 HIV–	$p < 0.001$	Cytology, histology	Women aged 18–50 years recruited from family health centres and family planning clinics in Harare

Table 70 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p* value	%	Odds ratio (95% CI)/ *p*-value		
Jamieson *et al.* (2002), USA	SBH (11, 16, 18, 51, 52, 53) and MY09/MY11 primers (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 66, 68, 70, Pap 155, Pap 291, W13B)	767	390	63.7 HIV+, 27.4 HIV–	2.3 (2.0–2.8)			Cytology	Cervicovaginal lavage specimen used for HPV testing. Women were participants in the HER.
Levi *et al.* (2002), Brazil	SPF10 primers with reverse line blot (6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68/73, 70, 74)	208		98.1 HIV+; most common types: 6, 39.2; 51, 31.9; 11, 26.0; 18, 24.0; 16, 22.5		13.4% Pap III on cytology		Cytology, histology	Cervical cytobrush specimens were used for HPV testing.
Tate & Anderson (2002), USA	No HPV DNA detection	43	103			73 HIV+, 27 HIV–	$p = 0.019$	Cytology, histology	
Hawes *et al.* (2003), Senegal	MY09/MY11 primers (16, 18, 31, 33, 35, 45, 51, 52, 56)	335 HIV-1 only, 69 HIV-2 only, 29 HIV-1 and HIV-2	3686	69.1 HIV-1 only, 61.8 HIV-2 only, 67.9 HIV-1 and 2, 25.3 HIV–		*SIL/ICC* 17.2 HIV-1 only, 19.5 HIV-2 only, 34.4 HIV-1 and 2, 4.0 HIV–	 2.2 (1.0–4.8) 6.0 (2.1–17.1) 8.0 (2.0–3.15)	Cytology, histology	Cervical cell samples obtained for HPV testing; instrument not specified

Table 70 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p* value	%	Odds ratio (95% CI)/ *p*-value		
Baay *et al*. (2004), Zimbabwe	GP5+/GP6+ primers with probing for HR types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) and LR types (6, 11, 34, 40, 42, 43, 44, 54)	61	174	54.0 HIV+ 27.0 HIV–	3.18 (1.67–6.10)	15.3 HIV+, 6.0 HIV–	$p = 0.037$	Cytology	Cervicovaginal lavage samples used for HPV testing
Branca *et al*. (2004), Italy	MY09/MY11 PCR plus type-specific priming (E6/E7) (6/11, 16, 18, 31, 33, 35, 45, 52, 53, 58, 66)	17	227	35.7 HIV+, 29.2 HIV–	$p = 0.43$	*HSIL on Pap* 50 HIV+, 18.2 HIV–	4.5 (1.08–18.8)	Cytology, histology	Women were patients referred for assessment of abnormal Pap smears; exocervical and endocervical specimens used for HPV testing
Levi *et al*. (2004), Brazil	HC2 and PGMY primers with line blot probing	255	36	87 HIV+, 100 HIV–				Cytology, histology	Cervical brush samples used for HPV testing; HIV+ women were enrolled during routine gynaecological visit; HIV–controls were all referred to the gynaecologist due to suspicion of CIN or condyloma.

Table 70 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p* value	%	Odds ratio (95% CI)/ *p*-value		
Strickler *et al.* (2005), USA	MY09/MY11/HMB01 (6, 11, 16, 18, 26, 31, 32, 33, 35, 39, 40, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 66, 68, 69, 70, 73, Pap 155, Pap 291, AE2)	1848	514		$p < 0.001$ incident HPV detection versus persistence of HPV infection			Cytology	Women were participants in the WIHS; HPV testing was performed on a cervicovaginal lavage specimen; relationship between HIV viral load, $CD4^+$ level and prevalence, incidence and persistence of HPV infection or SIL examined; interaction was stronger for prevalent and incident infection or SIL than persistent infection or SIL.

See Table 7 for a description of the primers used.

ALIVE, AIDS Link to Intravenous Drug Experience; CI, confidence interval; CIN, cervical intraepithelial neoplasia; DIANAIDS, Italian collaborative study on HIV/HPV; HC2, Hybrid Capture 2; HERS, HIV Epidemiology Research Study; HIV, human immunodeficiency virus; HIV+, HIV-positive; HIV–, HIV-negative; HR, high-risk; HSIL, high-grade squamous intraepithelial lesion; ICC, invasive cervical cancer; IDU, intravenous drug user(s); LCR, long control region; LR, low-risk; LSIL, low-grade squamous intraepithelial lesion; Pap, Papanicolaou test; PCR, polymerase chain reaction; REACH, Reaching for Excellence in Adolescent Care and Health; RFLP, restriction fragment length polymorphism; SBH, Southern blot hybridization; STD, sexually transmitted disease; VL, viral load; WIHS, Women's Interagency HIV Study; WITS, Women and Infant Transmission Study

Ferrera *et al*. (1997b) studied the prevalence of HPV infection in 23 HIV-positive and 28 HIV-negative prostitutes in Tegucigalpa, Honduras: 56.5% of HIV-positive and 18.0% HIV-negative women were positive for HPV DNA (odds ratio, 6.0; 95% CI, 1.5–26.7).

Frankel *et al*. (1997) characterized the prevalence of SIL among a group of HIV-positive women admitted to Yale-New Haven Hospital for an HIV-related illness. Of these, 55 women had cytology adequate for interpretation and 47.3% had evidence of cervical SIL.

Rezza *et al*. (1997) studied 135 HIV-positive and 101 HIV-negative women in Italy. SIL was diagnosed in 35.6% of HIV-positive and 8.9% of HIV-negative women (odds ratio, 5.64; 95% CI, 2.48–13.17). HPV DNA was detected in 72% of women with SIL and in 25% of women without SIL. HPV DNA was detected more often among HIV-positive (40%) than among HIV-negative (32%) women (not statistically significant). Multivariate analysis of risk factors for SIL included the use of oral contraceptives (adjusted odds ratio, 2.64; 95% CI, 1.16–6.00), being HIV-positive with < 200 $CD4^+$ cells (adjusted odds ratio, 7.29; 95% CI, 2.12–24.02) and having a high-risk HPV type (adjusted odds ratio, 17.53; 95% CI, 6.25–49.23).

In a young population (13–21 years) of 82 HIV-positive women in Brazil (Calore *et al.*, 1998), 21 (26%) showed characteristic features of HPV-infection and SIL.

La Ruche *et al*. (1998) reported on 2170 HIV-positive and HIV-negative women in Abidjan, Cote d'Ivoire. Women with LSIL, HSIL or cervical cancer were enrolled as were controls without cervical disease. Risk factors for cervical lesions that were studied included cervical HPV infection and HIV-1 and HIV-2 positivity. In a multivariate analyses, LSIL was associated with HPV positivity (adjusted odds ratio, 4.4; 95% CI, 2.6–7.4), HIV-1 positivity (adjusted odds ratio, 2.1; 95% CI, 1.2–3.7) and parity of more than three children (adjusted odds ratio, 2.1; 95% CI, 1.1–3.9). Risk factors for HSIL in the multivariate analysis included HPV positivity (adjusted odds ratio, 14.3; 95% CI, 6.6–30.9), HIV-1 positivity (adjusted odds ratio, 2.3; 95% CI, 1.1–4.7), tobacco chewing (adjusted odds ratio, 5.4; 95% CI, 1.3–22.9) and illiteracy (adjusted odds ratio, 2.1; 95% CI, 1.0–4.3). The only risk factor for cervical cancer that was significant was HPV positivity (adjusted odds ratio, 13.3; 95% CI, 3.2–55.5). A wide diversity of HPV types was found in LSIL and HSIL, but HPV 16 was the most common.

Maiman *et al*. (1998) examined risk factors for SIL in 253 HIV-positive and 220 HIV-negative women in the New York area, USA, and evaluated the sensitivity and specificity of cervical cytology in HIV-positive women. The sensitivity and specificity of cytology for all grades of CIN were 0.60 and 0.80 and those for high-grade CIN were 0.83 and 0.74, respectively. Abnormal cytology was found in 32.9% of HIV-positive women and 7.6% of HIV-negative women. In a multivariate analysis, risk factors for abnormal cytology were HIV infection with > 500 $CD4^+$ cells ($p = 0.002$), 200–499 $CD4^+$ cells ($p < 0.001$) and < 200 $CD4^+$ cells ($p < 0.001$) and HPV infection ($p < 0.001$).

Rezza *et al.* (1998) studied risk factors for cervical HPV infection in 135 HIV-positive and 101 HIV-negative Italian women at risk for HIV infection (intravenous drug users or sexual partners of men at risk for HIV infection). Women who were intravenous drug users

were at nearly threefold higher risk for HPV infection than heterosexual women (adjusted odds ratio, 2.7; 95% CI, 1.4–5.0), and this difference was not influenced by HIV serostatus.

Rugpao *et al*. (1998) studied 224 HIV-positive and 257 HIV-negative women who were partners of men with HIV-1 infection in Chiang Mai, Thailand. HIV-positive women were five times more likely to have an infection with a high-risk HPV type and cervical SIL. The prevalence of SIL increased with decreasing $CD4^+$ cell count, but the trend was not significant.

Six *et al.* (1998) investigated the impact of HIV infection on the prevalence, incidence and short-term prognosis of CIN in a prospective study among women from France and French Guyana with a 1-year follow-up. Prevalence of CIN was 7.5% among HIV-negative women and 26.5% among HIV-positive women. Factors associated independently with prevalence of CIN were lower level of $CD4^+$ cells, infection with HPV 16, 18 and 33 and related types, infection with HPV 31, 35 and 39 and related types, lifetime number of sexual partners, younger age, past history of CIN and lack of past cervical screening. Of 344 women, 278 were followed for 1 year at 6-month intervals. Incidence of CIN ranged from 4.9% in HIV-negative women to 27% in HIV-positive women with $< 500 \times 10^6$/L $CD4^+$ cells ($p < 0.001$). Progression from LSIL to HSIL during follow-up was detected in 38.1% of HIV-positive women with $\leq 500 \times 10^6$/L $CD4^+$ cells but not in HIV-negative women or HIV-positive women with $> 500 \times 10^6$/L $CD4^+$ cells. HPV 16, 18 and 33 and related types were also associated with higher incidence of CIN and progression from low-grade to high-grade CIN.

Uberti-Foppa *et al*. (1998) studied the prevalence of HPV and cytological abnormalities in HIV-positive women in Milan, Italy. Using Hybrid Capture 2, 66% of HIV-positive women and 15% of HIV-negative women were positive for HPV DNA ($p < 0.0001$). PCR gave positive results for HPV DNA in 91% and 48%, respectively ($p < 0.001$). No significant difference was observed with respect to prevalence of HPV between women who acquired it through intravenous drug use and women who acquired HIV through heterosexual contact ($p = 0.09$). LSIL and HSIL were both more common among intravenous drug users than among women who acquired HIV through sexual contact.

Cu-Uvin *et al*. (1999) examined the prevalence of cervical HPV infection in 851 HIV-positive women and 434 HIV-negative women at high risk in the HIV Epidemiology Research Study. HPV infection was more prevalent among HIV-positive women (64% versus 28%). This study showed no statistically significant difference in the prevalence of lower genital tract infections other than HPV between HIV-positive and HIV-negative women.

Goncalves *et al*. (1999) studied cervical cytology and HPV from cervical, vaginal and perianal scrapes from 141 HIV-positive women in Santos City, Brazil. One or more specimens that were positive for HPV DNA were found in 80.8% of patients. Two or more HPV types were detected in 45% of the samples. The most frequent HPV types detected were 16 and 18 (30.5%) and, overall, 34.8% had high-risk types; 65.7% of vaginal samples, 64.4% of cervical samples and 47.4% of perianal samples were positive for HPV DNA.

Hankins *et al*. (1999) examined risk factors for prevalent HPV infection in 375 women who participated in the Canadian Women's HIV Study: 67.2% of the women were positive for HPV DNA and 10.9% had SIL. Women with SIL were more likely to have HPV infection than those without SIL ($p = 0.002$). In a multivariate analysis, risk factors for HPV infection included $CD4^+$ cell count $< 0.20 \times 10^9$/L (adjusted odds ratio, 1.99; 95% CI, 1.17–3.37), non-white race (adjusted odds ratio, 2.00; 95% CI, 1.17–3.42), inconsistent use of condoms in the 6 months before study entry (adjusted odds ratio, 2.02; 95% CI, 1.16–3.50) and lower age; women aged 30–39 years (adjusted odds ratio, 0.51; 95% CI, 0.30–0.87) and 40 years or older (adjusted odds ratio, 0.52; 95% CI, 0.26–1.0) had lower risks than women aged < 30 years.

Kapiga *et al*. (1999) studied the prevalence of and risk factors for cervical SIL among 691 HIV-positive women who attended antenatal clinics in Dar es Salaam, Tanzania. Cytology was collected 3–6 months *post partum*. Mid-upper arm circumference was measured as an indicator of body wasting. The prevalence of SIL in the study population was 2.9%; 55% of the lesions were LSIL and 45% were HSIL. Risk factors for SIL in multivariate analysis included having a $CD4^+$ cell count < 200/mm^3 (odds ratio, 6.15; 95% CI, 1.19–41.37) and decreased by 68% for each 5-cm increase in mid-upper arm circumference (odds ratio, 0.32; 95% CI, 0.10–0.93), which indicated that more advanced HIV-related immunosuppression and body wasting were the primary risk factors for SIL in this population.

Leroy *et al*. (1999) studied the prevalence of SIL and its association with HIV-1 infection among 103 HIV-positive and 107 HIV-negative pregnant women in Kigali, Rwanda. The prevalence of SIL was higher in HIV-positive women than in HIV-negative women: 24.3% versus 6.5% (odds ratio, 4.6; 95% CI, 1.8–12.3); that of LSIL was 14.6% in HIV-negative and 4.6% in HIV-positive women; and that of HSIL was 9.7% and 1.9%, respectively.

Luque *et al*. (1999) included 93 HIV-positive women in upstate New York (USA) in a cross-sectional study to evaluate the relationship between plasma HIV-1 RNA levels and cervical HPV infection. HIV-1 RNA plasma levels of > 10 000 copies/mL were associated with the detection of high-risk HPV DNA types in cervical specimens (relative risk, 2.57; 95% CI, 1.29–13.56). In addition, similar HIV-1 RNA plasma levels were associated with abnormal Pap smears (relative risk, 2.11; 95% CI, 1.12–10.19).

Massad *et al*. (1999) studied the prevalence of and risk factors for abnormal cervical cytology among 1713 HIV-positive women and 482 risk-matched HIV-negative control women who participated in the Women's Interagency HIV Study. Cervical cytology was abnormal in 38.3% of HIV-positive women and 16.2% of HIV-negative women. HSIL was found in only 2.5% of the HIV-positive women. In a multivariate analysis, risk factors for abnormal cytology included HIV infection, lower $CD4^+$ cell count, higher level of HIV RNA, HPV positivity, previous history of abnormal cytology, being employed and the number of male sex partners within 6 months of enrolment. Having more than one abortion was associated with a decreased risk for cytological abnormality.

Palefsky *et al*. (1999) characterized the prevalence of 39 different HPV types in 1778 HIV-positive and 500 HIV-negative women participating in the Women's Interagency HIV Study. HIV-positive women had an increased risk for HPV infection (odds ratio, 4.08; 95% CI, 3.29–5.05). The distribution of HPV types was wide in both HIV-positive and HIV-negative women. HPV 16 was found in 5.2% of HIV-positive and 2.0% of HIV-negative women ($p < 0.001$). The prevalence of most HPV types increased with progressively lower $CD4^+$ cell strata. HPV 16 was not one of these types (see comments by Strickler *et al*., 2003; Section 2.8.3(*a*)(ii)). In a multivariate analysis, HIV-positive women with a $CD4^+$ cell count $< 200/mm^3$ were at highest risk for HPV infection compared with HIV-negative women, regardless of HIV RNA load (odds ratio, 10.13; 95% CI, 7.32–14.04), followed by women with a $CD4^+$ cell count $> 200/mm^3$ and an HIV RNA load > 20 000 copies/mL (odds ratio, 5.78; 95% CI, 4.17–8.08) and those with a $CD4^+$ cell count $> 200/mm^3$ and an HIV RNA load < 20 000 copies/mL (odds ratio, 3.12; 95% CI, 2.36–4.12), after adjustment for other factors. Other risk factors for HPV infection among HIV-positive women included racial/ethnic background (African-American versus Caucasian; odds ratio, 1.64; 95% CI, 1.19–2.28), current tobacco smoking (odds ratio, 1.55; 95% CI, 1.20–1.99) and younger age (age < 30 years versus ≥ 40 years; odds ratio, 1.75; 95% CI, 1.23–2.49).

Stratton *et al*. (1999) studied the prevalence of SIL in a cohort of 452 pregnant and 126 non-pregnant HIV-positive women who participated in the Women and Infant Transmission Study. The prevalence of SIL was similar for pregnant (17.0%) and non-pregnant women (23.8%) ($p = 0.09$). In a multivariate analysis, a lower percentage of $CD4^+$ cells ($p < 0.001$), HSV infection ($p = 0.03$) and inflammation on the Pap smear ($p < 0.001$) were all associated with SIL, but pregnancy status was not.

Temmerman *et al*. (1999) studied 51 HIV-positive and 469 HIV-negative women at a family planning clinic in Nairobi, Kenya. In a multivariate analysis, detection of HPV was associated with HIV-1 infection (odds ratio, 3.9; 95% CI, 2.0–7.7) and the number of pregnancies (for ≥ 3 pregnancies compared with 0 or 1; odds ratio, 0.4; 95% CI, 0.2–0.9). HPV infection was strongly associated with high-grade CIN (odds ratio, 14.9; 95% CI, 6.8–32.8); 17.6% of HIV-positive and 5.1% of HIV-negative women had CIN as detected by a Pap test (odds ratio, 4.77; 95% CI, 1.84–12.36). In a multivariate model, predictors of high-grade CIN included HIV-1 positivity (odds ratio, 4.8; 95% CI, 1.8–12.4), the number of lifetime sexual partners (for ≥ 4 partners compared with 0 or 1; odds ratio, 3.8; 95% CI, 1.1–13.5) and education (for secondary compared with primary; odds ratio, 0.38; 95% CI, 0.17–0.88).

Ahdieh *et al*. (2000) studied 184 HIV-positive and 84 HIV-negative women who participated in the ATDS Link to Intravenous Drug Experience cohort and were followed semi-annually over a 6-year period. Of the 187 participants who were positive for HPV at least once, the probability of subsequent HPV positivity was 47.5% for HIV-negative women, 78.7% for HIV-positive women with $CD4^+$ cell counts ≥ 200 and 92.9% for HIV-positive women with $CD4^+$ cell counts < 200 cells/μL ($p < 0.001$). Compared with HIV-infected participants, the relative incidence of HPV clearance was 0.29% and 0.10%

among HIV-positive women with $CD4^+$ cell counts ≥ 200 and < 200 cells/μL ($p < 0.001$), respectively.

Ammatuna *et al*. (2000) studied the presence of HPV DNA in cervical scrapings from 110 HIV-positive women. Using PCR, HPV DNA was found in 60.9% of the samples. Using Hybrid Capture 2, low-risk HPV types were found in 19.4% of the patients, high-risk HPV types in 41.8% and both low-risk and high-risk types in 38.8%. CIN was found in 53.6% of the women. HPV was associated with the detection of CIN (odds ratio, 3.55; 95% CI, 1.96–6.48).

Branca *et al*. (2000) studied 266 HIV-positive and 193 HIV-negative women at high risk in Italy. HIV-positive women were more likely to have SIL (odds ratio, 3.9; 95% CI, 2.2–7.0), most of which were low-grade, while a high prevalence of HPV DNA PCR genotypes was observed in both groups: 48.5% of HIV-positive women and 52% of HIV-negative women had one or more high-risk HPV types detected by PCR.

In a study from Scotland, United Kingdom (Cubie *et al.*, 2000) that included 63 HIV-infected women, high-risk HPV types were detected in 25% of those with normal cytology, while over 80% of women with abnormal cytology were high-risk HPV-positive.

French *et al.* (2000) explored the relationship between vitamin A (retinol) deficiency and SIL in 1314 HIV-positive women who participated in the Women's Interagency HIV Study. At the baseline visit, 15.5% had retinol concentrations consistent with deficiency (< 1.05 μmol/L). In a multivariate model, SIL was associated with retinol concentrations < 1.05 μmol/L (odds ratio, 1.62; 95% CI, 1.02–2.58) together with HPV infection (odds ratio, 11.45; 95% CI, 5.51–23.82), older age (per 10 years; odds ratio, 0.57; 95% CI, 0.44–0.76), being Hispanic/Latin American (odds ratio, 1.86; 95% CI, 1.03–3.37), higher $CD4^+$ levels (per 100 cells/mm^3; odds ratio, 0.80; 95% CI, 0.72–0.88), a body mass index < 18.5 (odds ratio, 2.16; 95% CI, 1.05–4.47) or a body mass index > 25.0 (odds ratio, 0.66; 95% CI, 0.46–0.93).

Heard *et al*. (2000) studied risk factors for CIN in 307 HIV-positive women. CIN was diagnosed in 27.0% and HPV infection in 52.8% of the women. Among all HPV-positive women, high HPV load was found in 55.6%. High HPV viral load was more common among women with $CD4^+$ cell counts < 200/μL compared with those with $CD4^+$ cell counts > 200/μL ($p = 0.002$). High HPV viral load was also associated with an increased risk for CIN in a multivariate analysis in comparison with HPV-negative women (adjusted odds ratio, 16.8; 95% CI, 7.0–40.3). Low HPV viral load was a risk factor for CIN only in women with $CD4^+$ cell counts < 200/μL (adjusted odds ratio, 7.4; 95% CI, 1.3– 43.0).

Marais *et al*. (2000) studied the prevalence of HPV infection in 47 HIV-positive and 52 HIV-negative sex workers in South Africa as well the prevalence of antibodies to HPV 16 VLP by ELISA in cervicovaginal lavage and serum specimens. HIV-positive women had a significantly higher prevalence of HPV DNA than HIV-negative women (85% versus 42%; $p = 0.00001$). They also had a lower rate of positivity than HIV-negative women for serum IgA antibodies ($p = 0.012$) but a higher rate of positivity for cervical anti-VLP 16 IgG antibodies ($p = 0.002$).

Moscicki *et al*. (2000) studied the prevalence of cervical HPV infection and SIL in 133 HIV-positive and 55 HIV-negative women aged 13–18 years who participated in the Reaching for Excellence in Adolescent Care and Health cohort. Few of the HIV-infected women (6.6%) had $CD4^+$ cell levels < 200/mm^3. HPV infection was found in 77.4% of HIV-positive and 54.5% of HIV-negative women (relative risk, 1.4; 95% CI, 1.1–1.8). Among those with HPV infection, 70.1% of the HIV-positive and 30% of the HIV-negative women had abnormal cytology ($p < 0.001$). In a multivariate analysis, HIV positivity was a significant risk factor for both HPV infection (odds ratio, 3.3; 95% CI, 1.6–6.7) and SIL (odds ratio, 4.7; 95% CI, 1.8–14.8). $CD4^+$ cell count and HIV viral load were not associated with HPV infection or SIL.

Torrisi *et al*. (2000) examined the prevalence of HPV in 104 HIV-positive women, 106 HIV-negative women with previously normal cytology (Group 2) and 112 HIV-negative women with previously abnormal cytology (Group 3). SIL was found in 50% of HIV-positive versus 5.66% of HIV-negative Group 2 ($p < 0.001$) and 56.3% of HIV-negative Group 3 women ($p = 0.433$). HPV DNA positivity was found in 53.8% of HIV-positive, 6.6% of HIV-negative Group 2 and 42% HIV-negative Group 3 women. Multiple HPV types were found in 21.4% of HIV-positive women.

Womack *et al*. (2000) characterized cervical HPV infection in 466 women at high risk for HIV infection during primary cervical cancer screening in Zimbabwe. Compared with HIV-negative women, HIV-positive women had a more than twofold prevalence of HPV (64.3% versus 27.6%), a nearly threefold higher prevalence of high-grade CIN (17.3% versus 5.9%) and more than sevenfold the amount of HPV DNA. The amount of HPV DNA increased with severity of disease in both HIV-negative and HIV-positive women.

Duerr *et al*. (2001) examined risk factors for SIL among 709 HIV-positive and 341 HIV-negative women who participated in the HIV Epidemiology Research Study. SIL was more common among HIV-positive than among HIV-negative women (18.8% versus 5.3%; $p < 0.001$) as was HPV infection (64.5% versus 29.2%; $p < 0.001$). In a multivariate analysis, the association with SIL was higher for high-risk HPV types (adjusted prevalence ratio, 27.0; 95% CI, 12.5–58.4) than for low-risk types (adjusted prevalence ratio, 10.5; 95% CI, 4.5–24.6). Intermediate-risk types showed little difference from high-risk types (adjusted prevalence ratio, 25.0; 95% CI, 11.6–54.2). Lower $CD4^+$ cell levels were also associated with SIL but more weakly than HPV infection ($CD4^+$ < 200; adjusted prevalence ratio, 1.9; 95% CI, 1.2–3.0; $CD4^+$ 200–500; adjusted prevalence ratio, 1.6; 95% CI, 1.0–2.5).

Hameed *et al.* (2001) studied 209 HIV-positive women for whom Hybrid Capture and cytology data were available. One hundred and one women (48%) were positive for HPV subtypes by DNA typing by this method; 19/9% had SIL according to cytology, most of which were low-grade.

Mayaud *et al*. (2001) studied the relationship between HPV infection, HIV infection and SIL in 100 HIV-positive and 555 HIV-negative pregnant women in Tanzania. There was no association between HPV and HIV (odds ratio, 1.02; 95% CI, 0.6–1.6). SIL was

associated with HPV (odds ratio, 3.66; 95% CI, 1.9–7.0), but not with HIV (odds ratio, 1.54; 95% CI, 0.7–3.4).

Thomas *et al.* (2001a) reported no significant association between HPV and HIV infection in a study of sex workers in Bangkok.

Volkow *et al*. (2001) studied the prevalence of HPV infection and SIL in 85 HIV-positive and 99 HIV-negative women at high risk in Mexico. Cases included women who were positive for HIV and accepted to participate. HPV DNA was detected by PCR in 69% of HIV-positive women and 29% of HIV-negative women ($p < 0.0001$).

Chirenje *et al*. (2002) performed a cross-sectional study of the prevalence of CIN among 207 HIV-positive women and 355 HIV-negative women who attended a family health centre and family planning clinics in Harare, Zimbabwe. Cervical cytology was abnormal in 25.6% of HIV-positive women compared with 6.7% of HIV-negative women ($p < 0.001$).

Jamieson *et al*. (2002) examined risk factors for HPV infection and its association with cytological abnormalities at baseline in 767 HIV-positive women and 390 HIV-negative women in the HIV Epidemiology Research Study. HIV-positive women were more likely to have HPV infection than HIV-negative women (prevalence ratio, 2.3; 95% CI, 2.0–2.8). The distribution of HPV types was similar between the HIV-positive and HIV-negative women. HPV viral loads as measured by PCR dot blot signal strength were higher among HIV-positive than among HIV-negative women as was the proportion of HPV-positive women with multiple HPV types. Among women with high HPV viral load, HIV infection was not associated with SIL.

Levi *et al*. (2002) examined the prevalence of HPV infection and multiplicity of HPV types in 208 HIV-positive women in Brazil. Almost all women (98%) were HPV-positive; 78.9% had multiple HPV types with an average of three per patient. HPV 6 was the most common genotype (39.2%) followed by types 51 (31.9%), 11 (26.0%), 18 (24.0%) and 16 (22.5%); 28 patients (13.4%) had a Pap III score. The prevalence of high-risk genotypes increased with the cytological classification. There were no significant associations between the number of HPV genotypes, abnormal cytology, HIV viral load and $CD4^+$ cell count.

Tate and Anderson (2002) compared recurrence rates of CIN after ablation and hysterectomy in 43 HIV-positive women with those in 103 HIV-negative women. All patients were followed up for at least 24 months. Recurrence was greater in the HIV-positive women for all treatment modalities (73% versus 27%; $p = 0.019$). Higher recurrence rates were seen in women with $CD4^+$ cell counts < 200 cells/mm^3 compared with women with $CD4^+$ cell counts > 200/mm^3 (55% versus 26%; $p = 0.002$). The mean HIV viral load was also higher among women who had recurring disease than among those who did not (18 384 versus 3892; $p = 0.002$).

Hawes *et al*. (2003) studied 4119 women who attended an outpatient clinic in Senegal, an area in which both HIV-1 and HIV-2 are highly prevalent in the population. Among women infected with high-risk HPV, those with HIV-1 (odds ratio, 2.2; 95% CI, 1.0–4.8), HIV-2 (odds ratio, 6.0; 95% CI, 2.1–17.1) or both HIV-1 and HIV-2 (odds ratio, 8.0;

95% CI, 2.0–31.5) were more likely to have HSIL or cervical cancer than HIV-negative women. This relationship was not detected among women without high-risk HPV infection. HIV-2-positive women were more likely to have HSIL (odds ratio, 3.3; 95% CI, 0.9–12.4) or cervical cancer (odds ratio, 7.9; 95% CI, 1.1–57) than HIV-1-positive women. The authors hypothesized that the increase in risk associated with HIV-2 infection may reflect the longer periods of mild immunosuppression than are typically seen with HIV-1, and this may be relevant to the effect of highly active antiretroviral therapy (HAART) on the natural history of CIN.

Baay *et al*. (2004) studied the prevalence of cervical HPV infection in a population of women from rural Zimbabwe. The prevalence of HPV was higher in HIV-positive (54%) than in HIV-negative women (27%) (odds ratio, 3.18; 95% CI, 1.67–6.10). The most common HPV types in HIV-positive women were 33 (5.2%), 35 (4.6%), 45 (4.6%) and 58 (4.6%); HPV 16 was found in only 3.4%. Among HIV-negative women, the most common types were HPV 35 (11.5%), 6 (9.8%) and 58 (8.2%); HPV 16 was found in only 3.3%.

Branca *et al.* (2004) assessed risk factors and HPV-related mechanisms of CIN in 17 HIV-positive and 227 HIV-negative women in Italy. HPV prevalence was 36% in HIV-positive and 29% in HIV-negative women. HIV-positive women had more frequent HSIL Pap tests ($p = 0.04$), CIN2 or higher in cervical biopsy ($p = 0.049$) and external genital warts ($p = 0.019$).

Levi *et al*. (2004) studied HIV-positive women from Sao Paulo, Brazil. HPV-DNA prevalence was 87% in HIV-positive women, and 45% were infected by more than two types, compared with 8.3% in HIV-negative women. HPV 16 was the most common type found in HIV-positive women (30.9%) followed by types 52 (22.8%) and 59 (20.6%). In HIV-negative women, the most common types were HPV 51 (19.4%), 16 (16.7%) and 73 (16.7%). The number of HPV types detected among HIV-positive women increased, but not significantly, with increasing grade of Pap smear, whereas HPV viral load as measured by Hybrid Capture 2 (Group B high-risk types) was significantly increased ($p < 0.001$).

Strickler *et al*. (2005) studied the effect of HIV RNA level and $CD4^+$ cell count on the natural history of type-specific HPV infection in 1848 HIV-positive and 514 HIV-negative women who participated in the Women's Interagency HIV Study cohort. A strong interaction between the $CD4^+$ cell count and plasma HIV viral load was found for both prevalent ($p = 0.002$) and incident ($p = 0.001$) detection of HPV. The hazard ratio for incident HPV detection was highest among women with a $CD4^+$ cell count < 200/mm^3 (hazard ratio range, 4.0–5.0) or an HIV RNA level > 100 000 copies/mL; the relationship was weaker for persistent HPV infection. Although incident HPV detection was associated with the number of recent sexual partners (p for trend < 0.001), 22% of sexually inactive HIV-positive women with a $CD4^+$ cell count < 200/mm^3 also had at least one incidentally detected HPV type. There was strong interaction between the effects of HIV RNA and $CD4^+$ cell count on incident SIL, but only a weak effect on its persistence. The weak effect of HIV RNA viral load and $CD4^+$ cell count on HPV and persistence of

SIL may explain the limited number of HIV-positive women who develop HSIL and cervical cancer. The data on sexual activity and incident detection of HPV are consistent with the possibility that at least some of the HPV detected in HIV-positive women reflects reactivation of previously acquired HPV infection rather than a newly acquired infection.

(ii) *Natural history of cervical HPV infection and SIL* (Table 71)

Heard *et al*. (1995) followed 43 HIV-positive women who had normal cytology or SIL at baseline every 6 months for up to 18 months; 18 of 19 (95%) women who had SIL at baseline and who were not treated and eight of 13 (61%) women who were treated with surgery had persistent lesions.

Spinillo *et al*. (1996) studied 48 HIV-positive and 38 HIV-negative women with a history of intravenous drug use who attended an antenatal clinic during their first trimester of pregnancy. Participants were re-examined during their second and third trimesters and 8–12 weeks *post partum*: 27.1% of HIV-positive women and 7.9% of HIV-negative women had CIN at their baseline visit ($p = 0.027$). None of the lesions progressed throughout pregnancy in either HIV-positive or HIV-negative women.

Sun *et al*. (1997) compared the persistence of cervical HPV infection among 220 HIV-positive and 231 HIV-negative women in the New York City area, USA. HPV DNA was detected at baseline in 56% of the HIV-positive and 31% of the HIV-negative women. After four examinations, the cumulative prevalence of HPV infection was 83% in the HIV-positive and 62% in the HIV-negative women ($p < 0.001$). Twenty per cent of the HIV-positive and 3% of the HIV-negative women had persistent infections with high-risk types (18 or 45) ($p < 0.001$). The detection of HPV DNA in women who had previously had negative tests was not associated with sexual activity during the interval since the preceding examination, which suggests the possibility of reactivation of a previously acquired HPV infection as an explanation for the detection of at least some of these infections. The risk for persistent HPV infection in HIV-seropositive compared with HIV-negative women was 7.5 (95% CI, 3.6–16).

Minkoff *et al*. (1998) characterized the relationship between HIV status and infection with high-risk HPV types in 268 HIV-positive and 265 HIV-negative women in the New York area, USA. The prevalence at baseline of any HPV type was 73% among HIV-positive and 43% among HIV-negative women ($p < 0.0001$). The respective prevalence of high-risk HPV types was 32.5 and 17.0% ($p < 0.001$). The rate of detection of new high-risk HPV types was almost three times higher among HIV-positive than among HIV-negative women ($p < 0.01$). However, there was no difference in the rate of loss of detection of high-risk HPV types.

Eckert *et al*. (1999) compared the prevalence and type of HPV infection in the genital tract of 23 HIV-positive and 23 HIV-negative women who were matched for cytology. After matching, the groups had a similar prevalence of HPV DNA and of high-risk HPV types at baseline. On follow up, HIV-positive women were more likely to develop SIL (38% versus 10%; $p = 0.03$), to have visits at which HPV DNA was detected (68% versus

Table 71. Natural history of cervical HPV infection and squamous intraepithelial lesions (SIL) in HIV-positive and HIV-negative women

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p*-value	%/incidence	Odds ratio (95% CI)/ *p*-value		
Heard *et al*. (1995), France	No HPV DNA detection	43 SIL				Untreated patients, 95; treated patients, 61		Cytology, histology	Participants followed every 6 months with a Pap smear for at least 18 months
Spinillo *et al*. (1996), Italy	ISH (6/11, 16/18, 31/33/35)	48 CIN1–3	38 CIN1–3	NR		27.1 HIV+, 7.9 HIV–	$p = 0.027$	Biopsy, cytology, histology	Participants were HIV+ women with history of IDU being seen for antenatal care in their first trimester; re-examined in second and third trimester and 8–12 weeks *post partum*.
Sun *et al*. (1997), USA	MY09/MY11 PCR and type-specific primers (16 and 18)	220	231	*At baseline* 56 HIV+, 31 HIV– *Persistent HPV infection* 83 HIV+, 62 HIV–	7.5 (3.6–16)	NR			Persistent HPV infection defined as detection of the same type of HPV at 2 or more examinations during the follow-up period of 3–12 months; HPV testing performed on cervicovaginal lavage; almost all CIN were low-grade.
Minkoff *et al*. (1998), USA	MY09/MY11 PCR primers (2, 6, 11, 13, 26, 32,34, 40, 42, 53, 54, 55, 57, 59, 61, 62, 64, 66, 69, 70, 72, Pap 155, Pap 291, AE2, AE5-8, W13B, 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 68, 73)	268	265	73 HIV+, 43 HIV– *HR HPV* 32.5 HIV+, 17.0 HIV–	$p < 0.0001$ $p < 0.001$			Cytology, histology	HPV testing performed on cervicovaginal lavage specimen; women followed every 6 months
Eckert *et al*. (1999), USA	MY09/MY11 PCR primers (6/11, 31/33/35/39, 16/16/45)	23	23	73.9 HIV+, 65.2 HIV–	$p = 0.4$	*Baseline* 39 HIV+, 39 HIV– *Follow-up* 38 HIV+, 10 HIV–	$p = 0.03$	Cytology, histology	Dacron swabs used to measure HPV in the cervix/ectocervix and vaginal wall; participants matched by cytology results at baseline and followed every 4 months for 56 (HIV+) and 53 visits (HIV–)

Table 71 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p*-value	%/incidence	Odds ratio (95% CI)/ *p*-value		
La Ruche *et al*. (1999), Cote d'Ivoire	MY09/MY11 PCR	38	56	83.3 HIV+ 58.8 HIV–	$p = 0.015$	*Persistent CIN* 76 HIV+, 18 HIV– *Progression to high-grade CIN* 18 HIV+, 0 HIV–	4.3* (2.4–7.7)	Cytology, histology on women with high-grade cytology referred for colposcopy	HPV testing using cervical Viba-Brush specimen; women followed after a median of 5 months from the initial smear; *relative risk
Petry *et al*. (1999), Germany	HC1	138		NR		15.9 CIN1, 12.3 CIN2 or -3		Cytology, histology	
Cubie *et al.* (2000), Scotland, United Kingdom	HC for HR HPV, HC2 with probes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68)	63		Normal cytology, 25; abnormal cytology, 8.0; persistent HPV infection, 42.9		NR			
Ellerbrock *et al*. (2000), USA	MY09/MY11 PCR primers with RFLP typing and E6 primers (16 and 18)	328	325			91% HIV+, 75% HIV–	3.2 (1.7–61)	Cytology, histology	Women had no SIL at enrollment; HPV testing performed on cervicovaginal lavage specimen; Women followed for approximately 30 months

Table 71 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p*-value	%/incidence	Odds ratio (95% CI)/ *p*-value		
Ahdieh *et al*. (2001), USA	MY09/MY11/ HMB01 PCR primers (16/18/31/45, 33/35/39/51/52/ 56/58/59/68, 6/11/26/40/42/53/54/55/ 66/73/82/ 83/84)	871	439	Cumulative pre-valence of HPV infection: increase from 73.4 to 90.2 HIV+ women with $CD4^+$ < 200, increase from 28.1 to 54.0 HIV–				Cytology	Women were participants in the HERS and were followed at 6-month intervals for assessment of type-specific HPV infection; Increased HPV viral load using PCR as indicated by increased dot blot signal strength from 1 to 4
Calore *et al*. (2001), Brazil		1587 (baseline), 409 (follow-up)				12.6 SIL or cervical cancer		Cytology	
Cohn *et al*. (2001), USA	HC 2, HC RLU	103		66 HIV+, 56.3 with HR types HIV+	*p* value = 0.0006	20 CIN after 1 year of follow-up		Cytology, biopsy	Women were participants in the American Foundation for AIDS Research Community Based Clinical Trials Network in 6 US cities; Cervical Dacron swabs used for HPV testing; Women studied at baseline, 6 months and 12 months; Cases had $CD4^+ \leq 500/mm^3$.
Massad *et al*. (2001), USA	MY09/MY11/ HMB01 PCR (6, 11, 16, 18, 26, 31, 32, 33, 35, 39, 40, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 66, 68, 69, 70, 73, Pap 155, Pap 291, AE2)	1639	452	NR		73 HIV+, 42 HIV–	4.0 (2.6–6.1)	Cytology	Women were participants in the WIHS; HPV testing performed on a cervicovaginal lavage specimen; Women had measurement of serum retinol AND were followed every 6 months; Median follow-up, 4.0 years

Table 71 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p*-value	%/incidence	Odds ratio (95% CI)/ *p*-value		
Conley *et al*. (2002), USA	PCR using L1 consensus primers and RFLP typing analysis; some samples studied with E6-specific primers (16 and 18)	481 (baseline), 385 (incidence analysis)	437 (baseline), 341 (incidence analysis)	54 HIV+, 32 HIV–	$p < 0.001$	*Vulvovaginal and perianal condyloma and dysplasia* 6.2 HIV+, 0.9 HIV– *Incident condyloma (all sites)* 7 HIV+, 1 HIV–	$p < 0.0001$ 13.8 (10.9–17.3)	Cytology, histology	Study examined incident condyloma of vulva, vagina and perianal region and multicentric lesions; HPV testing performed on cervicovaginal lavage specimen
Silverberg *et al*. (2002), USA	MY09/MY11 PCR primers (6, 11, 16, 18, 26, 31, 32, 33, 35, 39, 40, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 66, 68, 69, 70, 73, 83, 84)	2032 (WIHS)	551 (WIHS)	*HPV 6/11* 5.0 HIV+, 0.9 HIV– *Other HPV* 58.7 HIV+, 29.0 HIV–	$p < 0.001$	Genital warts *WIHS* 9.8 HIV+, 3.1 HIV– *HERS* 13.6 HIV+, 5.0 HIV–	$p < 0.001$ $p < 0.001$	Cytology, physical examination	Women were participants in the WIHS and HERS and were followed every 6 months.
		863 (HERS)	420 (HERS)	*HPV 6/11* 4.3 HIV+, 1.2 HIV– *Other HPV* 59.9 HIV+, 26.0 HIV–	$p < 0.001$				

Table 71 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p*-value	%/incidence	Odds ratio (95% CI)/ *p*-value		
Branca *et al*. (2003), Italy	MY09/MY11 PCR primers with RFLP genotyping and confirmation of typing by sequencing	89	48	*At baseline* 38.6 HIV+, 27.1 HIV– *New infection during follow-up* 27.1 HPV/HIV+, 3.1 (HPV/HIV+) *Cleared infection during follow-up* 22.8 HPV+/HIV+, 69.2 HPV+/HIV–	 8.8 (1.20–64.6) 0.33 (0.16–0.67)	NR			Women were participants in the DIANAIDS project and were followed for a mean of 14 months.
Ford *et al*. (2003), Indonesia	PCR (primers not specified) with hybridization (6, 11, 16, 18, 31, 33, 35, 45, 52)	631 (baseline), 618 (18 months)		38.3 at baseline, 29.7 at 18 months		NR			Study of female sex workers in Bali, Indonesia; HPV testing performed at baseline and 18 months later; HPV testing performed on cervical swab specimen
Strickler *et al*. (2003), USA	PCR MY09/MY11/ HMB01 PCR (6, 11, 16,18, 26, 31, 32, 33, 35, 39, 40, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 66, 68, 69, 70, 73, Pap 155, Pap 291, AE2)	2929		HPV 6 HPV 11 HPV 16 HPV 18 HPV 31	4.9 (2.0–12.02) 2.05 (0.93–4.50) 1.69 (1.01–2.81) 2.24 (1.23–4.08) 3.07 (1.55–6.07)	NR		Cytology	Hazard ratio estimates for association between $CD4^+ < 200$ versus ≥ 500 with incident detection of specific HPV types; women were participants in the WIHS and HERS; HPV testing was performed on a cervicovaginal lavage specimen.

Table 71 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p*-value	%/incidence	Odds ratio (95% CI)/ *p*-value		
Massad *et al*. (2004a), USA	PCR MY09/ MY11/HMB01 PCR (6, 11, 16, 18, 26, 31, 32, 33, 35, 39, 40, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 66, 68, 69, 70, 73, Pap 155, Pap 291, AE2)	202	21	*HR types* 49.5 HIV+, 19.1 HIV– *LR types* 19.8 HIV+, 9.5 HIV–	$p < 0.001$	*Progression of CIN 1* 3.8 HIV+, 0 HIV–	0.4 (0.25–0.66)	Cytology, histology	Women were participants in the WIHS. HPV testing performed on a cervicovaginal lavage specimen; women with a histological diagnosis of CIN1 were included in the analysis; women were followed for a mean of 3.3 years.
Moscicki *et al*. (2004), USA	PCR MY09/ MY11/HMB01 PCR primers (16-like, 16, 31/33/35, 52, 58, 67, 18-like, 18, 39, 45, 59/68/70, 56-like, 56, 53/66, 26/69, 51, 6/11/42/43/44, 54/40, 13/32, 67/72, 2/57, 55)	172	84	NR		*LSIL at baseline* 22.5 HIV+, 10.2 HIV– *HSIL at baseline* 5.3 HIV+, 0 HIV–	$p < 0.002$ $p = 0 .13$	Cytology	Cervicovaginal lavage samples used for HPV testing; participants were women aged 13–18 years in the REACH cohort.

See Table 7 for a description of the primers used.

CI, confidence interval; CIN, cervical intraepithelial neoplasia; DIANAIDS, Italian collaborative study on HIV/HPV; HC, Hybrid Capture; HERS, HIV Epidemiology Research Study; HIV, human immunodeficiency virus; HIV–, HIV-negative; HIV+, HIV-positive; HR, high-risk; HSIL, high-grade squamous intraepithelial lesion; IDU, intravenous drug user(s); ISH, in-situ hybridization; LR, low-risk; LSIL, low-grade squamous intraepithelial lesion; NR, not reported; Pap, Papanicolaou test; PCR, polymerase chain reaction; REACH, Reaching for Excellence in Adolescent Care and Health; RFLP, restriction fragment length polymorphism; RLU, relative light unit; WIHS, Women's Interagency HIV Study

40%; $p = 0.04$) and to have more visits at which multiple HPV DNA types were detected (18% versus 0%; $p = 0.02$) than HIV-negative women.

La Ruche *et al*. (1999) performed a short-term prospective study of CIN in Abidjan, Cote d'Ivoire. Of 94 women with a cytological diagnosis of SIL, 36 were infected with HIV-1 and two with HIV-2. The average follow-up period after the initial smear was 5 months. HIV-positive women had a higher percentage of persistent CIN (76%) than HIV-negative women (18%) (relative risk, 4.3; 95% CI, 2.4–7.7). Progression to high-grade CIN occurred more frequently among HIV-positive (18%) than HIV-negative women (0%). In a multivariate analysis, persistence of lesions was associated with HIV positivity and an undetermined grade of CIN at baseline; HPV infection was not a significant risk factor after adjustment for the other factors in the model.

Petry *et al*. (1999) studied the role of HPV testing as a screening tool for incident SIL among 138 HIV-positive women in Germany. The prevalence of high-grade neoplasia (≥ CIN2) was 12.3% (17/138), and the total prevalence of cervical neoplasia including CIN1 was 26.8% (37/138).

Cubie *et al*. (2000) performed a prospective observational cohort study of 63 HIV-positive women in Edinburgh, United Kingdom. Abnormal cervical cytology, particularly that of low grade, was common in these HIV-infected women. Using Hybrid Capture, high-risk HPV types were detected in 25% of the women with normal cytology. Over 80% of those with abnormal cytology of any grade were positive for HPV. Persistent high-risk HPV infection, as defined by two or more consecutive HPV-positive results, was common and found in 27 of 63 (42.9%) women from whom multiple samples were obtained. Progression of cervical disease, even among the more strongly immunosuppressed women, was a rare event in this cohort.

Ellerbrock *et al*. (2000) studied risk factors for incident SIL in 328 HIV-positive and 325 HIV-negative women in the New York area, USA. During follow-up, the incidence of SIL was 8.3 cases per 100 person–years in HIV-positive and 1.8 cases per 100 person–years in HIV-negative women ($p < 0.001$). Of the incident SILs, 91% were LSIL in HIV-positive women and 75% in HIV-negative women. In a multivariate analysis, risk factors for incident SIL included HIV infection (relative risk, 3.2; 95% CI, 1.7–6.1), transient HPV DNA detection (relative risk, 5.5; 95% CI, 1.4–21.9), persistent infection with HPV DNA types other than 16 or 18 (relative risk, 7.6; 95% CI, 1.9–30.3), persistent infection with HPV DNA types 16 and 18 (relative risk, 11.6; 95% CI, 2.7–50.7) and younger age (< 37.5 years versus ≥ 37.5 years of age; relative risk, 2.1; 95% CI, 1.3–3.4).

Ahdieh *et al*. (2001) performed a prospective study of HPV infection in women who participated in the HIV Epidemiology Research Study. In a multivariate analysis, increased signal strength on dot blot, but not viral risk category, was independently associated with persistence of HPV infection among HIV-positive women (odds ratio, 2.5; 95% CI, 2.1–2.9). Persistence was 1.9 (95% CI, 1.5–2.3) times more common among women with a $CD4^+$ cell count < 200 cells/μL compared with those with a count > 500 cells/μL. Among the HPV types examined, HPV 16 had the highest incidence rate among HIV-negative women (1.67 per 100 person–years). Among HIV-positive women, HPV 18 had the highest

incidence rate among the high-risk types (2.61 per 100 person–years) but low-risk HPV 53 had the highest incidence rate overall (6.23 per 100 person–years).

Calore *et al*. (2001) studied cytological specimens from 1587 HIV-positive women in Brazil: 12.6% had SIL or cervical cancer in at least one specimen; 24 women progressed from normal to LSIL within 3 years and 11 progressed from normal to HSIL within 3 years.

Cohn *et al*. (2001) studied the 1-year incidence of CIN in 103 women who participated in the AIDS Research Community Based Clinical Trials Network in six cities in the USA. Higher HPV viral loads as measured by higher Hybrid Capture RLU ratios were associated with high-grade CIN ($p = 0.0006$). Incident CIN occurred in 20% of women during 1 year of follow-up, and was associated with higher HPV RLU ratios at baseline ($p = 0.03$).

Massad *et al*. (2001) studied the incidence, progression and regression rates of abnormal cervical cytology among 1639 HIV-positive and 452 HIV-negative women who participated in the Women's Interagency HIV Study. At least one abnormal smear was found during the whole follow-up among 73.0% of HIV-positive and 42.3% of HIV-negative women ($p < 0.001$). The incidence of HSIL was low among HIV-positive women and only 5.9% ever developed HSIL during follow-up. The incidence of SIL was 8.9/100 person–years among HIV-positive and 2.2/100 person–years among HIV-negative women (relative risk, 4.0; 95% CI, 2.6–6.1). Progression 6 months after an abnormal smear was found in 14% of HIV-positive women. HIV positivity, HPV positivity, lower $CD4^+$ cell count and higher HIV RNA level predicted the incidence of abnormal cytology. HPV positivity and higher HIV RNA level predicted the progression of abnormalities found at baseline. HPV negativity, higher $CD4^+$ lymphocyte count and lower HIV RNA level predicted regression of disease.

Conley *et al*. (2002) studied the incidence of vulvovaginal and perianal condylomata acuminata and intraepithelial neoplasia in 925 HIV-positive and HIV-negative women in the New York area, USA. Vulvovaginal and perianal condylomata acuminata or intraepithelial neoplasia were found in 6% of HIV-positive and 1% of HIV-negative women at baseline. Among women without lesions at enrolment, 9% of HIV-positive and 1% of HIV-negative women developed vulvovaginal or perianal lesions over a median follow-up of 3.2 years. Risk factors for incident disease included HIV-1 infection ($p = 0.013$), HPV infection ($p = 0.0013$), lower $CD4^+$ cell counts ($p = 0.0395$) and history of frequent intravenous drug use ($p = 0.02$).

Silverberg *et al*. (2002) examined the relationship between HIV infection and incidence of genital warts and infection with HPV 6 or 11 in both the Women's Interagency HIV Study and HIV Epidemiology Research Study populations. The prevalence of HPV 6 or 11 was 5.6 times higher and that of genital warts was 3.2 times higher in HIV-positive than in HIV-negative women in the Women's Interagency HIV Study and 3.6 times and 2.7 times higher, respectively, in the HIV Epidemiology Research Study. In the former, the risk for HPV 6 or 11 infection increased from 5.1 (95% CI, 2.9–8.8) among HIV-negative women to 8.8 (95% CI, 6.1–12.8) among HIV-positive women with $CD4^+$ cell counts > 200/mm^3 and to 12.8 (95% CI, 8.8–18.8) among HIV-positive women with $CD4^+$ cell counts ≤ 200/mm^3. In this study, infection with HPV 6 or 11 was associated with an increased risk for genital warts

compared with HPV negativity in HIV-negative women (odds ratio, 2.7; 95% CI, 1.6–4.6), HIV-positive women with $CD4^+$ cell counts > 200/mm^3 (odds ratio, 4.9; 95% CI, 3.2–7.7) and HIV-positive women with $CD4^+$ cell counts ≤ 200/mm^3 (odds ratio, 5.3; 95% CI, 3.3–8.5). The incidence of infection with HPV 6 or 11 and the incidence of genital warts measured in cases per 100 person–years were higher among HIV-positive women than among HIV-negative women in both of these studies.

Branca *et al*. (2003) studied the natural history of cervical HPV infection in 89 HIV-positive and 48 HIV-negative women who participated in the DIANAIDS study. New HPV infections during follow-up were more common among HIV-positive than HIV-negative women (odds ratio, 8.8; 95% CI, 1.2–64.6), and clearance of HPV infection at baseline was less frequent among HIV-positive than HIV-negative women (odds ratio, 0.33; 95% CI, 0.16–0.67). In a multivariate analysis, risk factors for HPV positivity at the end of the study included HIV positivity ($p < 0.001$), PCR positivity at entry ($p = 0.009$), p53 polymorphism at aa-72 ($p = 0.01$), high-risk HPV type ($p = 0.02$) and significant Pap smear at entry ($p = 0.04$).

Ford *et al*. (2003) studied female sex workers in Bali, Indonesia, for cervical HPV infection at baseline and again 18 months later. The prevalence of HPV infection was 38.3% at baseline, which declined to 29.7% after 18 months. The prevalence of HPV infection declined with age ($p < 0.01$). Infection with *N. gonorrhoeae* was associated with HPV infection at baseline ($p = 0.03$). HPV infection declined in the study area that had the more intensive educational programme ($p < 0.01$).

Strickler *et al*. (2003) examined the relationship between prevalence and incidence of specific HPV types in HIV-positive women who participated in the Women's Interagency HIV and HIV Epidemiology Research Studies. In a cross-sectional analysis of data from the first study, HPV 16 had a weaker association with more advanced immune status as measured by $CD4^+$ cell counts than other HPV types. This largely reflected the observation that the prevalence of HPV 16 was higher among women with higher $CD4^+$ cell levels than that of other HPV types, the proportional prevalence of which was increased at lower $CD4^+$ cell strata. A summary prevalence ratio and incidence hazard ratio were estimated for each HPV type. Using data from both studies, the prevalence ratio for HPV 16 was low compared with that of other HPV types at every visit in both populations. The prevalence ratio was smallest for HPV 16 compared with that of all HPV types measured (1.25; 95% CI, 0.97–1.62; $p = 0.01$). The association of $CD4^+$ T-cell stratum with incidence of HPV 16 was also among the smallest measured. HPV types that had small summary prevalence ratios also had small incidence hazard ratios. The investigators concluded that the prevalence and incidence of HPV 16 is more weakly associated with immune status as measured by $CD4^+$ cell level in HIV-positive women than that of other HPV types, which suggests that HPV 16 may be more resistant to the effects of immune surveillance.

Massad *et al*. (2004a) studied the natural history of histologically confirmed CIN1 in 202 HIV-positive and 21 HIV-negative women who participated in the Women's Interagency HIV Study. The prevalence of high-risk HPV in HIV-infected women was 49.5% compared with 19.1% in HIV-negative women. Progression occurred in eight (3.8%) HIV-

positive women (incidence density, 1.2/100 person–years) but not in HIV-negative women. Regression occurred more often in HIV-negative than in HIV-positive women (relative risk, 0.40; 95% CI, 0.25–0.66; $p < 0.001$). In a multivariate analysis, regression was associated with HPV infection (hazard ratio for low-risk HPV, 0.28; 95% CI, 0.13–0.61; hazard ratio for high-risk HPV, 0.34; 95% CI, 0.20–0.55 versus no HPV detected) and Hispanic ethnicity (hazard ratio, 0.48; 95% CI, 0.23–0.98). The lesions of HIV-positive women with HPV infection at the time of diagnosis of CIN were less likely to regress than those of HIV-negative women (hazard ratio, 0.18; 95% CI, 0.05–0.62).

Moscicki *et al*. (2004) studied the incidence of HSIL diagnosed cytologically among HIV-positive and HIV-negative adolescent girls who participated in the Reaching for Excellence in Adolescent Care and Health cohort. The incidence of HSIL at the end of follow-up was higher for HIV-positive girls (21.5%) than for HIV-negative girls (4.8%). In a multivariate analysis, use of hormonal contraceptives (hazard ratio, 2.60; 95% CI, 1.25–5.40), high concentrations of IL12 in cervical mucous (hazard ratio, 2.28; 95% CI, 1.17–4.43), persistent LSIL diagnosed cytologically (hazard ratio, 1.67; 95% CI, 1.29–2.18) and infection with HPV type 16 only (hazard ratio, 3.69; 95% CI, 1.06–12.80), HPV type 18 only (hazard ratio, 4.49; 95% CI, 1.16–17.42) and multiple high-risk HPV types (16, 18 and 56) (hazard ratio, 3.69; 95% CI, 1.07–12.71) were significantly associated with the development of HSIL.

(iii) *Effect of highly active antiretroviral therapy (HAART) on CIN* (Table 72)

Heard *et al*. (1998) examined the natural history of cervical lesions in 49 HIV-positive women in Paris, France, before and 5 months after initiation of HAART. They examined the prevalence of HPV using southern blot hybridization (to define high-level infection) and PCR (to identify low-level infection). The prevalence of SIL decreased from 69 to 53% during follow-up ($p < 0.0001$). Two women with HSIL regressed to LSIL and one regressed to normal, and nine of 21 (43%) women with LSIL regressed to normal. In all patients except one, the prevalence of HPV infection detected by southern blot hybridization and PCR did not change. There was a greater increase in the absolute number of $CD4^+$ cells in the subgroup of patients whose lesions regressed (99 versus 50×10^6/L; $p = 0.03$) compared with those whose lesions did not regress.

Delmas *et al*. (2000) evaluated the natural history of SIL in HIV-positive women in a cohort that was followed in 12 European countries. Women with lower $CD4^+$ cell counts ($< 200 \times 10^6$/L) had twice the prevalence and incidence of SIL than women with higher $CD4^+$ cell counts ($> 500 \times 10^6$/L), and fewer had regression of CIN1. Regression of SIL in women with a low $CD4^+$ cell count was lower (20.5%) in those who received antiretroviral therapy than in those who did not (31.4%), but the difference was not significant ($p = 0.30$).

Lillo *et al*. (2001) studied the effect of HAART on high-risk HPV infections and related cervical lesions in 163 HIV-positive women in Italy. High prevalences of both high-risk HPV infection (68%) and SIL diagnosed cytologically were found at baseline

Table 72. Effect of highly active antiretroviral therapy (HAART) on cervical intraepithelial neoplasia (CIN) in HIV-positive women

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p* value	%	Odds ratio (95% CI)/ *p* value		
Heard *et al.* (1998), France	MY09/MY11 PCR primers (16, 18, 33) plus SBH (6/11/42, 16/18/33, 31/35/39, other types), sequencing of unidentifiable types	49		HIV+ *Before HAART* 81 by PCR, 54 by SBH *After HAART* 93 by PCR, 56 by SBH		9/21 women with LSIL at baseline regressed to normal cytology (3 with persistent colposcopic abnormalities); 2/13 women with HSIL at baseline regressed to LSIL and one regressed to normal.	$p < 0.0001$	Cytology, histology	HPV testing on cervical cotton swab and wooden spatula; women were followed prospectively at a median of 5 months after initiation of HAART.
Delmas *et al.* (2000), 12 European countries	SBH (6, 11, 42, 16, 18, 31, 33, 35, 39), PCR MY09/MY11 (16, 18, 33), GP1/GP2 primers to detect other types	467 (baseline), 229 (follow-up), 115 CIN1 (follow-up)				*SIL* 24.2 HIV+ at baseline, 23.6 at 1 year, 29.5 at 18 months *Progression of LSIL to HSIL* 8.1 at 1 year *Regression of LSIL to normal* 30.9 at 1 year *Cumulative regression rate among women with* $CD4^+ < 200$ 20.5 with HAART, 31.4 without HAART	$p = 0.30$	Cytology	Women were participants in a European cohort on natural history of HIV infection; HPV testing was performed on cervical brush specimens; women with normal cytology at baseline were followed every 6 months for a median of 2 years; women diagnosed with CIN1 were followed for a median of 18 months without treatment.
Lillo *et al.* (2001), Italy	HC 2 and MY09/MY11 PCR with E6 and L1 priming (6, 11, 16, 18, 31, 33, 35, 45)	163		68 HR HPV; persistent HPV infection in HAART-treated versus non-treated	1.18 (0.63–3.46)	6.2 HSIL, 20.2 LSIL; progression of cytological changes in HAART-treated versus non-treated	2.01 (0.44–9.20)	Cytology, histology	Participants followed at 6-month intervals; HPV infection defined as having the same type at baseline and follow-up, transitory as having a change in HPV status and HPV– as having consistently negative HPV tests; HPV samples obtained by brushing squamocolumnar junction

Table 72 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p* value	%	Odds ratio (95% CI)/ *p* value		
Minkoff *et al*. (2001), USA	MY09/MY11/ HMB01 PCR (6, 11, 16, 18, 26, 31, 32, 33, 35, 39, 40, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 66, 68, 69, 70, 73, Pap 155, Pap 291, AE2)	507		NR		*Regression of cytological abnormalities* 35.3 with HAART, 29.5 without HAART *Progression of cytological abnormalities* 15.6 with HAART, 22.3 without HAART	1.4 (1.04–1.82) 0.68 (0.52–0.88)	Cytology	Women were participants in the WIHS; HPV testing performed on a cervicovaginal lavage specimen; women had measurement of serum retinol and were followed every 6 months; all had HR HPV infection; progression and regression were measured using consecutive Pap smear pairs.
Robinson *et al*. (2001), USA	No HPV DNA detection	56	62			*Disease persistence among women not treated within 6 months of diagnosis* 60 HIV+, 32 HIV– *Persistent or recurrent CIN after therapy* 17.6 with HAART, 70.3 without HAART	$p < 0.05$ $p < 0.05$		Retrospective chart review of women treated for CIN; cervical conization or loop electrosurgical excision therapy used
Heard *et al*. (2002), France	Regression to normal cytology in HAART-versus non-HAART-treated	168 CIN (96 HAART-treated)		NR		Regression to normal, 34.1 Progression to high-grade, 22.7	1.93 (1.14–3.29)	Cytology, histology	Women were followed at 6-month intervals; 37 were treated with HAART at the time of study initiation, 59 initiated HAART after the baseline visit.

Table 72 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p* value	%	Odds ratio (95% CI)/ *p* value		
Moore, A.L. *et al*. (2002), United Kingdom	No HPV DNA detection	71				*Prevalence of CIN* 55 before HAART, 62 after HAART Regression, 13	$p = 0.20$	Cytology, histology	Women attended a gynaecology clinic, required cytology before HAART initiation and another at least 6 months after HAART initiation; median time between pre- and post-HAART smears, 10 months.
Schuman *et al*. (2003), USA	MY09/MY11 PCR primers (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 66, 68, 70, Pap 155, Pap 291, W13B)	774	391	64.0 HIV+, 28.7 HIV–				Cytology	Cervicovaginal lavage specimen used for HPV testing; women were participants in the HERS and were followed every 6 months.
Uberti-Foppa *et al.* (2003), Italy	HC2 PCR MY09/MY11 and SPF10	83 stable with no or standard HAART, 71 more potent HAART		NR		SIL baseline end-point *Standard HAART* LSIL, 30.1–13.3 HSIL, 14.5–1.2 *More potent HAART* LSIL, 15.5–24 HSIL, 12.7–2.8	$p < 0.0001$ $p = 0.06$	Histology	Mean age of patients was 32.3 ± 5.2 years (range, 21–45 years); histology classified according to the Bethesda system

Table 72 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence		Cervical abnormality		Pathology reading	Comments
				%	Odds ratio (95% CI)/ *p* value	%	Odds ratio (95% CI)/ *p* value		
Ahdieh-Grant *et al*. (2004), USA	MY09/MY11/ HMB01 PCR (6, 11, 13, 16, 18, 26, 31, 32, 33, 35, 39, 40, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 66, 68, 69, 70, 73, Pap 155, Pap 291, AE2)	141 with regression of SIL, 171 with no regression of SIL		NR		*Incidence of regression* 0 (95% CI, 0–2.4)/100 person–years before HAART, 12.5 (95% CI, 9.9–15.1)/ 100 person–years after HAART	$p = 0.002$	Cytology	Participants in the WIHS; HPV testing performed on a cervicovaginal lavage specimen; women had measurement of serum retinol and were followed every 6 months; all had HR HPV infection?; progression and regression were measured among women with a normal Pap smear at enrolment who developed SIL during at least 7 years of follow-up.
Massad *et al*. (2004b), USA	PCR MY09/MY11 and specific probes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and other low-risk types)	15 621	469	NR		HAART versus non-HAART Genital warts VIN1 VIN2–3	 0.76 (0.58–0.99) 0.65–0.49–0.88) 0.64 (0.40–1.04)	Cytology	Follow-up of 8 years

See Table 7 for a description of the primers used.

CI, confidence interval; HC, Hybrid Capture; HERS, HIV Epidemiology Research Study; HIV, human immunodeficiency virus; HIV+, HIV-positive; HIV–, HIV-negative; HR, high-risk; HSIL, high-grade squamous intraepithelial lesion; ISH, in-situ hybridization; LSIL, low-grade squamous intraepithelial lesion; NR, not reported; Pap, Papanicolaou test; PCR, polymerase chain reaction; SBH, southern blot hybridization; SIL, squamous intraepithelial lesion; VIN, vulvar intraepithelial neoplasia; WIHS, Women's Interagency HIV Study

(LSIL, 20.2%; HSIL, 6.2%). The women were followed every 6 months for a mean observation time of 15.4 months (range, 6–24 months). Although $CD4^+$ cell counts increased significantly in subjects who received HAART, persistence of high-risk HPV infection (adjusted odds ratio, 1.18; 95% CI, 0.63–3.46) and progression of SILs (adjusted odds ratio, 2.01; 95% CI, 0.44–9.20) were comparable among those who were and those who were not treated with HAART.

Minkoff *et al*. (2001) examined the relationship between the use of HAART and progression or regression of cervical cytology in the Women's Interagency HIV Study. Women with persistent HPV positivity were more likely to have lesions that progressed: 16.2% progressed with HPV positivity at one of three visits, 23.6% progressed with positivity at two of three visits and 24.6% progressed with positivity at all three visits ($p < 0.001$). After adjustment for $CD4^+$ cell count and cytological status, women who took HAART were more likely to experience regression (odds ratio, 1.4; 95% CI, 1.04–1.82) and less likely to show progression (odds ratio, 0.68; 95% CI, 0.52–0.88).

Robinson *et al*. (2001) performed a retrospective chart review of 56 HIV-positive and 62 HIV-negative women to determine the rates of recurrence, persistence and progression of CIN after excisional therapy with and without HAART. Among those who were not treated within 6 months of diagnosis, persistence occurred in 60% of HIV-positive and 32% of HIV-negative women ($p < 0.05$). Risk factors for recurrence among HIV-positive women after treatment included margin involvement of specimens obtained by loop electrosurgical excision ($p < 0.05$). Disease in women who took HAART that included protease inhibitors was less likely to recur or persist after treatment than that in HIV-positive women who did not take HAART ($p < 0.05$).

Heard *et al.* (2002) studied 168 HIV-positive women, of whom 37 were taking HAART at the time of study initiation and 59 began taking HAART after baseline, every 6 months. Overall, regression of CIN was observed in 39.9% of the women. The risk for regression of CIN was nearly twice as high in women who received HAART than in those who did not (relative hazard, 1.93; 95% CI, 1.14–3.29). HAART had a similar effect on the regression of low-grade CIN to normality (30/88 women; 1.99; 95% CI, 0.94–4.18) and on the reversion of high-grade to low-grade CIN (31/174 women).

Moore, A.L. *et al*. (2002) studied the prevalence of CIN in 71 HIV-positive women 10 months after initiation of HAART. The prevalence of CIN before HAART was 55%; a median of 10 months after treatment with HAART, this had increased to 62% ($p = 0.20$). Thirteen per cent of patients experienced regression of a CIN lesion, which was associated with a greater but non-statistically significant increase in $CD4^+$ cell count.

Schuman *et al*. (2003) examined risk factors for the progression and regression of cytological abnormalities in 774 HIV-positive and 391 HIV-negative women who participated in the HIV Epidemiology Research Study. HAART was not significantly associated with the probability of progression of cervical lesions.

Uberti-Foppa *et al*. (2003) assessed the long-term effect of HAART by comparing HPV and cytology/histology results at the beginning and end of a study of 83 women who were stable without HAART or who were taking HAART but did not change their therapy

(Group S), and 71 women who changed to a more potent HAART regimen due to failure of the treatment (Group W). Participants were followed at 6–12-month intervals for a mean of 36 months. HPV infection was defined as positivity for the same type at baseline and at follow-up; infection was described as transitory when a change in HPV status occurred; and HPV status was considered to be negative when HPV tests were consistently negative. Although treatment with HAART increased the $CD4^+$ cell level among women in both groups, HAART-associated increases in $CD4^+$ cell level did not affect the persistence of HPV. However, women who took HAART in Group S had fewer HPV-positive low-grade biopsies at the end of the study compared with baseline (30.1% versus 13.3%; $p = 0.00004$). No significant reduction was seen in Group W. The data suggest that long-term use of HAART may be beneficial in the regression of low-grade lesions.

Ahdieh-Grant *et al*. (2004) studied 312 HIV-positive women in the Women's Interagency HIV Study who had normal cervical cytology at baseline, who developed incidental SIL during 7 years of follow-up and who could be monitored for regression or progression of lesions in relation to the use of HAART. Of these, 141 (45.2%) had lesions that regressed to normal with a median time to regression of 2.7 years. The incidence of regression increased (p for trend = 0.002) after HAART was introduced. Women whose lesions did not regress had lower $CD4^+$ cell levels than those whose lesions regressed ($p < 0.01$). However, the majority of cervical lesions among HIV-positive women, whether they took HAART or not, did not regress to normal.

Massad *et al*. (2004b) followed the incidence and predictors of genital warts and VIN among HIV-positive and HIV-negative women at high risk who participated in the Women's Interagency HIV Study for up to 8 years. In a multivariate analysis, warts were associated with HAART (relative hazard, 0.76; 95% CI, 0.58–0.99), $CD4^+$ cell count (relative hazard, 0.91/100 cell/cm^2 increase; 95% CI, 0.86–0.96), history of AIDS (relative hazard, 1.25; 95% CI, 1.00–1.57), abnormal Pap test results (relative hazard, 2.18; 95% CI, 1.73–2.75), high- or medium-risk HPV types (relative hazard, 1.91; 95% CI, 1.48–2.47), low-risk HPV types (relative hazard, 1.48; 95% CI, 1.10–2.00), tobacco smoking (relative hazard, 1.43; 95% CI, 1.09–1.88), having one child (relative hazard, 1.54; 95% CI, 1.11–2.13) and age (relative hazard, 0.74/10 years; 95% CI, 0.64–0.86). VIN of any grade was linked to HAART (relative hazard, 0.65; 95% CI, 0.49–0.88), $CD4^+$ cell count (relative hazard, 0.92; 95% CI, 0.87–0.97), abnormal Pap test results (relative hazard, 16.03; 95% CI, 11.33–22.69), high- or medium-risk HPV types (relative hazard, 1.37; 95% CI, 1.06–1.77) and age (relative hazard, 0.85/10 years; 95% CI, 0.73–0.99). While HAART was associated with a reduced relative hazard for incidental genital warts and VIN of any grade, it was not significantly associated with a reduced relative hazard for VIN2–3 in a multivariate analysis (relative hazard, 0.64; 95% CI, 0.40–1.04).

Two studies analysed the incidence of cervical cancer before and after the introduction of HAART.

The International Collaboration on HIV and Cancer (2000) examined the relationship between HAART and incidence of cancer in HIV-positive adults. Rate ratios were estimated by comparing incidence rates from 1997 to 1999 with those from 1992 to 1996. The

rate ratio for cervical cancer was 1.87 (95% CI, 0.77–4.56), which indicated that there had been no significant change in the incidence of cervical cancer since the introduction of HAART.

Dorrucci *et al*. (2001) analysed the incidence of cervical cancer in Italian women before and after the introduction of HAART. They estimated the incidence per 1000 person–years of cervical cancer as a first AIDS-defining disease for the periods 1981–91, 1992–95 and 1996–98 in 483 women with a median follow-up of 7 years. Compared with 1981–95, the hazard ratio for cervical cancer for 1996–98 was 7.41 (95% CI, 1.21–45.44). After adjustment for age at HIV seroconversion, the hazard ratio decreased to 4.75 (95% CI, 0.80–28.24). The incidence of cervical cancer had not declined after the introduction of HAART in this population.

(*b*) *Studies of the anorectal region*

(i) *Prevalence of anal HPV infection and anal SIL* (Table 73)

Carter, P.S. *et al*. (1995) studied 90 HIV-positive men, 77 HIV-negative men and 43 men of unknown HIV status who attended a genitourinary medicine clinic in London, United Kingdom. The relative risk for AIN for HIV-positive men was 1.58 (95% CI, 1.01–2.48) compared with HIV-negative men. The relative risk for developing AIN for those with anal warts compared with those without anogenital warts was 4.70 (95% CI, 1.81–12.20).

Hillemans *et al*. (1996) examined the prevalence of anal HPV infection and anal disease in 102 HIV-positive and 96 HIV-negative women at high risk in the New York metropolitan area, USA. Using Hybrid Capture 2 on anal swabs, HPV DNA was found in 29.4% of HIV-positive and 2.1% of HIV-negative women. Anal cytological abnormalities were low-grade or atypical, and were found in 27.3% of HIV-positive and 6.4% of HIV-negative women with satisfactory smears. Of 33 women with anal cytological abnormalities, 19 (58%) had anal HPV DNA compared with 13 (8%) of 160 women without cytological abnormalities ($p < 0.001$). In a multivariate logistic regression analysis, HPV infection was the only risk factor associated with anal cytological abnormalities (adjusted odds ratio, 16.0; 95% CI, 8.9–3.2). In a logistic regression model of risk factors for anal cytological abnormalities that did not include HPV DNA positivity, HIV-positive women who had a $CD4^+$ cell count < 200/mm^3 were at higher risk than HIV-positive women who had a $CD4^+$ cell count > 200/mm^3 (adjusted odds ratio, 7.3; CI, 1.8–49.2). HIV positivity was found to be an independent risk factor for HPV infection and, similarly to anal cytological abnormalities, the strength of the association with anal HPV infection among HIV-positive women was greater in those who had $CD4^+$ T-lymphocyte counts < 200/mm^3 than in those who had $CD4^+$ counts > 200/mm^3 (adjusted odds ratio, 11.6; 95% CI, 2.1–64.5).

Melbye *et al*. (1996) studied 81 HIV-positive and 70 HIV-negative women in Copenhagen and Aarhus, Denmark. Using PCR, anal HPV was detected in 78% of HIV-positive women and 60% of HIV-negative women. Abnormal anorectal smears were found in 19% of HIV-positive women and in none of the HIV-negative women.

Table 73. Prevalence of anal HPV infection and anal squamous intraepithelial lesions (SIL) in HIV-positive and HIV-negative individuals

Reference, study location	Sex	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence (%)	Anorectal abnormality (%)	Pathology reading	Comments
Carter, P.S. *et al.* (1995), United Kingdom	Men		90	77 HIV–, 43 unknown HIV status		*AIN* 41.1 HIV+, 26.0 HIV–, 39.5 unknown HIV status *Anogenital warts* 58.9 (HIV+), 79.2 (HIV–), 95.3 unknown HIV status	Histology	Men were recruited from a genitourinary medicine clinic.
Hillemans *et al.* (1996), USA	Women	HC2 with typing of HC2-positives using MY09/MY11 PCR	102	96	29.4 HIV+ 2.1 HIV–	27.3 HIV+, 6.4 HIV–	Cytology	HPV testing on anal swab material
Melbye *et al.* (1996), Denmark	Women	MY09/MY11 PCR (6, 11, 16, 18, 31, 33, 35, 45, 39, 51, 52) and HC	81	70	*PCR* 78 HIV+, 60 HIV– *HC* 38 HIV+, 7 HIV–	19 HIV+, 0 HIV–	Cytology	HPV testing on anal Dacron swab material and cervical cotton swab material; women were recruited from HIV screening clinics.
Palefsky *et al.* (1997a), USA	Men	MY09/MY11 PCR (39 different HPV types) and HC	129		PCR, 93.2 HIV+ HC, 84.5 HIV+	35 ASCUS, 12 LSIL, 1 HSIL	Cytology	Testing on anal swab material; study subjects were MSM with CDC group IV HIV disease.
Friedman *et al.* (1998), USA	Men	HC2 and MY09/ MY11/HMB01 PCR	184	79	90.4 HIV+, 69.6 HIV–	60.0 HIV+, 29.6 HIV–	Cytology	HPV testing on anal swab material; patients were MSM.

Table 73 (contd)

Reference, study location	Sex	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence (%)	Anorectal abnormality (%)	Pathology reading	Comments
Palefsky *et al*. (1998a), USA	Men	No HPV DNA detection	346	262		*Abnormal cytology* 45.9 HIV+, 9.9 HIV– *AIN by cytology or histology* 35.8 HIV+, 7.3 HIV–	Cytology, histology	
Palefsky *et al*. (1998b), USA		MY09/MY11 PCR (39 different HPV types) and HC2	346	262	*PCR* 93.1 HIV+, 61.0 HIV– *HC* 87.3 HIV+, 37.3 HIV–	NR		Same cohort as above; testing on anal swab material; patients were MSM.
Sayers *et al*. (1998), United Kingdom	Men	PCR with E6 primers (6, 11, 16, 18)	66	232 (181 MSM), 51 hetero-sexual)	NR	*AIN in satisfactory smears* 30.0 HIV+ MSM, 4.7 HIV– MSM, 0 HIV– heterosexual		Anal cytology obtained with a cytobrush
Goldstone *et al*. (2001), USA	Men	No HPV DNA detection	131			*Biopsy* 60.0 high-grade AIN, 3 invasive anal cancer	Cytology, histology, biopsy	Patients referred for condyloma or other presumably benign anorectal disease
Holly *et al*. (2001), USA	Women	MY09/MY11 PCR (39 types)	251	68		*Anal cytology* 26.0 HIV+, 8.2 HIV–	Cytology, histology	WIHS study

Table 73 (contd)

Reference, study location	Sex	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence (%)	Anorectal abnormality (%)	Pathology reading	Comments
Palefsky *et al.* (2001), USA	Women	MY09/ MY11 PCR (39 types and HC2)	251	68	76.2 HIV+, 42.1 HIV– *Anal HPV* 79 HIV+ 63 HIV–			HPV testing on anal swab specimens
Sobhani *et al.* (2001), France	Men and women	In-situ PCR (6, 11, 16/18, 31, 33)	116	60	*Oncogenic HPV* 27 HIV+, 1.3 HIV–	*Condyloma recurrence* 75 HIV+, 6 HIV–	Histology	Eight women were diagnosed with condyloma at entry; fixed anal biopsy specimens; ISH or in-situ PCR for EBV and HSV; immunohistochemistry for CMV
Drobacheff *et al.* (2003), France	Men and women	HC2	50	50	58.0 HIV+, 6.0 HIV–	4 HIV+, 0 HIV–		HPV testing on anal swab material; the risk for HPV infection in HIV+ compared with HIV– was 9.7 (95% CI, 3.2–29.7).
Moscicki *et al.* (2003), USA	Men and women	MY09/MY11 PCR for HPV LR (6/11/42/44) and HR (16/18/31/33/ 35/39/42/51/52/56/5 8)	241	107	*Boys* 48.3 HIV+, 36.0 HIV– *Girls* 32.2 HIV+, 13.4 HIV–	*Boys* 52.5 HIV+, 16.7 HIV– *Girls* 21.3 HIV+, 5.7 HIV–	Cytology	Subjects were adolescents participating in the REACH cohort; HPV testing on anal swab material
Piketty *et al.* (2003), France	Men	MY09/MY11 PCR (39 different HPV types)	50 with no history of RAI, 67 with history of RAI		*No RAI* 46.0 any infection *RAI* 85	*No RAI* 16 LSIL, 19 HSIL *RAI* 49 LSIL, 18 HSIL	Cytology, histology	HPV testing from anal swab specimen

Table 73 (contd)

Reference, study location	Sex	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence (%)	Anorectal abnormality (%)	Pathology reading	Comments
Chin-Hong *et al*. (2004), USA	Men	MY09/ MY11 PCR (39 different HPV types)		1218	56.8, 25.6 HR types, 26.4 LR types	NR		Study of sexually active MSM in 4 US cities participating in the EXPLORE cohort

See Table 7 for a description of the primers used.

AIN, anal intraepithelial neoplasia; ASCUS, atypical squamous cells of undetermined significance; CDC, Centers for Disease Control; CI, confidence interval; CMV, cytomegalovirus; EBV, Epstein–Barr virus; EXPLORE, collaborative study among homosexual men in six US cities; HC2, Hybrid Capture 2; HIV, human immunodeficiency virus; HIV–, HIV-negative; HIV+, HIV-positive; HR, high-risk; HSIL, high-grade squamous intraepithelial lesion; HSV, herpes simplex virus; ISH, in-situ hybridization; LR, low-risk; LSIL, low-grade squamous intraepithelial lesion; MSM, men who had sex with men; NR, not reported; PCR, polymerase chain reaction; RAI, receptive anal intercourse; REACH, Reaching for Excellence in Adolescent Care and Health

Palefsky *et al*. (1997a) characterized the prevalence of anal HPV infection and anal lesions in 129 HIV-positive men who had sex with men and had Centers for Disease Control group IV HIV disease. Abnormal anal cytology was detected in 39% of subjects and anal HPV infection, as measured by PCR, in 93%. Risk factors for abnormal cytology in a multivariate analysis included HPV 16/18 infection (relative risk, 2.1; 95% CI, 1.2–3.5) and intravenous drug use (relative risk, 1.8; 95% CI, 1.2–2.7). Infection with HPV 6/11 also had significantly elevated relative risks in a separate model. Anal cytological abnormalities and HPV infection are common among homosexual/bisexual men with group IV HIV disease.

Friedman *et al*. (1998) studied the prevalence of anal HPV infection, using Hybrid Capture 2 and PCR, and anal disease, using cytology, in a cohort of HIV-positive and HIV-negative men who had sex with men. HPV was found more commonly in HIV-positive men (90.4% versus 69.6%), among whom the number of HPV types detected and Hybrid Capture RLU ratios were higher. Among HIV-positive men, the quantity of HPV DNA as measured by Hybrid Capture RLU ratio was inversely associated with $CD4^+$ cell count. Anal cytological abnormalities were more common among HIV-positive than HIV-negative men (60% versus 29.6%) and, among HIV-positive men, AIN was found more frequently among those with a $CD4^+$ cell count < 200/μL. Men with a high Hybrid Capture RLU ratio were 2.8 times more likely to have AIN (95% CI, 1.1–7.3) than those with samples that were both PCR- and Hybrid Capture 2-negative.

Palefsky *et al*. (1998a) examined the prevalence of AIN in a cohort of 346 HIV-positive and 262 HIV-negative men who had sex with men. Abnormal cytology was detected in 45.9% of HIV-positive and 9.9% of HIV-negative men. Using cytology or histology, AIN was diagnosed in 36% of HIV-positive and 7% of HIV-negative men (relative risk, 5.7; 95% CI, 3.6–8.9). High-grade AIN was found in 17 HIV-positive men (5%) and in only one HIV-negative man. Among HIV-positive men, the relative risk for AIN increased with lower $CD4^+$ cell levels but was elevated even in men with $CD4^+$ cell levels > 500/mm^3 (relative risk, 3.8; 95% CI, 2.1–6.7) compared with HIV-negative men. High-level HPV infection, as measured by detection of both low-risk and high-risk types using Hybrid Capture, was another significant risk factor for AIN in both HIV-positive (relative risk, 8.8; 95% CI, 2.3–35) and HIV-negative men (relative risk, 20; 95% CI, 5.5–71) compared with HPV-negative men.

Palefsky *et al*. (1998b) also studied the prevalence of anal HPV infection in the cohort described above. Anal HPV DNA was detected by PCR in 93% of HIV-positive and 61% of HIV-negative men. The spectrum of HPV types was similar in HIV-positive and HIV-negative men, and HPV 16 was the most common type. Infection with multiple HPV types was found in 73% of HIV-positive and 23% of HIV-negative men. Among HIV-positive men who were positive by Hybrid Capture for low-risk and high-risk types, lower $CD4^+$ cell levels were associated with higher levels of high-risk HPV DNA ($p = 0.004$) but not low-risk HPV DNA. These data suggest increased replication of the higher-risk HPV types with more advanced immunosuppression. For HIV-positive men, risk factors for the presence or absence of HPV DNA could not be assessed because most were positive for

HPV DNA using PCR or Hybrid Capture. Among the risk factors examined in univariate analyses for the HIV-negative men, the relative risks for HPV infection detected by PCR were: lifetime rectal drug use, 1.4 (95% CI, 1.1–1.7); lifetime history of rectal discharge, 1.3 (95% CI, 1.0–1.7); and lifetime level of receptive anal intercourse compared with no receptive anal intercourse: low, 1.3 (95% CI, 0.97–1.7); medium or high, 1.5 (95% CI, 1.1–2.1; p for trend = 0.03). The relative risks for HPV DNA positivity using Hybrid Capture were similar to those using PCR.

Sayers *et al*. (1998) studied 66 HIV-positive and 181 HIV-negative men who had sex with men and 51 HIV-negative heterosexual men who attended a genitourinary medicine clinic in Edinburgh, United Kingdom. AIN was noted in 30% of satisfactory anal smears from HIV-positive men, in 4.7% of satisfactory smears from HIV-negative men who had sex with men and in no smear from HIV-negative heterosexual men. There was no significant difference in the detection of HPV types 6, 11, 16 and 18 between HIV-positive and HIV-negative men.

Goldstone *et al.* (2001) determined the prevalence of anal HSILs and anal squamous-cell cancer in 131 HIV-positive and 69 HIV-negative men who had sex with other men. Ninety-three per cent had abnormal anal cytology. Biopsy results revealed that 60% of patients had high-grade AIN and 3% had invasive anal cancer. Four of five men with anal squamous-cell cancer were HIV-positive.

Holly *et al*. (2001) examined the prevalence of anal lesions in 251 HIV-positive and 68 HIV-negative women at high risk who participated in the San Francisco section of the Women's Interagency HIV Study. Abnormal anal cytology was diagnosed in 26% of HIV-positive and 8% of HIV-negative women. High-grade AIN was detected by histology or cytology in 6% of HIV-positive and 2% of HIV-negative women. HIV-positive women had an increased risk for anal disease as their $CD4^+$ cell counts decreased ($p < 0.0001$) and as their plasma HIV RNA viral load increased ($p = 0.02$). HIV-positive women with abnormal cervical cytology had an increased risk for abnormal anal cytology at the same visit (relative risk, 2.2; 95% CI, 1.4–3.3). In a multivariate analysis, an HIV viral load of > 100 000 copies/mL (relative risk, 2.4; 95% CI, 1.1–3.9), history of anal intercourse (relative risk, 2.3; 95% CI, 1.2–3.6) and concurrent abnormal cervical cytology (relative risk, 2.1; 95% CI, 1.0–3.6) were significantly associated with abnormal anal cytology.

Palefsky *et al*. (2001) studied the same population of women for the presence of anal HPV using PCR and Hybrid Capture 2 (relative risk, 1.8; 95% CI, 1.3–2.5). Among 200 women for whom there were concurrent data on anal and cervical HPV, anal HPV was more common than cervical HPV in both HIV-positive (79% versus 53%) and HIV-negative (43% versus 24%) women. In a multivariate analysis of HIV-positive women, $CD4^+$ cell counts ≤ 200 cells/mm^3 compared with counts > 500 cells/mm^3 (relative risk, 1.4; 95% CI, 1.1–1.5) and cervical HPV infection (relative risk, 1.3; 95% CI, 1.1–1.4) were associated with anal HPV infection. Women over 45 years of age had a lower risk than women under 36 years of age (relative risk, 0.80; 95% CI, 0.50–0.99), and African-American women had a lower risk (relative risk, 0.86; 95% CI, 0.72–1.0) than Caucasian women.

Sobhani *et al.* (2001) determined the prevalence of anal dysplasia and cancer in patients with anal condyloma with respect to HIV status and HPV positivity. The most important factors that differed significantly between HIV-positive and HIV-negative patients were the prevalence of oncogenic HPV and other current infections (44% versus 0%). During follow-up, condylomas recurred in 75% of HIV-positive patients but in only 6% of HIV-negative patients.

Drobacheff *et al*. (2003) studied 50 HIV-positive and 50 HIV-negative men and women in France. Using Hybrid Capture 2 on anal swab material, HPV DNA was found in 58% and 6% of the samples, respectively. There was no difference in the prevalence of high-risk HPV types between men with and without a history of anal intercourse. Risk factors for HPV infection were $CD4^+$ cell counts < 500/μL (relative risk, 2.13; 95% CI, 1.0–4.7) and history of anogenital warts (relative risk, 2.36; 95% CI, 1.2–4.6). A very low prevalence of anal lesions was found in this study but the authors did not mention the use of high-resolution anoscopy to detect lesions. Using the Hybrid Capture RLU ratio, HPV load was greater in patients with $CD4^+$ cell counts ≤ 500/μL than in patients with $CD4^+$ cell counts > 500/μL ($p < 0.04$). Similar to the results of Palefsky *et al*. (2001), HIV-positive women in this study had a rate of anal HPV infection that was similar to that of HIV-positive men. Overall, the data showed that HIV-positive patients with low $CD4^+$ cell counts have anal HPV infection, regardless of the route of HIV transmission.

One study reported anal HPV and cytology data among HIV-positive and HIV-negative adolescent boys with a history of sex with men and girls who participated in the Reaching for Excellence in Adolescent Care and Health cohort (Moscicki *et al.*, 2003). The prevalence of anal HPV infection was similar in HIV-infected [28/58 (48%)] and uninfected [9/25 (36%)] boys ($p = 0.3$), but was greater in HIV-positive girls [59/183 (32%)] than in HIV-negative girls [11/82 (13%)] ($p < 0.001$). Abnormal anal cytology was more common among boys (41.6%) than girls (16.5%; $p < 0.001$). Independent risk factors for abnormal anal cytology in boys included infection with low-risk HPV types, infection with high-risk HPV types, infection with unknown HPV types and HIV positivity. Among girls, independent risk factors for abnormal anal cytology included infection with high-risk or unknown HPV types and number of sexual partners within the past 3 months. The results suggest that anal cytology screening should be considered in HIV-positive homosexual/bisexual boys and possibly HIV-positive girls.

Piketty *et al*. (2003) compared the prevalence of anal HPV infection and SIL among 67 HIV-positive men who had sex with men and 50 HIV-positive heterosexual male intravenous drug users with no history of receptive anal intercourse; 46% of the heterosexual intravenous drug users had anal HPV infection and 18% had anal HSIL. Among the 67 men who had sex with men, 85% had anal HPV infection and 18% had anal HSIL. The data showed that anal HPV infection and anal SIL may be acquired in the absence of anal intercourse in HIV-positive men.

Chin-Hong *et al*. (2004) studied the age-related prevalence of anal HPV infection among 1218 sexually active, HIV-negative men who had sex with men aged 18–89 years who participated in the EXPLORE study in four cities in the USA. HPV DNA was found

in the anal canal of 57% of the study participants. The prevalence of anal HPV infection did not change with age or geographical location. In a multivariate analysis, anal HPV infection was associated with receptive anal intercourse during the preceding 6 months (odds ratio, 2.0; 95% CI, 1.5–2.8; $p < 0.0001$) and with having 6–30 sexual partners during the preceding 6 months (odds ratio, 1.4; 95% CI, 1.1–1.9), and more than 30 partners (odds ratio, 2.3; 95% CI, 1.5–3.6).

(ii) *Natural history of anal HPV infection and anal SIL* (Table 74)

In a prospective study of 158 HIV-positive and 147 HIV-negative men who had sex with men and did not have anal disease at baseline, high-grade AIN developed in 15.2 and 5.4%, respectively (Critchlow *et al.*, 1995). High-grade AIN among HIV-positive men was associated with the detection of high levels of HPV 16 or 18, detection of HPV types other than 16 or 18, $CD4^+$ cell count $\leq 500 \times 10^6$/L and the number of positive HPV tests.

To characterize the natural history of anal HPV infection, Critchlow *et al*. (1998) followed 287 HIV-negative and 322 HIV-positive men who had sex with men who attended a community-based clinic. Anal HPV DNA was detected at study entry in 91.6% of HIV-positive and 65.9% of HIV-negative men. Detection of HPV was associated with lifetime number of sexual partners and recent receptive anal intercourse (HIV-negative men), lower $CD4^+$ lymphocyte count (HIV-positive men) and anal warts (all men). Among the men who were negative for HPV at study entry, detection of HPV during follow-up was associated with HIV, unprotected receptive anal intercourse and any sexual contact since the last visit. Becoming HPV-negative during follow-up was less common among men with HIV infection or high levels of HPV at study entry. Men with low-risk HPV types at entry were more likely to become HPV-negative than men with intermediate- or high-risk HPV types ($p \leq 0.05$).

In a prospective study of the incidence of anal HSIL among 346 HIV-positive and 262 HIV-negative men who had sex with men, Palefsky *et al*. (1998a) showed that HIV-positive men were more likely to develop HSIL than HIV-negative men (relative risk, 3.7; 95% CI, 2.6–5.7). A life-table estimate of the 4-year incidence of HSIL was 49% (95% CI, 41–56%) among HIV-positive and 17% (95% CI, 12–23%) among HIV-negative men. The incidence of high-grade AIN within 2 years of follow-up was 20% in HIV-positive and 8% in HIV-negative men who were normal at baseline; 62% of HIV-positive and 36% of HIV-negative men with low-grade AIN at baseline progressed to high-grade AIN. Of the HIV-positive men who had ASCUS at baseline, 70.3% were diagnosed with low- or high-grade AIN within 2 years, as were 30.8% of HIV-negative men. Overall, the relative risk for progression of anal disease in HIV-positive men was 2.4 (95% CI, 1.8–3.2) compared with HIV-negative men. The relative risk increased to 3.1 (95% CI, 2.3–4.1) in HIV-positive men with $CD4^+$ cell counts < 200/mm^3. Infection with multiple HPV types was a risk factor for progression of anal disease in both HIV-positive (relative risk, 2.0; 95% CI, 1.0–4.1) and HIV-negative (relative risk, 5.1; 95% CI, 2.3–11) men. The incidence of anal HSIL and progression of LSIL to HSIL within 2 years

Table 74. Anal HPV infection and anal squamous intraepithelial lesions (SIL) in HIV-positive and HIV-negative patients

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence (%)	Anorectal abnormality (%)	Pathology reading	Comments
Critchlow *et al*. (1995), USA	SBH and MY09/ MY11 PCR	158	147	NR	High-grade AIN, 15.2 HIV+, 5.4 HIV–	Cytology, histology	Dacron anal swab specimens used for HPV testing. Participants had no anal disease at baseline. The study included MSM only.
Critchlow *et al*. (1998), USA	SBH, HC or MY09/MY11 PCR (LR 6/11/42/43/44, IR 31/ 33/35/39 or HR 16/18/45)	322	287	*All types* 91.6 HIV+, 65.9 HIV–, *LR types* 49.1 HIV+, 36.2 HIV–, *IR types* 39.1 HIV+, 14.6 HIV–, *HR types* 55.9 HIV+, 28.9 HIV–			Prospective cohort study of anal HPV infection. HPV testing on anal swab material; included MSM only

Table 74 (contd)

Reference, study location	Method of detection (types included)	No. of HIV-positive cases	No. of HIV-negative controls	HPV prevalence (%)	Anorectal abnormality (%)	Pathology reading	Comments
Palefsky *et al*. (1998a), USA	MY09/MY11/HMB 01 (6, 11, 16,18, 26, 31, 32, 33, 35, 39, 40, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 66, 68, 69, 70, 73, Pap 155, Pap 291, AE2)	346	262	NR	*4-year incidence of HSIL* 49 HIV+, 17 HIV– *2-year incidence of HSIL among men with no disease at baseline* 20 HIV+, 8 HIV– *2-year incidence of HSIL among men with LSIL at baseline* 62 HIV+, 36 HIV–	Cytology, histology	Dacron anal swab specimens used for HPV testing; HIV+ participants followed every 3–6 months; HIV– participants followed every 12 months; included MSM only
Lacey, H.B. *et al*. (1999) United Kingdom	GP5/GP6 PCR primers with type-specific primers for 6/11, 16, 18, 31, 33	57		84.2 HIV+	70 incident HSIL among men with no abnormality or LSIL at baseline	Cytology, histology	Dacron anal swab specimen used for HPV testing. Participants were followed every 4 months. The study included MSM only.
Sobhani *et al.* (2001), France	In-situ PCR (6, 11, 16/18, 31, 33)	114	60		*Recurrence during follow-up* 75 HIV+, 6 HIV–		Study included men and women who had been treated for anal canal condyloma.

See Table 7 for a description of the primers used.
AIN, anal intraepithelial neoplasia; CI, confidence interval; HC, Hybrid Capture; HIV, human immunodeficiency virus; HIV+, HIV-positive; HIV–, HIV-negative; HSIL, high-grade squamous intraepithelial lesion; IR, intermediate-risk; HR, high-risk; LR, low-risk; LSIL, low-grade squamous intraepithelial lesion; MSM, men who had sex with men; NR, not reported; PCR, polymerase chain reaction; SBH, Southern blot hybridization

of follow-up is high in HIV-positive homosexual or bisexual men and lower in HIV-negative men.

In a study in the United Kingdom, Lacey, H.B. *et al*. (1999) followed 57 HIV-positive men who had sex with men over an average of 17 months. High-risk HPV types were detected in 84% and high-grade disease in 10.5% at baseline; 70% of the men with no or low-grade disease at baseline developed high-grade disease during follow-up. The study was limited by the relatively small sample size and by the fact that no biopsies were taken at baseline, which raises the possibility of misclassification of disease in some participants.

Sobhani *et al*. (2001) followed 114 HIV-positive and 60 HIV-negative men and women who had been treated for anal canal condyloma. During follow-up, condylomata recurred in 75% of HIV-positive (19 high-grade AIN and one invasive carcinoma detected in the follow-up biopsy), but only 6% of HIV-negative patients (one high-grade AIN). Male sex (odds ratio, 2.9; 95% CI, 1.9–7.2) and HIV positivity (odds ratio, 10.3; 95% CI, 8.8–12.9) were among the independent risk factors for recurrence of condyloma. High serum HIV load was also associated with recurrence, whereas low $CD4^+$ T-lymphocyte counts were not. In-situ hybridization and in-situ PCR were also used to detect EBV, CMV, HSV and gonococcus in biopsy specimens at baseline. One or more of these agents was found in specimens from 21 HIV-positive but no HIV-negative patients.

(iii) *Effect of HAART on anal SIL*

Palefsky *et al*. (2001) studied the short-term effects of HAART on the natural history of AIN. AIN and the level of anal HPV DNA, measured using the Hybrid Capture RLU ratio, were evaluated among 98 HIV-positive men who had sex with men at least 6 months before initiation of treatment with HAART. The results were compared with those evaluated 6 months after initiation of HAART. Among men whose most severe pre-HAART diagnosis was ASCUS or low-grade AIN, 18% (95% CI, 6–31%) of the lesions progressed and 21% (95% CI, 8–34%) regressed 6 months after initiation of HAART. Seventeen per cent (95% CI, 0–38%) of men who had a normal diagnosis at the start of the study developed AIN. Only 4% (95% CI, 0–10%; 1/28) of men had high-grade AIN that regressed to normal. There was no reduction in the proportion of men who tested positive for HPV DNA or in the levels of HPV DNA after initiation of HAART. These results indicate that HAART has little effect on either AIN or HPV in the first 6 months after initiation of treatment.

Durante *et al*. (2003) studied the incidence of anal cytological abnormalities among 100 HIV-positive women who participated in the GRACE cohort. Fourteen had abnormal anal cytology at baseline; among the remaining 86 women, 40 (46.5%) had an HPV infection in both the anus and the cervix. Of these, 13 had at least one HPV type in common, while 27 had no HPV types in common. Cervical and anal HPV infection were compared and, as in earlier studies, anal HPV infection was found to be more common than cervical HPV infection. Anal infection was found in 57 women (66.3%) and cervical HPV infection was found in 45 women (52.3%). Among the 86 women who had normal baseline cytology, the incidence of an abnormality was 22 (95% CI, 14–33) per 100 person–years. In a

multivariate analysis, women were at increased risk if they had a baseline $CD4^+$ cell count of < 500 cells/mm^3 (relative hazard, 4.11; 95% CI, 1.18–14.25) or a high-risk anal HPV-type infection (relative hazard, 2.54; 95% CI, 0.91–7.14) or were current cigarette smokers at baseline (relative hazard, 3.88; 95% CI, 1.12–13.42). Use of HAART had no effect on incidental anal cytological abnormalities (relative hazard, 1.07; 95% CI, 0.40–2.85). The authors concluded that the incidence of anal cytological abnormalities was high among this cohort of HIV-infected women, which indicates that they are at high risk for anal SIL.

Horster *et al*. (2003) screened HIV-positive patients for visible anal condyloma between 1985–95 (before treatment with HAART) and 1996–2001 (after treatment with HAART). A total of 1472 patients were screened repeatedly for anal condyloma as a risk factor for AIN and anal cancer. The proportion of screens that were positive for anal condyloma was significantly higher after the treatment ($p < 0.001$) independent of $CD4^+$ cell counts.

Gonzales-Ruiz *et al*. (2004) compared 117 HIV-positive patients in 1994–95 with 109 HIV-positive patients in 2001–02 with respect to the prevalence and distribution of HIV-related anorectal pathologies, such as anal ulcer and anogenital condyloma, and non-HIV-related anorectal pathologies, including fissure, fistula *in ano*, haemorrhoids and perianal abscess. The prevalence and distribution of anorectal pathology seen in HIV patients did not change after the introduction of HAART.

Piketty *et al.* (2004) examined the effect of HAART-associated increases in $CD4^+$ cell count on the prevalence of AIN. Forty-five HIV-positive protease inhibitor-treated men who had sex with men were enrolled in a cross-sectional study in France. The patients had previously received HAART for a median of 32 months. Anal cytology was abnormal in 32 patients (71%), and HPV DNA was detected in 36 (80%). Baseline prevalence of anal HPV infection and AIN was not affected by baseline $CD4^+$ cell count. The prevalence of anal SIL and HPV infection was similar in patients who had a significant increase in $CD4^+$ cell count after initiation of HAART compared with those who did not. The results demonstrated a high prevalence of anal SIL, including HSIL, and anal HPV infection in HIV-positive men who had sex with men despite immunity associated with HAART.

Wilkin *et al*. (2004) assessed the association between HAART and the prevalence of anal HPV infection and AIN in 92 HIV-positive men, 40 of whom had no history of anal intercourse. High-risk HPV DNA was identified in 61%, and was associated with a history of receptive anal intercourse (78% versus 33%; $p < 0.001$); 47% had abnormal cytology and 40% had AIN on biopsy. Risk factors for anal HPV infection included history of receptive anal intercourse (odds ratio, 7.2; 95% CI, 2.8–18; $p < 0.001$) but not baseline $CD4^+$ cell count, age or use of HAART. Among men with HPV infection, in a multivariate analysis, higher baseline $CD4^+$ cell count was protective against AIN at biopsy (odds ratio, 0.5; 95% CI, 0.3–0.9; $p = 0.03$) as was current use of HAART (odds ratio, 0.09; 95% CI, 0.01–0.75; $p = 0.03$). The relationship between the use of HAART and AIN was apparent only after controlling for baseline $CD4^+$ cell count.

(*c*) *Invasive cervical and anal cancer among HIV-positive subjects*

Zanetta *et al*. (1995) evaluated retrospectively all patients referred for invasive cervical carcinoma from 1991 to 1994 at the San Gerardo Hospital, Milan, Italy. Six of 340 women (1.8%) were found to be HIV-positive. Five of the six HIV-positive patients with cervical cancer were known to have been infected 13–81 months before diagnosis of cancer, but none had undergone a Pap test in the previous year. HIV-positive women were younger than the general population ($p = 0.02$) and were more likely to have a history of intravenous drug use ($p = 0.000001$) and more advanced disease at presentation ($p = 0.04$).

In the USA and Puerto Rico, Goedert *et al*. (1998) matched people who had cancer, were under the age of 70 years and were taken from population-based cancer registries with people who had AIDS and were taken from population-based AIDS registries. AIDS-related cancers were defined as those that had a significantly increased incidence after diagnosis of AIDS and an increased prevalence between 5 years before and 2 years after diagnosis of AIDS. The relative risk for anal cancer after AIDS was 31.7 (95% CI, 11.6–69.2). However, the risk was also increased about 15-fold during the early pre-AIDS period. Thus, the *p* value for trend was not significant ($p = 0.085$). The relative risk for cervical carcinoma *in situ* was < 1 during the period before AIDS but increased significantly after AIDS to 1.7 (p for trend = 0.01); in contrast, invasive cervical cancer had a relative risk of 5.4 before AIDS that did not increase significantly after AIDS (relative risk, 2.9; 95% CI, 0.7–16.0).

Mayans *et al*. (1999) reported on the incidence of cervical cancer in Catalonia, Spain, as an AIDS-defining diagnosis using an AIDS surveillance system from 1994 to 1996. Age-specific incidence rates for invasive carcinoma of the cervix from a population-based cancer registry were used to calculate the population attributable risk per cent. Fifty-six women with cervical cancer were reported to the AIDS registry, with a mean age of 32 years. Cervical cancer was the sixth most common AIDS-defining illness. The age-specific rate among HIV-positive women aged 20–49 years was 186.7/100 000 and the attributable fraction of HIV among women in this age group was 94.5%. The incidence rate ratio among HIV-positive and HIV-negative women in this age group was 18.5 (95% CI, 11.2–29.2).

Serraino *et al*. (1999) studied the risk for cervical cancer in a longitudinal study of 1340 HIV-positive intravenous drug users, 811 HIV-negative intravenous drug users and 801 HIV-positive heterosexual women aged 15–49 years in northern Italy and south-eastern France. A total of 9070 person–years of observation were accumulated among HIV-positive women and 2310 among HIV-negative women. Overall, the standardized incidence ratio (SIR) was 12.8 (95% CI, 6.6–22.4) among HIV-positive women and was higher for HIV-positive intravenous drug users (SIR, 16.7; 95% CI, 5.2–28.2) than for HIV-positive heterosexual women (SIR, 6.7; 95% CI, 0.0–15.9). No case of invasive carcinoma of the cervix was diagnosed among HIV-negative intravenous drug users.

In the largest study on the relationship between HPV-related neoplasia and HIV infection, Frisch *et al*. (2000) performed an AIDS–cancer registry match to examine invasive HPV-associated cancers and carcinoma *in situ* among 257 605 men and 51 760 women with

HIV infection/AIDS from 5 years before the date of onset of AIDS to 5 years after this date. The incidences of all HPV-associated cancers in AIDS patients were significantly increased compared with the expected numbers of cancers. For invasive cancers, overall risks were significantly increased for cervical (relative risk, 5.4; 95% CI, 3.9–7.2), vulvovaginal (relative risk, 5.8; 95% CI, 3.0–10.2) and anal (relative risk, 6.8; 95% CI, 2.7–14.0) cancers in women. Among men, the risks for anal (relative risk, 37.9; 95% CI, 33.0–43.4), penile (relative risk, 3.7; 95% CI, 2.0–6.2), tonsillar (relative risk, 2.6; 95% CI, 1.8–3.8) and conjunctival (relative risk, 14.6; 95% CI, 5.8–30.0) cancers were significantly increased. The relative risks for these invasive cancers changed little during the 10 years spanning the AIDS onset. Relative risks were significantly increased for in-situ cervical (4.6; 95% CI, 4.3–5.0) and vulvovaginal (3.9; 95% CI, 2.0–7. 0) lesions in women. The relative risk for anal cancer among men was highest among those with a history of homosexual contact (59.5; 95% CI, 51.5–68.4), although men who were reported to have acquired HIV through intravenous drug use (relative risk, 5.9; 95% CI, 2.7–11.2) were also at a significantly increased risk. Women with a history of intravenous drug use were at higher risk for cervical cancer (relative risk, 7.0; 95% CI, 4.7–10.0) than those who acquired HIV through heterosexual contact (relative risk, 4.9; 95% CI, 2.7–8.2). The estimated incidence of anal cancer after AIDS was 18.2/100 000 person–years among men and 3.9/100 000 person–years among women. The estimated incidence of cervical cancer among women after AIDS was 85.7/100 000 person–years and that for vulvovaginal cancer was 7.9/100 000 person–years. Among men, the relative risk was increased for anal (60.1; 95% CI, 49.2–72.7) and penile (6.9; 95% CI, 4.2–10.6) in-situ lesions. In contrast to invasive cancers, relative risks for in-situ lesions increased during the 10 years spanning the AIDS onset for the cervix (p for trend < 0.001), vulvovaginal area (p for trend = 0.04) and penis (p for trend = 0.04).

In a case–control study, Sitas *et al*. (2000) examined the relationship between HIV and a number of cancer types or sites that are common in three tertiary referral hospitals in Johannesburg, South Africa. Significant excess risks associated with HIV infection were found for vulvar cancer (odds ratio, 4.8; 95% CI, 1.9–12.2) and cervical cancer (odds ratio, 1.6; 95% CI, 1.1–2.3).

Using a match between the New York State Cancer Registry and the New York City AIDS Registry, Gallagher *et al*. (2001) compared cancer incidence in patients who were diagnosed with AIDS between 1981 and 1994 and were 15–69 years of age in New York State with that in the New York State general population. Sex and HIV-risk group-specific SIRs, relative risks after AIDS and trends of relative risks were calculated to determine the risk for cancer. Among non-AIDS-related cancers, an elevated SIR for combined rectal, rectosigmoid and anal cancer was found for both men (SIR, 3.3; 95% CI, 2.60–4.15) and women (SIR, 3.0; 95% CI, 1.39–5.77). After the diagnosis of AIDS, the SIR for combined rectal, rectosigmoid and anal cancer was 4.0 (95% CI, 2.6–6.0) in men and 4.2 (95% CI, 0.9–12.2) in women. Among women, the SIR for invasive cervical cancer was 9.1 (95% CI, 6.9–10.8) and the relative risk after AIDS was 6.5 (95% CI, 4.1– 9.7). Among men, the SIR for rectal, rectosigmoid and anal cancer was significantly elevated only among men with a history of homosexual contact (SIR, 5.8; 95% CI, 4.4–7.4). Among women, a history of

intravenous drug use, heterosexual contact and unknown/other factors resulted in significantly increased SIRs for rectal, rectosigmoid and anal cancer as well as cervical cancer.

Newton *et al*. (2001) performed a case–control study of HIV infection and cancer risk in Kampala, Uganda. Of the 302 cases recruited, 190 had a cancer with a potential infectious etiology (cases); the remaining 112 adults who had a tumour not known to have an infectious etiology formed the control group. The odds ratios for HIV positivity among cases of specific cancers (other than Kaposi sarcoma in adults) were compared with those in controls, adjusted for age and sex and, in adults, for the number of lifetime sexual partners. In adults, HIV infection was associated with a significantly ($p < 0.05$) increased risk for non-Hodgkin lymphoma and conjunctival squamous-cell carcinoma but not for cervical cancer (odds ratio, 1.6; 95% CI, 0.7–3.6).

Gichangi *et al*. (2002) studied 3902 women who were diagnosed with reproductive tract malignancies at Kenyatta National Hospital from 1989 to 1998, a period when the Kenyan national prevalence of HIV rose from 5 to 15%; 85% of the women had invasive cervical cancer and the age at presentation and severity of cervical cancer were compared between HIV-positive and HIV-negative women. There was no significant difference in either age at presentation or severity of cervical cancer between HIV-positive and HIV-negative women. Of the 118 (5%) women who were tested for HIV, 36 (31%) were HIV-positive and were significantly younger (42 versus 47 years of age; $p < 0.001$) than HIV-negative women. Neither the proportion of women with cervical cancer under the age of 35 years nor the severity of cervical cancer changed during the study period. The authors concluded that HIV-positive women were younger at the time of diagnosis of cervical cancer than HIV-negative women, but that the profile of cervical cancer changed relatively little in Kenya despite the rapid rise in HIV prevalence in the population.

Serraino *et al*. (2002) analysed data from the national AIDS surveillance systems of 15 European countries that had 50 or more female AIDS cases and from population-based cancer registries of those countries. Female cases aged 20–49 years who were diagnosed between 1993 and 1999 were included in the study. The odds ratio for cervical cancer as an AIDS-defining illness increased with age, was significantly elevated in southern (odds ratio, 3.1; 95% CI, 1.8–5.4) and central Europe (odds ratio, 2.5; 95% CI, 1.4–4.4) compared with northern Europe and was also increased among intravenous drug users (odds ratio, 1.5; 95% CI, 1.2–1.9). The proportion of cervical cancer correlated directly with the proportion of intravenous drug users among female AIDS cases and was highest in areas where population-based cervical cancer screening programmes were less effective.

Cress and Holly (2003) reported on the age-adjusted incidence rates calculated by gender, race/ethnicity, county and year of diagnosis for over 2100 cases of cancer of the anus diagnosed between 1995 and 1999 in California, USA. Age-adjusted incidence rates by time period 1973–99 were calculated for San Francisco County, where HAART was introduced widely in 1996. For all of California, there was an average 2% annual increase in the incidence of anal cancer among non-Hispanic white men between 1988 and 1999. The incidence of anal cancer among white men who resided in San Francisco County more than doubled between 1984–90 and 1996–99 and, for men aged 40–64 years, rose

from 3.7 cases per 100 000 in 1973–78 and 8.6 cases per 100 000 in 1984–90 to 20.6 cases per 100 000 in 1996–99.

Dal Maso *et al*. (2003) performed a linkage study of people aged 15–69 years using records from the Italian Registry of AIDS and 19 cancer registries that covered 23% of the Italian population for the period 1995–98. Significantly increased SIRs were observed for cervical cancer (21.8; 95% CI, 12.9–34.6) and anal cancer (34; 95% CI, 12.1–73.6). SIRs were similar for both cervical and anal cancer among intravenous drug users and non-users.

In another case–control study, Gichangi *et al*. (2003) studied the association between HIV infection and cervical cancer in Kenyan women. Cases were 367 women who had invasive cervical cancer and controls were 226 women who had fibroids. HIV-positive women with cervical cancer were significantly younger than HIV-negative women with cervical cancer ($p < 0.001$) and were more likely to have poorly differentiated tumours (odds ratio, 3.1; 95% CI, 1.2–8.3) after adjustment for histological cell type and clinical stage. Risk factors for cervical cancer in a multivariate analysis included HIV positivity under the age of 35 years (adjusted odds ratio, 3.3; 95% CI, 1.0–10.8) and never having had a Pap smear (adjusted odds ratio, 5.1; 95% CI, 1.8–14.6).

Mbulaiteye *et al*. (2003) linked records from AIDS and cancer registries in 11 regions of the USA from 1990 to 1996 to examine the relationship between AIDS-related immunosuppression (measured by $CD4^+$ cell count) and the risk for cancer. The SIRs in AIDS patients were 8.8 (95% CI, 6.0–13.0) for invasive cervical cancer, 9.3 (95% CI, 7.4–11.6) for in-situ cervical cancer and 49.9 (95% CI, not reported) for anal cancer. These risks were not modified by $CD4^+$ cell count.

Bower *et al*. (2004) followed a cohort of 8640 HIV-positive individuals in London, United Kingdom, and found that the incidence of invasive anal cancer was 60/100 000 patient–years. In the period before the introduction of HAART (1984–95), the incidence of invasive anal cancer was 35 (95% CI, 15–72)/100 000 patient–years of follow-up. In the period after the introduction of HAART (1996–2003), the incidence was 92 (95% CI, 52–149)/100 000 patient–years of follow-up ($p > 0.05$). The relative risks for anal cancer in the HIV-positive cohort compared with the general population were 67 and 176 in the periods before and after the introduction of HAART, respectively. The data showed that, although the difference between the incidence of anal cancer in the periods before and after the introduction of HAART was not significant, the incidence of anal cancer has not declined since the introduction of HAART.

Sobhani *et al*. (2004) studied 164 French HIV-positive patients who had condylomata of the anus after treatment of their lesion from 1993 to 2002. At baseline, 16% of HIV-positive patients and 6% of HIV-negative patients had high-grade AIN. During follow-up, seven of 199 patients (3.5%; six HIV-positive, one HIV-negative) developed invasive anal cancer after 13–108 months. Six of seven patients who developed anal cancer had high-grade disease at baseline.

3. Studies of Animal Papillomaviruses

Due to the species specificity of papillomaviruses, infection of experimental animals with human papillomavirus (HPV) is not possible. However, understanding the natural history and carcinogenic potential of HPVs is assisted by the study of several animal papillomaviruses.

Whereas cancer is the end-point to assess carcinogenicity in the study of HPV, benign tumours (warts and papillomas) are often used as the end-point in the analysis of the association of papillomavirus with naturally occurring or experimentally induced neoplasia in animals. This is based on the grounds that: (*a*) the incidence of warts is higher than that of cancer and is therefore easier to monitor; (*b*) it is difficult to follow the course of disease in wild animals; (*c*) domestic animals, such as cattle, are usually killed before the onset of malignancy; and (*d*) papillomavirus-associated cancer ultimately derives from warts, and thus the presence of warts can be considered as an indication of possible incipient neoplastic progression.

For each of the animal papillomaviruses discussed below, naturally occurring warts and their progression to cancer are considered primarily, followed by experimental reproduction in natural and heterologous hosts and tumour production in transgenic animals.

3.1 Non-human primate papillomaviruses (Table 75)

Two different types of papillomavirus were isolated from papillomas of the colobus monkey (*Colobus guereza*): CgPV 1 from a penile papilloma (O'Banion *et al.*, 1987) and CgPV 2 from a cutaneous papilloma (Kloster *et al*., 1988). CgPV 1 is a typical genital alpha-papillomavirus, whereas CgPV 2 belongs to the cutaneous beta-papillomaviruses (Chan, S.Y. *et al*., 1997a).

Another papillomavirus was isolated from five of eight cases of focal epithelial hyperplasia in pygmy chimpanzees (*Pan paniscus*) and was called PCPV (van Ranst *et al*., 1991). This virus is evolutionarily related to HPV 13 (85% sequence homology), which is associated with oral focal epithelial hyperplasia in humans (van Ranst *et al*., 1992a). Recently, oral focal epithelial hyperplasia was also diagnosed in a neotropical primate, the howler monkey (*Alouatta fusca*). Whereas the group-specific papillomavirus antigen (denatured L1) was identified by immunohistochemistry, in-situ hybridization with various HPV probes did not reveal any DNA, which suggests the possible presence of a papillomavirus that is specific for the howler monkey (Sá *et al.*, 2000).

Cervical and vaginal epithelial neoplasms, including cervical cancer, were identified in 20/385 (5.2%) female cynomolgus macaques (*Macaca fascicularis*). All lesions were positive when stained with antibodies against bovine papillomavirus 1 (BPV 1), HPV 1, 6, 11,

Table 75. Papillomaviruses in non-human primates

Non-human primate	Papillomavirus	Genus and species[a]	Greatest homology to	Reference
Colobus guereza	CgPV 1	Alpha 9/7	HPV-16/18	O'Banion *et al*. (1987)
	CgPV 2	Beta 1	HPV-5/8	Kloster *et al*. (1988)
Pan paniscus	PCPV	Alpha 10	HPV-13	van Ranst *et al*. (1991)
Alouatta fusca	HMPV	Alpha[b]	–	Sá *et al.* (2000)
Macaca mulatta	RhPV 1	Alpha 12	HPV-16	Kloster *et al.* (1988)
	RhPV a to m	Alpha	–	Chan, S.Y. *et al*. (1997b)

CgPV, *Colobus guereza* papillomavirus; HMPV, howler monkey papillomavirus; PCPV, pygmy chimpanzee papillomavirus; RhPV, rhesus monkey papillomavirus
[a] For a definition of genus and species, see de Villiers *et al*. (2004a).
[b] Uncertain

16, 18 or 31 and HPV 16 E6. Although a DNA fragment was amplified by polymerase chain reaction (PCR) using degenerate primers from nine of 16 cases, sequencing was not successful and the nature of the amplified fragment remains doubtful (Wood *et al.*, 2004).

Rhesus monkey genital papillomavirus

Kloster *et al*. (1988) isolated and cloned an integrated papillomavirus genome (designated RhPV 1) from a lymph node metastasis of a penile squamous-cell carcinoma in a rhesus monkey (*Macaca mulatta*). Viral DNA was fully sequenced and it was determined that the integration site in the viral genome was within the L1 open reading frame (ORF; Ostrow *et al*., 1991). A series of different RhPV genomes (RhPV a to RhPV m) were subsequently isolated and, similarly to RhPV 1, these were found to belong to the alpha-papillomaviruses (Chan, S.Y. *et al*., 1997b).

Ostrow *et al.* (1990) performed a retrospective study of a colony of rhesus monkeys to assess the extent of RhPV 1 infection in individuals that had either mated with the index male or with intermediate sexual partners. Biopsies or scrapes were analysed from 30 females, the index male and four intermediate males that all belonged to the same group, from four mature females from a different group and from seven virgin females. The direct (6/12) and indirect (15/18) mates of the index male were found to be positive for viral DNA, clinical lesions or histopathology. One of the four intermediate males analysed by PCR was positive for RhPV 1 DNA; four intermediate males were all clinically positive. The lesions displayed various degrees of neoplasia, ranging from koilocytosis, grade 1 cervical intraepithelial neoplasia (CIN1) and koilocytosis plus CIN1 to invasive squamous-cell carcinomas of the penis and the cervix. Virgin females and those from the outside group showed no RhPV 1 infection. These results strongly indicated that infection by RhPV 1 is a cause of genital neoplasia. Subsequently, Ostrow *et al*. (1995) analysed a number of fresh or archival genital tissues of rhesus monkeys from three geographically

distinct regions for evidence of papillomavirus infection. By PCR, RhPV 1 DNA sequences were found in 12/59 (20.3%) animals from the three areas. The serological status of the animals was also investigated and 34/59 (57.6%) animals were positive for at least one RhPV antigen. There was concordance between viral DNA positivity and seropositivity in 10 cases. Histopathological analysis showed that the majority of the samples was clinically normal, with the occasional presence of mild-to-moderate chronic inflammation and focal squamous metaplasia. Four cases showed features of papillomavirus infection; of these, one was classified as CIN1 and another was the only case that concorded with seropositivity. All cases were RhPV DNA-negative. This situation parallels HPV infection in humans, in which most cases of infection are undetected clinically and the concordance between seropositivity and viral DNA positivity is not complete.

3.2 Bovine papillomavirus (BPV)

3.2.1 *Heterogeneity of BPV* (Table 76)

BPVs are a heterogeneous group of viruses that are distributed worldwide. They induce papillomatosis of the skin, the genital and paragenital area, the eye, the upper gastrointestinal tract and the urinary bladder. Six members (BPV 1–6) have been described in detail (Jarrett *et al*., 1984a,b; Jarrett, 1985), and a further 13 types were identified recently (Antonsson & Hansson, 2002; Ogawa *et al*., 2004), which more than trebles the heterogeneity of BPVs (Table 76).

The six well-characterized BPVs were originally classified into two subgroups (A and B), based on their genomic structure and recognized pathology. Subgroup A comprised BPV 1, 2 and 5, which were commonly defined as fibropapillomaviruses — that is, viruses that infect both the epithelium and the underlying derma and give rise to fibropapillomas. Subgroup B comprised BPV 3, 4 and 6, defined as purely epitheliotropic BPVs that infect only the epithelium and induce true papillomas. Papillomaviruses have recently been reclassified (de Villiers *et al*., 2004a) following the Greek letter nomenclature used for other virus families. According to the new nomenclature, the epitheliotropic BPVs 3, 4 and 6 are defined as xi-papillomaviruses and BPVs 1 and 2 as delta-papillomaviruses. The genome of BPV 5 appears to share homology with both xi- and delta-papillomaviruses (Bloch & Breen, 1997) but BPV 5 appears to have a dual pathology and causes both fibropapillomas and epithelial papillomas (see below; Bloch *et al*., 1994a). These two observations have led to the re-classification of BPV 5 as the only member of the epsilon-papillomavirus genus (de Villiers *et al*., 2004a).

The new BPV types were found in teat papillomas and in healthy teat skin but their pathology and whether they are delta-, xi- or epsilon-papillomaviruses are not yet known. BPVs 1, 3 and 6 were also found in healthy teat skin, which strongly suggests latent or subclinical infection (see Section 3.2.3(*b*)).

Table 76. Heterogeneity of bovine papillomaviruses (BVPs) and their tumours

BPV type	Old classification	New classification[a]	Benign tumours/healthy skin	Malignant tumours in natural host	Malignant tumours in experimental hosts
BPV 1	Subgroup A	Delta 4	Skin fibropapillomas, including penis and teats	Penile carcinoma	Hamsters; transgenic mice
BPV 2	Subgroup A	Delta 4	Skin and oesophageal fibropapillomas	Urinary bladder cancer	Hamsters; SCID mice
BPV 3	Subgroup B	Xi	Skin epithelial papillomas	NK	NT
BPV 4	Subgroup B	Xi	Upper gastrointestinal tract epithelial papillomas	Upper gastrointestinal tract carcinoma	Hamsters; nude mice
BPV 5	Subgroup A	Epsilon	Skin epithelial and fibropapillomas (teats, udder and face)	NO	NT
BPV 6	Subgroup B	Xi	Skin epithelial papillomas (teats and udder)	NO	NT
BAA 1 to 5 BAPV 1 to 10 BAPV 11MY	NA	Delta/Xi[c]	Skin papillomas, healthy skin	NK	NT
BPV[b]	NA	NA	Skin fibropapillomas	Meningiomas[d]	
BPV[b]	NA	NA	Epithelial papillomas	Eye carcinoma	
BPV[b]	NA	NA	Skin hyperkeratosis	Skin carcinoma and basaliomas	

NA, not applicable; NK, not known; NO, never observed; NT, not tested
[a] For new classification and a definition of genus and species, see de Villiers *et al*. (2004a)
[b] Unidentified type
[c] To be established
[d] Meningiomas were experimentally produced in calves by injecting the virus into the brain.

3.2.2 *BPV 1*

BPV 1 induces primarily fibropapillomas of the penis of bulls and of the teats and udders of cows but can also spread to adjacent skin and to the muzzle (Campo *et al*., 1981). It has been used extensively in transmission experiments, in which the rate of infection in cattle can be up to 100% (Jarrett, 1985). Olson *et al*. (1969) were among the first to perform transmission experiments with BPV. In addition to transmitting BPV to skin, Gordon and Olson (1968) induced meningiomas in 17/19 calves (89.5%) by injecting the virus into the brain. These tumours were found as early as 33 days after inoculation and the incidence of neoplasms in the brain was similar to that of warts in the skin.

(*a*) *BPV 1 in hamsters*

Inoculation of BPV 1 into Syrian golden hamsters (*Mesocricatus auratus*) induced fibromas and fibrosarcomas of the skin, chondromas of the ear and meningiomas of the brain, depending on the site of injection; metastases to internal organs were relatively frequent particularly in the lungs (10% of the animals) (Olson *et al*., 1969). Pfister *et al*. (1981) extracted BPV 1 from a cow udder fibropapilloma and inoculated 10^9 viral particles subcutaneously into the back of each of six 2-month-old hamsters. Fourteen months later, one of the animals developed a fibrous histiocytoma with some areas of fibrosarcoma at the site of injection, and another developed a fibroma with partially atypic fibroblasts. Both tumours were positive for BPV 1 DNA, which was present in multiple episomal copies, but not for structural viral antigens or virus particles.

(*b*) *BPV 1 in transgenic mice*

BPV 1 transgenic mice (BPV 1:69 mice) were first generated by Lacey *et al*. (1986). A partial tandem duplication of the BPV 1 genome that contained two copies of the early transforming region and one of the late structural genes was used. One of these transgenic mice had approximately five copies of integrated viral DNA in head-to-tail tandem structures. The heterozygous progeny of this mouse were used to generate homozygous animals. At 8 months of age, all animals developed tumours (initially benign fibromas) in multiple body locations. Tumours were most frequently found in the face and head area and on the end of the tails of heterozygotes where they had been clipped for DNA analysis. The tumours became malignant and locally invasive with age. No virion or viral structural antigens were detected in the fibromas or fibrosarcomas. Whereas in young normal mice and in normal skin the viral DNA was integrated into the cell DNA and transcriptionally inactive, the viral DNA in the tumours was extrachromosomal and transcriptionally active (Lacey *et al.*, 1986; Sippola-Thiele *et al.*, 1989). The same transgenic mice were further analysed for specific chromosomal abnormalities that emerged during the carcinogenic process. In contrast to fibromas, fibrosarcomas consistently showed trisomy or duplication of chromosome 8 and/or monosomy or translocation of chromosome 14, which suggests that these chromosomal losses and/or duplications accompany and contribute to neoplastic transformation (Lindgren *et al*., 1989).

3.2.3 *BPV 2*

BPV 2 induces classical skin warts (Campo *et al*., 1981) that are histologically similar to those induced by BPV 1 (Jarrett, 1985). It also induces fibropapillomas of the oesophagus and rumen, which, contrary to fibropapillomas of the skin, do not produce viruses and appear to be the result of abortive infection (Jarrett *et al.*, 1984a). In experiments in which BPV 2 is transmitted to the skin, the virus produces warts in 100% of the animals (Jarrett, 1985).

(*a*) *BPV 2 and cancers of the urinary bladder* (Table 77)

In Scotland, 30% of cattle that had squamous-cell carcinoma of the upper gastrointestinal tract (see below) had concurrent bladder tumours (Jarrett *et al*., 1978a): haemangioendotheliomas (23%), transitional-cell carcinomas (8%), fibromas (4%) and adenocarcinomas (1%). The same histological types of tumour, including the Pagetoid variant of urothelial carcinoma, have been found in cattle in other parts of the world and were associated with bracken fern in the diet, which contains highly immunosuppressive and mutagenic chemicals (Pamukcu, 1963; Rosenberger, 1971; Hirono, 1986; Borzacchiello *et al*., 2001).

Injections of a 10% suspension of homogenized bovine wart tissue, either alone or in combination with 3-hydroxy-kynurenine and/or 3-hydroxyanthranilic acid, into the wall of the urinary bladder of 2–3-month-old calves induced fibromas and polyps in 13/15 animals examined cystoscopically at intervals starting 14 days after inoculation. Simultaneous intradermal injections of the same suspensions or application on scarified skin in the same animals induced fibropapillomas in the skin of 12 calves in 33–83 days. No malignant tumours were observed in six calves examined histopathologically from 40 to 81 days after inoculation (Olson *et al*., 1959). In another experiment (Olson *et al*., 1965), suspensions of six naturally occurring bladder tumours (two haemangiomas, one haemangioma plus

Table 77. Bovine papillomavirus (BPV) in urinary bladder cancers

BPV type	Naturally occurring cancers (%)	Experimentally induced cancers (%)	Control cases	Reference
NK		13/15 (87)	NA	Olson *et al*. (1959)[a]
BPV 2	7/15 (46%)	9/13 (69%)	2/10 (20%)	Campo *et al*. (1992)
BPV 2	1[b]		NA	Borzacchiello *et al*. (2001)
BPV 2	46/60 (75%)*		17/34 (50%)*	Borzacchiello *et al*. (2003a)
BPV 2	11/27 (40%)		NK	Lioi *et al*. (2004)

NA, not applicable; NK, not known

[a] The cancers were induced with a suspension of bovine wart tissue.

[b] Single case report

* $p < 0.01$

papilloma, two papillomas, one papilloma plus adenocarcinoma plus squamous carcinoma — this latter case was accompanied by metastasis to the iliac node) were inoculated into the skin, vagina and urinary bladder of young calves. Of 17 inoculated calves, 10 developed skin fibropapillomas, seven developed fibropapillomas of the vagina and five developed polyps and fibromas of the urinary bladder. These experiments demonstrated both the presence of BPV in tumours of the urinary bladder and the ability of the virus to induce bladder tumours. [At that time, the heterogeneity of BPV was not known and the identity of the virus used in the above experiments is uncertain.]

Campo *et al*. (1992) and, more recently, Borzacchiello *et al*. (2003a) and Lioi *et al*. (2004) showed that the virus that is involved in bladder cancer in cattle in Italy and the United Kingdom is BPV 2. Campo *et al*. (1992) found multiple copies of episomal BPV 2 DNA in seven of 15 biopsies (46%) of naturally occurring bladder tumours from animals in bracken-infested areas. Eight of 10 normal bladder biopsies were negative and, of the remaining two biopsies, one was positive for BPV 2 DNA and the other contained an unidentified papillomavirus. Borzacchiello *et al*. (2003a) found BPV 2 DNA in 46/60 (75%) biopsies of bladder cancer and 17/34 (50%) biopsies of normal bladder epithelium. Despite the high incidence of BPV 2 DNA in normal urothelium, the difference between pathological and normal samples was statistically significant ($p < 0.01$). Lioi *et al*. (2004) found BPV 2 DNA in 11/27 (40%) bladder tumour biopsies; there was no information on BPV DNA positivity in normal samples of the urinary bladder.

In an experiment designed to reproduce the progression of papillomas to carcinomas of the upper gastrointestinal tract (see below), further evidence of the involvement of BPV 2 and its synergism with bracken fern in the induction of urinary bladder malignancies was obtained. Calves approximately 3–4 months of age were immunosuppressed by either treatment with azathioprine (10 animals) or a diet with bracken fern (12 animals). Some of the animals were infected with BPV 4 (see below), but not with BPV 2. All of the immunosuppressed calves developed urinary bladder tumours approximately two years after the beginning of the experiment. However, the animals immunosuppressed with azathioprine developed benign haemangiomas, whereas the animals fed bracken fern developed malignant tumours that were representative of the whole range of naturally occurring bladder cancers. Bladder biopsies from three animals in the azathioprine-treated group and 10 animals in the group fed bracken fern were analysed for the presence of BPV DNA. BPV 2 DNA was found in biopsies of tumours from nine of 13 animals (69%), including haemangiomas of the azathioprine-treated animals. Biopsies from four animals of the group fed bracken fern were negative. Biopsies from cases with multiple tumour types were either all positive or all negative. As in the naturally occurring bladder cancers, no virus or structural viral antigens, no evidence of abortive infection and no production of virus were detected in the experimental tumours, as in cases of fibropapillomas of the upper gastrointestinal tract (see above). It was concluded that immunosuppression favoured the establishment of premalignant viral lesions, and that mutagens present in the bracken fern promoted their malignant progression (Campo *et al*., 1992).

The viral DNA in bladder lesions is infectious and can initiate a replicative cycle in the permissive environment of the skin; extracts from urinary bladder cancers induced skin warts (Olson *et al*., 1965). The viral oncoprotein E5 is expressed in the tumour tissue (Borzacchiello *et al*., 2003a), the *Ras* gene is activated at early stages of ptaquiloside carcinogenesis (Shahin *et al*., 1998; Campo, 2002) and expression of the tumour suppressor fragile histidine tetrads (*FHIT*) locus is down-regulated (Borzacchiello *et al*., 2001). Fragile sites are often disrupted by integration of HPV DNA in cervical cancers (Butler *et al*., 2000) and alterations of *FHIT* expression have been observed in many cervical carcinomas (Takizawa *et al*., 2003). The immunosuppression induced by bracken fern prevents tumour rejection and the mutagens in fern contribute to destabilization of the genome, particularly when BPV is involved. Ingestion of bracken fern has been deemed to be the cause of chromosomal abnormalities, which include acentric fragments, rearrangements and chromatid and chromosome breaks and gaps (Moura *et al*., 1988; Stocco dos Santos *et al*., 1998; Lioi *et al*., 2004). The incidence of chromosomal abnormalities increases when the bladder is infected with BPV 2 (Lioi *et al*., 2004).

(*b*) *Latency of BPV 2*

Experimental reproduction of tumorigenesis of the urinary bladder also showed the presence of latent BPV 2 (Campo *et al.*, 1992), which could be reactivated by immunosuppressive treatment, as in the bladder, and/or by damage to the skin (Campo *et al*., 1994a). Four of 10 azathioprine-treated cattle developed skin warts, two contained BPV 1 and two showed the presence of BPV 2. One of 12 animals fed bracken fern developed a BPV 2 wart. All the warts developed at sites of damaged skin. Four fully immunocompetent animals developed BPV 1 warts at the site of damaged skin, which indicated that wounding, with the attendant cell proliferation, is sufficient for reactivation of the latent virus (Campo *et al.*, 1994a).

Epithelia may not be the only site where latent papillomavirus is located. BPV DNA has been found in the episomal form in circulating lymphocytes of three of five experimental cattle with warts and in lymphocytes of the general cattle population in the presence or absence of warts (seven of 18) (Campo *et al*., 1994a). Latent BPV 2 infection of lymphocytes has also been established in cattle (10/11) by transfection of blood from three donors that had BPV 2 in their lymphocytes (3/3) (Stocco dos Santos *et al*., 1998).

(*c*) *BPV 2 in mouse xenografts*

The ability of BPV 2 DNA to induce tumours was confirmed in xenografts. BPV 2 DNA extracted from bovine fibropapillomas was injected into bovine scrotal skin before it was grafted onto the back of severe combined immunodeficient (NOD- SCID) mice. All of the 14 grafts developed fibropapillomas that produced mature infectious virus. When the experiment was repeated with molecularly cloned BPV 2 DNA, only 50% of 140 grafts developed epithelial papillomas that had no fibroblastic component and did not produce virus. Similar results were observed with molecularly cloned BPV 1 DNA. The differences between the tumours induced by ‘natural’ BPV DNA and recombinant BPV DNA was

ascribed to a different methylation pattern of the viral genome and it was concluded that production of the virus requires a fibroblastic component (Pawellek *et al.*, 2002).

(*d*) *BPV 2 in hamsters*

A 10% suspension of a spontaneous fibropapilloma that contained BPV 2 and was removed from the neck of a cow was injected subcutaneously into the back of a hamster. After 2 years, a subcutaneous fibrous tissue nodule was excised from the injection site. The induced tumour was diagnosed as a fibrosarcoma that contained multiple extrachromosomal copies of complete BPV 2 DNA, but no virus or structural viral antigens (Moar *et al*., 1981).

3.2.4 *BPV 3*

BPV 3 was isolated from epithelial skin papillomas in Australian cattle (Pfister *et al*., 1979). It produced warts on the skin but not in the conjunctiva or at other sites. Nothing is known about its natural history and no transmission experiments have been performed.

3.2.5 *BPV 4*

BPV 4 is the causative agent of papillomas of the upper gastrointestinal tract in cattle (Campo *et al*., 1980). In a survey of 7746 cattle from local abattoirs, Jarrett *et al*. (1978b) found upper gastrointestinal tract tumours in 19% of the animals. One hundred unselected serial papillomas were taken for histological examination; 78% were found to be true epithelial squamous papillomas and 22% were fibromas or fibropapillomas; 79% of the affected animals had papillomas at one site and the remaining 21% had papillomas at more than one site. BPV 2 DNA but no virus was present in the fibropapillomas (Jarrett *et al*., 1984a), while the BPV 4-induced epithelial papillomas produced virus (Campo *et al*., 1980). BPV 4 was also found in 47/75 (62.2%) papillomas of the upper gastrointestinal tract in cattle (Borzacchiello *et al*., 2003b).

(*a*) *BPV 4 and cancer of the upper gastrointestinal tract*

In the above-mentioned survey, 80 cases of squamous-cell carcinomas of the upper gastrointestinal tract [7% tongue, 5% palate, 8% pharynx, 50% oesophagus, 30% rumen] were selected in so-called ‘cancer farms’ [total incidence not given], the grazing grounds of which were infested with bracken fern. Of 366 cattle from ‘cancer farms’, 39% had squamous papillomas. Ninety six per cent of the animals that had squamous-cell carcinomas also had squamous papillomas; 36% of the animals that had squamous-cell carcinomas had metastases (liver and/or spleen), 56% had tumours of the large intestine (polyps, adenomas and adenocarcinomas) and 30% had urinary bladder cancers. All stages of progression from papilloma to squamous-cell carcinoma were observed (Jarrett, 1978; Jarrett *et al.*, 1978a,b). It was concluded that, in animals that grazed on bracken fern, BPV 4 papillomas were widespread, persistent and prone to progress to cancer, probably

due to the synergistic interaction between the virus and the chemicals present in the bracken fern (Jarrett *et al*., 1978a).

Widespread and persistent papillomatosis of the upper gastrointestinal tract can also occur in the absence of bracken fern when cattle are immunosuppressed by other factors, such as infection with bovine diarrhoea virus (Tsirimonaki *et al*., 2003).

The progression from papilloma to carcinoma was reproduced by Campo *et al*. (1994b) in an experiment that lasted 13 years. Of 32 calves, 3–5 months of age, six were infected in the palate with BPV 4, six were infected with BPV 4 and immunosuppressed with azathioprine, four were immunosuppressed with azathioprine, six were kept on a diet of bracken fern, six were infected with BPV 4 and fed bracken fern and four were kept as untreated controls. The virus-infected azathioprine-treated animals had to be killed after 2 years. All calves infected with BPV 4 developed squamous papillomas at the site of injection. However, the animals that were immunosuppressed either by azathioprine or by bracken fern developed florid and persistent papillomatosis with papillomas that spread away from the inoculation site, particularly in the azathioprine-treated animals. The last surviving animal from the group treated with BPV 4 and bracken fern still had papillomas 13 years after infection which had spread from the mouth to the lower oesophagus and the rumen. No progression from papilloma to carcinoma was observed. Two of six animals from the virus-treated group fed bracken fern developed cancers of the upper gastrointestinal tract and lower intestine 6 and 10 years, respectively, after the start of the experiment. Both animals had typical papillomas, foci of carcinoma in the oesophagus that infiltrated the underlying tissue and polyps and adenomas and adenocarcinomas of the duodenum, jejunum and colon. No malignancies of the gastrointestinal tract were detected in animals of the other groups. As observed previously for naturally occurring upper gastrointestinal tract cancers (Campo *et al*., 1985), in which only one of approximately 100 cases of cancer examined was found to be positive for BPV 4 DNA, no viral DNA could be detected in the experimental cancers. It was concluded from this experiment that immunosuppression prevented rejection of papillomas and allowed their expansion, while mutagens present in the bracken fern were responsible for neoplastic conversion of papilloma cells and promoted their neoplastic progression. The almost total absence of BPV 4 DNA in the cancers suggests that the continuous presence and expression of the viral genome is not necessary for maintenance of the neoplastic state.

(*b*) *BPV 4 in mouse xenografts*

The tumorigenic potential of BPV 4 was studied in nude mouse xenografts. Chips of fetal bovine palate tissue infected with BPV 4 were implanted into nude mice either under the kidney capsule or subcutaneously and induced virus-producing papillomas (Gaukroger *et al*., 1989). One of the xenograft papillomas underwent spontaneous transformation to squamous-cell carcinoma with metastasis to the spleen (Gaukroger *et al*., 1991). The malignant cells were confirmed to be of bovine origin by major histocompatibility complex typing and by the nucleotide sequence of the bovine *Ras* gene. No BPV 4 DNA was detected in either the primary or metastatic cancer. Spontaneous conversion of papillomas in the

xenograft system is very rare and was observed only once in approximately 100 mice bearing papillomas generated in different experiments. Neoplastic progression was, however, greatly accelerated by the implantation in the recipient mice of slow-releasing pellets of either the initiator 7,12-dimethylbenz[*a*]anthracene (DMBA) or the promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The progression of BPV 4 papillomas to carcinomas was observed in 13/20 (65%) implants in mice exposed to DMBA and in four of 33 (12%) implants in mice exposed to TPA (Gaukroger *et al*., 1993).

(*c*) *BPV 4 in hamsters*

Six young Syrian hamsters received injections of a 10% suspension of a homogenized BPV 4 papilloma into the right buccal pouch and intradermally on the skin of the back. One hamster developed a liposarcoma at the site of injection 20 months later. The tumour had no evidence of fibrocytic transformation which concorded with the inability of the virus to transform fibroblasts *in vivo*; it was positive for BPV 4 DNA, which was present in multiple extrachromosomal copies, but not for virions or structural antigens (Moar *et al*., 1986).

3.2.6 *BPV 5 and BPV 6*

BPV 5 induces 'rice grain' fibropapillomas (so called because of their appearance) on the teats and udders of cattle (Campo *et al*., 1981) and BPV 6 induces epithelial papillomas (Jarrett *et al*., 1984b). In the United Kingdom, these two viruses have not been found in any other location in the body and the tumours they produce have not been reported to undergo malignant conversion, although BPV 6 papillomas are very persistent and natural regression has not been observed (Jarrett, 1985).

In a survey of 1657 cattle from local abattoirs (Lindholm *et al*., 1984), 37% of the animals were found to have papillomas on the teats and udders. Of the affected animals, 28% had BPV 1 warts, 88% had BPV 5 warts and 92% had BPV 6 warts; 58% had double infections with BPV 5 and 6 and 23% had triple infections with BPV 1, 5 and 6; only 14% were infected by only one virus, and this was most frequently BPV 6 (8.7%) followed by BPV 5 (4.4%) and then by BPV 1 (0.8%). BPV 4 was never found and it was therefore concluded that there is no association between alimentary and teat papillomas.

Although BPV 5 had been detected only in fibropapillomas in the United Kingdom (Jarrett, 1985), a later survey conducted in Australia revealed that it can cause both fibropapillomas and epithelial papillomas (Bloch *et al*., 1994a).

3.2.7 *Unknown BPV types that cause cancer in cattle*

(*a*) *Bovine ocular squamous-cell carcinoma*

In Australia, approximately 10–20% of some herds are affected by ocular squamous-cell carcinoma (Spradbrow & Hoffman, 1980). The carcinomas derive from papillomas, the malignant transformation of which is particularly noticeable in lightly pigmented ani-

mals and implicates the role of ultraviolet (UV) light as a co-carcinogen. Viral particles that strongly resemble papillomavirus were detected in eight of 25 early lesions, including one conjunctival plaque, five conjunctival papillomas, one eyelid papilloma and one eyelid keratinized horn (Ford *et al*., 1982).

(*b*) *Bovine skin carcinoma*

Australian herds are affected commonly by skin cancer (Spradbrow *et al*., 1987). Similarly to ocular squamous-cell carcinomas, the cancers derive from precursor lesions. Of a herd of 4–15-year-old cattle, all 13 had lesions with differing degrees of severity, from early lesions, such as cutaneous horns with acanthosis and hyperkeratosis, to advanced lesions, such as squamous-cell carcinomas and basaliomas. In two of four animals that were observed for 3 years, progression of early lesions to squamous-cell cancer was observed. Viral DNA that hybridized to BPV 1 under low-stringency conditions was found in 10/11 keratotic lesions and five of eight neoplasms (two squamous cancers and three basaliomas). Similarly to bovine ocular squamous-cell carcinomas, it was concluded that an unknown papillomavirus in conjunction with UV light was responsible for the skin cancers.

3.2.8 *BPV in equine sarcoids* (Table 78)

Equids can be infected by BPV and the infection leads to a fibroblastic tumour called a sarcoid. Sarcoids are a common disease of horses (*Equus equus*) and donkeys (*Equus asinus*); they are locally invasive, non-metastatic tumours that are rarely rejected by the host.

The histological similarity between equine sarcoids and bovine fibromas suggested a link between BPV and the equine disease. Infection of horses with BPV induced sarcoids

Table 78. Bovine papillomavirus (BPV) DNA in equine sarcoids

No. of positive cases	Predominant (like)-type	Reported equine 'variants'	Geographical location	Reference
12/14 (86%)	BPV 1	Yes	Australia	Trenfield *et al*. (1985)
12/13 (92%)	BPV 1	Yes	USA	Angelos *et al*. (1991)
17/20 (85%)	BPV 1	Yes	Switzerland	Angelos *et al*. (1991)
24/24 (100%)	BPV 1	Yes	United Kingdom	Reid & Smith (1992); Reid *et al*. (1994)
58/58 (100%)	BPV 1	No	Switzerland	Otten *et al*. (1993)
56/76 (73%)	BPV 1	Yes	Australia	Bloch *et al*. (1994b)
41/41 (100%)	BPV 1	No	Belgium	Martens *et al*. (2001a)
94/96 (98%)	BPV 2	No	USA	Carr *et al.* (2001a)
39/41 (95%)	BPV 1	Yes	United Kingdom, Switzerland	Chambers *et al*. (2003a,b)

similar to those that occur naturally; however, in contrast to natural sarcoids, experimental sarcoids regressed (Olson & Cook, 1951).

Lancaster *et al*. (1977) first detected BPV DNA in natural equine sarcoids in the USA. Neither natural nor experimental sarcoids contained virus or structural viral antigens. More recent analyses of these equine tumours throughout the world have confirmed the original findings (Table 78).

Trenfield *et al*. (1985) reported the presence of BPV DNA in 12/14 (86%) equine sarcoids from Australia. The restriction enzyme pattern of the BPV sequences was not identical to that of BPV 1 or BPV 2, which suggested the presence of variants or subtypes.

Angelos *et al*. (1991) found BPV DNA in 12/13 (92%) sarcoids from horses from New York State and in 17/20 (85%) sarcoids from horses from Switzerland. The viral DNA was similar to BPV 1 in 22 biopsies and similar to BPV 2 in seven biopsies. BPV DNA was also found in one biopsy each of a fibrosarcoma, a fibropapilloma and a pyogranulomatous dermatitis. No biopsy showed a restriction enzyme pattern of viral DNA identical to reference BPV 1 or BPV 2 DNA, which indicated the presence of BPV subtypes or variants.

Reid and Smith (1992) and Reid *et al*. (1994) analysed 24 sarcoid samples from six horses and 18 donkeys from the United Kingdom. All the biopsies contained BPV DNA and BPV 1-like sequences were more prevalent than BPV 2-like sequences; however, the sequences were not identical to either BPV 1 or BPV 2. There was no correlation between viral type, clinical type or anatomical location of the lesions or sex of the animals.

Otten *et al*. (1993) analysed 58 sarcoids from 32 horses and two donkeys. BPV 1 DNA was found in 55 biopsies and BPV 2 in three biopsies. One horse had two sarcoids, one with BPV 1 DNA and the other with BPV 2 DNA. The BPV sequences in the sarcoids had the same restriction enzyme patterns as those found in BPV 1 and BPV 2 isolates from cattle from the same geographical area, and the relative incidence of BPV 1 and BPV 2 infection was the same in cattle and horses, which suggested that the BPV variants found in equine sarcoids are not specific for horses.

Bloch *et al*. (1994b) conducted a retrospective analysis of equine sarcoids from Australian horses. BPV DNA was detected in 56/76 (73%) samples; of these, 82% were similar to BPV 1 and 18% were similar to BPV 2.

Martens *et al*. (2001) found BPV DNA in all of 41 samples of sarcoids from 19 Belgian horses. Thirty-four sarcoids harboured BPV 1 DNA and seven contained BPV 2 DNA.

Carr *et al*. (2001a) found BPV DNA in 94/96 (98%) sarcoid samples from American horses; BPV 2 DNA was present in 62% of the positive samples and the viral nucleotide sequences had 100% homology with reference BPV 1 or 2.

More recently, Chambers *et al*. (2003a,b) found BPV DNA in 39/41 (95%) samples of sarcoids from horses in the United Kingdom and Switzerland. BPV 1 DNA was present in 34/37 positive samples, although variations from the established nucleotide sequences of BPV 1 or BPV 2 were consistently observed. BPV DNA was also found in four of five samples of granulomatous dermatitis, as reported previously by Angelos *et al*. (1991). It is unclear what role this condition might play in sarcoid pathogenesis.

In all surveys but one (Carr *et al*., 2001a), BPV 1-like DNA has been found more often than BPV 2 DNA (Table 78). Furthermore, the absence in most surveys of BPV 1 or BPV 2 DNA sequences identical to those of the reference genomes suggests the existence of 'equine-adapted' variants of BPV that specifically infect horses.

The causal involvement of BPV in equine sarcoids has been confirmed by the expression of viral oncogenes. Nasir and Reid (1999) analysed sarcoids for the expression of BPV *E2*, *E5*, *E6*, *E7* and *L1* genes and found that 18/20 tumour samples examined were positive for E2 expression and 10 were positive for L1 expression. Viral oncogene *E5*, *E6* and *E7* transcripts were detected in 16, nine and 12 tumours, respectively. Carr *et al*. (2001b) found that 23/23 sarcoids were positive for expression of the E5 oncoprotein. Chamber *et al*. (2003b) detected genomic BPV E5 DNA in 39/41 (95%) sarcoids and BPV E5 mRNA transcripts in 34/41 (85%) samples which confirmed active viral transcription.

3.3 Equine papillomavirus (EqPV)

Horses can also develop genital, cutaneous, ocular, and oral papillomas and squamous-cell carcinomas. Papillomavirus antigen was detected in papillomas but not in carcinomas (Junge *et al*., 1984; Olson, 1987). An EqPV was isolated from cutaneous papillomas of one pony and one horse. The same viral type was found in papillomas of the muzzle and the leg but not in penile papillomas, which suggests the existence of two different types of EqPV (O'Banion *et al.*, 1986).

3.4 Papillomavirus in cervidae

Papillomavirus DNA was isolated from fibropapillomas or fibromas of different members of the cervidae family, such as European elk (*Alces a. alces*) (EEPV or EPV; Moreno-Lopez *et al*., 1981), reindeer (*Rangifer tarandus*) (RPV; Moreno-Lopez *et al*., 1987), red deer (*Cervus elaphus*) (RDPV; Moar & Jarrett, 1985), mule deer (*Odocoileus hemionus*) and white-tailed deer (*Odocoileus virginianus*) (DPV; deer fibromavirus subtypes a and b, respectively; Groff *et al*., 1983). While DPV and EEPV have been reported to induce tumours with a prominent fibroblastic component *in vivo*, RPV mainly induces fibromas in its natural host.

The genomes of EEPV or RPV induced fibrosarcomas after experimental infection of young Syrian hamsters by subcutaneous injection. The tumours contained multiple, non-integrated copies of the EEPV or RPV genome (Stenlund *et al*., 1983; Moreno-Lopez *et al*., 1987).

3.5 Cottontail rabbit papillomavirus (CRPV)

The discovery of the viral etiology of naturally occurring warts in cottontail rabbits (Shope & Hurst, 1933) and the subsequent demonstration that benign papillomas induced by the virus progress into carcinomas (Rous & Beard, 1935) constitute hallmarks in the

history of viral oncology. A number of important properties of papillomaviruses such as the role of E6 and E7 viral genes in the development of papillomas and carcinomas (Georges *et al*., 1984; Nasseri & Wettstein, 1984; Danos *et al*., 1984) and the synergism between virus and chemical co-carcinogens (Rous & Beard, 1934; Rous & Kidd, 1938; Rous & Friedwald, 1944) were established for the first time using CRPV.

3.5.1 *Species specificity*

In nature, CRPV infects primarily cottontail rabbits (*Sylvilagus floridanus*) and occasionally jackrabbits (*Lepus californicus*), and rabbit papillomatosis is endemic in certain parts of the USA (Syverton, 1952; Stevens & Wettstein, 1979; Kreider & Bartlett, 1981). Infectious virus has been obtained from papillomas induced in jackrabbits and snowshoe hares (*Lepus americanus*) (Beard & Rous, 1935), and the host range has even been extended to rats under experimental conditions (Kreider & Bartlett, 1981). Warts of naturally infected cottontail rabbits usually contain large amounts of virion, in spite of a great variation in viral content (Beard, 1956).

3.5.2 *Viral multiplication and tumour induction*

The first phase of infection in cottontail rabbits lasts from 1 to 6 weeks, during which time papillomas grow; 95–100% of the infected animals develop papillomas that are permanently benign in 71%, regress in 6% and progress to squamous cancer within 12–18 months in 23% of the animals. Experimental infection of domestic rabbits (*Oryctolagus cunniculus*) follows a different course: 95–100% of domestic rabbits developed papillomas after 1–6 weeks and regression of the papillomas occurred in 9% of the rabbits after 1–3 months. In 25% of the rabbits, papillomas remained permanently benign but, in 66% of the animals, they progressed to squamous-cell carcinomas within 6–12 months and were mainly accompanied by metastatic spread to the lung (Wettstein, 1987). A larger number of papillomas progress more rapidly to cancer in domestic rabbits than in cottontail rabbits, which implies that the genetic background of the host is involved in malignant conversion. Infectious virus is found in papillomas of cottontail rabbits but not in those of domestic rabbits or in cancers in either species; however, viral DNA is present in the non-productive lesions at low copy numbers (Shope & Hurst, 1933; Beard, 1956; Noyes & Mellors, 1957; Orth *et al*., 1971; Nasseri & Wettstein, 1984; Wettstein, 1987; Zeltner *et al*., 1994).

3.5.3 *Co-factors for tumour induction and progression*

Malignant transformation of CRPV-induced papillomas is accelerated and its frequency is increased when scarified skin is exposed to chemical carcinogens.

The application of tar to the ears of rabbits produces generalized epidermal hyperplasia and papillomatosis, but the papillomas never become malignant and often regress after cessation of application. However, when administered intravenously to rabbits,

CRPV induces carcinomas on tarred skin. Rapid malignant progression was also observed when CRPV-induced papillomas were treated with methylcholanthrene (Rous & Kidd, 1938; Rous & Friedewald, 1944; Rogers & Rous, 1951). In addition, the much higher frequency of tumour progression observed in domestic rabbits compared with cottontail rabbits implies that the genetic background of the host is an intrinsic co-factor in the malignant progression of persistent warts, which has been linked to genes in the class II region of the major histocompatibility index (Han *et al.*, 1992, 1994).

3.5.4 *Latency of CRPV*

In an experiment designed to study the latency and reactivation of CRPV, Amella *et al*. (1994) scarified and inoculated domestic rabbits with serial dilutions of CRPV. With undiluted virus, six of seven injection sites in seven rabbits developed papillomas. With virus diluted from 1:1000 to 1:100 000, none of 14 sites in 14 animals developed papillomas. PCR on tissue from injection sites that did not develop papillomas showed the presence of viral DNA. It was concluded that infection with low doses of virus results in the establishment of viral latency, and that the virus can be reactivated by skin injury.

Zhang *et al*. (1999) reported that highly diluted preparations of CRPV lead to the establishment of a subset of latent infections in New Zealand white rabbits that can be activated by UV-radiation shortly after infection. Sites that did not form papillomas within 3 months after irradiation were CRPV DNA-positive and showed transcripts from the E1 region, but were E6/E7 RNA-negative. Attempts to activate infections that remained latent by repeating UV irradiation at the end of the 3-month observation period were, however, unsuccessful.

3.5.5 *CRPV in transgenic rabbits*

CRPV in conjunction with activated *Ras* was used to generate three transgenic New Zealand white rabbits. Two rabbits had CRPV DNA only and one had both CRPV DNA and activated *Ras*. The two CRPV transgenic rabbits were phenotypically normal up to 2 weeks after birth, but then started to develop epidermal hyperkeratosis. When the animals were 20–30 days of age, small papillomas appeared and spread all over the body. The rabbits died of pneumonia and septicaemia at 40 and 75 days, respectively. No malignant changes were detected in the papillomas. The third rabbit that was transgenic for both CRPV DNA and *Ras*, had thickened skin at birth and died at day 3. It was covered with epidermal papillomas that had already undergone highly malignant progression. The entire skin was described by the authors as ‘an extended squamous carcinoma’. No neoplasia was detected in other organs. Integrated CRPV DNA was detected in all tissues but was episomal and greatly amplified in tumours in all three rabbits. In contrast, there was no difference in *Ras* transgene copy number between normal and tumour tissues. CRPV DNA was transcribed in papillomas and carcinomas but not in normal tissues, while *Ras* was transcribed only in the cancers. It was concluded

by the authors that the rapid progression of papillomas to carcinomas was due to synergism between CRPV oncogenes and activated *Ras* (Peng *et al.*, 1993).

3.6 Domestic rabbit oral papillomavirus (ROPV)

Oral papillomas were initially found in 31% of 51 New Zealand white rabbits from two commercial sources. The virus was isolated and inoculated into the tongue, vulva and bulbar conjunctiva of three non-infected rabbits. All rabbits developed papillomas of the tongue but not of the conjunctiva or vulva. No cross-immunity was observed between the cutaneous (CRPV) and oral viruses and it was concluded that they have separate identities. Nine of 10 neonatal hamsters inoculated in the thoracombular region with oral papillomavirus developed fibromas (Sundberg *et al*., 1985). Tissue fragments from New Zealand white rabbit tongue, larynx, cervix, vulva/vagina and penis that were infected with extracts prepared from oral papillomas induced by ROPV and subsequently placed subrenally into athymic mice were tested by southern blot analysis and found to be positive for ROPV. Viral production was observed in subrenal xenografts from penile and vulvar tissue. After direct penile inoculation of adult rabbits with ROPV, 10/17 rabbits produced small raised lesions (papillomas) of approximately 1 mm^3 that were ROPV-positive by both in-situ hybridization and southern blot analyses and were also positive for viral capsid antigen by immunohistological staining. These lesions quickly regressed within 50–60 days (Christensen *et al.*, 1996c; Harvey *et al*., 1998). In another experiment, ROPV-induced benign papillomas at oral and genital sites regressed in 100% of infected domestic rabbits approximately 60 days after infection (Christensen *et al*., 2000).

3.7 Ovine papillomatosis (OVP) (Table 79)

In Australia, Hawkins *et al.* (1981) first described squamous-cell carcinomas in sheep. They occurred more commonly on areas that were poorly covered by wool and lacking pigmentation — the vulva, tail and perineum — which implied that UV light played as an important role as a co-factor in their etiology.

Vanselow *et al*. (1982) reported the apparent transformation of ovine facial papillomas into carcinomas and the presence of virions that resembled papillomaviruses in one of them. Further support for UV as a co-factor came from a study that correlated the increased use of the total and partial removal of tails with an increased prevalence of neoplasias in sheep in Australia. These procedures expose the entire perineal area, including the vulval labia, to direct sunlight. These observations suggested that the progression from virally induced papilloma to carcinoma first demonstrated with CRPV also occurs in sheep (Vanselow & Spradbrow, 1983).

Trenfield *et al*. (1990) analysed 67 benign precancerous cutaneous ear lesions (cutaneous horns, papillomas, fibropapillomas) from 51 sheep and 16 lesions from other cutaneous sites from 15 sheep. Ten ear lesions and one vulvar lesion were analysed for viral DNA using BPV 1 DNA as a probe; the vulvar lesion and eight of the 10 lesions were posi-

Table 79. Papillomavirus in tumours of sheep and goats

Lesion	Viral DNA[a]	Viral antigen[a,b]	Virus[a]	Reference
Cutaneous papillomas	ND	ND	2/3	Vanselow *et al*. (1982)
Hyperkeratotic scales	ND	ND	0/1	Vanselow & Spradbrow (1983)
Rumenal fibropapilloma	0/30[c]	6/10	0/20	Norval *et al*. (1985)
Cutaneous and vulvar lesions[d]	11/83	ND	ND	Trenfield *et al*. (1990)
Perineal squamous-cell carcinomas and papillomas	20/26	0/17	ND	Tilbrook *et al*. (1992)
Cutaneous filiform papillomas	1/1	9/9	Yes (NR)	Hayward *et al*. (1993)
Mammary papillomas of the goat udder	3/20	0/20	ND	Manni *et al*. (1998)
Papillomatosis	ND	5/5	5/5	Uzal *et al*. (2000)

ND, not determined; NR, incidence not reported
[a] Positive lesions/analysed lesion
[b] Common structural antigen
[c] Only tested with HPV 1 DNA
[d] Keratinized horns, papillomas and fibropapillomas

tive. The viral DNA had a BPV-like restriction enzyme pattern similar to that of equine sarcoids (see Section 3.2.8). A similar survey was performed by Tilbrook *et al*. (1992) who found that five of 10 premalignant biopsies and 15/16 squamous-cell carcinomas, all from the perineal region of sheep, contained papillomavirus-like DNA using both BPV probes and HPV probes. The filiform squamous papillomas on sheep reported by Hayward *et al*. (1993) were not of the fibropapilloma type but histologically resembled verruca vulgaris; they were present in less than 1% of 2660 young sheep and were always found on the lower forelegs. Papillomavirus was visualized by electron microscopy and viral DNA was detected by low-stringency hybridization with an HPV 16 DNA probe. All papillomas analysed were positive for the common viral antigen. Similar observations have been made in Patagonia and support the possible existence of a second OPV (Uzal *et al*., 2000). The occurrence of rumenal fibropapillomas in 25/200 sheep from local abattoirs in Scotland was reported by Norval *et al*. (1985), who also described one animal with a squamous-cell carcinoma of the rumen.

Manni *et al*. (1998) reported evidence for the existence of papillomavirus-like sequences in mammary papillomas of goats. No viral particles were found in papillomatous lesions from the mammary skin of goats, but reverse blot hybridization revealed cross-hybridization between DNA extracted from goat mammary papillomas and HPV 8, 10 and 16. Southern blot, using OPV and BPV 4 DNA probes under conditions of reduced stringency, detected homologous sequences in 40% of the biopsies.

3.8 *Mastomys natalensis* papillomavirus (MnPV)

Mastomys natalensis is a common rodent in southern Africa. Interest in these animals was stimulated by the early observation of Oettlé (1957) that 28–53% of *Mastomys* that were older than 1 year suffered from stomach cancer. As the incidence of stomach cancer is extremely low in other laboratory rodents and was highly variable in the different *Mastomys* laboratory colonies, exogenous causal factors were postulated. Extrachromosomal MnPV DNA was found in various tissues from animals of such colonies, whereas colonies that did not display a high rate of spontaneous stomach tumour formation were free from MnPV (Amtmann *et al*., 1984; Amtmann & Wayss, 1987).

In addition, a high rate of spontaneous epithelial skin tumours in colonies of *Mastomys* was first described by Burtscher *et al*. (1973). Malignant conversion rates of 11% were detected in some colonies (Giessen colony), whereas inbred strains derived from other colonies (Heidelberg colony) were free from malignancies. The animals of the Giessen and the Heidelberg colonies were found to harbour the latent papillomavirus, MnPV, the genomic sequence (7687 base pairs) of which was determined by Tan *et al*. (1994). Almost half of the tumours failed to give a uniform picture by histological examination. They were composed of keratoacanthomas, papillomas and epithelial proliferation. MnPV-infected animals developed keratoacanthomas and papillomas of the skin, in an age-dependent manner. The tumours never appeared in animals under 50 weeks of age but, by 16 months of age, 80% of the animals had tumours. The viral genome copy number increased markedly (30 000-fold) during tumour formation (Amtmann *et al.*, 1984; Amtmann & Wayss, 1987; Tan *et al.*, 1994). Amtmann *et al*. (1984) showed that treatment of the skin with TPA increased the DNA copy number (100-fold) and lowered the age at tumour appearance to as early as 14 weeks. Similar results were obtained when the skin was irritated with sandpaper, which indicated that the wound healing processes are prequisite for activation of latent papillomavirus genomes (Siegsmund *et al*., 1991). When the purified virions isolated from benign as well as from malignant tumours were used to infect the scarified skin of young *Mastomys*, 11/30 infected animals developed tumours (Müller & Gissmann, 1978).

Extrachromosomal MnPV DNA was found in all cutaneous DNA samples. In addition, viral DNA persisted in tissues other than the skin. After studying the effects of different chemical carcinogens, Amtmann *et al*. (1984) concluded that the activation of MnPV *in vivo* is mediated by a cellular mechanism that is correlated to second-stage tumour promotion, since transition from benign keratoacanthomas to malignant tumours was not induced by tumour promoters or DMBA.

Recently, transgenic mice that carry the oncogene E6 of MnPV were generated (Helfrich *et al*., 2004) and used in two-stage skin carcinogenesis experiments with DMBA and TPA. In this system, squamous-cell carcinomas developed in nearly 100% of MnPV E6 transgenic mice compared with 10% of their non-transgenic littermates, from which it can be concluded that the MnPV *E6* transgene favours malignant progression of chemically induced tumours.

3.9 Mouse papillomavirus (MmPV)

The only known MmPV was isolated from a zoological colony of European harvest mice (*Micromys minutus*) (Sundberg *et al*., 1988). Adult mice of each sex developed acanthomas, papillomas, inverted papillomas, sebaceous carcinomas and pulmonary keratinaceous cysts. MmPV was detected in 28/28 benign and malignant biopsies and structural antigen in 20/31 biopsies. MmPV could be transmitted to one of two harvest mice but not to laboratory mice (CAF or C3H strains) or to wild deer mice (*Peromyscus maniculatus gambeli*).

3.10 Canine oral papillomavirus (COPV)

Dogs can be affected by oral papillomatosis, particularly if kept in kennels in large numbers. The incubation period for oral papillomas varies from 4 to 10 weeks and regression usually follows in 3–14 weeks (Olson, 1987). Progression to squamous cancer is rare (Watrach *et al*., 1970), but has been observed. Analysis of the lesions by light and electron microscopy (Watrach *et al*., 1969) showed features typical of papillomavirus infection. The genomic sequence of COPV was determined by Delius *et al.* (1994). In addition to benign lesions, squamous-cell carcinomas of both cutaneous (Bregman *et al*., 1987) and oral mucosal tissue (Teifke *et al*., 1998) have been associated with COPV. Vaccination with 'live' COPV extract occasionally resulted in the development of various epithelial neoplasms at the injection site. Although a large majority of dogs were protected from natural infection, 12/5400 dogs developed cancers at the site of vaccine inoculation. The cancers comprised 10 highly invasive squamous-cell carcinomas, one basal-cell epithelioma and one epidermal pseudocarcinomatous hyperplasia. Five of 12 cancers were positive for COPV structural antigen, but all were negative for viral particles by electron microscopy (Bregman *et al*., 1987). More recent investigations included 19 cutaneous and mucocutaneous papillomas, as well as 29 oral and 25 non-oral squamous-cell carcinomas in dogs. Immunohistological analysis provided evidence for the presence of papillomavirus antigens in more than 50% of the oral and cutaneous papillomas, while no papillomavirus antigen were demonstrated in venereal papillomas. In addition, one squamous-cell carcinoma was papillomavirus antigen-positive and overexpression of p53 was detectable in approximately 35% of all squamous-cell carcinomas (Teifke *et al*., 1998).

3.11 Feline papillomas

Two Persian cats, 10 and 13 years of age, respectively, both of which received steroid immunosuppressive therapy, developed sessile hyperkeratotic skin lesions that were positive for papillomavirus, group-specific viral structural antigen (denatured L1) and viral BPV 1 DNA (Carney *et al*., 1990). Both cats were negative for feline leukaemia virus and feline immunodeficiency virus (FIV). Twelve years later, the *Felis domesticus* papillomavirus was cloned from hyperkeratotic cutaneous lesions of a Persian

domestic cat and sequenced (Tachezy *et al*., 2002a). In another study, a 6-year-old cat that was positive for FIV developed skin lesions consisting of slightly raised pigmented plaques, 2-7 mm in diameter, with a rough, slightly verrucous surface. The lesions were positive for papillomavirus and for viral structural antigen (Egberink *et al*., 1992). From both studies, it could be concluded that cats display clinical papillomaviral lesions when immunosuppressed either by FIV infection or by steroid therapy.

3.12 Avian papillomavirus

In a large survey of 25 000 captured chaffinches (*Fringilla coelebs*) in the Netherlands, papillomas were found on the bare part of the leg of 1.3% of the birds (Lina *et al*., 1973). The DNA of a *Fringilla* papillomavirus (FPV) was isolated from such cutaneous papillomas (Osterhaus *et al.*, 1977) but could not be transmitted to other chaffinches, canaries or hamsters (Moreno-Lopez *et al*., 1984). From that DNA, two partial sequences of FPV were determined (Osterhaus *et al*., 1977; Moreno-Lopez *et al*., 1984).

Recently, an avian papillomavirus genome has been cloned from a cutaneous exophytic papilloma from an African grey parrot, *Psittacus erithacus* (PePV; Tachezy *et al*., 2002b). The PePV genome (7304 base pairs) represents the first complete avian papillomavirus genome.

4. Molecular Mechanisms of HPV-induced Carcinogenesis

4.1 Experimental data that support the carcinogenicity of specific HPV genotypes and analyse the mechanism of HPV-linked carcinogenesis

In this section, the molecular mechanisms that contribute to HPV-induced carcinogenesis are described. Numerous studies have ascribed many biochemical activities, e.g. the ability of the viral proteins E6 or E7 to bind cellular factors, and biological properties, e.g. the immortalization of cells, to other HPV proteins. Section 4.1.1 provides a general introduction to molecular studies of cells that established the importance of HPV in cervical and skin cancers. Section 4.1.2 describes the numerous biochemical activities ascribed to HPV proteins. Section 4.1.3 describes the biological properties of HPV gene products in tissue culture, and the role of individual biochemical activities in mediating these properties. Subsequent sections describe other mechanistic aspects of HPV-associated cancers, including the contribution of viral DNA integration, cellular chromosomal alterations and co-factors.

4.1.1 *Transforming capacity of HPV*

HPVs are small DNA viruses that infect various epithelial tissues including the epidermis (cutaneous types) and the epithelial linings of the upper respiratory system and anogenital tract (mucosotropic types). The difference in their ability to promote malignant

transformation is the basis for the classification of HPVs into low- and high-risk types. This concept emanates from observations made more than 20 years ago that some HPV types were more frequently found in cancers than in benign lesions, and was followed by a large number of studies that demonstrated that these risk categories reflect the inherent and differential abilities of the viruses to interfere with proliferation and stability of the genome of the infected cell. A number of assays have been used to evaluate the ability of the E6 and E7 proteins to transform cells *in vitro*. Three types of cell were mainly used: (*a*) established rodent cell lines, e.g. NIH 3T3, (*b*) primary rodent cells (rat embryo fibroblasts, neonatal mouse or rat kidney cells) and (*c*) primary human keratinocytes, the natural host of the virus. The evidence for transformation included immortalization, formation of foci and growth in soft agar, cell proliferation and differentiation.

(*a*) *Mucosal HPV*

A subset of mucosotropic HPVs that belong to the alpha genus, including the high-risk HPV types 16 and 18, are associated with more than 99% of cervical carcinomas (Walboomers *et al.*, 1999). In these cancers, the papillomaviral DNA genome is often found integrated into the host chromosome (Boshart *et al.*, 1984; Schwarz *et al.*, 1985; Yee *et al.*, 1985). Cervical epithelial cells that harbour integrated HPV 16 DNA have a selective growth advantage over cells that harbour normal extrachromosomal viral genomes; this growth advantage correlates with the increased expression of two viral genes in particular, *E6* and *E7* (Jeon *et al.*, 1995). The early proteins, E6 and E7, bind and inactivate the tumour-suppressor gene product, p53, and the retinoblastoma tumour-suppressor protein (pRb), respectively (Dyson *et al.*, 1989; Münger *et al.*, 1989a; Werness *et al.*, 1990). In cell lines derived from HPV-positive cervical cancers, these genes are not inactivated mutationally, whereas they are mutated in cell lines derived from HPV-negative cervical cancers (Scheffner *et al.*, 1991). The expression of the *E6* and *E7* viral genes is required for the continued growth of cell lines derived from cervical cancers (Hwang *et al.*, 1993; Francis *et al.*, 2000; Goodwin & DiMaio, 2000; Goodwin *et al.*, 2000; Nishimura, A. *et al.*, 2000; Wells *et al.*, 2000). These facts support the hypothesis that E6 and E7 are causally related to the onset and maintenance of human cervical cancers. In addition, continuous expression of these early proteins can lead to the accumulation of mutations in the cellular genome that are required for malignant conversion (reviewed in zur Hausen, 1999). Both E6 and E7 co-operate to induce transformation of epithelial cells (Münger *et al.*, 1989b); however, a fully malignant phenotype is only observed after prolonged cultivation of the transformed cells (Hurlin *et al.*, 1991; Dürst *et al.*, 1995), which supports the multistep nature of HPV-induced transformation. In a transgenic mouse model, the expression of HPV 16 E6 alone has been shown to be sufficient to induce carcinomas (Song *et al.*, 1999). In contrast, E6 and E7 of the low-risk mucosal types have very low transforming activities *in vitro* (Farr *et al.*, 1991; Sang & Barbosa, 1992). Exceptions include immortalization by the low-risk type HPV 6 of human mammary epithelial cells which are not the natural host cells of these viruses (Band *et al.*, 1993). A recent report demonstrated the presence of integrated genomes of the low-risk type HPV 11, but not those of HPV 6, in cancers that deve-

loped in patients with early-onset recurrent respiratory papillomatosis (Reidy *et al.*, 2004). This points to the relevance of integration of the viral genome in malignant transformation, as discussed in Section 4.1.4.

(*b*) *Cutaneous HPVs*

Epidemiological studies have clearly demonstrated that a subset of cutaneous HPV types classified into the beta genus (approximately 25 types, also called epidermodysplasia verruciformis (EV)-HPV types, have been sequenced so far) are commonly and consistently found in non-melanoma skin cancers. These skin tumours arise predominantly at sites exposed to the sun and, contrary to mucosal types, the EV-HPV DNA copy number appears to be much lower than one copy/cell (de Villiers, 1998; Iftner *et al.*, 2003). Cutaneous HPVs have been studied less extensively than mucosal types and their capacity for cell transformation and molecular mechanisms are still largely unknown. Most cutaneous HPV types express E6 and E7 gene products that are structurally similar to those of the mucosal types but their genome does not harbour an identifiable E5 open-reading frame (ORF) (Pfister, 2003).

Schmitt *et al.* (1994) performed a comparative analysis of various properties of the E6 and E7 proteins of EV-associated type 8 and non-EV-associated type 1 cutaneous HPVs by transfecting the genes into different cell lines. HPV 8 E6, HPV 16 E6 and E7 and HPV 1 E7 but not cottontail rabbit papillomavirus (CRPV) long E6 or HPV 8 E7 were able to transform immortalized mouse fibroblasts (C127 cell line) while cells that expressed HPV 1 E6 or CRPV short E6 exhibited a weak transformed phenotype. The in-vitro retinoblastoma protein (Rb)-binding affinity (relative to that of HPV 16 E7) was 66% for HPV 1 E7, 34% for HPV 8 E7 and 11% for CRPV E7. None of the E6 or E7 proteins of the cutaneous HPV types 1 or 8 or CRPV revealed true immortalizing activities in primary human keratinocytes. In these cells, only a weak induction of proliferation was observed with HPV 8 E7, and only HPV 8 E7 transformed primary rodent cells co-transfected with the EJ-*Ras* oncogene.

HPV 5 and 8 E7s were shown to form complexes with the Rb protein, but with lower affinities than that of HPV 16 E7 (Yamashita *et al.*, 1993). Ciccolini *et al.* (1994) found that HPV 1 E7 binds to pRb with an affinity similar to that of high-risk E7 proteins but has no transforming activity in primary rodent cells. HPV 8 E6 protein expressed *in vitro* was shown not to bind murine p53 (Steger & Pfister, 1992). Similarly, HPV 1 and 8 E6 proteins bound to neither human p53 nor E6-associated protein (E6-AP) (Elbel *et al.*, 1997). Furthermore, HPV 8 E2, E4 and E6 were shown to interact with the TATA box-binding protein (TBP) and a number of TBP-associated factors (Enzenauer *et al.*, 1998).

Caldeira *et al.* (2003) analysed the in-vitro properties of E7 proteins of cutaneous EV- (HPV 20 and 38) and non-EV- (HPV 10) HPV types that are frequently detected in the skin. It was shown that HPV 38 E7 binds to and inactivates the tumour suppressor pRb and induces loss of G1/S transition control. In contrast, HPV 10 and HPV 20 E7 proteins do not display in-vitro transforming activities. Moreover, E6 and E7 of HPV 38 were shown to immortalize primary human keratinocytes, which suggests a role of HPV 38 infection in skin carcinogenesis.

4.1.2 *Biochemical properties of HPV proteins*

In this section, the biochemical properties of the HPV proteins E5, E6 and E7 are reviewed. Much of this information has been acquired for mucosal high-risk HPV (mainly HPV 16 and 18) proteins. Description of differences in the biochemical and biological properties of proteins of high-risk versus low-risk HPVs is largely provided in Section 4.1.3.

(*a*) *E5*

The study of the HPV E5 protein and recognition of its tumorigenic potential arose from the analysis of the transforming potential of BPV 1 in mouse C127 cells (see Section 3.3) (Yang *et al.*, 1985; DiMaio *et al.*, 1986; Schiller *et al.*, 1986; Schlegel *et al.*, 1986; Settleman *et al.*, 1989). The E5 ORF and the hydrophobic nature of its gene product are conserved in many papillomaviruses, although the degree of conservation of the primary amino acid sequence is variable (Bubb *et al.*, 1988). High-risk HPV E5 is considered to be tumorigenic because it transforms murine fibroblasts and keratinocytes in tissue culture (Leechanachai *et al.*, 1992; Pim *et al.*, 1992; Straight *et al.*, 1993), enhances the immortalization potential of E6 and E7 (Stöppler *et al.*, 1996) and, in cooperation with E7, stimulates the proliferation of human and mouse primary cells (Bouvard *et al.*, 1994b; Valle & Banks, 1995). Two major biochemical activities have been attributed to HPV 16 E5 *in vitro*: the ability to enhance the activity of the epidermal growth factor receptor (EGFR) in the presence of ligand (Leechanachai *et al.*, 1992; Pim *et al.*, 1992; Straight *et al.*, 1993) and the ability to bind and inactivate the 16-kDa pore-forming membrane component of the vacuolar H^+ adenosine triphosphatase (v-ATPase) (Conrad *et al.*, 1993; Adam *et al.*, 2000; Briggs *et al.*, 2001). Multiple studies have suggested that EGFR, a 170-kDa tyrosine kinase receptor, mediates the biological activities of HPV 16 E5 protein, and purport that E5 activates EGFR and induces mitogenic signalling and transformation of cells via this receptor (Straight *et al.*, 1993; Crusius *et al.*, 1998; Tomakidi *et al.*, 2000). Co-immunoprecipitation studies have indicated that HPV 16 E5 can form a complex with growth factor receptors when both proteins are overexpressed (Hwang *et al.*, 1995), but this binding has not always been observed (Conrad *et al.*, 1994). The binding of HPV 16 E5 to the 16-kDa subunit of the v-ATPase (Conrad *et al.*, 1993) is thought to delay endosomal acidification in human keratinocytes (Straight *et al.*, 1995), which has been implicated in the enhancement of EGFR phosphorylation in keratinocytes, since failure to acidify endosomes may result in decreased receptor degradation and increased receptor recycling to the cell surface (Straight *et al.*, 1993, 1995).

(*b*) *E6*

Mucosal high-risk E6 proteins are best known for their ability to associate with the cellular tumour suppressor p53 (Werness *et al.*, 1990). Association of E6 with p53 leads to degradation of p53 via recruitment of an ubiquitin ligase, E6-AP (Scheffner *et al.*, 1990; Huibregtse *et al.*, 1991; Scheffner *et al.*, 1993), and results in the inhibition of the transcriptional regulatory activities of the p53 protein in tissue culture cells (Lechner

et al., 1992; Mietz *et al.*, 1992). E6 proteins from multiple human and animal papillomaviruses bind to cellular proteins other than p53 and E6-AP. These include (*a*) transcription factors such as p300 (Patel *et al.*, 1999; Zimmermann *et al.*, 1999), myc (Gross-Mesilaty *et al.*, 1998), interferon regulatory factor 3 (IRF3) (Ronco *et al.*, 1998) and autocrine motility factor 1 (AMF-1/Gps2) (Degenhardt & Silverstein, 2001); (*b*) factors that determine adhesion, cytoskeleton and polarity, such as paxillin (Tong & Howley, 1997; Tong *et al.*, 1997; Vande Pol *et al.*, 1998), the mammalian homologue of *Drosophila* disk–large tumour-suppressor gene product (DLG) (Kiyono *et al.*, 1997; Lee *et al.*, 1997), Scribble (Nakagawa & Huibregtse, 2000), membrane-associated guanylate inverted-1 (MAGI-1) (Glaunsinger *et al.*, 2000) and multiple PDZ protein 1 (MUPP1) (Lee *et al.*, 2000); (*c*) apoptosis factors such as the pro-apoptic Bcl2 protein, Bak (Thomas & Banks, 1998, 1999); (*d*) replication factors and DNA repair factors such as mcm7 (Kühne & Banks, 1998; Kukimoto *et al.*, 1998) and XRCC1 (Iftner *et al.*, 2002); and (*e*) other proteins such as E6 target protein 1 (E6TP1) (Gao *et al.*, 1999), E6 binding protein 1 (E6BP1) (Chen *et al.*, 1995) and protein kinase PKN (Gao *et al.*, 2000). In addition, E6 can induce telomerase activity by inducing the expression of human telomerase reverse transcriptase (hTERT) (Klingelhutz *et al.*, 1996; Gewin & Galloway, 2001; Oh *et al.*, 2001; Veldman *et al.*, 2001). A more complete compendium of factors that interact with E6 is available (Mantovani & Banks, 2001).

Several E6 targets (paxillin, IRF3, E6BP1, E6TP1 and E6-AP) share a common α-helical structural motif that is known or suspected to mediate their binding to E6 (Tong *et al.*, 1997; Chen *et al.*, 1998; Vande Pol *et al.*, 1998; Be *et al.*, 2001). Mutations in E6 that disrupt its ability to bind α-helical partners also cause defects in multiple biological properties (Liu *et al.*, 1999). One of these partners, E6-AP, has attracted considerable attention because it is believed to be responsible for mediating high-risk HPV E6-dependent destabilization of multiple targets including p53 (Scheffner *et al.*, 1993), myc (Gross-Mesilaty *et al.*, 1998) and the PDZ domain protein, Scribble (Nakagawa & Huibregtse, 2000). This ubiquitin ligase, however, probably does not account completely for the ability of E6 to target cellular factors for proteasome-mediated degradation. Among the cellular partners of high-risk HPV E6 that are destabilized independently of E6-AP are human DLG and members of the MAGI family (Grm & Banks, 2004). It is also important to note that cutaneous HPV E6s, including the EV-associated HPV 8 E6, do not bind E6-AP (Elbel *et al.*, 1997). Another putative α-helical partner, E6TP1, a Rap guanosine triphosphatase-activating protein, has been implicated in the capacity of E6 to immortalize mammary epithelial cells (Gao *et al.*, 2001). It has been demonstrated that, for bovine papillomavirus (BPV) 1 E6, the selective binding of E6, but not of E6-AP, to paxillin mediates its transforming potential (Vande Pol *et al.*, 1998); however, it remains unclear whether an interaction with paxillin is of biological importance for the transforming potential of HPV E6 protein.

The interaction of high-risk E6 proteins with proteins that contain the PDZ domain, such as DLG, Scribble, MAGI1–3 and MUPP-1, has drawn a lot of interest. E6 can target PDZ-domain proteins for degradation (Gardiol *et al.*, 1999; Nakagawa & Huibregtse,

2000; Massimi *et al.*, 2004). The intracellular location and levels of DLG are altered in cervical cancers and their precursor lesions (Watson *et al.*, 2002; Cavatorta *et al.*, 2004; Lin *et al.*, 2004). Disruption of the C-terminal domain in E6 that mediates its interaction with PDZ-domain proteins leads to defects in the transforming potential of E6 in certain tissue culture-based assays (Kiyono *et al.*, 1997) and its tumorigenic potential in mice (Nguyen *et al.*, 2003), but not its capacity for immortalization.

(*c*) *E7*

High-risk HPV E7 proteins are best known for their ability to associate with the cellular tumour suppressor, pRb (Dyson *et al.*, 1989; Münger *et al.*, 1989a; Gage *et al.*, 1990). Association of high-risk E7 with pRb also promotes the degradation of pRb (Boyer *et al.*, 1996; Jones *et al.*, 1997a) through a proteasome-mediated pathway (Berezutskaya & Bagchi, 1997; Gonzalez *et al.*, 2001) and disrupts the capacity of pRb to bind and inactivate functionally cellular E2F transcription factors (Phelps *et al.*, 1991; Chellappan *et al.*, 1992). In addition to binding pRb, high-risk E7 proteins can bind to other pocket proteins (p107 and p130) that are related to pRb (Dyson *et al.*, 1992; Davies *et al.*, 1993) and also interact with different members of the E2F family of transcription factors (Dyson *et al.*, 1993; Classon *et al.*, 2000). The inactivation of pocket proteins by E7 is necessary but not sufficient to elicit the transforming potential of E7 (Heck *et al.*, 1992; Phelps *et al.*, 1992; Kiyono *et al.*, 1998). High-risk E7 is also purported to complex with cyclins (Dyson *et al.*, 1992; Arroyo *et al.*, 1993; Tommasino *et al.*, 1993; McIntyre *et al.*, 1996) and to inactivate the cyclin-associated kinase inhibitors p21 and p27 (Funk *et al.*, 1997; Jones *et al.*, 1997b). Thus, E7 can associate with and/or alter the activities of multiple cellular factors that normally contribute to the regulation of the cell cycle. Other interactions have been identified between high-risk E7 and cellular factors including 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.*, 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.*, 1996, 1997; Phillips & Vousden, 1997), TBP-associated factor-110 (Mazzarelli *et al.*, 1995) and a novel human DnaJ protein, hTid-1 (Schilling *et al.*, 1998).

4.1.3 *Biological properties of HPV proteins*

(*a*) *Immortalization*

The E6 and E7 proteins of mucosal high-risk HPVs have transforming activity in tissue culture. They act independently or synergistically to immortalize multiple cell types including human foreskin keratinocytes, cervical epithelial or mammary epithelial cells (Dürst *et al.*, 1987a; Pirisi *et al.*, 1987, 1988; Hawley-Nelson *et al.*, 1989; Kaur *et al.*, 1989; Band *et al.*, 1990; Hudson *et al.*, 1990; Halbert *et al.*, 1991; Wazer *et al.*, 1995). To date, the E6 and E7 proteins of only one cutaneous EV-associated HPV type, HPV 38, have shown to

immortalize human primary keratinocytes (Caldeira *et al.*, 2003). Mucosal high-risk (HPV 16, 18, 31) E7s but not low-risk (HPV 6, 11) E7s cooperate with an activated *ras* to transform neonatal rat kidney or human cervical epithelial cells (Matlashewski *et al.*, 1987; Crook *et al.*, 1988; Phelps *et al.*, 1988; Storey *et al.*, 1988). The contribution of HPV 16 E7 to immortalization correlates with its disruption of the p16/pRb pathway (Kiyono *et al.*, 1998; Jarrard *et al.*, 1999). While E7 appears to be critical for efficient immortalization of multiple human epithelial cell types (Hawley-Nelson *et al.*, 1989; Halbert *et al.*, 1991), it may be dispensible depending on the method by which the cells are cultured (Ramirez *et al.*, 2001). Low-risk mucosal HPV 6 E7 and cutaneous EV-HPV 8 E7 demonstrate weak immortalizing activity compared with that of high-risk HPV 16 E7 (Halbert *et al.*, 1992; Schmitt *et al.*, 1994). This difference potentially correlates with the relative capacity of these E7 proteins to induce the degradation of pRb rather than their affinity for pRb (Giarrè *et al.*, 2001). The E5 protein can enhance the immortalization of keratinocytes effected by the combination of E6 and E7 (Stöppler *et al.*, 1996). The mechanism by which E6 contributes to immortalization is controversial. Some studies have correlated its potential for immortalization with its ability to induce the expression of telomerase (Kiyono *et al.*, 1998), since telomerase activity is clearly induced by E6 (Klingelhutz *et al.*, 1996). Whereas E6 modulates the transcription of hTERT through a direct stimulation of myc-mediated transactivation of the hTERT promoter (Veldman *et al.*, 2003), another study argued that induction of myc was not found (Gewin & Galloway, 2001). An alternative hypothesis that was recently put forward is that E6 relieves the repression of the telomerase promoter by inducing degradation of the transcriptional repressor, NFX1-91 (Gewin *et al.*, 2004). The level of telomerase activity in E6-positive cells increases further when they become immortalized although levels of E6 expression do not change (Fu *et al.*, 2003), which indicates that other events contribute to telomerase activation. Other studies have linked the immortalization potential of E6 in mammary epithelial cells and keratinocytes to its inactivation of p53 (Dalal *et al.*, 1996; McMurray & McCance, 2004).

(*b*) *Genomic instability*

Another hallmark of cells that express E6 and E7 is genomic instability, which has been observed in multiple epithelial cell types (Smith *et al.*, 1989; Hashida & Yasumoto, 1991; Reznikoff *et al.*, 1994; White *et al.*, 1994; Coursen *et al.*, 1997; Steenbergen *et al.*, 1998; Duensing & Münger, 2002; Shen *et al.*, 2002b). Abnormalities included monosomies and trisomies, chromatid gaps and breaks, double minutes and aberrant chromosomes. Structural changes are more commonly detected in chromosomes 1, 3 and 5 and less frequently in chromosomes 7, 8, 10, 12, 13, 16 and 22. Some of these allelic losses have been associated with particular genes that could be involved in malignant conversion and/or progression. Among these, losses in 3p and 10p have been associated with telomerase activation (Steenbergen *et al.*, 1998), which is a crucial step for cell immortalization mediated by high-risk HPVs (Klingelhutz *et al.*, 1996; Coursen *et al.*, 1997). Mitotic abnormalities can be induced by high-risk HPV 16 (but not low-risk HPV 6) E6 and E7 proteins by direct subversion of the mitotic spindle checkpoint (Thomas & Laimins, 1998; Duensing *et al.*, 2000).

The ability of E6 to induce genomic instability probably reflects its ability to inhibit the function of p53 (Havre *et al.*, 1995), which leads to the disruption of normal DNA repair processes and a consequent accumulation of genetic change. The genomic instability induced by E7 may reflect its effect on centrosome biogenesis and the consequent defects in segregation of daughter chromosomes during cell division (Duensing *et al.*, 2000; Duensing & Münger, 2001; Duensing *et al.*, 2001a,b). However, the manner in which E7 induces genomic instability remains unclear. While studies in mice have indicated that inactivation of pRb is sufficient to induce centrosome abnormalities (Balsitis *et al.*, 2003), other studies have demonstrated that E7 can induce centrosome abnormalities through a pRb-independent mechanism (Duensing & Münger, 2003).

Using an HPV 16-positive cell line (W12) derived from a low-grade squamous intraepithelial lesion (LSIL), Pett *et al.* (2004) recently suggested that acquisition of chromosomal instability is correlated with integration of the viral genome. The contrary has been shown in raft cultures of human keratinocytes that contain episomal HPV 16 in which genomic instability was observed in the absence of viral integration (Duensing *et al.*, 2001b). Progress in this area has been hampered by the lack of experimental models and methods to determine HPV integration that are suitable for use on large series in human biological specimens.

(*c*) *DNA damage responses*

Both E6 and E7 can abrogate normal DNA damage responses (Kessis *et al.*, 1993; Slebos *et al.*, 1994; Demers *et al.*, 1996). This is thought to reflect the activity of both E6 and E7 in inhibiting p53-mediated cell-cycle arrest. This correlates at least in part with the ability of E6 to bind and inactivate p53 (Song *et al.*, 1998); for E7, this correlates not only with its ability to disrupt the function of the cell-cycle regulator pRb, but also with its ability to inactivate p21 (Funk *et al.*, 1997; Jones *et al.*, 1997b), the cyclin-dependent kinase inhibitor that is induced when p53 is activated in response to DNA damage (Helt *et al.*, 2002). Abrogation of the DNA-damage responses is hypothesized to contribute to the accumulation of genetic alterations in HPV-positive cells, which include those that might contribute to tumorigenicity.

(*d*) *Cell proliferation and differentiation*

Whereas suprabasal cells are withdrawn from the cell cycle in normal stratified epithelia, HPV can re-programme suprabasal cells to sustain DNA synthesis which may contribute to the production of progeny virus through amplification of the viral genome. E7 is necessary (Flores *et al.*, 2000) and sufficient (Cheng *et al.*, 1995; Herber *et al.*, 1996) for this phenotype. It remains uncertain whether the ability of E7 to induce DNA synthesis in differentiated cells reflects the failure of cells that express E7 to withdraw from the cell cycle (Sacco *et al.*, 2003) or an ability of E7 to re-programme the differentiated cell to re-enter the cell cycle (Cheng *et al.*, 1995; Chien *et al.*, 2002). Inactivation of pRb alone can cause suprabasal cells to sustain DNA synthesis (Balsitis *et al.*, 2003); however, other properties of E7 may also contribute to this phenotype, notably its recognition by casein

kinase as a substrate for phosphorylation (Chien *et al.*, 2000). E6 has also been shown to induce suprabasal DNA synthesis (Song *et al.*, 1999), a p53-independent activity that correlates with the ability of E6 to bind PDZ-domain proteins (Nguyen *et al.*, 2003). A potentially related activity of E6 and E7 is their ability to inhibit keratinocyte differentiation (Schlegel *et al.*, 1988; Barbosa & Schlegel, 1989; Pan & Griep, 1994; Herber *et al.*, 1996; Gulliver *et al.*, 1997; Sherman *et al.*, 1997; Pei *et al.*, 1998; Song *et al.*, 1999). For E7, this probably reflects its inactivation of pRb (Gulliver *et al.*, 1997; Balsitis *et al.*, 2003), but inactivation of p53 by E6 is not sufficient for the inhibition of keratinocyte differentiation (Sherman *et al.*, 1997). It remains unclear whether the re-programming of suprabasal cells to sustain DNA synthesis by E6 and E7 or their inhibition of differentiation contribute to the tumorigenic potential of HPV; however, recent studies indicate that the ability of E6 to bind PDZ-domain proteins, which is required for the induction of both of these acute effects on stratified squamous epithelia, correlates with its contribution to the promotion phase of carcinogenesis (Simonson *et al.*, 2005).

High-risk E6 and E7 proteins modulate apoptosis. E7 can induce apoptosis in mouse lens and retina (Howes *et al.*, 1994; Pan & Griep, 1994; Nakamura, T. *et al.*, 1997). E7 can also sensitize human keratinocytes, mammary epithelial cells and uroepithelial cells to agents that induce apoptosis such as tumour necrosis factor (TNF) (Stöppler *et al.*, 1998; Basile *et al.*, 2001), ionizing radiation (Puthenveettil *et al.*, 1996) and ultraviolet (UV) radiation (Carlson & Ethier, 2000) but it has the opposite effect on TNF-induced apoptosis in human fibroblasts (Thompson *et al.*, 2001) or hydrogen peroxide-induced apoptosis in astrocytes (Lee, W.T. *et al.*, 2001). In mouse lens fibre cells and mouse fibroblasts, apoptosis is mediated through the inactivation of pRb by E7 (Alunni-Fabbroni *et al.*, 2000) and the consequent up-regulation of E2F activity (McCaffrey *et al.*, 1999). In hepatocytes, however, it is hypothesized that E7 mediates apoptosis through inactivation of pRb and induction of p21 (Park *et al.*, 2000a). The apoptosis induced by E7 occurs through p53-dependent as well as p53-independent pathways (Pan & Griep, 1995). E7 induces expression of p53 (Song *et al.*, 1998; Seavey *et al.*, 1999), but this p53 is not fully transcriptionally active (Eichten *et al.*, 2002). Whereas E7 can inhibit p53-induced cell-cycle arrest, it does not inhibit p53-induced apoptosis (Wang, Y. *et al.*, 1996) which indicates that these two pathways are separable, and that E7-induced p53 is capable of triggering apoptosis.

4.1.4 *Experimental evidence for a role of mucosal high-risk HPV in malignant conversion and in human cervical cancer*

(*a*) *Requirement of HPV gene expression for cell growth and invasion*

Expression of HPV E6 and E7 proteins is essential for cellular immortalization, but other factors are required for the acquisition of a fully transformed phenotype. HPV-immortalized cells have been shown to become tumorigenic either spontaneously (Pecoraro *et al.*, 1991) or after treatment with chemical carcinogens (Garrett *et al.*, 1993) or exposure to γ-irradiation (Dürst *et al.*, 1995). The necessity of expression of viral proteins for

transformation to the malignant phenotype has been demonstrated using various cell lines in these experimental models. Using an inducible promoter, it has been shown that continued expression of HPV 16 E7 in the presence of activated *Ras* is necessary for the maintenance of a transformed phenotype in primary rodent cells (Crook *et al.*, 1989). Non-malignant revertants were obtained in a model in which transcription of E6 and E7 was impaired (von Knebel Doeberitz *et al.*, 1992, 1994). In another study, stimulation of non-tumorigenic HeLa (HPV 18)-fibroblast hybrids to invasive growth was shown to involve loss of TNFα-mediated repression of viral transcription and participation of AP-1 (Soto *et al.*, 1999). Moreover, a key role for the constitutive expression of c-fos in the transformation of cervical cancer cells has been demonstrated (van Riggelen *et al.*, 2005).

Interference with the expression of HPV 16 E6 and E7 was studied in the HPV 16-positive cervical cancer cell line SiHa using E6 short-interfering RNA (siRNA). Yoshinouchi *et al.* (2003) showed that E6siRNA decreased the levels of mRNA that encode E6 and E7 and induce nuclear accumulation of p53. Moreover, E6siRNA suppressed monolayer and anchorage-independent growth of SiHa cells, which was associated with induction of p21 and hypophosphorylation of pRb.

The contribution of HPV genes to the development of malignancy has also been studied *in vivo* through the generation and characterization of HPV transgenic mice. These studies are described in Section 4.1.6.

(*b*) *Integration of HPV sequences*

In most invasive cancers, high-risk HPV genomes are integrated into the host genome. Integration of HPV can also be found in premalignant lesions, particularly in grade 2/3 cervical intraepithelial neoplasia (CIN2/3). In contrast, HPV DNA is commonly found extrachromosomally in benign and low-grade lesions. Low-risk HPV types are very rarely found integrated in tumours. However, Reidy *et al.* (2004) studied tissue specimens from patients with a history of benign early-onset recurrent respiratory papillomatosis who developed laryngeal cancer. Integrated HPV 11 was found in these specimens as judged by the absence of full-length E2 transcripts measured by real time-polymerase chain reaction (PCR) in a manner similar to that of high-risk HPVs in cervical cancers. An increased ability of high-risk HPV types to integrate into host DNA compared with low-risk types also has been suggested *in vitro* (Kessis *et al.*, 1996).

Integration is considered to be an important molecular event in HPV-induced carcinogenesis. Integrated sequences of DNA have been consistently identified in cervical cancers by southern blot hybridization (Dürst *et al.*, 1985; Cullen *et al.*, 1991). A systematic analysis of large series of HPV-infected cells and tissues has been hampered by a lack of less time-consuming and labour-intensive methods to determine integration of HPV. Since integration of HPV often disrupts the E2 gene (Schwarz *et al.*, 1985; Romanczuk & Howley, 1992), determination of a lack of amplification of E2 sequences by PCR-based protocols has been considered; however, results obtained with these methods are ambiguous because of the concomitant presence of non-integrated and integrated molecules in the biological specimens and to technical failures due to presence of inhibitors or other

factors. However, detection of early gene transcripts by reverse-transcription PCR is more sensitive both in cancers (Park *et al.*, 1997) and in benign or dysplastic cervical swabs, in which the presence of integrated genomes has been shown to correlate with severity of disease, particularly for HPV 18 (Hudelist *et al.*, 2004). Recently, alternative methods for the accurate determination of the physical status of HPV genomes have been proposed (Klaes *et al.*, 1999; Luft *et al.*, 2001; see Section 1.3.3). In the study by Klaes *et al.* (1999), transcripts derived from integrated HPV were more frequently detected in high-grade lesions and cervical cancer than in normal or low-grade dysplastic tissues. Integration of HPV 16 and 18 in high-grade lesions is often accompanied by chromosomal abnormalities (Hopman *et al.*, 2004). This supports the potential use of measurements of HPV integration as markers of progression in cervical cancer. In tonsillar cancers, the presence of extrachromosomal HPV 16 genomes and high viral loads were correlated with better prognosis (Mellin *et al.*, 2002).

Integration of HPV genomes affects both viral and host gene expression. HPV gene expression is regulated by viral and cellular transcriptional activators and repressors (see Section 1.1.6). Normal regulation is altered by viral integration and leads to the continuous expression of E6 and E7 proteins and, consequently, selective growth advantage, as shown by Jeon *et al.* (1995), who used clonal populations of the W12 cell line that harbour non-integrated or integrated HPV 16 DNA. Integration correlated with increased E7 protein synthesis; cells with integrated viral DNA had growth advantages and phenotypic changes compatible with those of high-grade neoplasia compared with cells that harboured extrachromosomal viral DNA.

This consistent pattern of disruption seen in the viral genome does not seem to occur in the host genome. The occurrence of integrated HPV DNA sequences at preferential sites of human chromosomes has been reported and suggests a non-random pattern of integration. In cervical carcinomas, Ferber *et al.* (2003a) observed HPV integration into and around the *hTERT* gene, which resulted in an increase in hTERT expression. Furthermore, HPV 18 DNA was found integrated in the proximity of *c-myc* in several cervical cancers (Ferber *et al.*, 2003b) but no up-regulation of endogenous proto-oncogene expression was observed. Cytogenetic and molecular studies have shown that HPV 16 and 18 DNA sequences can be found integrated in particular chromosomal loci known as common fragile sites in cervical cancers (Ferber *et al.*, 2003b; Thorland *et al.*, 2003), in HPV 16-immortalized keratinocytes (Popescu & diPaolo, 1990) and in an HPV 16-positive cell line derived from a cancer of the tongue (Ragin *et al.*, 2004). One of these regions, the *FRA*3B common fragile site that encompasses the fragile histidine tetrads (*FHIT*) tumour-suppressor gene is mapped on chromosome 3p. Butler *et al.* (2002) showed a clear association between the loss of *FHIT* expression and progression of HPV 16-positive CIN. Invasive cervical cancers that express high-risk HPV E6 and E7 transcripts were shown to contain normal *FHIT* transcription, while fewer viral transcripts were detected when *FHIT* was abnormally expressed, which suggests that E6 and E7 could be repressed in the presence of *FHIT* aberrations (Segawa *et al.*, 1999).

The studies described above favour the concept that HPV genomes may interfere with critical cellular functions by insertional mutagenesis. However, this has not been confirmed in recent studies that used HPV transcript and genome-based amplification techniques (see Section 1.3.3). A comprehensive analysis of integration sites of HPV 16 and 18 in 21 anogenital cancerous and pre-cancerous lesions revealed only single integration events in which E6 and E7 transcripts could be detected (Ziegert *et al.*, 2003). This could be an indication that the major function of HPV integration is the conservation and stabilization of HPV gene expression. A thorough review of integration sites of HPV in cervical dysplasia and cancer (Wentzensen *et al.*, 2004) concluded that these are randomly distributed over the whole genome with a clear predilection for genomic fragile sites. The relative impact of physical and functional disturbance of viral and cellular genes in HPV-mediated carcinogenesis needs further study. Many observations have demonstrated that the malignant phenotype cannot be attributed exclusively to the expression of HPV genes. It has been hypothesized that modification of host cell genes that interfere with the expression or function of viral genes will eventually contribute to immune evasion, and tumour progression and invasion (zur Hausen, 1999).

(*c*) *Chromosomal abnormalities in HPV-associated cancers*

Numerical chromosomal changes have been described in several HPV-associated cancers including cervical (see below), vulvar (Pinto *et al.*, 1999; Rosenthal *et al.*, 2001) and head and neck tumours (Braakhuis *et al.*, 2004).

Aneuploidy has been observed in cervical cancers and their precursor lesions (reviewed in Lazo, 1999). Loss of heterozygosity (LOH), which most frequently involves chromosomes 1, 3, 6, 11, 17 and 18 has been reported. Losses in the short (p) arm of chromosome 3 and gain on the long (q) arm are among the most frequent events associated with progression from high-grade lesions to cervical cancer (Heselmeyer *et al.*, 1996; Larson *et al.*, 1997; Wistuba *et al.*, 1997; Lin, W.M. *et al.*, 2000; Nishimura, M. *et al.*, 2000). An association between the severity of anal intraepithelial neoplasia (AIN) and chromosomal changes detected by comparative genomic hybridization has been described (Haga *et al.*, 2001). The most common alteration involved 3q, similar to the commonest alteration seen in cervical cancer, which suggests that a common molecular pathway for these HPV-associated malignancies exists. Moreover, LOH at 3p was more frequent in HPV 16- and 18-positive cervical tumours, whereas LOH at the 5p regions was more frequent in HPV-negative tumours (Mitra, 1999). Kersemaekers *et al.* (1999) analysed the CIN component, invasive carcinoma and lymph node metastases from 10 patients with primary squamous-cell carcinoma of the cervix for LOH. In CIN lesions, LOH was frequently found at 3p, 6p and 11q. During progression to an invasive tumour, losses of genes were observed on 6q, 17p and 18q. It was suggested that loss of an additional locus on the X chromosome and activation of the *erb*B2 oncogene are important in progression to metastases. Evidence that integration of high-risk HPV is associated with genomic alterations measured by comparative genomic hybridization was provided in a study that examined different degrees of cervical squamous intraepithelial lesions (SIL): more numerical chromosomal aberrations

were found in high-grade lesions with integrated HPV DNA than in low-grade lesions (Alazawi *et al.*, 2004). ELhamidi *et al.* (2004) analysed 164 CIN for LOH at 12 microsatellite loci and found that LOH at D3S1300, D3S1260, D11S35 and D11S528 was associated with CIN, which showed a tendency to persist and/or progress. An indication of geographical distribution of genetic alterations in oesophageal carcinomas has been reported by Si *et al.* (2004): HPV-positive tumours from Hong Kong, but not from Sichuan, had a higher frequency of LOH at D5S82, D6S397 and D13S260 than HPV-negative tumours.

Alterations in the *pRb* and *p53* genes have also been studied. Kim *et al.* (1997) reported infrequent LOH at *pRb* (14%) and *p53* (5.5%) loci in 55 primary cervical carcinomas. The genes *p53* and *pRb* are less frequently mutated in HPV-positive than in HPV-negative cervical cancer cell lines and tumours (reviewed in Tommasino *et al.*, 2003). Similarly, expression of HPV 16 E6 in head and neck cancers correlated with the absence of mutations within the *p53* gene (Braakhuis *et al.*, 2004; Dai *et al.*, 2004).

Continuous expression of papillomavirus early genes may directly promote genetic abnormalities that often result in impaired function of genes that are critical for cell homeostasis. Furthermore, expression of high-risk HPV *E6* and *E7* genes in the basal cell layer induces chromosomal instability and aneuploidy. It has recently been suggested that this event precedes integration of the viral genomes which in turn triggers the continuous expression of early genes (Melsheimer *et al.*, 2004). It could therefore be speculated that the physical status of the HPV genome and testing for aneuploidy could be used as prognostic tools (Kashyap & Das, 1988).

(*d*) *Alterations of specific proto-oncogenes*

Several investigations have addressed the structural or functional alteration of different proto-oncogenes. Most of these were descriptive in nature and few were designed to correlate the observed alteration with progression of disease. Therefore, a direct role for these genetic alterations in HPV-associated carcinogenesis is difficult to establish.

(i) RAS

Mutations in the *RAS* family of oncogenes have been described in both premalignant and malignant cervical lesions. Levels of H- and N-*RAS* mRNA were significantly higher in cervical carcinomas than in normal tissue or CIN; however, no correlation was found between levels of expression of each *RAS* gene and the presence of HPV (Mammas *et al.*, 2004). This contrasts with the results of Golijow *et al.* (1999) who found higher rates of mutation in K-*RAS* codon 12 in non-cancerous cervical smears that contained high-risk HPV types than in samples infected with low-risk HPV. One study *in vitro* showed that activation of Ha-*RAS* genes and inhibition of protein phosphatases by okadaïc acid stimulated HPV 18 p105 promoter activity (Medina-Martínez *et al.*, 1997). This activation depended on the presence of an intact AP-1 binding site. Leis *et al.* (1998) reported a missense mutation within c-*RAS*^{Ha} codon 61 in the metastasis of an HPV 18-positive penile squamous-cell carcinoma that was absent in the primary tumour and previous metastases. This suggests that alterations in *ras* may be associated with late-stage disease.

(ii) MYC

HPVs have been shown to integrate in the proximity of *c-MYC*, which justifies the search for alterations of this proto-oncogene in HPV-associated lesions. However, the results have not been consistent. Recently, Abba *et al.* (2004) described *c-MYC* amplification in a high proportion of cervical cancers compared with benign and premalignant cervical lesions. Moreover, a significant association between *c-MYC* amplification and HPV 16 infection was observed. Elevated levels of *c-MYC* have been found in several HPV-positive cervical carcinoma cell lines (Dürst *et al.*, 1987b). More recently, Hukku *et al.* (2000) described genetic changes associated with progression to a malignant phenotype of a non-tumorigenic HPV 18-immortalized human prostate cancer cell line, which included amplification of *c-myc* that was considered to be central to this process. However, the significance of these events in HPV-mediated transformation is not clear. The involvement of the Myc protein in HPV-induced immortalization was recently addressed (Veldman *et al.*, 2003). High-risk HPV E6 was shown to associate with Myc complexes (Myc/Max) and activate the hTERT promoter. The specific Myc antagonist, Mad, represses E6-transactivation of hTERT.

(iii) ERB

HER-2/*NEU* mRNA and protein were detected in a large proportion of cervical adenocarcinomas. Preferential expression of this proto-oncogene was more strongly associated with lesions that contained HPV 16 than with those that contained HPV 18 (Roland *et al.*, 1997). Similar patterns of expression were previously observed in different mammary and cervical tumours compared with normal tissues: increased levels of *c-ERB*B-2 mRNA expression were found in two advanced and poorly differentiated endometrial adenocarcinomas, two ductal mammary carcinomas and three carcinomas *in situ* of the cervix (Brumm *et al.*, 1990). An increase in ERBB2 expression was also correlated with progression of disease and has been considered to be a late event in cervical carcinogenesis (Kersemaekers *et al.*, 1999).

(iv) *Other*

Epigenetic events that involve the methylation of viral genes or the long control region (LCR) of high-risk HPVs have been described. The LCR of HPV 16 (Badal *et al.*, 2003) and 18 (Badal *et al.*, 2004) is hypermethylated in normal and low-grade cervical smears and is gradually methylated to a lesser degree in high-grade smears. This correlates with an increased transcriptional activity of the early region and consequently greater availability of the E6 and E7 proteins.

Hypermethylation of cellular gene promoters has also been observed in cervical carcinomas (Dong *et al.*, 2001; Virmani *et al.*, 2001; Steenbergen *et al.*, 2004), and down-regulation of the expression of tumour-suppressor genes could therefore be considered of importance in tumour progression.

4.1.5 *Interactions between HPV and environmental agents*

(*a*) *Effects of other infectious agents*

The proposed mechanisms through which infectious agents might act as co-factors in HPV-associated tumorigenesis include direct biological interactions, such as modification of HPV replication and transcription, and indirect effects, such as inflammation and damage to the epithelial barrier that protects against HPV infection. Herpes simplex virus-2 (HSV-2) is one of the infectious agents that has been most frequently studied as a potential co-factor for cervical cancer. However, epidemiological studies have provided conflicting results (see Section 2.7.2) for an association of HSV-2 with cervical cancer.

Because of the lytic behaviour of many herpesviruses, including HSV-2, abortive infections would need to be involved for HSV-2 to have a direct effect on HPV-associated tumorigenesis. Accordingly, UV-inactivated HSV-2 can transform rodent cells *in vitro* (Duff & Rapp, 1971a,b, 1973). Several studies have demonstrated an interaction between HSV-2 and HPV in transformation *in vitro* (Dhanwada *et al.*, 1993), whereas others have found that HSV-2 can suppress HPV gene expression (Fang *et al.*, 2003). Thus, laboratory data similarly to the epidemiological data are not consistent with regard to a possible interaction of HSV-2 in HPV-associated tumorigenesis.

Laboratory studies conducted during the 1970s demonstrated the ability of UV-inactivated HSV-2 and HSV-1 to transform hamster cells, and showed that these transformed cell lines caused tumours in newborn rodents (Duff & Rapp, 1971a,b, 1973). The continued presence of the HSV genome was observed in some tumorigenic cell lines, but the transformed phenotype was also found to persist in the absence of detectable HSV viral sequences (Davis & Kingsbury, 1976). These findings and the inconsistent detection of HSV DNA in specimens of human cervical cancer gave rise to hypotheses of a possible 'hit and run' mechanism (Davis & Kingsbury, 1976; Skinner, 1976; Galloway & McDougall, 1983), i.e. the concept that a virus may be involved in the initiation or promotion of cancer without being required for the maintenance of the transformed phenotype.

At least two separate genomic regions of HSV have been shown to transform rodent cells *in vitro*: the morphological transforming region II in the *Bgl*II N fragment and the morphological transforming region III in the *Bgl*II C fragment (Jones, 1995). In contrast to rodent cells, the *Bgl*II N fragment in human keratinocytes was found to induce tumorigenic clones in cells that had been immortalized by HPV but not in normal cells (DiPaolo *et al.*, 1990), a finding that is consistent with the hypothesis that HSV is a co-factor in HPV-associated cervical tumorigenesis, but is not itself an important etiological agent. Moreover, although *Bgl*II N sequences were not detected in tumour-derived cell lines (DiPaolo *et al.*, 1990, 1998), when only the Xho2 segment of the *Bgl*II N fragment was used, HPV-immortalized cells could still be transformed and the Xho2 segment was found to be maintained stably in an integrated form in the host cell genome (DiPaolo *et al.*, 1998). These data were interpreted as evidence that the Xho2 fragment contains the transforming sequences of the *Bgl*II N fragment, and that the remaining sequences have an inhibitory effect on stable integration. However, a study of 200 specimens of human

cervical cancer failed to detect any HSV-2 sequences using sensitive PCR methods (Tran-Thanh *et al.*, 2003). In-vivo studies of the detection of HSV and other infectious agents in cervical specimens are mainly reviewed in Section 2.7.2.

Other herpesviruses that are reported to infect the cervix have also been shown to transform epithelial cells in tissue culture, including cytomegalovirus (CMV) (Galloway *et al.*, 1984; Doniger *et al.*, 1999), human herpesvirus (HHV) 6 (Razzaque, 1990; Kashanchi *et al.*, 1997) and Epstein-Barr virus (EBV) (Lopes *et al.*, 2003; Busson *et al.*, 2004; Thompson & Kurzrock, 2004), but there is no strong evidence that these viruses are involved in cervical cancer.

Chlamydia trachomatis is a microbial agent that has most consistently been shown to be associated with cervical cancer in epidemiological studies that controlled statistically for HPV infection (see Section 2.7.2). Although it is not believed to have a direct effect on host DNA or on the transcription of HPV genes, several biological mechanisms by which *C. trachomatis* may increase the risk for cervical cancer have been described. First, it may have anti-apoptotic effects (Fan *et al.*, 1998): resistance of infected cells to apoptosis ensures the persistence of *C. trachomatis* infection, while cell death at the end of the infection cycle triggers release and initiates a new infection cycle (Fan *et al.*, 1998, Perfettini *et al.*, 2003a,b). These anti-apoptotic effects could result in increased persistence of epithelial cells that are co-infected with HPV and/or reduce the probability of cell death following the development of chromosomal abnormalities, which increase in frequency with increasing grade of cervical neoplasia (Lorenzato *et al.*, 2001, Melsheimer *et al.*, 2001). Second, infection by *C. trachomatis* is associated with squamous metaplasia and hypertrophic ectopy, which have been shown to be a risk factor for cervical neoplasia (Moscicki *et al.*, 1999). Third, *C. trachomatis* may cause human cervical epithelial cells to separate from each other due to the breakdown of the cadherin–catenin junctions in the epithelium (the N-cadherin/β-catenin complex) and thereby increase the exposure of basal cells to HPV (Prozialeck *et al.*, 2002). Fourth, it may increase the risk for HPV infection and its persistence through modulation of immune factors: *C. trachomatis* is reported to inhibit the expression of interferon (IFN) γ-inducible major histocompatibility complex (MHC) class II (Zhong *et al.*, 1999), as well as the expression of MHC class I (Zhong *et al.*, 2000; Hook *et al.*, 2004). If this is true, it could impair the adaptive immune response to HPV. Quantitative or qualitative alterations in expression of MHC class I (e.g. due to the presence of viral antigens) can result in stimulation of natural killer (NK) cells (core effector cells of the innate immune system that can kill a broad range of intracellular microbially infected cells without prior sensitization). *C. trachomatis* may, however, inhibit NK cell function and result in a decrease in the lytic capability of NK cells, reduced NK cell production of TNFα and IFNγ and a decrease in antibody-dependent cellular cytotoxicity (Mavoungou *et al.*, 1999). Last, chronic infection with *C. trachomatis* is associated with a predominantly T-helper (Th)2 (humoral immune) cytokine pattern, whereas Th1 (cellular immune) cytokines are important in the control of intracellular microbes such as *C. trachomatis* and HPV (Stephens, 2003).

Overall, the immune response to microbial infection (i.e. cervical inflammation) may play a role in HPV-associated tumorigenesis and help explain the possible associations of cervical cancer with a range of pathogens, including herpesviruses, *C. trachomatis*, *Trichomonas vaginalis, Neisseria gonorrhoeae, Candida albicans* and others (Castle & Giuliano, 2003). The mechanisms by which inflammation might cause an increased risk for cervical cancer have been best described for *C. trachomatis*. Many of the cytokines that are secreted during *C. trachomatis* infection, including TNFα and IFNγ, could cause tissue damage by inducing apoptosis of uninfected cells (Perfettini *et al.*, 2000), and infiltrating macrophages may cause further tissue damage through release of reactive oxygen species (Castle & Giuliano, 2003). Together, these effects probably result in partial disruption of the tissue barrier and exposure of basal cells to HPV infection. Furthermore, it has been hypothesized that the reactive oxygen species released by infiltrating macrophages could cause host cell DNA damage that leads to increased risk for cervical cancer in cells that are protected against apoptosis by HPV (Gravitt & Castle, 2001; Smith *et al.*, 2004). Support for this proposed mechanism has come from laboratory studies that showed an association between inflammatory host responses and oxidative DNA damage (Zhuang *et al.*, 2002; Touati *et al.*, 2003).

The incidence of cervical cancer is significantly increased in women who have human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) (Mbulaiteye *et al.*, 2003), and biomarkers of host immune status in HIV-positive women, including HIV RNA level and $CD4^+$ T-cell count, are associated with risk for HPV infection and cervical neoplasia (see Section 2.8.3). It is uncertain, however, whether there might also be a direct biological interaction between HIV and HPV. In-vitro studies have shown that HIV TAT protein can co-activate HPV (Tornesello *et al.*, 1993; Vernon *et al.*, 1993; Buonaguro *et al.*, 1994). Whereas some studies have reported that epithelial cells can be infected by HIV *in vitro* (Moore *et al.*, 2003; Yeaman *et al.*, 2003), there is little evidence of this *in vivo* (Spira *et al.*, 1996; Greenhead *et al.*, 2000; Miller & Shattock, 2003; Wu, Z. *et al.*, 2003). Taken together, it seems improbable that HPV-infected cervical epithelial cells could be co-infected with HIV, which limits the opportunity for the two viruses to interact directly at the molecular level.

Adeno-associated virus (AAV) may have a protective effect against HPV-associated cervical tumorigenesis. AAV is a helper-dependent parvovirus that requires co-infection with other DNA viruses, such as adenovirus, for its replication (Leonard & Berns, 1994). In tissue culture, AAV inhibits the tumorigenic effects of HPV (Hermonat, 1994a) and BPV (Hermonat, 1989) and furthermore, HPV can support replication of AAV (Walz *et al.*, 1997; Meyers *et al.*, 2001), a finding that is consistent with possible HPV/AAV co-infection in nature. Initial laboratory studies found that AAV suppressed papillomavirus replication (Hermonat, 1992) and attributed this effect to Rep 78, the major non-structural regulatory protein of AAV. Rep 78 can interfere with transcription factors and HPV promoter activity (Zhan *et al.*, 1999; Su *et al.*, 2000; Prasad *et al.*, 2003). However, recent studies have found that the effects of AAV on HPV replication are complex (Agrawal *et al.*, 2002). High levels of AAV decreased but low levels increased HPV replication

(Meyers *et al.*, 2001) and, under certain culture conditions, AAV actually increased the tumorigenicity of papillomavirus (Hermonat *et al.*, 1998). The significance of these recent findings is not yet clear, but AAV also has anti-neoplastic effects that are independent of its proposed biological interaction with HPV. Through the direct interaction of AAV proteins with cellular genes, AAV has been shown to induce differentiation of tumour cell lines (Bantel-Schaal, 1995), down-regulate *c-FOS* and *c-MYC* (Hermonat, 1994b), inhibit cell proliferation (Walz & Schlehofer, 1992) and reduce carcinogen-induced mutagenicity (Schlehofer & Heilbronn, 1990).

(*b*) *Hormones and anti-estrogens*

Epidemiological data suggest an association between hormonal status, use of hormonal contraception and parity and the risk for preneoplastic lesions of the cervix and cervical cancer (see Section 2.7). The experimental evidence for such an association derives primarily from the fact that the endogenous level of the steroidal hormone, progesterone — the major ingredient of oral contraceptives and injectable hormonal contraceptives — increases during pregnancy (Pater *et al.*, 1994), and experimental studies indicate the presence of hormonal recognition elements in the LCR of high-risk mucosal HPV and increased production of the E6 protein in response to exogenous hormonal stimulation *in vitro* (reviewed by Pater *et al.*, 1994; Moodley *et al.*, 2003; de Villiers, 2003). There is also experimental evidence that hormones may mediate changes in the immune status of the cervical mucosa (Roche & Crum, 1991).

In a review of the literature, Pater *et al.* (1994) summarized the evidence that supports an association between hormones and HPV-mediated tumorigenesis. Several reports before 1994 observed hormone-enhanced transformation of primary rodent cells, immortalization of genital keratinocytes and enhanced expression of HPV in cervical cells. In addition, inhibition of transformation and expression by anti-hormones (e.g. RU-486) had been observed in several reports. Since that time, the evidence to support a role of hormones, including estrogen and progesterone, in the increase in HPV expression has accumulated (Chen *et al.*, 1996; Khare *et al.*, 1997; Webster *et al.*, 2001).

In addition to the above-mentioned effects of hormones on the expression of HPV and the ensuing carcinogenesis, there is evidence for other mechanisms of action of hormones in the development of HPV-related cervical cancer. Auborn *et al.* (1991) observed enhanced 16α-hydroxylation of estradiol activity in both cervical and foreskin cells immortalized with HPV 16. As 16α-hydroxyestron is known to be a risk factor for other estrogen-sensitive cancers, increased concentrations of this metabolite in target cells in the cervix may enhance cervical carcinogenesis by increasing cell proliferation in the presence of HPV 16. Monsonego *et al.* (1991) examined estrogen and progesterone receptor profiles in CIN and invasive cervical cancers and found high levels of expression of progesterone receptors in the underlying stromal cells of preneoplastic lesions of the cervix. These data suggest that, *in vivo*, sex steroid hormones, particularly progesterone, may act indirectly on HPV-infected epithelial cells and be implicated as co-factors in HPV-related cervical neoplasia. These

results could also explain the relative predisposition to malignant transformation of the cervical mucosa compared with vulvar and penile mucosa.

More recently, evidence of a co-carcinogenic role for hormones in cervical cancer has accumulated from studies conducted in HPV-infected transgenic mice. Arbeit *et al.* (1996) demonstrated that exposure of K14-HPV 16 transgenic mice to 17β-estradiol increased the incidence of proliferating cells in the cervical and vaginal squamous epithelium and resulted in a concomitant up-regulation of *E6*/*E7* gene expression through all stages of carcinogenesis. In addition, exposure of these K14-HPV 16 transgenic mice to estrogen induced hyperplasia in the lower uterine gland, and continuous exposure to estrogen resulted in the development of squamous metaplasia and neoplastic progression (Elson *et al.*, 2000). In transgenic mice that express HPV 16 E6 or E7 alone, Riley *et al.* (2003) showed that E7 and estrogen combined are sufficient to induce cervical cancer, and that E6 contributes to increased tumour growth. In a more recent study, estrogen was found to contribute not only to the genesis but also to the maintenance and malignant progression of cervical cancers in HPV 16 transgenic mice (Brake & Lambert, 2005). Michelin *et al.* (1997) observed significant activation of the viral upstream regulatory region in response to exogenous estrogen and progesterone and pregnancy in HPV 18 transgenic mice.

(*c*) *Nutrients*

A large number of different food constituents and nutrients have been associated with a reduction in the persistence of HPV and the development of preneoplastic lesions and invasive cancer of the cervix. The mechanisms by which these food-derived compounds confer protection are not entirely clear. Three main types of nutrient and/or nutrient metabolite have been identified: those that are involved in oxidation reactions (e.g. carotenoids, vitamins C and E), those that are involved in methylation or one-carbon transfer reactions (e.g. folic acid, vitamin B12, vitamin B6, cysteine and the biological marker, serum homocysteine) and nutrient metabolites that have hormone-like activity (e.g. retinoic acid and its isomers).

Epidemiological research over the past few decades has indicated that anti-oxidant nutrients such as carotenoids that are found in fruit and vegetables as well as vitamins C and E may confer protection against the persistence of HPV and the development of preneoplastic lesions and invasive cervical cancer (see Section 2.7.1). The mechanism by which these nutrients might prevent cervical cancer remains unclear. Reactive oxygen species appear to play a central role in cell signalling by activating transcription factors, AP-1 and nuclear factor (NF)-κB, cell proliferation and apoptosis (Palmer & Paulson, 1997). In animal and in-vitro models, reactive oxygen species increased viral titres (Peterhans, 1997) and the infectivity of influenza virus (Hennet *et al.*, 1992). As anti-oxidants, carotenoids and vitamins C and E have a multitude of effects that may be chemopreventive. These compounds have been shown to quench reactive oxygen species that can lead to cellular damage and dysregulation of cell signalling (Palmer & Paulson, 1997). Carotenoids and vitamins C and E may also potentiate host cellular and humoral immunity (Meydani *et al.*, 1995). Studies of HIV and influenza virus also indicate a role for

anti-oxidants (in particular nutrient anti-oxidants) in the down-regulation of viral replication and expression.

Administration of anti-oxidants to animals infected with influenza virus protected against the lethal effects of influenza (Oda *et al.*, 1989). *In vitro*, increases in the cellular oxidant load have been shown to increase the replication of HIV (Pace & Leaf, 1995; Peterhans, 1997). This effect is thought to be due to the fact that reactive oxygen species activate NF-κB, a nuclear transcriptional factor that is obligatory for HIV replication (Pace & Leaf, 1995), and in-vitro studies have consistently demonstrated inhibition of NF-κB activation by anti-oxidants (see review by Epinat & Gilmore, 1999). Further molecular epidemiological studies are required to delineate to what extent anti-oxidant nutrients have a protective effect *in vivo* and the mechanisms by which they act.

Evidence has accumulated to suggest that reactive oxygen species and their down-regulation by anti-oxidants may have a similar effect on HPV infection. Activation of AP-1, a central transcription factor for the expression of E6 and E7 proteins of high-risk HPV types (Cripe *et al.*, 1990; Offord & Beard, 1990), has been shown *in vitro* to be inhibited by anti-oxidants. Rösl *et al.* (1997) demonstrated that the anti-oxidant pyrrolidine-dithiocarbamate selectively suppressed AP-1-induced HPV 16 gene expression in HPV 16-immortalized human keratinocytes, and suggested that manipulation of the redox potential may be a novel therapeutic approach to interfere with the expression of high-risk HPVs.

In addition to its effects on target cells and cell signalling, the oxidant–anti-oxidant balance is an important determinant of immune cell function, and affects the maintenance of immune cell membrane lipids, control of signal transduction and gene expression of immune cells (Meydani *et al.*, 1995; Anderson & Theron, 1990), events that are important for the loss of HPV infection and regression of CIN.

Whitehead *et al.* (1973, 1989) proposed a correlation between folic acid status and the use of oral contraceptives and increased risk for cytological abnormalities among women in the USA. This increased risk was thought to be due to a deficiency of folic acid in local tissues, which could not be detected by measuring folate in serum or in the diet. In support of this hypothesis, several laboratory studies have delineated a role for folic acid in the prevention of cancer at several sites. Folic acid is essential for the synthesis of purine nucleotides and thymidilate, which are essential for the synthesis of DNA during cell replication and repair. In addition, folic acid is necessary for the synthesis of *S*-adenosylmethionine, the main donor of methyl groups in various methylation reactions, such as methylation of the DNA base cytosine (Poirier, 2002). Low tissue levels of folate increase the frequency of fragile sites on DNA (Ames & Wakimoto, 2002), enhance the risk of attack on DNA by carcinogens and viruses (Hsieh *et al.*, 1989), and decrease DNA repair (Ames & Wakimoto, 2002) and DNA methylation (Wainfan *et al.*, 1988). Kim *et al.* (1994) observed significant increases in global DNA methylation in cervical tissue with increasing grade of cervical lesion, which suggested that the change in methylation status may be an early event in cervical carcinogenesis. Extending the work to genital HPV types, DNA methylation within the upstream regulatory region has been shown to regulate expression of high-risk HPV *in vitro* (Rösl *et al.*, 1993; Thain *et al.*, 1996). One

in-vitro study demonstrated that sequence-specific methylation of CpG sites in the constitutive enhancer region of the HPV 18 upstream regulatory region resulted in a down-regulation of transcriptional activity (Rösl *et al.*, 1993). Methylation of a novel transcription factor-binding site decreased the activity of the HPV 16 enhancer and suppressed viral transcription (List *et al.*, 1994). Additional in-vitro studies demonstrated that methylation of specific CpG sites within the HPV 18 E2-binding site abolishes binding (Thain *et al.*, 1996) and leads to a direct effect on E6 and E7 transcription. In summary, changes in the methylation of host and viral DNA may result in an increase in the production of viral proteins and hence in the risk for carcinogenesis. However, it remains unclear whether nutritional status of methyl donors such as folic acid and vitamin B12 influence the methylation patterns of host and viral DNA.

As described in Section 2.7.1, topical all-*trans*-retinoic acid was found to effect regression of CIN2 lesions in one placebo-controlled clinical trial (Meyskens *et al.*, 1994). Chemoprevention studies that used retinoic acid to prevent preneoplastic lesions or make them regress at other epithelial sites have been successful. A growing body of basic experimental research indicates that retinoic acid and related compounds also have chemopreventive activity in the cervix. Retinoic acid is essential for terminal differentiation of cervical epithelial cells because it decreases cellular proliferation and DNA replication. It differentially inhibits the growth and differentiation of HPV 16-immortalized cervical epithelial cells (Agarwal *et al.*, 1991; Eckert *et al.*, 1995) and low-passage human foreskin keratinocytes (Pirisi *et al.*, 1992; Khan *et al.*, 1993; Creek *et al.*, 1994) compared with normal human keratinocytes in the absence of an HPV infection. In addition to decreasing cellular proliferation in HPV 16-immortalized low-passage human keratinocytes, physiological concentrations of retinoic acid inhibit the expression of HPV 16 E6 and E7 (Pirisi *et al.*, 1992; Khan *et al.*, 1993; Creek *et al.*, 1994). Retinoic acid may indirectly reduce levels of HPV mRNA by influencing the activity of AP-1 (Schüle *et al.*, 1991) or the expression of transforming growth factor β (TGFβ) (Batova *et al.*, 1992). In addition, retinoic acid suppresses cell growth. However, this suppression appears to be lost in late stages of HPV 16-induced transformation of human keratinocytes (Borger *et al.*, 2000) and cervical carcinoma cell lines. In several in-vitro model systems, cells in the late stages of HPV 16-induced transformation acquire resistance to retinoic acid-induced differentiation through several different mechanisms, including loss of growth inhibition, loss of sensitivity to TGF β (Borger *et al.*, 2000), continued growth stimulation (Higo *et al.*, 1997; Sizemore *et al.*, 1998) and loss of expression of the retinoid receptor (Bartsch *et al.*, 1992). This resistance to retinoic acid is consistent with observations that therapy with retinoic acid does not reduce recurrence rates of invasive cervical cancer (Wadler *et al.*, 1997; Look *et al.*, 1998; Weiss *et al.*, 1998) nor does it increase regression of CIN3 (Meyskens *et al.*, 1994). Taken together, these data suggest that retinoic acid may only be effective in the early stages of cervical carcinogenesis by modulating the clearance and persistence of HPV, viral load and the regression of moderate CIN.

(*d*) *Tobacco smoke*

The evidence from epidemiological studies is sufficiently strong to conclude that, in the presence of HPV infection, tobacco smoking is a co-factor in the development of pre-neoplastic lesions of the cervix and invasive cervical cancer (see Section 2.7.1). Currently, there appear to be two different mechanisms by which tobacco smoking can increase the risk for cervical diseases. Cigarette smoke contains mutagens, carcinogens and other components that may act as initiators and/or promoters of uterine cervix carcinogenesis. These components can either affect immune function and allow HPV infection to persist and progress or act directly as co-carcinogens in cervical tissue or both.

Products of cigarette smoke have been found in body fluids outside the lung such as in the breast fluid of lactating smokers, the amniotic fluid of smokers, the urine and saliva of infants of mothers who smoke and secretions of the cervical mucus of smokers (Holly *et al.*, 1986). Both nicotine and cotinine are measurable in the cervical tissue of smokers (Sasson *et al.*, 1985; Schiffman *et al.*, 1987; Hellberg *et al.*, 1988; McCann *et al.*, 1992) and nonsmokers exposed to secondhand tobacco smoke (Jones *et al.*, 1991). On average, concentrations of nicotine were more than 45 times higher in cervical tissue than in serum among women who smoked (Sasson *et al.*, 1985).

Nicotine is metabolized by oxidative *N*-nitrosation to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone that is in turn metabolized by α-hydroxylation to nitrosamine, which is considered to be one of the most potent carcinogens in cigarette smoke (Hellberg *et al.*, 1988). Farin *et al.* (1995) demonstrated that both HPV 16-immortalized oral and cervical cell lines express cytochrome P450 enzymes that are necessary for the activation of nitrosamines and polycyclic aromatic hydrocarbons. Significantly higher concentrations of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Prokopczyk *et al.*, 1997) and benzo[*a*]pyrene metabolites (Melikian *et al.*, 1999a) have been observed in the cervical mucus of smokers compared with that of nonsmokers.

As determined by the Ames *Salmonella* mutagenicity test, smokers are at an increased risk for having mutagenic cervical fluid (Holly *et al.*, 1986). Cervical tissues from smokers have significantly elevated levels of DNA damage as measured by DNA adducts compared with nonsmokers (Simons *et al.*, 1993, 1995; Melikian *et al.*, 1999b). In-vitro experiments (Nakao *et al.*, 1996; Yang *et al.*, 1996) suggest that cigarette-smoke condensate induces faster growth in serum, higher saturation density, anchorage-independent growth and tumorigenicity in cells immortalized by HPV 16 and 18. Melikian *et al.* (1999b) also suggested that HPV 16-infected cells *in vitro* are more susceptible to DNA damage by benzo[*a*]pyrene than non-HPV-infected cells. Finally, tobacco smoke has been associated with aberrant hypermethylation of the tumour-suppressor gene *p16* which significantly correlated with the grade of cervical disease (Lea *et al.*, 2004). Collectively, these data provide biochemical evidence that components of cigarette smoke have a carcinogenic potential on cervical tissue which, in an HPV-infected cervix, would increase the risk for progression to cervical carcinoma.

In addition to its direct role in carcinogenesis, tobacco smoking has been associated with a generalized suppression of the immune system, including a significant decrease in NK cells and NK cell activity, in circulating levels of immunoglobulin (Ig)G and IgA (Ferson *et al.*, 1979) and in Langerhans cells (Barton *et al.*, 1988; Poppe *et al.*, 1996). Langerhans cells are dendritic cells that are localized in the epithelium and present antigen to T lymphocytes. A reduction in the number of Langerhans cells available to detect and present viral antigens may facilitate the establishment and persistence of local viral infection. Giuliano *et al.* (2002c) demonstrated that tobacco smoking was associated with an increased risk for persistence and duration of high-risk HPV infection. The resultant viral persistence may increase the probability of the development of virally induced neoplastic transformation.

(*e*) *Radiation*

(i) *Ionizing radiation*

Carcinomas in EV patients usually show very slow progression, are only destructive locally and show very low invasive and metastatic potential. However, treatment of EV patients with ionizing radiation (γ-rays, X-rays) provokes rapid metastasis, which is probably due to its co-carcinogenic effect (IARC, 2002) and/or the release of large amounts of TNFα (Jablonska & Orth, 1985; Jablonska & Majewski, 1994). These findings have led to strict regulations of the use of ionizing radiation in the therapeutic treatment of EV patients.

Similarly, a 16-fold increase in the risk for malignant transformation has been reported after X-ray radiation therapy for multiple laryngeal papillomas, with a latency period of 5–40 years (summarized in Lindeberg & Elbrond, 1991). A later report showed relatively high frequencies of anaplastic transformation after irradiation of primary laryngeal carcinomas, and recommended a surgical approach rather than radiotherapy for the treatment of these tumours (Hagen *et al*., 1993).

The effect of radiation *in vitro* on human epithelial cells that contain HPV provides a useful model to study the genetic alterations that contribute to transformation. The exposure of HPV 16-immortalized human foreskin cells to X-radiation resulted in malignant conversion after approximately 100 additional tissue culture passages (Dürst *et al*., 1995). HPV 18-immortalized human bronchial epithelial cells exposed to ionizing radiation showed several chromosomal alterations but were not tumorigenic in nude mice despite their ability to grow in soft agar (Willey *et al*., 1993). HPV 18-immortalized bronchial cells irradiated with a single dose of radon-simulated α-particles and maintained in culture for a period of up to 3 months became tumorigenic. No mutation in the K-, H- or N-*RAS* genes was found in four of the tumours (Hei *et al*., 1994).

Since HPV DNA is occasionally found in bronchogenic carcinomas (see Section 2.6.2), it may be of interest to assess the risk of environmental or occupational exposure to radon on the progression of HPV lesions of the respiratory epithelium.

(ii) *Ultraviolet radiation (UV)*

EV has been regarded as a model for the development of non-melanoma skin cancer on sites that are exposed to the sun. Infection with a specific group of HPV types (classified in the beta genus and also termed EV-HPV types) has been associated with the benign and malignant lesions that occur in EV patients. The presence of EV-HPV DNA was also demonstrated in non-melanoma skin cancer in immunocompetent patients (Iftner *et al*., 2003), as well as in up to 90% of non-melanoma skin cancers in organ transplant recipients. EV-associated and other cutaneous HPV types were also demonstrated in normal skin biopsies (35%) and in a small number of melanomas. The frequent presence of more than one HPV type within a lesion was noted in immunosuppressed transplant recipients, and at least one type was EV-associated. The data indicate that primary infection with the majority of HPV types apparently occurs early in life, after which it remains latent. Prolonged UV radiation is needed either to activate viral gene functions and/or to inactivate the cellular genes responsible for controlled cell growth (de Villiers, 1998; Pfister, 2003).

UV activation of the HPV promoter

It is well known that UV radiation stabilizes and activates p53 in the skin. A consensus p53-responsive element was identified in the LCR of the non-EV cutaneous HPV type 77. This virus type is found in warts and skin cancers of renal transplant recipients and has a high degree of sequence homology with other common HPVs that are found in warts in the general population. Consistent with the presence of the p53-responsive element, the HPV 77 LCR was transcriptionally activated by p53. Thus, UV radiation can stimulate expression of HPV 77 genes through activation of p53 (Purdie *et al.*, 1999).

The effect of UV on the LCR from cutaneous HPV types 1, 2, 3, 5, 7, 20, 23, 27, 38, 41 and 77 was evaluated in three cell lines that harbour wild-type or mutant p53 or lack p53. Each of the HPV types reacted differently to the irradiation, and reactions varied from strong inhibition to strong activation of LCR activity (de Villiers *et al.*, 1999b). In another study, a transient transfection assay was conducted in primary human epithelial keratinocytes to determine whether UVB radiation modulates LCR promoter activity of the EV-HPV types 5, 8, 9, 14, 23, 24 and 25. The LCR promoters of HPV types 5 and 8 were activated by UVB in these cells, which suggests a role of this interaction in the development of non-melanoma skin cancer (Akgül *et al.*, 2005).

UV radiation also influences pathways of pro-inflammatory cytokines and mitogen-activated protein kinases. Ruhland and de Villiers (2001) analysed the influence of interleukin (IL)-1 receptor antagonists and inhibitors of the p38 and JUN N-terminal kinase pathways in the presence or absence of UV on the LCRs of EV-HPV types 20 and 27 in RKO, HaCaT and H1299 cell lines that express wild-type or mutated p53 or that lack p53, respectively. The results showed that IL-1α, IL-1β, IL-6, IL-17, TNFα and IFNα, -β and -γ activated the promoter in the HPV 20 LCR but inhibited the HPV 27 LCR promoter. UV radiation induced a prolonged activation of JUN N-terminal kinase in HaCaT and H1299 but not in RKO cells, and the dephosphorylation of this protein was enhanced in the presence of p53 and the HPV LCRs. UV is known to induce expression of cellular genes

that encode cell-cycle inhibitory and apoptotic factors. However, the overall consequence to the cell of the induction of viral genes by UV radiation is not known.

Anti-apoptotic effect of viral protein E6

The E6 proteins of cutaneous HPV types were shown to inhibit apoptosis *in vitro* in response to UV-induced damage. This occurs in both p53-null and wild-type cells and does not require degradation of p53 (Jackson & Storey 2000). A recent study demonstrated that expression of HPV 77 E6 can effectively block UV-induced apoptosis in cells that have UV-activated p53, by selective attenuation of the *trans*-activation of the p53-regulated pro-apoptotic genes *FAS*, *PUMAbeta*, *APAF-1* and *PIG3*. This suggests that HPV 77 E6 may play an important role in specifically deregulating p53-dependent apoptosis upon UVB irradiation (Giampieri *et al.*, 2004).

One of the cellular responses to UVB damage in the skin is the induction of apoptosis, which involves a number of signalling factors that include the pro-apoptotic Bak protein. In a study to investigate the role of HPV proteins in UV-induced apoptosis, Bak was found to be targeted by the E6 proteins of cutaneous HPV type 77 and EV-associated type HPV 5 for degradation *in vitro* and in regenerated epithelium. These data support a link between the virus and UVB radiation in the induction of HPV-associated skin cancer and suggest a survival mechanism for virally infected cells (Jackson *et al.*, 2000). It is conceivable that individuals who are infected by these HPV types are at an increased risk for developing actinic keratoses and squamous-cell carcinomas, possibly through their chronic prevention of UV light-induced apoptosis (Bouwes Bavinck *et al.*, 2001). However, because this activity is shared among E6 proteins of HPV types that are exclusively associated with benign skin lesions (e.g. HPV 10), it is probably not sufficient to confer carcinogenic properties *in vivo*.

UV-induced immunosuppression

It is well known that UV radiation modifies the immune system in the skin (Kripke & Morison, 1985). Local photo-immunosuppression occurs when the skin is exposed to low doses of UVB and may prevent an inflammatory reaction that could damage skin that is exposed to the sun. For immunocompromised patients, the additional role of photo-immunosuppression is particularly important. Renal transplant recipients have an increased risk for squamous-cell carcinomas, the great majority of which are present on skin that has been exposed to the sun. HPV DNA is found in many of these skin lesions, which suggests that UVB affects the local immune response and renders the skin incapable of clearing the infection (Vermeer & Hurks, 1994). This effect may contribute to the postulated interaction between HPV infection and exposure to UV.

Psoralen–UV treatment and HPV

Patients who have psoriasis and are treated with psoralen–UV (PUVA) are at an increased risk for skin cancer, but the exact cause of this increased incidence is not well understood. It has been suggested that PUVA may increase expression of HPV in the skin

by directly stimulating virus replication, immune suppression or both, and thereby leads to the development of skin cancer. The prevalence of HPV, as measured by PCR, in the skin (hair follicles) is increased in patients who have psoriasis and a history of exposure to PUVA compared with those who do not (Wolf *et al.*, 2004).

4.1.6 *Transgenic models for HPV-associated cancers*

Numerous HPV transgenic mouse models have been generated and characterized and provide a wealth of information regarding the in-vivo biological properties of HPV genes, in particular *E6* and *E7* of HPV 16. In this section, the tumorigenic properties of viral genes that have been discovered through studies in mice, are discussed, as well as the value that HPV transgenic mice have provided to the assessment of host immune responses to these viral antigens.

(*a*) *Tumorigenic properties of HPV genes in mouse skin*

The most common type of cancer caused by HPVs in humans is squamous carcinoma of the anogenital tracts and oral cavity. The first evidence in HPV transgenic mice that HPV 16 genes could induce squamous carcinoma derived from the analysis of αAcryE6/E7 mice (in which the viral genes were placed under transcriptional control of the αA crystallin promoter) that expressed these genes ectopically in the skin (Lambert *et al.*, 1993). The mice developed squamous carcinomas as adults (Lambert *et al.*, 1993), as well as tumours of the lens (Griep *et al.*, 1993) and retina (Griep *et al.*, 1998). Transgenic mice in which the expression of HPV 16 *E6* (Song *et al.*, 1999), HPV 16 E7 (Herber *et al.*, 1996) and all early HPV 16 genes (Coussens *et al.*, 1996) was directed to the basal compartment of stratified squamous epithelia by the keratin 14 (K14) promoter developed cancers. The severity of the cancers that arose in mice that expressed *E6* or *E7* differed individually. In K14E7 mice, the tumours were primarily benign or low-grade carcinomas, whereas in the K14E6 mice, the majority of tumours were malignant carcinomas of higher grade (Song *et al.*, 2000). These differences were also seen in the synergy between HPV genes and chemical carcinogens in the induction of skin cancer. *E6* was found to contribute to two stages in carcinogenesis — promotion, which is a required step in the formation of benign papillomas, and progression, which is the process that converts a benign tumour to a malignant cancer — whereas *E7* only contributed to promotion (Song *et al.*, 2000). Exclusively benign skin tumours were observed in other strains of mice in which the expression of HPV 16 or 18 *E6* and *E7* was directed by the tyrosinase or human keratin (hK) 1 promoters (Greenhalgh *et al.*, 1994; Kang *et al.*, 2000). Activated forms of the *Ras* proto-oncogene acted synergetically with HPV 16 *E6* and *E7* to produce tumours (Schreiber *et al.*, 2004).

The carcinogenic properties of a cutaneous HPV in mice have now been described (Schaper *et al.*, 2005). The early region of the EV-HPV 8 genome was placed under transcriptional control of the hK14 promoter. While multiple independent lines of HPV 8 transgenic mice did not display acute phenotypes of epithelial hyperplasia as seen in mice that expressed HPV 16 *E6* and *E7* genes separately or together (see above), they deve-

loped both benign and malignant tumours of the skin at frequencies of 91 and 6%, respectively. The frequency of tumours was higher and tumours occurred at an earlier age than those in HPV 16 transgenic mice. This provides the first evidence that a cutaneous HPV can cause tumours *in vivo* in an animal model.

(*b*) *Tumorigenic properties of HPV genes in the reproductive tracts of mice*

The first mouse model for HPV-induced cervical cancer used a recombinant retrovirus to transduce HPV 16 *E6* and *E7* genes into the mouse cervix (Sasagawa *et al.*, 1992). Cervical cancer developed when the mice exposed to the E6/E7 recombinant retrovirus were treated with the tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate or the mutagen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. A more tractable model for HPV-induced carcinogenesis was developed in which K14HPV16 transgenic mice were treated with exogenous estrogen. These mice developed a progressive disease that led to the formation of squamous carcinoma of the cervix over a 6-month period and closely reflected the histopathological characteristics of the progressive disease that leads to cervical cancer in humans (Arbeit *et al.*, 1996; Elson *et al.*, 2000). The individual role of *E6* and *E7* in cervical cancer was found to differ from that observed in the skin; *E7* played a more dominant role in the cervix and *E6* in the skin (Riley *et al.*, 2003).

The validity of these HPV 16 transgenic mice as models for human cervical cancer has been demonstrated at several levels. First, the histopathological progression of disease in mice closely parallels that in human cervical cancer (Riley *et al.*, 2003). Second, the role of estrogen as a co-factor in the development and progression of cervical cancers in mice (Brake & Lambert, 2005) parallels the epidemiological evidence for a role of estrogen in human cancers (see Section 2.7.1). Third, there is a close parallel in the expression pattern of biomarkers for human and murine cervical cancer (Brake *et al.*, 2003). Last, the demonstration that the anti-estrogenic drug, indole-3-carbinol, inhibits cervical cancer in the HPV-transgenic mouse model (Auborn *et al.*, 1991) has led to its successful use in the clinical treatment of CIN2/3 (Bell *et al.*, 2000).

(*c*) *Use of HPV transgenic mice for immunological studies*

Many investigators have used HPV transgenic mice to investigate host immune responses to viral antigens and to develop protocols to induce antigen-specific therapeutic immune responses against viral antigens. Although viral genes that are expressed as transgenes should be considered as self-antigens, many studies have demonstrated that HPV transgenic mice can mount immune responses to E7 when stimulated appropriately. When immunized with E7 protein, HPV transgenic mice (line 19 αAcryE6E7) that develop squamous-cell carcinomas (Griep *et al.*, 1993) produced antibody and showed Th responses to E7 that were indistinguishable from those seen in immunized syngeneic, non-transgenic mice (Frazer *et al.*, 1995; Herd *et al.*, 1997). Non-immunized line 19 mice, when allowed to age and develop skin lesions, showed spontaneous E7-specific immune responses (Frazer *et al.*, 1995). While these antigen-specific responses neither contributed

to nor modulated the skin diseases that occurred in these mice, non-specific local inflammatory responses did contribute positively to skin disease (Hilditch-Maguire *et al.*, 1999). K14E7 mice that express E7 from the K14 promoter display split tolerance to E7, which is characterized by the ability to mount normal Th and B-cell responses to E7, but the inability to mount E7-specific CTL responses on genetic backgrounds (i.e. H-2b) in which E7-specific CTL-restricted epitopes exist (Doan *et al.*, 1998; Frazer *et al.*, 1998; Doan *et al.*, 2000).

The absence of CTL responses in K14E7 mice was associated with a non-specific down-regulation of CD8-positive T cells (Tindle *et al.*, 2001) that was probably attributable to transgene-associated disturbance of the K14E7 thymic architecture (Malcolm *et al.*, 2003). Similar split tolerance was observed in HPV transgenic mice in which the expression of E7 was directed from the K10 promoter (Borchers *et al.*, 1999). The underlying reason for this split tolerance is not understood, but could reflect the fact that keratin promoters are active in thymic epithelial cells (Frazer *et al.*, 1998).

The generation of HPV transgenic mouse models that express viral antigens in the skin offered the opportunity to use graft studies to investigate immune recognition of these antigens, when expressed in clinically relevant amounts in keratinocytes by naïve, immunologically intact animals. E7 did not function as a classical minor transplantation antigen with regard to its expression in mouse epidermis. When grafted onto non-transgenic mice, skin from K14E7 mice was not rejected even when these mice were immunized against E7 and developed E7-specific CTL responses (Dunn *et al.*, 1997; Frazer *et al.*, 2001). Similarly, grafts in which E6 antigen was expressed from the K14 promoter were also accepted (Matsumoto *et al.*, 2004). However, recipient animals were induced to reject the skin grafts in an E7-specific manner after stimulation of systemic pro-inflammatory responses (Frazer *et al.*, 2001) or through passive transfer of E7-specific CTLs in combination with E7-specific immunization (Matsumoto *et al.*, 2004). The reason why the immune system has difficulty in recognizing E7 antigen in the skin remains unclear, but E7 may possibly inhibit host immune responses. In one study in transgenic mice, it was suggested — on the basis of a comparison of expression levels of various IFN-responsive genes using real-time PCR in E7 transgenic versus non-transgenic mice — that E7 can suppress innate immune responses through its interaction with IRF1 (Um *et al.*, 2002). In another study, however, no effect of E7 was noted on the levels of MHC class I protein, a major IFN-response gene product, following induction by IFN when keratinocytes from K14E7 transgenic mice were compared with those from non-transgenic mice (Leggatt *et al.*, 2002). An alternative explanation for the poor recognition of E7 by the host immune response is that, as a non-secreted antigen, it is poorly cross-presented by antigen-presenting cells and/or that the local immune environment of the skin is not conducive to recognition by the host immune system of keratinocyte-expressed antigens. Regardless of the mechanism, the insights gained from these graft studies have clear implications with regard to the design of effective therapeutic vaccines to treat patients with HPV-associated disease.

4.2 Immune mechanisms and HPV-associated neoplasia

4.2.1 *Immunosuppression*

Impaired immunity is a host factor that has been associated with increased numbers of HPV-related lesions, as reported in studies of various populations of immunosuppressed patients. Renal transplant patients, who suffer from cell-mediated immune suppression, have an increased risk for cutaneous and genital HPV lesions (Rüdlinger *et al.*, 1986; Bouwes Bavinck & Berkhout, 1997). Generalized T-cell deficiency has also been associated with an increased incidence of anogenital neoplasia (Tindle & Frazer, 1994), as demonstrated by the increased relative risk of 5.4 (95% confidence interval, 3.9–7.2) for cervical cancer in the AIDS/National Cancer Registry study in the USA (Frisch *et al.*, 2000). Among patients with EV, NK cell-mediated cytotoxicity against keratinocytes that harbour EV-HPV DNA was markedly decreased (Majewski *et al.*, 1990). In healthy individuals, most HPV-associated lesions regress spontaneously (Stern *et al.*, 2000). Occasionally, virus persists and lesions progress, although it is not understood why some immunocompetent individuals fail to clear the infection. Both the innate and adaptive arms of cell-mediated immunity play a critical role in the determination of the outcome of an initial infection and the prevention of recurrences. A better understanding of these areas is critical for the development of therapeutic vaccines against HPVs (for reviews, see Konya & Dillner, 2001; Stern *et al.*, 2000; Melief *et al.*, 2002).

4.2.2 *Histological studies*

As HPV infection is restricted to the epithelium, clearance must be mediated by local immune defences. To address the question which effectors mediate clearance, several studies have performed histochemical analyses of genital lesions and in some cases have compared lesions from patients who responded to IFN therapy with those from patients who did not.

In naturally regressing genital warts, an increase in the infiltration of macrophages, NK cells and both $CD4^+$ and $CD8^+$ T cells into the epithelial layer was observed (Coleman *et al.*, 1994). Warts that responded to treatment with IFNg and IFNa2a showed increases in Th1 inflammatory cells, macrophages and $CD4^+$ T cells (Arany & Tyring, 1996; Hong *et al.*, 1997). Furthermore, endothelial cells that line capillaries in the underlying stroma expressed the up-regulated adhesion molecules VCAM and E-selectin and the cytokine RANTES. In contrast, warts that did not respond had fewer infiltrating T cells and reduced levels of IL-1, granulocyte macrophage colony-stimulating factor and TNF. These results suggest that a Th1 response is important in the generation of a milieu that clears HPV infection.

CIN and cancers show somewhat different features. Notably, there is a decrease in Langerhans cells in low-grade lesions and a more pronounced depletion in high-grade lesions (Viac *et al.*, 1990). Langerhans cells are specialized epithelial dendritic cells that function in the uptake of antigens. Upon migration to draining lymph nodes, they change into potent antigen-presenting cells with enhanced expression of MHC and co-stimulatory

molecules that are necessary to generate a vigorous T-cell response. Recent studies have shown that activation (or maturation) of dendritic cells depends on signals from either innate immunity triggers such as Toll-like receptors or adaptive immunity triggers such as the CD40 ligand on $CD4^+$ T cells (Melief *et al.*, 2002). In the absence of antigen presentation by fully activated dendritic cells, the resulting $CD8^+$ T-cell response is more likely to be tolerogenic than cytolytic. A study that examined Langerhans cells in low- and high-grade cervical lesions with two markers, CD1 antigen and S-100 protein (Connor *et al.*, 1999), showed a significant reduction in S100-positive Langerhans cells in high-grade compared with low-grade lesions and normal tissues. The lack of S100 expression suggested an early failure of activation of Langerhans cells in HPV-infected tissue. Lesions that contain HPV 16 have reduced numbers of Langerhans cells in the infected epidermis (Matthews *et al.*, 2003), which was attributed to reduced levels of E-cadherin on the surface of basal keratinocytes. In culture, the E6 protein mediated the reduction in levels of E-cadherin. Tobacco smoking, which has been shown to be a risk factor for the development of several anogenital cancers (Daling *et al.*, 1996), also results in the depletion of Langerhans cells in the cervical mucosa (Poppe *et al.*, 1996).

Several groups have investigated the numbers and types of T cell in cervical dysplasia with varying results (IARC, 1995). Such discrepancies may arise because cross-sectional studies cannot determine whether the lesion is progressing, regressing or static. Taken together, the results indicate that early lesions show little change in the numbers of intraepithelial T cells, whereas high-grade lesions and cancers show an increase in $CD8^+$ T cells. However, although human leukocyte antigen (HLA) class II molecules are up-regulated in late-stage lesions (see Section 4.2.4(*b*)), there is no corresponding increase in $CD4^+$ T cells.

4.2.3 *Cell-mediated immunity*

The evidence that cell-mediated immunity plays a role in the control of HPV infections derives from several lines: (*a*) most HPV-infected tissues show an inflammatory response at the time of regression; (*b*) individuals who have genetic or acquired immune deficiency are at increased risk for persistent HPV infections that progress to cancer; (*c*) efforts at immunomodulation, e.g. the use of IFN and imiquimod (which indirectly enhance cell-mediated immunity), can promote the regression of HPV lesions; and (*d*) numerous animal models have shown that inoculation of viral proteins resulted in a delayed-type hypersensitivity response that required the presence of $CD4^+$ T cells and that clearance of tumours depended on the presence of $CD8^+$ T cells (IARC, 1995; Konya & Dillner, 2001). The host immune system develops a multifactorial response that involves innate immune cells as well as helper and cytolytic T-cell responses.

(*a*) *T-Helper cell responses*

Most priming of $CD4^+$ T cells occurs in the lymph nodes following the transit of Langerhans cells from infected tissues. Most infiltrating T lymphocytes carry HLA-DR

antigens — a late activation marker — but only a few express CD25 — an early activation marker (Coleman *et al.*, 1994), which indicates that they are probably activated at distant sites. T-Helper cells largely function through the up-regulation of CD40 ligand which interacts with CD40 on dendritic cells and leads to their maturation (Melief *et al.*, 2002). CD40L on $CD4^+$ T cells can also directly signal to $CD8^+$ CTL. In addition, $CD4^+$ cells play a crucial role by interacting with B cells to induce antibody production. The interaction of T-helper cells with dendritic cells determines whether they secrete Th1 or Th2 cytokines.

Activation of $CD4^+$ occurs through recognition of MHC class II/peptide complexes present on antigen-presenting cells. Much effort has been made to identify which viral peptides can be recognized by $CD4^+$ T cells and what are the consequences of having viral-specific $CD4^+$ T-cell clones. In theory, all viral antigens can serve as targets that will be presented by class II antigens. Numerous studies have shown that $CD4^+$ T cells proliferate in response to both early and late antigens (Tindle *et al.*, 1991; Kadish *et al.*, 1994; Luxton *et al.*, 1997), and the responses tended to be type-specific. Which T-cell epitopes are presented is governed by the ability of a particular MHC molecule to bind a given peptide.

In addition to their critical function in priming other cells in the cell-mediated immune response, $CD4^+$ T cells deliver cytokines to the infected tissue, thereby influencing the outcome of the infection. The production of Th1-type cytokines is benefical for the reduction of lesions, whereas the elaboration of Th2 cytokines has a deleterious effect. Under the influence of IL-12, a Th1 response yields TNF and IFNg, whereas under the influence of IL-10, the Th2 cytokines IL-4 and IL-5 are generated, which favour B-cell development. The extent to which $CD4^+$ T cells kill keratinocytes that have up-regulated HLA-DR is unclear.

Assays to detect $CD4^+$ T-cell responses have relied on the use of peptides to stimulate T-cell proliferation, as measured by incorporation of tritium. More sensitive assays now measure the release of IL-2 from stimulated cells. Numerous studies have detected $CD4^+$ T-cell responses in sera of patients. In some studies, Th1 responses to either E7 or E2 correlated with the clearance of lesions (Kadish *et al.*, 1997). However, in other studies, the frequency of responses was similar in cases with low-grade lesions and in controls (de Gruijl *et al.*, 1996), even in studies that used virgin women as controls (Nakagawa *et al.*, 1996).

(*b*) *Cytotoxic T-cell responses*

The role of $CD8^+$ T cells in killing HPV-infected cells is extremely important as most therapeutic vaccine strategies are aimed at induction of this type of T cell. It is now recognized that the ability of CTL to kill is not based solely on the interaction of the $CD8^+$ T-cell receptor with antigenic peptides presented by MHC class I. These interactions, in the absence of other signalling cascades, frequently lead to tolerance. In contrast, productive CTL memory and killing requires signalling from highly activated dendritic cells and $CD4^+$ T cells (Melief *et al.*, 2002).

Numerous mouse models have shown that tumours that express HPV 16 or 18 E6 or E7 antigens are killed by generating a CTL response that is antigen- and MHC-restricted (Chen

et al., 1991; Feltkamp *et al.*, 1993; Tindle & Frazer, 1994). In these cases, the epitopes that served as recognition sequences were specified by the mouse MHC class I loci.

CTLs to E7 have been difficult to detect in the peripheral blood of patients known to have been exposed to HPV 16 or 18 (Borysiewicz *et al.*, 1996); however, they can be detected at higher frequency in lesions that contain infiltrates from tumours (Evans *et al.*, 1997). Oligopeptide epitopes or HLA-matched cervical cancer cell lines rarely stimulated peripheral blood CTLs, whereas viral vectors with recombinant E6 and/or E7 stimulated CTLs from 30-60% of HPV 16-positive CIN patients *in vitro* (Nakagawa *et al.*, 1997; Nimako *et al.*, 1997).

In a longitudinal study, more CTL responses to HPV 16 E6 were detected in women who had HPV 16 infection without SIL than in women who had SIL (Nakagawa *et al.*, 2000). Fifty-one women who had HPV 16 infection and three HPV 16-negative control women were enrolled; 22 (55%) of 40 women who cleared HPV 16 infection had an E6 CTL response at least once compared with none of nine women who had persistent HPV 16 ($p = 0.003$). No such difference was demonstrated for E7; 25 (63%) of 40 women who cleared HPV 16 infection responded versus five (56%) of nine women with persistent HPV 16 ($p = 0.720$). It appears that the lack of response to E6 is important in the persistence of HPV 16 infection. More studies on the CTL response to HPV antigens are needed to understand fully the role of CTLs in mediating regression.

4.2.4 *Major histocompatability complex (MHC)*

(*a*) *MHC class I*

MHC class I antigens are expressed ubiquitously on human cells, including cervical keratinocytes. They present peptides that are derived from the processing of endogenous antigens of both host and viral origin to $CD8^+$ T cells. When a vigorous $CD8^+$ T-cell response is generated through stimulation by activated dendritic cells and by CD40 signalling pathways, cytolytic $CD8^+$ T cells can kill cells that present viral peptides on the MHC class I antigen.

Early results on the expression of class I antigens gave conflicting results, probably due to the antibodies that were used for immunostaining. However, more recent studies consistently observed down-regulation of class I expression in cervical cancers (IARC, 1995). HLA class I A and B genes are highly polymorphic and inherited alleles for each gene are expressed. Examination of cervical tumours revealed that down-regulation of MHC class I expression was variable (Keating *et al.*, 1995). Certain alleles were more frequently down-regulated including A2, A3, the A9 group, the B5 group, B7, B8 and B44. Several studies have examined whether certain HLA class I genotypes confer a risk for progression to cancer (see Section 4.2.5). The risk associated with some of these alleles is probably due to their more frequent down-regulation, although it is unclear whether this is an early event that would affect progression. Down-regulation of MHC class I has also been observed in laryngeal papillomas (Vambutas *et al.*, 2000).

Many viruses target various steps in the class I antigen presentation pathway to escape immune detection. The mechanism by which class I down-regulation occurs in HPV infection is not clear and probably involves multiple mechanisms. Cromme *et al.* (1994) showed that directly diminished expression of class I genes was a rare event. The defect in class I expression in some tumours was found to be due to a loss of the peptide transporter 1 associated with antigen processing (TAP1) which occurred through down-regulation of the transcription of TAP1. HPV 16 and 18 E7 have been reported to down-regulate the promoters of both the TAP1 and MHC class I heavy chain (Georgopoulos *et al.*, 2000). Other studies have reported that the HPV 11 E7 binds to the TAP transporter protein and blocks the loading of peptides onto class I antigens (Vambutas *et al.*, 2001). HPV 16 E5 was recently reported to cause the retention of HLA class I complexes in the Golgi apparatus and impede their transport to the cell surface; this was rectified by treatment with IFN. Unlike BPV E5, HPV 16 E5 did not affect the synthesis of HLA class I heavy chains or the expression of the transporter associated with the antigen that processes TAP (Ashrafi *et al.*, 2005).

(*b*) *MHC class II*

MHC class II antigens are generally only expressed on antigen-presenting cells, and present antigens derived from an exogenous antigen-presenting pathway to $CD4^+$ T cells. However, in the majority of cervical premalignant and malignant neoplasias, HLA class II molecules are up-regulated on keratinocytes (Glew *et al.*, 1992; Cromme *et al.*, 1993). Normal ectocervical epithelium does not express detectable levels of class II antigens, nor did expression of HPV genes in cultured epithelium result in class II expression (Coleman & Stanley, 1994), which suggests that the up-regulation of class II was indirect and was mediated by the pro-inflammatory cytokine IFNγ and in part by TNFα (Majewski *et al.*, 1991). Consistent with this explanation is the finding that cultured keratinocytes that expressed HPVs also expressed class II antigens when treated with IFNγ and the fact that class II expression is increased in various inflammatory skin diseases. Also consistent with this hypothesis is the correlation between increased inflammatory response in the stroma and increased infiltrating inflammatory cells and the extent of class II upregulation (Coleman & Stanley, 1994). The DR, DP and DQ class II MHC subloci are differentially expressed on keratinocytes within cervical squamous tumours, which suggests independent regulation (Glew *et al.*, 1992). It has also been reported that the HPV 16 E5 protein can block INF-induced surface expression of MHC class II by preventing degradation of the invariant chain (Zhang *et al.*, 2003).

4.2.5 *Modulation of innate immune responses by HPV*

HPVs display multiple activities that may contribute to down-modulation of both antigen-specific and innate immune responses. Previous sections have described studies that demonstrated an ability of HPV gene products to modulate MHC class I and class II cell surface expression, as well as the expression of TAPs, all of which are components of

the cellular machinery that mediates antigen-specific responses. This section reviews studies that demonstrate that HPV gene products can modulate innate immune responses. For a more in-depth summary of the effects of HPVs on the host immune response the reader is referred to the review by O'Brien and Campo (2002).

A number of laboratories have identified the effects of HPV proteins E6 and E7 on specific cellular factors that modulate the cellular response to IFNs. Ronco *et al.* (1998) discovered that HPV 16 E6 can bind to and destabilize IRF3 and thereby inhibit the induction of IFNβ. Li *et al.* (1999) provided evidence that HPV 18 E6, but not HPV 11 E6, binds tyrosine kinase 2 and impairs activation of the Jak-signal transducer and activator of transcription pathway by IFNα. Thus, E6 proteins of high-risk but not low-risk HPVs studied to date can inhibit IFN responses.

Barnard and McMillan (1999) proposed that a direct interaction of HPV 16 E7 with the p48 component of the IFN-stimulated gene factor 3 transcription complex contributes to the inhibition by E7 of IFNα-inducible genes. In a subsequent study, it was proposed that E7 from both high- and low-risk HPVs can inhibit IFNα-inducible genes, and that this activity required the region in E7 that is involved in Rb-binding (Barnard *et al.*, 2000). Other studies found that HPV 16 E7 can impair the function of IRF1 (Park *et al.*, 2000b; Perea *et al.*, 2000) and that this correlates with the ability of E7 to interact with IRF1 at least in part through its Rb-binding domain (Park *et al.*, 2000b).

Indirect evidence for an effect of HPVs on the innate immune response derives from gene expression profiling studies on human epithelial cell lines that harbour intact high-risk HPV genomes or express high-risk HPV genes, in which a down-regulation of the expression of many IFN-responsive genes was observed (Chang & Laimins, 2000; Nees *et al.*, 2001).

5. Summary of Data Reported and Evaluation

5.1 Human papillomavirus (HPV) infection

Papillomaviruses are a family of DNA viruses that have a double-stranded, closed, circular genome of 7000–8000 base pairs and a non-enveloped T=7 icosahedral capsid. Approximately 100 human papillomaviruses (HPVs) have been molecularly cloned and sequenced, and other putative types have been identified based on polymerase chain reaction products that represent partial genomes. HPVs have a strict species-specific tropism and infect only mucosal or cutaneous epithelia. Analysis of HPV genomes, and those of a number of animal papillomaviruses, has led to the development of robust phylogenetic trees that form a stable framework for the placement of additional HPV types as they arise. A newly proposed taxonomy and nomenclature follows generally accepted criteria. Higher order phylogenetic assemblages are considered to be a genus; for example, the genital HPVs belong to the genus alpha-papillomavirus and the cutaneous epidermodysplasia verruciformis-associated HPVs belong to the genus beta-papillomavirus. Clusters of lower order

are known as species, which are closely related phylogenetically; while members of the species have distinct genomes, they have identical or very similar biological or pathological properties. For example, the species alpha-9 includes HPV types 16, 31, 33, 35, 52 and 58. Among genital HPVs, the nomenclatures 'high-risk' and 'low-risk' are widely used and refer to types that are frequently found in cervical cancers versus types that are rarely or never found in cervical cancers. Sequencing of a hypervariable region from many isolates of HPV 16 has provided evidence that HPVs are ancient viruses that have co-evolved with their hosts. A continuing area of investigation is the determination of how variation within a type affects the pathogenic potential of that type.

The structure of the viral genome can be divided into three parts: a non-coding region that contains the origin of viral replication and multiple elements that regulate transcription of the viral genes; an early region that encodes non-structural viral proteins; and a late region that encodes two capsid proteins, L1 and L2. HPVs are thought to infect cells in the basal layer of the epithelium and establish maintenance of the viral genome as a low-copy number nuclear plasmid. The E6 and E7 gene products promote cellular replication of at least some suprabasal cells that harbour the HPV genome. As infected cells move further up in the epithelium, epithelial differentiation promotes replication of productive viral DNA, expression of the late genes, assembly of the capsid and encapsidation of a chromatinized viral genome.

The repetitive structure of 72 capsomers that forms the papillomavirus capsid is highly immunogenic. Combined use of monoclonal antibodies, mutagenesis and the recently derived crystallographic structure of a capsomer has localized neutralization epitopes to type-specific surface-exposed loops. Virus-like particles that originate from the expression and self-assembly of L1 provide an antigen target to measure naturally occurring antibodies. In response to HPV infection, most, but not all, women develop immunoglobulin G antibodies directed towards type-specific conformational epitopes on L1. These antibodies have a low titre and are slow to develop, but generally persist for many years. Serum and cervical immunoglobulin A antibodies to L1 also develop, but are lost quickly. While antibodies to other HPV proteins may be elicited, no reproducible assays have been developed for their measurement, except for antibodies to HPV 16 and 18 E6 and E7, which are seen quite frequently in individuals who have invasive cancer.

During the past decade, many techniques have been applied for the detection of HPV infection and disease. Visual inspection of the cervix or genital tract, colposcopy, cytology and histology are all used to detect clinical manifestations of HPV-associated disease. The most reliable confirmation of current HPV infection is by assays that detect HPV nucleic acids. Hybrid Capture 2™, which employs cocktails of probes, is widely used clinically. Most studies use polymerase chain reaction methods that permit determination of HPV types, and many protocols use primers that amplify a conserved region of the L1 gene that can then be hybridized to (or interrogated with) type-specific probes. Primer pairs for both genital and cutaneous HPV types have been developed. Real-time polymerase chain reaction assays facilitate the quantitation of viral load in a specimen, and sequencing allows the identification of viral variations. Serological assays that use virus-like particles

can provide a measure of past HPV infection. Taken together, these methods have contributed greatly to understanding the natural history of genital HPV infections.

Transmission of genital HPVs occurs primarily through sexual intercourse. Annual rates of incident infection in young women are approximately 5–15%, and infections by high-risk types, particularly HPV 16, are the most frequent. Overall HPV positivity in cytologically normal women has been reported at levels of between 1.5% and 39%. The incidence and prevalence of HPV infections peak in young adults in most study populations, and the prevalence of specific HPV types has been reported in numerous studies. Although the age of the women who were tested and the type of polymerase chain reaction methods used could introduce some variability, clear geographical differences in prevalence exist, and, as anticipated, the prevalence of HPV infection was also higher in populations of commercial sex workers or women and men who were infected with human immunodeficiency virus. However, there is a paucity of studies on the natural history of HPV infection in men and on HPV infection at non-genital sites.

Viral DNA persists for a median of approximately 1 year, and high-risk types persist somewhat longer than low-risk types. As HPVs induce cellular proliferation, HPV infection may result in a range of morphological manifestations. While the development of more severe lesions such as stage 3 cervical intraepithelial neoplasia may be due to accumulated events, some infections may cause the rapid appearance of this lesion. When HPV DNA is no longer detectable by polymerase chain reaction amplification, it is uncertain how often the viral infection has been cleared rather than remaining either latent or at extremely low levels of persistence. In addition, viral clearance is associated with regression of the lesions.

The classification of the cytological and histological changes that precede invasive cervical cancer has changed over time as understanding of the link between HPV infection and the appearance of lesions has improved. In 2001, the Bethesda cytological classification was revised. The dichotomous division of squamous intraepithelial lesions into low-grade and high-grade is based on virological, molecular and clinical observations. The term low-grade more frequently reflects a transient HPV infection, whereas the term high-grade implies a lesion in which high-risk HPVs are present. The terms cervical intraepithelial neoplasia 1, 2 and 3 are used for cytological classification in some European countries, but are generally used for histological classification. Grade 3 cervical intraepithelial neoplasia, which subsumes the diagnoses of severe dysplasia and carcinoma *in situ*, has been proposed as the proximal precursor to invasive cervical cancer. In addition to cervical intraepithelial neoplasia, HPVs cause similar lesions in glandular cervical epithelium, as well as in the epithelia of the vulva, vagina, anus, penis and some other non-genital sites. Benign lesions are also found in a variety of genital and non-genital sites.

The treatment of benign genital lesions may be accomplished using cytotoxic treatments, e.g. with trichloroacetic acid, podophyllin, podofilox, 5-fluorouracil; immunomodulation with agents such as imiquimod or interferons; or physical ablation, e.g. with a laser, through excision by surgery or with laser electrocautery excision. Numerous efforts are under way to develop therapeutic vaccines that could be used as adjuvant therapies. These approaches attempt to stimulate powerful cell-mediated immune responses and most

frequently target the viral E6 and/or E7 proteins. While some preliminary studies provide evidence of immunological and, in some cases, clinical responses, no strategy has yet been tested in large, blinded trials. In contrast, the realisation of a prophylactic vaccination to prevent HPV infection has been accomplished. Three trials have provided proof of the principle that vaccination with virus-like particles can elicit strong antibody responses that seemingly protect against genital infection and clinical disease in a type-specific manner. The next decade should determine whether durable protection against HPV infection can be achieved, and large randomized controlled trials should provide evidence of a reduction in HPV-induced neoplasia.

5.2 Human carcinogenicity data

Cancer of the cervix

The evidence evaluated for determination of the carcinogenic potential of individual HPV types derived primarily from three lines of epidemiological data that include results predominantly from HPV type-specific case–control studies, as well as prospective cohort studies and case series from five continents. Traditionally, prospective cohort studies are considered to provide the highest level of evidence; however, in the case of HPV, they have limitations because of the small number of cancer end-points and the need to focus on the surrogate end-point of grade 3 cervical intraepithelial neoplasia. For this reason, results from case–control studies are emphasized because of the far larger number of invasive cancers that have been evaluated. Virtually all cervical cancers contained HPV DNA. The large and comprehensive case series permitted consideration of the relative frequency of different HPV types across cervical lesions of increasing severity. Finally, the classification of risk for HPV types derived from epidemiological evidence was considered in the light of phylogenetic classifications.

Strong epidemiological evidence confirmed the previous evaluation that HPV types 16 and 18 are carcinogenic to humans. The evidence for carcinogenicity was strongest for HPV 16. In addition, a convincing association, mainly from case–control studies, was found for HPV types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66. HPV types 26, 68, 73 and 82 were associated with cervical cancer in some case–control studies but were rarely found in case series and were not associated with an increase in risk in prospective studies; overall, the epidemiological data for these types were not considered to show a consistent association. Despite a large amount of HPV type-specific data that failed to demonstrate an association, HPV 6 and 11 have been reported in very rare cases, but they did not contribute meaningfully to the burden of cervical cancer. Fewer data were available for other HPV types.

Anogenital cancers other than those of the cervix

Cancer of the vulva

Vulvar cancers include basaloid, warty and squamous-cell cancers. Case series showed that the prevalence of HPV DNA in vulvar cancers was higher in basaloid and warty tumours (> 50% in most studies) and lower in keratinizing squamous-cell carcinomas (approximately 5% in most studies). Seroepidemiological studies were consistent with HPV DNA studies and supported a causal role for HPV 16 in the pathogenesis of these subsets of vulvar cancers. The evidence for HPV 18 was less strong, but was suggestive. Studies of the carcinogenicity of HPV 6, 11, 31 or 33 in basaloid and warty tumours of the vulva were limited by the small number of tumours reported. Most verrucous carcinomas were not associated with HPV. However, HPV 6 or 11 have consistently been detected in the rare verrucous carcinoma that arises from Buschke-Löwenstein tumours.

Cancer of the vagina

HPV DNA was detected in a high proportion of vaginal cancers (> 50% in most studies), particularly that of HPV 16. Combined with the seroepidemiological data, the studies of vaginal cancer consistently showed a strong association with HPV 16 and a less consistent association with HPV 18.

Cancer of the penis

Similarly to vulvar cancers, penile cancers include basaloid, warty and keratinizing squamous-cell cancers. Case series showed that the prevalence of HPV DNA in penile cancers was higher in basaloid and warty tumours (> 50% in most studies) and lower in keratinizing squamous-cell carcinomas. Studies of the carcinogenicity of HPV 6, 11, 31 or 33 in penile cancer were limited by the small number of tumours reported. However, HPV 6 or 11 have consistently been detected in the rare verrucous carcinoma that arises from Buschke-Löwenstein tumours.

Cancer of the anus

As in vaginal and cervical cancers, HPV DNA was detected in a high proportion of anal cancers using polymerase chain reaction (> 50% in most studies), particularly that of HPV 16. The data on HPV DNA prevalence combined with the seroepidemiological data on anal cancer indicated that there was a consistent association with HPV 16 and a less consistent association with HPV 18. Studies of the carcinogenicity of HPV 6, 11, 31 or 33 in anal cancer were limited by the small number of tumours reported. However, HPV 6 or 11 have consistently been detected in the rare verrucous carcinoma that arises from Buschke-Löwenstein tumours.

Cancers of the aerodigestive tract

Cancer of the oral cavity

HPV DNA was detected in a limited number of cancers of the oral cavity (tongue, floor of the mouth, gum, palate and other sites of the mouth). The range of detection was wide with an estimated average of approximately 25%. HPV 16 was detected in about 70% of HPV-positive cases and HPV 18 in a smaller fraction. Several studies that compared tumours with normal tissue detected HPV DNA more frequently in the tumours. Several seroepidemiological case–control studies and one prospective study indicated an increase in risk for oral cavity cancer associated with HPV 16.

Cancer of the oropharynx

HPV DNA was consistently detected in a substantial fraction of cancers of the oropharynx with an estimated average prevalence of 35%. HPV 16 DNA was detected in approximately 80% of HPV-positive cases. Several studies that compared tumours with normal tissue revealed large differences in HPV DNA detection, particularly that of HPV 16 in cancer of the tonsil. Seroepidemiological case–control studies and one prospective study showed marked increases in risk associated with serological markers of expression of HPV. These associations were much stronger than those observed for cancer of the oral cavity.

Cancer of the oesophagus

Some studies detected HPV DNA in cancers of the oesophagus, but others reported negative results. Seroepidemiological studies also gave contradictory results, although some prospective studies showed a positive association.

Cancer of the larynx

HPV DNA was detected in a variable fraction of cancers of the larynx. Limited and contradictory data resulted from comparisons of tumours and normal tissue. Some cross-sectional and prospective seroepidemiological data suggested a modest association with HPV 16 and 18. In patients with recurrent papillomatosis, some well documented reports pointed to an involvement of HPV 6 and 11.

Cancer of the skin and conjunctiva

The prevalence of HPV DNA was high in squamous-cell skin carcinomas, especially among immunosuppressed patients. HPV DNA was, however, frequently detected at very low copy number in normal skin specimens from the general population and immunosuppressed patients. Case–control studies consistently showed an association between HPV types of genus beta, also known as epidermodysplasia verruciformis-associated HPV, and squamous-cell carcinoma of the skin. Some HPV types from the genus beta were considered to be carcinogenic in patients who have epidermodysplasia verruciformis.

HPV 16 DNA was highly prevalent in rare squamous-cell carcinomas of periungual skin, which provides evidence for a carcinogenic role of HPV in these tumours.

The presence of HPV in some conjunctival squamous-cell carcinomas and the results of a small case–control study from Uganda suggested an association between HPV and these carcinomas.

The Working Group recognized that assays and sampling methods that can detect epidermodysplasia verruciformis-related and cutaneous HPV types are still in an early phase of development; this prevented more detailed conclusions on the role of individual HPV types in non-melanoma skin cancers.

Cancer at other sites

High- and low-risk types of HPV were reported, mainly in a number of case reports, in cancers of other organs including the colon, ovary, breast, prostate, urinary bladder and nasal and sinonasal cavity. The significance of these findings is questionable, since other studies that analysed cancers of the same sites failed to confirm these data. Data on the relationship of HPV with lung cancer were also equivocal, although it was noted that, in the setting of recurrent papillomatosis, a small number of case series consistently reported detection of HPV 11 DNA. At present, there is a lack of evidence from case–control studies to support a possible involvement of HPV infections in tumours of these organs.

Co-factors

Tobacco smoking

Regardless of differences in the prevalence of current and past use of tobacco across the many studies and populations evaluated, tobacco smoking was consistently associated with risk for stage 3 cervical intraepithelial neoplasia and invasive squamous-cell carcinoma of the cervix. The risk estimates for this association were consistently in the range of 2.0 regardless of the study design (retrospective versus prospective), the restriction criteria employed (any HPV type-positive versus high-risk HPV-positive) and the covariates included in the model. Concordant with the vast amount of evidence that indicates that tobacco constituents are carcinogens and that smoking reduces immunological function, these data demonstrated that smoking is a co-factor with HPV in the development of invasive cervical cancer.

Hormonal contraceptives

The association between the use of oral contraceptives and squamous-cell cancer of the cervix was complicated by the different formulations used across geographical regions and changes in the formulations that have occurred within populations over time. In addition, it may not be possible to account adequately for the effect of Papanicolaou test smear screening and the consequent bias from the detection of lesions on the estimates of the association between the use of oral contraceptives and cervical cancer.

It was not possible to assess the strength of the association between the use of other hormonal contraceptive formulations such as injectable contraceptives and Norplant and the risk for cervical intraepithelial neoplasia and cancer. More research is needed to evaluate

adequately the risk associated with these commonly used formulations in developed and developing countries.

Parity

The data that support an association between parity (three or more children) and increased risk for cervical cancer were consistent.

Nutrients

Although numerous studies to assess the association between nutrient status and cervical disease have been carried out in the past few decades, very few restricted their analyses to HPV-positive women and most had small sample sizes. Therefore, the evidence in support of an association is too limited to draw conclusions for any one nutrient in the circulation or in the diet.

Infectious co-factors

Other than the human immunodeficiency virus (see below), *Chlamydia trachomatis* was the co-infection most consistently reported to have an epidemiological association with cervical neoplasia and invasive cervical cancer, after statistical control for the effects of HPV infection. The actual point(s) in the multistage process of tumorigenesis that might be affected by *C. trachomatis* remains uncertain. As residual confounding by HPV (due to shared sexual risk factors) is a potential source of bias, the possible association between *C. trachomatis* and HPV-associated cervical tumorigenesis must be viewed as an intriguing but unproven relationship. There was less consistent epidemiological evidence supporting a role of other sexually transmitted infections in cervical cancer, including herpes simplex virus 2. In contrast, cervical inflammation in general might play a role in HPV infection and cervical cancer, a possibility that could explain in part some of the conflicting findings reported by epidemiological studies conducted on an agent-specific basis and requires further investigation.

Human immunodeficiency virus (HIV)

A large number of studies consistently demonstrated an association between cervical HPV infection and HIV infection and between cervical intraepithelial neoplasia and HIV infection. A high proportion of HIV-positive women were infected with multiple HPV types and cervical HPV infection was more likely to persist in HIV-positive women than in HIV-negative women. Similarly, there was a strong association between anal HPV infection and HIV infection and between anal intraepithelial neoplasia and HIV infection in both men and women. The prevalence of anal HPV infection was very high in HIV-positive men who had sex with men and approached 100% in some studies. The prevalence of anal HPV infection exceeded that of cervical HPV infection in studies of HIV-positive women and HIV-negative women at high risk for HIV infection.

Studies of the effect of highly active antiretroviral therapy on the natural history of cervical HPV infection and cervical intraepithelial neoplasia used a wide variety of

measures of outcome and methodologies, which rendered definitive conclusions difficult. Some studies showed no effect of this therapy on the natural history of cervical intraepithelial neoplasia while others showed a modest reduction in its incidence and progression and an increase in its regression. This therapy appeared to have little or no effect on the natural history of anal HPV infection and anal intraepithelial neoplasia.

Data from cancer and acquired immune deficiency syndrome registry matches from developed countries consistently showed an increase in the incidence of cervical and anal cancer as well as that of vulvar, vaginal, penile and non-melanoma skin cancers in HIV-positive individuals compared with the general population. The degree to which the incidence of cervical cancer is increased among HIV-positive women in developing countries (i.e. Africa) was not as clear. No data were reported on the prevalence of different HPV types in invasive cancers in HIV-positive individuals. It is therefore not known whether this differs from those in HIV-negative individuals. Similar to the limited beneficial effect of highly active antiretroviral therapy on cervical and anal intraepithelial neoplasia, emerging data suggest that this therapy has not led to a reduction in the incidence of HPV-associated cervical and anogenital cancers.

5.3 Animal carcinogenicity data

Several animal papillomaviruses are carcinogenic in their natural hosts. Malignancies include cancer of the genital tract in monkeys, cancer of the upper gastrointestinal tract and urinary bladder in cattle, skin cancer in rabbits, dogs and rodents, cancer of the oral cavity in dogs, cancer of the vulva and perianal region in sheep and sarcoids in equids.

The rhesus monkey papillomavirus 1 is the most relevant to high-risk alpha-HPV: it infects the genital mucosa, is sexually transmitted and induces lesions that can progress to squamous-cell carcinomas.

Among the various bovine papillomaviruses (BPV), BPV-2 and BPV-4 are associated with cancer. Co-factors in pasture bracken fern that have been identified as chemical mutagens and immunosuppressants are prerequisite for the occurrence of cancer. BPV-2 has consistently been associated with cancer of the urinary bladder, and the E5 protein, which has strong transforming activity, is expressed in cancer cells.

BPV-4 causes papillomas of the upper gastrointestinal tract that can develop into carcinomas. Viral DNA is gradually lost during carcinogenesis and alterations in cellular proto-oncogenes (*ras* and *EGF-R*) and tumour-suppressor genes (*p53*) accumulate during progression.

Infection of equids as the heterologous host by BPV-1, and occasionally BPV-2, causes aggressive and persistent sarcoids. Viral DNA is present in the tumour and the E5 oncoprotein is consistently expressed. BPV infection of equids is the only documented case of natural cross-species infection by a papillomavirus.

Cross-species transmission also takes place between cottontail rabbits (*Sylvilagus floridanus*) infected with cottontail rabbit papillomavirus (CRPV) and domestic rabbits (*Oryctalagus cunniculus*), but only when the two species are housed together.

CRPV-induced papillomas progress to carcinomas at a much higher frequency in domestic rabbits than in cottontail rabbits, which implicates the genetic background of the host in the neoplastic process. Increased malignant progression of persistent warts is linked to alleles within the hypervariable region of class II DQ alpha genes.

The transforming proteins SE6, LE6 and E7 of CRPV are consistently expressed in all cancers. Experiments on overexpression in rodent cells also determined a transforming role for E8, which may represent an orthologue of the HPV E5 protein. Most genes, except for *E4* and *E5*, are necessary for the induction of papillomas in domestic rabbits. Single amino acid mutations in the context of the full genome within the trans-activation domain of E2, that maintain the replication function of E2, caused a dramatic loss in the efficiency of the induction of papillomas and carcinomas.

BPV-associated carcinogenesis has highlighted the interaction between the virus and environmental co-factors, and the functional analysis of BPV-transforming proteins has helped elucidate the proteins of HPVs. CRPV-associated carcinogenesis has features that may be relevant to HPV-associated non-melanoma skin cancer in humans.

In addition, animal papillomaviruses are highly valuable for the study of the interaction between the virus and the host immune system. BPV, CRPV and canine oral papillomavirus have been used extensively in vaccination experiments that first proved that prophylactic vaccination with capsid proteins or virus-like particles prevents infection. The minor capsid protein L2 of BPV and CRPV presents a virus neutralization epitope which is common to the L2 proteins of many papillomavirus types. This raises the possibility of developing polyvalent vaccines against HPV.

The observation in different animal papillomavirus models that therapeutic vaccination with viral early proteins can induce tumour regression is of major importance for the development of therapies against HPV-induced disease.

5.4 Other relevant data

Studies have been designed to determine how HPVs cause cancer. One hallmark of HPV-associated cancers is the frequent integration of the viral genome into random sites within the human genome, which leads to a clonal selection of cells in which HPV oncogenes E6 and E7 are up-regulated. HPVs play an active role in cervical cancer, because the expression of E6 and E7 is required for the continued growth and tumorigenicity of cervical cancer-derived cell lines. Another hallmark of cervical cancers is the accumulation of specific genetic alterations, most notably on chromosomes 1, 3 and 5, that are predicted to contribute to carcinogenesis. Their accumulation is probably the direct consequence of the expression of HPV oncoproteins that induce genomic instability through the dysregulation of cell-cycle regulatory machinery during G2/M and/or induction of centrasome abnormalities.

HPVs do not infect animals. Therefore, other experimental systems, including biochemical and tissue culture assays and transgenic animal-based models, have been used to determine the biological properties of HPVs that relate to their carcinogenic potential.

Relevant biochemical properties of the HPV-encoded oncoproteins E5, E6 and E7 include the inactivation of tumour suppressors, the modulation of cell-cycle regulatory, DNA-repair and apoptotic processes, the de-regulation of gene expression and the activation of signal transduction pathways. Properties bestowed on cells in tissue culture by HPVs that relate to their carcinogenicity include immortalization and genomic instability, as well as alterations in cell proliferation, differentiation, responses to DNA damage and apoptosis. In transgenic mice, HPV oncogenes, either individually or together, can induce cancers of the skin and cervix, two sites at which HPVs are implicated in human cancer. Good correlations have been found between the biochemical activities of the individual viral gene products that contribute to the formation of cancer in these animal model systems and those that contribute to their tumorigenic potential in tissue culture. Of particular importance are the interaction of E7 with the tumour-suppressor proteins pRb, p21 and p27, the interaction of E6 with p53, Bak and PDZ domain proteins, the ability of E6 to activate telomerase and the ability of E5 to stimulate the activity of the growth factor receptor.

Many of the experimental studies to date have focused on the analysis of high-risk HPVs, particularly HPV 16. There is a strong correlation between the tumorigenic activity of these high-risk HPVs in experimental systems and their carcinogenic activity in humans. Experimental studies carried out on other HPVs provide valuable insight into their carcinogenic potential. Of particular importance are studies of epidermodysplasia verruciformis-associated cutaneous HPVs, such as HPV 8 and HPV 38, that suggest that they play a role in human cancer.

The immunobiology of HPV infections, the role of the humoral and cellular components of the immune system in the control of these infections and HPV-associated cancers and the modulating effect of HPVs on the immune system were also assessed. Studies in these areas provide evidence that the immune system can respond to and may provide control over HPV infections and perhaps also HPV-associated cancer, and that HPVs in turn have the potential capacity to modulate the immune system at multiple levels.

A role of the human immune system in HPV infections is demonstrated by the fact that inflammation is commonly observed in regressing lesions, that immunosuppressed patients are at an increased risk for HPV infections and associated neoplasia and that HPV lesions respond positively to non-specific immune modulation.

With regard to specific cellular immune responses to HPVs, $CD4^+$ T-helper and $CD8^+$ cytotoxic T-lymphocyte immune responses against viral proteins can be induced in animal models. Moreover, in women infected with high-risk HPVs, cytotoxic T-lymphocyte responses to E7 can be detected in tumour infiltrates. Responses of these lymphocytes to HPV 16 E6 also are detected in women who have HPV 16-positive premalignant lesions. In contrast, a lack of cytotoxic T-lymphocyte responses to HPV 16 E6 was noted in women who had persistent HPV 16 infections. The specific role of cytotoxic T-lymphocytes in mediating the regression of HPV-associated lesions remains unclear.

With regard to immunomodulation by HPVs, tissue culture-based studies suggest that E5 and E7 oncoproteins of high-risk HPVs can modulate the cell-surface levels of major histocompatibility complex class I and class II molecules and inhibit the function of transporters associated with antigen presentation, respectively. In human cervical cancers, major histocompatibility complex class I is down-regulated whereas class II is up-regulated. In addition, the E6 proteins of both high- and low-risk HPVs and the E7 protein of high-risk HPVs can modulate the activity of several factors that regulate interferon-responsive pathways, which mediate the innate immune response and modulate antigen-specific responses. Furthermore, the risk for cervical cancer could be affected by genetic polymorphisms in the major histocompatibility complex class I and II genes.

5.5 Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of HPV 16 in the cervix, vulva (basaloid and warty tumours), vagina, penis (basaloid and warty tumours), anus, oral cavity and oropharynx.

There is *sufficient evidence* in humans for the carcinogenicity of HPV 18 in the cervix.

There is *sufficient evidence* in humans for the carcinogenicity of HPV types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 in the cervix.

There is *limited evidence* in humans for the carcinogenicity of HPV 16 in the larynx and periungual skin (squamous-cell carcinoma).

There is *limited evidence* in humans for the carcinogenicity of HPV 18 in the vulva (basaloid and warty tumours), vagina, penis (basaloid and warty tumours), anus, oral cavity and larynx.

There is *limited evidence* in humans for the carcinogenicity of HPV 6 and HPV 11 in the larynx (squamous-cell carcinoma) and in the vulva, penis and anus (verrucous carcinomas of the latter three sites).

There is *limited evidence* in humans for the carcinogenicity of HPV genus-beta types in the skin (squamous-cell carcinoma). In the rare case of patients with epidermodysplasia verruciformis, there is compelling evidence for the carcinogenicity of HPV genus-beta types 5 and 8 in the skin (squamous-cell carcinoma).

There is *limited evidence* in humans for the carcinogenicity of HPV in the conjunctiva (squamous-cell carcinoma).

There is *inadequate evidence* in humans for the carcinogenicity of HPV in the oesophagus, lung, colon, ovary, breast, prostate, urinary bladder and nasal and sinonasal cavities.

Overall evaluation

HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 are *carcinogenic to humans (Group 1)*.

HPV 6 and HPV 11 are *possibly carcinogenic to humans (Group 2B)*.

Some types of HPV genus beta are *possibly carcinogenic to humans (Group 2B)*, with the notable exception that HPV 5 and HPV 8 are carcinogenic to patients with epidermodysplasia verruciformis.

Evaluations in the *IARC Monographs* provide a qualitative assessment of carcinogenicity. The HPV types that have been classified as *carcinogenic to humans* can differ by an order of magnitude in risk for cervical cancer. The Working Group cautions that the design of HPV screening tests must also consider other factors that are discussed in the General Remarks.

6. References

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LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
Ad	Adenovirus
ADC	Adenocarcinoma
AIDS	Acquired immune deficiency syndrome
AIN	Anal intraepithelial neoplasia
ALIVE	AIDS Link to Intravenous Drug Experience
ALTS	ASCUS/LSIL Triage Study
AMF-1/Gps2	Autocrine motility factor 1
AP-1	Activator protein 1
ASCUS	Atypical squamous cells of undetermined significance
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
bp	Base pair
BPV	Bovine papillomavirus
BS	Binding site
CC	Carcinoma cuniculatum
CgPV	*Colobus guereza* papillomavirus
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
CIS	Carcinoma *in situ*
CMV	Cytomegalovirus
COPV	Canine oral papillomavirus
CRPV	Cottontail rabbit papillomavirus
CTL	Cytotoxic T lymphocytes
DBD	DNA-binding domain
DLG	*Drosophila* disc–large tumour-suppressor gene product
DMBA	7,12-Dimethylbenz[*a*]anthracene
DPV	Deer papillomavirus
DVI	Direct visual inspection
E6-AP	E6-associated protein
E6BP1	E6 binding protein 1
E6TP1	E6 target protein 1
EBV	Epstein-Barr virus
EC	Epithelioma cuniculatum

EEPV	European elk papillomavirus
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EqPV	*Equus caballus* (horse) papillomavirus
EV	Epidermodysplasia verruciformis
FcPV	*Frignilla coelebs* (chaffinch) papillomavirus
FdPV	*Felix domesticus* (cat) papillomavirus
FHIT	Fragile histidine tetrads
FIV	Feline immunodeficiency virus
GAG	Glycosaminoglycan
GFP	Green fluorescent protein
GST	Glutathione *S*-transferase
HAART	Highly active antiretroviral therapy
HaOPV	Hamster oral papillomavirus
HERS	HIV Epidemiology Research Study
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HRA	High-resolution anoscopy
HSIL	High-grade squamous intraepithelial lesion
HSV	Herpes simplex virus
hTERT	Human telomerase reverse transcriptase
ICTV	International Committee on the Taxonomy of Viruses
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory factor
ISH	In-situ hybridization
LCR	Long control region
LEEP	Loop electrosurgical excision procedure
LIPA	Reverse line probe assay hybridization
LLETZ	Large loop electrosurgical excision of the transformation zone
LOH	Loss of heterozygosity
LSIL	Low-grade squamous intraepithelial lesion
MAGI	Membrane-associated guanylate kinase inverted protein
MHC	Major histocompatibility complex
MmPV	*Micromys minutus* papillomavirus
MnPV	*Mastomys natalensis* papillomavirus
mRNA	Messenger RNA
MTHFR	Methylene tetrahydrofolate reductase
MUPP1	Multiple PDZ protein 1

NASBA	Nucleic acid sequence-based amplification
Nd:YAG	Neodymium:yttrium–aluminium garnet
ND10	Nuclear domain 10
NES	Nuclear export sequence
NF-κB	Nuclear factor-κB
NK	Natural killer
NLS	Nuclear localization signal
NURD	Nuclease remodelling and deacetylase
OvPV	Ovine papillomavirus
ORF	Open-reading frame
PI3K	Phosphatidylinositol-3′-kinase
Pap test	Papanicolaou test
PARP	Poly(ADP-ribose) polymerase
PCNA	Proliferating-cell nuclear antigen
PCPV	Pygmy chimpanzee papillomavirus
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDZ	PSD-95/Disc-large/ZO1 protein
PePV	*Psittacus erithacus timneh* (parrot) papillomavirus
PIN	Penile intraepithelial neoplasia
PML	Promyelocytic leukaemia protein
pRb	Retinoblastoma tumour-suppressor protein
PsPV	*Phocoena spinipinnis* papillomavirus
Rb	Retinoblastoma
RDPV	Red deer papillomavirus
REACH	Reaching for Excellence in Adolescent Care and Health
RFLP	Restriction fragment length polymorphism
RhPV	Rhesus monkey papillomavirus
RLB	Reverse line blotting
RLU	Relative light unit
ROPV	Domestic rabbit oral papillomavirus
RPA	Replication protein A
RPV	Reindeer papillomavirus
RT-PCR	Reverse transcriptase polymerase chain reaction
SCC	Squamous-cell carcinoma
SIL	Squamous intraepithelial lesion
SIR	Standardized incidence ratio
siRNA	Short-interfering RNA
SMR	Standardized mortality ratio
SPP	Suprapubic resection of the prostate
STD	Sexually transmitted disease
TAP	Transporter associated with antigen processing

TBP	TATA box-binding protein
TGF	Transforming growth factor
Th	T-helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
α-Toc	α-Tocopherol
TopBP1	Topoisomerase II beta-binding protein 1
TPA	12-*O*-Tetradecanoylphorbol 13-acetate
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TURP	Transurethral resection of the prostate
UV	Ultraviolet
VAIN	Vaginal intraepithelial neoplasia
v-ATPase	Vacuolar H^+ adenosine triphosphatase
VC	Verrucous carcinoma
VIA	Visual inspection with acetic acid
VILI	Visual inspection with Lugol's iodine
VIN	Vulvar intraepithelial neoplasia
VLP	Virus-like particle
VSV	Vesicular stomatis virus
WIHS	Women's Interagency HIV Study
WITS	Women and Infant Transmission Study
YB1	Y box-binding transcription factor
YY1	Ying Yang 1 transcription factor

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