

**MALARIA AND SOME
POLYOMAVIRUSES (SV40, BK, JC, AND
MERKEL CELL VIRUSES)**

VOLUME 104

IARC MONOGRAPHS
ON THE EVALUATION
OF CARCINOGENIC RISKS
TO HUMANS

**MALARIA AND SOME
POLYOMAVIRUSES (SV40, BK, JC, AND
MERKEL CELL VIRUSES)**

VOLUME 104

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, 7-14 February 2012

LYON, FRANCE - 2014

**IARC MONOGRAPHS
ON THE EVALUATION
OF CARCINOGENIC RISKS
TO HUMANS**

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, lifestyle 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 since 1982 by Cooperative Agreement U01 CA33193 with the United States National Cancer Institute, Department of Health and Human Services. Additional support has been provided since 1986 by the Health, Safety and Hygiene at Work Unit of the European Commission Directorate-General for Employment, Social Affairs and Equal Opportunities, and since 1992 by the United States National Institute of Environmental Health Sciences, Department of Health and Human Services. The contents of this volume are solely the responsibility of the Working Group and do not necessarily represent the official views of the National Cancer Institute, the National Institute of Environmental Health Sciences, the United States Department of Health and Human Services, or the European Commission Directorate-General for Employment, Social Affairs and Equal Opportunities.

This volume was made possible, in part, through Cooperative Agreement CR 834012 with the United States Environmental Protection Agency, Office of Research and Development. The contents of this volume do not necessarily reflect the views or policies of the Environmental Protection Agency.

Published by the International Agency for Research on Cancer,
150 cours Albert Thomas, 69372 Lyon Cedex 08, France
©International Agency for Research on Cancer, 2014
(published online, 2013)

Distributed by WHO Press, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland
(tel.: +41 22 791 3264; fax: +41 22 791 4857; e-mail: bookorders@who.int).

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The IARC Monographs Working Group alone is responsible for the views expressed in this publication.

IARC Library Cataloguing in Publication Data

Malaria and some Polyomaviruses (SV40, BK, JC, and Merkel cell viruses) / IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (2012: Lyon, France)

(IARC monographs on the evaluation of carcinogenic risks to humans ; v. 104)

1. Malaria – complications 2. Malaria – parasitology 3. Carcinogens 4. Polyomavirus – pathogenicity
5. Simian virus 40 6. BK Virus 7. JC Virus 8. Merkel cell Polyomavirus

I. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans II. Series

ISBN 978 92 832 0142 7
ISSN 1017-1606

(NLM Classification: W1)

PRINTED IN FRANCE

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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. 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. Similarly, identification of cancer sites with *sufficient evidence* or *limited evidence* in humans should not be viewed as precluding the possibility that an agent may cause cancer at other sites.

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 Section of IARC Monographs, 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 Section of IARC Monographs, so that corrections can be reported in future volumes.

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⁵ Yuan Chang's laboratory identified Merkel cell polyomavirus in 2008 and the University of Pittsburgh has USA and European patent coverage for methods to diagnose and immunize against the virus causing human Merkel cell carcinoma.

⁶ Mauro Tognon is a co-inventor of one pending patent application for an indirect ELISA assay for the detection of SV40-specific large T antigen antibodies.

⁷ Each Observer agreed to respect the Guidelines for Observers at *IARC Monographs* meetings. Observers did not serve as meeting chair or subgroup chair, draft any part of a Monograph, or participate in the evaluations. They also agreed not to contact participants before the meeting, not to lobby them at any time, not to send them written materials, and not to offer them meals or other favours. IARC asked and reminded Working Group Members to report any contact or attempt to influence that they may have encountered, either before or during the meeting.

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PREAMBLE

The Preamble to the *IARC Monographs* describes the objective and scope of the programme, the scientific principles and procedures used in developing a *Monograph*, the types of evidence considered and the scientific criteria that guide the evaluations. The Preamble should be consulted when reading a *Monograph* or list of evaluations.

A. GENERAL PRINCIPLES AND PROCEDURES

1. Background

Soon after IARC was established in 1965, it received frequent requests for advice on the carcinogenic risk of chemicals, including requests for lists of known and suspected human carcinogens. It was clear that it would not be a simple task to summarize adequately the complexity of the information that was available, and IARC began to consider means of obtaining international expert opinion on this topic. In 1970, the IARC Advisory Committee on Environmental Carcinogenesis recommended ‘... that a compendium on carcinogenic chemicals be prepared by experts. The biological activity and evaluation of practical importance to public health should be referenced and documented.’ The IARC Governing Council adopted a resolution concerning the role of IARC in providing government authorities with expert, independent, scientific opinion on environmental carcinogenesis. As one means to that end, the Governing Council recommended that IARC

should prepare monographs on the evaluation of carcinogenic risk of chemicals to man, which became the initial title of the series.

In the succeeding years, the scope of the programme broadened as *Monographs* were developed for groups of related chemicals, complex mixtures, occupational exposures, physical and biological agents and lifestyle factors. In 1988, the phrase ‘of chemicals’ was dropped from the title, which assumed its present form, *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*.

Through the *Monographs* programme, IARC seeks to identify the causes of human cancer. This is the first step in cancer prevention, which is needed as much today as when IARC was established. The global burden of cancer is high and continues to increase: the annual number of new cases was estimated at 10.1 million in 2000 and is expected to reach 15 million by 2020 ([Stewart & Kleihues, 2003](#)). With current trends in demographics and exposure, the cancer burden has been shifting from high-resource countries to low- and medium-resource countries. As a result of *Monographs* evaluations, national health agencies have been able, on scientific grounds, to take measures to reduce human exposure to carcinogens in the workplace and in the environment.

The criteria established in 1971 to evaluate carcinogenic risks to humans were adopted by the Working Groups whose deliberations resulted in the first 16 volumes of the *Monographs* series. Those criteria were subsequently updated by further ad hoc Advisory Groups ([IARC, 1977, 1978, 1979, 1982, 1983, 1987, 1988, 1991](#); [Vainio et al., 1992](#); [IARC, 2005, 2006](#)).

The Preamble is primarily a statement of scientific principles, rather than a specification of working procedures. The procedures through which a Working Group implements these principles are not specified in detail. They usually involve operations that have been established as being effective during previous *Monograph* meetings but remain, predominantly, the prerogative of each individual Working Group.

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* represent the first step in carcinogen risk assessment, which involves examination of all relevant information to assess the strength of the available evidence that an agent could alter the age-specific incidence of cancer in humans. The *Monographs* may also indicate where additional research efforts are needed, specifically when data immediately relevant to an evaluation are not available.

In this Preamble, the term ‘agent’ refers to any entity or circumstance that is subject to evaluation in a *Monograph*. As the scope of the programme has broadened, categories of agents now include specific chemicals, groups of related chemicals, complex mixtures, occupational or environmental exposures, cultural or behavioural practices, biological organisms and physical agents. This list of categories may expand

as causation of, and susceptibility to, malignant disease become more fully understood.

A cancer ‘hazard’ is an agent that is capable of causing cancer under some circumstances, while a cancer ‘risk’ is an estimate of the carcinogenic effects expected from exposure to a cancer hazard. The *Monographs* are an exercise in evaluating cancer hazards, despite the historical presence of the word ‘risks’ in the title. The distinction between hazard and risk is important, and the *Monographs* identify cancer hazards even when risks are very low at current exposure levels, because new uses or unforeseen exposures could engender risks that are significantly higher.

In the *Monographs*, an agent is termed ‘carcinogenic’ if it is capable of increasing the incidence of malignant neoplasms, reducing their latency, or increasing their severity or multiplicity. The induction of benign neoplasms may in some circumstances (see Part B, Section 3a) contribute to the judgement that the agent is carcinogenic. The terms ‘neoplasm’ and ‘tumour’ are used interchangeably.

The Preamble continues the previous usage of the phrase ‘strength of evidence’ as a matter of historical continuity, although it should be understood that *Monographs* evaluations consider studies that support a finding of a cancer hazard as well as studies that do not.

Some epidemiological and experimental studies indicate that different agents may act at different stages in the carcinogenic process, and several different 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, 1991](#); [Vainio et al., 1992](#); [IARC, 2005, 2006](#); see also Part B, Sections 4 and 6). As mechanisms of carcinogenesis are elucidated, IARC convenes international scientific conferences to determine whether a broad-based consensus has emerged

on how specific mechanistic data can be used in an evaluation of human carcinogenicity. The results of such conferences are reported in IARC Scientific Publications, which, as long as they still reflect the current state of scientific knowledge, may guide subsequent Working Groups.

Although the *Monographs* have emphasized hazard identification, important issues may also involve dose–response assessment. In many cases, the same epidemiological and experimental studies used to evaluate a cancer hazard can also be used to estimate a dose–response relationship. A *Monograph* may undertake to estimate dose–response relationships within the range of the available epidemiological data, or it may compare the dose–response information from experimental and epidemiological studies. In some cases, a subsequent publication may be prepared by a separate Working Group with expertise in quantitative dose–response assessment.

The *Monographs* are used by national and international authorities to make risk assessments, formulate decisions concerning preventive measures, provide effective cancer control programmes and decide among alternative options for public health decisions. The evaluations of IARC Working Groups are scientific, qualitative judgements on the evidence for or against carcinogenicity provided by the available data. These evaluations represent only one part of the body of information on which public health decisions may be based. Public health options vary from one situation to another and from country to country and relate to many factors, including different socioeconomic and national priorities. Therefore, no recommendation is given with regard to regulation or legislation, which are the responsibility of individual governments or other international organizations.

3. Selection of agents for review

Agents are selected for review on the basis of two main criteria: (a) there is evidence of human exposure and (b) there is some evidence or suspicion of carcinogenicity. Mixed exposures may occur in occupational and environmental settings and as a result of individual and cultural habits (such as tobacco 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. Ad hoc Advisory Groups convened by IARC in 1984, 1989, 1991, 1993, 1998 and 2003 made recommendations as to which agents should be evaluated in the *Monographs* series. Recent recommendations are available on the *Monographs* programme web site (<http://monographs.iarc.fr>). IARC may schedule other agents for review as it becomes aware of new scientific information or as national health agencies identify an urgent public health need related to cancer.

As significant new data become available on an agent for which a *Monograph* exists, a re-evaluation may be made at a subsequent meeting, and a new *Monograph* published. In some cases it may be appropriate to review only the data published since a prior evaluation. This can be useful for updating a database, reviewing new data to resolve a previously open question or identifying new tumour sites associated with a carcinogenic agent. Major changes in an evaluation (e.g. a new classification in Group 1 or a determination that a mechanism does not operate in humans, see Part B, Section 6) are more appropriately addressed by a full review.

4. Data for the *Monographs*

Each *Monograph* reviews all pertinent epidemiological studies and cancer bioassays in experimental animals. Those judged inadequate or irrelevant to the evaluation may be cited but not summarized. If a group of similar studies is not reviewed, the reasons are indicated.

Mechanistic and other relevant data are also reviewed. A *Monograph* does not necessarily cite all the mechanistic literature concerning the agent being evaluated (see Part B, Section 4). Only those data considered by the Working Group to be relevant to making the evaluation are included.

With regard to epidemiological studies, cancer bioassays, and mechanistic and other relevant data, only reports that have been published or accepted for publication in the openly available scientific literature are reviewed. The same publication requirement applies to studies originating from IARC, including meta-analyses or pooled analyses commissioned by IARC in advance of a meeting (see Part B, Section 2c). Data from government agency reports that are publicly available are also considered. Exceptionally, doctoral theses and other material that are in their final form and publicly available may be reviewed.

Exposure data and other information on an agent under consideration are also reviewed. In the sections on chemical and physical properties, on analysis, on production and use and on occurrence, published and unpublished sources of information may be considered.

Inclusion of a study 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 each study description (see Part B). The reasons for not giving further consideration to an individual study also are indicated in the square brackets.

5. Meeting participants

Five categories of participant can be present at *Monograph* meetings.

(a) *The Working Group*

The Working Group is responsible for the critical reviews and evaluations that are developed during the meeting. The tasks of Working Group Members 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 mechanisms of carcinogenesis; and (vi) to make an overall evaluation of the carcinogenicity of the exposure to humans. Working Group Members generally have published significant research related to the carcinogenicity of the agents being reviewed, and IARC uses literature searches to identify most experts. Working Group Members are selected on the basis of (a) knowledge and experience and (b) absence of real or apparent conflicts of interests. Consideration is also given to demographic diversity and balance of scientific findings and views.

(b) *Invited Specialists*

Invited Specialists are experts who also have critical knowledge and experience but have a real or apparent conflict of interests. These experts are invited when necessary to assist in the Working Group by contributing their unique knowledge and experience during subgroup and plenary discussions. They may also contribute text on non-influential issues in the section on exposure, such as a general description of data on production and use (see Part B, Section 1). Invited Specialists do not serve as meeting chair

or subgroup chair, draft text that pertains to the description or interpretation of cancer data, or participate in the evaluations.

(c) *Representatives of national and international health agencies*

Representatives of national and international health agencies often attend meetings because their agencies sponsor the programme or are interested in the subject of a meeting. Representatives do not serve as meeting chair or subgroup chair, draft any part of a *Monograph*, or participate in the evaluations.

(d) *Observers with relevant scientific credentials*

Observers with relevant scientific credentials may be admitted to a meeting by IARC in limited numbers. Attention will be given to achieving a balance of Observers from constituencies with differing perspectives. They are invited to observe the meeting and should not attempt to influence it. Observers do not serve as meeting chair or subgroup chair, draft any part of a *Monograph*, or participate in the evaluations. At the meeting, the meeting chair and subgroup chairs may grant Observers an opportunity to speak, generally after they have observed a discussion. Observers agree to respect the Guidelines for Observers at IARC *Monographs* meetings (available at <http://monographs.iarc.fr>).

(e) *The IARC Secretariat*

The IARC Secretariat consists of scientists who are designated by IARC and who have relevant expertise. They serve as rapporteurs and participate in all discussions. When requested by the meeting chair or subgroup chair, they may also draft text or prepare tables and analyses.

Before an invitation is extended, each potential participant, including the IARC Secretariat, completes the WHO Declaration of Interests to report financial interests, employment and

consulting, and individual and institutional research support related to the subject of the meeting. IARC assesses these interests to determine whether there is a conflict that warrants some limitation on participation. The declarations are updated and reviewed again at the opening of the meeting. Interests related to the subject of the meeting are disclosed to the meeting participants and in the published volume ([Cogliano et al., 2004](#)).

The names and principal affiliations of participants are available on the *Monographs* programme web site (<http://monographs.iarc.fr>) approximately two months before each meeting. It is not acceptable for Observers or third parties to contact other participants before a meeting or to lobby them at any time. Meeting participants are asked to report all such contacts to IARC ([Cogliano et al., 2005](#)).

All participants are listed, with their principal affiliations, at the beginning of each volume. 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.

6. Working procedures

A separate Working Group is responsible for developing each volume of *Monographs*. A volume contains one or more *Monographs*, which can cover either a single agent or several related agents. Approximately one year in advance of the meeting of a Working Group, the agents to be reviewed are announced on the *Monographs* programme web site (<http://monographs.iarc.fr>) and participants are selected by IARC staff in consultation with other experts. Subsequently, relevant biological and epidemiological data are collected by IARC from recognized sources of information on carcinogenesis, including data storage and retrieval systems such as PubMed. Meeting participants who are asked to prepare preliminary working papers for specific sections

are expected to supplement the IARC literature searches with their own searches.

Industrial associations, labour unions and other knowledgeable organizations may be asked to provide input to the sections on production and use, although this involvement is not required as a general rule. Information on production and trade is obtained from governmental, trade and market research publications and, in some cases, by direct contact with industries. Separate production data on some agents may not be available for a variety of reasons (e.g. not collected or made public in all producing countries, production is small). 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 to prepare preliminary working papers. The working papers are compiled by IARC staff and sent, before the meeting, to Working Group Members and Invited Specialists for review.

The Working Group meets at IARC for seven to eight days to discuss and finalize the texts and to formulate the evaluations. The objectives of the meeting are peer review and consensus. During the first few days, four subgroups (covering exposure data, cancer in humans, cancer in experimental animals, and mechanistic and other relevant data) review the working papers, develop a joint subgroup draft and write summaries. Care is taken to ensure that each study summary is written or reviewed by someone not associated with the study being considered. During the last few days, the Working Group meets in plenary session to review the subgroup drafts and develop the evaluations. As a result, the entire volume is the joint product of the Working Group, and there are no individually authored sections.

IARC Working Groups strive to achieve a consensus evaluation. Consensus reflects broad

agreement among Working Group Members, but not necessarily unanimity. The chair may elect to poll Working Group Members to determine the diversity of scientific opinion on issues where consensus is not readily apparent.

After the meeting, the master copy is verified by consulting the original literature, edited and prepared for publication. The aim is to publish the volume within six months of the Working Group meeting. A summary of the outcome is available on the *Monographs* programme web site soon after the meeting.

B. SCIENTIFIC REVIEW AND EVALUATION

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 that directly impinges on its interpretation should be brought to the attention of the reader, a Working Group comment is given in square brackets.

The scope of the *IARC Monographs* programme has expanded beyond chemicals to include complex mixtures, occupational exposures, physical and biological agents, lifestyle factors and other potentially carcinogenic exposures. Over time, the structure of a *Monograph* has evolved to include the following sections:

1. Exposure data
2. Studies of cancer in humans
3. Studies of cancer in experimental animals
4. Mechanistic and other relevant data
5. Summary

- Evaluation and rationale

In addition, a section of General Remarks at the front of the volume discusses the reasons the agents were scheduled for evaluation and some key issues the Working Group encountered during the meeting.

This part of the Preamble discusses the types of evidence considered and summarized in each section of a *Monograph*, followed by the scientific criteria that guide the evaluations.

1. Exposure data

Each *Monograph* includes general information on the agent: this information may vary substantially between agents and must be adapted accordingly. Also included is information on production and use (when appropriate), methods of analysis and detection, occurrence, and sources and routes of human occupational and environmental exposures. Depending on the agent, regulations and guidelines for use may be presented.

(a) General information on the agent

For chemical agents, sections on chemical and physical data are included: the Chemical Abstracts Service Registry Number, the latest primary name and the IUPAC systematic name are recorded; other synonyms are given, but the list is not necessarily comprehensive. Information on chemical and physical properties that are relevant to identification, occurrence and biological activity is included. 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.

For biological agents, taxonomy, structure and biology are described, and the degree of variability is indicated. Mode of replication,

life cycle, target cells, persistence, latency, host response and clinical disease other than cancer are also presented.

For physical agents that are forms of radiation, energy and range of the radiation are included. For foreign bodies, fibres and respirable particles, size range and relative dimensions are indicated.

For agents such as mixtures, drugs or lifestyle factors, a description of the agent, including its composition, is given.

Whenever appropriate, other information, such as historical perspectives or the description of an industry or habit, may be included.

(b) Analysis and detection

An overview of methods of analysis and detection of the agent is presented, including their sensitivity, specificity and reproducibility. Methods widely used for regulatory purposes are emphasized. Methods for monitoring human exposure are also given. No critical evaluation or recommendation of any method is meant or implied.

(c) Production and use

The dates of first synthesis and of first commercial production of a chemical, mixture or other agent are provided when available; for agents that 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 when available. 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.

The countries where companies report production of the agent, and the number of companies in each country, are identified. Available data on production, international trade and uses are

obtained for representative regions. 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.

(d) *Occurrence and exposure*

Information on the occurrence of an agent in the environment is obtained from data derived from the monitoring and surveillance of levels in occupational environments, air, water, soil, plants, foods and animal and human tissues. When available, data on the generation, persistence and bioaccumulation of the agent are also included. Such data may be available from national databases.

Data 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 reported. Information is presented on the range of human exposure, including occupational and environmental exposures. This includes relevant findings from both developed and developing countries. Some of these data are not distributed widely and may be available from government reports and other sources. In the case of mixtures, industries, occupations or processes, information is given about all agents known to be 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 date and place. For biological agents, the epidemiology of infection is described.

(e) *Regulations and guidelines*

Statements concerning regulations and guidelines (e.g. occupational exposure limits, maximal levels permitted in foods and water, pesticide registrations) are included, 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 vaccination and therapy, are described.

2. Studies of cancer in humans

This section includes all pertinent epidemiological studies (see Part A, Section 4). Studies of biomarkers are included when they are relevant to an evaluation of carcinogenicity to humans.

(a) *Types of study considered*

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

Cohort and case-control studies relate individual exposures under study to the occurrence of cancer in individuals and provide an estimate of effect (such as relative risk) as the main measure of association. Intervention studies may provide strong evidence for making causal inferences, as exemplified by cessation of smoking and the subsequent decrease in risk for lung cancer.

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

under study. In correlation studies, individual exposure is not documented, which renders this kind of study more prone to confounding. In some circumstances, however, correlation studies may be more informative than analytical study designs (see, for example, the *Monograph on arsenic in drinking-water*; [IARC, 2004](#)).

In some instances, case reports and case series have provided important information about the carcinogenicity of an agent. These types of study 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 and case series 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 that surround the interpretation of case reports, case series 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, these types of study may add materially to the judgement that a causal relationship exists.

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

(b) *Quality of studies considered*

It is necessary to take into account the possible roles of bias, confounding and chance in the interpretation of epidemiological studies. Bias is the effect of factors in study design or execution that lead erroneously to a stronger or weaker association than in fact exists between an agent and disease. Confounding is a form of bias

that occurs when 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. The role of chance is related to biological variability and the influence of sample size on the precision of estimates of effect.

In evaluating the extent to which these factors have been minimized in an individual study, consideration is given to several aspects of design and analysis as described in the report of the study. For example, when suspicion of carcinogenicity arises largely from a single small study, careful consideration is given when interpreting subsequent studies that included these data in an enlarged population. 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.

First, 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.

Second, the authors should have taken into account — in the study design and analysis — 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 or may not be more appropriate than those with national rates. Internal comparisons of frequency of disease among individuals at different levels of exposure are also desirable in cohort studies, since they minimize the potential

for confounding related to the difference in risk factors between an external reference group and the study population.

Third, 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. These methods have been reviewed for case–control studies ([Breslow & Day, 1980](#)) and for cohort studies ([Breslow & Day, 1987](#)).

(c) *Meta-analyses and pooled analyses*

Independent epidemiological studies of the same agent may lead to results that are difficult to interpret. Combined analyses of data from multiple studies are a means of resolving this ambiguity, and well conducted analyses can be considered. There are two types of combined analysis. The first involves combining summary statistics such as relative risks from individual studies (meta-analysis) and the second involves a pooled analysis of the raw data from the individual studies (pooled analysis) ([Greenland, 1998](#)).

The advantages of combined analyses are increased precision due to increased sample size and the opportunity to explore potential

confounders, interactions and modifying effects that may explain heterogeneity among studies in more detail. A disadvantage of combined analyses is the possible lack of compatibility of data from various studies due to differences in subject recruitment, procedures of data collection, methods of measurement and effects of unmeasured co-variables that may differ among studies. Despite these limitations, well conducted combined analyses may provide a firmer basis than individual studies for drawing conclusions about the potential carcinogenicity of agents.

IARC may commission a meta-analysis or pooled analysis that is pertinent to a particular *Monograph* (see Part A, Section 4). Additionally, as a means of gaining insight from the results of multiple individual studies, ad hoc calculations that combine data from different studies may be conducted by the Working Group during the course of a *Monograph* meeting. The results of such original calculations, which would be specified in the text by presentation in square brackets, might involve updates of previously conducted analyses that incorporate the results of more recent studies or de-novo analyses. Irrespective of the source of data for the meta-analyses and pooled analyses, it is important that the same criteria for data quality be applied as those that would be applied to individual studies and to ensure also that sources of heterogeneity between studies be taken into account.

(d) *Temporal effects*

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, peak exposure (when appropriate) and time since cessation of exposure, are reviewed and summarized when available. Analyses of temporal relationships may be useful in making causal inferences. In addition, 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 mechanisms of carcinogenesis.

(e) *Use of biomarkers in epidemiological studies*

Biomarkers indicate molecular, cellular or other biological changes and are increasingly used in epidemiological studies for various purposes ([IARC, 1991](#); [Vainio et al., 1992](#); [Toniolo et al., 1997](#); [Vineis et al., 1999](#); [Buffler et al., 2004](#)). These may include evidence of exposure, of early effects, of cellular, tissue or organism responses, of individual susceptibility or host responses, and inference of a mechanism (see Part B, Section 4b). This is a rapidly evolving field that encompasses developments in genomics, epigenomics and other emerging technologies.

Molecular epidemiological data that identify associations between genetic polymorphisms and interindividual differences in susceptibility to the agent(s) being evaluated may contribute to the identification of carcinogenic hazards to humans. If the polymorphism has been demonstrated experimentally to modify the functional activity of the gene product in a manner that is consistent with increased susceptibility, these data may be useful in making causal inferences. Similarly, molecular epidemiological studies that measure cell functions, enzymes or metabolites that are thought to be the basis of susceptibility may provide evidence that reinforces biological plausibility. It should be noted, however, that when data on genetic susceptibility originate from multiple comparisons that arise from subgroup analyses, this can generate false-positive results and inconsistencies across studies, and such data therefore require careful evaluation. If the known phenotype of a genetic polymorphism can explain the carcinogenic mechanism of the agent being evaluated, data on this phenotype may be useful in making causal inferences.

(f) *Criteria for causality*

After the quality of individual epidemiological studies of cancer has been summarized and assessed, a judgement is made concerning the strength of evidence that the agent in question is carcinogenic to humans. In making its judgement, the Working Group considers several criteria for causality ([Hill, 1965](#)). A strong association (e.g. a large relative risk) is more likely to indicate causality than a weak association, although it is recognized that estimates of effect of small magnitude do not imply lack of causality and may be important if the disease or exposure is common. Associations that are replicated in several studies of the same design or that use 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 exposure), and results of studies that are judged to be of high quality are given more weight than those of studies that are judged to be methodologically less sound.

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

Several scenarios may increase confidence in a causal relationship. On the one hand, an agent may be specific in causing tumours at one site or of one morphological type. On the other, carcinogenicity may be evident through the causation of multiple tumour types. Temporality, precision of estimates of effect, biological plausibility and coherence of the overall database are considered. Data on biomarkers may be employed in an

assessment of the biological plausibility of epidemiological observations.

Although rarely available, results from randomized trials that show different rates of cancer 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, a judgement may be made that, in the aggregate, they show evidence of lack of carcinogenicity. Such a judgement requires first that the studies 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 (a) be consistent with an estimate of effect of unity for any observed level of exposure, (b) when considered together, provide a pooled estimate of relative risk that is at or near to unity, and (c) have 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 that the relative risk of cancer increases with increasing level of exposure. It is important to note that evidence of lack of carcinogenicity obtained from several epidemiological studies can apply only to the type(s) of cancer studied, to the dose levels reported, and to the intervals between first exposure and disease onset observed in these studies. Experience with human cancer indicates that the period from first exposure to the development of clinical cancer is sometimes longer than 20 years; latent periods substantially shorter than 30 years cannot provide evidence for lack of carcinogenicity.

3. Studies of cancer in experimental animals

All known human carcinogens that have been studied adequately for carcinogenicity in experimental animals have produced positive results in one or more animal species ([Wilbourn *et al.*, 1986](#); [Tomatis *et al.*, 1989](#)). For several agents (e.g. aflatoxins, diethylstilbestrol, solar radiation, 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 that cause cancer in experimental animals also cause cancer in humans, it is biologically plausible that agents for which there is *sufficient evidence of carcinogenicity* in experimental animals (see Part B, Section 6b) also present a carcinogenic hazard to humans. Accordingly, in the absence of additional scientific information, these agents are considered to pose a carcinogenic hazard to humans. Examples of additional scientific information are data that demonstrate that a given agent causes cancer in animals through a species-specific mechanism that does not operate in humans or data that demonstrate that the mechanism in experimental animals also operates in humans (see Part B, Section 6).

Consideration is given to all available long-term studies of cancer in experimental animals with the agent under review (see Part A, Section 4). In all experimental settings, the nature and extent of impurities or contaminants present in the agent being evaluated are given when available. Animal species, strain (including genetic background where applicable), sex, numbers per group, age at start of treatment, route of exposure, dose levels, duration of exposure, survival and information on tumours (incidence, latency, severity or multiplicity of neoplasms or preneoplastic lesions) are reported. Those studies in experimental animals that are judged to be irrelevant to the evaluation or judged to be inadequate

(e.g. too short a duration, too few animals, poor survival; see below) may be omitted. Guidelines for conducting long-term carcinogenicity experiments have been published (e.g. [OECD, 2002](#)).

Other studies considered may include: experiments in which the agent was administered in the presence of factors that modify carcinogenic effects (e.g. initiation–promotion studies, co-carcinogenicity studies and studies in genetically modified animals); studies in which the end-point was not cancer but a defined precancerous lesion; experiments on the carcinogenicity of known metabolites and derivatives; and studies of cancer in non-laboratory animals (e.g. live-stock and companion animals) exposed to the agent.

For studies of mixtures, consideration is given to the possibility that changes in the physicochemical properties of the individual substances may occur during collection, storage, extraction, concentration and delivery. Another consideration is that chemical and toxicological interactions of components in a mixture may alter dose–response relationships. The relevance to human exposure of the test mixture administered in the animal experiment is also assessed. This may involve consideration of the following aspects of the mixture tested: (i) physical and chemical characteristics, (ii) identified constituents that may indicate the presence of a class of substances and (iii) the results of genetic toxicity and related tests.

The relevance of results obtained with an agent that is analogous (e.g. similar in structure or of a similar virus genus) to that being evaluated is also considered. Such results may provide biological and mechanistic information that is relevant to the understanding of the process of carcinogenesis in humans and may strengthen the biological plausibility that the agent being evaluated is carcinogenic to humans (see Part B, Section 2f).

(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, schedule and duration of exposure, species, strain (including genetic background where applicable), sex, age and 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.

Considerations of importance 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 monitored adequately, particularly in inhalation experiments; (iii) whether the doses, duration of treatment and route of exposure were appropriate; (iv) whether the survival of treated animals was similar to that of controls; (v) whether there were adequate numbers of animals per group; (vi) whether both male and female animals were used; (vii) whether animals were allocated randomly to groups; (viii) whether the duration of observation was adequate; and (ix) whether the data were reported and analysed adequately.

When benign tumours (a) occur together with and originate from the same cell type as malignant tumours in an organ or tissue in a particular study and (b) appear to represent a stage in the progression to malignancy, they are usually combined in the assessment of 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 induces only benign neoplasms that appear to be end-points that do not readily undergo transition to malignancy, the agent

should nevertheless be suspected of being carcinogenic and requires further investigation.

(b) *Quantitative aspects*

The probability that tumours will occur may depend on the species, sex, strain, genetic background and age of the animal, and on the dose, route, timing and duration of the exposure. Evidence of an increased incidence of neoplasms with increasing levels 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. Mechanisms such as induction of DNA damage or inhibition of repair, altered cell division and cell death rates and changes in intercellular communication are important determinants of dose–response relationships for some carcinogens. Since many chemicals require metabolic activation before being converted to their reactive intermediates, both metabolic and toxicokinetic 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 ([Hoel *et al.*, 1983](#); [Gart *et al.*, 1986](#)), as could saturation of processes such as DNA repair. The dose–response relationship can also be affected by differences in survival among the treatment groups.

(c) *Statistical analyses*

Factors considered include the adequacy of the information given for each treatment group: (i) number of animals studied and number examined histologically, (ii) 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](#); [Portier & Bailer, 1989](#); [Bieler &](#)

[Williams, 1993](#)). The choice of the most appropriate statistical method requires consideration of whether or not there are differences in survival among the treatment groups; for example, reduced survival because of non-tumour-related mortality can preclude the occurrence of tumours later in life. When detailed information on survival is not available, comparisons of the proportions of tumour-bearing animals among the effective number of animals (alive at the time the first tumour was discovered) can be useful when significant differences in survival occur before tumours appear. The lethality of the tumour also requires consideration: for rapidly fatal tumours, the time of death provides an indication of the time of tumour onset and can be assessed using life-table methods; non-fatal or incidental tumours that do not affect survival can be assessed using methods such as the Mantel-Haenzel test for changes in tumour prevalence. Because tumour lethality is often difficult to determine, methods such as the Poly-K test that do not require such information can also be used. When results are available on the number and size of tumours seen in experimental animals (e.g. papillomas on mouse skin, liver tumours observed through nuclear magnetic resonance tomography), other more complicated statistical procedures may be needed ([Sherman *et al.*, 1994](#); [Dunson *et al.*, 2003](#)).

Formal statistical methods have been developed to incorporate historical control data into the analysis of data from a given experiment. These methods assign an appropriate weight to historical and concurrent controls on the basis of the extent of between-study and within-study variability: less weight is given to historical controls when they show a high degree of variability, and greater weight when they show little variability. It is generally not appropriate to discount a tumour response that is significantly increased compared with concurrent controls by arguing that it falls within the range of historical controls, particularly when historical

controls show high between-study variability and are, thus, of little relevance to the current experiment. In analysing results for uncommon tumours, however, the analysis may be improved by considering historical control data, particularly when between-study variability is low. Historical controls should be selected to resemble the concurrent controls as closely as possible with respect to species, gender and strain, as well as other factors such as basal diet and general laboratory environment, which may affect tumour-response rates in control animals ([Haseman et al., 1984](#); [Fung et al., 1996](#); [Greim et al., 2003](#)).

Although meta-analyses and combined analyses are conducted less frequently for animal experiments than for epidemiological studies due to differences in animal strains, they can be useful aids in interpreting animal data when the experimental protocols are sufficiently similar.

4. Mechanistic and other relevant data

Mechanistic and other relevant data may provide evidence of carcinogenicity and also help in assessing the relevance and importance of findings of cancer in animals and in humans. The nature of the mechanistic and other relevant data depends on the biological activity of the agent being considered. The Working Group considers representative studies to give a concise description of the relevant data and issues that they consider to be important; thus, not every available study is cited. Relevant topics may include toxicokinetics, mechanisms of carcinogenesis, susceptible individuals, populations and life-stages, other relevant data and other adverse effects. When data on biomarkers are informative about the mechanisms of carcinogenesis, they are included in this section.

These topics are not mutually exclusive; thus, the same studies may be discussed in more than one subsection. For example, a mutation in a

gene that codes for an enzyme that metabolizes the agent under study could be discussed in the subsections on toxicokinetics, mechanisms and individual susceptibility if it also exists as an inherited polymorphism.

(a) *Toxicokinetic data*

Toxicokinetics refers to the absorption, distribution, metabolism and elimination of agents in humans, experimental animals and, where relevant, cellular systems. Examples of kinetic factors that may affect dose-response relationships include uptake, deposition, biopersistence and half-life in tissues, protein binding, metabolic activation and detoxification. Studies that indicate the metabolic fate of the agent in humans and in experimental animals are summarized briefly, and comparisons of data from humans and animals are made when possible. Comparative information on the relationship between exposure and the dose that reaches the target site may be important for the extrapolation of hazards between species and in clarifying the role of in-vitro findings.

(b) *Data on mechanisms of carcinogenesis*

To provide focus, the Working Group attempts to identify the possible mechanisms by which the agent may increase the risk of cancer. For each possible mechanism, a representative selection of key data from humans and experimental systems is summarized. Attention is given to gaps in the data and to data that suggests that more than one mechanism may be operating. The relevance of the mechanism to humans is discussed, in particular, when mechanistic data are derived from experimental model systems. Changes in the affected organs, tissues or cells can be divided into three non-exclusive levels as described below.

(i) Changes in physiology

Physiological changes refer to exposure-related modifications to the physiology and/or response of cells, tissues and organs. Examples of potentially adverse physiological changes include mitogenesis, compensatory cell division, escape from apoptosis and/or senescence, presence of inflammation, hyperplasia, metaplasia and/or preneoplasia, angiogenesis, alterations in cellular adhesion, changes in steroidal hormones and changes in immune surveillance.

(ii) Functional changes at the cellular level

Functional changes refer to exposure-related alterations in the signalling pathways used by cells to manage critical processes that are related to increased risk for cancer. Examples of functional changes include modified activities of enzymes involved in the metabolism of xenobiotics, alterations in the expression of key genes that regulate DNA repair, alterations in cyclin-dependent kinases that govern cell cycle progression, changes in the patterns of post-translational modifications of proteins, changes in regulatory factors that alter apoptotic rates, changes in the secretion of factors related to the stimulation of DNA replication and transcription and changes in gap-junction-mediated intercellular communication.

(iii) Changes at the molecular level

Molecular changes refer to exposure-related changes in key cellular structures at the molecular level, including, in particular, genotoxicity. Examples of molecular changes include formation of DNA adducts and DNA strand breaks, mutations in genes, chromosomal aberrations, aneuploidy and changes in DNA methylation patterns. Greater emphasis is given to irreversible effects.

The use of mechanistic data in the identification of a carcinogenic hazard is specific to the mechanism being addressed and is not readily

described for every possible level and mechanism discussed above.

Genotoxicity data are discussed here to illustrate the key issues involved in the evaluation of mechanistic data.

Tests for genetic and related effects are described in view of the relevance of gene mutation and chromosomal aberration/aneuploidy to carcinogenesis ([Vainio et al., 1992](#); [McGregor et al., 1999](#)). The adequacy of the reporting of sample characterization is considered and, when necessary, commented upon; with regard to complex mixtures, such comments are similar to those described for animal carcinogenicity tests. The available data are interpreted critically according to the end-points detected, which may include DNA damage, gene mutation, sister chromatid exchange, micronucleus formation, chromosomal aberrations and aneuploidy. The concentrations employed are given, and mention is made of whether the use of an exogenous metabolic system in vitro affected the test result. These data are listed in tabular form by phylogenetic classification.

Positive results in tests using prokaryotes, lower eukaryotes, insects, plants and cultured mammalian cells suggest that genetic and related effects could occur in mammals. Results from such tests may also give information on the types of genetic effect produced and on the involvement of metabolic activation. Some end-points described are clearly genetic in nature (e.g. gene mutations), while others are associated with genetic effects (e.g. unscheduled DNA synthesis). In-vitro tests for tumour promotion, cell transformation and gap-junction intercellular communication 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. Critical appraisals of these tests have been published ([Montesano et al., 1986](#); [McGregor et al., 1999](#)).

Genetic or other activity manifest in humans and experimental mammals is regarded to be of

greater relevance than that in other organisms. The demonstration that an agent can induce gene and chromosomal mutations in mammals *in vivo* indicates that it may have carcinogenic activity. 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 that rules out the carcinogenicity of agents that act through other mechanisms (e.g. receptor-mediated effects, cellular toxicity with regenerative cell division, peroxisome proliferation) ([Vainio et al., 1992](#)). Factors that may give misleading results in short-term tests have been discussed in detail elsewhere ([Montesano et al., 1986](#); [McGregor et al., 1999](#)).

When there is evidence that an agent acts by a specific mechanism that does not involve genotoxicity (e.g. hormonal dysregulation, immune suppression, and formation of calculi and other deposits that cause chronic irritation), that evidence is presented and reviewed critically in the context of rigorous criteria for the operation of that mechanism in carcinogenesis (e.g. [Capen et al., 1999](#)).

For biological agents such as viruses, bacteria and parasites, other data relevant to carcinogenicity may include descriptions of the pathology of infection, integration and expression of viruses, and genetic alterations seen in human tumours. Other observations that might comprise cellular and tissue responses to infection, immune response and the presence of tumour markers are also considered.

For physical agents that are forms of radiation, other data relevant to carcinogenicity may include descriptions of damaging effects at the physiological, cellular and molecular level, as for chemical agents, and descriptions of how these effects occur. 'Physical agents' may also be considered to comprise foreign bodies, such as

surgical implants of various kinds, and poorly soluble fibres, dusts and particles of various sizes, the pathogenic effects of which are a result of their physical presence in tissues or body cavities. Other relevant data for such materials may include characterization of cellular, tissue and physiological reactions to these materials and descriptions of pathological conditions other than neoplasia with which they may be associated.

(c) *Other data relevant to mechanisms*

A description is provided of any structure–activity relationships that may be relevant to an evaluation of the carcinogenicity of an agent, the toxicological implications of the physical and chemical properties, and any other data relevant to the evaluation that are not included elsewhere.

High-output data, such as those derived from gene expression microarrays, and high-throughput data, such as those that result from testing hundreds of agents for a single end-point, pose a unique problem for the use of mechanistic data in the evaluation of a carcinogenic hazard. In the case of high-output data, there is the possibility to overinterpret changes in individual end-points (e.g. changes in expression in one gene) without considering the consistency of that finding in the broader context of the other end-points (e.g. other genes with linked transcriptional control). High-output data can be used in assessing mechanisms, but all end-points measured in a single experiment need to be considered in the proper context. For high-throughput data, where the number of observations far exceeds the number of end-points measured, their utility for identifying common mechanisms across multiple agents is enhanced. These data can be used to identify mechanisms that not only seem plausible, but also have a consistent pattern of carcinogenic response across entire classes of related compounds.

(d) *Susceptibility data*

Individuals, populations and life-stages may have greater or lesser susceptibility to an agent, based on toxicokinetics, mechanisms of carcinogenesis and other factors. Examples of host and genetic factors that affect individual susceptibility include sex, genetic polymorphisms of genes involved in the metabolism of the agent under evaluation, differences in metabolic capacity due to life-stage or the presence of disease, differences in DNA repair capacity, competition for or alteration of metabolic capacity by medications or other chemical exposures, pre-existing hormonal imbalance that is exacerbated by a chemical exposure, a suppressed immune system, periods of higher-than-usual tissue growth or regeneration and genetic polymorphisms that lead to differences in behaviour (e.g. addiction). Such data can substantially increase the strength of the evidence from epidemiological data and enhance the linkage of in-vivo and in-vitro laboratory studies to humans.

(e) *Data on other adverse effects*

Data on acute, subchronic and chronic adverse effects relevant to the cancer evaluation are summarized. Adverse effects that confirm distribution and biological effects at the sites of tumour development, or alterations in physiology that could lead to tumour development, are emphasized. Effects on reproduction, embryonic and fetal survival and development are summarized briefly. The adequacy of epidemiological studies of reproductive outcome and genetic and related effects in humans is judged by the same criteria as those applied to epidemiological studies of cancer, but fewer details are given.

5. Summary

This section is a summary of data presented in the preceding sections. Summaries can be found on the *Monographs* programme web site (<http://monographs.iarc.fr>).

(a) *Exposure data*

Data are summarized, as appropriate, on the basis of elements such as production, use, occurrence and exposure levels in the workplace and environment and measurements in human tissues and body fluids. Quantitative data and time trends are given to compare exposures in different occupations and environmental settings. Exposure to biological agents is described in terms of transmission, prevalence and persistence of infection.

(b) *Cancer in humans*

Results of epidemiological studies pertinent to an assessment of human carcinogenicity are summarized. When relevant, case reports and correlation studies are also summarized. The target organ(s) or tissue(s) in which an increase in cancer was observed is identified. Dose–response and other quantitative data may be summarized when available.

(c) *Cancer in experimental animals*

Data relevant to an evaluation of carcinogenicity in animals are summarized. For each animal species, study design and route of administration, it is stated whether an increased incidence, reduced latency, or increased severity or multiplicity of neoplasms or preneoplastic lesions were observed, and the tumour sites are indicated. If the agent produced tumours after prenatal exposure or in single-dose experiments, this is also mentioned. Negative findings, inverse relationships, dose–response and other quantitative data are also summarized.

(d) Mechanistic and other relevant data

Data relevant to the toxicokinetics (absorption, distribution, metabolism, elimination) and the possible mechanism(s) of carcinogenesis (e.g. genetic toxicity, epigenetic effects) are summarized. In addition, information on susceptible individuals, populations and life-stages is summarized. This section also reports on other toxic effects, including reproductive and developmental effects, as well as additional relevant data that are considered to be important.

6. Evaluation and rationale

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

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 to a higher or lower category than a strict interpretation of these criteria would indicate.

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). A classification may change as new information becomes available.

An evaluation of the degree of evidence 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 the degree of evidence.

(a) Carcinogenicity in humans

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 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. A statement that there is *sufficient evidence* is followed by a separate sentence that identifies the target organ(s) or tissue(s) where an increased risk of cancer was observed in humans. Identification of a specific target organ or tissue does not preclude the possibility that the agent may cause cancer at other sites.

Limited evidence of carcinogenicity: A positive association has been observed between exposure to the agent 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 humans are known to encounter, which are mutually consistent in not showing a positive association between exposure to the agent and any studied cancer at any observed level of exposure. The results from these studies alone or combined should have narrow confidence intervals with an upper limit close to the null value (e.g. a relative risk of 1.0). Bias and confounding should be ruled out with reasonable confidence, and the studies

should have an adequate length of follow-up. 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.

When the available epidemiological studies pertain to a mixture, process, occupation or industry, the Working Group seeks to identify the specific agent 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.

(b) *Carcinogenicity in experimental animals*

Carcinogenicity in experimental animals can be evaluated using conventional bioassays, bioassays that employ genetically modified animals, and other in-vivo bioassays that focus on one or more of the critical stages of carcinogenesis. In the absence of data from conventional long-term bioassays or from assays with neoplasia as the end-point, consistently positive results in several models that address several stages in the multistage process of carcinogenesis should be considered in evaluating the degree of evidence of 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 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) two or more independent studies in one species carried out at different times or

in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well conducted study, ideally conducted under Good Laboratory Practices, can also provide *sufficient evidence*.

A single study in one species and sex 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, or when there are strong findings of tumours at multiple sites.

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; (b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the studies; (c) the agent increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential; or (d) the evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs.

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 is not carcinogenic. A conclusion of *evidence suggesting lack of carcinogenicity* is inevitably limited to the species, tumour sites, age at exposure, and conditions and levels of exposure studied.

(c) *Mechanistic and other relevant data*

Mechanistic and other evidence judged to be relevant to an evaluation of carcinogenicity and of sufficient importance to affect the overall

evaluation is highlighted. This may include data on preneoplastic lesions, tumour pathology, genetic and related effects, structure–activity relationships, metabolism and toxicokinetics, physicochemical parameters and analogous biological agents.

The strength of the evidence that any carcinogenic effect observed is due to a particular mechanism is evaluated, using terms such as ‘weak’, ‘moderate’ or ‘strong’. The Working Group then assesses whether that particular mechanism is likely to be operative in humans. The strongest indications that a particular mechanism operates in humans derive 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.

The conclusion that a mechanism operates in experimental animals is strengthened by findings of consistent results in different experimental systems, by the demonstration of biological plausibility and by coherence of the overall database. Strong support can be obtained from studies that challenge the hypothesized mechanism experimentally, by demonstrating that the suppression of key mechanistic processes leads to the suppression of tumour development. The Working Group considers whether multiple mechanisms might contribute to tumour development, whether different mechanisms might operate in different dose ranges, whether separate mechanisms might operate in humans and experimental animals and whether a unique mechanism might operate in a susceptible group. The possible contribution of alternative mechanisms must be considered before concluding that tumours observed in experimental animals

are not relevant to humans. An uneven level of experimental support for different mechanisms may reflect that disproportionate resources have been focused on investigating a favoured mechanism.

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.

(d) Overall evaluation

Finally, the body of evidence is considered as a whole, to reach an overall evaluation of the carcinogenicity of the agent to humans.

An evaluation may be made for a group of agents that have been evaluated by the Working Group. In addition, when supporting data indicate that other related agents, for which there is no direct evidence of their 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 agents if the strength of the evidence warrants it.

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

Group 1: The agent is carcinogenic to humans.

This category is used when there is *sufficient evidence of carcinogenicity* in humans. Exceptionally, an agent 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 acts through a relevant mechanism of carcinogenicity.

Group 2.

This category includes agents 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 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 mechanistic and other relevant data. The terms *probably carcinogenic* and *possibly carcinogenic* have no quantitative significance and are used simply as descriptors of different levels of evidence of human carcinogenicity, with *probably carcinogenic* signifying a higher level of evidence than *possibly carcinogenic*.

Group 2A: The agent is 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 may be classified in this category when there is *inadequate evidence of carcinogenicity* in humans and *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 may be classified in this category solely on the basis of *limited evidence of carcinogenicity* in humans. An agent may be assigned to this category if it clearly belongs, based on mechanistic considerations, to a class of agents for which one or more members have been classified in Group 1 or Group 2A.

Group 2B: The agent is possibly carcinogenic to humans.

This category is used for agents 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 for which there is *inadequate evidence of carcinogenicity* in humans and less than *sufficient evidence of carcinogenicity* in experimental animals together with supporting evidence from mechanistic and other relevant data may be placed in this group. An agent may be classified in this category solely on the basis of strong evidence from mechanistic and other relevant data.

Group 3: The agent is not classifiable as to its carcinogenicity to humans.

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

Exceptionally, agents 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 that do not fall into any other group are also placed in this category.

An evaluation in Group 3 is not a determination of non-carcinogenicity or overall safety. It often means that further research is needed, especially when exposures are widespread or the cancer data are consistent with differing interpretations.

Group 4: The agent is probably not carcinogenic to humans.

This category is used for agents for which there is *evidence suggesting lack of carcinogenicity* in humans and in experimental animals. In some instances, agents 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 mechanistic and other relevant data, may be classified in this group.

(e) Rationale

The reasoning that the Working Group used to reach its evaluation is presented and discussed. This section integrates the major findings from studies of cancer in humans, studies of cancer in experimental animals, and mechanistic and other relevant data. It includes concise statements of the principal line(s) of argument that emerged, the conclusions of the Working Group on the strength of the evidence for each group of studies, citations to indicate which studies were pivotal to these conclusions, and an explanation of the reasoning of the Working Group in weighing data and making evaluations. When there are significant differences of scientific interpretation among Working Group Members, a brief summary of the alternative interpretations is provided, together with their scientific rationale and an indication of the relative degree of support for each alternative.

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GENERAL REMARKS

1. Background

This one-hundred-and-fourth volume of the *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans* is the eighth volume devoted to infectious biological agents. The biological agents that have been considered to date by the *IARC Monographs* are listed in [Table 1](#). Several have been recognized as major risk factors involved in the burden of cancer.

In Volume 104, the *IARC Monographs* consider the following agents for the first time: malaria (a disease caused by infection with the *Plasmodium* parasite) and four polyomaviruses: the simian virus SV40, and the BK, JC, and Merkel cell polyomaviruses. Infection by these microorganisms concerns a very large proportion of the world population.

A summary of the findings of this volume appears in *The Lancet Oncology* ([Bouvard et al., 2012](#)).

2. Malaria

The global burden of malaria is enormous; about 50% of the world's population is at risk of developing malaria, with an estimated annual incidence of 219 million cases worldwide (range, 154–289 million), mostly in sub-Saharan Africa and Papua New Guinea ([WHO, 2011](#)). A role for malaria in the etiology of endemic Burkitt lymphoma in areas where malaria is highly endemic (holoendemic) has long been suspected ([Burkitt, 1961](#)). In these areas, this type of non-Hodgkin lymphoma can account for up to 70% of childhood cancers, with the highest incidence at age 5–9 years ([Parkin et al., 2008](#)).

While malaria is caused by several species of *Plasmodium*, the majority of studies investigating the link between malaria and cancer have been conducted in areas where *P. falciparum* is highly prevalent, or have focused deliberately on *P. falciparum* (the species linked to the most severe form of malaria).

2.1 Ecological studies

Major epidemiological studies of malaria (infection with *P. falciparum*) and cancer, mainly Burkitt lymphoma, include ecological studies and fewer case-control and cohort studies. For most carcinogens, causal inference is primarily based on case-control and cohort studies, with little, if any, support from ecological studies. For malaria, in contrast, ecological studies provide important

Table 1 Evaluation of the carcinogenicity to humans of biological agents from this and previous *IARC Monographs* volumes

Biological agent	IARC Group	<i>IARC Monographs</i> volume (year)
Hepatitis B virus	1	Vol 59 (IARC, 1994a); Vol 100B (IARC, 2012)
Hepatitis C virus	1	Vol 59 (IARC, 1994a); Vol 100B (IARC, 2012)
Hepatitis D virus	3	Vol 59 (IARC, 1994a)
HIV-1	1	Vol 67 (IARC, 1996); Vol 100B (IARC, 2012)
HIV-2	2B	Vol 67 (IARC, 1996)
HTLV-I	1	Vol 67 (IARC, 1996); Vol 100B (IARC, 2012)
HTLV-II	3	Vol 67 (IARC, 1996)
EBV	1	Vol 70 (IARC, 1997); Vol 100B (IARC, 2012)
KSHV	1	Vol 70 (IARC, 1997); Vol 100B (IARC, 2012)
HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59	1	Vol 64 (IARC, 1995); Vol 90 (IARC, 2007); Vol 100B (IARC, 2012)
HPV type 68	2A	Vol 64 (IARC, 1995); Vol 90 (IARC, 2007); Vol 100B (IARC, 2012)
HPV types 26, 30, 34, 53, 66, 67, 69, 70, 73, 82, 85 and 97	2B	Vol 64 (IARC, 1995); Vol 90 (IARC, 2007); Vol 100B (IARC, 2012)
HPV types 6 and 11	3	Vol 64 (IARC, 1995); Vol 90 (IARC, 2007); Vol 100B (IARC, 2012)
Some HPV of genera beta and gamma	3	Vol 64 (IARC, 1995); Vol 90 (IARC, 2007); Vol 100B (IARC, 2012)
HPV types 5 and 8 of genera beta	2B	Vol 64 (IARC, 1995); Vol 90 (IARC, 2007); Vol 100B (IARC, 2012)
SV40 simian polyomavirus	3	Vol 104 (this volume)
BK polyomavirus (BKV)	2B	Vol 104 (this volume)
JC polyomavirus (JCV)	2B	Vol 104 (this volume)
Merkel cell polyomavirus (MCV)	2A	Vol 104 (this volume)
Malaria (infection by <i>Plasmodium falciparum</i> in holoendemic areas)	2A	Vol 104 (this volume)
<i>Schistosoma haematobium</i>	1	Vol 61 (IARC, 1994b); Vol 100B (IARC, 2012)
<i>Schistosoma mansoni</i>	3	Vol 61 (IARC, 1994b)
<i>Schistosoma japonicum</i>	2B	Vol 61 (IARC, 1994b)
<i>Helicobacter pylori</i>	1	Vol 61 (IARC, 1994b); Vol 100B (IARC, 2012)
<i>Opisthorchis viverrini</i>	1	Vol 61 (IARC, 1994b); Vol 100B (IARC, 2012)
<i>Opisthorchis felinus</i>	3	Vol 61 (IARC, 1994b)
<i>Chlonorchis sinensis</i>	1	Vol 61 (IARC, 1994b); Vol 100B (IARC, 2012)

EBV, Epstein–Barr virus; HIV, human immunodeficiency virus; HPV, human papillomavirus; HTLV, human T-lymphotropic virus; KSHV, Kaposi sarcoma-associated herpesvirus

information on causal inference because of large differences in exposure in areas with different burdens of infection with *P. falciparum* (particularly in children) and limited population migration. Particularly in areas of holoendemic malaria, ecological measures provide a strong indication of individual exposure. However, the Working Group found that there were often limitations in the assessment of exposure in pertinent analytical studies (see below). Hence, in the *Monograph* on malaria, ecological studies were described in detail and assessed with consideration of the complexity of the parameters that influence the global epidemiology of malaria.

The burden of infection with *P. falciparum* within a population is described ecologically on the basis of transmission intensity. The definition of transmission intensity for falciparum malaria is based on exposure in children aged 2–10 years and measured by entomological inoculation rate (EIR), parasite reproductive numbers, prevalence of parasites in peripheral blood, and frequency of splenomegaly. Holoendemic malaria transmission has been defined as parasite prevalence of > 70% and splenomegaly in > 80% of children.

2.1.1 Environmental ecology, vector-species identification and locally dominant parasites

Malaria is a mosquito-borne infection and so the biology of this disease is closely linked to the environmental ecology of the species of *Anopheles* mosquito responsible for transmission of the parasite. This explains the geographical distribution of malaria, the differences in transmission intensity that are mostly dependent on climatic variables (e.g. altitude, season), and the differences seen in rural versus urban populations.

2.1.2 Diagnosis of malaria

The correct diagnosis of malaria remains a challenge in many countries. The burden of malaria can be overestimated when diagnosis is based on symptoms alone, since the symptoms typical of malaria (e.g. fever, chills) are also exhibited during other microbial infections ([Ari et al., 2011](#)). In sub-Saharan countries, the burden of infectious disease is high, and co-infection by *P. falciparum* and a second infectious agent (e.g. hookworm or *Mansonella perstans*) in the same individual is common ([Hillier et al., 2008](#)). In contrast, reliance on passive national reporting of malaria has likely led to underestimation of the true burden of malaria worldwide ([Snow et al., 2005](#)).

2.1.3 Variation in host genetic susceptibility

Genetic polymorphisms known to influence the severity of malaria (e.g. sickle-cell trait) are widespread in African people and may also have a role in susceptibility to cancer.

2.1.4 Modifiable factors

Modifiable factors such as intense use of chemically treated bednets, use of indoor spraying with insecticides, and availability of effective antimalarial therapy may also have a strong influence on the global burden of malaria.

2.2 Analytical epidemiological studies

Only one cohort study and few case–control studies on the link between malaria and Burkitt lymphoma have been reported, and even fewer have looked at other cancers (e.g. Kaposi sarcoma). For these studies, uncertainty about the accuracy of the methods used for the assessment of exposure to *P. falciparum* infection was a major limitation. Although the use of serology for the detection of antimalaria antibodies may appear to be a better means of exposure assessment than questionnaires, accuracy of the results is highly dependent on the choice of antibodies used as markers of infection. It has been shown that immunoglobulin G (IgG)-specific antimalaria antibodies are often short-lived in young children and levels only increase with age and repeated infections. Therefore, use of such antibodies tends to underestimate past infection with *P. falciparum*. The choice of malaria antigen against which such antibodies are targeted can also be problematic because of the well documented genetic variation in the surface antigens of *P. falciparum*. Measurement of antibodies to antigens derived from whole schizont extracts is thought to provide a good estimate of past exposure to *P. falciparum* ([Marsh & Kinyanjui, 2006](#)).

2.3 Burkitt lymphoma, Epstein–Barr virus and malaria

Epstein-Barr virus (EBV) has been established as an essential etiological agent for endemic Burkitt lymphoma ([IARC, 1997, 2012](#)). More than 90% of the population worldwide is infected with this highly oncogenic virus. However, infection is asymptomatic in most carriers since the virus usually remains latent. Most studies on Burkitt lymphoma have looked at the role of EBV reactivation per se, without looking at the potential effect of malaria status; likewise, most studies reporting on the association between malaria and Burkitt lymphoma did not consider EBV status. Based on two recent case–control studies looking at the effects of co-infection with EBV and *P. falciparum*, and on strong mechanistic data showing clear interactions between EBV and *P. falciparum*, the Working Group concluded that endemic Burkitt lymphoma is likely to be caused by the combined effects of early co-infection with EBV and malaria in a holoendemic area.

3. Polyomaviruses

In the 1950s and early 1960s, millions of people worldwide received poliovirus vaccines that were contaminated with SV40, a polyomavirus that naturally infects the rhesus macaque. Shortly after the discovery of this contamination, SV40 was shown to induce tumours in newborn hamsters; there have thus been serious concerns that SV40 may cause cancer in humans who received the contaminated virus.

In 1971, BK polyomavirus (BKV) and JC polyomavirus (JCV) became the first naturally human-tropic polyomaviruses to be isolated. Merkel cell polyomavirus (MCV) was discovered more recently, in 2008, in a rare skin cancer, Merkel cell carcinoma. Infection with human polyomaviruses is widespread in the general population, with about 50% to > 90% of adults being infected worldwide.

During the evaluation process, the Working Group raised several issues inherent to the characteristics of the polyomaviruses evaluated in this volume and that are important to consider when interpreting the data. These issues are summarized below.

3.1 Specificity of the detection methods

In epidemiological studies, detection of infection by polyomaviruses has been mostly based on the amplification of viral DNA by polymerase chain reaction (PCR), and past infections have been assessed by the detection of antibodies raised against the viral capsid. Concerns have been raised regarding both types of method.

3.1.1 PCR-based methods

PCR techniques are extremely sensitive; laboratory contamination, lack of specificity or cross-amplification with other polyomaviruses might explain some discrepancies observed between studies.

BKV and JCV are very closely related to one another and to SV40; the three viruses share about 70–75% identity at the nucleotide level across the entire genome. Indeed, primers initially designed to amplify SV40 DNA sequences can cross-amplify BKV or JCV sequences. Furthermore, some PCR primers used have been shown to co-amplify contaminating SV40 sequences present in common laboratory plasmids and in some laboratory cell lines. Consequently, and also because of the ubiquitous presence of BKV and JCV viruses in the human population, it was necessary to apply caution in the interpretation of the PCR results e.g. false-positive results in studies assessing the presence of SV40 in human cancers.

3.1.2 Serological methods

Detection of antibodies to polyomaviruses in human sera is performed mostly by neutralization tests or enzyme-linked immunosorbent assays (ELISA) using recombinant virus-like particles (VLPs). Because the structural organization of some polyomaviruses is highly conserved, competitive-inhibition studies are necessary to ensure the specificity of the antibodies detected. Indeed, it has been suggested that reactivity to SV40 in human sera reported in some studies is due to cross-reactivity to BKV and JCV, since reactivity to SV40 is often eliminated by pre-incubation with BKV or JCV VLPs.

Assays for haemagglutination inhibition (HAI) have also been used to measure titres of antibodies to BKV in human sera, since BKV cause agglutination of human erythrocytes of group O; however, since JCV has also now been shown to be capable of haemagglutination, HAI is not completely specific for BKV.

3.2 Biomarkers of infection

The widespread presence of BKV, JCV and MCV in a healthy population is one of the main difficulties when studying the potential association of these viruses with human cancer. Infection by these viruses is asymptomatic and mainly latent in the general population, although shedding of viral particles in urine has been observed. Reactivation of latent infection (e.g. by immunosuppression) of polyomaviruses can result in high viraemia and viruria. It is however unclear whether viruria is an appropriate biomarker for use in retrospective case–control studies, particularly when cancer (or other conditions) for which the cases are being treated, or hospitalization itself, could be associated with viral reactivation and shedding.

3.3 Notion of subtypes, variants and mutations

Genetic variants of BKV, JCV and MCV have been isolated. However, most epidemiological studies that were available to the Working Group did not consider this additional level of complexity, which may greatly influence the detection of polyomaviruses. Examples of genetic variants of each virus are described below.

3.3.1 BKV

Four BKV genotypes (I–IV) have been isolated and shown to be fully distinct serotypes ([Knowles et al., 1989](#); [Pastrana et al., 2012](#)); some of the subgenotypes identified also behave as fully distinct serotypes ([Pastrana et al., 2013](#)). The different BKV genotypes have different biological characteristics, and may have different cellular tropism and different pathogenic potential *in vivo* ([Pastrana et al., 2013](#)).

In > 80% of BKV-positive healthy subjects, BKV belongs to genotype I, although geographical variation has been reported.

Many of the primers first developed for the detection of BKV in PCR assays were based on the sequence of genotype I and did not detect all genotypes. Consequently, variant BKV genotypes lower the sensitivity of detection by PCR and may also have a substantial effect on viral load quantitation ([Randhawa et al., 2011](#)).

Most serological assays are not designed to distinguish between the four main serotypes of BKV, e.g. serum antibodies specific for BKV-I are poorly neutralizing against BKV-IV and vice versa ([Pastrana et al., 2012](#)).

3.3.2 JCV

The JCV archetype and several variants classified according to rearrangements in the structure of the viral non-coding regulatory region (NCCR) have been isolated. Variants have exclusively been found in lesions associated with progressive multifocal leukoencephalopathy (PML), a JCV-induced lethal disease in immunosuppressed patients. This suggests that these JCV variants only may cause progressive multifocal leukoencephalopathy. It has been reported that in patients with progressive multifocal leukoencephalopathy, the archetype NCCR was detected in the urine, while NCCR variants were found in cerebrospinal fluid and in blood ([Ryschkewitsch et al., 2013](#)). These data suggest that coincident infections with different JCV variants having different biological behaviour may occur, and highlight the importance of the choice of marker of infection.

The role of JCV NCCR variants in human cancer has received limited attention.

3.3.3 MCV

Most MCV genomes analysed so far isolated from Merkel cell carcinoma have contained mutations within large T antigen sequences leading to the expression of a C-terminal truncated protein deficient for viral replication. Additionally, mutations in other parts of the viral genome have been reported in Merkel cell carcinoma that may also lead to defective viral replication or assembly (e.g. within the *VPI* gene or within the origin of replication). This is in contrast to the wildtype MCV

genome commonly detected in the skin of healthy adults that expresses full-length large T-antigen and can actively produce virions.

3.4 Lytic infection versus transformation; species specificity; permissivity and non-permissivity

Since 1960, when SV40 was isolated and shown to induce tumours when injected into newborn hamsters, a huge amount of research has been carried out on this, the first oncovirus to be discovered, and provided many of the important paradigms in our understanding of normal cell biology and tumorigenesis (e.g. discovery of tumour suppressor genes such as *TP53* and *RB*).

The mechanism by which infection with SV40 causes cell transformation and the formation of malignant tumours is well established in rodents and in systems *in vitro*. SV40, like most polyomaviruses, gives rise to either lytic (productive) infections or incomplete (abortive) infections, depending on the host cell. Not all cells support the natural replicative and productive viral cycle and hosts can be divided into ‘permissive’ and ‘non-’, or ‘semi-permissive’.

Cell transformation by a polyomavirus appears to result from an infection that fails to be lytic (productive), either because of the properties of the host cell, or because of the defective nature of the infecting virus. SV40 can easily transform mouse, rat, and hamster cells that are non-permissive for the virus, and induces tumours in these rodents. In contrast, many simian cell lines are permissive for SV40 and die when infected, liberating virions, and so cannot be transformed.

On the other hand, transformation of permissive cells is not impossible as it could result from failure of the lytic cycle owing to defects in the replicative capacity of the infecting polyomavirus. This raises the potential importance of variants of polyomavirus in human cancers (e.g. mutations of MCV large T-antigen found in Merkel cell carcinoma).

When considering the data from cancer bioassays in animals and studies in humans in its evaluation of the carcinogenicity of polyomaviruses in humans, the Working Group took into account the notions of species specificity, permissivity *versus* non-permissivity and lytic infection *versus* transformation.

3.5 Newly discovered human polyomaviruses

Since the discovery of MCV in 2008, seven additional human polyomaviruses have been identified: KI polyomavirus, WU polyomavirus, trichodysplasia spinulosa-associated polyomavirus, and human polyomavirus types 6, 7, 9 and 10. None have yet been clearly associated with human diseases, with the exception of trichodysplasia spinulosa-associated polyomavirus, which was isolated from a rare hyperplastic but non-neoplastic skin tumour that can occur in transplant patients (see “Introduction to polyomaviruses,” in this Volume). Serological studies have revealed that types 6, 7 and 9 are circulating in the human population. Like the other polyomaviruses, the novel human polyomaviruses encode small and large T-antigens and thus are potentially oncogenic ([Dalianis & Hirsch, 2013](#); [Ehlers & Wieland, 2013](#)).

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1. Exposure Data

Malaria is a human disease caused by infection with a parasite of the genus *Plasmodium*.

1.1 Taxonomy, structure, and biology of *Plasmodium*

1.1.1 Taxonomy

Plasmodium is a genus of parasites belonging to the family Plasmodiidae, order Haemosporidia and phylum Apicomplexa. The genus *Plasmodium* is subdivided into 10 subgenera. Malaria parasites in humans are all classified in the subgenera *Plasmodium* and *Laverania*. Four are well-characterized, strict human pathogens (e.g. *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*), and one (*P. knowlesi*) is a recently identified human pathogen ([Cox-Singh et al., 2008](#); [Kantele & Jokiranta, 2011](#)).

Plasmodium has two obligate hosts in its life cycle: a mosquito host, which also serves as vector to a vertebrate host. The insect vector of the parasite is used as one biological criterion for classification of the different species of *Plasmodium*. Other biological criteria include the host range, the type of host cell infected, the length of the different stages of the life cycle, the presence or absence of relapse/recrudescences, and geographical distribution.

The morphology of the parasite is also used to characterize species. Morphological criteria

include the shape of the trophozoite, the gametocyte and the oocyst, the number of nuclei in the erythrocytic and exo-erythrocytic schizonts, the aspect and distribution of the hemozoin pigment from the metabolism of the haemoglobin, and the nature of the damage induced by the parasite in the host cell. As more *Plasmodium* species are sequenced, newer taxonomic criteria based on molecular characteristics such as the 18S small subunit rRNA, the genes for the circumsporozoite protein and for cytochrome b are now being included to define *Plasmodium* species and generate phylogenetic trees ([Outlaw & Ricklefs, 2011](#)).

All *Plasmodium* species examined to date have 14 chromosomes, one mitochondrion and one plastid. Sequencing data have resolved the question of the origin of *P. falciparum* and its relationship with other *Plasmodium* parasites of primates and humans. Extensive sequence analysis of primate *Plasmodium* DNA indicates that *P. falciparum* is genetically related to a gorilla *Plasmodium* parasite in the *Laverania* subgenera ([Liu et al., 2010](#)).

1.1.2 Structure

Specialized complexes of apical organelles known as micronemes, rhoptries and dense granules are distinguishing morphological features of parasites belonging to the phylum Apicomplexa. In addition, apicomplexan parasites have a vestigial plastid organelle, the apicoplast, which has its own genome and gene-expression

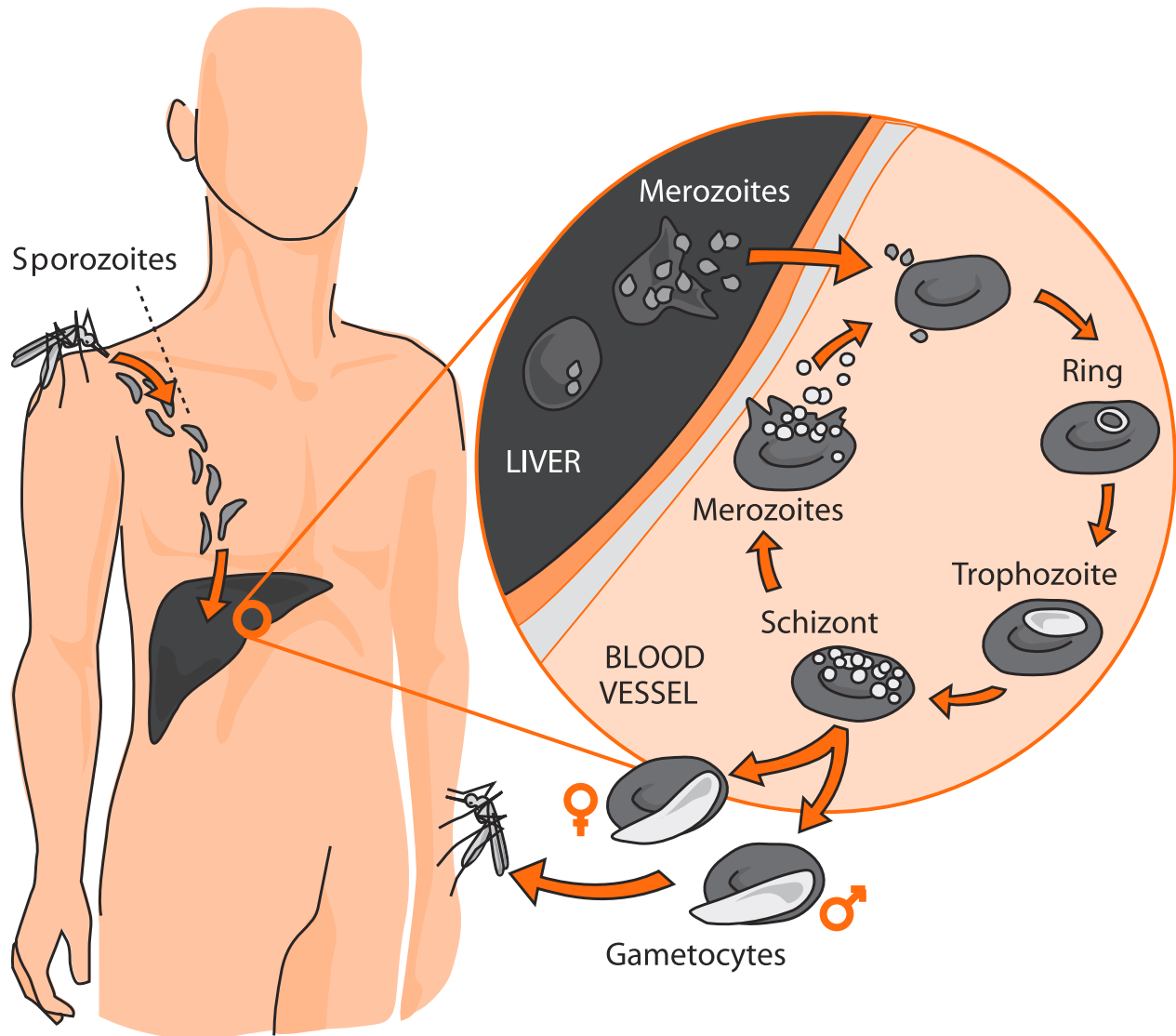
machinery ([Waller & McFadden, 2005](#)). Apical organelles have been implicated in the process of host-cell invasion. *Plasmodium* parasites have a complicated life cycle as they infect both mosquitoes and humans and, within each host, there are several stages of development. In the human host, the parasite first invades and replicates in hepatocytes. In this hepatic development phase, the parasite is known as a liver schizont. Liver schizonts appear as clusters of small basophilic bodies (merozoite nuclei) located in vacuoles within host hepatocytes, measuring 40–80 μm in diameter when mature. After replication in the hepatocyte, the parasite is released as a merozoite, which then infects erythrocytes. Four developmental stages are found in erythrocytes: trophozoites, schizonts, merozoites, and gametocytes. Each of these stages has well-defined morphological features and, combined with characteristic modifications of the host erythrocyte, these features are used to distinguish between the four primary species of human *Plasmodium* during infection of the human host via microscopic examination of Giemsa-stained peripheral-blood smears. The trophozoites are small and rounded, known as ring forms, and measure 1–2 μm in diameter. The schizont stage is amorphous and multinucleated, measuring up to 7–8 μm in length. The schizont can either divide into merozoites and repeat the erythrocyte-infection cycle, or differentiate into gametocytes. Gametocytes are the sexually reproductive stage and are approximately 1.5 times the diameter of the erythrocyte in length.

1.1.3 Life cycle, natural history of infection, persistence, latency

The life cycle of *Plasmodium* species in humans is shown in [Fig. 1.1](#). The parasite transits between the *Anopheles* mosquito vector and the human host. The pre-erythrocytic stage begins when an infected female *Anopheles* mosquito inoculates *Plasmodium* sporozoites into the skin

or into the bloodstream of humans during a blood meal. The sporozoites circulate transiently in the bloodstream before invading hepatocytes, where an asexual life cycle occurs. Recent studies have shown that sporozoites can remain for up to 6 hours at the site of injection ([Yamauchi et al., 2007](#)), and that 25% of those leaving the injection site may enter the draining lymph nodes via the lymphatic vessels ([Amino et al., 2006](#)). When sporozoites reach the liver parenchyma, they migrate through several hepatocytes before definitive infection. This migration seems to be advantageous for malaria infections in at least two different ways: to activate the sporozoites for infection and to increase the susceptibility of the host hepatocytes ([Mota & Rodriguez, 2004](#)). It is thought that invasion of the hepatocytes first requires invasion of Küpffer cells found in the liver ([Pradel & Frevert, 2001](#)). A hepatic phase of development begins that lasts approximately 1–2 weeks. Replication of *Plasmodium* in the liver is known as the exo-erythrocytic stage. The merozoites develop in unique vacuoles in the hepatocytes known as “parasitophorous vacuoles”. For *P. falciparum* and *P. malariae*, this stage of the life cycle always proceeds to rupture and release of merozoites. However, the liver stages of *P. vivax* and *P. ovale* can either result in release of merozoites or the establishment of the hypnozoite stage. Hypnozoites are a latent phase of infection in the liver and can remain so for years ([Markus, 2011](#)). Very little is known about the biology of the hypnozoite phase. Malarial relapse due to emergence of hypnozoites is characteristic of *P. vivax* and *P. ovale* and distinguishes infections with these pathogens from those with *P. falciparum* and *P. malariae*.

Plasmodium parasites are highly species-specific and this has limited understanding of the biology of these parasites in their human cellular targets, e.g. hepatocytes and erythrocytes. Of the human parasites, most is known about *P. falciparum* in the erythrocytic phase of replication, because this parasite can be cultured

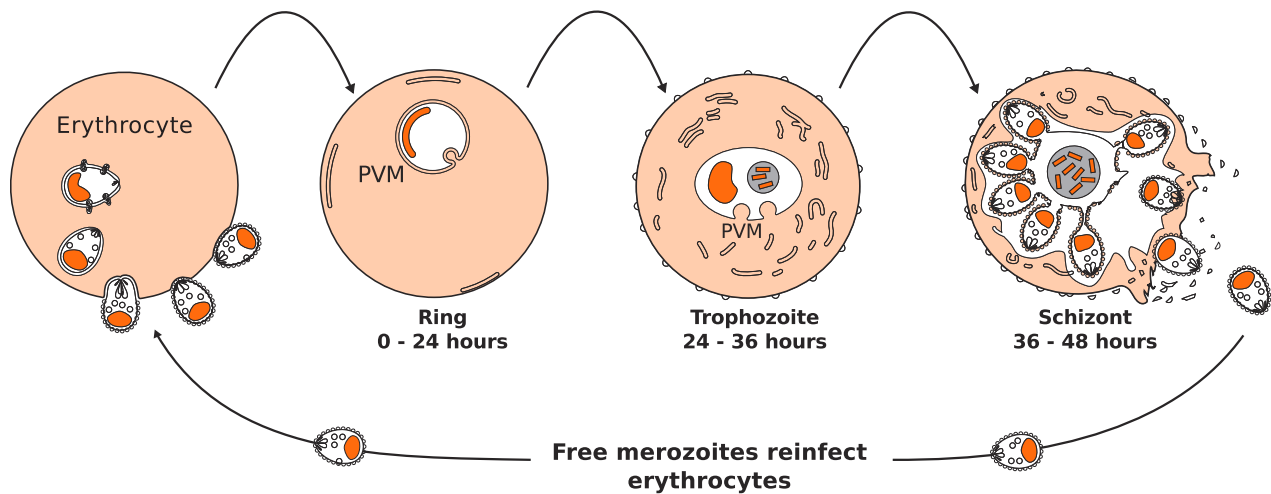
Fig. 1.1 Life-cycle of *Plasmodium* species in humans

Prepared by the Working Group

in erythrocytes *ex vivo*. *P. vivax* preferentially infects reticulocytes, the immature form of erythrocytes; only recently has a system of culture *in vitro* been developed that allows for limited continuous culture of *P. vivax* (Panichakul *et al.*, 2007).

The *P. falciparum* merozoite can use many erythrocyte receptors for invasion; in contrast, invasion by *P. vivax* requires the Duffy antigen/chemokine receptor (as known as FY glycoprotein

or cluster of differentiation 234, CD234) (Miller *et al.*, 1975). The detailed life cycle of *P. falciparum* within erythrocytes (the intra-erythrocytic stage) is shown in Fig. 1.2. In the newly infected erythrocyte, the parasite becomes enclosed in a vacuolar membrane and microscopic examination shows the clear presence of a ring in infected cells (i.e. the “ring” stage). The parasite remains in the ring stage for 24–32 hours and then matures to the trophozoite stage.

Fig. 1.2 Life-cycle of *Plasmodium falciparum* in erythrocytes

P. falciparum infects the erythrocyte and proceeds through several stages of maturation.

PVM, parasitophorous vacuole membrane

Prepared by the Working Group

What makes *P. falciparum* different from other malaria parasites in the trophozoite stage is that the infected erythrocytes become sequestered in the microvasculature by adhering to endothelial cells (Sherman *et al.*, 2003). Parasite-induced membrane changes are thought to be responsible for the adherence of infected erythrocytes.

The trophozoites mature into schizonts, which then divide into merozoites. Rupture of the erythrocyte releases merozoites into the bloodstream and repeated cycles of erythrocyte invasion and rupture result in exponential increases in parasite burden. These high levels of parasite burden are linked to the pathogenesis of *P. falciparum*, which is characterized by haemolytic anaemia. In contrast, because *P. vivax* preferentially targets immature erythrocytes, which are found at low levels in the peripheral blood, the parasite burden is significantly less than 1% of peripheral erythrocytes (Panichakul *et al.*, 2007). In some infected erythrocytes, instead of maturing into merozoites, the schizont differentiates into male and female gametocytes. These can be ingested by a *Anopheles* mosquito during a blood meal to complete the life cycle of *Plasmodium* in humans

(the life cycle in mosquitoes is not described in this *Monograph*).

Infection of erythrocytes by *P. falciparum* results in extensive remodelling of the erythrocytic membrane, especially during the trophozoite phase (Cooke *et al.*, 2004). This results in functional changes in the erythrocyte, including increased membrane rigidity, reduced cell deformability, increased permeability and increased adhesiveness to the endothelium and other host cells. Although many of the parasite proteins essential for these alterations are unknown, several key proteins have been identified in recent years. For example, the parasite erythrocyte membrane protein 1 (PfEMP1) is a surface adhesin detected in the membrane of infected erythrocytes and is thought to be responsible in part for the adherent properties of the infected erythrocyte by forming knob-like structures on the surface of the infected cell. Proteomic analysis of *P. falciparum* has identified more than 400 proteins that could be exported to the erythrocyte (Marti *et al.*, 2004). A common export domain was identified on an additional 72 *P. falciparum* genes, suggesting that the proteins

encoded by these genes could be exported to the surface of the erythrocyte ([Sargeant et al., 2006](#)).

1.2 Epidemiology of *Plasmodium* infection

1.2.1 Global distribution of *Plasmodium* infection in humans

Assessing the global epidemiology of malaria is a complex process involving evaluation of environmental ecology, vector-species identification, the local dominant parasites, dynamics of the exposed population, chemotherapeutic trends in clinical malaria, and the outcome of control strategies ([Ototo et al., 2011](#)). Variation in the genetics of host susceptibility also contributes to the distribution of malaria globally. Overestimates of the burden of malaria can result when diagnosis is based on symptoms alone, since the symptoms typical of malaria (e.g. fever, chills, malaise) are also exhibited by other microbial infections characterized by acute febrile illness and the correct diagnosis of clinical malaria remains a challenge ([Ari et al., 2011](#)). In contrast, reliance on passive national reporting of malaria has likely led to an underestimate of the true burden of malaria worldwide ([Snow et al., 2005](#)).

Globally, populations in 107 countries and territories (about 50% of the world population) are at risk of malaria, with an estimated annual incidence of 216 million cases of malaria ([WHO, 2011](#)), and up to 1.2 million deaths due to malaria in 2010 ([WHO, 2011](#); [Lancet, 2012](#)). An estimated 86% of all deaths occur in children aged less than 5 years ([WHO, 2011](#)). The *Plasmodium* species that infect humans are confined to the tropical and subtropical areas of the world where their insect vectors, mosquitoes of the genus *Anopheles*, are found. The large majority of infections can be linked to *P. falciparum*, with the remainder predominantly caused by *P. vivax*, and a very small number caused by *P. malariae*, *P. ovale* and *P. knowlesi* ([WHO, 2011](#)). The geographical

distribution of *Plasmodium* species infecting humans is shown in [Table 1.1](#).

In sub-Saharan African, the highest rates of morbidity and mortality are associated with infection with *P. falciparum*. *P. vivax* is less frequent than *P. falciparum* in Africa, but is the dominant *Plasmodium* species causing malaria in many localities outside Africa. Infection by *P. falciparum* and *P. malariae* occurs worldwide, while *P. ovale* is limited to Africa and parts of Asia.

Malaria is endemic in many countries within the tropical regions of the world. The greatest burden of falciparum malaria occurs in Africa ([Table 1.2](#); [WHO, 2011](#)).

1.2.2 Transmission of *Plasmodium*

Plasmodium parasites are transmitted to humans by an infective bite from the *Anopheles* mosquito. The distribution of *Anopheles* species is highly variable from region to region, with considerable species variation between proximate geographical areas due to differences in environmental and climatic variables (e.g. altitude) that support or limit vector burden. Out of 390 species of *Anopheles* mosquitoes, only 50 species are known to transmit *Plasmodium*, with 20 species showing more localized global geographical distribution ([Sinka et al., 2010a, b, 2011](#)). The most common vectors of human *Plasmodium* parasites in many parts of Africa are *Anopheles gambiae* and *Anopheles funestus* ([Sinka et al., 2010a](#)).

The burden of infection with *P. falciparum* within a population is described ecologically on the basis of transmission intensity ([Gething et al., 2011](#)). Transmission intensity can be measured in numerous ways, including the entomological inoculation rate (EIR). EIR is an estimate of the annual number of bites by infectious mosquitoes received by one person ([WHO, 2010](#)). In Africa, the average EIR due to *P. falciparum* is 112 and ranges from < 1 in Sudan, to 814 in Equatorial

Table 1.1 Geographical distribution of *Plasmodium* species that infect humans

Parasite	Host	Geographical distribution
<i>P. falciparum</i>	Human	Africa, Asia, South/Central America
<i>P. vivax</i>	Human	Africa, Asia, South/Central America
<i>P. malariae</i>	Human	Africa, Asia, South/Central America
<i>P. ovale</i>	Human	Africa
<i>P. knowlesi</i>	Human/monkeys	Asia

Data from [WHO \(2011\)](#)

Guinea ([Kelly-Hope & McKenzie, 2009](#)), and 1594 in Uganda ([Yeka et al., 2005](#)). There can be great diversity in EIR rates even within limited geographical areas ([Kelly-Hope & McKenzie, 2009](#)). Alternative indicators of transmission intensity have been suggested, such as prevalence of antimalaria antibodies ([Corran et al., 2007](#)) (see Section 1.2.3), or multiplicity of infection (e.g. as defined by the measurement of number of *P. falciparum* strains infecting a single host) ([Arnot, 1998](#); [Babiker et al., 1999](#)), but neither of these methods has been widely adopted in large-scale epidemiological studies.

Categories of transmission intensity for *P. falciparum* malaria have been described based on exposures in children aged 2–10 years and measured by EIR rates, parasite reproductive numbers, the prevalence of parasites in peripheral blood, and frequency of splenomegaly. Holoendemic malaria transmission can be defined as parasite prevalence of > 70% and splenomegaly in > 80% of children. Shown in [Fig. 1.3](#) is a world map of *P. falciparum* endemicity ([Gething et al., 2011](#)). In this map, malaria endemicity has been categorized based on the age-standardized annual mean *P. falciparum* parasite rate in children aged 2–10 years (PfPR_{2–10}). Four endemicity classes are shown, with the highest being holoendemic (e.g. PfPR_{2–10} > 40%, intense, stable year-round transmission). The highest endemicity of *P. falciparum* transmission is seen across equatorial Africa and in Papua New Guinea. The categorization of transmission intensity is especially relevant for understanding the burden of disease

due to *P. falciparum* malaria, for which there is an epidemiological correlation between holoendemic malaria and increased risk of endemic Burkitt lymphoma (see Section 2). Papua New Guinea is the only other region of the world where the intensity of *P. falciparum* transmission approaches that seen in Africa ([Gething et al., 2011](#)).

The ability of the mosquito vector to survive in a given environment is a critical factor in determining the transmission intensity of malaria. For example, malaria transmission has long been known to decrease with increasing altitude. Transmission of *P. falciparum* is common at altitudes of < 1500 m. However, episodic transmission of malaria has been reported in areas at altitudes above 2000 m, depending on vector dynamics and environmental influences ([Cooper et al., 2009](#)). Modifiable factors that influence the existence of the disease include climate change, effective and available antimalarial therapy, intense use of chemically treated bednets, and residual indoor spraying. Recent reports of reduction in the levels of *P. falciparum* transmission in Africa have been attributed to implementation of control strategies ([WHO, 2011](#)), but the risk of infection rebounds if vector-control strategies are relaxed.

Table 1.2 Estimated number of cases of malaria and percentage due to *Plasmodium falciparum* in 2010

WHO Regional Office	Total no. of cases of malaria	Cases of malaria due to <i>P. falciparum</i> (%)	Total no. of malaria deaths due to <i>P. falciparum</i>
Africa	174 000 000	98	596 000
Americas	1 000 000	34	1000
Eastern Mediterranean	10 000 000	82	15 000
Europe	200	5	0
South-east Asia	28 000 000	54	38 000
Western Pacific	2 000 000	77	5 000
All	216 000 000	91	655 000

Data from [WHO \(2011\)](#)

1.2.3 Biological markers of infection and susceptibility

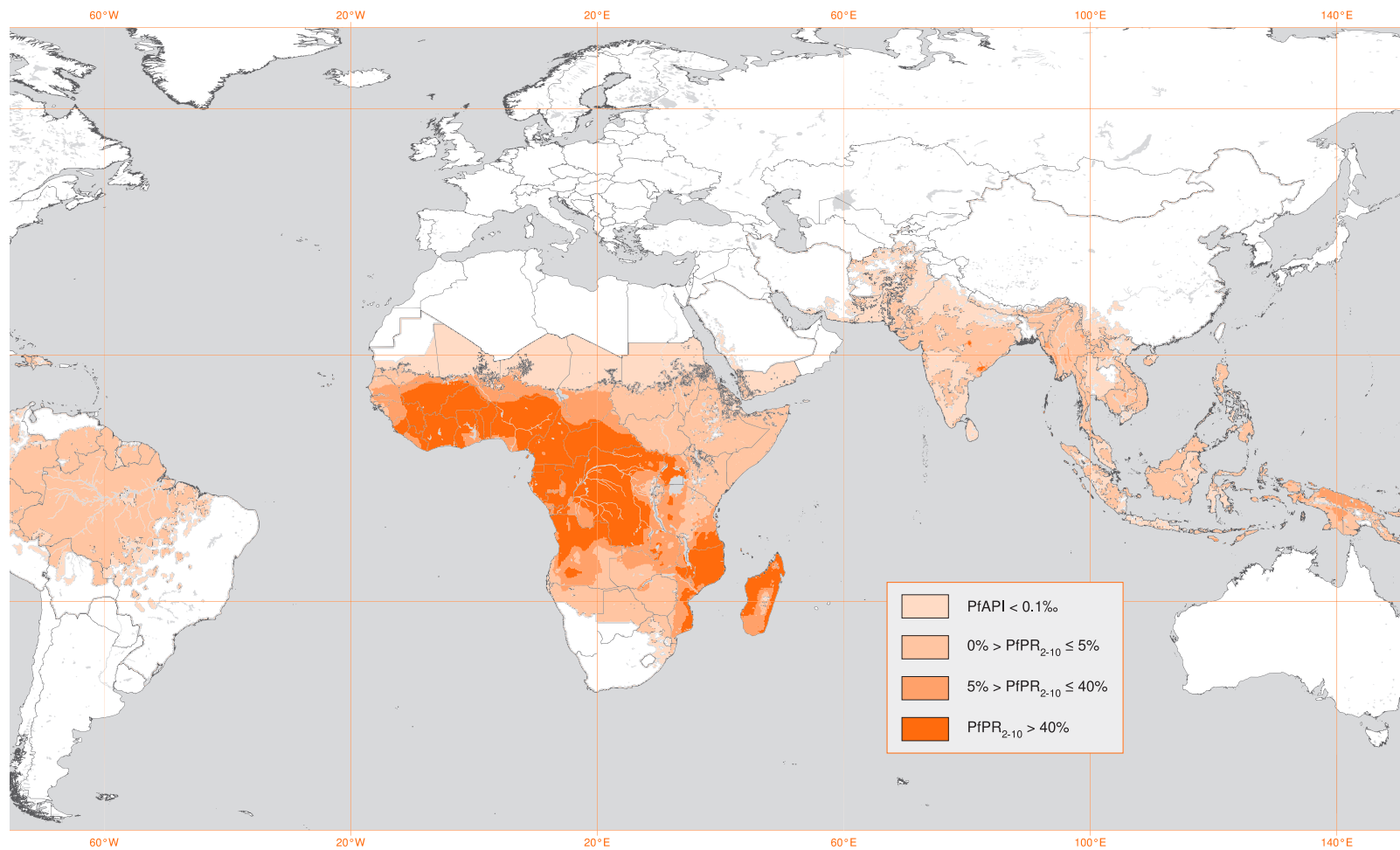
Diagnosis of the erythrocytic stage of infection can be a challenge in many countries. Routine laboratory diagnosis is done by microscopic examination to detect parasitized erythrocytes in Giemsa-stained thick and thin films, from samples of blood obtained by finger prick ([Krafts et al., 2011](#)); however, this method relies on the availability of good microscopes, power sources, and trained microscopists. Because of these limitations, WHO has recommended that biomarker-based rapid diagnostic tests become the standard if feasible ([WHO, 2010](#)). These tests are based on presence of the parasite lactose dehydrogenase antigen or histidine-rich protein 2 (HRP-2) antigen in the blood ([Wongsrichanalai et al., 2007](#)). The sensitivity and specificity of these tests were found to be similar to those of microscopic detection ([Abba et al., 2011](#)). In the research setting, quantitative polymerase chain reaction (PCR) is used alongside microscopy ([Malhotra et al., 2005](#)). While serology is useful in understanding the epidemiology of malaria, it is not used diagnostically.

Defining the correlations of protective humoral immunity to *P. falciparum* has presented a challenge to scientists for several reasons (reviewed in [Marsh & Kinyanjui, 2006](#)). This is even more evident in malaria holoendemic

regions, where antibodies to *P. falciparum* are often short-lived in children ([Kinyanjui et al., 2007](#); [Crompton et al., 2010](#)) and only increase with age and repeated infections ([Crompton et al., 2010](#)). Thus, because of the short half-life of immunoglobulin G (IgG)-specific antimalaria antibodies in children, use of antibodies as a marker of past exposure to *P. falciparum* is not always a reliable indicator. For example, in one study, children with a documented infection with *P. falciparum* and a *P. falciparum* IgG-antibody response to merozoite surface antigen 1 (MSP-1₁₉), (MSP-2) type A and B, apical merozoite antigen 1 (AMA-1) ectodomain, and region II of the 175 kDa erythrocyte-binding antigen (EBA-175II) was detected, but follow-up of the same children showed that the specific antibody response was lost ([Kinyanjui et al., 2007](#)). In addition, the choice of malaria antigen to be used as a marker of infection can be problematic because of the well-documented genetic variation in the surface antigens of *P. falciparum* ([Marsh & Kinyanjui, 2006](#)).

Several different assays have been used to measure antibody responses to *P. falciparum* ([Marsh et al., 1989](#)), but the most common is currently an enzyme-linked immunosorbent assay (ELISA). New technologies involving multiplex bead-based immunoassays have also been used and have the advantage of measuring more

Fig. 1.3 Spatial distribution of *Plasmodium falciparum* malaria in 2010 stratified by endemicity class



Estimates of the age-standardized annual mean *Plasmodium falciparum* parasite rate in children aged 2–10 years (PfPR₂₋₁₀), within the spatial limits of stable transmission, stratified into four levels of risk. Areas of no risk and unstable risk (PfAPI < 0.1%) are also shown.

Adapted from © [Malaria Atlas Project \(2010\)](#) and © [Gething et al. \(2011\)](#)

than one antigen in a limited sample volume ([Asito et al., 2010](#)). While no direct comparison was made in this particular study, other reports have found that sensitivity is similar to that of ELISA-based methods ([Smits et al., 2012](#)). Most epidemiological studies of *P. falciparum* infection and susceptibility are based on candidate vaccine antigens, because most research has focused on development of a vaccine against this parasite. Several different antibodies against antigens derived from either the blood stage [e.g. merozoite surface protein 1 (MSP-1), serine repeat antigen 5 (SERA5, SE36) and apical membrane antigen 1 (AMA-1)] or the liver stage of infection [e.g. liver stage antigen 1, LSA-1; circumsporozoite protein 1, CSP-1] have been evaluated, but the value of using these antibodies as markers of exposure to *P. falciparum* in children living in areas where holoendemic transmission occurs is unclear. However, measurement of antibodies to antigens derived from whole schizont extracts is thought to be a good estimate of past exposure to *P. falciparum*. The serology for detection of malaria infection is complicated, and no clear consensus has emerged about which antibodies are protective ([Asito et al., 2010](#)).

The evolutionary burden of malaria on the human population is seen in the large number of inheritable genetic mutations occurring in erythrocytes including, for example, a variant of the haemoglobin beta gene, haemoglobin S (*HbS*), that causes sickle-cell disease in homozygotes. The advantage for the host is suggested by epidemiological studies demonstrating that heterozygotes for the sickle-cell gene (i.e. genotype *HbAS*; who exhibit the sickle-cell trait) are protected from the high parasite densities and severe disease that characterize infection with *P. falciparum* ([Lell et al., 1999](#); [Aidoo et al., 2002](#)). Other human haemoglobinopathies such as thalassaemias and glucose 6-phosphate dehydrogenase deficiency also provide protection against infection. [Table 1.3](#) summarizes genetic polymorphisms that are known to be involved

in attenuating the severity of disease (see also Section 4.1.3).

Unlike *P. falciparum*, the distribution of *P. vivax* in African populations may be restricted by the absence of the erythrocytic Duffy antigen in many Africans. It has been suggested that the Duffy antigen is necessary for the entry of the *P. vivax* merozoite into an erythrocyte ([Howes et al., 2011](#)), although a very recent study has cast doubt on the strict requirement for this antigen for infection ([Mendes et al., 2011](#)).

1.2.4 Pathology of infection: malaria

The pathology associated with exposure to *Plasmodium* differs significantly by species of *Plasmodium* parasite, age of the infected person, and intensity of transmission of the parasite. All infected hosts experience fever and chills associated with the periodicity of the rupture of the infected erythrocytes. Differences in the symptoms and severity of disease caused by the different *Plasmodium* species are described in [Table 1.4](#).

Uncomplicated malaria occurs after infection with any *Plasmodium* species and is characterized by fever, chills and sweating in the majority of patients. If host immunity is adequate the infection can be cleared, but without treatment with effective antimalarial drugs, recrudescence of parasitaemia and relapse of symptoms can occur. Other symptoms include headache, nausea, myalgia, and vomiting ([Trampuz et al., 2003](#)). In contrast, complicated or severe malaria is characterized by anaemia, cerebral malaria and metabolic acidosis, the main causes of death due to malaria infection. Other complications of severe malaria include pulmonary oedema, acute renal failure, and hypoglycaemia ([Newton et al., 1998](#)).

The greatest burden of morbidity and mortality is associated with complicated falciparum malaria and the most vulnerable

populations are pregnant women (Section 4.1.2), children aged less than 5 years, and the elderly.

In sub-Saharan Africa where *P. falciparum* is holoendemic, more than 50% of children are parasitaemic at any given point in time ([Høgh, 1996](#)). Under these conditions, *Plasmodium* infection in children is recurrent, and the child's immune system is under constant stress from repeated *Plasmodium* infections (see Section 4.1.2). Complicated malaria is associated with severe anaemia, the major reason for malaria-related hospital admissions in Africa. Severe malarial anaemia is characterized by: haemoglobin, < 5 g/dL; haematocrit (erythrocyte volume fraction), < 15%; respiratory distress; and peripheral parasitemia ([Marsh et al., 1995](#)). The mechanisms of pathogenesis are poorly understood, but are linked to enhanced clearance of erythrocytes in the spleen from the periphery ([Weatherall et al., 2002](#)). Cerebral malaria is another complication of severe falciparum malaria and is linked to sequestration of the infected erythrocytes in the cerebral vasculature ([Weatherall et al., 2002](#)).

In sub-Saharan Africa where malaria transmission is endemic, pregnant women can be infected with *P. falciparum* and have evidence of infection of the placenta at the time of birth ([Desai et al., 2007](#)). Infection of pregnant women with *P. falciparum* has considerable adverse effects on maternal health and fetal morbidity and mortality ([Desai et al., 2007](#); [Rogerson et al., 2007](#)). Infection of the placenta can cause intrauterine growth retardation and premature delivery, resulting in low-birth-weight infants or stillbirth ([Desai et al., 2007](#)). Although the greatest burden of placental malaria is in primigravida women, women of all gravidities are at risk ([Desai et al., 2007](#)).

While initial studies on placental malaria focused on birth outcomes, more recent studies have found that infants born to mothers with malaria have altered innate and adaptive immune responses. Several groups have reported that sensitization to parasitic antigens occurs *in*

utero. This exposure affects the development of specific antimalaria antibodies to prevent infection and also infant T-cell responses at birth, with a shift towards T-helper 2 (Th2) mediated responses in cord blood mononuclear cells from parasitized placenta ([Ismaili et al., 2003](#); [Dent et al., 2006](#); [Metenou et al., 2007](#); [Flanagan et al., 2010](#)). Skewing of the gamma delta T-cell repertoire and altering responses to toll-like receptor (TLR) signalling in cord blood of infants born to mothers with placental malaria also indicate an effect of placental malaria on innate immunity ([Adegnika et al., 2008](#); [Cairo et al., 2008](#)). Another mechanism by which placental malaria could affect infant immunity was suggested by the recent finding that tolerance to antigens encountered *in utero* develops through establishment of regulatory T cells ([Mold et al., 2008](#)). In support of this, cord blood of infants born to mothers with placental malaria showed an expansion of both malaria-specific and general regulatory CD4⁺ T cells, as characterized by expression of FOXP3 ([Malhotra et al., 2009](#); [Flanagan et al., 2010](#)). Long-term follow-up of these infants found that they had a greater risk of malaria over time, and evidence of T-cell anergy ([Malhotra et al., 2009](#)). Thus a consequence of exposure to malaria *in utero* could be significantly reduced ability of an infant to respond to *Plasmodium* and to other infections encountered early in life, and an increased susceptibility to infectious diseases encountered subsequently during infancy.

Placental malaria could have an additional role in increasing infant susceptibility to heterologous infections. An important factor in protection against infections during infancy is the transfer of protective maternal antibodies across the placenta. However, several studies have demonstrated that placental malaria and maternal hyper-gammaglobulinaemia are associated with a significant reduction in the transfer of maternal IgG, including antibodies specific for measles, herpes simplex virus 1 (HSV-1), respiratory syncytial virus (RSV), tetanus, and

Table 1.3 Some genetic polymorphisms involved in resistance to malaria caused by *Plasmodium falciparum*

Condition	Gene [variant]	Proposed protective mechanisms	References
Sickle cell	Haemoglobin beta [Haemoglobin C] [Haemoglobin E]	Reduced cyto-adherence of infected erythrocytes Reduced erythrocyte invasion by merozoites, lower intra-erythrocytic parasite growth, and enhanced phagocytosis of infected erythrocytes.	Agarwal et al. (2000) , Modiano et al. (2001) Hutagalung et al. (1999) , Chotivanich et al. (2002)
	[Haemoglobin S]	Selective sickling of infected sickle-trait erythrocytes leading to enhanced clearance by the spleen. Reduced erythrocyte invasion, early phagocytosis, and inhibited parasite growth under oxygen stress in venous microvessels. Enhancement of innate and acquired immunity.	Cholera et al. (2008)
α -Thalassaemia β -Thalassaemia	Haemoglobin alpha Haemoglobin beta	Reduced rosetting. Increased micro-erythrocyte count in homozygotes reduces the amount of haemoglobin lost for given parasite density, thus protecting against severe anaemia.	Allen et al. (1997) , May et al. (2007) , Fowkes et al. (2008)
-	Glucose-6-phosphate dehydrogenase	G6PD deficient erythrocyte sensitive to oxidant stress causes its protection against parasitization.	Allison & Clyde (1961) , Bienzle et al. (1972) , Ruwende et al. (1995) , Tishkoff et al. (2001)
-	Pyruvate kinase	Invasion defect of erythrocytes and preferential macrophage clearance of ring-stage-infected erythrocytes.	Durand & Coetzer (2008)
Ovalocytosis	Solute carrier family 4 anion exchanger 1	Inhibition of merozoite entry into the erythrocyte, impairment of intracellular parasite growth and prevention of the erythrocyte lysis that occurs with parasite maturation, leading to release of merozoites into the blood stream.	Cortés et al. (2004, 2005)
Elliptocytosis	Alpha spectrin	Erythrocytes resistant to invasion	Facer (1995)
-	Glycophorins A, B, E	Erythrocytes resistant to invasion	Wang et al. (2003)
-	ABO blood groups	Reduced <i>P. falciparum</i> resetting in group O individuals	Rowe et al. (2007) , Barragan et al. (2000) , Paré et al. (2008)
-	HLA-B	Development of specific immunity	Hill et al. (1991) , Gilbert et al. (1998) ; Young et al. (2005)
-	Haptoglobin	Oxidative damage to uninfected cells might be more marked in haptoglobin-polymorphic individuals since haptoglobin proteins bind less efficiently to haemoglobin, increasing premature destruction of erythrocytes and stimulating cytokine release by these circulating cells.	Elagib et al. (1998) , Quaye et al. (2000) , Cox et al. (2007)

Table 1.3 (continued)			
Condition	Gene [variant]	Proposed protective mechanisms	References
-	Nitric oxide synthase 2	Increased NO production induces T-helper 1 cytokines, which activate macrophages and could thus be an antimalarial resistance mechanism.	Kun et al. (2001) , Hobbs et al. (2002)
-	Haemoxygenase I	Release of free haem in the bloodstream	Pamplona et al. (2007) , Garcia-Santos & Chies (2010)
-	TLR1, TLR4, TLR9	<i>P. falciparum</i> glycosylphosphatidylinositol induces signalling via TLR4 and hemozoin-induced immune activation involves TLR9.	Coban et al. (2005) , Mockenhaupt et al. (2006) , Leoratti et al. (2008a)

Adapted from [Driss et al. \(2011\)](#)
HLA, human leukocyte antigen; TLR, tool-like receptor; NO, nitric oxide

Table 1.4 Clinical course of infection with *Plasmodium* in humans

Parasite	Liver schizogony period (days)	Relapse	Erythrocytes parasitized	Severe malaria	More severe complications
<i>P. falciparum</i>	5–6	No	All	Yes	Cerebral malaria, acute renal failure, severe anaemia, adult respiratory distress syndrome
<i>P. vivax</i>	8	Yes	Reticulocytes	Yes (occasionally)	Splenomegaly
<i>P. ovale</i>	8	Yes	Reticulocytes	Rare	Rare
<i>P. malariae</i>	13	No	Mature erythrocytes	Rare	Nephrotic syndrome

Prepared by the Working Group

varicella zoster virus (VZV) ([Okoko et al., 2001a](#), [b](#); [Cumberland et al., 2007](#)).

The parasite burden associated with *P. vivax* infection is lower than that associated with *P. falciparum* infection, and the morbidity associated with *P. vivax* infection is thus less severe.

1.2.5 Prophylaxis and treatment of malaria

The treatment of malaria targets primarily the blood stage of *Plasmodium* infection. While chloroquine has been the therapeutic drug of choice, increasing resistance among all *Plasmodium* spp., particularly *P. falciparum*, has led to the adoption of artemisinin-based therapy ([Burki, 2011](#)). However, resistance to artemisinins has now been reported in a growing number of countries in south-east Asia, and WHO recommends that oral artemisinin-based monotherapies be withdrawn from the market and replaced with artemisinin-based combination therapy ([WHO, 2011](#)).

Intermittent preventive treatment of malaria has been recommended by WHO for preventive treatment of pregnant women and infants living in areas of high transmission of *P. falciparum*. Currently, 35 of 45 countries in sub-Saharan Africa and Papua New Guinea have adopted this policy. While intermittent preventive treatment is also recommended for infants living in regions of moderate to high malaria transmission, no countries have adopted this policy ([WHO, 2011](#)).

The past decades have seen the introduction of insecticide-treated bednets ([WHO, 2011](#)), and it is estimated that the number of bednets in sub-Saharan Africa increased from 5.6 million in 2004 to 145 million in 2010.

Studies on the efficacy of new drugs continue because the number of drugs that treat malaria effectively is limited and because of resistance issues ([Burrows et al., 2011](#)). Primaquine is the only known drug that targets the liver stage of infection and eradicates hypnozoites; however, primaquine can cause haemolytic anaemia in individuals with glucose-6-phosphate dehydrogenase deficiency, so widespread use of this drug for malaria-elimination campaigns is not feasible ([Beutler & Duparc, 2007](#)).

There is no currently licensed vaccine for any of the human *Plasmodium* pathogens. A vaccine targeting the blood stage of *P. falciparum* was recently tested in phase III trials and found to be about 50% efficacious in preventing clinical and severe malaria in infants and children in Africa ([Agnandji et al., 2011](#)).

2. Cancer in Humans

While malaria in humans is caused by several species of *Plasmodium*, including *P. falciparum*, *P. malariae*, *P. vivax*, *P. ovale*, and *P. knowlesi* (see Section 1), the majority of studies investigating

the link between malaria and cancer have focused on *P. falciparum* (the species linked to the most severe forms of malaria), or been conducted in areas where *P. falciparum* is highly prevalent. Unless otherwise stated, references to malaria in this chapter refer to malaria attributable to *P. falciparum*. The cancers investigated for a possible association with malaria include lymphoma ([Ross et al., 1982](#); [Cook-Mozaffari et al., 1998](#); [Vineis et al., 2000](#); [Tavani et al., 2000](#)), especially Burkitt lymphoma ([O'Connor & Davies, 1960](#); [Burkitt, 1961, 1969](#); [Burkitt & O'Connor, 1961](#); [Burkitt & Wright, 1963, 1966](#); [Wright, 1963](#)), Kaposi sarcoma ([Geddes et al., 1995](#); [Cottoni et al., 1997, 2006](#); [Ascoli et al., 2001](#); [Serraino et al., 2003](#)), cancer of the cervix ([Odida et al., 2002](#)), cancer of the prostate ([Thomas, 2005](#); [Elson et al., 2011](#)), nasopharyngeal carcinoma ([Yadav & Prasad, 1984](#); [Chen et al., 1990](#)), and cancer of the liver ([Welsh et al., 1976](#); [Lu et al., 1988](#)). Most research has focused on endemic Burkitt lymphoma and classical Kaposi sarcoma, both caused by gamma herpes viruses, Epstein–Barr virus (EBV) and Kaposi sarcoma-associated herpesvirus (KSHV), respectively. Research relating to other cancers is scant.

Exposure to malaria has been assessed by a variety of different methods: questionnaire, serology, or indirectly by measuring carriage of haemoglobin genotype abnormalities (e.g. sickle cell–haemoglobin C). The haemoglobin genotypes, because they are associated with partial protection against mild and severe malaria, are appropriate genetic markers for assessing lifetime exposure to malaria in populations living in areas where malaria is endemic. [Questionnaire measurement of exposure to malaria is subject to interviewer and recall bias, and asymptomatic malaria, if relevant, is impossible to measure by questionnaire. Measurement of antimalaria antibodies provides a more objective measure than the questionnaire approach, but is limited by several factors: the levels are subject to reverse-causality bias, misclassification, non-reproducibility, and

incomplete knowledge of the relevant markers of cancer risk. The use of haemoglobin genotypes, or any other genetic marker of susceptibility to malaria, is reproducible and not subject to reverse causality. It should be noted that the controls should have comparable environmental exposure to malaria. General limitations of the malaria case–control studies included limited understanding of how to measure malaria exposure (patent malaria, malaria genotype, or malaria diversity) and how to compare results for different assays from different time periods].

2.1 Burkitt lymphoma

2.1.1 Background

(a) Historical aspects

The earliest mention of tumours consistent with Burkitt lymphoma in the medical literature can be traced back to the medical notes of Albert Cook, shortly after his arrival in Uganda in 1894. Anecdotal reports suggest that doctors who worked in the region were familiar with the different manifestations of the disease, and wood carvings depicting jaw tumours suggest that the disease preceded colonial intervention. However, it was a paper by Denis Parsons Burkitt describing “a sarcoma involving the jaws in African children” that established that “sarcomas” previously thought to be independent entities were in fact a single disease ([Burkitt, 1958](#)). Subsequently, the clinical ([Burkitt & O'Connor, 1961](#)), radiological ([Davies & Davies, 1960](#)), and pathological ([O'Connor, 1961](#)) features of the disease were described, and the eponym “Burkitt lymphoma” applied to tumours with similar presentation. Description of the morphological, histochemical and cytological features of Burkitt lymphoma ([O'Connor & Davies, 1960](#); [Burkitt & O'Connor, 1961](#); [Wright, 1963](#)) led to cases with histology consistent with Burkitt lymphoma in Africa being reported worldwide, including in Papua New Guinea ([Reay-Young, 1974](#); [Lavu](#)

et al., 2005), Brazil ([Luisi et al., 1965](#)), Colombia ([Beltrán et al., 1966](#)), India ([Bai & Agrawal, 1967](#); [Date et al., 1970](#)), England, the USA ([Dorfman, 1965](#); [O'Connor et al., 1965](#); [Ziegler & Miller, 1966](#); [Burkitt, 1967](#); [Levine et al., 1982](#)), Malaysia ([Krishnappa & Burke, 1967](#)), China ([Ji & Li, 1992](#)), and the Republic of Korea ([Myong et al., 1990](#); [Choi et al., 2009](#)).

(b) Disease characteristics

A monoclonal B-cell non-Hodgkin lymphoma, Burkitt lymphoma is considered to be a single disease that occurs as three epidemiological and clinical subtypes, namely: endemic, sporadic and immunodeficiency-associated ([Leoncini et al., 2008](#)). Endemic Burkitt lymphoma is defined as affecting children in equatorial Africa and New Guinea, sporadic Burkitt lymphoma affects children and young adults throughout the world, and immunodeficiency-associated Burkitt lymphoma is primarily associated with HIV infection. These subtypes are indistinguishable by routine histopathological techniques. Endemic Burkitt lymphoma characteristically involves the jaw or other facial bones, distal ileum, caecum, ovaries, kidney or the breast. In the sporadic type, the ileum and caecum are the most common sites of involvement, and the jaw is less commonly involved.

(c) Molecular characterization

All three subtypes of Burkitt lymphoma are characterized by deregulation of the *MYC* gene, a master regulator of cellular differentiation, growth, and apoptosis ([Klein, 2009](#)). Deregulation of *MYC* is caused by chromosomal translocations that place the *MYC* coding sequence on chromosome 8 (8q24) next to the promoter sequences of genes encoding immunoglobulin heavy chains on chromosome 14 (14q32) in 80–90% of cases, or genes encoding kappa or lambda immunoglobulin light chains on chromosome 2 (2p12) and 22 (22q11), respectively, in 10–15% of cases ([Leoncini et al., 2008](#)). Although

translocation cannot be demonstrated in about 10% of cases ([Leucci et al., 2008](#)), these cases still show evidence of *MYC* deregulation.

It has been reported that Epstein–Barr virus (EBV) is detected in nearly 100% of cases of endemic Burkitt lymphoma; this proportion is smaller in cases of sporadic and immunodeficiency-associated Burkitt lymphoma ([Carbone et al., 2008](#)).

Endemic and sporadic Burkitt lymphomas differ at the molecular level, principally in the regions where chromosomal break-points occur ([Gutiérrez et al., 1992](#)). No specific epidemiological differences, other than geographical origin of the tumours, have been linked to the molecular differences in chromosomal break-points. One idea is that chromosomal break-points might be related to the timing of the translocation relative to B-cell differentiation and/or infection of the B cell with EBV ([Kuhn-Hallek et al., 1995](#)). No correlation has been shown between EBV positivity and specific chromosomal break-points ([Gutiérrez et al., 1992](#)).

2.1.2 Epidemiology of endemic Burkitt lymphoma

(a) Geographical clusters

Denis Burkitt was the first to comment on the uneven geographical distribution of Burkitt lymphoma ([Burkitt, 1961](#); [Burkitt et al., 1963](#); [Burkitt & Wright, 1966](#)). His maps showed that the highest incidence of Burkitt lymphoma was found in a broad belt around equatorial Africa, spanning from 10 degrees north to 10 degrees south, with an extension of the belt southward along a thin coastal rim in Mozambique ([Burkitt, 1963](#)). Within this belt, cases showed climatic restriction. Few cases were recorded at altitudes higher than 4000 m above sea level, where temperatures can fall below 16 °C, in places such as Kigezi in Western Uganda, Kilimanjaro in the United Republic of Tanzania, Rwanda, or Burundi. Many cases were recorded

in lowland regions with yearly rainfall of 50 cm or less ([Burkitt, 1962a, b, c](#); [Haddow, 1963](#)). Outside Africa, cases of Burkitt lymphoma were seen in Papua New Guinea ([Booth *et al.*, 1967](#); [Lavu *et al.*, 2005](#)), which has a tropical climate, and in tropical regions of Brazil ([Burkitt, 1967](#)).

The discovery of EBV in cultured cells from Burkitt lymphoma examined by electron microscopy in 1964 ([Epstein *et al.*, 1964](#)) suggested that EBV could be involved in Burkitt lymphoma etiology. EBV was also discovered to be ubiquitous and to be spread *via* contact with saliva ([IARC, 2012](#)). Dalldorf first proposed an etiological link between Burkitt lymphoma and malaria ([Dalldorf, 1962](#); [Dalldorf *et al.*, 1964](#)), based on data from Kenya, Papua New Guinea, Malaysia, and Brazil, which suggested that Burkitt lymphoma was not common in any area where holoendemic or hyperendemic malaria did not exist. This hypothesis was supported by biological evidence showing that chronic infection with malaria stimulated the reticuloendothelial system and caused polyclonal B-cell stimulation and immunosuppression ([Greenwood *et al.*, 1970](#); [Barker & Powers, 1971](#)), which could influence the risk of developing Burkitt lymphoma.

The geographical correlation between Burkitt lymphoma and holoendemic malaria persisted at a country level. For example, in Uganda, the highest incidence of Burkitt lymphoma was observed in low-lying districts of Lango, West Nile, Madi, and Acholi, and the lowest incidence was observed in high-lying districts of Kigezi ([Wright & Roberts, 1966](#); [Wright, 1973](#)). Similar patterns were observed even at a micro-geographical level, such as in studies restricted to the districts of West Nile ([Williams *et al.*, 1974](#)). This geographical pattern in Uganda was also apparent in a recent study from the country ([Ogwang *et al.*, 2008](#)). In Kenya, the incidence is low in highland tribes, such as the Kalenjin, and high in lowland tribes, like the Luo in Kisumu ([Dalldorf *et al.*, 1964](#); [Rainey *et al.*, 2007a, b](#)). In the United Republic of Tanzania, cases were less

frequent in the Kilimanjaro highlands ([Kitinya & Lauren, 1982](#)), but more common in the lower regions of Mara ([Brubaker, 1984](#)). Similar patterns were also observed in Cameroon in West Africa ([Wright *et al.*, 2009](#)).

The geographical patterns of distribution of Burkitt lymphoma in Africa support the role of a mosquito-borne infection, now widely believed to be malaria ([Fig. 2.1](#); [Burkitt, 1962a, b, c](#)). The evidence supporting a link between Burkitt lymphoma and malaria is summarized in [Table 2.1](#).

[Cook & Burkitt \(1971\)](#) analysed the proportional distribution of Burkitt lymphoma across large geographical areas using data on several selected tumour types at hospitals in Kenya, Uganda, and United Republic of Tanzania. The relative frequency of Burkitt lymphoma, as a fraction of the seven tumours investigated, was highest around the lake shores of Lake Victoria and in northern Uganda along the River Nile. Burkitt lymphoma was rare or not reported in areas where malaria transmission season was shorter than 6 months, suggesting that the incidence of Burkitt lymphoma is influenced both by duration and intensity of exposure lasting for 6 months or more. Because this study covered many malaria-free areas that were densely populated, but remained tumour-free, the proportional distribution of cases was not explained by the underlying population distribution ([Kafuko & Burkitt, 1970](#)).

[Kafuko & Burkitt \(1970\)](#) conducted a detailed analysis of malaria and incidence of Burkitt lymphoma at the country level in Uganda between 1963 and 1966. Patterns of malaria distribution were obtained by examining thin and thick blood smears for malaria parasitaemia, supplemented by spleen surveys, in children in 100 schools and in 86 mass surveys in the general population [limited detail was provided on how schools and survey villages were selected]. Malaria endemicity was classified as hypoendemic (absolute parasite prevalence

in the age group 2–10 years, < 10%), mesoendemic (parasite prevalence, 11–50%), hyperendemic (if constantly > 50%), and holoendemic (if constantly > 75%), and the proportion of areas in the area with each level of malaria endemicity was calculated. Although no statistical comparisons were made, there was an obvious gradient in the incidence of Burkitt lymphoma from 0 per 100 000 children in the Kigezi Highlands (where 80% of the areas surveyed were malaria-free and 20% were hypoendemic), to 5.49 per 100 000 in the West Nile district (where 59% of the areas were holoendemic and 35% of the areas were hyperendemic).

[Schmauz *et al.* \(1990\)](#) found similar geographical patterns of incidence of Burkitt lymphoma and malaria endemicity in 18 districts of Uganda between 1966 and 1973.

[Kafuko *et al.* \(1969\)](#) investigated the pattern of Burkitt lymphoma and malaria at a micro-geographical level within the West Nile district of Uganda. Data on malaria were obtained from children in 17 schools and from 11 mass surveys. Data on Burkitt lymphoma were obtained from the Kuluva cancer registry. A strong geographical correlation was observed between areas with a high incidence of Burkitt lymphoma and areas with a high prevalence of malaria. In addition, a correlation was observed between the age at which malaria parasite prevalence peaked (age 0–4 years) and the age at which Burkitt lymphoma cases peaked. However, the overall prevalence of malaria parasitaemia was generally > 70% at most times in all areas and increased to 91% during some of the months in children in some age groups. These results highlighted the fact that only a few children among those exposed repeatedly to very high levels of malaria parasitaemia develop Burkitt lymphoma.

[Morrow *et al.* \(1976\)](#) investigated micro-geographical patterns of Burkitt lymphoma using data on 123 patients residing in the Mengo district of Uganda, diagnosed during 1959–68. The incidence rate of Burkitt lymphoma was lower in the

counties at higher altitude, presumably because the prevalence of malaria was lower. In addition, there was seasonal variation in the incidence of Burkitt lymphoma, which was attributed to seasonal changes in the prevalence of malaria ([Morrow *et al.*, 1977](#)). The incidence of Burkitt lymphoma declined during the period of observation and was attributed to a fall in the burden of malaria in the Mengo district caused by socio-economic and health-care improvements and widespread distribution of chloroquine. The fall in the incidence of Burkitt lymphoma appeared to be specific, since no changes were noted for other cancer diagnoses during the same period.

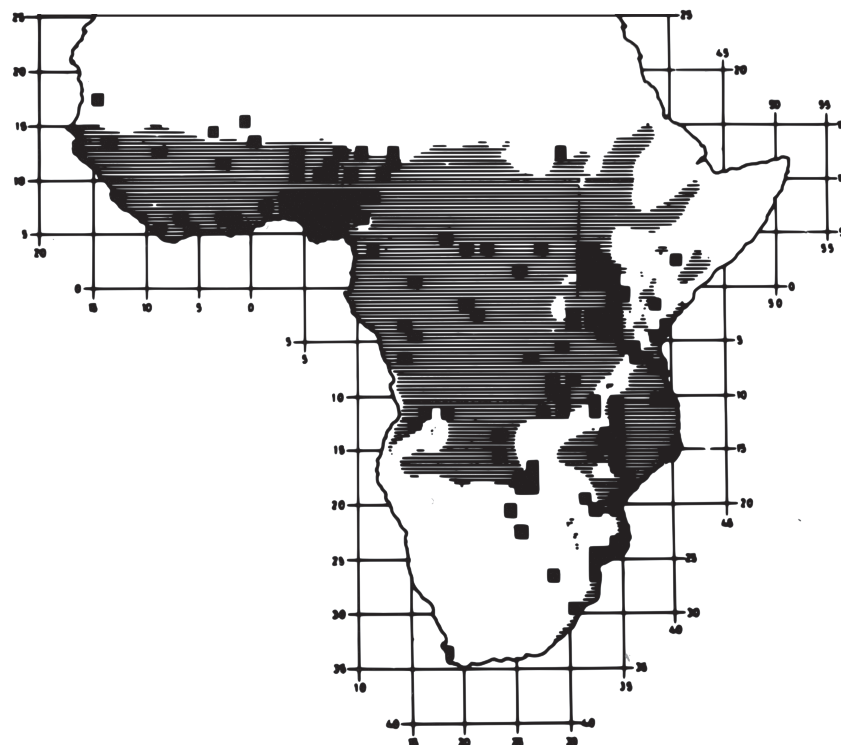
A role for malaria in the etiology of Burkitt lymphoma was also suggested by studies in migrants in Uganda. [Burkitt & Wright \(1966\)](#) reported that migrants from Rwanda, Burundi, and Kigezi district in Uganda (areas where both malaria and Burkitt lymphoma are rarely if ever seen) to Buganda (a subnational kingdom within Uganda), where both malaria and Burkitt lymphoma are endemic, showed susceptibility to Burkitt lymphoma that was approximately equal to that of the indigenous population. Notably, the incidence of Burkitt lymphoma was 0.49 per 100 000 among adult immigrants aged 16–45 years *versus* 0.10 per 100 000 in this age group among the locally born, further suggesting that malaria infection serves as the trigger mechanism of onset of Burkitt lymphoma ([Morrow *et al.*, 1976](#)).

The age at diagnosis of Burkitt lymphoma varied inversely according to malaria endemicity. In Mengo district, in southern Uganda, the median age was 8.2 years for areas of Mengo where malaria transmission was mesoendemic, and 7.8 years in Acholi and 6.6 years in Lango, two areas where malaria transmission is holoendemic ([Morrow *et al.*, 1977](#)).

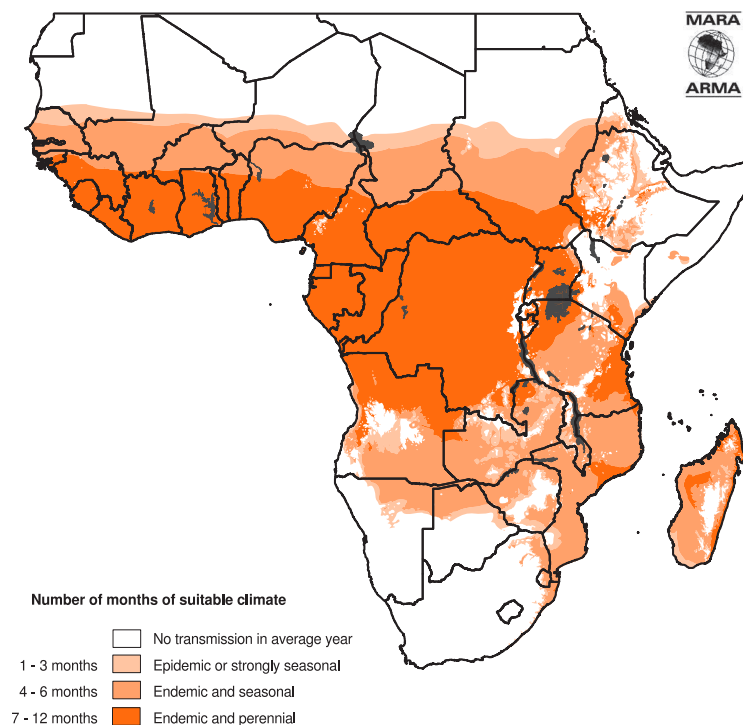
In Ghana, [Biggar & Nkrumah \(1979\)](#) observed that cases of Burkitt lymphoma originated from predominantly rural areas, based on a study of 236 cases treated at Korle Bu Hospital, Accra, in

Fig. 2.1 Estimated distribution of Burkitt lymphoma and of malaria in Africa

A: The Burkitt lymphoma belt of Africa



B: Duration of the malaria transmission season



A. The shaded area represents that in which, on climatic grounds, Burkitt lymphoma might be expected to occur. The black squares show the distribution of the series of cases compiled by Denis Burkitt ([Haddow, 1963](#))

B. The map is based on theoretical stably local climatic, and therefore the potential duration, onset and end of the malaria transmission season, in the average year.

The dark orange areas represent regions where hyperendemic or holoendemic transmission of *P. falciparum* malaria is expected to occur 7–12 months per year.

Note: Based on a theoretical model from available long-term climate data, the map is reasonably accurate but since it is not based on actual malaria data it may not reflect the real malaria status.

From [MARA/ARMA \(2001\)](#)

Table 2.1 Ecological evidence supporting a link between malaria and endemic Burkitt lymphoma

1	Geographical coincidence of Burkitt lymphoma and holoendemic or hyperendemic <i>P. falciparum</i> malaria (see Fig. 2.1 , Panel A and Panel B)
2	Lower risk of Burkitt lymphoma in malaria-protected areas, e.g. urban areas or highlands, or areas with cool temperatures
3	Coincidence of peak age of maximum titres of antimalarial immunoglobulin and peak age at which Burkitt lymphoma develops
4	Coincidence of malaria splenomegaly, a marker of chronic exposure to malaria, and Burkitt lymphoma
5	Elevated risk of Burkitt lymphoma in migrants from areas with a low intensity of malaria to areas of high intensity, with the age at developing Burkitt lymphoma reflecting age at migration (Burkitt & Wright, 1966)
6	Protective effect of sickle-cell trait against development of Burkitt lymphoma
7	Protective effect of malaria suppression, eradication, or use of malaria prevention strategies
8	Time-space clustering and reports of seasonal variation in Burkitt lymphoma correlated with seasonal malaria

Prepared by the Working Group

1970–75. This pattern was consistent with a role for repeated exposure to malaria in the etiology of Burkitt lymphoma. In a separate cross-sectional survey of malaria, [Biggar et al. \(1981\)](#) reported a malaria prevalence of 1.4% in urban areas and 22% in rural areas. Unlike Uganda, where seasonal variation in the incidence of Burkitt lymphoma was reported ([Morrow et al., 1977](#)), no seasonal variation was observed in Ghana ([Biggar & Nkrumah, 1979](#)).

[Rainey et al. \(2007a, b\)](#), have reported ecological evidence linking the incidence of Burkitt lymphoma to risk of malaria at a population level in two studies conducted in Kenya. In the first study ([Rainey et al., 2007a](#)), the incidence of paediatric Burkitt lymphoma in Kenya was estimated using data on 960 histologically verified cases diagnosed between 1988 and 1997. Risk of malaria was classified based on recent estimates of transmission intensity at the district level as low risk, arid/seasonal, highland, endemic coast, and lakeside endemic. The 10-year average annual incidence rate varied by malaria endemicity from 0.39 per 100 000 in low-risk regions, 0.25 in arid/seasonal regions, 0.66 in highland, 0.68 in endemic coast and 1.23 in endemic lake areas ($P = 0.002$). The odds ratio (OR) for Burkitt lymphoma in regions with chronic and intense malaria transmission compared with regions

with no or sporadic transmission was 3.47 (95% CI, 1.30–9.30).

In the second study, focusing on a smaller geographical region, [Rainey et al. \(2007b\)](#) conducted an analysis of micro-geographical variation of Burkitt lymphoma in Nyanza province, including areas with holoendemic as well as seasonal malaria. Data was obtained for cases diagnosed between 1999 and 2004 at New Nyanza Province General Hospital, the largest hospital with facilities to diagnose and treat Burkitt lymphoma in the province. Analysis was performed using two appropriate cluster-detection methods (Anselin's Local Moran test for spatial autocorrelation and a spatial scan test statistic) to identify significant clustering or "hot spots" with a very high incidence of Burkitt lymphoma and "cold spots" with a low incidence of Burkitt lymphoma. Significant clustering was identified in five locations in the western region of Kisumu district: Central Kisumu, East Seme, South Central Kisumu, South West Kisumu and West Kisumu, with incidence rates of 2.1–8.0 cases per 100 000 children; and in two locations in Nyando District: Kakola/East Kano and North Nyakach, with incidence rates of 3.1 and 4.4 cases per 100 000 children, respectively. A cold spot was identified in Nyamira, Kisii and Gucha districts, including some areas where

malaria transmission was holoendemic and some areas where malaria transmission was seasonal. [Although the hot spots may be influenced by access to infrastructure, including roads to the hospital, this factor was not considered an important cause of bias because a large administrative area (directly east of Kisumu city) with a large population with good access to roads had few cases. These results support a role of environmental cofactors in “hot spot” activity, possibly micro-geographical malaria endemicity.] This variation in the incidence of Burkitt lymphoma at a micro-geographical level is a consistent feature that was reported by Ogwang *et al.* in Uganda (Ogwang *et al.*, 2008). [Case ascertainment was incomplete.]

(b) Time–space clusters

Time–space clusters of Burkitt lymphoma (defined as cases occurring closer in time and space than expected by chance) were first reported by Pike *et al.* (1967) and Williams *et al.* (1969) in the West Nile district in Uganda. The first report included 36 cases of histologically confirmed Burkitt lymphoma diagnosed in 1961–65, and the second report included an additional 29 cases diagnosed in 1966–67. Clustering was assessed and the results were consistent with significant time clusters, at intervals ranging from 30 to 360 days, and distance clusters, ranging from 2 to 40 km. At least five cases were diagnosed in one village (Aliba) in West Nile within 2 years (Pike *et al.*, 1967). No epidemiological evidence of personal contact between the cases was demonstrated, [suggesting clustering was likely to be due to area-wide changes in cofactors rather than person-to-person contact]. Clustering activity was apparent in a larger analysis including 200 cases from West Nile in 1965–75 (Williams *et al.*, 1978).

Morrow *et al.* reported a cluster of seven cases occurring over 27 months (October 1966 to December 1968) in Bwamba county in Uganda (Morrow *et al.*, 1971). However, no evidence of

clustering was observed in other parts of southern Uganda, including Mengo district (Morrow *et al.*, 1976), or elsewhere in Africa, including in the Mara region in northern United Republic of Tanzania (Brubaker *et al.*, 1973; Siemiatycki *et al.*, 1980), or in Ghana (Biggar & Nkrumah, 1979). Observation of clusters ignited interest in discovering cofactors that might influence prevalence and intensity of etiological exposures over larger areas or longer time intervals that might be responsible for area-wide drift of Burkitt lymphoma in Africa. However, negative data from other parts of Africa, coupled with lack of epidemiological data supporting interpersonal contact between affected individuals in the clusters, and the large time and space intervals in the reported clusters reduced enthusiasm for characterizing clusters of Burkitt lymphoma as a means of elucidating shared environmental etiology.

Van den Bosch *et al.* (1993b) investigated clustering in 146 cases of Burkitt lymphoma diagnosed during July 1987 and October 1989 in Malawi. Cases in children aged > 8 years were closer together in time and space than would be expected by chance.

2.1.3 Correlation between age at diagnosis of Burkitt lymphoma and malaria biomarkers

Emmanuel *et al.* (2011) correlated proportional age distribution of Burkitt lymphoma with age distribution of malaria biomarkers. The authors hypothesized that, given the rapid growth rate of Burkitt lymphoma (Iversen *et al.*, 1972), and estimates that the latent interval from onset to diagnosis might be as short as 6–8 months (Williams *et al.*, 1974), the malaria exposures that influence onset of disease should have an age-specific pattern similar to that of Burkitt lymphoma. Data on Burkitt lymphoma were obtained from four well-characterized data sets from Ghana (1965–89) (Nkrumah & Olweny, 1985), Uganda (1991–2006) (Parkin

[et al., 2010](#)), and (1997–2006) ([Ogwang et al., 2008](#)), and the United Republic of Tanzania (1960–2009) ([Geser et al., 1989](#)). Data on malaria were compiled age-specifically from published studies conducted in the same countries ([Smith et al., 1999](#); [Peyerl-Hoffmann et al., 2001](#); [Owusu-Agyei et al., 2002](#)). Data on malaria included prevalence of malaria parasites in peripheral blood, geometric mean parasite density, and mean multiplicity of malaria genotypes, defined as the number of malaria genotypes per positive blood sample based on the merozoite surface protein-2 (MSP-2) ([Smith et al., 1999](#); [Peyerl-Hoffmann et al., 2001](#); [Owusu-Agyei et al., 2002](#)). Strong and significant correlations were observed between the age-specific pattern of Burkitt lymphoma and the age-specific mean number of multiplicity of *P. falciparum* genotypes in all study regions (Pearson correlation coefficients ranging from 0.77 to 0.91 ([Fig. 2.2](#) for two study regions in Ghana and United Republic of Tanzania)). Both the incidence of Burkitt lymphoma and parasite multiplicity peaked between age 5 and 9 years, and then declined gradually. This was in contrast to the prevalence of parasites in peripheral blood and geometric mean parasite density, which both peaked at ages 2 and 3 years and decreased thereafter, gradually for the former and rapidly for the latter ([Fig. 2.2](#)). [The Working Group noted that these results were ecological, were based on data spanning many years when epidemiological techniques for the study of malaria were changing, the populations correlated may not have been overlapping, and the completeness and accuracy of the cases were uncertain. This study was valuable for highlighting the limits in our understanding of the exact nature of malaria exposure relevant to the pathogenesis of Burkitt lymphoma and hence the most appropriate biological measure of malaria exposure most proximally linked to risk of Burkitt lymphoma.]

[Taken together, correlation studies have provided the most consistent evidence for a link between Burkitt lymphoma and falciparum

malaria, but at a population level. Interpretation of the results must be cautious because the results do not measure relationship between exposure and risk at the individual level.]

2.1.4 Cohort studies

Only one cohort study has examined the link between Burkitt lymphoma and malaria. [De-Thé et al. \(1978\)](#) conducted a prospective study to examine the impact of infection with EBV on the risk of Burkitt lymphoma. In northern Uganda, 42 000 children aged 4–8 years were recruited and followed over time for development of Burkitt lymphoma. At recruitment, blood was collected from every child and presence of the malaria parasite was evaluated by thick and thin blood films. Subsequently, 14 cases of Burkitt lymphoma were diagnosed. Within this cohort, EBV was analysed in 14 cases and 69 representative controls from the same population. There were three types of control (four or five per case) selected as follows: (1) serum from a neighbour of the same age and sex selected at random from the main survey; (2) four controls from the serum bank from children of the same age, sex, and locality as the child with Burkitt lymphoma and bled at the same time; (3) serum from a random sample of the surveyed population. There were no marked differences between the number of malarial parasites in children with Burkitt lymphoma before diagnosis and in controls, but cases at diagnosis had significantly fewer parasites than controls [possibly because cases had been given antimalarial drugs. No numbers or levels of parasitaemia were available in the publication].

2.1.5 Case-control studies

Ten case-control studies have investigated the link between Burkitt lymphoma and malaria ([Williams, 1966](#); [Pike et al., 1970](#); [Ziegler et al., 1972](#); [Feorino & Mathews, 1974](#); [Nkrumah &](#)

[Perkins, 1976](#); [Nkrumah et al., 1979](#); [Carpenter et al., 2008](#); [Mutalima et al., 2008](#); [Asito et al., 2010](#); [Guech-Ongey et al., 2011](#)) ([Table 2.2](#), [Table 2.3](#)). Most studies were previously reviewed in *IARC Monograph Volume 70* ([IARC, 1997](#)) and in *IARC Monograph Volume 100B* ([IARC, 2012](#)). Two studies, conducted since the last IARC evaluation ([Asito et al., 2010](#); [Guech-Ongey et al., 2011](#)) are reviewed here for the first time.

(a) *Exposure assessed using antibody assays*

[Ziegler et al. \(1972\)](#) investigated the frequency, type of malaria, and immunological response to malaria in 100 patients with Burkitt lymphoma treated at the Uganda Cancer Institute between 1967 and December 1970. No significant differences were noted in titres of antimalaria antibodies measured by immunofluorescence assay at admission among 17 cases and 18 controls [selection of controls was not fully described] ([Table 2.2](#)). [The Working Group noted that there were few controls in the study and the source population was not described, suggesting that neither may have been ideal. Although the controls were matched by tribe, age, and sex to the cases, no further information was provided on age and sex, both important risk factors for Burkitt lymphoma. The Working Group was uncertain how to evaluate the malaria markers, especially given that antibodies were not correlated with parasitaemia and splenomegaly, both measures of high malaria burden.]

[Feorino & Mathews \(1974\)](#) compared titres of antimalaria antibodies, measured by indirect immunofluorescence assay and indirect haemagglutination assay in 60 patients with Burkitt lymphoma aged 4–15 years *versus* 60 controls matched on age (aged 3–16 years), sex, tribe and residence. Titres of > 1:16 were considered as positive. No statistically significant difference was found in prevalence between cases and controls by indirect immunofluorescence (95% in cases *versus* 93% in controls) or by indirect haemagglutination (65% *versus* 67%) or in the

geometric mean titres. [Antimalaria antibodies were measured in blood samples taken after use of chloroquine in the cases, which could explain the null association. In addition, the cellular immune response was not measured, which may have consequences for understanding the role of immunity to malaria infection. The limitations of this study included small sample size. The Working Group noted that the patients with Burkitt lymphoma, perhaps even controls, from Feorino & Mathews were a subset of those described in the study by [Ziegler et al. \(1972\)](#), but it was not possible to determine whether the 17 patients with Burkitt lymphoma evaluated for antimalaria antibodies were included in [Ziegler et al. \(1972\)](#). The subject population was not adequately described, so potential for selection bias, lack of information on potential confounders and the appropriateness of the exposure measures could not be evaluated.]

[Nkrumah et al. \(1979\)](#) investigated patterns of total immunoglobulin (Ig) as well as malaria-specific IgG, IgA, and IgM levels in 56 patients with newly diagnosed Burkitt lymphoma (age 4–14 years) and 56 apparently healthy, nearest-neighbour controls, individually matched on age, sex, tribe, and residence in Ghana. The malaria-specific IgG and IgM antibodies for the schizont antigen of *P. falciparum* and *P. malariae* were measured using the indirect immunofluorescence assay in cases and controls. Children with Burkitt lymphoma had lower values of total immunoglobulin (IgG, IgA, and IgM) than did the nearest-neighbour controls (Mann–Whitney U test statistic, $P < 0.001$ for IgG, IgA, and IgM). Antibody titres for *P. falciparum* were higher than those for *P. malariae*, reflecting higher intensity of transmission of *P. falciparum* and tendency of the human host to respond to higher antibodies to *P. falciparum* than *P. malariae*. IgG antimalaria antibody titres to *P. falciparum* (and *P. malariae*) did not differ between cases and controls. However, IgM antibody titres to *P. falciparum*, but not *P. malariae*, were statistically

significantly lower in cases of Burkitt lymphoma than controls ($P < 0.001$). The inverse association between Burkitt lymphoma and IgM levels was unclear. [The Working Group noted that measurement of serum total, as well as malaria-specific, IgA, IgG, and IgM levels at 1–2 months and again at 12 months in 18 patients revealed no change in Ig levels in samples from pre-treatment and post-remission periods, suggesting that the low levels of Ig found might be related to abnormalities in Ig synthesis or catabolism in the patients, but not due to a disease effect. The Working Group noted small sample size and uncertainty about the reliability of assays for antimalaria antibodies as limitations of these early studies.]

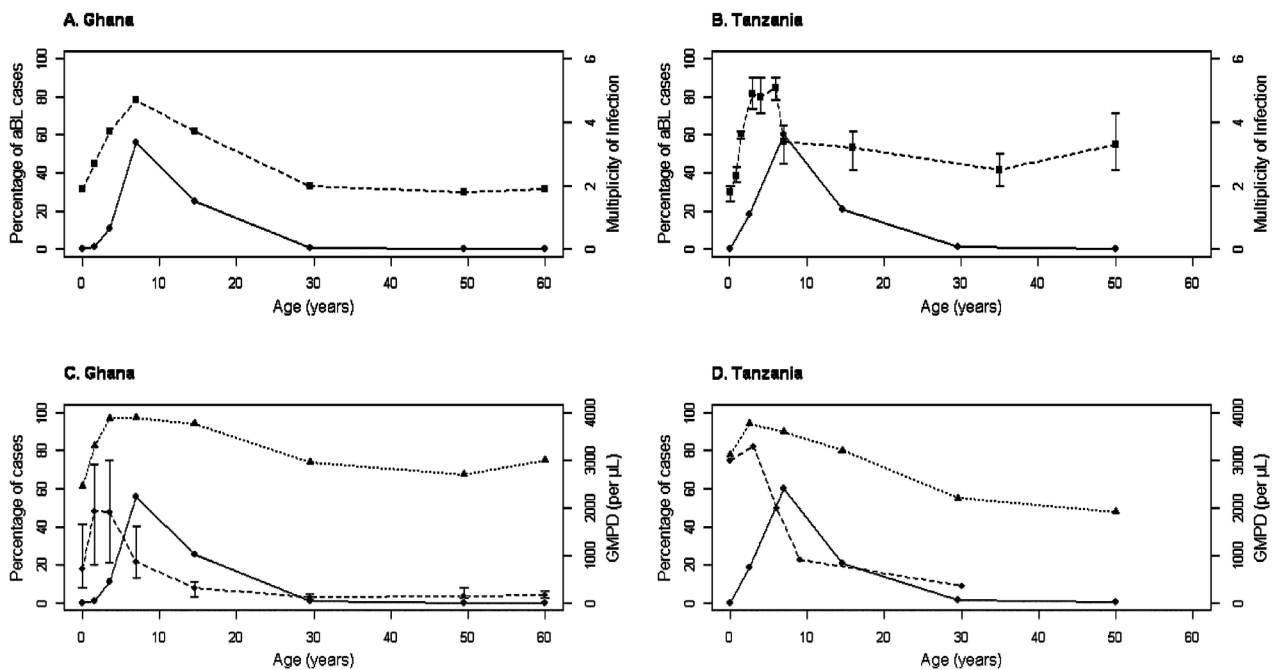
[Carpenter et al. \(2008\)](#) investigated the association between Burkitt lymphoma and malaria in 325 cases and 579 controls aged 0–14 years in Uganda who were seronegative for HIV. The cases were clinically diagnosed and histologically confirmed as Burkitt lymphoma at the Uganda Cancer Institute and the controls were other children diagnosed with non-malignant conditions or non-lymphatic cancers at the same hospitals. Compared with controls, cases were more likely to report a history of frequent treatment for malaria in the past year (OR, 2.0; 95% CI, 1.3–3.1) and less likely to report use of insecticides at home (OR, 0.2; 95% CI, 0.1–0.3). Among 126 cases and 70 controls with data available on antibodies, compared with those with negative values for antimalaria antibodies (9.5% of cases *versus* 22.8% of controls), the odds ratio for Burkitt lymphoma was 2.5 (95% CI, 1.6–3.6) for children with low titres of antimalaria antibodies and it was 3.4 (95% CI, 1.7–6.7) for children with high titres of antimalaria antibodies (P for trend = 0.05). When stratified by urban *versus* rural districts, these associations were stronger and statistically significant in the rural districts. [A test for interaction was not provided; the stratum-specific analyses were sparse. The strengths of the study included using a combination of questionnaire methods and antibody

measurement to assess exposure to malaria; restriction of analyses to HIV-negative subjects, and an effort to histologically verify diagnosis in many cases (recent studies of lymphoma pathology in low-resource settings have indicated variable accuracy of clinical and local pathology diagnosis, thus, misclassification is a concern. However, the results are typical for Africa). The limitations included the relatively small sample with results for antimalaria antibodies, which reduced the effective power of the study to observe interactions, and the possibility of reverse causality and biases in the study could not be excluded. The Working Group noted that all children in this study were definitely exposed to malaria and negative results for antibodies may reflect false-negative results or low sensitivity of the assays currently used.]

[Mutalima et al. \(2008\)](#) conducted a case-control investigation of 148 children aged 0–14 years and diagnosed with Burkitt lymphoma in Malawi. The controls were 104 children admitted to the same hospital with non-malignant conditions or cancers other than haematological malignancies and Kaposi sarcoma. Malaria infection was assessed by questionnaire and ELISA for antibody to the whole schizont. The mean age for cases was 7.1 (SD, 2.6 years) and for controls it was 5.1 (SD, 3.9 years). Among children with data on use of mosquito nets (20% of cases and 24% of controls), the odds ratio for Burkitt lymphoma was 0.2 (95% CI, 0.03–0.9) for those who reported using a mosquito net compared with those who did not. Compared with children who had negative or low titres of antimalaria antibodies (cases, 29%; controls, 53%), the odds ratio for Burkitt lymphoma was 2.4 (95% CI, 1.2–4.4) in those with medium or high antimalaria antibodies (P for trend, < 0.01). [The limitations of the study included incomplete diagnostic verification of cases, and uneven age distribution of cases *versus* controls.]

[Asito et al. \(2010\)](#) compared levels of anti-bodies to three *P. falciparum* antigens: the merozoite

Fig. 2.2 Characteristics of cases of Burkitt lymphoma in Africa correlated with mean multiplicity of infection, prevalence, and geometric mean parasite density for *Plasmodium falciparum* malaria



Age group intervals for the cases were plotted according to the age groups used in the malaria papers.

A-B: The percentage of Burkitt lymphoma cases by age (—) and mean multiplicity of *P. falciparum* malaria parasites (---), defined as the average number of distinct genotypes per positive blood sample based on the merozoite surface protein-2 (MSP-2) assessed by polymerase chain reaction in Ghana and the United Republic of Tanzania.

C-D: The percentage of Burkitt lymphoma cases per age (—), the prevalence of *P. falciparum* malaria (...), and the geometric mean parasite density (---) in the general population in Ghana and United Republic of Tanzania. (Age group intervals for the cases are plotted according to the age groups used in the malaria papers.)

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surface protein-1 (MSP-1), liver stage antigen-1 (LSA-1), and apical membrane antigen-1 (APM-1) in 32 cases of Burkitt lymphoma obtained from a large referral hospital and 25 controls enrolled from a region in Western Kenya where malaria is endemic and individually matched on age and sex. No differences between cases and controls were noted in levels of IgG to all *P. falciparum* antigens. This result was specific for anti-malaria antibodies because differences were noted between cases and controls for anti-EBV antibodies. [The strengths of this study included examining multiple antibodies to malaria, including those linked to protective immunity to malaria (MSP-1, LSA-1) and histological verification of cases. The limitations included the small sample size, which could have reduced the

power of the study to observe statistically significant results, and lack of geographical matching of cases to controls. The cases were more likely to have been treated with antimalarial medication than were the controls and this could explain the inverse association. Also, the focus of the study was EBV, not malaria.]

[Guech-Ongey et al. \(2011\)](#) compared antibodies to SE36 antigen in 657 cases of Burkitt lymphoma (92% microscopically confirmed; age 0–14 years) and 498 controls. SE36 is a recombinant protein based on the Pf-SERA5 gene, and a target for protective malaria immunity ([Aoki et al., 2002](#); [Okech et al., 2006](#)). The cases were enrolled at the Korle-Bu Teaching Hospital, Accra, Ghana, during 1965–94. The controls were apparently healthy children enrolled from

Table 2.2 Case-control studies examining the association between endemic Burkitt lymphoma and malaria

Reference, Study location, and period	Characteristic of cases of Burkitt lymphoma	Characteristics of controls	Detection method	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjusted potential confounders Comments
Ziegler et al. (1972) Uganda 1967–70	100 histologically confirmed cases (age 0–14 yr); malaria results available only for 17 cases	18 age-, sex-, and tribe-matched hospital-based controls	Malaria parasitemia by microscopy; FAT	FAT ≤ 1:4 FAT 1:8–1:32 FAT ≥ 1:64	3 8 6	1.0 [7.11 (0.82–69.34)] ^a [2.29 (0.32–19.10)] ^a	FAT performed on samples obtained at admission. Interpretation of FAT levels is uncertain as levels were unrelated to presence of parasitaemia, malaria splenomegaly, disease stage, or response to therapy. The selection of controls was not ideal as matching for age, sex, and residence was not performed. Overlaps partially with Feorino & Mathews (1974) ^b
Feorino & Mathews (1974) Uganda	60 histologically confirmed cases (age 4–15 yr)	60 matched on sex, age, tribe, and residence; age 3–16 yr	IIF and IHA	<i>IIF</i> Negative Positive <i>IHA</i> Negative Positive	3 57 21 39	1.0 ^a [1.35 (0.22–9.66)] ^a 1.0 [0.93 (0.41–2.11)] ^a	The subjects were a subset of those included in the study by Ziegler et al. (1972) , thus the results do not represent a totally independent experiment ^b
Nkrumah et al. (1979) Ghana	56 histologically confirmed cases (age 4–14 yr)	56 matched on sex, age, tribe and residence	Antimalarial IgG against <i>P. falciparum</i> schizont by IIF	< 1:16 dilution factor 1:16 1:64 1:256 1:1024 1:4096	0 0 1 8 18 29	- - - 1.0 ^a [0.90 (0.24–3.40)] [1.04 (0.29–3.66)]	This study carefully individually matched the controls to the case on age, sex, tribe and residence and used population-based controls.
			Antimalarial IgM against <i>P. falciparum</i> schizont by IIF	< 1:4 dilution factor 1:4 1:16 1:64 1:256	35 9 11 0 1	1.0 ^a [0.32 (0.10–1.00)] [0.28 (0.10–0.80)] - -	

Table 2.2 (continued)

Reference, Study location, and period	Characteristic of cases of Burkitt lymphoma	Characteristics of controls	Detection method	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjusted potential confounders Comments
Carpenter <i>et al.</i> (2008) Uganda 1994–99	325 HIV-negative cases confirmed by local histology or cytology (age ≤ 15 yr)	579 HIV-negative controls (age ≤ 15 yr) admitted for orthopaedic conditions (447) or cancers other than lymphoma or leukaemia (132)	Antibodies to <i>P. falciparum</i> malaria by IIF; 126 cases and 70 controls	<i>Antimalaria IIF antibody levels:</i> Negative Low (1:64 or 1:256) High (≥ 1:1024)	12 72 42	1.0 (0.4–2.5) 2.5 (1.6–3.6) 3.4 (1.7–6.7) <i>P</i> = 0.05	Age, sex, district, household income, and tribe/slight differences in rural/urban distribution of the cases and age distribution of cases. Questionnaire data on use of mosquito bed net, past history of malaria showed associations compatible with malaria antibody levels.
Mutalima <i>et al.</i> (2008) Malawi 2005–06	148 children (age ≤ 15 yr) (including 9 HIV-positive cases), 109 confirmed by local histology or cytology	104 children (age ≤ 15 yr) admitted for non-malignant conditions (11) or cancers other than lymphoma or leukaemia (93)	ELISA for <i>P. falciparum</i> schizont extract (PfSE) for 139 cases and 96 controls	<i>OD readings used as a surrogate for malaria antibody titres categorized as:</i> Negative/low (OD < 0.2) Medium/high (OD ≥ 0.2) <i>P</i> -value <i>Bed net use</i> No Yes	38 91 12 17	1.0 2.4 (1.2–4.4) <i>P</i> < 0.01 0.2 (0.03–0.9)	Age, sex, and residence/the controls were younger than cases. Similar to the Uganda study, questionnaire data with mosquito bed net use were consistent with a role for malaria.

Table 2.2 (continued)

Reference, Study location, and period	Characteristic of cases of Burkitt lymphoma	Characteristics of controls	Detection method	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjusted potential confounders Comments
Asito <i>et al.</i> (2010) 2007–08	32 children (age ≤ 15 yr) (1 HIV-positive case), all cases confirmed by local histology or cytology	25 children (age ≤ 15 yr) randomly selected from the community age and sex matched to the case	Malaria measured by microscopy of thick smear, and by luminex bead assay for IgG to AMA-1, MSP-1, and LSA-1 antigens at 1:6400 dilution	Difference in levels of <i>P. falciparum</i> IgG titres compared by Mann–Whitney U test.	13% of cases positive for parasites in blood vs 68% in controls; Relative titres of IgG to <i>P. falciparum</i> antigens similar in cases and controls		The patients were probably treated with antimalarial drugs before admission
Guech-Ongey <i>et al.</i> (2011) 1965–94	657 children (age ≤ 15 yr) (none was HIV-positive), 92% confirmed by local histology or cytology	498 children (age ≤ 15 yr) nearest neighbour control from age and sex matched to the case	ELISA for SE36 antibodies; end dilution titres categorized as tertiles:	<i>Anti-SE36 IgG antibody titres</i> High Medium Low <i>P</i> -trend	168 217 271	1.0 1.33 (0.96–1.86) 1.67 (1.21–2.32) <i>P</i> = 0.002	Age, sex, enrolment calendar period, and test plate. SE36 is recombinant protein under evaluation as a blood-stage malaria vaccine candidate. High antibody titres have been linked with lower risk of severe malaria and lower mean parasite levels. High titres of anti-SE36 antibodies may be considered as partially protective against severe malaria.

^a Odds ratios are approximate because matching was ignored.

^b 60 cases in the Feorino & Mathews study were part of the study by [Ziegler *et al.* \(1972\)](#)

AMA-1, apical merozoite antigen 1; ELISA, enzyme-linked immunosorbent assay; FAT, immunofluorescent antimalarial antibody titre; Ig, immunoglobulin; IHA, indirect haemagglutination assay; IIF, indirect immunofluorescence; LSA-1, liver stage antigen 1; MSP-1, merozoite surface protein; NR, not reported; OD, optical density; SE36, serine repeat antigen 5; vs, *versus*

Table 2.3 Case–control studies examining the association between endemic Burkitt lymphoma and haemoglobin electrophoretic patterns

Reference, Study location, and period	Characteristic of cases of Burkitt lymphoma	Characteristics of controls	Haemoglobin genotype	No. of exposed cases	Adjusted potential confounders/comments
Williams (1966) Nigeria 1960–65	100 Yoruban cases histologically confirmed (age 5–15 yr); results presented for 95 cases only with HbAA and HbAS.	331 hospital Yoruban children attending the same hospital over 5 yr with known haemoglobin results, same age and living in a comparable area; results based on 320 controls with only HbAA and HbAS (sickle-cell trait)	HbAA	78	Retrospective study using routinely collected data; hospital controls may have conditions associated with abnormal haemoglobin; study limited to one tribe, although Burkitt lymphoma occurs in other tribes.; results suggest decreased incidence of Burkitt lymphoma in children with HbAS (sickle-cell trait)
			HbAS	17	
Pike et al. (1970) Uganda	36 histologically confirmed cases	50 neighbourhood controls matched on age, sex, tribe, and place of residence	HbAA HbAS	30 6	Small sample size; limited power to observe significant associations; results suggest decreased incidence of Burkitt lymphoma in children with HbAS (sickle-cell trait)
Nkrumah & Perkins (1976) Ghana	110 histologically confirmed cases	112 neighbour controls matched by same age, sex, and tribe	HbAA	90	Cases individually matched with one or more unrelated nearest-neighbourhood children of the same age, sex, tribe, and residence; results suggest decreased incidence of Burkitt lymphoma in children with HbAS (sickle-cell trait)
			HbAS	11	
		110 patient-sibling controls	HbAA HbAS	85 12	Siblings of the case, closest in age, were chosen as sibling controls; results suggest decreased incidence of Burkitt lymphoma in children with HbAS (sickle-cell trait)

HbAA; wild-type haemoglobin gene AA; HbAS, variant haemoglobin gene AS (sickle-cell trait)

the neighbourhood of the cases, and matched for age, sex and calendar time. Children with Burkitt lymphoma had statistically significantly decreased mean log end-point dilution titres [0.63 logs lower] than controls (Student *t*-test, $P = 0.019$). Compared with children with high anti-SE36 IgG antibody tertiles, the odd ratios for Burkitt lymphoma were 1.33 (95% CI, 0.96–1.86) for children with medium SE36 tertiles and 1.67 (95% CI, 1.21–2.31) for children with low anti-SE36 tertiles (P for trend = 0.002). The children with Burkitt lymphoma in Ghana may have had lower titres of antibodies to SE36 antigen than matched controls from the same community [suggesting that high anti-SE36 titres may be protective for Burkitt lymphoma, akin to the protective effect suggested for severe malaria] ([Aoki et al., 2002](#); [Okech et al., 2006](#)). [The strength of this study was the large size, selection of age-, sex- and residence-matched controls, and the use of anti-SE36 antimalaria antibodies, which have been associated with reduced risk of severe malaria in epidemiological studies in Africa ([Aoki et al., 2002](#); [Okech et al., 2006](#)), to assess exposure to malaria. An inverse association would suggest that antibodies that are protective for severe malaria or malaria parasitaemia may be protective for Burkitt lymphoma. Lack of data on antibody to the whole schizont was a limitation.]

(b) *Exposure assessed indirectly by haemoglobin genotype*

Three studies indirectly assessed the role of malaria in Burkitt lymphoma by determining haemoglobin type, including HbAS, which is responsible for the sickle-cell trait. Young children with HbAS are substantially protected against severe falciparum malaria ([Allison, 1963](#)).

[Williams \(1966\)](#) conducted a retrospective comparison of electrophoretic patterns of haemoglobin protein (which reflect haemoglobin genotypes for sickle-cell disease) in 100 Yoruban children (aged 5–15 years) with microscopically

confirmed Burkitt lymphoma during 1960–1965 and 331 Yoruban children of the same age and living in a comparable area treated at the hospital during the same period. The P value for the association between carriage of protective genotypes (HbAS, HbSS, HbAC, HbCC) and Burkitt lymphoma was < 0.10 . Restricted to 95 cases and 320 controls with HbAA and HbAS abnormalities (excluding HbSS, HbCC, HbAC, HbCC), children with Burkitt lymphoma had 18% carriage of HbAS compared with 30% in controls ([Williams, 1966](#)). [The limitations of the study included use of a retrospective design, use of controls selected from children attending the hospital, which may be related to malaria or abnormal haemoglobin genotypes. Haemoglobin electrophoresis results were based on routine tests, but these may have varied over the 5 years covered by the study. The Working Group noted that authors' decision to exclude children aged 0–4 years was considered reasonable because of survivor bias among the children with the sickle-cell trait. The hospital controls may be biased to children with conditions associated with malaria or HbAS.]

[Pike et al. \(1970\)](#) compared haemoglobin electrophoretic patterns among 36 cases of microscopically confirmed Burkitt lymphoma from the Uganda Cancer Institute with those of 50 control children individually matched on age, sex, tribe and residence. The results suggest a decreased incidence of Burkitt lymphoma among HbAS children.

[Nkrumah & Perkins \(1976\)](#) compared haemoglobin electrophoretic patterns in 110 microscopically confirmed cases with controls individually matched to the nearest neighbouring child of the same age, sex, and tribe ($n = 112$), or to the sibling closest in age to the case ($n = 110$). In subgroup analysis, carriage of haemoglobin C (HbAC) was associated with a slight, but non-significant, protective effect against Burkitt lymphoma (one-sided $P < 0.1$).

2.1.6 *Intervention studies*

Only one study to control Burkitt lymphoma through malaria intervention has been implemented. [Geser *et al.* \(1989\)](#) reported the results of a study the aim of which was to prevent Burkitt lymphoma through suppression of malaria in children in the Mara region in northern United Republic of Tanzania [the population varied between 177 000 and 200 000 during the study period] where the incidence of Burkitt lymphoma had been monitored through careful registration of cases since 1964 ([Eshleman, 1966](#); [Brubaker *et al.*, 1973](#); [Siemiatycki *et al.*, 1980](#); [Geser & Brubaker, 1985](#)). Malaria suppression was implemented in the North Mara region through mass distribution of bi-weekly doses of chloroquine to children aged < 10 years, starting in 1978. The burden of malaria was monitored by survey, and the impact of the programme on the incidence of Burkitt lymphoma was monitored by registering all new cases in North Mara [intervention region] as well as in neighbouring South Mara [non-intervention region].

The prevalence of malaria parasitaemia in the intervention region fell from [24%, prevalence in lowland and highland areas of North Mara] in the baseline survey in May 1976 to 11% in 1977 and 13% in 1978. The prevalence in non-intervention communities was higher and did not fall during the intervention (28% in 1976 to 37% in 1978) ([Geser *et al.*, 1989](#)). The fall in the prevalence of malaria parasitaemia was transient and started rising, in part, due to programme failures in the drug distribution system ([MacCormack & Lwihula, 1983](#)), and, in part, due to development of chloroquine resistance ([Draper *et al.*, 1985](#)), and surpassed the high levels that had prevailed before the programme before 1977. The programme was abandoned in 1981.

Notably, the incidence of Burkitt lymphoma fell rapidly in the intervention villages from about 4 per 100 000 during 1964 and 1977 (range, 2.6–6.9 per 100 000 children) before intervention

and fell to about 1 per 100 000 children during 1977 to 1982 (range, 0.5–3 per 100 000). The lowest incidence of Burkitt lymphoma during the entire observation period (0.5 per 100 000) was recorded during the intervention period. However, the incidence of Burkitt lymphoma was falling before the malaria-suppression trial. The fall in incidence was transient, and although it continued falling for about 1–2 years after malaria prevalence started rising, the incidence rose to 7.1 per 100 000 to surpass levels before the malaria-suppression intervention. [Some aspects of this analysis supported the association between malaria and Burkitt lymphoma, but others did not. A causal interpretation would suggest that the lag times would be similar between when incidence of Burkitt lymphoma started to fall after the malaria-suppression intervention was introduced and when it started increasing after malaria suppression was lost. The results were susceptible to errors in case ascertainment due to the short period of intervention and the small numbers of cases of Burkitt lymphoma, despite the large numbers of people participating in the intervention.]

2.1.7 *Cofactors*

(a) *EBV*

See [Table 2.4](#)

EBV is an established cause of Burkitt lymphoma and is a common childhood infection in sub-Saharan Africa ([IARC, 1997, 2012](#)). [Carpenter *et al.* \(2008\)](#) reported an odds ratio of 3.6 (95% CI, 2.3–5.6) and 4.5 (95% CI, 2.3–8.7) for Burkitt lymphoma in children with medium and high titres of anti-EBV antibody, respectively, compared with those with low or negative antibody titres in Uganda. The joint effects of EBV and malaria were examined by estimating the odds ratio for Burkitt lymphoma in children with raised levels of EBV antibody only, antimalaria antibody only, or both EBV and antimalaria antibody. Cases were five times more likely than

controls to have jointly elevated anti-EBV and antimalaria antibodies (OR, 5.0; 95% CI, 2.8–8.9), but not more likely to have elevated antibodies to either EBV or malaria only [but the confidence intervals associated with these odds-ratio estimates were wide].

[Mutalima et al. \(2008\)](#) reported odds ratios of 4.1 (95% CI, 1.6–10.1) and 14.8 (95% CI, 5.8–38.5) for medium and high anti-EBV antibody titres compared with low titres. Cases were 13.2 times more likely than controls to have jointly elevated anti-EBV and antimalaria antibodies (95% CI, 3.8 – 46.6, $P < 0.001$) compared with those with low antibodies to both EBV and malaria. The odds ratios were 1.4 (95% CI, 0.3–63) for those with elevated malaria antibodies only and 5.7 for those with elevated EBV antibodies only.

[Asito et al. \(2010\)](#) [described in Section 2.1.5] compared levels of IgG to EBV antigens – viral capsid antigen (VCA), EA_d, EBNA-1 and Zta – and EBV viral load in plasma in 32 children with histologically confirmed Burkitt lymphoma compared with 25 children matched on age and sex from one village in the study area. Cases of Burkitt lymphoma had significantly increased titres of anti-Zta ($P = 0.0017$) and VCA IgG levels ($P < 0.0001$), and plasma EBV viral loads ($P < 0.0001$) indicative of viral reactivation. Levels of EBNA-1 and EA_d-specific IgG were not different between cases and controls. Among the cases, those with tumours in the abdomen had significantly elevated anti-Zta IgG levels ($P < 0.0065$) and plasma EBV viral loads ($P < 0.033$) compared with patients with jaw tumours. [While malaria and EBV are the most widely accepted cofactors for endemic Burkitt lymphoma, the role of a “third” cofactor has been considered to account for several unexplained epidemiological features of endemic Burkitt lymphoma. For example, endemic Burkitt lymphoma predominates in males, but neither malaria nor EBV show significant disparity between the sexes. No additional cofactor has yet been found.]

The findings on the joint effects of EBV and malaria are biologically plausible given that malaria induces polyclonal stimulation of B cells ([Chêne et al., 2007](#)) and impairs anti-EBV T-cell responses ([Kataaha et al., 1984](#); [Facer & Playfair, 1989](#); [Langhorne et al., 1989](#)). The effect of malaria on EBV control, however, are transient because the EBV load in peripheral blood returns to lower levels when malaria is successfully treated ([Lam et al., 1991](#)). Two other issues remain unresolved: (1) are certain EBV strains more lymphomagenic than others? and (2) what is the relevant measurement of malaria exposure? [The Working Group noted that the risk of Burkitt lymphoma is only linked to the more fatal *P. falciparum*.]

(b) HIV

HIV is an established risk factor for AIDS-related Burkitt lymphoma ([IARC, 2008](#)). The risk of Burkitt lymphoma in people who are HIV-positive is 50 times higher than in the general population; however, the data relating HIV to endemic Burkitt lymphoma is scant and most cases are HIV-negative ([Newton et al., 2001](#); [Mutalima et al., 2008](#)). Furthermore, there are scant published data investigating the joint effect of HIV and malaria on Burkitt lymphoma ([IARC, 2012](#)). Despite a high prevalence of HIV in malaria-endemic areas, no epidemic increase in endemic Burkitt lymphoma has been described in Africa. [This might be due to low survival of HIV-infected children in Africa, or underascertainment.] Cases were more likely than controls to be HIV-positive in the study by [Mutalima et al. \(2008\)](#) (nine cases and two controls were HIV-positive), but they were not able to examine differences in antibodies to malaria or EBV by HIV status in Malawi.

2.1.8 Other factors

Several studies have investigated other factors or surrogates for malaria in relation to Burkitt lymphoma in malaria-endemic regions, but the interaction with malaria was not specifically examined. These include family/household characteristics ([Rainey et al., 2008](#); [Morrow et al., 1974a](#)), rural or urban status ([Biggar & Nkrumah, 1979](#)), arthropod-borne viruses ([Van den Bosch & Lloyd, 2000](#)), and exposures to plants ([Van den Bosch et al., 1993a](#)). In none of these studies was the evidence of malaria infection examined or controlled for.

2.2 Kaposi sarcoma

Kaposi sarcoma is a systemic disease presenting with cutaneous lesions that can also involve the oral cavity, lymph nodes, and viscera. It is caused by Kaposi sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV8). It was originally described by Moritz Kaposi, in 1872.

Infection with KSHV alone is not sufficient to cause Kaposi sarcoma. The most important cofactor predisposing a KSHV-infected person to Kaposi sarcoma is HIV co-infection or, to a lesser extent, other immunodeficient states such as iatrogenic immune suppression in organ transplant recipients. Before the HIV epidemic, classic (sporadic) Kaposi sarcoma represented up to 9% of all cancers in parts of sub-Saharan Africa, such as Uganda, in both men and women. The incidence of Kaposi sarcoma in specific geographic areas before the HIV epidemic points to a role of as-yet-unknown cofactors in the etiology of this cancer ([IARC, 2012](#)).

Only two cancer registry-based studies have examined the link between classical Kaposi sarcoma and malaria in Italy using case-control methodology ([Table 2.5](#); [Geddes et al., 1995](#); [Cottoni et al., 1997](#)). The studies looking at Kaposi sarcoma measured malaria ecologically,

using birth in a formerly malaria-endemic area to measure exposure to malaria. [The Working Group noted that the malaria infection relevant in these studies, although unknown, was likely due to infection by *P. vivax*, which was prevalent in Italy. Malaria was declared eradicated in Italy in the 1960s, thus, many individuals may have been exposed from childhood into adulthood. The lack of direct measurement of malaria and conducting the study many years after exposure to malaria complicates interpretation of the results of these studies.]

[Geddes et al. \(1995\)](#) investigated the association between classical Kaposi sarcoma and birth in a formerly malaria-endemic area in a case-control study. The cases were 204 patients with histologically confirmed classical Kaposi sarcoma (139 men, 65 women) aged 50 years or older (mean age, 70 years) identified from 11 cancer registries in Italy. The controls were 777 subjects with other cancers, matched for age (± 2 years), sex, and year of diagnosis, identified from the same registry. The odds ratio for Kaposi sarcoma in people born in an area where malaria was endemic was 2.98 (95% CI, 1.97–4.51), and was elevated both in northern (OR, 2.2) and southern (OR, 2.0) Italy, and remained statistically significant in multivariable analyses, including age, sex, registration area, age group, population density in 1936, and altitude. [The weakness of this study was the use of birth region as the best measure for exposure to malaria, which may not reflect actual individual exposure to malaria. The Working Group noted that there was malaria/geographical correlation with Kaposi sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), the established cause of Kaposi sarcoma, which may confound results. However, the strong associations in both northern and southern Italy suggest results may be valid.]

[Cottoni et al. \(1997\)](#) investigated the association between a past history of malaria and classical Kaposi sarcoma in a case-control study in northeastern Sardinia. The cases were 40 patients with

Table 2.4 Case-control studies examining the association between Burkitt lymphoma, malaria, and infection with Epstein-Barr virus

Reference, study location, and period	Characteristics of cases of Burkitt lymphoma	Characteristics of controls	Detection method	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjusted potential confounders Comments
Carpenter et al. (2008) Uganda 1994–99	325 HIV-negative cases, confirmed by local histology or cytology (age ≤ 15 yr)	579 HIV-negative controls (age ≤ 15 yr) admitted for orthopaedic conditions (<i>n</i> = 447) or cancers other than lymphoma or leukaemia (<i>n</i> = 132)	Antibodies to <i>Plasmodium falciparum</i> measured by IIF; 126 cases and 70 controls; EBV measured by ELISA	<i>Antimalarial antibody levels/ EBV antibodies</i> Low EBV titre: Low antimalarial titre (≤ 1:64) High antimalarial titre (≥ 1:256) High EBV titre: Low antimalarial titre (≤ 1:64) High antimalarial titre (≥ 1:256) <i>P</i> -trend	12 15 22 77	1.0 (0.4–2.4) 1.1 (0.5–2.4) 1.0 (0.5–2.2) 5.0 (2.8–8.9) <i>P</i> = 0.003	Age, sex, district, household income, and tribe. Malaria assessed in a smaller subset because of loss of samples during transit [related to 09/11/2001 disruptions], but demographical characteristics similar in tested <i>vs</i> not tested subjects.
Musalima et al. (2008) Malawi 2005–06	148 children (age ≤ 15 yr) (9 HIV-positive cases), 109 confirmed by local histology or cytology	104 children (age ≤ 15 yr) admitted for non-malignant conditions (<i>n</i> = 11) or cancers other than lymphoma or leukaemia (<i>n</i> = 93)	ELISA for <i>P. falciparum</i> schizont extract for 139 cases and 96 controls; EBV measured by IIF	<i>Antimalarial antibody levels/EBV antibodies</i> Low EBV titre: Low antimalarial titre (≤ 1:64) High antimalarial titre (≥ 1:256) High EBV titre: Low antimalarial titre (≤ 1:64) High antimalarial titre (≥ 1:256) <i>P</i> -trend	5 7 32 82	1.0 1.4 (0.3–63) 5.7 (1.6–20.7) 13.2 (3.8–46.6) <i>P</i> = 0.001	Age, sex, and residence

EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; IIF, indirect immunofluorescence; *vs*, *versus*

classical Kaposi sarcoma and the controls were 120 members of the general population matched on age, sex, and geographical residence. The matched odds ratio associated with a history of malaria was 1.21 (95% CI, 0.51–2.84; 20 exposed cases). The results were similar to those obtained in 1980 from the same group, where a history of malaria was reported by 19 out of 39 patients with classical Kaposi sarcoma, which was similar to that reported by adults of a similar age in the general population (about 50%). [Malaria in this study was likely to be attributable to *P. vivax*. The strength of this study was the ability to directly measure malaria exposure by questionnaire. However, the reliability and specificity of recall for historically remote infections is uncertain, raising concern about exposure misclassification. The study was small (40 cases) and was underpowered.]

2.2.1 Cofactors

KSHV/HHV-8 is a necessary cause of Kaposi sarcoma (IARC, 2012) and HIV is an established risk factor for Kaposi sarcoma. However, no studies have examined the link between malaria, KSHV, HIV, and Kaposi sarcoma.

2.2.2 Other factors

Several studies have investigated other factors or surrogates for malaria exposure in relation to Kaposi sarcoma in malaria-endemic regions, but evidence for interaction with malaria infection has not been examined specifically. The risk factors examined were exposure to volcanic soils (Montesu *et al.*, 1995; Simonart, 2006; Pelser *et al.*, 2009a), plants (Whitby *et al.*, 2007; Pelser *et al.*, 2009b), steroids (Goedert *et al.*, 2002), smoking (Goedert *et al.*, 2002), other parasites (Lin *et al.*, 2008), and genetic polymorphisms (Contu *et al.*, 1984).

2.3 Cancers at other sites

A few sporadic reports have reported associations between malaria and cancer at other sites, including the cervix (Odida *et al.*, 2002), prostate (Thomas, 2005; Elson *et al.*, 2011), nasopharyngeal carcinoma (Yadav & Prasad, 1984; Chen *et al.*, 1990), lymphoma (Ross *et al.*, 1982; Schmauz *et al.*, 1990; Cook-Mozaffari *et al.*, 1998; Tavani *et al.*, 2000; Vineis *et al.*, 2000) and liver (Welsh *et al.*, 1976; Lu *et al.*, 1988).

Odida *et al.* (2002) reported a geographical correlation between malarial endemicity and high-grade cervical cancer in Uganda among 457 cases ascertained in 1968–73. Based on 304 cases with complete data, the relative risk of high-grade cervical cancer in areas with severe malaria was 2.04 (95% CI, 1.37–3.04) compared with cases from an area with low malaria endemicity [the completeness of the cancer and population data in this study were uncertain].

Yadav & Prasad (1984) measured titres of antibodies to *P. falciparum* antigens in 22 patients with nasopharyngeal carcinoma and 43 controls. Antimalaria antibodies were present in all except one patient, and 14 patients had titres of > 80 and 4 patients > 640. Compared with controls, the mean titres of antimalaria antibodies for most age groups were higher in the patients. Those patients with high titres of antimalaria antibodies also had high titres of IgA anti-VCA, an antibody that is diagnostic for nasopharyngeal carcinoma [the role of reverse causality and small size of the study were concerns].

Chen *et al.* (1990) investigated the role of malaria in nasopharyngeal carcinoma in Taiwan, China, among 347 cases and in population-based controls enrolled from a household register and matched to cases on age and sex. Self-reported history of malaria was associated with an increased risk of nasopharyngeal carcinoma in males (OR, 2.1; 95% CI, 1.27–3.66) and females (OR, 2.17; 95% CI, 0.82–5.70).

[Elson et al. \(2011\)](#) investigated the association between prostate cancer and Duffy antigen/receptor for chemokines (DARC) in a case-control study of 81 patients *versus* age-matched controls enrolled from a phlebotomy laboratory in Jamaica. No association was found between lack of DARC expression and prostate cancer (OR, 1.0; 95% CI, 0.48–2.1). [DARC is required by *P. vivax* for infection, thus lack of expression is protective against infection by this species of *Plasmodium*. The relevance of this study for DARC as a marker of malaria-related exposure was uncertain because direct exposure of patients to malaria was not considered.] [The Working Group noted that the few reports of associations between malaria and cancer for isolated sites were interesting, but insufficient to inform about carcinogenicity attributable to malaria for these sites.]

2.4 Susceptible populations

Populations susceptible to endemic Burkitt lymphoma include children who are born into and live continuously in areas where malaria is holoendemic. Duration and intensity of exposure is important because episodic or seasonal occurrence of malaria, lasting for less than 6 months, does not appear to be closely related to the incidence of Burkitt lymphoma ([Burkitt, 1969](#)). Susceptibility to Burkitt lymphoma peaks between ages 5 and 8 years, when peak titres of antimalaria antibodies are attained [given the postulation that latency of Burkitt lymphoma is relatively short, the peak age may be related to a biological window when children are most vulnerable owing to their B-cell physiology, or when the probability of exposure to a particular malaria strain peaks]. In a few small studies in Uganda, adults migrating from areas where malaria was not endemic to hyperendemic or holoendemic areas were at increased risk of the acute consequences of malaria and increased risk

of Burkitt lymphoma ([Burkitt & Wright, 1966](#); [Morrow, 1985](#)).

3. Cancer in Experimental Animals

There is no animal model for carcinogenesis associated with *P. falciparum*. However, *P. berghei*-infected rodents develop pathologies with symptoms comparable to a certain degree with human malaria. *P. berghei* is highly homologous to *P. falciparum* in most essential aspects of structure, gene content, biochemistry and life cycle. A few studies of carcinogenicity were performed with *P. berghei*, the results of which are summarized in [Table 3.1](#).

3.1 Mouse

A group of 25 Swiss mice [sex not reported] received an intraperitoneal injection of mouse erythrocytes parasitized with *P. berghei* strain K 173. Two weeks after infection, they were fed a diet lacking *para*-aminobenzoic acid (a parasite growth factor) for 2 months and became parasite-free. Immunity was tested and immune mice were killed after 7 months. Seventeen of the 25 animals were reinfected 2 months before euthanasia. A group of 18 unexposed Swiss mice served as controls and were killed at age 8–14 months. An increased incidence of lymphoma (11 out of 25 versus 0 out of 18, [$P < 0.005$]), all involving the thymus, was observed. An immune mouse and a control mouse developed a Rauscher type leukaemia ([Jerusalem, 1968](#)). [The Working Group noted the confusing study design, that there was a probable virus contamination in the colony and that spontaneous leukaemias occurred in both groups. This made the study difficult to interpret.]

Four groups of 10–13 male Balb/c mice received (i) an intraperitoneal injection of mouse erythrocytes parasitized with *P. berghei*

Table 2.5 Case–control studies examining the association between Kaposi sarcoma and malaria

Reference, Study location, and period	Characteristic of cases with Kaposi sarcoma	Characteristics of controls	Detection method	Exposure categories	No. of exposed cases	Odds ratio (95% CI)	Adjusted potential confounders. Comments
Geddes <i>et al.</i> (1995) Italy 1976–91	204 incident cases with classical Kaposi sarcoma (age ≥ 50 yr), histologically confirmed from 11 cancer registries	777 with other cancers, matched on age (± 2 yr), sex, registry, and calendar year	Birth in an area endemic for malaria (data available on 188 cases and 746 controls)	Birth in an area endemic for malaria No Yes	83 105	1.0 2.98 (1.97–4.51)	Age, sex, and registration area Only cases of classical Kaposi sarcoma were evaluated
Cottoni <i>et al.</i> (1997) Italy 1991–96	40 cases	120 selected from an electoral register matched on sex, age (± 5 yr), and residence	Malaria history by questionnaire	A history of malaria? No Yes <i>P</i> -heterogeneity	19 20	1.0 1.207 (0.513–2.842) <i>P</i> = 0.67	Matching factors Evaluated classical Kaposi sarcoma

yr, year

yoelii; or (ii) an intraperitoneal injection of an extract of spleen and thymus from mice with lymphoma induced by Moloney leukaemogenic virus (MLV); or (iii) an injection of parasitized erythrocytes followed by an injection of the extract within 5 minutes; or (iv) an injection of parasitized erythrocytes followed by an injection of the extract 10 days later. After 26 weeks, an increased incidence of MLV-induced lymphoma was observed in mice injected with parasitized erythrocytes and injected with the extract within 5 minutes, when compared with mice injected with the extract only (10 out of 12 *versus* 1 out of 11, [$P < 0.001$]). Six of 13 mice injected with parasitized erythrocytes and injected with the extract 10 days later developed MLV-induced lymphoma, but this increase was not statistically significant ([Wedderburn, 1970](#)).

Three groups of 10–12 neonatal CFW mice received an intravenous injection of simian virus 40 (SV40), or an intraperitoneal injection of mouse erythrocytes parasitized with *P. berghei yoelli*, or an injection of SV40 followed by an injection of parasitized erythrocytes after 3 months. After up to 12 months, 11 out of 11 mice (100%) injected with both SV40 and parasitized erythrocytes developed sarcomas of the liver and/or spleen compared with 7 out of 10 mice (70%) injected with SV40 only. This increase was not statistically significant. No such tumours were observed in mice injected with parasitized erythrocytes only. In a similar experiment in adult mice, no sarcomas were observed in any group ([Hargis & Malkiel, 1979](#)).

3.2 Rat

Four groups of 21–24 male Buffalo rats received: (i) an intraperitoneal injection of mouse erythrocytes parasitized by *P. berghei* and normal diet; or (ii) a diet containing aflatoxin B₁ at a dietary concentration of 2 ppm for 10 weeks; or (iii) an intraperitoneal injection of parasitized erythrocytes 12 days before starting

a diet containing aflatoxin B₁ for 10 weeks; or (iv) a normal diet and an intraperitoneal injection of uninfected blood. After 60–82 weeks, the incidence of hepatocellular carcinoma was: 0 out of 22, 13 out of 19 (68%), 5 out of 14 (36%) and 0 out of 19, respectively, in the four groups. The incidence of aflatoxin B₁-induced hepatocellular carcinoma in the group injected concurrently with parasitized erythrocytes was not statistically significant compared with that in the group treated with aflatoxin B₁ only ([Angsubhakorn *et al.*, 1988](#)).

4. Mechanistic and Other Relevant Data

4.1 Malaria and the host immune system

4.1.1 Immune response to malaria

Despite extensive efforts, the factors involved in immunity to malaria and pathogenesis of malaria infection are still poorly understood. It is well established that people residing in regions where malaria is endemic acquire immunity to malaria through natural exposure to the parasite. This naturally acquired immunity, which is a non-sterile state of immune protection against the parasite and the clinical disease, results from and is maintained by a continuous exposure to multiple infections. In the human host, the malaria parasite develops in antigenically distinct stages, targeting different cells and tissues, such as the parenchymal cells in the liver and erythrocytes in the blood (see Section 1.1.3 and [Fig. 1.1](#)). Several features of malaria and the ensuing immune response suggest that the parasite and the host immune system interact through a myriad of complex mechanisms, which result in the ability of an otherwise functional immune system to eliminate the parasite and protect the

individuals against subsequent infection ([Riley et al., 2006](#); [Doolan et al., 2009](#)).

The intensity of malaria transmission determines the development of immunity as a function of age ([Fig. 4.1](#)), and the nature of the symptoms therefore depends upon the level of transmission. For example, clinical symptoms related to *P. falciparum* infection become increasingly rare with age in areas where malaria is highly endemic, and where the individual can receive several infected bites each day (up to 1500 infected bites per year). The acquisition of clinical immunity develops step-wise, to severe malaria and subsequently to the clinical symptoms, but immunity to the parasite itself only develops in part and develops slowly ([Fig. 4.1](#)). For instance, children aged 5–10 years still frequently carry high levels of parasites in their blood without having symptoms. Adolescents and adults carry the parasite throughout their life, although at very low levels; sterile immunity may not exist. As a consequence, in areas where malaria is holoendemic or hyperendemic, the spleens of exposed children become massively enlarged at an early age, and a correlation between parasite-levels and the size of the spleen has been established ([Fig. 4.1](#)).

Natural immune responses to the pre-erythrocytic stages of *P. falciparum* (sporozoite and liver) have been suggested to have limited involvement in malarial immunity ([Hoffman et al., 1987](#); [Marsh & Kinyanjui, 2006](#); [Langhorne et al., 2008](#); [Borrmann & Matuschewski, 2011](#); [Schwenk & Richie, 2011](#)). However, other studies have demonstrated that antibodies may contribute to protection against the pre-erythrocytic stages during malaria infection ([Hollingdale et al., 1984](#); [Kebaier et al., 2009](#)). Antibodies to sporozoites may act through neutralizing opsonization of sporozoites and/or block the invasion of hepatocytes ([Schofield et al., 1987](#); [Nussenzweig & Nussenzweig, 1989](#); [Fidock et al., 1997](#); [Nardin et al., 1999](#)).

The infected hepatocyte is an important target of protective immunity, since the presence

of parasites in this MHC class I-expressing cell may render processed parasite antigens more accessible to cell-mediated immune responses ([Speake & Duffy, 2009](#)).

During the liver stage, effector functions of CD4⁺ and CD8⁺ T-cells have been implicated ([Tsuji & Zavala, 2003](#); [Overstreet et al., 2008](#); [Doolan et al., 2009](#); [Wykes & Good, 2009](#); [Tsuji, 2010](#)). There is evidence that the presence of T cells specific for liver-stage antigens and secreting interferon-gamma (IFN- γ) is associated with reduced incidence of malaria ([Perlaza et al., 2008](#); [Todryk & Bejon, 2009](#)).

Protective immunity to the asexual blood forms of malaria parasites, the pathogenic stage of the life cycle, involves both cellular and antibody-mediated mechanisms. For *P. falciparum*, a role for antibodies was originally demonstrated by passive transfer of adult immune immunoglobulin G (IgG) to infected children ([McGregor, 1964](#)). Antibodies can be effective in protection against blood-stage parasites by various mechanisms. These include opsonization of merozoites before uptake through Fc receptors and/or complement receptors on phagocytes, blocking of invasion of erythrocytes, complement-mediated lysis of infected erythrocytes, inhibition of adherence to the endothelium or neutralization of malaria toxins ([Bolad & Berzins, 2000](#)), or by antibody-dependent cell-mediated responses that may kill the parasites ([Bouharoun-Tayoun & Druilhe, 1992](#); [Shi et al., 1999](#)). The major IgG subclasses involved in parasite elimination are of the cytophilic type (i.e. IgG1 and IgG3), while the non-cytophilic types (i.e. IgG2 and IgG4) seem to counteract the protective ones ([Garraud et al., 2003](#)). However, in some malaria-endemic areas, elevated levels of IgG2 have been related to decreased risk of infection, especially in individuals carrying a specific allele variant of Fc γ RIIA, which binds IgG2 ([Aucan et al., 2000](#); [Garraud et al., 2003](#); [Israelsson et al., 2008](#); [Nasr et al., 2009](#)).

Table 3.1 Studies of carcinogenicity in experimental animals infected with *Plasmodium berghei*

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	For each target organ: incidence (%) and/or multiplicity of tumours	Significance	Comments
Mouse, Swiss (NR) 9 mo Jerusalem (1968)	- <i>P. berghei</i> strain K 173 (1.2×10^6 parasitized mouse erythrocytes), i.p. injection; 2 wk after infection, mice were fed <i>para</i> -aminobenzoic acid-free diet for 2 mo and became uninfected. Immunity was tested and immune animals were killed after 7 mo. Some were reinfected 2 mo before killing. <i>n</i> = 25 (17 immune reinfected + 8 immune not reinfected) - Unexposed controls <i>n</i> = 18	Lymphoma: 11/25 ^a , 0/18 Rauscher-type leukaemia: 1/25, 1/18	[<i>P</i> < 0.005] [NS]	^a Five additional infected mice had tumours suspected of being lymphoma. All lymphomas involved the thymus. Virus particles (median, 157 nm; not further described) were found in the spleen in 28.5% of exposed mice and 4.5% of controls. Confusing study design.
Mouse, Balb/c (M) up to 6 mo Wedderburn (1970)	- <i>P. berghei yoelii</i> (10^6 parasitized mouse erythrocytes in 0.1 mL), i.p. injection <i>n</i> = 10 - Extract of enlarged spleens and thymuses of mice with MLV-induced lymphoma in 0.5 mL, i.p. injection <i>n</i> = 11 - <i>P. berghei yoelii</i> + extract within 5 min, i.p. injection <i>n</i> = 13 (1/13 mice died after 24 d) - <i>P. berghei yoelii</i> followed 10 days later by extract, i.p. injection <i>n</i> = 13	Lymphoma: 0/10, 1/11, 10/12*, 6/13**	*[<i>P</i> < 0.001], **[NS]; vs extract only- injected group	
Mouse (neonatal), CFW (NR) up to 12 months Hargis & Malkiel (1979)	- SV40 (strain VA45–54, 10^4 TCID ₅₀ /0.1 mL, intravenous injection in the anterior facial vein) <i>n</i> = 10 - SV40 followed by 5×10^6 <i>P. berghei yoelii</i> -infected mouse erythrocytes (i.p. injection after 3 mo) <i>n</i> = 11 - <i>P. berghei yoelii</i> only (i.p. injection) <i>n</i> = 12	Sarcomas of the liver and/or spleen: - 7/10 (70%; 2 reticulum cell sarcoma, 2 spindle cell sarcoma, 1 pleomorphic sarcoma, 1 poorly differentiated sarcoma, 1 lymphosarcoma) - 11/11 (100%; 2 reticulum cell sarcoma, 2 spindle cell sarcoma, 3 pleomorphic sarcoma, 2 poorly differentiated sarcoma, 2 lymphosarcoma) - 0/12 Tumour latency: 11 months, 9 mo, - Lung metastases: 1, 4, 0	[NS], <i>P. berghei yoelii</i> + SV40 group vs SV40 group	All tumours expressed SV40 T-antigen

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	For each target organ: incidence (%) and/or multiplicity of tumours	Significance	Comments
Rat, Buffalo (M) 60–82 wk Angsubhakorn et al. (1988)	- 0.5 mL uninfected blood (i.p. injection) + normal diet <i>n</i> = 24 - <i>P. berghei</i> (10 ⁶ parasited mouse erythrocytes, i.p. injection) + normal diet <i>n</i> = 23 - 0.5 mL uninfected blood (i.p. injection) followed by diet containing aflatoxin B ₁ at 2 ppm (for 10 wk) after 12 d <i>n</i> = 23 - <i>P. berghei</i> (i.p. injection) followed by diet containing aflatoxin B ₁ (for 10 wk) after 12 d <i>n</i> = 21	Hepatocellular carcinoma: 0/19, 0/22, 13/19 (68%), 5/14 (36%)	[NS], <i>P. berghei</i> + aflatoxin B ₁ group vs aflatoxin B ₁ group	

d, day; i.p., intraperitoneal; M, male; min, minute; MLV, Moloney leukaemogenic virus; mo, month; NR, not reported; NS, not significant; SV40, simian virus 40; TCID₅₀, tissue culture infectious dose 50; vs, versus; wk, week

Another subclass of great interest in malaria is immunoglobulin E (IgE), for which some reports indicate an association with protection ([Bereczky et al., 2004](#); [Farouk et al., 2005](#); [Duarte et al., 2007](#)), and other data suggest an association with pathogenesis ([Perlmann et al., 1994, 1997, 2000](#); [Seka-Seka et al., 2004](#); [Leoratti et al., 2008b](#)). Various immune complexes, which consist of either IgE and antigen aggregates, or IgE with IgG-anti-IgE could bind to Fc receptors expressed on monocytes that become activated, giving rise to secretion of tumour necrosis factor (TNF) ([Perlmann et al., 1999](#)). The production of TNF is genetically regulated: certain nucleotide polymorphisms in the promoter give rise to overproduction of TNF, which leads to a pathogenic effect, while low production of TNF is associated with protection against malaria ([McGuire et al., 1999](#), [Gimenez et al., 2003](#)).

(a) Antigen-presenting cells

Antigen-presenting cells (APCs) are crucial initiators of adaptive immunity. There are three types of professional APCs, namely monocytes, dendritic cells and B cells. APCs interact with pathogens through specialized receptors termed pattern-recognition receptors that have evolved to detect danger signals such as pathogen-associated molecular patterns. A well studied family of pattern-recognition receptors are the toll-like receptors (TLRs), which are expressed differently in different APC populations ([Akira et al., 2006](#); [Kawai & Akira, 2010](#)).

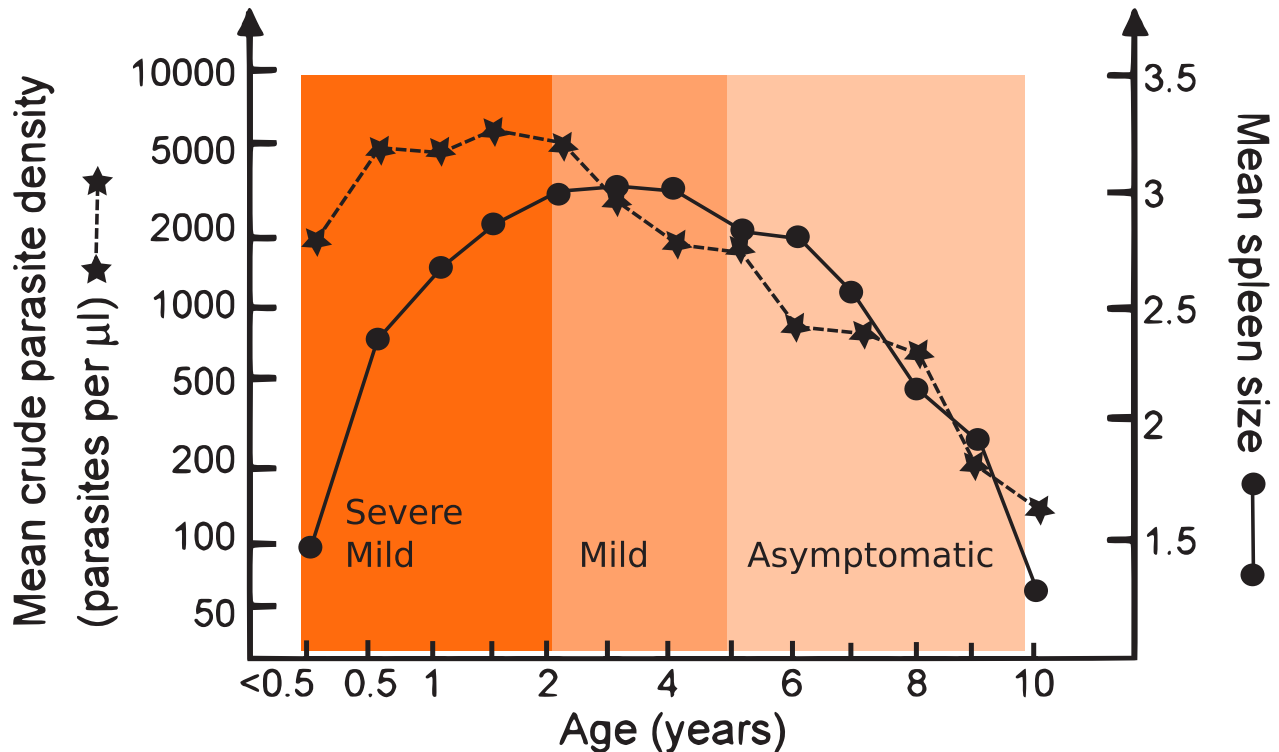
In humans, monocytes and myeloid dendritic cells subsets express mainly TLR1 through TLR6, while TLR7 and TLR9 are expressed in plasmacytoid dendritic cells ([Kadowaki et al., 2001](#), [Jarrossay et al., 2001](#)). Recent evidence suggests that TLRs are central mediators of pro-inflammatory responses during malaria ([Coban et al., 2007](#)). For example it has been shown that hemozoin, which is a by-product generated after the degradation of haemoglobin by the parasite, impairs both antigen presentation

and immunomodulatory functions of the monocytes/macrophages ([Schwarzer et al., 1998](#); [Skorokhod et al., 2004](#)) and that *P. falciparum* glycosylphosphatidylinositol activates the TLR9 pathway ([Coban et al., 2007](#)). It has been shown that the malaria glycosylphosphatidylinositol anchor activates monocytes to release TNF, IL-6 and IL-12 through the TLR2 pathway ([Schofield et al., 2002](#); [Gowda, 2007](#), [Zhu et al., 2009](#)). However, the role of TLRs in the development of immunity to malaria and in the pathology of malaria is not well understood.

Dendritic cell-mediated suppression of T-cell functionality has been suggested to be the cause of the poor T-cell mediated immune responses seen in malaria ([Ocaña-Morgner et al., 2003](#); [Urban & Roberts, 2003](#)). Observations *in vivo* have shown that children with acute malaria have a lower expression of the human leukocyte antigen (HLA) DR on peripheral blood dendritic cells compared with healthy children ([Urban et al., 2001](#)). Moreover, a possible immunomodulatory role has been suggested for the BDCA-3⁺ subset of myeloid dendritic cells upon malaria infection in Kenyan children ([Urban et al., 2006](#)). Observations *in vitro* have shown that exposure of APCs to hemozoin increases the production of both pro- and anti-inflammatory mediators ([Coban et al., 2005](#)). Taken together, existing data suggest that the function of various APC subsets is influenced by *P. falciparum* and that immune responses to malaria may be hampered by modulation of APC functions.

(b) Natural killer cells

During human malaria infections, activation of natural killer (NK) cells *in vivo* has been inferred from the observation that NK-cell activity was increased in malaria-infected children and that this activity correlated with parasitaemia and plasma levels of IFN- γ ([Ojo-Amaize et al., 1981](#); [Hansen et al., 2007](#)). Another study has shown that NK cells are the first cells to respond by producing IFN- γ after exposure

Fig. 4.1 Occurrence of symptoms of clinical malaria

Average suggestive occurrence of clinical malaria symptoms as a function of age, mean parasite density and mean spleen. Mean parasite density (***) and mean spleen size (ooo) of children in a malaria-afflicted area of holoendemic transmission. The spleen was palpated according to Hackett, with the examiner's hand being pressed gently against the abdominal wall of the child until the spleen is felt. At a size of 3, the spleen was felt inbetween the umbilical level and half-way to the lower border of the thoracic cage, at a size of 2, the spleen was felt inbetween halfway to the lower border of the thoracic cage and the lower border of thoracic cage and at a size of 1 the spleen was felt under the thoracic cage. Reprinted from [Chène et al. \(2009\)](#), with permission from Elsevier. From [Björkman, 1985](#).

of human peripheral blood mononuclear cells to *P. falciparum*-infected erythrocytes *in vitro* ([Artavanis-Tsakonas & Riley, 2002](#)). In contrast, others have shown that the secretion of IFN- γ by NK cells in response to infected erythrocytes is not detected in all malaria-naïve or previously exposed donors. These authors showed that NK cells comprise only a small proportion of IFN- γ -producing cells *in vitro* and that the depletion of NK cells does not affect IFN- γ production after exposure for 24 hours to live *P. falciparum*-infected erythrocytes at the schizont stage. Instead, the predominant source of innate IFN- γ was attributed to gamma/delta T-cells ($\gamma\delta$ T-cells) that express NK receptors ([D'Ombrain et al., 2007](#)). Moreover, a study in Papua New Guinea showed high levels of IFN- γ production *ex vivo*

associated with protection from high parasite densities and clinical disease ([D'Ombrain et al., 2008](#)). The release of IFN- γ by NK cells seems to require both a direct contact between the infected erythrocytes and the NK cells and be dependent on the presence of accessory cells (monocytes and myeloid dendritic cells), while TGF- β suppresses NK-cell activation ([Newman et al., 2006](#)).

There is accumulating evidence that NK T cells play a role in protective immunity against malaria as shown in experimental systems (e.g. malaria in mice). An early study found that the humoral immune response against circumsporozoite protein was strongly diminished in CD1d-deficient mice compared with wild-type mice ([Schofield et al., 1999](#)). Since the CD1d is a

lineage-commitment marker of NK T cells, mice lacking this marker have a deficiency in NK T cells. The study further demonstrated that glycosylphosphatidylinositol purified from the asexual blood stages of *P. falciparum* was able to stimulate murine CD4⁺ NK T cells *in vitro*, suggesting that CD4⁺ NK T cells act as helper T cells facilitating the production of anti-circumsporozoite protein antibody by B cells *in vivo* ([Schofield et al., 1999](#)). The role of NK T cells as helper T cells in the regulation of anti-malarial humoral responses *in vivo* was later confirmed by two independent studies ([Molano et al., 2000](#); [Romero et al., 2001](#)). Overall, NK T cells do not seem to have a clear physiological role in inhibiting the pre-erythrocytic stages of the parasite. However, when the NK T cells have been activated by the artificial ligand α -galactosylceramide (α -GalCer), these cells do display an inhibitory effect against the development of liver stages of malaria *in vivo*. This anti-plasmodial activity of α -GalCer-activated NK T cells seems to be mediated by IFN- γ , since the activity is abolished in mice lacking IFN- γ or IFN- γ receptors ([Gonzalez-Aseguinolaza et al., 2000](#)).

(c) Gamma/delta T cells

Gamma/delta T cells share characteristics with both NK and $\alpha\beta$ NK T cells, such as the capacity to secrete high amount of pro-inflammatory cytokines and cytolytic activity against tumours and pathogens. Increasingly, it is accepted that $\gamma\delta$ T cells form part of the protective immune response directed against *P. falciparum*. It has been shown that $\gamma\delta$ T cells inhibit the growth of the asexual blood stages of *P. falciparum* by the granule exocytosis-dependent cytotoxic mechanism ([Farouk et al., 2004](#); [Costa et al., 2011](#)). Moreover, studies have shown that *P. falciparum* infection induces expansion of $\gamma\delta$ T cells to reach up to 30% of circulating T cells in humans ([Roussilhon et al., 1990](#); [Ho et al., 1994](#); [Worku et al., 1997](#)).

Subpopulations of $\gamma\delta$ T cells have been shown to be upregulated to cytolytic functions after interaction with virus-infected cells, like EBV ([Häcker et al., 1992](#), [Harly et al., 2011](#)) and cytomegalovirus (CMV) ([Knight et al., 2010](#)).

(d) Regulatory T cells

An increase in CD4⁺CD25⁺FoxP3⁺ regulatory T cells has been observed in human infected with *P. falciparum* ([Walther et al., 2005](#); [Scholzen et al., 2009](#)) and with *P. vivax* ([Jangpatarapongsa et al., 2008](#)). The increase in regulatory T cells during malaria infections appears to be associated directly with parasite load ([Walther et al., 2005, 2009](#); [Scholzen et al., 2010](#)). Increased levels of CD4⁺CD25^{hi} have been correlated with the risk of malaria infection in rural Kenya ([Todryk et al., 2008](#)), and *Foxp3* mRNA levels during acute infection with *P. falciparum* in Gambian children inversely correlated with parasite-specific IFN- γ responses after parasite clearance, indicating that induction of regulatory T cells during acute infection could limit the extent of the memory Th1 response ([Walther et al., 2009](#)). It is obvious that multiple mechanisms govern the induction, expansion and activation of regulatory T cells during malaria infection, including host-immune mediators such as APCs and immunoregulatory cytokines ([Scholzen et al., 2010](#)).

(e) B cells

B cells are important effector cells of the immune system through the production of antibodies against invading microbes and vaccine antigens. In addition to their established effector function as antibody-producing cells, recent studies indicate that B cells also modulate T-cell responses by antibody-independent mechanisms.

Plasma cells reside in specialized niches in lymphoid organs and the bone marrow, where stromal cells provide an environment conducive to survival. Memory B cells preserve and recall previous antigenic experience to prevent or limit reinfection, thus ensuring long-term protection

for each individual and protection of the species through passive transfer from mother to child during pregnancy and after birth.

B cells play a major role in immunity to malaria as demonstrated by early experiments of serum Ig transfer, as mentioned above ([Cohen et al., 1961](#)). In mice, memory B cells are generated during primary infection with *P. chabaudi* ([Ndungu et al., 2009](#)) and these cells rapidly differentiate to plasma cells upon reinfection with the parasite ([Nduati et al., 2010](#)). Acute infection with *P. falciparum* has a profound effect on B-cell function, resulting in hypergammaglobulinaemia, increased plasma concentrations of the B-cell activating factor, and an increase in atypical memory B cells ([Asito et al., 2008](#); [Weiss et al., 2009](#); [Kumsiri et al., 2010](#)). In children, antibody responses to malaria antigens are often short-lived and only maintained in the presence of the parasites; the mechanisms underlying changes in B-cell function during acute malaria are incompletely understood. Important receptors for the regulation of antigen-independent activation of B cells are TLRs. In humans, circulating memory B cells express higher levels of TLR6, TLR7, TLR9 and TLR10, whereas naive B cells were shown to express low levels of TLR and to respond poorly to TLR ligands. Transitional B cells in cord blood and in peripheral blood express the highest levels of TLR9 and respond to TLR9 ligation with proliferation, differentiation and antibody production ([Capolunghi et al., 2008](#)). Polyclonal B-cell activation during malaria infection is probably due to activation of TLR9 by parasite molecules and ligation of the B-cell receptor by malaria antigens ([Bernasconi et al., 2003](#)). Furthermore, high concentrations of B-cell activating factor may inhibit the differentiation of memory B cells to plasma cells in the presence of TLR9 ligands ([Darce et al., 2007](#)). [Modulation of the B-cell compartment during and after an acute episode of malaria may contribute to enhanced susceptibility to bacterial pathogens and reduced antibody responses to

vaccines included in the Expanded Programme on Immunization (EPI) for children living in malaria-endemic areas.]

B cells secrete cytokines and chemokines, either constitutively or in response to antigens, upon triggering by TLR ligands, T cells, or combinations thereof ([Lund & Randall, 2010](#)).

(f) Cytokines

For a successful elimination of parasites, it is important to have an early innate response, including secretion of pro-inflammatory cytokines like IL-12 and TNF- α . For optimal clearance of the parasite, this initial response should be followed by an effective adaptive immune response including high titres of anti-malaria antibodies to clear the parasitaemia. On the other hand, pro-inflammatory cytokines may also play an important role in determining pathology, in particular in the development of severe malaria. In fact, low levels of IL-10 and high levels of TNF- α have been associated with disease severity. In severe cases of malaria among Gabonese children, levels of IL-12 were found to be lower than in the mild cases ([Luty et al., 2000](#)). In addition, the development of cerebral malaria has been associated with high plasma levels of IL-1 β ([Prakash et al., 2006](#)) and higher levels of IL-6 and IL-10 were associated with severe malaria than with uncomplicated malaria ([Lyke et al., 2004](#)).

4.1.2 Immunity to malaria in infants

Development of immunity is dependent on exposures to pathogens as well as the age of the host. During childhood, there are several changes in the lymphocyte compartment and these are especially evident from birth until age 2 years. Healthy infants have significantly higher numbers of peripheral CD19+ B cells and CD3+ T cells compared with adults ([Denny et al., 1992](#); [Comans-Bitter et al., 1997](#)). Within the lymphocyte compartment, the percentage

of memory B and T cells expands over time and reflects the infants' antigenic history. The relative lack of maturity due to limited exposure to antigens, compounded with an environment that promotes a Th2-like response characterized by minimal inflammatory reactions, leads to a reduced capacity of infants to adequately mount an effective immune response in the first year of life. In addition, infants are unable to respond to thymus-independent antigens until about age 2 years, leaving them especially vulnerable to encapsulated bacteria.

Falciparum malaria only elicits protective immunity after several years of continuous exposure, during which time recurring infections and illness occur ([Marsh et al., 1989](#)). Thus, children living in malaria-endemic regions of sub-Saharan Africa have the burden of early age of exposure and repeated exposure to falciparum malaria, while their immune system is still developing. That this is problematic is shown by the fact that not only do children aged less than 5 years suffer the highest morbidity and mortality due to infection with *P. falciparum*, they also have the highest all-cause mortality of any age group living in malaria-endemic regions ([Snow & Omumbo, 2006](#)). Infants living in a malaria-endemic region of Kenya were found to have elevated levels of transitional B cells and reduced non-class-switched memory B cells compared with infants living in a non-malaria-endemic region ([Asito et al., 2011](#)). Expansion of a peripheral memory B-cell subset characterized by expression of the inhibitory ligand FCRL4 and hyporesponsiveness was described in infants as young as age 2 years living in a malaria-epidemic region of Mali ([Weiss et al., 2009](#)).

Plasmodium-specific antibody levels rapidly decline in infants after clearance of parasitaemia ([Kinyanjui et al., 2007](#); [Akpogheneta et al., 2008](#)) and immunity is acquired only after several years of exposure ([Akpogheneta et al., 2008](#); [Crompton et al., 2010](#)). The delay in development of protective immunity to malaria results in repeated

infection of infants and young children with *P. falciparum*, which involves continual exposure of their B cells to parasitic antigens.

4.1.3 Malaria and host genetic polymorphisms

Malaria is a complex disease with an etiology that shows a large variation in severity of infection, which is attributable to environmental factors, virulence of the parasite, and host genetics ([Mackinnon et al., 2005](#); [Sakuntabhai et al., 2008](#)). Variation in the severity of *P. falciparum* infections includes hypersymptomatic or asymptomatic parasitaemia, severe malarial anaemia, and cerebral malaria, and is suggested to be caused by genetic factors of parasite and host ([Garcia et al., 1998](#); [Rihet et al., 1998](#)). Host genetic polymorphisms known to influence susceptibility/resistance to *P. falciparum* include polymorphisms leading to haemoglobinopathies and other erythrocyte disorders. The involvement of polymorphisms in immune-response genes (e.g. HLA, cytokines) is also suspected. This subchapter gives only a brief overview of these polymorphisms, which have been reviewed extensively elsewhere ([Verra et al., 2009](#); [Driss et al., 2011](#)).

(a) Haemoglobinopathies/erythrocyte disorders

The genetic basis of some important erythrocyte disorders in the modulation of the clinical course of malaria was first discovered in the middle of the last century. They include the sickle-cell trait ([Allison, 1954](#)) and haemoglobinopathies such as thalassaemias and glucose-6-phosphate dehydrogenase deficiency (reviewed by [Hill, 1999](#); [Williams, 2006](#)) (see also [Table 1.3](#) and Section 1.2.3).

(b) *Immune-response genes*(i) *HLA polymorphisms*

The immune response and susceptibility to malaria have been the object of extensive investigation using twin studies with the aim of dissecting genetic regulation ([Sjöberg et al., 1992](#); [Jepson et al., 1997a, b](#)). These investigations showed that monozygotic twins were always more concordant than dizygotic twins in their responses to malaria, even more so than individuals with identical HLA class II; this implies that genes both inside and outside the HLA class II region are involved in susceptibility to malaria. Further evidence that genetics plays a role in shaping individual immune responses to malaria has come from inter-ethnic comparisons of susceptibility to malaria in West Africa. Epidemiological studies conducted in Burkina Faso and Mali have shown that the Fulani people have lower parasite rates and densities and fewer attacks of malaria than their sympatric ethnic counterparts who live under similar socioeconomic conditions and with comparable intensity of malaria transmission. This is most likely due to their more efficient immune response ([Modiano et al., 1996, 1998, 1999, 2001a](#); [Dolo et al., 2005](#); [Verra et al., 2009](#)). The involvement of the classical erythrocyte disorders mentioned above in the stronger protection of the Fulani against malaria seems unlikely, since they are present at lower frequencies in the Fulani ([Modiano et al., 2001a](#)). Typing of HLA class I alleles revealed that the Fulani are genetically distant from sympatric groups ([Modiano et al., 2001b](#)).

Regarding HLA, associations between the class I haplotype HLA-B53 and the class II haplotype HLA-DRB1*1302-DQB1*0501 and protection against severe malaria have been reported ([Hill et al., 1991](#)). These authors identified an epitope of the liver-surface antigen (LSA-1) that binds HLA-B53, providing a possible functional explanation for the association between HLA-B53 and protection from severe malaria

([Hill et al., 1992](#)). However, this association could not be reproduced in other countries ([Gilbert et al., 1998](#); [Young et al., 2005](#)). The influence of the HLA system on the immune response has been extensively studied in the Gambia. In a comparison of humoral and cellular responses in HLA-identical and HLA-non-identical individuals, non-HLA genes played a more substantial role than the HLA genes ([Jepson et al., 1997b](#)). Taken together, the available evidence suggests that it is unlikely that the HLA loci are a major genetic determinant of immune reactivity to malaria ([Verra et al., 2009](#)).

(ii) *Cytokines and related gene polymorphisms*

TNF has been shown to have an important role in the pathogenesis of malaria ([Kwiatkowski et al., 1990](#)). In a study conducted in the Gambia, the presence of the 308A allele in the *TNF* promoter region, known to increase the transcription of the gene compared with the 308G allele, was associated with a higher risk of death or severe neurological sequelae due to cerebral malaria. This risk was independent of variation in the nearby HLA class I and II genes ([McGuire et al., 1994](#)).

Many other single-nucleotide polymorphisms have been reported, including those in IFN- γ , the IFN- γ receptor, IFN regulatory factor-1 (IRF-1), CD40 ligand, interleukins IL-1 and IL-4, Fc γ receptors, TLRs, mannose-binding protein, complement receptor 1, and intercellular adhesion molecule 1 (ICAM-1). For detailed references to these polymorphisms see [Verra et al. \(2009\)](#).

Importantly, results for all single-nucleotide polymorphisms have been difficult to reproduce in different countries, suggesting that different combinations of allelic variants may determine the complex clinical outcomes of severe malaria in different populations.

At present, the multicountry collaborative Malaria Genomic Epidemiology Network (Malaria-GEN) is trying to gain fundamental

new insights into the effects of genetic variations on susceptibility to malaria and molecular mechanisms involved in protection from and pathogenesis of malaria ([Malaria Genomic Epidemiology Network, 2008](#)).

4.2 Biological properties of *P. falciparum* antigens relevant to immune dysfunction

The parasite *P. falciparum* resides most of its lifetime in the bloodstream, in the midst of the immune system. When an *Anopheles* mosquito takes a blood meal, the parasite – in its infective stage – is expelled into the circulation and rapidly migrates to the liver where it infects and divides in hepatocytes. On their way to the liver, however, some parasites are trapped in the local lymphoid system, which promotes immune recognition. Subsequently, the infected hepatocyte bursts, and thousands of merozoites are released into the blood stream and swiftly invade erythrocytes. The parasite further develops and divides inside the erythrocyte, which upon rupture releases parasites, initiating 48-hour cycles that may perpetuate for 9 months or longer (see Section 1).

Efforts to develop an effective vaccine against malaria are challenged by the complex life cycle of *P. falciparum*, its capacity to parasitize and hide within the host cells, and its masterful ability to avoid clearance by the innate and adaptive immune responses of the host. The virulence of *P. falciparum* is related to its ability to evade host immunity through clonal antigenic variation of antigens at the surface of the parasitized erythrocyte, and through tissue-specific adhesion of parasitized erythrocytes.

During intra-erythrocytic growth of *P. falciparum*, parasite-derived proteins such as PfEMP1, RIFIN, STEVOR and SURFIN are successively expressed, exported and presented at the surface of the human erythrocyte ([Helmby](#)

[et al., 1993](#); [Baruch et al., 1995](#); [Cheng et al., 1998](#); [Fernandez et al., 1999](#); [Kyes et al., 1999](#); [Winter et al., 2005](#)). PfEMP1, the dominant polypeptide, is an adhesin that enables erythrocytes infected with mature parasite stages to adhere in the microvasculature and sequester, thereby avoiding clearance by the spleen. The parasite thrives in the hypoxic environment of the post-capillary vessels. Consequently, only erythrocytes infected with early-stage parasites are seen in the peripheral blood. Sequestration, depending on endothelial adhesion and binding of parasitized erythrocytes to uninfected erythrocytes and/or parasitized erythrocytes in the context of inflammation, may lead to complete clogging of the microvasculature and to severe malaria. Furthermore, by presenting ligands such as PfEMP1 at the surface of the parasitized erythrocyte, the parasite exposes an Achilles' heel, allowing for host immune recognition of the altered erythrocyte surface ([Wahlgren et al., 1999](#)). To evade the mounting immune response, the polypeptides display extensive antigenic variation, simultaneously changing the receptor recognition and the tissue tropism of the infected cell. Thus, the adhesive properties of the exposed variant surface antigen also determine the outcome of the disease.

4.2.1 Antigenic variation

PfEMP1 is a large protein (200–360 kDa) encoded by a family of approximately 60 *var* genes ([Scherf et al., 2008](#)). Each individual parasite only expresses a single variant gene at a time, maintaining all other members of the family in a transcriptionally silent state according to a very precise, but yet to be determined, counting mechanism ([Chen et al., 1998a](#), [Scherf et al., 1998](#)). The same *var* gene is expressed during many parasite generations. Switching of expression to another variant PfEMP1 species occurs at a rate of about 1%, and allows the parasite to avoid immune clearance and to prolong the period of infection.

The *var* genes are composed of two exons, a conserved intron and a 5' upstream flanking sequence (ups). Almost all PfEMP1s share some conservation in their structure: an N-terminal segment, variable numbers of Duffy binding-like domains, one or two cysteine-rich interdomain regions (CIDR), a transmembrane domain, sometimes a C2 domain; and a conserved intracellular acidic terminal segment ([Baruch et al., 1995](#); [Chen et al., 2000](#); [Kraemer & Smith, 2006](#)). Although the PfEMP1 proteins differ extensively in their amino-acid composition and binding properties, they have related protein architectures. As an example of PfEMP1 organization and binding phenotype, the PfEMP1 expressed by the parasite clone FCR3S1.2 is shown in [Fig. 4.2](#) (see also [Chen et al., 2000](#)).

4.2.2 B-cell dysfunction

Chronic infection with *P. falciparum* leads to a severely dysregulated B-cell compartment. The existence of malarial B-cell mitogens was suggested decades ago by the clinical presence of hypergammaglobulinaemia ([Abele et al., 1965](#); [Greenwood & Vick, 1975](#)), elevated titres of auto-antibodies ([Adu et al., 1982](#)), and the frequent occurrence of Burkitt lymphoma ([Greenwood et al., 1970](#)). Moreover, extracts derived from cultured *P. falciparum* induce B-cell proliferation and antibody production *ex vivo* ([Greenwood et al., 1979](#); [Gabrielsen & Jensen, 1982](#); [Kataaha et al., 1984](#)).

During intra-erythrocytic proliferation of *P. falciparum* (for details see above, and [Fig. 4.2](#)), PfEMP1 is successively expressed, exported and presented at the surface of the human erythrocyte membrane. Parasitized erythrocytes have the ability to bind to several receptors present on B cells, such as non-immune human immunoglobulins (IgG and IgM) ([Scholander et al., 1996, 1998](#); [Flick et al., 2001](#); [Rasti et al., 2006](#); [Barfod et al., 2011](#)), heparan sulfate ([Chen et al., 1998b](#); [Barragan et al., 2000](#)), CD31/PECAM1 and

CD36 ([Treutiger et al., 1997](#); [Chen et al., 1998b](#)). The parasitized erythrocytes and the cysteine-rich interdomain region 1 α (CIDR1 α) domain of PfEMP1 from the parasite clone FCR3S1.2 harbour a multiadhesive phenotype that is able to bind to CD31/PECAM-1, CD36 and IgM ([Fig. 4.2](#); [Chen et al., 2000](#)). Heparan sulfate is present on all B cells, but CD31/PECAM-1 expression is mainly associated with early developmental stages of B cells in the secondary lymphoid organs ([Jackson et al., 2000](#)), while CD36 seems to be differentially expressed on B-cell subsets during development and in response to antigens ([Won et al., 2008](#)).

PfEMP1 is included in the family of Ig-binding proteins in which the binding activity resides in different parts of the molecule, depending on the particular PfEMP1-species ([Donati et al., 2004](#)). For example, the Duffy binding-like domain 2 β (DBL2 β) is the adhesive ligand of the parasite clone TM284, while CIDR1 α is the ligand for the FCR3S1.2 clone ([Chen et al., 2000](#); [Flick et al., 2001](#); [Donati et al., 2004](#)). PfEMP1-CIDR1 α binds to both non-immune IgM and IgG, and to IgG from human and other animal species, and is also able to bind to the Fc and Fab parts of both IgM and IgG ([Donati et al., 2004](#)). Characterization of the crystal structure of the PfEMP1-CIDR1 α subdomain from the “Malayan-camp parasite” strain indicates that it is composed of a bundle of three α -helices that are connected by a loop to three additional helices ([Klein et al., 2008](#)). Although there is very wide diversity in PfEMP1 sequences, there is still structural similarity between the CIDR and Duffy binding-like domains ([Singh et al., 2006](#); [Singh et al., 2008](#)), suggesting that PfEMP1 is a polymer of three-helix bundles. Interestingly, the binding pattern and crystal structure of CIDR are similar to those of another microbial Ig-binding protein, protein A of *Staphylococcus aureus* ([Graille et al., 2000](#)).

The structural and functional similarities between protein A and PfEMP1 are important for the effect of malaria infection in the B-cell

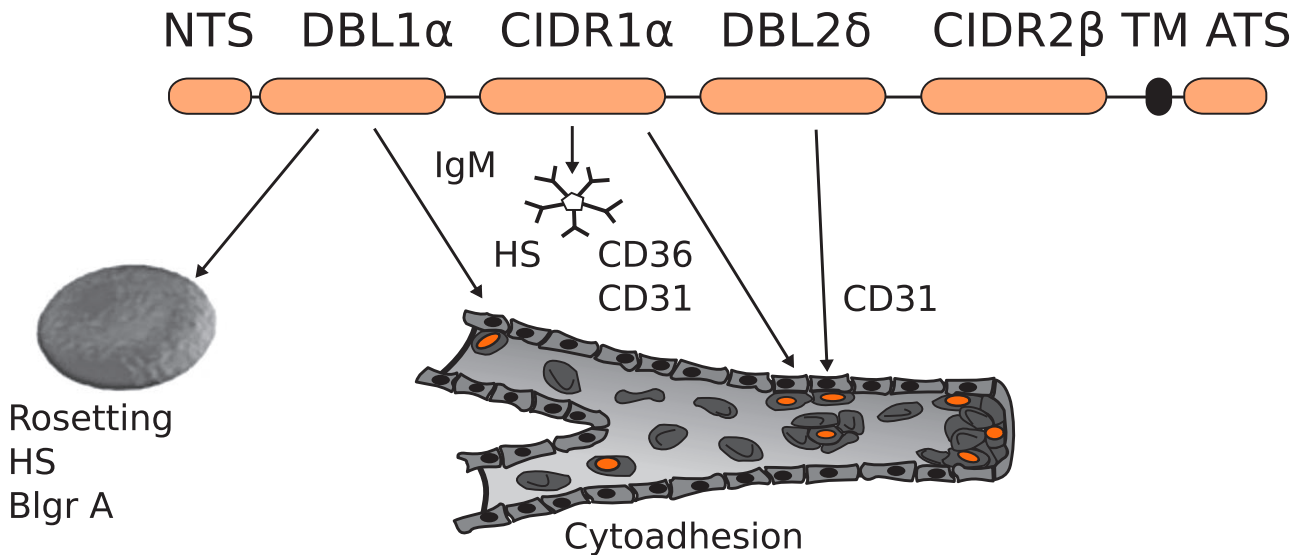
compartment. Microbial Ig-binding proteins are produced by protozoa, viruses, and bacteria, and play important physiological roles ([Chen et al., 1998b](#); [Graille et al., 2000](#)). During the infectious process, Ig-binding proteins may act in an evasion mechanism to divert specific antibody responses ([Daniel-Ribeiro et al., 1989](#); [Léonetti et al., 1999](#)). CIDR1 α is a polyclonal B-cell activator that binds to purified B lymphocytes from non-immune donors, inducing activation, proliferation, and antibody secretion ([Donati et al., 2004, 2006a](#)). These effects are predominantly observed in memory B cells. The CIDR–B-cell interaction is partially mediated through the binding to surface Ig ([Donati et al., 2006a](#)). PfEMP1 affects the dynamics of germinal-centre B cells by increasing the proportion of cycling cells, inducing proliferation, and protecting B cells from apoptosis ([Donati et al., 2006a](#)). The mechanisms include upregulation of genes involved in immunological signalling pathways, cell growth/apoptosis, and transcription. The known anti-apoptotic genes that were upregulated included *CALB1*, *DNAJB1* (previously called *Hsp40*), *GSTM2*, and *BNIP1*, which encodes the BCL2/adenovirus E1B 19 kDa interacting protein. CIDR1 α also downregulated the expression of *BCL2L1* (previously known as *BCL-xl*) and *BCL2*, and increased the expression of *XIAP* ([Donati et al., 2006a](#)).

During the erythrocytic growth phase, the *P. falciparum* parasite contains TLR9 ligands ([Parroche et al., 2007](#)) known to induce proliferation of memory B cells ([Bernasconi et al., 2003](#); [Kumsiri et al., 2010](#)). CIDR increases the expression of TLR7 and TLR10, and sensitizes B cells to TLR9 signalling ([Simone et al., 2011](#)). Despite its ability to bind surface Ig, CIDR-induced B-cell activation does not activate LYN and/or phosphotyrosine pathways associated with B-cell receptor signalling. It rather induces phosphorylation of downstream kinases such as ERK1/2, p38 and the IKK complex ([Simone et al., 2011](#)). B-cell activation can also be potentiated by the presence of elevated levels of IL-10, IL-6 and

TNF ([Lyke et al., 2004](#)) during severe malaria; it can also be produced *in vitro* by lymphocytes exposed to parasitized erythrocytes ([Wahlgren et al., 1995](#)).

In vitro, exposure of B cells to *P. falciparum* schizont extracts induces mRNA expression of activation-induced deaminase (AID), and Ig class switching ([Potup et al., 2009](#)). Infants living in malaria-endemic regions have alterations in their B-cell subset distribution, B-cell homeostasis, longevity, quantity and quality of humoral responses based on age and on the dynamics of malaria transmission ([Asito et al., 2008](#); [Weiss et al., 2009](#)). Impaired humoral immune protection associated with prenatal or chronic exposure to *P. falciparum* is common in children living in malaria-endemic regions ([Chelimo et al., 2005](#); [Scott et al., 2005](#)). A recent study demonstrated that children from a malaria-endemic area had higher overall numbers of B cells, reduced populations of circulating memory B cells, and an expanded population of immature transitional B cells, compared with infants living in an area with unstable malaria transmission ([Asito et al., 2011](#)). Expansion of transitional B-cell populations has been demonstrated to correlate to impaired humoral immunity ([Cuss et al., 2006](#)).

The marked effect of malaria infection on B cells not only pertains to the nature of the malarial antigens, but also to the biology of the infection. A large proportion (83%) of parasitized erythrocytes in fresh isolates bind non-immune Igs ([Scholander et al., 1998](#)) and could therefore interact in the peripheral blood with B cells, *via* their surface Igs. Erythrocytes parasitized by *P. falciparum* have the potential to directly interact with B cells at different anatomical sites, and to induce activation and differentiation into antibody-secreting cells. Mature stages of infected erythrocytes sequester in the vasculature to avoid clearance by the spleen. Under stringent blood-flow conditions, large numbers of infected erythrocytes fail to adhere to the vascular endothelium and end-up trapped in the spleen

Fig. 4.2 Molecular architecture of PfEMP1

The molecular architecture of PfEMP1 encoded by the *var1* gene of the parasite clone FCR3S1.2 depicting its receptor specificities including those present on B cells such as heparan sulfate (HS), CD31/PECAM1 and CD36.

ATS, acidic terminal segment; Blgr A, blood group A antigen; CIDR, one or two cysteine-rich interdomain regions; DBL, Duffy binding-like domain; PfEMP1, *Plasmodium falciparum* erythrocyte membrane protein 1; HS, heparan sulfate; IgM, immunoglobulin M; NTS, N-terminal segment

Reprinted from [Chène et al. \(2009\)](#) with permission from Elsevier. From [Pettersson \(2005\)](#).

where B lymphocytes can represent up to 50% of the splenocytes. Parasitized erythrocytes and their constituent Ags could thus interact in the spleen with B cells displaying a variety of surface phenotypes, Ag-binding repertoires and signalling profiles ([Engwerda et al., 2005](#)). Noteworthy, during malaria infection the splenic architecture is profoundly changed, and the splenomegaly observed after malaria infection partially results from a prominent migration of macrophages into the white pulp regions of the spleen. These changes may alter the homeostasis of the B-cell compartment ([Urban et al., 2005](#)).

In summary, *P. falciparum* affects the B-cell compartment through mechanisms that include: (a) polyclonal activation *via* B-cell receptor- and TLR9-engagement leading to increased AID activation, Ig production and switching, proliferation and survival, resulting in expansion of the B-cell compartment; (b) diversion of specific antibody responses, both as result of polyclonal activation and persistent activation;

(c) dysregulation of the development of B-cell subsets, which leads to higher numbers of total B cells, a reduced population of memory B cells and higher numbers of transitional B cells; and (d) these mechanisms contribute to a persistent activation of B cells, which may result in impairment of B-cell functions.

4.2.3 Immune evasion

The parasitized erythrocytes of children and those in the placenta of pregnant women frequently carry non-immune IgM and IgG at their surfaces, where they have been found to participate in the sequestration of the parasitized erythrocytes ([Scholander et al., 1996, 1998](#); [Flick et al., 2001](#); [Rasti et al., 2006](#)). The PfEMP1 protein VAR2CSA was found to be involved in immunoglobulin binding, placental adhesion and pathogenesis ([Rasti et al., 2006](#)). Antibodies generated to this adhesion inhibit binding *in vitro* (reviewed in [Scherf et al., 2008](#)). Further, recent evidence indicates that the capacity of the

parasitized erythrocytes to bind non-immune IgM also allows them to evade immune recognition through the masking of PfEMP1-specific IgG epitopes by nonspecific IgM. Binding of non-immune IgM to erythrocytes infected by parasites expressing the PfEMP1 protein VAR2CSA blocked subsequent specific binding of human monoclonal IgG to the Duffy binding-like domains of this PfEMP1 variant. Taken together, the results indicate that the affinity of VAR2CSA for non-specific IgM has evolved not only to allow placental sequestration of the parasitized erythrocyte, but also to evade acquired protective immunity without compromising VAR2CSA function or increasing the susceptibility of the parasitized erythrocytes to complement-mediated lysis ([Barfod et al., 2011](#)).

4.3 Other infectious agents associated with falciparum malaria and human cancer

4.3.1 EBV and Burkitt lymphoma

EBV is a ubiquitous oncogenic gamma-herpes virus that infects and persists in more than 90% of the adult population worldwide. African children are infected early in life and nearly all have sero-converted by age 3 years, while in affluent countries primary infection is often delayed until adolescence ([Biggar et al., 1978a, b](#); [Pirou et al., 2012](#)). EBV was originally identified in the tumour cells of a Burkitt lymphoma and was among the first viruses to be associated with the pathogenesis of a human cancer; its etiological role in the causal association with Burkitt lymphoma is established ([IARC, 2012](#)). After primary infection, EBV enters into a lifelong persistent infection, characterized by low-level periodic lytic reactivation and viral shedding into saliva for transmission. This persistence is usually benign and only causes disease when the host-virus balance is upset; it relies on a balance between viral latency, viral replication, and host

immune responses. The EBV genome contains approximately 100 open reading frames (ORFs) of which only few have been characterized with regard to expression and function. EBV establishes various forms of latency in which different sets of EBV-encoded antigens are expressed, depending on the differentiation stage, location and origin of the infected cell. Latently infected B lymphocytes express nine proteins, i.e. six nuclear antigens (EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, EBNA-LP) and three membrane proteins (LMP-1, LMP-2A, LMP-2B). In addition, two types of non-coding RNA are expressed, the EBV-encoded RNAs and the BamH1A rightward-transcript micro-RNAs (miR-BARTs) (see review, [Rickinson & Kieff, 2007](#); [Forte & Luftig, 2011](#)). In contrast, the majority of Burkitt lymphomas express only one virally encoded protein, EBNA-1. These two patterns of gene expression are often referred to as latency III and latency I, respectively, and they are achieved by the use of different promoters to generate alternative primary transcripts from which the different EBNA mRNAs are spliced (reviewed in [Rowe et al., 2009](#)). *In vivo*, EBV latency is established in resting memory B cells, infectious virus is produced when memory B cells switch from the latent to the lytic phase, which seems to occur after the differentiation from memory to plasma cells ([Laichalk & Thorley-Lawson, 2005](#)). These memory B cells seem to express none of the viral latent proteins and are invisible to the immune system.

Endemic Burkitt lymphoma, the most frequent paediatric cancer in sub-Saharan Africa, is a high-grade B-cell lymphoma characterized by the consistent presence of EBV ([Epstein et al., 1964, 1965](#); [zur Hausen et al., 1970](#)), and a chromosomal translocation involving the human proto-oncogene *c-MYC* on chromosome 8 and the *Ig* loci (located either on chromosome 2, 14 or 22) that result in deregulated expression of the *c-MYC* protein (reviewed by [Klein, 2009](#)). Endemic Burkitt lymphoma seems to originate

from a germinal centre-derived B cell or a memory B cell. The hypermutation undergone by tumour cells is a feature of germinal-centre reaction during B-cell activation and differentiation, and the Ig gene break-point to which *MYC* is transferred in endemic Burkitt lymphoma occurs at the V(D)J region, suggesting that the translocation occurs during the process of somatic hypermutation and class-switch recombination (Thorley-Lawson & Allday, 2008). The somatic hypermutation and Ig recombination involve the use of a mutation-generating enzyme, AID (reviewed by Pavri & Nussenzweig, 2011).

The role of EBV in lymphomagenesis seems to relate to different mechanisms and properties of EBV-related products. EBV latent proteins induce expression of AID (He *et al.*, 2003). Another suggested role for EBV has been to confer resistance to apoptosis, with evidence of anti-apoptotic potential being reported for several genes, including EBV-encoded RNAs (Komano *et al.*, 1998; Nanbo *et al.*, 2002), EBNA-1 (Kennedy *et al.*, 2003), and BHRF1 (a BCL2 homologue protein usually associated with the lytic virus cycle; Kelly GL *et al.*, 2009), and with evidence of virally mediated epigenetic repression of BIM (also known as BCL2L11), a proapoptotic human tumour suppressor that is a member of the BCL2 family (Paschos *et al.*, 2009).

(a) Malaria and Burkitt lymphoma

The endemicity of *P. falciparum* malaria varies greatly, as does the incidence of endemic Burkitt lymphoma. Indeed, endemic Burkitt lymphoma only occurs where malaria transmission is high, within the so-called “lymphoma belt” of sub-Saharan Africa, and the high-transmission areas of Papua New Guinea. Furthermore, in the areas and countries where endemic Burkitt lymphoma occurs, it only arises among people living in areas with the highest transmission, the so-called holoendemic or hyperendemic areas. Conversely, the incidence of endemic Burkitt lymphoma is lower in areas where malaria is

less endemic (mesoendemic, hypoendemic). For example, there is little malaria or endemic Burkitt lymphoma in the northern and southern parts of Africa. This suggests a dose–response relationship between the presence of *P. falciparum* malaria and endemic Burkitt lymphoma. In order to develop Burkitt lymphoma, a child has to be exposed to continuous infection, and chronic malaria since birth, as occurs in holoendemic or hyperendemic areas of Africa or New Guinea. Thus, having few or even multiple infections with *P. falciparum* does not predispose a child to endemic Burkitt lymphoma, it is the chronic nature of infection that is likely to increase the risk of developing this disease. There is no evidence that the other malaria parasites (*P. vivax*, *P. ovale*, *P. malariae*, *P. knowlesi*) are involved in the genesis of Burkitt lymphoma (see Section 2).

Antimalarial treatment in holoendemic areas has been associated with a decrease in the incidence of endemic Burkitt lymphoma (Burkitt, 1969; Morrow *et al.*, 1976). A malaria intervention trial was conducted in United Republic of Tanzania from 1977 to 1982 to monitor the prevalence of endemic Burkitt lymphoma and malaria, but the results were inconclusive due to methodological issues (Geser *et al.*, 1989). Still, a high average annual rate of incidence (4.7 per 100 000 population) of Burkitt lymphoma prevailed in the holoendemic area. All cases were detected in the lowlands, with none occurring on the high plateau situated more than 1500 m above sea level, although 17 cases would have been expected if the rate found in the lowlands had also prevailed in the highlands. Correspondingly, it was also found that malaria endemicity was considerably higher in the lowlands (Geser *et al.*, 1989). Other attempts to determine whether relative peaks in malaria incidence act as a trigger for endemic Burkitt lymphoma – by analysing space–time clustering or seasonality of cases – have been inconclusive (Ogwang *et al.*, 2008). However, analytical models have found high-risk clusters

of incidence of endemic Burkitt lymphoma within a malaria holoendemic region, indicating the heterogeneity of malaria exposure within the same area ([Rainey et al., 2007b](#)). These data support the role of intense malaria transmission in the etiology of endemic Burkitt lymphoma ([Rainey et al., 2007b](#)).

The heterogeneity in the relationship between malaria endemicity and incidence of endemic Burkitt lymphoma suggests that the timing of co-infection and the duration of infection with *P. falciparum* at the individual level may determine the outcome of EBV dysregulation leading to endemic Burkitt lymphoma ([Moormann et al., 2011](#)). This assumption is supported by the fact that the average age of patients with endemic Burkitt lymphoma in hyperendemic areas is between 6 and 8 years *versus* 16.2 years in low-transmission areas ([Burkitt & Wright, 1966](#)). The chronology of EBV and *P. falciparum* co-infections in relation to incidence of endemic Burkitt lymphoma in malaria holoendemic areas is depicted in [Fig. 4.3](#). Immigrants from areas where malaria is hypoendemic, who migrated to holoendemic areas, developed endemic Burkitt lymphoma in late adolescence and adulthood ([Burkitt & Wright, 1966](#)). Moreover, development of endemic Burkitt lymphoma before age 2 years is extremely rare, although the reason for this is unclear.

P. falciparum has been clearly shown to be a powerful B-cell mitogen. As a consequence of polyclonal B-cell activation, hyper-gammaglobulinaemia is induced by the infection and is likely to be responsible for the high levels of IgM and IgG in children at age 5–10 years ([Greenwood & Vick, 1975](#); [Whittle et al., 1990](#)); these levels are three to four times higher in children in highly endemic areas than in children in non-endemic areas. This age range is also the age at which the incidence of endemic Burkitt lymphoma peaks ([Mwanda et al., 2004](#)).

No other parasite, prion, bacterium, or helminth has been associated with the

development of endemic Burkitt lymphoma, although some of these agents are very common in the underprivileged populations that are affected by this form of cancer.

(b) Malaria and EBV

Although co-infection with *P. falciparum* and EBV has long been implicated in the genesis of endemic Burkitt lymphoma, the relative contribution of these various mechanisms is not yet fully understood. A prospective study on more than 40 000 Ugandan children found very high antibody titres of antibodies to the EBV viral capsid antigen (VCA) in children who subsequently developed endemic Burkitt lymphoma. This led to the hypothesis that infection with EBV early in life results in an infection that is poorly controlled and increases the risk of developing the tumour ([De-Thé et al., 1978](#)). Recent studies compared EBV load and time of EBV primary infection in two Kenyan districts with different patterns of malaria transmission. The mean age of infection in the holoendemic area was 7.28 months, with 35.3% of the children infected before age 6 months *versus* 8.39 months, with 12.2% being infected before age 6 months, in a district with limited transmission of malaria. Modelling analysis showed that residence in holoendemic areas and younger age at first EBV infection were significant predictors for having a higher EBV load throughout the observation period ([Pirion et al., 2012](#)).

The contribution of *falciparum* malaria to the pathogenesis of endemic Burkitt lymphoma depends on the nature of the malaria antigens and their direct effect on the B-cell compartment and persistence of EBV, and on the impairment of EBV-specific T-cell immunity during infection. Infection with *P. falciparum* has immunosuppressive effects, as reflected by the impairment of macrophage function and antigen presentation (dendritic-cell inhibition), diminished specific T-cell response, induction of regulatory T cells and high plasma levels of pro-inflammatory

cytokines (IL-6, TNF) and regulatory cytokines (IL-10, TGF β) (reviewed by [Cunnington & Riley, 2010](#)). IL-10 and TGF β decrease EBV-specific T-cell responses ([Altioek et al., 1990](#); [Bejarano & Masucci, 1998](#)).

In immunocompetent individuals, EBV-specific T cells restrict viral replication, prevent the switch to lytic production, and limit the number of latently infected B cells (reviewed in [Rickinson & Kieff, 2007](#)). EBV is activated when the immune system is compromised (reviewed by [Hopwood & Crawford, 2000](#)). During malaria infection, the virus/host balance is changed in favour of the virus. Impairment of immune control of EBV in patients with acute malaria infection leads to increased numbers of circulating EBV-carrying B cells ([Whittle et al., 1984](#); [Lam et al., 1991](#)), viral reactivation, and increased viral burden, as demonstrated by quantitative measurement of EBV DNA ([Moormann et al., 2005](#); [Rasti et al., 2005](#); [Donati et al., 2006b](#); [Njie et al., 2009](#)). Comparison of levels of EBV DNA in blood from children living in two areas with different malaria-transmission intensities showed a correlation between viral load and the endemicity of malaria ([Moormann et al., 2005](#)). Detection rates and levels of EBV DNA were also shown to be higher in children with acute malaria than in control children without malaria ([Donati et al., 2006b](#)).

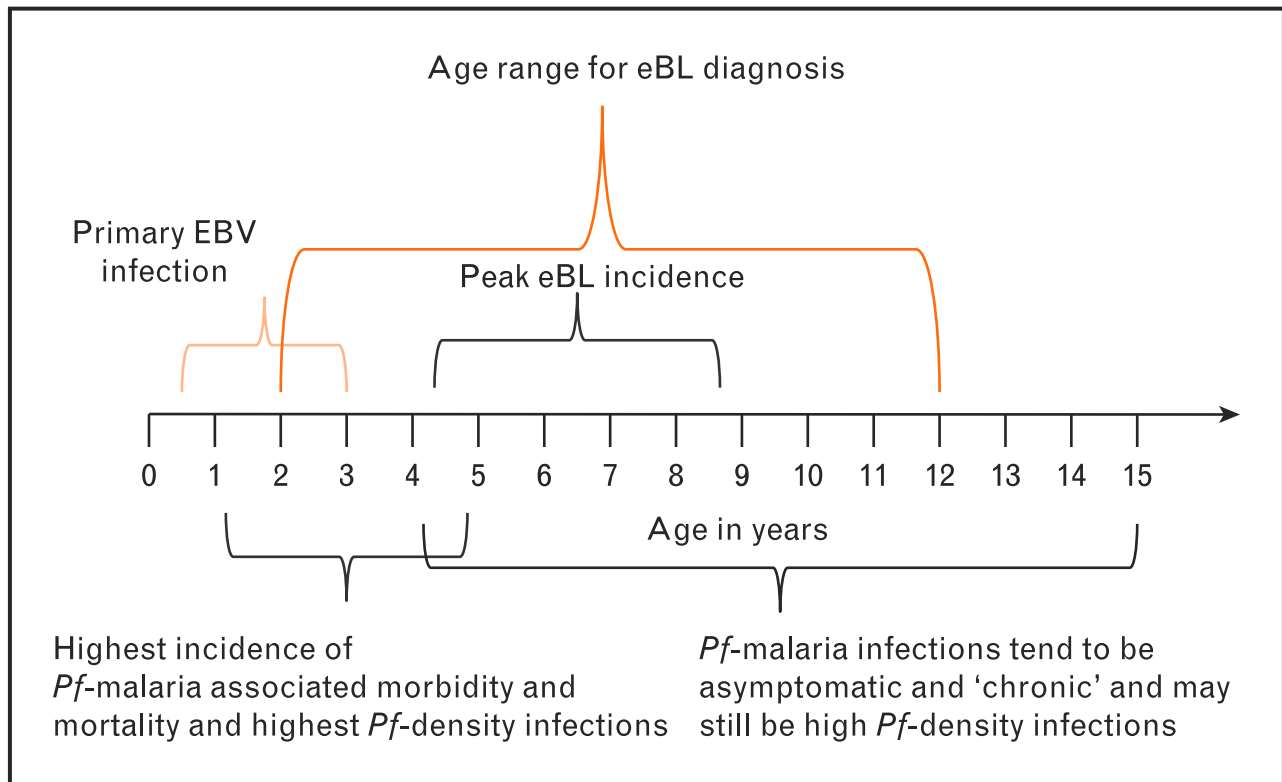
Studies in Kenya demonstrated an age-related loss of T-cell IFN- γ responses to lytic and latent EBV antigens, but predominantly to lytic antigens, in children living in areas of holoendemic malaria ([Moormann et al., 2007](#)). T-cell responses against lytic, but not latent, EBV antigens seem to decrease gradually among malaria-exposed children aged 5–9 years, compared with other age groups and non-malaria-exposed children ([Moormann et al., 2011](#)).

The impact of malaria on the control of EBV persistence seems to be evident only before immunity to malaria is fully acquired. Children, but not adults, living in malaria-endemic areas have high

serum levels of EBV DNA, an indicator of active EBV replication ([Rasti et al., 2005](#)). Endemic Burkitt lymphoma is a childhood tumour with a peak incidence at between age 5 and 8 years, an age range at which malaria infection may impair the virus/host balance. Moreover, the onset of endemic Burkitt lymphoma is marked by high antibody titres, indicative of viral replication ([Henle et al., 1969](#)). EBV reactivation can be monitored by the appearance of antibodies against VCA and BZLF1, which are markers of EBV reactivation ([Yamauchi et al., 1998](#); [Dardari et al., 2000](#)). A study that examined the effect of chronic infection with *P. falciparum* on EBV antibody titres reported higher and more persistent IgG titres to EBNA-1, VCA and BZLF1 in children from a malaria holoendemic area than in children from a hypoendemic area ([Piriou et al., 2009](#)).

In vivo, latent EBV in infected B cells can be reactivated to enter the lytic cycle by various cellular signalling pathways, including ligation to B-cell receptors and plasma-cell differentiation ([Laichalk & Thorley-Lawson, 2005](#)). Viral reactivation can also be triggered *in vitro* in EBV-carrying cell lines by anti-human Ig and by several chemical agents and drugs ([IARC, 2012](#)). Lytic replication begins by the expression of the intermediate-early EBV lytic activator genes *BZLF1* and *BRLF1*, followed by translation of late genes encoding structural components such as VCA. The first demonstration that EBV can be reactivated directly by another pathogen was published by [Chène et al. \(2007\)](#), who showed that PfEMP1 is able to directly drive a latently infected B cell into EBV replication. Infected erythrocytes and the PfEMP1 domain CIDR1 α bound to the EBV-carrying B-cell line Akata led to a lytic-cycle reactivation and an increase in viral load, independent of cell proliferation ([Chène et al., 2007](#)). Viral reactivation was also induced in latently infected B cells derived from the blood of healthy EBV carriers and patients with endemic Burkitt lymphoma.

Fig. 4.3 Chronology of *Plasmodium falciparum* malaria and Epstein–Barr virus infections in relation to incidence of endemic Burkitt lymphoma



eBL, endemic Burkitt lymphoma; EBV, Epstein–Barr virus; *Pf*, *Plasmodium falciparum*
 From [Moormann et al. \(2011\)](#). Reprinted with permission from Wolters Kluwer Health.

The molecular mechanisms underlying the EBV switch from latency to the lytic cycle may result in part from the interaction of PfEMP1 with surface Igs, and possibly other molecules, mimicking the effect achieved by B-cell receptor cross-linking with anti-human Igs ([Chêne et al., 2007](#)) and triggering viral reactivation in a similar way as anti-human Ig induces viral production in EBV-carrying cell lines ([Takada, 1984](#)). The main interactions between B lymphocytes and infected erythrocytes/malarial antigens most likely occur in the secondary lymphoid tissues *in vivo*, such as the spleen where memory B cells, potentially latently infected by EBV, could return to undergo additional rounds of maturation. The disruption of the secondary lymphoid organs that characterize malaria infections may favour the

triggering of viral reactivation in EBV-carrying lymphocytes by malarial antigens.

The preferential activation of the memory B-cell compartment by malarial antigens is of importance given that EBV resides in a latent state in memory B cells, and that differentiation from memory to plasma cells results in switching the latent to the lytic phase ([Babcock et al., 1998](#); [Laichalk & Thorley-Lawson, 2005](#)). Therefore, the increased Ig production and differentiation induced by stimulation with malarial antigens may also contribute to the increased viral production that characterizes malaria infection.

4.3.2 KSHV

KSHV, also known as HHV-8, is the etiological agent of Kaposi sarcoma ([IARC, 2012](#)). The risk of Kaposi sarcoma is significantly increased by immune suppression, such as that caused by HIV, leading to increased viral load and shedding ([Engels et al., 2003](#); [IARC, 2012](#)). Geographical and temporal variation in the incidence of Kaposi sarcoma and the prevalence of KSHV among immunocompetent individuals, indicate that other (co)factors may be important in facilitating both transmission and disease, e.g. environmental cofactors ([Ziegler, 1993](#); [Dedicoat & Newton, 2003](#); [Whitby et al., 2007](#); [Dollard et al., 2010](#); [IARC, 2012](#)) and presence of mosquitoes and other blood-sucking arthropods ([Coluzzi et al., 2003](#); [Ascoli et al., 2006](#)).

The association between malaria and KSHV was suggested by epidemiological analysis of the distribution of Kaposi sarcoma in Africa before the AIDS epidemic ([Cook-Mozaffari et al., 1998](#)). However, cross-sectional analysis of the incidence of Kaposi sarcoma in Latina (a central province of Italy where malaria was endemic until the 1930s), and in Malta (where malaria was not endemic), showed similar results, suggesting that malaria endemicity is not likely to increase the risk of disease ([Serraino et al., 2003](#)). Studies in Ugandan women found that seropositivity to KSHV was significantly associated with malaria parasitaemia, hookworm and *Mansonella pestans* ([Wakeham et al., 2011](#)). It seems therefore that other infections that are also prevalent in malaria-endemic areas may be contributing factors.

A recent hypothesis suggests that the immunosuppressive effect of antimalarial drugs e.g. chloroquine and quinine derivatives, may play a role in the high incidence of Kaposi sarcoma in malaria-endemic areas ([Ruocco et al., 2011](#)). The iatrogenic immunosuppression caused by these drugs is long-lasting; traces of chloroquine can be found several years after the last ingestion ([Meyer et al., 2010](#)).

The evidence for host immune dysregulation in the pathogenesis of Kaposi sarcoma is based on evaluation of high-risk populations (e.g. HIV-infected and post-transplant patients) ([Grulich et al., 2007](#)), as well as an increasing understanding of specific immune defects. Risk of Kaposi sarcoma is strongly associated with defects in cellular immunity. Epidemiological and clinical studies suggest that KSHV viraemia is a risk factor for development of Kaposi sarcoma (reviewed by [Uldrick & Whitby, 2011](#)). KSHV-infected patients who develop Kaposi sarcoma showed a reduced KSHV-specific CD4 response as measured by an IFN- γ enzyme-linked immunosorbent spot assay, specific for KSHV-derived peptides ([Guihot et al., 2006](#)).

There are insufficient data available to evaluate the precise role of malaria in KSHV infection. However, it is possible that in the same way that malaria impairs the immune control of EBV persistence, malaria could also lead to KSHV reactivation and increased prevalence of KSHV, with increased incidence of Kaposi sarcoma and other lymphoproliferative disorders.

4.3.3 Other human herpes viruses

Human herpes viruses are widely distributed pathogens. In immunocompetent individuals the clinical outcomes of infection are generally benign, but in immunocompromised hosts, primary infection, reinfection, or extensive viral reactivation can lead to critical diseases.

It has been suggested that acute infection with *P. falciparum* induces HSV-1 and VZV reactivation ([Cook 1985](#); [Regunath et al., 2008](#); [Sowunmi et al., 2008](#)). Recently, the effect of acute *P. falciparum* malaria infection on reactivation and shedding of all known HHVs including HSV-1, HSV-2, EBV, VZV, CMV, HHV-6, HHV-7 and KSHV (HHV-8) was analysed in a retrospective study among Ugandan children. Quantification of viral loads in blood and saliva demonstrated that malaria infection increased the load of EBV

in the blood and of HSV-1 in the oral cavity. HHV-6, HHV-7 and CMV were frequently detected in the malaria-infected children, but acute malaria was not found to be associated with reactivation of the β herpes viruses (Chêne *et al.*, 2011). [The sample size in this study was too small to draw any conclusion on the impact of malaria on reactivation of VZV and HHV-8.]

4.4 *Plasmodium* infection in experimental animals

Murine models for malaria infection do exist, employing parasites such as *P. yoelii*, *P. berghei*, and *P. chabaudii* (Serguiev & Demina, 1957; Landau & Killick-Kendrick, 1966), but there is a lack of studies on the induction of B-cell tumours or B-cell hyperplasia. A major limitation of these models is that repeated infection with *P. falciparum* cannot be reproduced because the murine *Plasmodium* induces sterilizing immunity. On the other hand, *P. falciparum*, the parasite associated with endemic Burkitt lymphoma, is specific for humans. Injection of human erythrocytes into immunodeficient mice results in greater than 90% of circulating erythrocytes that are human and can be infected by *P. falciparum* (Badell *et al.*, 2000; Angulo-Barturen *et al.*, 2008; Jiménez-Díaz *et al.*, 2009). This model is useful for the study of antimalarial drugs.

Transplantation of human cord-blood CD34⁺ progenitor cells to newborn Rag2^{-/-}γ_c^{-/-} mice leads to *de novo* development of major functional components of the human adaptive immune system (Chicha *et al.*, 2005). The model has already been developed for EBV infection and the results support the model of EBV persistence *in vivo*. Intriguingly, in cases that were characterized by nodular and diffuse proliferation similar to infectious mononucleosis, EBV also induced clonal expansion and ongoing somatic mutations without germinal-centre reactions (Cocco *et al.*, 2008). More recently, the same model was used to

investigate the effects of HTLV-1 infection *in vivo*, where alterations in human T-cell development were observed (Villaudy *et al.*, 2011). No animal model has been developed for efficient development of the human erythrocyte, and there is no suitable model available that incorporates all the key facets of both *P. falciparum* and EBV infections. The mouse transplanted with the human adaptive immune system could be further developed to support the injection of *P. falciparum*-infected erythrocytes selected *in vivo* to grow in immunodeficient mice and escape phagocytosis. Another possibility could be the use of murine malaria in CD34⁺Rag2^{-/-}γ_c^{-/-} mice, or the use of purified malarial antigens such as CIDR. Other aspects could also be tested, like the functional consequences of the interaction of *P. falciparum* with TLR9 on EBV-infected B cells.

4.5 Susceptible populations

In human malaria, certain processes are critical to disease development, and genetic variants that disrupt these processes can protect against the disease. The invasion of erythrocytes by malaria parasites plays a central role here. However, the few studies that evaluated potential effects of haemoglobin polymorphisms on the risk of developing Burkitt lymphoma have not been conclusive (see Section 2.1.5(b)).

4.6 Mechanisms of lymphomagenesis

It is in the context of an uninterrupted presence of malaria parasites, a continuous activation of the immune system, splenomegaly and concurrent infections by viruses, bacteria and parasites that endemic Burkitt lymphoma emerges at high frequency. The determining factors that bring about endemic Burkitt lymphoma are, as currently understood, the malaria parasite and EBV.

Different mechanisms may explain the incremental contribution of chronic and acute infection with *P. falciparum* in the lymphomagenesis of endemic Burkitt lymphoma.

On the one hand, malaria infection induces B-cell proliferation and activation, and protects against apoptosis, thereby increasing the latently infected B-cell pool. On the other hand, malaria infection increases virus production, and impairs EBV-specific responses. Increased viral reactivation may result from modulation of T cells, NK cells and humoral responses that limit the progression to lytic replication ([Precopio et al., 2003](#); [Moormann et al., 2007](#)), and from direct activation of the lytic cycle resulting from specific interaction between malarial antigens and latently infected B cells involving CIDR, and TLR9 engagement. After expansion, memory B cells can differentiate to antibody-secreting plasma cells. *P. falciparum* schizonts and infected erythrocytes have the potential to interact directly with B cells, thereby inducing proliferation and differentiation into antibody-secreting cells, concomitant with viral reactivation. *P. falciparum* has a TLR9 ligand ([Parroche et al., 2007](#)) and TLR9 signalling synergistically enhances proliferation of EBV-infected B cells ([Zauner & Nadal, 2012](#)).

The association of EBV with endemic Burkitt lymphoma is probably determined by the age at which EBV infection occurs. The timing of co-infection and duration of *P. falciparum* infections at the individual level seems to determine the outcome of EBV dysregulation leading to endemic Burkitt lymphoma. This assumption is supported by the fact that the average age of patients in hyperendemic areas was found to be 8.1 years, whereas in low-risk areas it was 16.2 years, and immigrants from malaria hypoendemic areas who migrated to holoendemic areas developed endemic Burkitt lymphoma in late adolescence and adulthood ([Burkitt & Wright, 1966](#)). Moreover, development of endemic Burkitt lymphoma before age 2 years is an extremely rare

event. These effects are more pronounced in children living in areas with perennial transmission where they are repeatedly infected with *P. falciparum* and harbour chronic, asymptomatic, and often untreated infections.

Increased viral load may increase the risk by multiple non-exclusive mechanisms at different levels:

- infecting bystander B cells and augmenting the pool of latently infected B cells;
- increasing activity of AID, which enhances the possibility that translocations will occur;
- providing an anti-apoptotic environment that tolerates the *MYC* translocation ([Kelly & Rickinson, 2007](#));
- exhaustion of T-cell immune responses. In mouse models it has been shown that the frequency of infections has a greater effect on immune function since the greater the number of antigenically heterologous infections, the greater the decrease in overall T-cell activity. Acute and persistent infection can induce loss of memory T-cell function to previously encountered non-cross-reactive viruses ([Doherty, 1993](#); [Kim & Welsh, 2004](#)).

Endemic Burkitt lymphoma is one of the most rapidly dividing tumours, thus the triggering event must occur shortly before clinical presentation. The frequency of the molecular event leading to malignancy must be low, as the annual incidence of endemic Burkitt lymphoma in holoendemic areas is on average only 5–10 (ranging up to 22) per 100 000 children, implying that the oncogenic event is rare.

Thus, EBV and malaria together exert effects on the human host that increase the likelihood of *MYC* translocation, and that synergize with the de-regulated *MYC* protein to enhance the proliferative capacity and survival of the tumour. The contribution of malaria-induced activation and enhanced survival of B cells, plus the EBV-driven proliferation and facilitation of mutation in

memory germinal-centre B cells may assist the MYC-Ig translocation to persist, giving rise to a Burkitt lymphoma progenitor cell.

5. Summary of Data Reported

5.1 Exposure data

Malaria in humans is caused by four well-characterized strict human *Plasmodium* pathogens: *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. A fifth pathogen, *P. knowlesi*, infects both humans and monkeys. The greatest burden of morbidity and mortality is associated with *P. falciparum*. While all people living in equatorial regions of the world are at risk of malaria, most cases (> 90%) of falciparum malaria and death occur in children aged < 5 years in sub-Saharan Africa. Across sub-Saharan Africa and in Papua New Guinea, malaria transmission is highly endemic, although there is great local variation. Transmission of endemic malaria is categorized according to several parameters, including entomological inoculation rate, parasite prevalence and splenomegaly in children aged 2–10 years. Holoendemic malaria transmission can be defined as parasite prevalence of > 70% and splenomegaly in > 80% of children, and there is no sterilizing immunity from a primary infection. Host genetic variation influences disease susceptibility. Where malaria transmission is endemic, *Plasmodium* infection in children recurs frequently. Antibodies are clearly important in protection against *P. falciparum*; however, correlations of humoral protection have not been well defined.

P. falciparum has a complex life cycle that involves both human and *Anopheles* mosquito hosts. After the bite of a *Plasmodium*-infected mosquito, the parasite travels to the liver; this stage of development is asymptomatic. It is only when the parasite is released from infected liver cells and invades erythrocytes that clinical illness

occurs. Falciparum malaria can be uncomplicated, with fever and chills, or associated with complications such as severe acute anaemia, cerebral malaria, adult respiratory distress syndrome and acidosis. Interventions such as use of bednets and intermittent prevention and treatment for malaria have been widely used to reduce the burden of malaria. Drug resistance remains a problem, and no vaccines are currently available.

5.2 Human carcinogenicity data

5.2.1 Endemic Burkitt lymphoma

All the studies reviewed for endemic Burkitt lymphoma were conducted in sub-Saharan Africa, where infection with Epstein–Barr virus (EBV), an established cause of the disease, is largely ubiquitous and occurs at an early age.

Evidence consistent with an etiological role for holoendemic malaria caused by infection with *P. falciparum* in relation to endemic Burkitt lymphoma comes from a variety of sources. In 1962, Dennis Parsons Burkitt highlighted a very strongly specific geographical association between Burkitt lymphoma and holoendemic malaria on the African continent. Since then, a substantial body of evidence has accumulated from multiple correlation studies strongly linking the incidence of Burkitt lymphoma to areas where *P. falciparum* transmission is more intense. These ecological studies carry substantial weight in this context because of global and local contrasts in exposure (e.g. there are areas where either everybody or nobody is exposed).

Early case–control studies were inconclusive due to small sample size, inadequate exposure assessment at the individual level, or poor methodology. Uncertainties in some of the serological measurements employed continue to be a limitation, even in recent case–control studies. Nevertheless, two studies that measured antibodies to the whole schizont (considered as a

measure of exposure) found an increasing risk of endemic Burkitt lymphoma with increasing titres of antimalaria antibody; they also demonstrated the combined effect of malaria and EBV infection.

5.2.2 Other cancers

There was no clear evidence of an association between infection with *P. falciparum* and cancer at any other site.

There were no data available on cancer associated with species of *Plasmodia* other than *P. falciparum*.

In summary, evidence on the association between holoendemic malaria caused by infection with *P. falciparum* and cancer is strongly supportive, but not conclusive.

5.3 Animal carcinogenicity data

In a study in male Balb/c mice, intraperitoneal injection of *Plasmodium berghei yoelli* promoted induction of lymphoma by Moloney leukaemogenic virus. In a limited study in Swiss mice, intraperitoneal injection of mouse erythrocytes parasitized with *P. berghei* strain K173 increased the incidence of lymphoma. In a third study in neonatal CFW mice, intraperitoneal injection of mouse erythrocytes parasitized with *P. berghei yoelli* resulted in a non-statistically significant promotion of SV40-induced sarcomas of the liver and/or spleen. In one study in male Buffalo rats, intraperitoneal injection of mouse erythrocytes parasitized with *P. berghei* did not promote aflatoxin B₁-induced hepatocellular carcinoma.

5.4 Mechanistic and other relevant data

Different mechanisms may explain the incremental contributions of EBV and of chronic infection with *P. falciparum* in the lymphomagenesis of endemic Burkitt lymphoma:

- The timing of co-infection and the duration of malarial infections at the individual level seem to determine the outcome of EBV dysregulation, leading to endemic Burkitt lymphoma. The endemicity of malaria infection correlates with the viral load, while it inversely correlates with the average age at which primary infection with EBV occurs.
- EBV is recognized as a necessary agent in the pathogenesis of endemic Burkitt lymphoma.
- The malaria parasite *P. falciparum* reactivates EBV both *in vitro* and *in vivo*.
- *In vitro*, malarial antigens such as PfEMP1 (*P. falciparum* erythrocyte membrane protein), cause polyclonal B-cell activation, enhance B-cell proliferation and differentiation, and alter different cellular pathways that inhibit apoptosis. These mechanisms lead to increased survival of the B cells that are the target for the specific chromosomal *MYC* translocation that characterizes endemic Burkitt lymphoma.
- *In vivo*, infection with *P. falciparum* modifies the B-cell subset composition, with expansion of immature transitional B cells and an increase in the activity of the enzyme activation-induced deaminase (AID), which generates mutations involved in immunoglobulin affinity and immunoglobulin class-switching. The increased survival and maturation further augment the EBV load.
- Several latent and lytic EBV-associated proteins increase the activity of AID and prevent apoptosis, and thus these viral proteins can additionally augment the likelihood of developing endemic Burkitt lymphoma by preventing apoptosis of B cells that carry the *MYC* translocation.

6. Evaluation and Rationale

6.1 Cancer in humans

There is *limited evidence* for an association between malaria caused by infection with *P. falciparum* in holoendemic areas and cancer. A positive association has been observed with endemic Burkitt lymphoma.

6.2 Cancer in experimental animals

There is *inadequate evidence* in experimental animals for the carcinogenicity of *P. berghei*.

6.3 Mechanistic and other relevant data

There is strong evidence that the malaria parasite *P. falciparum* reactivates EBV, which is the known cause of endemic Burkitt lymphoma. In addition, infection with *P. falciparum* augments the pool of B cells in which this tumour arises.

6.4 Overall evaluation

Malaria caused by infection with *P. falciparum* in holoendemic areas is *probably carcinogenic to humans (Group 2A)*.

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INTRODUCTION TO POLYOMAVIRUSES

1. Discovery

In 1953, Ludwik Gross reported that a filterable infectious agent could cause salivary cancer in laboratory mice ([Gross, 1953](#); [Stewart et al., 1957](#)). The cancer-causing agent was found to be a non-enveloped DNA virus that was named murine polyomavirus (from the Greek roots poly-, which means “many,” and -oma, which means “tumours”), for its ability to cause tumours in multiple tissues in experimentally infected rodents (reviewed in [Sweet & Hilleman, 1960](#)). The discovery spurred renewed interest in the idea that viral infections might be a major cause of cancer in humans.

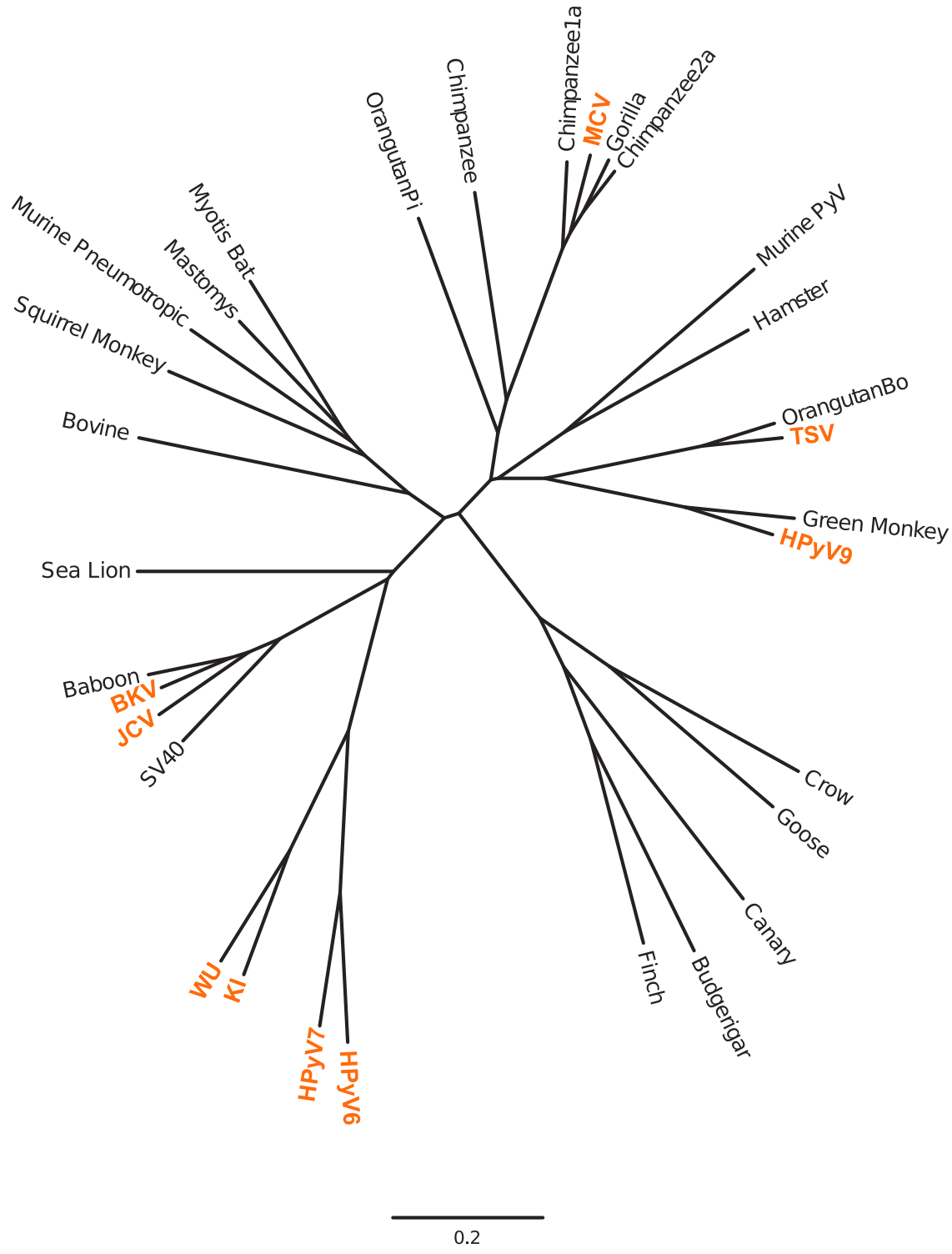
By the late 1950s, various investigators had succeeded in developing cell culture systems for analysing the transforming activities of murine polyomavirus *in vitro*. This work set the stage for the discovery of the primate polyomavirus simian virus 40 (SV40), which was identified as a contaminant in primary cultures of monkey kidney cells used to produce vaccines against poliovirus ([Sweet & Hilleman, 1960](#); [Eddy et al., 1962](#)). Millions of individuals were exposed to infectious SV40 virions present in contaminated polio vaccines administered between 1955 and 1963 (reviewed in [Shah & Nathanson, 1976](#); [Dang-Tan et al., 2004](#)).

In 1971, the first two naturally human-tropic polyomaviruses were discovered in specimens from immunocompromised patients ([Gardner et al., 1971](#); [Padgett et al., 1971](#)). The two viruses,

BK polyomavirus (BKV) and JC polyomavirus (JCV), were eventually found to chronically infect the great majority of humans worldwide (reviewed in [Abend et al., 2009](#); [Maginnis & Atwood, 2009](#)). The apparent ubiquity of BKV and JCV makes it difficult to correlate seropositivity for BKV- or JCV-specific antibodies with specific disease states, such as cancer.

Reports in the past four years have revealed the existence of seven more human polyomaviruses. Perhaps the most intriguing of the new species, named Merkel cell polyomavirus (MCV), was discovered through a directed genomic search of an unusual form of skin cancer, Merkel cell carcinoma (MCC) ([Feng et al., 2008](#)). Another new polyomavirus, trichodysplasia spinulosa-associated polyomavirus (TSV), was isolated from a rare hyperplastic (but non-neoplastic) trichodysplasia spinulosa skin tumour that can occur in transplant patients ([van der Meijden et al., 2010](#)). Little is currently known about the cancer-causing potential of TSV. Five other recently discovered human polyomaviruses – named WU polyomavirus (WUV), KI polyomavirus (KIV), human polyomavirus 6 (HPyV6), HPyV7, and HPyV9 – have not so far been clearly associated with human disease states ([Allander et al., 2007](#); [Gaynor et al., 2007](#); [Schowalter et al., 2010](#); [Sauvage et al., 2011](#); [Scuda et al., 2011](#)). The remaining *Monographs* in this Volume will focus on SV40, BKV, JCV, and MCV.

Fig. 1.1 Phylogenetic tree of known polyomavirus species



Human-derived species are in bold type.
Prepared by the Working Group.

2. Taxonomy and phylogeny

The exterior structure of the non-enveloped capsids of members of the viral family Polyomaviridae is strikingly similar to the capsids of a different family of non-enveloped viruses called the Papillomaviridae. Both families carry circular, double-stranded DNA (dsDNA) genomes. Based on these considerations, the two virus groups were originally classified within a single family, the Papovaviridae (a sigla condensation of PAPillomavirus POLyomavirus simian VACuolating). However, sequencing of the genomes of various polyomaviruses and papillomaviruses revealed essentially no detectable sequence homology between the two virus groups and furthermore showed that the two groups had dramatically different genetic organization. Since the sequencing results indicate that polyomaviruses and papillomaviruses probably never shared a common viral ancestor, they were officially split into separate viral families.

Interestingly, several recent reports suggest that polyomaviruses and papillomaviruses may occasionally recombine with one another to produce viable chimeric viruses with mixed genetic characteristics of both families ([Woolford et al., 2007](#)). Another recent report documenting a novel viral species that infects eels suggests that polyomaviruses might also recombine with members of other families of DNA viruses to produce viable chimeric progeny ([Mizutani et al., 2011](#)). The taxonomy of these apparently chimeric viral species is currently undefined.

A recent proposal that is currently being reviewed by the International Committee on Taxonomy of Viruses suggests that the various members of the family Polyomaviridae be grouped into separate genera ([Johne et al., 2011](#)). If approved, this would result in the division of the current sole genus *Polyomavirus* into three genera: *Orthopolyomavirus*, *Wukipolyomavirus*, and *Avipolyomavirus*. The first two genera

encompass all the currently known human- and primate-tropic species, while the third genus includes species thought to be tropic only for birds. The four primate polyomavirus species that have been proposed to be potentially implicated in human cancer (SV40, BKV, JCV, and MCV) are all members of the proposed genus *Orthopolyomavirus*.

Although SV40, BKV, and JCV are separate viral species, they are very closely related to one another, sharing about 70–75% identity at the nucleotide level across the entire genome. MCV is only distantly related to the SV40 cluster, sharing less than 35% nucleotide identity across the entire genome ([Fig. 1.1](#)). Phylogenetic analyses of BKV and JCV isolates from different populations worldwide have shown that the two virus species exhibit a geographical pattern of genetic drift that closely resembles proposed patterns of prehistoric human migration (reviewed in [Yogo et al., 2004, 2009](#)). MCV is somewhat more closely related to murine polyomavirus, with the two species sharing about 50% nucleotide identity across the complete viral genome. Phylogenetic trees based on the nucleotide or amino acid sequences of individual viral gene products give similar results, suggesting that the members of the SV40 cluster diverged from one another relatively recently, while the split between MCV and members of the SV40 cluster occurred in the much more distant past.

3. Structure of the virion

The exterior surface of the polyomavirus virion is a naked protein capsid composed entirely of a single virally encoded protein called capsid viral protein 1 (VP1) ([Stehle et al., 1996](#)) (see cover photograph). The virion contains a total of 72 pentameric VP1 capsomers arranged on a $T = 7d$ icosahedral lattice ([Yan et al., 1996](#)). VP1 subunits are folded into a classic eight-stranded β

jellyroll fold that is shared among a wide variety of viral capsid proteins and the cellular protein nucleoplasmin (Stehle *et al.*, 1994; Carrillo-Tripp *et al.*, 2009). The jellyroll, which forms the core of the pentameric VP1 interface, is arranged perpendicular to the spherical virion, such that the assembled virion has a distinctive knobby appearance. An additional capsid protein, VP2, as well as its N-truncated isoform VP3, associate with the central lumen of each VP1 capsomer (Chen *et al.*, 1998).

In all extensively studied examples, it appears that polyomavirus infectious entry requires interactions between VP1 and one or more cellular glycans that carry at least one sialic acid residue. In several distantly related polyomaviruses, the binding site for the sialylated glycan receptor is formed by a pocket along the outer rim of the apical portion of the VP1 capsomer knob (Stehle and Harrison, 1996; Neu *et al.*, 2008; Neu *et al.*, 2010). The loops that form the receptor binding pocket vary extensively between polyomavirus species, and even among closely related polyomavirus subspecies (Luo *et al.*, 2012). This variation may reflect selective pressure to evade recognition by antibodies that occlude the receptor binding site.

The floor of the canyons between the capsomer knobs is formed primarily by N- and C-terminal arms of VP1. The arms are stabilized by disulfide bonds between neighbouring VP1 molecules. The fully mature virion shows a remarkably high degree of stability and can remain fully infectious even after aggressive insults, such as heating at 75 °C for 1 hour (Lelie *et al.*, 1987; Sauerbrei & Wutzler, 2009). This high degree of virion stability raises the possibility that polyomaviruses might be transmitted environmentally or via lightly cooked meat products (reviewed in zur Hausen, 2009, 2012).

Purified VP1 can spontaneously self-assemble into virus-like particles (VLPs) that closely resemble the native virion (Salunke *et al.*, 1986). Such VLPs have been widely used for

serological analyses of polyomavirus epidemiology (Hamilton *et al.*, 2000; Carter *et al.*, 2003; de Sanjose *et al.*, 2003; Stolt *et al.*, 2003). Purified polyomavirus VLPs can be immunogenic when administered to laboratory animals, raising serum antibody responses capable of neutralizing native virions *in vitro* (Goldmann *et al.*, 1999; Velupillai *et al.*, 2006; Randhawa *et al.*, 2009). VLPs can even be humorally immunogenic in mice in the absence of functional T-cell immunity (Vlastos *et al.*, 2003), suggesting that VLP-based immunogens might be effective in immunocompromised human subjects who may be at greater risk of polyomavirus-associated disease. This suggests that polyomaviruses might be a suitable target for the development of VLP-based preventive vaccines, similar to the highly successful VLP-based vaccines against hepatitis B virus and human papillomaviruses (HPV).

4. Genomic organization, gene products, and replication

The circular ~5 kb dsDNA polyomavirus genome is roughly divided into two oppositely oriented transcriptional units separated by a non-coding control region (NCCR) (reviewed in Gu *et al.*, 2009) (Fig. 1.2). One transcriptional unit encodes several T-antigens, such as small T-antigen (sT) and large T-antigen (LT). The other transcriptional unit encodes the VP1 and VP2/3 capsid proteins and the agnoprotein. The term T-antigen historically derives from their expression in SV40-induced tumours (Rapp *et al.*, 1964, 1965). Although T-antigen sequences vary among the mammalian polyomavirus species, the overall arrangement of major functional domains of the singly spliced LT gene is broadly conserved and sT is expressed from an unspliced open reading frame encoding a protein of low relative molecular mass (18–20 kDa). The splice

donor used for mRNAs encoding LT is within the sT open reading frame, such that sT and all LT isoforms share a common leader peptide sequence, typically about 80 amino acids long. In addition, many polyomavirus species express multiply spliced transcripts encoding various T-antigen isoforms. Although murine polyomavirus expresses an additional membrane-bound T-antigen protein called middle T, critical for its transforming properties ([Fluck & Schaffhausen, 2009](#)), this gene product does not appear to be expressed by the human polyomaviruses.

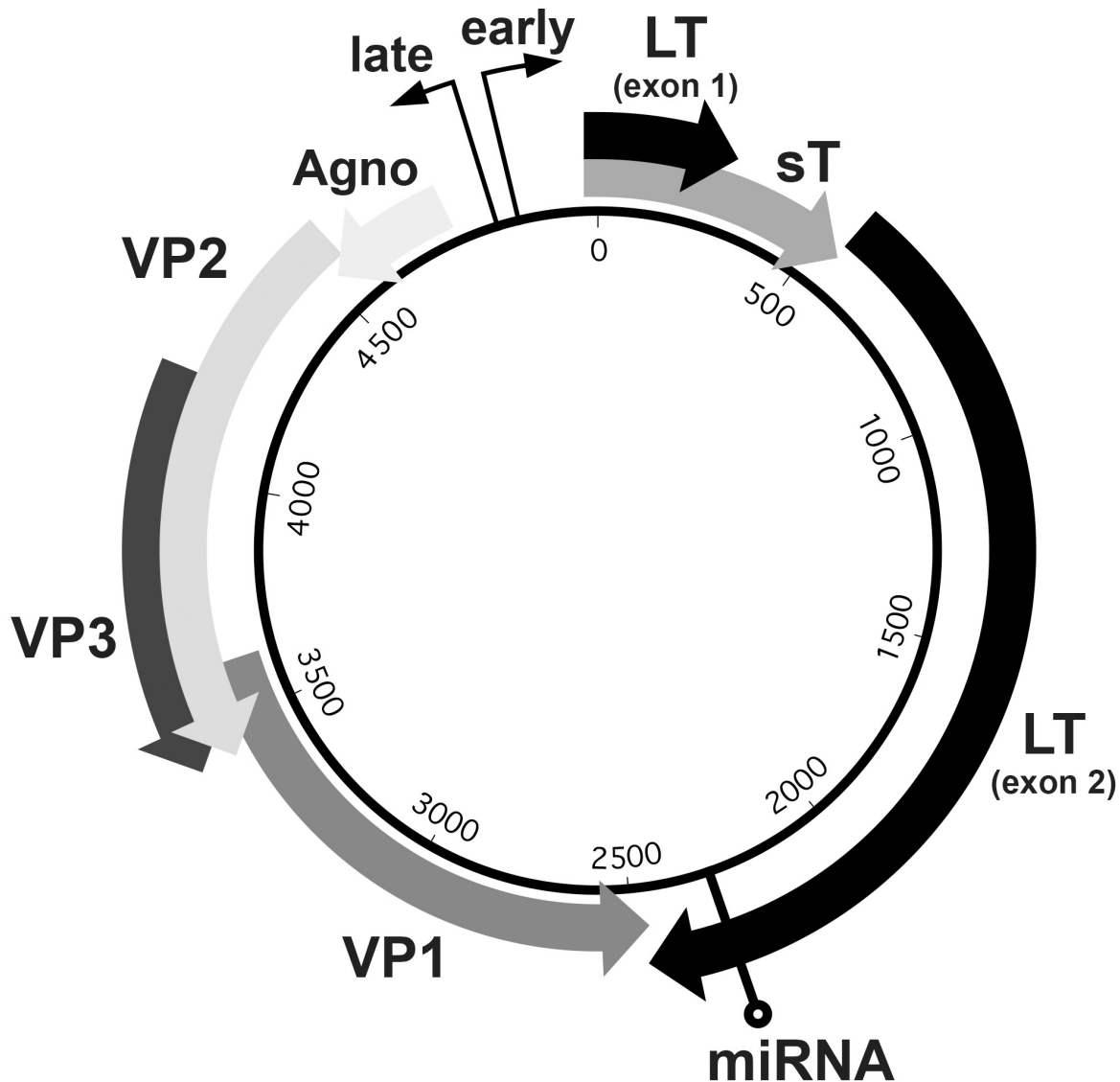
Genes in the T-antigen region are generally expressed at early time points after infectious entry, while the capsid proteins are expressed at high levels only at later time points ([Atkin et al., 2009](#); [Feng et al., 2011](#); [Neumann et al., 2011](#)). Early studies of the SV40 NCCR provided some of the first information on the nature of eukaryotic promoters and enhancers, and the SV40 promoter/enhancer system remains one of the most extensively studied examples of eukaryotic transcriptional regulation. The NCCR is highly variable between, and even within, polyomavirus species. Observations indicate that rearrangements in NCCR sequences have important functional consequences for SV40, BKV, and JCV transcriptional regulation, replication, and pathogenesis (reviewed in [White et al., 2009a](#); [Yaniv, 2009](#)) (see Section 4 of the *Monographs* on SV40, BKV, and JCV in this Volume).

The 3' end of the early and late regions is separated by a short segment that contains bi-directional polyadenylation signals. In the case of murine polyomavirus (and likely all other polyomaviruses), termination of transcription is inefficient, particularly for late mRNAs (encoding VP1/2/3). The expression of un-terminated late mRNAs with perfect complementarity to early (T-antigen) mRNAs is thought to play an important role in the regulation of the shift from expression of the T-antigens early after infection to the expression of capsid proteins during the productive late phase of infection (reviewed in

[Gu et al., 2009](#)). The early-to-late shift may also be partially controlled by expression of microRNAs that appear to be encoded on late strand transcripts transiting the early region ([Sullivan et al., 2005](#); [Seo et al., 2008](#); [Seo et al., 2009](#)). These microRNAs may be capable of antagonizing early gene expression and might also exert regulatory effects on the host cell ([Bauman et al., 2011](#)).

The polyomavirus NCCR also encompasses the viral origin of replication (Ori). The origin of mammalian polyomaviruses contains multiple pentameric sequences (G(A/G)GGC) that serve as the binding sites for the LT protein. Twelve LT molecules load onto the origin to form a double-hexameric head-to-head ring structure, forming the active helicase for unwinding the origin and recruiting cellular DNA replication machinery ([Fanning & Zhao, 2009](#)). Origin unwinding ("melting") occurs adjacent to an A/T-rich sequence. The structure of these conformational changes and DNA binding for LT double hexamers has been solved by crystallography ([Li et al., 2003](#); [Gai et al., 2004](#); [Harrison et al., 2011](#)). In addition to its helicase function, LT also serves to recruit various cellular DNA replication factors ([Gannon & Lane 1987](#); [Dornreiter et al., 1990](#); [Melendy & Stillman, 1993](#); [Simmons et al., 1996](#)). The species-specific ability of LT to recruit DNA polymerase α -primases of various hosts appears to be a major determinant of polyomavirus host range (see below). Polyomavirus sT proteins also play an unclear, possibly indirect role in facilitating the replication of the viral DNA ([Berger & Wintersberger, 1986](#); [Cicala et al., 1994](#); [Kwun et al., 2009](#)).

A small gene product called agnoprotein is encoded 5' of the VP2 open reading frame in SV40, BKV, and JCV. Proposed roles for agnoproteins include regulation of viral gene expression, virion assembly, cell lysis, and dysregulation of a wide variety of cellular processes ([Suzuki et al., 2010](#); [Johannessen et al., 2011](#); [Sariyer et al., 2011](#); reviewed in [Khalili et al., 2005](#); [Moens et al., 2007](#)).

Fig. 1.2 Polyomavirus family genome map

Protein coding sequences are displayed as large arrows. Early transcripts encode small T-antigen (sT) and large T-antigen (LT) proteins. In most polyomaviruses for which transcript maps are available, additional T-antigen isoforms (not shown) are encoded by multiply spliced mRNAs. The late promoter controls the expression of transcripts encoding the VP2, VP3, and VP1 capsid proteins. In SV40, BKV, and JCV, the late transcript also encodes an agnoprotein (Agno). Other polyomaviruses (including MCV) are not known to encode agnoproteins. The origin of replication (Ori, not shown) overlaps with the early promoter. Many polyomaviruses encode a microRNA (miRNA, lollipop) on late region read-through transcripts.

Prepared by the Working Group.

5. Viral life-cycle

Like other non-enveloped virus families, polyomaviruses are believed to breach host-cell membranes after internalization via endocytic pathways. For polyomaviruses whose infectious entry pathways have been extensively studied, a common feature appears to be the engagement of cell-surface glycans that carry at least one sialic acid residue ([Tsai et al., 2003](#); reviewed in [Neu et al., 2009](#)). In the cases of murine polyomavirus, SV40, and BKV, the entry receptor is one or more types of sialylated lipids called gangliosides. The target gangliosides appear to be widely distributed on a variety of cell types since these viruses, or reporter vectors based on them, can infect or transduce a wide range of cell lines from various species ([Nakanishi et al., 2008](#)). Thus, receptor binding and subsequent steps in the infectious entry process are unlikely to be major determinants of host or tissue tropism. For MCV, there is controversy about whether the sialylated glycans required for infectious entry are displayed in the form of gangliosides or in association with protein ([Erickson et al., 2009](#); [Schowalter et al., 2011](#)). In addition to the sialylated glycan co-receptor, MCV also appears to require interactions with a different highly widespread form of cellular glycan called heparan sulfate. Like murine polyomavirus, SV40, and BKV, MCV can bind to and successfully infect (or transduce) a wide range of cell lines from various species ([Feng et al., 2011](#); [Neumann et al., 2011](#); [Schowalter et al., 2011](#)). JCV differs from the other polyomavirus species in that it has been proposed to require a serotonin (5-hydroxytryptamine) 2A receptor (5-HT_{2A}R) in addition to the linear sialylated target LS-tetrasaccharide c (LSTc) ([Elphick et al., 2004](#); [Neu et al., 2010](#)). The expression of the unique entry receptors of JCV may dictate its tropism for cells of the central nervous system *in vivo* (see Section 1.1 of the *Monograph* on JCV in this Volume), as well as its relatively restricted

cellular entry tropism *in vitro* ([Nakanishi et al., 2008](#)).

After engagement of the cognate receptor, polyomaviruses are endocytosed and traffic through the endoplasmic reticulum, where cellular factors facilitate compromise of the cellular lipid bilayer, allowing escape of the viral genome into the cytoplasm. The viral DNA, which may remain at least partially associated with the capsid proteins ([Kuksin & Norkin, 2012](#)), then traffics to the nucleus, where transcription factors are recruited to allow early gene expression.

In permissive cells, the early genes target host-cell signalling to drive cell-cycle progression. By promoting cellular expression of DNA replication factors, replication of the episomal viral DNA is facilitated, possibly through a rolling circle replication mechanism ([Bjursell, 1978](#)). Expression of the LT antigen can concurrently lead to expression of late gene capsid proteins. Assembly of high levels of encapsidated virus results in active lysis of the host cell.

In many cell types that support the infectious entry of polyomaviruses, the late phase of the viral life-cycle is blocked. Laboratory-adapted BKV and JCV strains with rearranged NCCRs can successfully complete the viral life-cycle in some cultured cell lines. Primary isolates of these viruses with un-rearranged “archetype” NCCRs cannot readily be cultured in conventional cell lines ([Broekema & Imperiale, 2012](#)). MCV also appears to replicate very poorly in culture. This may reflect a tendency of these virus species to establish a form of viral latency in many cell types. Under this hypothesis, the viral genome is stably maintained as a low-copy-number episome, expressing few or no viral gene products. In this state, the virus would presumably be resistant to immune clearance, allowing durable long-term maintenance of the infection. This concept is reminiscent of the standard model for the papillomavirus life-cycle, in which the virus is stably maintained in undifferentiated

keratinocytes in the skin and the late phase of the life-cycle is closely tied to the eventual terminal differentiation of the keratinocyte (reviewed in [Doorbar, 2005](#)).

6. Host range

Early studies with murine polyomavirus showed that cell lines from non-native hosts, such as Chinese hamsters or Norway rats, do not support high-level replication of the viral genome (reviewed in [Atkin et al., 2009](#)). Interestingly, adult hamsters and rats exposed to murine polyomavirus show a greater tendency to develop tumours than do adult mice exposed to murine polyomavirus. SV40 also does not replicate in mouse cells. However, SV40 may pose an exception to the generally narrow host restriction of *Orthopolyomaviruses*, in that it has been shown to replicate in experimentally infected hamsters and can be vertically transmitted to pups *in utero* ([Patel et al., 2009](#)). This study also showed that the most robust replication was observed using laboratory-adapted SV40 isolates carrying complex NCCR rearrangements.

Consistent with the apparently limited host range of murine polyomavirus, phylogenetic analysis suggests that members of the proposed *Orthopolyomavirus* genus tend to co-speciate with their host mammals. For example, various chimpanzee and gorilla subspecies each appear to harbour host-specific relatives of MCV ([Leendertz et al., 2011](#)). Given the apparently slow rate of genetic divergence of polyomaviruses ([Yogo et al., 2009](#)), it appears unlikely that members of this cluster of MCV-related viruses are transmitted between their great ape hosts.

In contrast to *Orthopolyomaviruses*, it appears that at least one member of the proposed genus *Avipolyomavirus* is naturally transmitted among distantly related bird species ([Johne et al., 2011](#)). There are currently no comparable examples of

productive, naturally occurring transmission of a polyomavirus from one host mammal to another.

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SIMIAN VIRUS 40

1. Exposure Data

1.1 Host range and tissue tropism

The natural host of simian virus 40 (SV40) is the rhesus macaque, in which the virus is commonly maintained as a chronic infection of the kidney epithelium and other tissues. Although SV40 infection is not associated with known disease sequelae in immunocompetent animals, in immunodeficient animals SV40 can cause central nervous system disease as well as renal and pulmonary disease ([Sheffield *et al.*, 1980](#); [Lednicky *et al.*, 1998](#); [Axthelm *et al.*, 2004](#); [Dang *et al.*, 2008](#)). Under laboratory conditions, SV40 can be transmitted to a variety of non-native hosts. The outcome of SV40 infection is classified as either permissive or non-permissive. In the permissive case, the full replicative life-cycle of the virus is observed, with lysis of infected cells. In the non-permissive case, viruses gain entry and may begin to express early viral proteins, but the full infection cycle is blocked at viral DNA replication steps and late gene expression does not occur. Whether the outcome of SV40 infection is permissive or non-permissive is determined largely by the species of host cells used. Productive infections are elicited in African green monkey kidney cell lines (BSC or CV1) and some human cell lines, whereas non-permissive infections are seen in mice and mouse-derived cell lines (reviewed in [Atkin *et al.*, 2009](#)). The inability of SV40 to complete its life-cycle and

lyse infected cells from some species can have important biological consequences. For example, non-permissive mouse and rat cells can survive SV40 infection and go on to be stably transformed by the virus. The ability to support virus replication is also cell-type-specific, even within susceptible hosts. Detection of SV40 or SV40-like DNA sequences and proteins in human tissues is discussed in Section 1.2.

1.2 Methods for the detection of SV40 infection

The presence of SV40 infection has been examined by detection of the SV40 genome by polymerase chain reaction (PCR) techniques, and by detection of anti-capsid antibodies by neutralization tests or by enzyme-linked immunosorbent assay (ELISA) using recombinant virus-like particles (VLPs). Immunohistochemical staining and in situ hybridization methods are important to confirm the presence of SV40; however, these methods have not been used in epidemiological studies on SV40.

1.2.1 PCR-based methods

The methods for detection of SV40 DNA are not standardized, and differences in methods may be responsible for some of the discrepancies observed between studies ([Strickler, 2001](#), see also Section 4.3.1). Laboratory contamination, lack of specificity of PCR techniques, or

cross-amplification with other polyomaviruses might also explain some of the varying results. To reduce false-positive results, PCR primers should be able to differentiate between the SV40 genome and SV40 sequences present in common laboratory plasmids and in some cell lines ([López-Ríos et al., 2004](#)). [These lower-risk primers might still amplify contaminating SV40 DNA from cell lines carrying the integrated SV40 genome ([Cohen & Enserink, 2011](#)).] Using such primers dramatically reduced the number of samples detected containing SV40 DNA. These findings were confirmed by [Manfredi et al. \(2005\)](#). Since most investigators used primer sets (e.g. SV5/SV6, TA1/TA2) that did not amplify BK polyomavirus (BKV) and JC polyomavirus (JCV) DNA but that recognized SV40 sequences present in many laboratory plasmids, contamination from cloning and expression vectors could have occurred if appropriate precautions were not taken ([López-Ríos et al., 2004](#)). The absence of standardized PCR methods and quality control procedures, including appropriate blinding and relevant controls, may explain some of the varying findings reported for SV40 DNA detection. When detected, SV40 is present at low copy number ([David et al., 2001](#)).

1.2.2 Detection of SV40 antibodies

Serological studies on SV40 have for many years had to rely on neutralization tests (plaque neutralization or microwell neutralization). The recent detection of viral capsid antibodies has been greatly facilitated by the development of recombinant VLPs. ELISAs have been developed using VLPs or capsomers produced in baculovirus systems, and recombinant yeasts or bacteria, and these have become common serological tests for SV40.

In humans, anti-SV40 antibody reactivity is observed largely in BKV- and/or JCV-positive samples, and in those samples with BKV and JCV antibody titres higher than those observed

against SV40. The reactivity of rhesus macaque sera and human sera with VLPs of SV40, BKV, and JCV provides unambiguous evidence of immunological cross-reactivity between these viruses ([Carter et al., 2003](#); [Engels et al., 2004a](#)).

Competitive inhibition studies have shown that the reactivity to SV40 in human sera is often eliminated by pre-incubation with BKV and JCV VLPs, suggesting that all of the SV40 reactivity observed in humans is due to cross-reactivity to BKV and JCV ([Carter et al., 2003](#); [de Sanjosé et al., 2003](#); [Viscidi et al., 2003](#); [Rollison et al., 2005a](#); [Viscidi & Clayman, 2006](#); [Kjaerheim et al., 2007](#)). In contrast, in SV40-infected monkeys, SV40 reactivity was completely blocked by SV40 VLPs but not by BKV and JCV VLPs ([Carter et al., 2003](#); [Engels et al., 2004a](#)). To identify specific SV40 reactivity, it is necessary to perform competitive inhibition assays by pre-incubation of SV40-reactive human sera with an excess of BKV and JCV VLPs.

1.3 Epidemiology of infection

1.3.1 Transmission of SV40 infection to humans

(a) Transmission from animals to humans

The understanding of the transmission and pathogenesis of SV40 in humans is poor and largely incomplete. Natural exposure to SV40 in humans is considered a rare event, and antibodies to SV40 can be demonstrated only in people who have been in contact with monkeys ([Shah, 1966](#); [Engels et al., 2004a](#)) or who had received, in the past, SV40-contaminated poliovirus vaccine.

Infection with SV40 is common in rhesus macaques, where it causes a silent but lifelong infection ([Sweet & Hilleman, 1960](#)). Initial lytic infection is controlled by the immune system, and later the virus persists in the kidney, where it can be reactivated under conditions of immunosuppression ([Horvath et al., 1992](#)). There is evidence that SV40 is shed in the urine of infected

animals, which have higher titres of neutralizing antibodies ([Shah et al., 1969](#); [Lednicky et al., 1998](#); [Carter et al., 2003](#); [Minor et al., 2003](#)). About 80–100% of captive adult rhesus macaques and 50% of captive baboons ([Jones-Engel et al., 2006](#); [Simon 2008](#); [Westfall et al., 2008](#)) are SV40-seropositive. SV40 has been recovered from urine, faeces, and food residues taken from infected animals. Transmission seems to occur through the environment rather than through direct contact between animals (vertical or perinatal transmission or contact within the gang) ([Minor et al., 2003](#); [Bofill-Mas et al., 2004](#)).

The route of human exposure to SV40 is unclear. SV40 shedding in macaque urine or occupational injuries or specific incidents (bites, scratches, or splashes) suggest that humans could be at risk of infection with SV40. However, human cells are less susceptible to SV40 replication than are monkey cells ([Shein & Enders, 1962a](#); [Shah et al., 1969](#); [O'Neill & Carroll, 1981](#); [O'Neill et al., 1990](#)). Low levels of neutralizing antibodies have been reported in 27% of workers at monkey-export companies in India, suggesting that humans can be exposed through contact with infected animals ([Shah, 1966](#)). SV40 seroreactivity confirmed by competitive inhibition experiments was also detected in 10% of zoo workers with regular exposure to monkeys, compared with 3% of workers with infrequent exposure ([Engels et al., 2004a](#)). However, [Carter et al. \(2003\)](#) showed that although 6.6% of subjects from a normal population were SV40-positive, the reactivity disappeared after pre-incubation with BKV or JCV VLPs; hence, SV40 infection of humans via monkeys remains controversial.

(b) *Transmission through vaccines to humans*

SV40 was discovered as a contaminant in poliovirus vaccine. Formalin-inactivated poliovirus vaccine (injected) and live poliovirus vaccine (oral) were prepared in primary kidney cells of rhesus and cynomolgus macaques, some of which were from monkeys naturally infected

with SV40. Safety testing of the vaccine preparations led to the identification of a new virus called SV40, in 1959 ([Sweet & Hilleman, 1960](#); [Eddy et al., 1961, 1962](#)), 5 years after the formalin-inactivated vaccine was licensed. Moreover, although poliovirus vaccine batches approved in 1961 and later were required to be free of SV40, batches approved earlier were not recalled. Thus, the use of inactivated poliovirus vaccine (IPV) containing SV40 may have continued until 1963 ([Shah & Nathanson, 1976](#)).

The extent of contamination of poliovirus vaccine stocks with viable SV40 has not been established. For the IPV (Salk vaccine), the formalin inactivation process used for inactivation of poliovirus was shown to also inactivate the SV40 virions ([Sweet & Hilleman, 1960](#)), although residual infectious SV40 survived at low levels in some vaccine preparations ([Gerber et al., 1961](#)). Live SV40 could still be cultured after formaldehyde treatment, although titres were lower ([Gerber et al., 1961](#); [Fraumeni et al., 1963](#); [Engels et al., 2003a](#)). It is estimated that up to 30% of the killed vaccine lots contained live SV40 ([Shah & Nathanson, 1976](#)). Pre-licensure candidate oral poliovirus vaccines (OPVs) were presumably all contaminated with SV40. The candidate OPVs were field-tested from 1958 to 1960 at certain sites in various countries, including Mexico (Sabin OPV); Colombia, Costa Rica, Nicaragua, and Uruguay (Lederle Laboratories OPV); and Croatia, Poland, and the Republic of the Congo (Koprowski OPV). Only small trials were carried out in the USA. The commercially licensed Sabin OPV was supposedly SV40-free after 1963.

The Russian OPV was prepared from the pre-licensure Sabin viral strains and was used widely in 1959 and later in the Russian Federation and several countries in eastern Europe ([Butel, 2012](#)). The Russian vaccine was subsequently also provided to other countries and was likely contaminated with SV40 until the late 1970s ([Cutrone et al., 2005](#)). In these vaccines, the heat treatment in the presence of $MgCl_2$ used

to attenuate poliovirus did not completely inactivate SV40 ([Cutrone et al., 2005](#)). In addition, military recruits in the USA from 1959 to 1961 who received SV40-contaminated adenovirus vaccines and the several thousand individuals in the USA who received the experimental live poliovirus vaccine by the oral route in earlier clinical trials were at risk of exposure to live SV40 ([Cutrone et al., 2005](#)).

Briefly after vaccination with contaminated OPV, small amounts of infectious SV40 were identified in stools of some of the immunized neonates and infants ([Melnick & Stinebaugh, 1962](#)). However, no SV40 antibody response was observed in recipients, suggesting either that the SV40 virions present in the attenuated poliovirus vaccine were not infectious in humans or that the virions had little or no infectivity in humans ([Morris et al., 1961](#); [Shah & Nathanson, 1976](#)). Seroconversion was documented after parenteral vaccination with contaminated vaccines ([Sweet & Hilleman, 1960](#); [Gerber, 1967](#); [Engels et al., 2003a](#)). [However, it is unclear whether the development of SV40 antibodies represented an infection or instead an exposure to inactivated viral proteins.]

(c) *Transmission from humans to humans*

Many routes of SV40 circulation in humans have been speculated, including faecal–oral, respiratory, and mother-to-child, but there is scant evidence to support any of these routes. [Patel et al. \(2008\)](#) reported that SV40 DNA was detected in 9.1% of tonsils in immunocompetent children ([Table 1.1](#)). However, the detection of SV40 DNA was not confirmed using low-contamination-risk primers as defined by [López-Ríos et al. \(2004\)](#), since SV40 DNA was not detected in tonsil tissues from 57 children and was detected in adenoid tissue in only 1 (1.3%) of 80 children and at a very low copy number ([Comar et al., 2010](#)). Thus, there are no supporting data that SV40 is transmitted by the respiratory route.

In a recent study ([Abedi Kiasari et al., 2011](#)), SV40 was identified in blood and/or urine of two transplant patients, suggesting that SV40 could cause infection in such patients.

1.3.2 *Prevalence of SV40 infection in the general population*

(a) *Prevalence of SV40 DNA*

Detection of SV40 DNA in immunocompromised or immunocompetent subjects was reported in many studies. The most recent studies, all of which used DNA detection by PCR, are presented in [Table 1.1](#).

About half of the studies did not detect SV40 DNA in the examined groups. In other studies, the SV40 prevalence varied from 1.3% to 25.6%, with SV40 being detected in the urine, stools, blood, lungs, and tonsil tissues. The heterogeneity of the findings is illustrated by the results of SV40 DNA detection in the urine or kidneys ([Shah et al., 1997](#); [Li et al., 2002a](#); [Manfredi et al., 2005](#); [Vanchiere et al., 2005a](#)). SV40 was not detected in two studies (with 166 and 20 patients, respectively) ([Shah et al., 1997](#); [Manfredi et al., 2005](#)) and was detected in 1 (4.5%) of 22 patients and 4 (5.6%) of 72 patients in the other two studies ([Li et al., 2002a](#); [Vanchiere et al., 2005a](#)). SV40 DNA has been found in the kidneys, in the urine, in stools, in peripheral blood cells, in the pituitary, and in lung/pleural samples ([Woloschak et al., 1995](#); [Galateau-Salle et al., 1998](#); [Martini et al., 1998, 2002](#); [Yamamoto et al., 2000](#); [Li et al., 2002a, b](#); [Vanchiere et al., 2005a, b, 2009](#)). The mean SV40 viral load in the urine of transplant patients was 0.001 times that of BKV and JCV, and the frequency of SV40 viruria was much lower than that of BK and JC viruria ([Thomas et al., 2009](#)).

[One reason for the differences between studies may be that the numbers of subjects investigated were generally low, with characteristics that were variable due to the fact that most

Table 1.1 Detection of SV40 DNA in control subjects from recent studies (past 15 years)

Reference	Study location	Type of specimen	Method	No. of samples	Detection of SV40 DNA <i>n</i> (%)
Shah et al. (1997)	USA	Urine	PCR + SB	166	0
Galateau-Salle et al. (1998)	France	Lung/pleural	PCR + SB	25	4 (16.0%)
Griffiths et al. (1998)	United Kingdom	Blood	PCR + SB + Seq	10	0
Martini et al. (1998)	Italy	Blood	PCR + SB + Seq	50	3 (6.0%)
Procopio et al. (1998)	Italy	Pleural	PCR + SB	20	0
Strizzi et al. (2000)	Italy	Pleural	PCR + SB	7	0
Yamamoto et al. (2000)	Japan	Blood	PCR + SB + Seq	64	3 (4.7%)
David et al. (2001)	USA	Blood	PCR + SB + Seq	115	18 (15.7%)
Strickler (2001)	USA	Lung tissue	PCR + SB + Seq	25	0
Li et al. (2002a)	USA	Blood	PCR + SB + Seq	22	5 (22.7%)
Li et al. (2002a)	USA	Urine	PCR + SB + Seq	22	1 (4.5%)
Martini et al. (2002)	Italy	Blood/bone tissue	PCR + SB + Seq	43	11 (25.6%)
Shivapurkar et al. (2002)	USA	Blood/lymphoid tissue	PCR + SB + Seq	28	0
Vilchez et al. (2002)	USA	Blood/lymph nodes	PCR + SB + Seq	107	0
Vivaldi et al. (2003)	Italy	Blood	PCR + SB + Seq	20	5 (25.0%)
Ozdarendeli et al. (2004)	Turkey	Thyroid tissue	PCR + Seq	83	0
Heinsohn et al. (2005)	Germany	Blood	qPCR	149	2 (1.3%)
Manfredi et al. (2005)	United Kingdom	Kidney	PCR + SB	20	0
Vanchiere et al. (2005a)	USA	Urine	PCR + Seq	72	4 (5.6%)
Meneses et al. (2005)	Costa Rica	Lymph nodes, tonsils	PCR + SB	51	0
Kjaerheim et al. (2007)	Norway	Blood	PCR + Seq	147	0
Ziegler et al. (2007)	Switzerland	Blood	qPCR	39	0
Heinsohn et al. (2009)	Hungary	Blood	qPCR + Seq	166	30 (18%)
Patel et al. (2008)	USA	Tonsils	PCR	220	20 (9.1%)
Pancaldi et al. (2009)	Italy	Blood	qPCR	148	24 (16.2%)
Vanchiere et al. (2009)	USA	Stool	qPCR + Seq	110	2 (1.8%)
Comar et al. (2010)	Italy	Tonsil/adenoid tissues	qPCR	80	1 (1.3%)
Campello et al. (2010)	Italy	Blood and intestine	RT-qPCR	91	0 (0.0%)
Bolognesi et al. (2005)	Italy	Lymphocytes	PCR	22	0 (0%)

PCR, polymerase chain reaction; qPCR, quantitative PCR; RT-qPCR, real-time qPCR; SB, Southern blot hybridization; Seq, sequencing; SV40, simian virus 40

studies were designed for the evaluation of the prevalence of SV40 in different cancers.]

There is no clear evidence of variations in prevalence according to the country, the sex of the subjects, or the presence of immunosuppression. However, in some studies SV40-positive subjects were older than SV40-negative subjects ([Patel et al., 2008](#)). Most of the discrepancies observed are thought to be due to variations in the PCR techniques used, either producing false-positive results or not being sensitive to detect SV40 when present. False-positive results have been reported to be due to the use of high-contamination-risk primers as defined by [López-Ríos et al. \(2004\)](#). High-contamination-risk primers are primers that amplify a region (nucleotides 4100–4713) of the gene encoding the SV40 large T-antigen (LT) present in many common laboratory plasmids. In contrast, primers not included in this region are considered as low-contamination-risk primers. In particular, the high SV40 prevalence reported in three studies was from one group from Italy that used high-contamination-risk primers ([López-Ríos et al., 2004](#)). In two recent studies using low-contamination-risk primers, SV40 was detected in 24 of 148 buffy coats of healthy blood donors ([Pancaldi et al., 2009](#)) and in none of 78 samples of peripheral blood mononuclear cells or 57 tonsil tissues from Italian children, but was found in only 1 (1.3%) of 80 adenoid samples ([Comar et al., 2010](#)).

With the recent advent of high-throughput sequencing technologies, it has become possible to perform random-primed deep sequencing of microbial nucleic acids from human specimens. This approach can reveal the presence of known, as well as previously undiscovered, viral sequences. Human “metagenomics” studies have not reported the presence of SV40 in a variety of specimen types, including faecal and sewage samples from developing countries, although numerous papillomaviruses and other polyomaviruses, including JCV, human polyomavirus 6 (HPyV6), HPyV7, and HPyV9, have been

detected ([Jones et al., 2005](#); [Finkbeiner et al., 2008](#); [Blinkova et al., 2009](#); [Victoria et al., 2009](#); [Reyes & Jiang, 2010](#); [Cantalupo et al., 2011](#); [Minot et al., 2011](#); [Sauvage et al., 2011](#)).

(b) *Prevalence of SV40 antibodies*

(i) *Detection by neutralization assays*

In recent years (since 1998), nine studies have investigated anti-SV40 antibodies by plaque neutralization or microwell neutralization tests ([Table 1.2](#)). Seroprevalence varied from 2% to 12%, with the highest values observed in the USA ([Jafar et al., 1998](#); [Rollison et al., 2003](#); [Viscidi et al., 2003](#)) and with higher values in adults than in infants and children. In addition, significantly lower titres of neutralizing SV40 antibodies are detected in humans compared with monkeys, the natural host. In one study ([Kjaerheim et al., 2007](#)), pre-incubation of all positive sera with BKV and JCV VLPs abolished the neutralization activity, indicating that neutralization assays are not specific.

(ii) *Detection by ELISA using VLPs or peptides*

In six serological studies using immunoenzymatic assays using VLPs and without pre-incubation or pre-adsorption with BKV and JCV VLPs, SV40 antibodies have been detected in a limited percentage of subjects (7.7–10.5%) ([Table 1.3](#)). In one of these studies ([Engels et al., 2004c](#)), the SV40 reactivity was further analysed by pre-incubation of sera with SV40, BKV, and JCV VLPs. The findings indicated that specific SV40 reactivity was detected in only 8 (22.9%) of the 35 SV40-reactive human sera investigated, compared with all 8 (100%) of the SV40-reactive macaque sera used as positive controls. In one recent study, SV40 antibodies were investigated using capsid viral protein 1 (VP1) and VP2/VP3 synthetic peptides ([Corallini et al., 2012](#)). Although the specificity of the test is unknown, ELISA reactivity was observed in 18% of the blood donors investigated.

Table 1.2 Detection of SV40 antibodies in healthy control subjects using neutralization tests (1998–2007)

Reference	Study population	Study location	Method	No. of subjects	Anti-SV40 antibodies	
					<i>n</i> (%)	Titre (range)
Jafar et al. (1998)	Adults	USA	Plaque neutr.	180	21 (11.7%)	1:20–1:320
Butel et al. (1999)	Children	USA	Plaque neutr.	337 ^a	20 (5.9%)	1:40–1:320
Butel et al. (2003)	All ages	Hungary	Plaque neutr.	589	17 (3.0%)	1:20–1:300
Butel et al. (2003)	All ages	Czech Republic	Plaque neutr.	350	7 (2.0%)	1:10–1:50
Knowles et al. (2003)	All ages	United Kingdom	Microwell neutr.	2435	79 (3.2%)	1:8–1:256
Engels et al. (2004c)	Mothers	USA	Plaque neutr.	187	8 (4.3%)	≥ 1:10
Minor et al. (2003)	Blood donors (age 9–96 yr)	United Kingdom	Microwell neutr.	2054	93 (4.5%)	≥ 1:8
Minor et al. (2003)	?	Sierra Leone	Microwell neutr.	62	2 (3.2%)	≥ 1:8
Minor et al. (2003)	Infants (age < 1 yr)	Democratic Republic of the Congo	Microwell neutr.	168	4 (2.4%)	≥ 1:8
Minor et al. (2003)	Children (age 3–4 yr)	Morocco	Microwell neutr.	419	14 (3.3%)	≥ 1:8
Minor et al. (2003)	Children (age 1–6 yr)	Poland	Microwell neutr.	923	26 (2.8%)	≥ 1:8
Rollison et al. (2003)	Adults	USA	Plaque neutr.	88	10 (11.4%)	≥ 1:10
Viscidi et al. (2003)	Blood donors	USA	Plaque neutr.	130	13 (10.0%)	≥ 1:40
Nurgalieva et al. (2005)	Individuals (age 11–69 yr)	Kazakhstan	Plaque neutr.	307	(4.9%)	1:10–1:500
Kjaerheim et al. (2007)	Adults	Norway	Microwell neutr.	147	4 (2.7%) ^b	≥ 1:50

^a All the subjects in this study were patients with a clinical diagnosis of disease.

^b None of the samples were found positive after pre-incubation with BKV and JCV VLPs.

BKV, BK polyomavirus; JCV, JC polyomavirus; neutr., neutralization; SV40, simian virus 40; VLPs, virus-like particles; yr, year

Table 1.3 Detection of SV40 antibodies using SV40 VLPs, or capsomers, or peptides in healthy control subjects in recent studies (since 2003)

Reference	Study population	Study location	Method	No. of subjects	Anti-SV40 antibodies <i>n</i> (%)
de Sanjosé <i>et al.</i> (2003)	Adults	Spain	VLP-based ELISA	587	56 (9.5%)
Carter <i>et al.</i> (2003)	Adults	USA	VLP-based ELISA	415	32 (7.7%)
Viscidi <i>et al.</i> (2003)	Healthy adults	USA	VLP-based ELISA	128	13 (10.2%)
Engels <i>et al.</i> (2004a)	Adults (2 groups)	USA	VLP-based ELISA	622	65 (10.5%)
			VLP-based ELISA	615	59 (9.6%)
Engels <i>et al.</i> (2004c)	Pregnant women	USA	VLP-based ELISA	200	6 (3.0%)
Rollison <i>et al.</i> (2005a)	General population controls	USA	VLP-based ELISA	434	41 (9.5%)
Corallini <i>et al.</i> (2012)	Blood donors	Italy	Synthetic peptides from capsid, ELISA	855	154 (18.0%)
Rollison <i>et al.</i> (2005a)	General population controls	USA	VLP-based ELISA adsorbed BKV/JCV	434	7 (1.6%)
Lundstig <i>et al.</i> (2005)	Pregnant women and hospital-based adults	Sweden	VLP-based ELISA adsorbed BKV/JCV	241	19 (7.9%)
Lundstig <i>et al.</i> (2005)	Children (age 1–13 yr)	Sweden	VLP-based ELISA adsorbed BKV/JCV	288	22 (7.6%)
Kjaerheim <i>et al.</i> (2007)	General population and blood donors	Norway	Capsomers/Luminex adsorbed SV40	147	16 (10.9%)
Carter <i>et al.</i> (2003)	Adults	USA	VLP-based ELISA blocked BKV/JCV	415	0 (0.0%)
Engels <i>et al.</i> (2004b)	Adults	North America	VLP-based ELISA blocked SV40/BKV/JCV	145	4 (2.8%)
Kean <i>et al.</i> (2009)	Adults	USA	Capsomers/Luminex blocked BKV/JCV	1501	32 (2.1%)
Kean <i>et al.</i> (2009)	Individuals (age 1–20 yr)	USA	Capsomers/Luminex blocked BKV/JCV	721	16 (2.2%)

BKV, BK polyomavirus; ELISA, enzyme-linked immunosorbent assay; JCV, JC polyomavirus; SV40, simian virus 40; VLP, virus-like particle; yr, year

SV40 antibodies were investigated in three studies by pre-adsorption of the sera with BKV and JCV VLPs, and in one study by pre-adsorption with SV40 ([Lundstig et al., 2005](#); [Rollison et al., 2005a](#); [Kjaerheim et al., 2007](#)). The reported seroprevalence of 1.6–10.9% suggests that the tests were not as specific as tests performed by blocking the SV40 reactivity with high concentrations of VLPs.

In four other studies ([Carter et al., 2003](#); [Engels et al., 2004a](#); [Kean et al., 2009](#)), the nature of the SV40 immunoreactivity was examined by competitive inhibition studies in which SV40-reactive human sera were pre-incubated with VLPs or capsomers of BKV, JCV, and SV40. Specific SV40 seroprevalence of 0–2.8% was reported in these studies. It should be noted that in the large study by [Kean et al. \(2009\)](#), no variation was observed according to age or sex.

1.3.3 Diseases associated with SV40 infection

(a) Rhesus monkeys

Progressive multifocal leukoencephalopathy (PML) and severe nephritis ([Horvath et al., 1992](#); [Chrétien et al., 2000](#); [Dang et al., 2005](#)) as meningoencephalitis ([Newman et al., 1998](#); [Simon et al., 1999](#)) were observed in simian immunodeficiency virus (SIV)-infected rhesus macaques that were seropositive for SV40 before SIV inoculation, or in SIV-infected animals that were inoculated with SV40.

(b) Humans

No disease has been clearly associated with SV40 infection in either immunocompetent or immunocompromised humans. However, SV40 DNA has been identified in PML and other diseases.

[Peters et al. \(1980\)](#) identified SV40 by indirect immunofluorescence in one case of PML using anti-virion antibodies. [The cerebrospinal fluid cell sample was not investigated by immunofluorescence with anti-JCV antibodies, and data were

not confirmed by virus isolation.] [Scherneck et al. \(1981\)](#) identified SV40 in a patient with PML by cell culture with CV1 monkey cells inoculated with homogenates of brain of the patient. SV40 antigen was also detected immunohistochemically in one case of PML ([Hayashi et al., 1985](#)), but attempts at viral isolation by cultivation on human brain tumour cells were unsuccessful. Brain tissue from two of the PML cases thought to be associated with SV40 was re-examined for the presence of SV40 VP1 DNA sequences by in situ hybridization and PCR using specific primers ([Stoner & Ryschkewitsch, 1998](#)). All these techniques failed to confirm the presence of SV40 but identified the presence of JCV. [Eizuru et al. \(1993\)](#) also identified the JCV genome in a previously identified case of SV40-associated PML based on LT immunostaining of cells infected with PML tissue. The original identification of SV40 was suspected to be due to the lack of specificity of the antibody used for immunostaining.

In addition, SV40 was recovered by monkey kidney cell culture inoculated with cerebrospinal fluid from a child with anatomical and neurological anomalies ([Brandner et al., 1977](#)). However, the SV40 infection could not be confirmed due to the absence of detection of SV40 neutralizing antibodies up to 60 days after detection of the virus. A lung transplant recipient developed end-stage renal failure potentially related to SV40 infection ([Milstone et al., 2004](#)). The diagnosis was documented by detecting SV40 DNA sequences (but not BKV or JCV) in the patient's kidney biopsy and urine sample by PCR, Southern blot, and DNA sequencing. Positive immunohistochemistry for SV40 was found in the kidney, and neutralizing antibodies for SV40 were detected in the serum.

SV40 DNA was also identified by PCR in kidney tissue and urine samples of patients with focal segmental glomerulosclerosis ([Li et al., 2002b](#)).

2. Cancer in Humans

Methodological considerations: case–control versus case-series study designs

Numerous studies have reported the prevalence of markers of infection by polyomaviruses in tumour tissues or blood obtained from humans with cancer. Many of these studies included specimens from individuals without cancer as “controls,” but such studies were not generally considered by the Working Group as case–control studies, given the convenience sampling strategies used or the lack of comparability of exposure measurement between comparison groups. Specifically, convenience sampling of controls led to the possibility that the control subjects were not representative of the source population. Also, the comparison of tumour tissues in cases with normal tissues (such as blood, urine, or biopsies of normal tissues) in controls may also be biased because it is uncertain whether polyomaviruses are uniformly present in these normal tissues or can be reliably detected by the assays used. However, because these studies contributed information on cancer sites not investigated by the case–control studies, included comparisons with both normal and pre-malignant control tissues, compared tumour tissue with a convenience sample of controls, compared different tissues in cases or controls, and/or presented findings for susceptible populations (i.e. transplant patients), they are considered here as case series.

2.1 Prospective studies

Prospective studies include cohort studies that followed up individuals who received SV40-contaminated vaccines, as well as case–control studies that prospectively evaluated biomarkers of SV40 infection using samples obtained before cancer diagnosis/control selection. These studies are summarized in [Table 2.1](#) (cohort studies) and

[Table 2.2](#) (prospective case–control studies) and reviewed below.

The premise of the cohort studies is that if SV40 causes cancer, cancer incidence or mortality will be higher in vaccinated cohorts than in unvaccinated cohorts. These studies are strongest when there is convincing documentation that the vaccine under consideration was contaminated with live SV40 and investigators can determine which individuals received the vaccine.

Some investigators have actively followed cohorts that are known to have received SV40-contaminated vaccines. More commonly, however, researchers have used cancer registry data to evaluate cancer risk for different birth cohorts with varying exposure to SV40-contaminated poliovirus vaccines. Defining cohorts on the basis of birth year is a reliable method to assign vaccination status because SV40 was present in killed poliovirus vaccines for a limited period (i.e. from 1955, when widespread vaccination campaigns were initiated, until early 1963, when the remaining contaminated vaccine lots were last used). Poliovirus vaccination campaigns targeted infants and school-age children during these years, and vaccine coverage rates were typically quite high. Candidate live-attenuated oral poliovirus vaccine (OPV), contaminated with higher titres of infectious SV40, were field-tested from 1958 to 1960 at selected sites in countries in the southern hemisphere, eastern Europe, and Africa; the contaminated Russian OPV was used much more widely and for a longer period of time.

A related assumption of the cohort studies of vaccine recipients is that infection with SV40 was less frequent, or had fewer health consequences, in people who did not receive SV40-contaminated vaccines (e.g. individuals born after 1963 for studies of poliovirus vaccination). As reviewed in Section 1, uncertainties remain about routes of SV40 transmission other than via receipt of a contaminated vaccine. Serology-based studies have suggested that SV40 infection

Table 2.1 Cohort studies of people exposed to SV40-contaminated vaccines

Reference, location, follow-up period	Total subjects	Exposure assessment	Organ site	Exposure categories	Exposed cases	Relative risk (95% CI)	Covariates Comments
Fraumeni <i>et al.</i> (1963) , USA 1950–59	9 489 100	State of residence associated with varying levels of IPV contamination	Leukaemia (mortality)	High-level, low-level, and no contamination	NR	NR Results are presented graphically. Leukaemia mortality was highest in states with vaccine contamination, but differences were apparent before the vaccine was introduced.	Calendar year Results are relevant for short-term risk. Report did not present measures of statistical uncertainty.
Geissler (1990) , German Democratic Republic 1959–86	1 777 104	Birth cohort with varying exposure to SV40-contaminated OPV	Glioma / glioblastoma Oligodendroglioma Medulloblastoma Spongioblastoma	Born 1959–61 (exposed) vs 1962–64 (unexposed)	52 11 79 93	NR Results are presented graphically, with suggestion of higher incidence in exposed birth cohort.	Age Report did not present measures of statistical uncertainty.
Olin & Giesecke (1998) , Sweden 1960–93	Not stated	Birth cohort with varying exposure to SV40-contaminated IPV	Brain cancers Ependymoma Osteosarcoma Mesothelioma	Born 1946–52 (exposed) vs bracketing years (unexposed)	NR	Varying by age and calendar year	Age, calendar year Report did not present measures of statistical uncertainty.
Strickler <i>et al.</i> (1998) , 9 representative areas of USA 1973–93	NR	Birth cohort with varying exposure to SV40-contaminated IPV	Ependymoma Brain cancers Osteosarcoma Mesothelioma	Born 1956–62 (exposed as infants) vs 1964–69 (unexposed)	NR NR NR NR	1.06 (0.69–1.63) 0.90 (0.82–0.99) 0.87 (0.71–1.06) 3.00 (0.67–13.11)	Age Results for 1947–52 birth cohort (exposed as children) were similar. The number of mesothelioma events was small, limiting the power of this analysis. The same cancer registry data were analysed separately by Fisher <i>et al.</i> (1999) .

Table 2.1 (continued)

Reference, location, follow-up period	Total subjects	Exposure assessment	Organ site	Exposure categories	Exposed cases	Relative risk (95% CI)	Covariates Comments
Strickler et al. (1999) , 9 representative areas of USA After 1973	NR	Birth cohort with varying exposure to SV40-contaminated IPV	Medulloblastoma	Born 1956–62 (exposed as infants) vs 1964–69 (unexposed)	NR	0.742 (0.55–1.00)	Age Results for 1947–52 birth cohort (exposed as children) were similar. Report also presents results for Connecticut during 1950–69 for children 0–4 years old.
Fisher et al. (1999) , 9 representative areas of USA 1973–93	3 886 342	Birth cohort with varying exposure to SV40-contaminated IPV	All cancers	Born 1955–59 (exposed) vs 1963–67 (unexposed)	5512	[0.89 (0.86–0.93)]	Age Analysis was limited to ages 18–26 years. Report did not present measures of statistical uncertainty [but these were calculated by the Working Group]. This report used essentially the same cancer registry data as Strickler et al. (1999) .
			Ependymoma and choroid plexus tumour		18	[1.20 (0.60–2.45)]	
			Other brain tumours		328	[0.92 (0.79–1.07)]	
			Osteosarcoma		53	[1.10 (0.74–1.64)]	
			Other bone tumours		89	[1.17 (0.85–1.60)]	
Carroll-Pankhurst et al. (2001) , Cleveland, Ohio, USA 1969–96	1073	Culture of live SV40 from IPV and OPV used in trial	Mesothelioma		6	[2.78 (0.64–19.0)]	Sex, age, race, calendar year Cancer deaths in cohort compared with general population expected. Study is a follow-up of Fraumeni et al. (1970) and Mortimer et al. (1981) .
			All cancers (mortality)	Receipt of SV40-contaminated vaccine	4	1.26 (0.34–3.23)	
			Leukaemia (mortality)		2	4.19 (0.51–15.73)	
			Testis (mortality)		2	36.98 (4.47–133.50)	

Table 2.1 (continued)

Reference, location, follow-up period	Total subjects	Exposure assessment	Organ site	Exposure categories	Exposed cases	Relative risk (95% CI)	Covariates Comments
Engels <i>et al.</i> (2003a) , Denmark	NR	Birth cohort with varying exposure to SV40-contaminated IPV	All cancers	Born 1955–61 (exposed as infants) <i>vs</i> 1964–70 (unexposed)	11 105	0.86 (0.81–0.91)	Age Results for 1946–52 birth cohort (exposed as children) were similar. The number of mesothelioma and choroid plexus tumour events was small, limiting the power of this analysis.
			Mesothelioma		6	0.48 (0.12–1.83)	
			Brain cancers		630	0.81 (0.74–0.90)	
			Ependymoma		45	1.25 (0.79–1.98)	
			Choroid plexus tumour		3	0.26 (0.06–1.24)	
			Bone tumour		153	1.00 (0.78–1.28)	
			Osteosarcoma		26	0.95 (0.53–1.71)	
			Leukaemia		711	0.96 (0.85–1.08)	
			NHL		480	0.93 (0.78–1.11)	
Strickler <i>et al.</i> (2003) , 9 representative areas of USA 1975–97	NR	Birth cohort with varying exposure to SV40-contaminated IPV	Mesothelioma	Born 1948–57 <i>vs</i> 1936–47 (men)	NR	[0.98 (0.84–1.15)]	Age, calendar year Inclusion of early birth cohorts allowed assessment of mesothelioma risk at older ages. Report includes comparisons of additional birth cohorts not presented in this table.
				Born 1948–57 <i>vs</i> 1936–47 (women)		[1.12 (0.94–1.32)]	
Thu <i>et al.</i> (2006) , Norway 1953–97	NR	Birth cohort with varying exposure to SV40-contaminated IPV	NHL	Multiple categories defined by prevalence of vaccine exposure	NR	Differences in birth cohort effects were not correlated with differences in vaccine exposure.	Sex, age, calendar year Results were also null for lymphocytic leukaemia and plasma cell neoplasms.
Price <i>et al.</i> (2007) , Great Britain 1968–2004	NR	Birth cohort with varying exposure to SV40-contaminated IPV	Mesothelioma (mortality)	Born 1951–55 <i>vs</i> 1962–66 (unexposed)	22	Men: 2.4 (1.2–5.0)	Sex, age The number of mesothelioma deaths was small, limiting the power of this analysis.
					9	Women: 3.8 (1.0–14)	
				Born 1956–60 <i>vs</i> 1962–66 (unexposed)	9	Men: 0.93 (0.39–2.3)	
					9	Women: 3.5 (0.93–13)	

CI, confidence interval; IPV, inactivated poliovirus vaccine; NHL, non-Hodgkin lymphoma; NR, not reported; OPV, oral poliovirus vaccine; SV40, simian virus 40; *vs*, *versus*

Table 2.2 Nested case–control studies of cancers and SV40

Reference, study location and period	Total cases Total controls	Control source (hospital, population)	Detection method	Organ site	Exposure categories	Exposed cases	Relative risk (95% CI)	Covariates Comments
Rollison <i>et al.</i> (2003) , USA, 1974–2000	44 88	Population (same cohort as cases)	Plaque neutralization assay for SV40 antibodies	Brain	Positive	5	1.00 (0.30–3.32)	Age, race, sex, date of blood draw, freeze/thaw history of sample 80% of tumours were glioblastomas or astrocytomas. Samples were obtained before cancer diagnosis.
Rollison <i>et al.</i> (2005a) , USA, 1974–2002	170 340	Population	ELISA for antibodies against SV40 VLPs	NHL	Positive (any positivity)	33	1.97 (1.03–3.76)	Sex, race, age, data of blood draw, freeze/thaw status of sample, cohort Some subjects contributed two serum samples. Antibodies were measured before NHL diagnosis. Most SV40 antibodies were non-specific.
					Positive (low reactivity)	9	1.74 (0.67–4.51)	
					Positive (medium reactivity)	17	3.30 (1.38–7.91)	
					Positive (high reactivity)	7	1.16 (0.43–3.13)	
					Positive after competitive blocking with BKV and JCV VLPs	4	1.51 (0.41–5.52)	

Table 2.2 (continued)

Reference, study location and period	Total cases Total controls	Control source (hospital, population)	Detection method	Organ site	Exposure categories	Exposed cases	Relative risk (95% CI)	Covariates Comments
Kjaerheim et al. (2007) , Norway, 1973–2003	49 147	Population (same cohort as cases)	ELISA for antibodies to SV40 VP1	Mesothelioma	Positive	32	1.5 (0.8–2.9)	Age, sex, sample date, county Samples were obtained before cancer diagnosis.
					Positive after competitive blocking with BKV and JCV VP1	7	1.5 (0.6–3.7)	
			ELISA for antibodies to SV40 LT		Positive	9	1.4 (0.6–3.2)	
			Neutralizing antibodies to SV40		Positive	1	0.8 (0.1–6.7)	
			PCR detection of SV40 DNA in serum		Positive	0	Not defined	

BKV, BK polyomavirus; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; JCV, JC polyomavirus; LT, large T-antigen; NHL, non-Hodgkin lymphoma; PCR, polymerase chain reaction; SV40, simian virus 40; VLPs, virus-like particles; VP1, capsid viral protein 1

is not common in the general populations of the USA and Europe (see Section 1). To the extent that SV40 does not circulate widely in the general population, receipt of contaminated vaccines represents a uniquely informative exposure. Even if SV40 can be acquired by routes other than vaccine-based exposures, individuals who received SV40-contaminated poliovirus vaccines usually received multiple doses, and exposures to SV40-contaminated poliovirus vaccines typically occurred in infancy or early childhood, when animal models suggest that the greatest susceptibility to cancer occurs (Section 3) ([Girardi *et al.*, 1963](#)).

An additional assumption of the cohort studies is that there is no other important cancer risk factor that varies across groups defined by vaccination status. For such confounding to obscure a measurable effect of SV40, the proposed risk factor would have to be common and have a sufficiently strong effect on cancer risk. For example, changes in asbestos exposure over time may affect comparison of mesothelioma incidence rates across birth cohorts. Also, cohort studies that rely on cancer registries for follow-up may be affected by changes over time in the completeness of cancer ascertainment or the classification of cancer subtypes. Finally, most studies have followed cohorts only through the 1990s, and since most poliovirus-vaccine recipients were young children, cohorts of poliovirus vaccine recipients have not reached the age of 50 years. The number of outcomes is thus limited for some cancers, especially mesothelioma, that increase in incidence with age.

For prospective case-control studies, the cases and controls are compared with respect to biomarkers of infection with SV40, typically serum or plasma antibodies. One issue for studies that use assays for SV40 antibodies is that natural infections with BKV and JCV, which are very common in the general population, can lead to production of cross-reacting antibodies. To address this issue, some studies have taken

measures to remove cross-reacting BKV and JCV antibodies. The prospective studies have assessed SV40 biomarkers using blood samples before case diagnosis, which eliminates the possibility that the development of cancer affects the biomarkers. However, if the samples were obtained many years before cancer diagnosis, such testing might miss subsequent SV40 infections.

[Fraumeni *et al.* \(1963\)](#) described short-term trends in leukaemia mortality in the USA after exposure to SV40-contaminated inactivated poliovirus vaccine (IPV). In the USA, IPV vaccination campaigns targeting children (mostly aged 6–8 years) began in 1955, and because vaccine supply was limited at that time, only a small number of lots of IPV were distributed to each state. The researchers grouped states in the USA into three categories of SV40 exposure based on subsequently measured contamination of these vaccine lots: none (14 states, with 1.9 million children), low-level (16 states, with 3.7 million children), and high-level (19 states, with 3.9 million children). Among children who were aged 6–8 years in 1955, leukaemia-related mortality during 1950–59 was highest in states with high-level exposure, intermediate in states with low-level exposure, and lowest in states with no exposure to SV40-contaminated vaccine. However, these differences in leukaemia-related mortality were apparent even during 1950–54, before IPV was introduced [pointing to explanations other than vaccine exposure]. After 1955, IPV was made available to individuals outside the age range of 6–8 years. Based on national mortality data, Fraumeni *et al.* did not observe a temporal increase during 1950–59 in leukaemia-related mortality among people in various age groups < 25 years.

[Geissler \(1990\)](#) compared cancer incidence in two birth cohorts in the German Democratic Republic. The 1959–61 birth cohort was considered exposed to SV40, because 86% of these individuals had received OPV as infants, beginning in 1960. No results of testing of OPV lots for

SV40 were presented by Geissler, but if SV40 was present, the titre would have been high, given the absence of any formalin treatment of OPV ([Shah & Nathanson, 1976](#)). The 1962–64 birth cohort received OPV beginning in 1963, when SV40 contamination was no longer present. Based on national cancer registry data, the overall incidence of brain tumours was similar in the SV40-exposed birth cohort (28.7 per 10 000) and the unexposed birth cohort (30.1 per 10 000). The incidence also appeared similar in the two cohorts for multiple subtypes of brain tumours. Geissler presented age-specific incidence rates of four types of brain tumour (glioma and glioblastoma, oligodendroglioma, medulloblastoma, and spongioblastoma). The incidence of these cancers appeared higher in the SV40-exposed birth cohort, but the investigator did not present relative risks, confidence intervals, or other data that would allow an assessment of statistical uncertainty. [[Cutrone *et al.* \(2005\)](#) detected SV40 in an OPV used in eastern Europe after the early 1960s, suggesting that people born in 1962–64 in the former German Democratic Republic could have been exposed to SV40 through vaccination.]

[Olin & Giesecke \(1998\)](#) described cancer incidence in Sweden after a 1957 vaccination campaign using IPV from the USA that was potentially contaminated with SV40. This vaccination campaign targeted school-age children born in 1946–53. Beginning in 1958, Sweden used IPV produced in Swedish laboratories, which was claimed to be free of SV40. The investigators assessed cancer registry data for 1960–93, comparing incidence in an exposed birth cohort (born 1946–52) with that in unexposed birth cohorts born slightly earlier or later. The evaluated malignancies included brain cancers (and specifically, ependymoma), osteosarcoma, and mesothelioma. Results were presented for different attained ages, without an overall summary relative risk, and no measures of statistical uncertainty were included. [Therefore, it is not possible to determine whether there

is evidence for an effect of SV40 exposure on cancer risk.] Nonetheless, most of the age-specific relative risk estimates were close to 1.00, and the investigators interpreted their findings as indicating no elevation in cancer risk in the SV40-exposed birth cohort. [There were no consistent patterns in the relative risks, and no summary relative risks or confidence intervals were provided. There would likely be very few cases within this cohort of Swedish schoolchildren. It is unknown whether the Swedish vaccine contained SV40.]

[Strickler *et al.* \(1998\)](#) evaluated cancer incidence using cancer registry data from nine areas of the USA that participated in the Surveillance, Epidemiology and End Results (SEER) programme and covered approximately 10% of the national population. The investigators cited the report by [Shah & Nathanson \(1976\)](#), which estimated that live SV40 was present in 10–30% of IPV used in the USA before 1963. By 1961, in the USA 88% of children aged < 20 years, and 55% of infants aged < 1 year, had received one or more doses of IPV. Since 1963, IPV in the USA has been free of SV40 contamination. On this basis, Strickler *et al.* considered three cohorts defined by birth year and thus with varying exposure to SV40-contaminated IPV: the 1947–52 birth cohort (exposed as children in 1955 or soon thereafter), the 1956–62 birth cohort (exposed as infants), and the 1964–69 birth cohort (born after 1963, and thus unexposed). Using SEER data, Strickler *et al.* found that incidence was not elevated for any of the included cancers (ependymoma, brain cancers overall, osteosarcoma, and mesothelioma) in either of the two SV40-exposed birth cohorts compared with the unexposed birth cohort. [The Working Group mentions above that studies that use cancer registries may be affected by changes in the quality of cancer registration over time. Nonetheless, a strength of this study is its use of SEER data, which are believed to be of consistently very high quality over the period evaluated. This study used largely the

same cancer registry data as [Fisher *et al.* \(1999\)](#), although the analysis methods differed.]

In a subsequent brief report, [Strickler *et al.* \(1999\)](#) extended their results, using SEER data to evaluate the association between exposure to SV40-contaminated IPV and risk of medulloblastoma. The 1956–62 birth cohort (exposed as infants to SV40-contaminated IPV) and the 1947–52 birth cohort (exposed as children) did not have elevated risk compared with the 1964–69 birth cohort (unexposed). Using data for the state of Connecticut, the investigators also reported that medulloblastoma incidence among those aged 0–4 years did not increase during 1950–69 in association with use of SV40-contaminated IPV.

[Fisher *et al.* \(1999\)](#) also used SEER data to examine cancer incidence in the USA, for two birth cohorts with varying exposure to SV40-contaminated IPV: the 1955–59 birth cohort (exposed) and the 1963–67 birth cohort (unexposed). To control for differences in ages attained during follow-up, the researchers restricted the analysis to cancer risk among those aged 18–26 years in these cohorts. [The results were similar to those presented in [Strickler *et al.* \(1998\)](#). One limitation of this study is that no confidence limits or *P*-values were presented for the comparisons of cancer incidence in the two birth cohorts.] An increased incidence of mesothelioma was suggested in the exposed cohort (relative risk [RR], 2.78 [95% confidence interval (CI): 0.64–19.0], based on 6 exposed cases); for comparison, [Strickler *et al.* \(1998\)](#) found a relative risk of 3.00 comparing similar exposed and unexposed birth cohorts, with a wide 95% confidence interval (0.67–13.11). [There is significant overlap with the study population of [Strickler *et al.* \(1999\)](#), and the results are consistent.]

[Carroll-Pankhurst *et al.* \(2001\)](#) reported 35–37-year follow-up of a cohort in the USA who were known to have been exposed to SV40-contaminated vaccines as neonates. In 1960–62, a total of 1073 neonates in Cleveland, Ohio,

participated in a study of the immunogenicity of poliovirus vaccine. Of them, 86% received OPV and 14% received IPV. Subsequent testing revealed that all vaccine lots used in this trial contained live SV40 at varying titre. An earlier report by [Fraumeni *et al.* \(1970\)](#) noted no deaths from cancer through 1968, and a report by [Mortimer *et al.* \(1981\)](#) described that only one of these individuals had developed a neoplasm (a salivary gland tumour of “low degree of malignancy”) through 1979. In their updated report, [Carroll-Pankhurst *et al.* \(2001\)](#) compared cancer mortality in this cohort with expected rates based on the United States national death certificate registry. Only four cancer-related deaths were found, which did not represent an elevation (RR, 1.26; 95% CI, 0.34–3.23). Two deaths were due to leukaemia, but these were of different types and did not comprise a significant excess (RR, 4.19; 95% CI, 0.51–15.73). Two deaths were from testicular cancer (RR, 36.98; 95% CI, 4.47–133.50) [which could be due to late diagnosis or poor treatment of testicular cancer due to socioeconomic factors, because the subjects had been recruited from an urban impoverished community. Testicular cancer has not otherwise been linked to SV40. A limitation of this study is its small size and that there was no replication. Strengths include the prospective documentation of SV40 exposure from contaminated vaccines and that this exposure occurred immediately after birth, when SV40 would be predicted to have the strongest effect on cancer based on animal studies. This study is unique in following people known to have received poliovirus vaccines with specifically documented contamination. The number of deaths from multiple cancers was not increased, nor was the total cancer mortality. A bias is that relative risks are reported only for those cancers that were observed, which would result in a high proportion of elevated risks, and no relative risks are reported for multiple cancers for which there were no observed cases.]

[Engels *et al.* \(2003a\)](#) reported on cancer risk associated with receipt of SV40-contaminated IPV in Denmark. IPV was first administered in Denmark in April 1955. Danish public health officials mounted a concerted vaccination campaign, especially targeted at children, and these efforts were maintained through the early 1960s. As of April 1962, approximately 90% of children aged 9 months or older had received at least one dose of IPV. Danish poliovirus vaccine, unlike the vaccine in the USA, was grown using a monolayer tissue culture method, which pooled kidney tissue from multiple macaques and increased the likelihood of SV40 contamination. Testing of Danish IPV in 1961 revealed that all nine evaluated lots of IPV, previously used in vaccination campaigns, contained live SV40. Beginning in 1963, all Danish IPV was free of SV40. Based on the period when SV40-contaminated IPV was used in Denmark, three birth cohorts of interest were identified: the 1946–52 birth cohort, who were vaccinated in 1955, soon after the vaccine first became available (exposed to SV40-contaminated IPV as young children); the 1955–61 birth cohort, who were vaccinated at age approximately 9 months or soon thereafter (exposed as infants); and the 1964–70 birth cohort (born after vaccines were cleared of SV40, and thus unexposed). Compared with the unexposed cohort, there was similar incidence in the two vaccine-exposed birth cohorts for every examined cancer, including mesothelioma, ependymoma, choroid plexus tumour, bone tumour, leukaemia, and non-Hodgkin lymphoma (NHL). [The strength of this study was the high contamination rate of the vaccines and its contemporaneous documentation.]

[Strickler *et al.* \(2003\)](#) evaluated mesothelioma incidence in the USA in relation to birth year. Assessment of SV40 exposure was based on a 1961 census describing the age-specific proportions of the USA population that had previously received at least one dose of potentially contaminated IPV. Using cancer registry follow-up for

1975–97, Strickler *et al.* described a temporal increase in mesothelioma incidence among men and women who were aged 75–84 years or > 85 years, although these age groups were unlikely to have received IPV during the years when SV40 contamination was present. In comparison, among younger people, who would have had greater IPV exposure during 1955–61, mesothelioma incidence was substantially lower and either constant or declining over time. For adjacent birth cohorts, differences in mesothelioma incidence were not correlated with differences in the prevalence of exposure to potentially contaminated IPV. For example, a comparison of the 1948–57 birth cohort with the 1936–47 birth cohort, which was less frequently exposed, revealed no difference in mesothelioma incidence [RR, 0.98; 95% CI, 0.84–1.15 for men; RR, 1.12; 95% CI, 0.94–1.32 for women]. Because mesothelioma incidence increases with age, a strength of this study is that it evaluated birth cohorts that had aged into late adulthood during the period of follow-up. [Exposure to asbestos was not assessed in these analyses and thus could obscure the effect of SV40. However, the estimated effect of SV40 was similar in both sexes and therefore the risk of mesothelioma is unlikely to be due to asbestos exposure. The effect of SV40 would be expected to be stronger in women, who were less frequently exposed to asbestos than men were.]

[Rollison *et al.* \(2003\)](#) described results of a prospective case-control study nested in a general population cohort in Washington County, Maryland, USA. The study included 44 cases with brain tumours, most of which were glioblastomas. Serum samples were evaluated for SV40 neutralizing antibodies. The prevalence of antibodies was identical in cases and controls (odds ratio [OR], 1.00; 95% CI, 0.30–3.32). [Serum samples were obtained before cancer diagnosis (in this study, 0.6–22.3 years before diagnosis), so associations with brain tumours could have been missed if some SV40 infections occurred subsequent to the draw date.]

[Rollison *et al.* \(2005a\)](#) described results of a prospective case-control study of NHL nested within the same general population cohort in Maryland, USA, as described in [Rollison *et al.* \(2003\)](#). Serum antibodies to SV40 were measured using a VLP ELISA. Compared with cancer-free controls, NHL cases (ascertained through a cancer registry) were more likely to manifest SV40 seropositivity (OR, 1.97; 95% CI, 1.03–3.76). However, no dose-response relationship between SV40 antibody levels and NHL risk was observed. [Antibody levels were evaluated based on the ELISA absorbance at a single dilution, an approach that does not provide a robust quantitative assessment.] Furthermore, the majority of SV40 antibody reactivity could be blocked by addition of BKV or JCV VLPs. SV40-specific reactivity (i.e. reactivity that could not be blocked by BKV or JCV VLPs) was not significantly associated with NHL (OR, 1.51; 95% CI, 0.41–5.52; based on 4 positive cases). Serum samples were obtained from cases a median of 10.8 years before NHL diagnosis. [As noted below for [Kjaerheim *et al.* \(2007\)](#), it is possible that an association with SV40 infection could have been missed if infection occurred subsequent to the blood draw, closer in time to the development of cancer. Nonetheless, a strength of this study is that the antibody measurements before diagnosis were not affected by disease status.]

[Thu *et al.* \(2006\)](#) reported on cancer incidence in Norway during 1953–97 in relation to pre-1964 exposure to IPV. They noted that in 1956–57, Norway used IPV produced in Denmark, which, as described in [Engels *et al.* \(2003a\)](#), was widely contaminated with SV40. From 1957 until 1963, Norway used IPV from the USA, which also may have been contaminated. Exposure to these vaccines varied according to birth year, with the highest vaccinated proportions (> 85%) for individuals born in 1943–62. The investigators used national cancer registry data to evaluate NHL incidence in relation to birth cohort. They estimated curvature effects

for various birth years, which correspond to the ratio of the rates of change in incidence for later *versus* earlier birth cohorts. Thu *et al.* observed a positive curvature effect (i.e. an acceleration) in NHL incidence in the 1928–32 birth cohort among both men (curvature effect, 1.18; 95% CI, 0.97–1.43) and women (curvature effect, 1.34; 95% CI, 1.08–1.66). However, these two birth cohorts had similar prevalence of exposure to SV40-contaminated IPV (i.e. 20–40% prevalence for the 1928–32 birth cohort *versus* 40–50% for the 1933–37 birth cohort). Also, there were no other significant curvature effects observed between adjacent birth cohorts related to differences in exposure to SV40-contaminated IPV. [Therefore, there is no consistent pattern associating curvature effects with differences in the prevalence of exposure to SV40-contaminated IPV]. Likewise, no curvature effects were observed for lymphocytic leukaemia or plasma cell neoplasms in the exposed birth cohorts.

[Price *et al.* \(2007\)](#) describe mesothelioma mortality rates in the United Kingdom for birth cohorts with varying exposure to SV40-contaminated IPV. IPV was used in vaccination campaigns in the United Kingdom during 1956–61. Price *et al.* do not describe testing of British IPV for SV40 contamination. In 1962, the United Kingdom switched to OPV that was free of SV40 contamination. Price *et al.* considered several birth cohorts: 1951–55 and 1956–60, considered as potentially exposed to SV40-contaminated IPV, and 1962–66, considered as unexposed. Using data on mesothelioma deaths in a national registry, they found that the age-standardized mortality from mesothelioma was greater in the 1951–55 birth cohort than the 1962–66 birth cohort, for both men (RR, 2.4; 95% CI, 1.2–5.0) and women (RR, 3.8; 95% CI, 1.0–14). Mesothelioma mortality was also borderline elevated in the 1956–60 birth cohort among women (RR, 3.5; 95% CI, 0.93–13) but not among men (RR, 0.93; 95% CI, 0.39–2.3). [The pattern is not clearly consistent with an increased

risk associated with vaccine exposure. A limitation of this study is that the cohorts were followed only to age 38 years and therefore mesothelioma mortality rates were very low. Also, because exposure to asbestos likely varied by birth year, a confounding effect due to asbestos could not be excluded.]

[Kjaerheim et al. \(2007\)](#) reported results of a prospective case-control study in Norway, nested within the Janus Serum Bank, a large cohort drawn from the general population. Mesothelioma cases from this cohort (diagnosed during 1973–2003) were identified using data in the Norwegian cancer registry and were matched to cancer-free controls in a 3:1 ratio on age, sex, date of blood sampling, and county of residence. Several measures of exposure to or infection with SV40 were evaluated using serum samples obtained in 1972, i.e. 0.4–30 years before case diagnosis. Using an ELISA that measured antibodies to the SV40 VP1 protein, the investigators found that a high proportion of subjects exhibited reactive antibodies, but the difference in prevalence was not significant (65% of cases *versus* 56% of controls; OR, 1.5; 95% CI, 0.8–2.9). SV40 VP1 antibodies were weaker than seen for BKV and JCV, and most SV40 antibodies were blocked by competition with VP1 proteins from those viruses. After blocking, the prevalence of SV40 VP1 seroreactivity did not differ between cases and controls (14% *versus* 11%; OR, 1.5; 95% CI, 0.6–3.7). Furthermore, only one case exhibited SV40 neutralizing antibodies. In addition, cases and controls did not differ with respect to the proportion with antibodies against SV40 LT measured by ELISA (18% *versus* 14%; OR, 1.4; 95% CI, 0.6–3.2). Finally, no subject had SV40 DNA detectable in serum by PCR and sequencing. [Strengths of this study include the sampling from a well-defined population and measurement of SV40 infection status before the development of cancer in cases (so that there is no disease effect). Another strength is that the study used multiple complementary measures of

SV40 infection, and the associations with cancer were similar according to the different measures. Poliovirus vaccination campaigns using potentially contaminated vaccines were carried out in Norway during 1956–62. Thus, if SV40 was acquired through vaccination, one would have expected cases to manifest serological evidence for infection. However, if SV40 infection occurred closer to mesothelioma diagnosis, after the blood draw in 1972 for some subjects, the study may have missed an association.]

2.2 Case-control studies

Two approaches have generally been used by case-control studies that have examined the association between SV40 and cancer. In one approach, some case-control studies have compared the prevalence of prior exposure to SV40-contaminated vaccines between cancer cases and controls. This approach mirrors that taken in cohort studies of vaccine recipients (reviewed in Section 2.1) and, as for the cohort studies, the relevance of these studies depends in large part on the quality of the assessment of vaccine exposure and evidence that the vaccine contained live SV40. In the second approach, cases and controls are compared with respect to biomarkers of infection with SV40, typically serum or plasma antibodies. As noted above for prospective case-control studies, one issue for studies that use assays for SV40 antibodies is that natural infections with BKV and JCV, which are very common in the general population, can lead to production of cross-reacting antibodies. Use of samples obtained after diagnosis of the cancer in the cases may result in artefacts in assessing SV40 infection status (i.e. a disease effect), but it has the advantage of capturing any prior exposure to or infection with SV40.

2.2.1 Mesothelioma

Three case-control studies have evaluated the association between SV40 exposure or infection and mesothelioma ([Table 2.3](#)). A general limitation of these studies is the small number of mesothelioma cases.

[Strickler *et al.* \(1996\)](#) reported results of a study in the USA that used serum samples from patients with mesothelioma and control subjects who had non-malignant gastrointestinal disease. The prevalence of SV40 neutralizing antibodies did not differ significantly between mesothelioma cases (9%) and controls (3%) [crude OR, 3.2; $P = 0.62$ by Fisher exact test]. [This study did not measure or adjust for asbestos exposure.]

[Rollison *et al.* \(2004\)](#) evaluated United States army veterans who had entered military service in 1959–61. During some intervals of this 3-year enrolment period, all army recruits were inoculated with an inactivated adenovirus vaccine, whereas during other intervals the vaccine was not used. Recruits could be divided into vaccine-exposed and unexposed groups based on date of entry into the army. [It is likely that this adenovirus vaccine contained live SV40, because in the tissue culture system that was used, adenovirus grows poorly without the presence of SV40 as a “helper virus,” and because formalin added during manufacture did not completely inactivate SV40. Furthermore, later testing of three lots of adenovirus vaccine yielded live SV40, and 100% of 9 recruits who received the vaccine in an early trial exhibited SV40 seroconversion.] Rollison *et al.* used a national Veterans Administration database to identify cases with mesothelioma and control subjects (patients with colon or lung cancer) during 1969–96, i.e. up to 35 years after entry into military service. Receipt of adenovirus vaccine was not associated with mesothelioma risk (OR, 1.49; 95% CI, 0.38–5.88). [A limitation of this study is that all subjects were likely exposed to SV40-contaminated IPV. However, IPV in the USA was not as uniformly contaminated with

SV40 as adenovirus vaccine was, and as noted in Section 2.1, cohort studies of recipients of SV40-contaminated IPV have not shown clear evidence of elevated risk of cancer, including mesothelioma. Therefore, it is unclear whether IPV exposure would have attenuated the association between receipt of SV40-contaminated adenovirus vaccine and mesothelioma. Lung cancer and, to a lesser extent, colon cancer are associated with asbestos exposure. Thus, use of individuals with these cancers as controls somewhat mitigates the possible confounding effects of asbestos.]

[Bolognesi *et al.* \(2005\)](#) recruited mesothelioma patients in a case-control study in Italy during 1996–2000. The researchers also evaluated control subjects, described as healthy or with benign lung disease, and patients with lung cancer. SV40 DNA was detected by PCR in peripheral blood T cells from 2 (11%) of 19 mesothelioma cases and 0 (0%) of 22 controls, who were healthy or had benign lung disease. The odds ratio is thus infinite. However, this difference in prevalence is not significant [$P = 0.21$ by Fisher exact test]. Also, a similar prevalence of detectable SV40 DNA in peripheral blood T cells (2 of 18, 11%) was found among the lung cancer cases [even though lung cancer has not been thought to be associated with SV40]. [There is possible confounding by age; controls were younger than cases, although differences are not statistically significant. SV40 DNA results were available on only a subset of subjects, and it is not clear how the evaluated subjects were selected. The majority of the cases reported asbestos exposure, but only a minority of the controls reported asbestos exposure.]

2.2.2 Haematological malignancies

Case-control studies evaluating the association between SV40 and haematological malignancies are summarized in [Table 2.4](#). Most of these studies have addressed NHL, but limited data are

Table 2.3 Case–control studies of mesothelioma and SV40

Reference, study location, and period	Total cases Total controls	Control source (hospital, population)	Detection method	Exposure categories	Exposed cases	Relative risk (95% CI)	Covariates Comments
Strickler <i>et al.</i> (1996) , USA, 1975–91	35 35	Hospital (patients with gastrointestinal disease)	Neutralizing antibodies to SV40	Positive	3	[3.2; $P = 0.62$ by Fisher exact test]	Sex, age Did not measure or adjust for asbestos exposure.
Rollison <i>et al.</i> (2004) , United States army veterans, 1969–96	10 221	Hospital (army veterans with colon or lung cancer)	Receipt of SV40-contaminated adenovirus vaccine, based on army enrolment date	Exposed to vaccine	4	1.49 (0.38–5.88)	Age, race Subjects were also exposed to SV40-contaminated poliovirus vaccine, which could have attenuated the association.
Bolognesi <i>et al.</i> (2005) , Italy, 1996–2000	19 22	Mixed (healthy individuals, patients with benign lung disease)	PCR for SV40 DNA in peripheral blood T cells	PCR positive	2	[Infinity; $P = 0.21$ by Fisher exact test]	None A similar prevalence of PCR positivity was observed in lung cancer cases (2 of 18; 11%). Possible confounding by age. Nearly all mesothelioma cases had asbestos exposure.

CI, confidence interval; PCR, polymerase chain reaction; SV40, simian virus 40

also available for other lymphoid neoplasms and leukaemias.

In an early study based in England, [Stewart & Hewitt \(1965\)](#) compared vaccination histories for case children who died from leukaemia during 1956–60 and control children from the same period. During the period of evaluation, England used the inactivated poliovirus vaccine ([Rodén, 1964](#)). A similar proportion of cases and controls had received poliovirus vaccination. [The report is very brief, and few details are provided by the investigators. Based on the dates of death among the cases, all vaccinations would have occurred before 1963, but no information was included on the level of SV40 contamination in poliovirus vaccines.]

[Holly & Bracci \(2003\)](#) reported results of a population-based case–control study in the USA. NHL cases were identified through a cancer registry, and controls were selected from the general population by random-digit dialling or from health administrative records. Study subjects provided questionnaire responses to medical history items, including whether they had received poliovirus vaccine and the date of vaccination. Self-reported history of poliovirus vaccination in 1963 or earlier, when live SV40 could have been present in poliovirus vaccines, was not associated with an elevated risk for any of the NHL subtypes examined, including the two most common subtypes, follicular lymphoma (OR, 1.0; 95% CI, 0.78–1.4) and diffuse large-cell lymphoma (OR, 0.81; 95% CI, 0.63–1.0). [A limitation of this study is that subjects' recall of vaccination events from early childhood could have been inaccurate, which might have biased the results. It is likely that both cases and controls were frequently vaccinated since the USA had a national vaccination programme.]

[De Sanjosé et al. \(2003\)](#) conducted a case–control study of lymphoma in Spain. Cases with B-cell neoplasm ($n = 485$, mostly NHL but also including plasma cell myeloma), Hodgkin lymphoma ($n = 57$), or T-cell lymphoma ($n = 35$)

were enrolled from four hospitals. Controls ($n = 587$) were patients treated for other medical conditions at the same hospitals. Antibodies against SV40 were measured in these individuals using an ELISA incorporating SV40 VLPs. Overall, SV40 seroprevalence was lower in cases than controls (OR, 0.61; 95% CI, 0.38–0.95). SV40 seroprevalence was not significantly elevated among cases with individual subtypes of B-cell neoplasm, Hodgkin lymphoma, or T-cell lymphoma. Furthermore, the levels of SV40 antibody present in seropositive subjects were much lower than seen in SV40-infected macaques, and these levels decreased when subjects' sera were incubated with BKV VLPs [suggesting that much of the SV40 reactivity was due to cross-reactivity to BKV].

A USA-based case–control study is described in two related reports, [Engels et al. \(2004b, 2005\)](#). In [Engels et al. \(2004b\)](#), the investigators evaluated 724 cases with NHL and 622 general population controls. Cases and controls were similar demographically and in terms of education, and analyses were adjusted for sex, race, birth year, and study site. Sera were tested for antibodies using SV40 VLP ELISAs in two independent laboratories. In one laboratory, SV40 seropositivity was less common in cases than controls (OR, 0.68; 95% CI, 0.46–1.00), and in the other laboratory the cases and controls had similar seroprevalence (OR, 1.02; 95% CI, 0.71–1.47). Associations were of comparable magnitude across major NHL subtypes. Among SV40 seropositive subjects, the majority of SV40 antibodies were non-specific, i.e. the SV40 reactivity could be competed away through incubation of sera with BKV or JCV VLPs. The proportion of cases and controls with SV40-specific antibodies was low in the evaluated subjects and did not differ between cases and controls.

In the related case–control study, [Engels et al. \(2005\)](#) measured antibodies to the SV40 LT in a sample of the participants from the parent case–control study ([Engels et al., 2004b](#)). The

Table 2.4 Case–control studies of haematological malignancies and SV40

Reference, study location, and period	Total cases Total controls	Control source (hospital, population)	Detection method	Organ site	Exposure categories	Exposed cases	Relative risk (95% CI)	Covariates Comments
Stewart & Hewitt (1965) , United Kingdom, 1956–60	999 999	NR	Immunization history	Leukaemia (mortality)	Poliovirus vaccination	270	[1.06 (0.86–1.30)]	NR Brief report provided few details. There was no information on contamination of poliovirus vaccines.
Holly & Bracci (2003) , USA, 1988–95	352 510 2402 controls	Population	Self-report of childhood vaccination	Follicular lymphoma Diffuse large-cell lymphoma	Poliovirus vaccination in 1963 or earlier	179 227	1.0 (0.78–1.4) 0.81 (0.63–1.0)	Age, sex Results were similar for less common NHL subtypes. Recall of vaccination may have been inaccurate.
de Sanjosé et al. (2003) , Spain, 1998–2002	520 485 57 35 587 controls	Hospital (benign conditions)	ELISA for antibodies against SV40 VLPs	Lymphoid neoplasms overall B-cell neoplasm Hodgkin lymphoma T-cell lymphoma	Positive	31 28 11 3	0.61 (0.38–0.95) 0.59 (0.37–0.94) 2.04 (0.96–4.33) 0.76 (0.22–2.57)	Age, sex Most cases were subtypes of NHL. Associations were similar across NHL subtypes. Antibody reactivity was reduced after incubation with BKV VLPs.

Table 2.4 (continued)

Reference, study location, and period	Total cases Total controls	Control source (hospital, population)	Detection method	Organ site	Exposure categories	Exposed cases	Relative risk (95% CI)	Covariates Comments
Engels <i>et al.</i> (2004c) , USA, 1998–2000	724 622	Population	ELISA for antibodies against SV40 VLPs in laboratory A	NHL	Positive	52	0.68 (0.46–1.00)	Sex, race, birth year, study site Associations were similar across NHL subtypes. Antibody reactivity was reduced after incubation with BKV or JCV VLPs.
			ELISA for antibodies against SV40 VLPs in laboratory B		Positive	70	1.02 (0.71–1.47)	
Engels <i>et al.</i> (2005) , USA, 1998–2000	85 95	Population	ELISA for antibodies to SV40 LT	NHL	Positive	5	1.2 (0.3–4.6)	Sex, race, age, study site Antibodies to SV40 LT were low-level.
Rollison <i>et al.</i> (2004) , United States army veterans, 1969–96	220 221	Hospital (army veterans with colon or lung cancer)	Receipt of SV40-contaminated adenovirus vaccine, based on army enrolment date	NHL	Exposed to vaccine	69	0.98 (0.65–1.47)	Age, race Subjects were also exposed to SV40-contaminated poliovirus vaccine, which could have attenuated the association.

BKV, BK polyomavirus; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; JCV, JC polyomavirus; LT, large T-antigen; NHL, non-Hodgkin lymphoma; NR, not reported; SV40, simian virus 40; VLPs, virus-like particles

investigators evaluated all SV40 VLP seropositive subjects, and a sample of SV40 VLP seronegative subjects sampled in strata defined by BKV VLP serostatus and case-control status, who were selected on the basis of SV40 and BKV VLP antibody status. Only 6% of NHL cases had measurable antibodies to LT, and the levels of antibodies were low. Furthermore, the presence of antibodies to SV40 LT was not associated with NHL (OR, 1.2; 95% CI, 0.3–4.6). The ELISA used by the investigators was able to demonstrate strong antibody responses to SV40 LT among hamsters with SV40-related tumours, demonstrating its sensitivity. [A strength of this study is measurement of antibody to LT, expression of which is thought necessary for SV40-mediated transformation.]

NHL was also evaluated in [Rollison *et al.* \(2004\)](#), the case-control study mentioned in Section 2.2.1 in conjunction with mesothelioma. Cases with NHL and control subjects with colon or lung cancer were identified using a military veterans health database. The investigators then compared cases and controls with respect to date of entry into the United States army, which tracked with the army's use of SV40-contaminated adenovirus vaccine. Receipt of adenovirus vaccine was not associated with NHL risk (OR, 0.98; 95% CI, 0.65–1.47).

2.2.3 Brain tumours

Case-control studies evaluating the association between brain tumours and SV40 are presented in [Table 2.5](#). These studies evaluated a range of different brain tumours but did not specifically examine ependymomas or choroid plexus tumours, two rare subtypes suggested by case series to be related to SV40 ([Bergsagel *et al.*, 1992](#)).

[Brenner *et al.* \(2003\)](#) conducted a hospital-based case-control study in the USA that included adult patients with glioma, meningioma, or acoustic neuroma. Controls were

patients admitted to the same hospitals with various non-malignant conditions. The investigators assessed SV40 exposure status through study questionnaire items regarding history of poliovirus vaccination, including year and route of vaccination. No significant association between poliovirus vaccination and cancer was observed for any of the three types of brain tumours, either overall or according to type of vaccine (inactivated or oral) or calendar year of vaccination. [A limitation of this study is that adult subjects would have had difficulty accurately recalling their vaccination status as young children. Supporting the possibility of inaccurate recall, a substantial proportion of subjects reported receipt of poliovirus vaccine before 1955, although large-scale campaigns did not begin in the USA until that year.]

[Rollison *et al.* \(2004\)](#) examined the association between brain tumours and receipt of SV40-contaminated adenovirus vaccine among United States army veterans. [Additional details are provided in Sections 2.2.1 and 2.2.2 above, as the study also evaluated mesothelioma and NHL.] The investigators found no association between brain tumours and previous receipt of adenovirus vaccine (OR, 0.76; 95% CI, 0.48–1.20). No data were available on histological subtypes of brain tumours.

2.2.4 Miscellaneous cancers

[Table 2.6](#) summarizes results of case-control studies that evaluated associations of other malignancies with SV40.

In the same report in which they evaluated leukaemia (see Section 2.2.2), [Stewart & Hewitt \(1965\)](#) also evaluated non-leukaemia cancers in England. Immunization histories were compared for children who had died from cancers other than leukaemia and control children. There was no difference in the proportion of case and control children who had received poliovirus vaccination. This brief report provides no details

Table 2.5 Case–control studies of brain tumours and SV40

Reference, study location, and period	Total cases Total controls	Control source (hospital, population)	Detection method	Organ site	Exposure categories	Exposed cases	Relative risk (95% CI)	Covariates Comments
Brenner <i>et al.</i> (2003) , USA, 1994–98	489 197 96 799 controls	Hospital (non-malignant conditions)	Self-report of childhood vaccination	Glioma Meningioma Acoustic neuroma	Poliovirus vaccination in 1954–62	133 53 33	1.08 (0.71–1.66) 0.95 (0.53–1.70) 1.30 (0.54–3.41)	Age, sex, race/ethnicity, distance of residence from hospital Recall of vaccination may have been inaccurate.
Rollison <i>et al.</i> (2004) , United States army veterans, 1969–96	181 221	Hospital (army veterans with colon or lung cancer)	Receipt of SV40-contaminated adenovirus vaccine, based on army enrolment date	Brain tumours	Exposed to vaccine	50	0.76 (0.48–1.20)	Age, race No data were available on brain tumour subtypes. Subjects were also exposed to SV40-contaminated poliovirus vaccine, which could have attenuated the association.

CI, confidence interval; SV40, simian virus 40

Table 2.6 Case–control studies of miscellaneous cancers and SV40

Reference, study location, and period	Total cases Total controls	Control source (hospital, population)	Detection method	Organ site	Exposure categories	Exposed cases	Relative risk (95% CI)	Covariates Comments
Stewart & Hewitt (1965) United Kingdom, 1956–60	1108 1108	NR	Immunization history	Non-leukaemia cancers (mortality)	Poliovirus vaccination	259	[0.97 (0.79–1.19)]	NR Brief report provided few details. There was no information on contamination of poliovirus vaccines. Cancer outcomes were not described.
Innis (1968) , Australia, 1958–67	706 706 controls 110 110 controls	Hospital	Poliovirus vaccination documented in medical record	All cancers, children > 1 year old All cancers, children < 1 year old	Receipt	618 29	[1.69 (1.25–2.29)] [1.61 (0.81–3.25)]	Matched on sex, age, hospital No data on date of poliovirus vaccination and likelihood of SV40 contamination. Cancer outcomes were not described.
Strickler et al. (1996) , USA, 1975–91	35 35	Hospital (patients with gastrointestinal disease)	Neutralizing antibodies to SV40	Osteo-sarcoma	Positive	1	[1.00 (0.012–80.77)]	Report also described results for mesothelioma.
Carter et al. (2003)	90 72	Population	VLP ELISA for SV40 antibodies	Prostate cancer	Positive	5	[0.65 (0.15–2.68)]	NR Report also described results for 122 paediatric osteosarcoma cases. SV40 antibody reactivity could be blocked by BKV and JCV VLPs.

BKV, BK polyomavirus; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; JCV, JC polyomavirus; NR, not reported; SV40, simian virus 40; VLPs, virus-like particles

on the types of non-leukaemia cancers that were included, the method of selecting controls, or the likelihood that poliovirus vaccines were contaminated with SV40. During the period of evaluation, England used the inactivated poliovirus vaccine ([Roden, 1964](#)).

[Innis \(1968\)](#) conducted a hospital-based case-control study during 1959–67 in Australia. Cases included children with a variety of unspecified malignancies. Controls were drawn from children admitted to the same hospital and were matched to cases on sex and age. Review of medical records provided data on vaccine exposure. An association was observed between poliovirus vaccination and cancer risk among children: this association was statistically significant among children aged > 1 year [crude OR, 1.69; 95% CI, 1.25–2.29; $P < 0.0005$] but not among younger children [crude OR, 1.61; 95% CI, 0.81–3.25]. [Although it is likely that some of the children aged > 1 year would have been vaccinated before 1963, a limitation of this study is that the investigator did not describe the dates of poliovirus vaccination, nor did he describe the frequency or level of contamination of poliovirus vaccines in Australia. Therefore, it is unclear whether children received poliovirus vaccine likely contaminated with SV40. Another limitation is that the types of cancer were not characterized. Also, there may be confounding by socioeconomic status if children with lower socioeconomic status were less likely to receive poliovirus vaccination. The control series may also have been biased if hospitalization would have been related to likelihood of vaccination.]

[Strickler et al. \(1996\)](#) reported results for osteosarcoma. The researchers evaluated serum samples collected in the USA from osteosarcoma cases and controls with non-malignant gastrointestinal diseases. The prevalence of SV40 neutralizing antibodies was identical in the two groups. [A limitation of this study is the small number of osteosarcoma cases. Also, the osteosarcoma cases were younger than the controls,

who had been matched to another case series. See Section 2.2.1.]

[Carter et al. \(2003\)](#) measured antibodies against SV40 VLPs in prostate cancer cases and controls in the USA. SV40 seroreactivity was observed in 5.6% of cases and 8.3% of controls. The study also included 122 children with osteosarcoma, among whom 2.5% had SV40 antibodies, but similarly aged controls were not evaluated. In competitive inhibition experiments, the researchers demonstrated that SV40 reactivity could all be blocked by addition of BKV or JCV VLPs, indicating that the SV40 antibodies were non-specific. [Limitations of this study include an inadequate description of how subjects were selected from the parent case-control study of prostate cancer.]

2.3 Susceptible populations

2.3.1 Children

Young children may comprise an especially susceptible population for any carcinogenic effects of SV40. As noted in Section 3, experimental results in animals suggest that the carcinogenic effect is strongest when exposure occurs in the neonatal period and drops off sharply with age ([Girardi et al., 1963](#)). Such results would suggest that children who received SV40-contaminated poliovirus vaccines, especially as infants, would have an elevated cancer risk. As reviewed in Section 2.2.1 and presented in [Table 2.1](#) and [Table 2.2](#), most cohort studies of young children who received SV40-contaminated IPV have not identified an elevated cancer risk. Many of these children received multiple inoculations (i.e. parenteral exposures) with SV40-contaminated IPV. The study by [Carroll-Pankhurst et al. \(2001\)](#) deserves special mention because it described a cohort of 1073 people exposed in the neonatal period (in the first 3 days of life). All of these neonates received poliovirus vaccines later documented to contain live SV40. After 35

years of follow-up, only 4 deaths from cancer were documented, which did not represent an excess, and the cancer deaths that occurred (2 from leukaemia and 2 from testicular cancer) did not suggest an effect of SV40 infection (see also Section 2.2.1 and [Table 2.1](#)).

2.3.2 People potentially exposed to SV40 in utero

Cancer risk in children born to women who received potentially contaminated poliovirus vaccines during pregnancy was evaluated in two studies (one case-control study and one cohort study combined with a nested case-control study). It is possible to consider these studies as providing information on cancer risk after *in utero* infection with SV40. [However, it is not established that infection can occur *in utero*.] Case-control studies (both retrospective and nested within a cohort) and cohort studies of this topic are presented in [Tables 2.7](#) and [Table 2.8](#), respectively.

[Farwell et al. \(1979\)](#) evaluated the association between childhood brain tumours and maternal exposure to IPV during pregnancy in a case-control study. Children with brain tumours who were born in 1956–62 (the period when children could have been exposed *in utero* through vaccination of the mother) and were aged 0–19 years at diagnosis were identified through the Connecticut Tumor Registry in the USA. Control children were identified through a search of birth certificates. The investigators asked the obstetricians who had delivered these children whether the mothers had received poliovirus vaccination during pregnancy. Based on the birthdates, vaccinations would have all been before 1963 when poliovirus vaccines in the USA were contaminated with SV40. Ten (67%) of 15 children with medulloblastoma, 8 (35%) of 23 children with glioma, and 1 (7%) of 14 children with other brain tumours had mothers who had been vaccinated during pregnancy, compared

with 8 (21%) of 38 control children. Overall, there was a borderline association between mothers' vaccine exposure and the occurrence of brain tumours in children [crude OR, 2.15; 95% CI, 0.76–6.54]. The difference in vaccine exposure was significant for medulloblastoma [crude OR, 7.5, 95% CI, 1.68–35.39] but not for glioma [crude OR, 2.0, 95% CI, 0.53–7.45] or other brain tumours.

[Engels et al. \(2004a\)](#) included both a cohort analysis in relation to poliovirus vaccine exposure and a nested case-control study evaluating associations with maternal SV40 antibody status. In the cohort study component, the investigators evaluated cancer risk among 54 796 children in the Collaborative Perinatal Project. The Collaborative Perinatal Project is a cohort study that enrolled pregnant women in the USA during 1959–66 and subsequently followed their children. The children were classified according to whether their mothers had received poliovirus vaccine (mostly IPV) during pregnancy and, if so, whether that vaccine had been administered before 1963. During 8 years of follow-up, children whose mothers had received pre-1963 poliovirus vaccine had an increased risk for haematological malignancies (mostly leukaemias; RR, 2.5; 95% CI, 1.1–5.6) and neural tumours (RR, 2.5; 95% CI, 1.0–6.3), compared with children whose mothers had not been vaccinated or had received poliovirus vaccine only in 1963 or later. Risk was not elevated for other miscellaneous types of cancer (RR, 1.2; 95% CI, 0.3–4.6). The most common type of neural tumour in the children was neuroblastoma, and maternal poliovirus vaccine exposure was associated with an elevated risk of this cancer (RR, 8.2; 95% CI, 1.6–43). [A strength of this study is the contemporaneous documentation of poliovirus vaccination during the mother's pregnancy, which eliminates differential recall of vaccination status between mothers of cases and controls.]

Of note, in the same report, [Engels et al.](#) described a nested case-control study testing sera

Table 2.7 Case-control studies of cancer in relation to potential exposure *in utero* to SV40

Reference, study location, and period	Total cases Total controls	Control source (hospital, population)	Detection method	Organ site	Exposure categories	Exposed cases	Relative risk (95% CI)	Covariates Comments
Farwell <i>et al.</i> (1979) , USA, 1956 and later	15	Population	Physician report of maternal vaccination during pregnancy	Medulloblastoma	Poliovirus vaccination of mother in 1956–62	10	[7.5 (1.68–35.39)]	Sex, birthdate, town of birth
	23			Glioma		8	[2.0 (0.53–7.45)]	
	14 38 controls			Other brain tumours		1	[0.29 (0.006–2.60)]	
Engels <i>et al.</i> (2004b) , USA, 1959–74	50	Population (same cohort as cases)	Plaque neutralization assay for SV40 antibodies VLP ELISA for SV40 antibodies	All cancers	Maternal seroconversion during pregnancy	3	1.5 (0.3–6.3)	Maternal receipt of poliovirus vaccine, timing of poliovirus vaccination Cases and controls were drawn from a cohort in which maternal receipt of poliovirus vaccine was associated with cancer. Study extended report by Rosa <i>et al.</i> (1988) .
	200					4	4.0 (1.0–15.7)	

CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; SV40, simian virus 40; VLP, virus-like particle

Table 2.8 Cohort studies of susceptible populations exposed to SV40

Reference, location, follow-up period	Total subjects	Detection method	Organ site	Exposure categories	Exposed cases	Relative risk (95% CI)	Covariates Comments
People potentially exposed in utero							
Engels et al. (2004a) , children in the USA 1959–74	54 796	Vaccination status of mothers followed during pregnancy	Neural tumours	Children whose mothers received pre-1963 polio vaccine	8	2.5 (1.0–6.3)	Sex, race, maternal age at delivery Evaluation of maternal sera did not reveal association of SV40 seroconversion with cancer in children. Study improves on exposure and outcome assessment and extends follow-up of Heinonen et al. (1973) .
			Neuroblastoma	vs children whose mothers received poliovirus vaccine in 1963 or later, or no vaccine.	NR	8.2 (1.6–43)	
			Haematological malignancies		10	2.5 (1.1–5.6)	
			Miscellaneous tumours		3	1.2 (0.3–4.6)	
Immunosuppressed populations							
Engels et al. (2003b) , people with AIDS in the USA 1980–96	56 808	Birth cohort with varying exposure to SV40-contaminated IPV	NHL	Born 1958–61 (exposed) vs 1964–67 (unexposed)	616	0.97 (0.79–1.20)	Sex, HIV risk group, ethnicity, age, registry area No association was seen with exposure for any lymphoma subtype.
Paracchini et al. (2006) , Italian transplant recipients NR	387	PCR testing of peripheral blood DNA from organ donors	All cancers	Organ donor with PCR-amplifiable SV40 DNA in peripheral blood	0	0	None The number of exposed transplant recipients was small.

CI, confidence interval; IPV, inactivated poliovirus vaccine; NHL, non-Hodgkin lymphoma; NR, not reported; PCR, polymerase chain reaction; SV40, simian virus 40; *vs*, *versus*

from these mothers for SV40 antibody seroconversion during pregnancy. Results were presented for all cancer types combined. During pregnancy, 3 of 46 case mothers exhibited seroconversion according to an SV40 plaque neutralization assay (the children developed neuroblastoma, leukaemia, or fibrosarcoma), and 4 of 50 case mothers exhibited seroconversion according to an SV40 VLP ELISA (2 children with neuroblastoma, 1 with astrocytoma, and 1 with leukaemia). Only 1 case mother, whose child developed neuroblastoma, seroconverted according to both assays. The seroconversion rates did not differ significantly between case and control mothers, although the results according to the VLP ELISA were borderline significant (OR, 4.0; 95% CI, 1.0–15.7). [Because SV40 seroconversion was not associated with cancer among the children, and seroconversion was seen in few of the mothers whose children developed cancer, the study does not provide strong support for the conclusion that the positive associations between vaccination and cancer seen in the cohort study were due to SV40 infection transmitted via maternal vaccination. Neuroblastoma is a unique tumour of early childhood and has not otherwise been associated with SV40. This study extends an earlier report by [Rosa et al. \(1988\)](#) by including additional cases ascertained during longer follow-up. A limitation of this case–control study was the small number of outcomes, which precluded a separate assessment by cancer type.]

2.3.3 Immunosuppressed people

Immunosuppressed individuals are a third potentially susceptible population. HIV-infected people and solid organ transplant recipients have an increased risk for a range of virus-related malignancies, including NHL ([Grulich et al., 2007](#)). Two cohort studies are summarized in [Table 2.8](#).

[Engels et al. \(2003b\)](#) examined the association between childhood exposure to potentially

contaminated IPV and risk of NHL among HIV-infected people in the USA. The investigators used linked data between HIV and cancer registries in the USA to evaluate NHL risk among 56 808 people with AIDS. People with AIDS were categorized by birth year into two groups based on likely exposure to SV40-contaminated IPV in childhood: an exposed birth cohort (born in 1958–61) and an unexposed birth cohort (born in 1964–67). After adjustment for potentially confounding demographic factors, the SV40-exposed and unexposed birth cohorts had similar NHL risk (RR, 0.97; 95% CI, 0.79–1.20). In addition, the SV40-exposed birth cohort did not have elevated risk for any NHL subtype defined by site or histology. [The Working Group noted that this study is important, showing that in this population with a very high NHL risk, there was no elevated risk for NHL associated with prior vaccination-related exposure.]

[Paracchini et al. \(2006\)](#) described cancer risk in a cohort of 41 transplant recipients in Italy who received organs from 12 donors who had SV40 DNA amplified from peripheral blood. The premise of the study was that SV40 might be transmissible to such transplant recipients and that immunosuppression administered to recipients would facilitate the development of cancer. During an average follow-up of 671 days, none of these recipients developed cancer. In comparison, cancer developed in 11 of 346 recipients who received organs from donors who did not have SV40 DNA detectable in blood. [The 11 cancers in the comparison group were not specified. Nonetheless, it is striking that none of the SV40-exposed recipients developed cancer, especially NHL, which is common in the early period after transplantation. Limitations of this study include the short follow-up and the small number of recipients who received organs from donors considered to be SV40-positive. Also, the sensitivity and specificity of detection of SV40 DNA in donor blood is uncertain, and it

is unknown whether SV40 can be transmitted through organ transplantation.]

2.3.4 Asbestos-exposed people

Asbestos exposure is a recognized cause of mesothelioma, and it would be of interest to assess the contribution of SV40 in asbestos-exposed individuals. However, no epidemiological study has used appropriate measures of SV40 infection to assess whether the effect of SV40 on mesothelioma risk is independent of asbestos exposure, or whether any effect of SV40 varies by asbestos exposure. [Mayall et al. \(1999\)](#) and [Cristaudo et al. \(2005\)](#) reported on the prevalence of SV40 DNA detected in mesothelioma tumours in relation to asbestos exposure, but the studies were too small to be informative ($n = 7$ and $n = 13$ asbestos-exposed cases, respectively).

2.4 Case series

Numerous studies have reported on the detection of SV40 in human tumours. These case series are not reviewed systematically in this report, because of methodological issues mentioned below and because other epidemiological study designs were judged to provide a higher level of evidence. Recent reviews that include summaries of case series, and which reached conflicting conclusions regarding the interpretation of those data, include [Institute of Medicine of the National Academies \(2002\)](#), [Vilchez & Butel \(2004\)](#), [Shah \(2007\)](#), and [Butel \(2010, 2012\)](#).

In the published case series, most investigators have used PCR to identify SV40 DNA in archived formalin-fixed or frozen tumour tissues. Positive studies that have been influential in stimulating additional research include [Carbone et al. \(1994\)](#), which found SV40 DNA in 60% of mesothelioma tumours; [Bergsagel et al. \(1992\)](#), which found SV40 DNA in 91% of ependymomas and 50% of choroid plexus tumours; [Vilchez et al. \(2002\)](#) and [Shivapurkar et al. \(2002\)](#), which found SV40

DNA in 42–43% of NHL tumours; and [Carbone et al. \(1996\)](#), which found SV40 DNA in 32% of osteosarcoma tumours. In subsequent studies, some investigators confirmed detection of SV40 DNA in these tumour types, but some studies were largely or entirely negative, for example, [Strickler et al. \(1996\)](#) and [Gordon et al. \(2002\)](#) for mesothelioma; [Weggen et al. \(2000\)](#), [Engels et al. \(2002\)](#), and [Rollison et al. \(2005a\)](#) for ependymomas and choroid plexus tumours; and [MacKenzie et al. \(2003\)](#) and [Schüler et al. \(2006\)](#) for NHL.

Reasons for the discrepant results across these case series are uncertain. One possibility is that geographical variability in the epidemiology of SV40 has resulted in differing contributions of SV40 to cancer in different regions. However, no clear geographical pattern has been identified, and studies conducted using tissues from the same country (e.g. USA) have produced conflicting results. Some studies were poorly designed or conducted (e.g. lacking negative or positive controls, testing of samples under unblinded conditions) or inadequately described in the published reports. The discrepancies across studies have prompted debate about whether appropriately sensitive methods were used in the negative studies, or whether false-positive results could explain the results of positive studies (see also Section 4.3.1). Few studies have used quantitative PCR to assess the amount of SV40 DNA present in tumour samples, but several studies have found that SV40 DNA, when detectable, is present at less than one copy per tumour cell ([Gordon et al., 2002](#); [Rollison et al., 2005b](#); [Ziegler et al., 2007](#)). Such low-level detection is inconsistent with a model whereby SV40 DNA is integrated into host DNA and present in every tumour cell.

Likewise, it has been difficult to interpret reports of the detection of SV40 DNA in a wide range of other tumour types, including, in addition to the types already mentioned, thyroid cancer ([Vivaldi et al., 2003](#)), colon cancer

([Shivapurkar et al., 2002](#); [Campello et al., 2010](#)), breast cancer ([Shivapurkar et al., 2002](#)), lung cancer ([Shivapurkar et al., 2002](#)), and prostate cancer ([Shivapurkar et al., 2002](#)). SV40 DNA has also reportedly been detected in additional histologically defined tumour subtypes, including astrocytomas and gliomas ([Martini et al., 1996](#); [Suzuki et al., 1997](#)), Hodgkin lymphoma ([Shivapurkar et al., 2002](#)), and chondrosarcomas and giant cell bone tumours ([Carbone et al., 1996](#); [Gamberi et al., 2000](#)).

Three multicentre studies have evaluated the reproducibility of SV40 DNA detection in human tumour tissues. In the first such study, [Testa et al. \(1998\)](#) reported results for four laboratories that tested DNA extracted from 12 mesothelioma cases. The PCR testing was performed in a blinded fashion, although the panel did not include tissues other than the mesothelioma cases as negative controls. Using each of two sets of PCR primers, 9 (75%) of the 12 tumours were positive in all four laboratories, and the remaining 3 tumours were variably positive.

The second interlaboratory comparison was reported by Strickler (2001). Nine testing laboratories performed blinded PCR evaluation of specimens prepared by a separate processing laboratory. The specimens included DNA extracts from 25 mesothelioma tumours (included in duplicate) and 25 specimens of normal lung, which were randomly sorted within the test batch. All samples were PCR positive for DNA from the human β -globin gene, indicating the presence of amplifiable DNA. Across the nine laboratories, the proportion of mesothelioma specimens that were PCR positive for SV40 DNA, using a variety of testing protocols, ranged from 0% to 40%. In the majority of cases, PCR positive reactions were not confirmed in the blinded testing of the replicate mesothelioma sample by the same laboratory or by testing in other laboratories. The highest detection rate (40%) was found by a laboratory that reported contamination of its PCR primers, and this laboratory

also found 28% of the normal lung extracts to be PCR positive for SV40. Unexpectedly, eight of the nine testing laboratories found some samples from a negative control batch to be PCR positive for SV40 DNA. This SV40 positivity was traced to contamination of the negative control batch during preparation by the processing laboratory.

In the third study, [Rollison et al. \(2005a\)](#) compared detection of SV40 DNA by PCR in two laboratories that independently assessed a panel of tumours under masked conditions. Among a total of 225 human brain tumours evaluated, most were astrocytomas or glioblastomas, but 29 cases were ependymomas and 14 were choroid plexus tumours. The majority of tumours had amplifiable human cellular DNA, but SV40 detection was uncommon. One laboratory detected SV40 DNA in three cases, and the second laboratory detected SV40 DNA in a different case. The second laboratory quantified the amount of SV40 DNA in the single PCR positive tumour as 0.12 copies in approximately 7000 cells, inconsistent with the clonal presence of SV40 in each tumour cell. Blinded positive and negative controls yielded expected results and were concordant in both laboratories.

The possibility that false-positive results arising from PCR contamination could explain some published positive reports is supported by [López-Ríos et al. \(2004\)](#). The investigators described results for 71 mesothelioma tumours. Application of two widely used PCR primer sets yielded positive results for SV40 DNA in 56–62% of cases. However, results were discordant in 14 cases, and the authors noted occasional PCR positivity of negative controls. These results led López-Ríos et al. to sequence the PCR products, and comparison with GenBank revealed that the amplified sequences were also present in commonly used laboratory plasmids. Repeated experiments, using alternative PCR primers that would be less likely to amplify plasmid sequences, yielded only 4 mesothelioma cases (6%) with weakly positive results. Finally, in

experiments that used PCR primers engineered to span a junction site between SV40 and plasmid DNA sequences, 16 (23%) of 69 mesothelioma cases were positive for the junction site found in plasmids rather than naturally occurring viral sequences. López-Ríos *et al.* also re-analysed data from a previously published study and demonstrated that the SV40 sequences amplified from human tumours in that study were unknowingly plasmid-derived contaminants.

Studies that have used other detection methods for SV40 in human tumour tissues have also produced inconsistent results. Examples of studies that have yielded differing results for detection of SV40 RNA or proteins include [Carbone *et al.* \(1994\)](#), [Testa *et al.* \(1998\)](#), [López-Ríos *et al.* \(2004\)](#), and [Brousset *et al.* \(2005\)](#) for mesothelioma; [Bergsagel *et al.* \(1992\)](#) and [Sabatier *et al.* \(2005\)](#) for brain tumours; and [Brousset *et al.* \(2004\)](#) and [Vilchez *et al.* \(2005\)](#) for NHL. No study has reported on whether patients who have tumours that manifest molecular evidence for SV40 also exhibit serum antibodies for SV40 infection.

[Although case series that have reported on detection of SV40 DNA in human tumours have been influential in motivating research on the role of SV40 in causing human malignancies, there are difficulties in interpreting these results that preclude giving them substantial weight in the assessment of the human evidence. Studies have produced widely conflicting results on the presence of SV40 DNA in human tumours, and many studies are of poor quality. There are few rigorous comparisons of detection of SV40 across laboratories, and the existing comparisons do not support that detection is consistent. Contamination of tumour specimens by low levels of SV40 DNA (present in plasmids or from other sources) cannot be ruled out as an explanation for detection.]

3. Cancer in Experimental Animals

3.1 Infection with SV40

Animal models for SV40 include hamsters (Syrian golden hamsters [*Mesocricetus auratus*], unless otherwise specified) and transgenic mice. Experimental studies of SV40 tumorigenicity *in vivo* dating from the 1960s (when the virus was first studied) to the present are summarized below.

3.1.1 Hamster

The oncogenic potential of SV40 was first demonstrated by [Eddy *et al.* \(1961\)](#) by the subcutaneous inoculation of rhesus monkey kidney cell extracts into newborn (NB) hamsters. [NB hamsters in this and other studies are ≤ 3 days old.] Among the injected animals ($n = 154$), 70% developed subcutaneous tumours, over 3.5–9 months, as well as occasional lung and kidney tumours. Tumours were identified as undifferentiated sarcomas. Control hamsters ($n = 65$), inoculated with extracts of human and feline tumours, failed to develop any tumours. The oncogenic substance was soon identified as SV40 ([Eddy *et al.*, 1962](#)). That follow-up study showed that SV40 strain 776 inoculated subcutaneously in NB hamsters ($n = 13$) induced subcutaneous sarcomas with latency periods of 7–9 months in 85% of animals. Some lung tumours were also induced (33%). Controls included animals injected with SV40 mixed with SV40 neutralizing antibody ($n = 17$); none of those controls developed tumours.

The oncogenic potential of SV40 has been confirmed and characterized in numerous reports in the decades after the studies of [Eddy *et al.* \(1961, 1962\)](#). It has been established that both host and viral factors influence the development of SV40-mediated neoplasia. Factors include age at the time of infection, route of exposure, dose of inoculum (amount of infectious virus), and

viral genetic variation. Reported studies have varied in the numbers of experimental animals and the duration of observation, but they have consistently and reproducibly shown that SV40 has the capacity to transform a variety of target cell types *in vivo*. Subcutaneous, intravenous, and intraperitoneal routes of injection with different strains of SV40 give rise to specific types of tumours. In these studies, cumulative controls numbering more than 1000 have shown that hamsters did not develop these tumours spontaneously.

(a) Subcutaneous inoculation

See [Table 3.1](#)

Subcutaneous inoculation of NB hamsters with SV40 usually results in the formation of subcutaneous tumours identified as fibrosarcomas. SV40 strain VA45-54 induced tumours in 45% of animals ($n = 94$), as well as peritoneal mesothelioma with lung metastases (1%) over a time period of 4–10 months ([Girardi *et al.*, 1962](#)). Controls, uninoculated or inoculated with medium or tissue culture fluids ($n = 409$), remained tumour-free. In a subsequent study, [Girardi *et al.* \(1963\)](#) showed that NB hamsters were more susceptible than older animals to SV40-induced tumours after subcutaneous inoculation. With a uniform inoculum of 3×10^5 TCID₅₀ (50% tissue culture infective dose) of SV40 strain VA45-54 and with groups ranging in size from $n = 54$ to $n = 11$, 96% of animals inoculated as newborns developed tumours, as did 60% of animals inoculated at age 7 days, 23% of those inoculated at age 1 month, and none of those inoculated at age 3 months. In addition, tumour latencies were shorter in younger animals. The effect of virus dose on tumour induction in NB animals was demonstrated, and the following subcutaneous tumour incidences were observed: 3×10^5 TCID₅₀, 96%; 3×10^3 TCID₅₀, 18%; and 30 TCID₅₀, 0%. Also, whereas SV40 treated with pre-immune serum remained tumorigenic (96%), SV40 neutralized with SV40 antiserum

induced no tumours. The subcutaneous route of SV40 inoculation in NB hamsters resulted in more tumorigenic activity (86%) than the intraperitoneal (4%) or intrapulmonary (11%) route. Control animals ($n = 371$) remained tumour-free. [Girardi \(1965\)](#) also tested the potential inhibitory effect of SV40-transformed human cells on SV40-induced tumorigenesis. NB hamsters were inoculated subcutaneously with SV40 strain Rh911 at 10^7 – 10^8 TCID₅₀ ($n = 46$). Then, 35 days later, half were inoculated intraperitoneally with 7×10^6 control human WI-26 cells and half with SV40-transformed WI-26 cells. Ninety-two percent of the SV40-infected hamsters treated with the control cells developed subcutaneous tumours, whereas only 4% of those treated with the SV40-transformed human cells developed tumours.

[Ashkenazi & Melnick \(1963\)](#) showed that SV40 that had been purified by terminal dilution and inoculated at 10^6 TCID₅₀ subcutaneously into NB hamsters induced undifferentiated subcutaneous sarcomas in 79% of animals, with latency periods of 4–8 months. [Black & Rowe \(1964\)](#) tested SV40 strain 777; subcutaneous inoculation of $10^{6.5}$ TCID₅₀ into NB hamsters ($n = 56$) resulted in subcutaneous sarcomas in 63% of recipients. Uninoculated control hamsters ($n = 24$) did not develop tumours. Three doses of three plaque variants of SV40 received from the Eddy laboratory ([Eddy *et al.*, 1961, 1962](#)) were tested in groups of 17–23 NB hamsters for relative tumorigenicity ([Takemoto *et al.*, 1966](#)). The dose of virus able to cause tumours in 50% of animals over an 11-month period after subcutaneous injection was as follows: large-plaque variant, $10^{4.2}$ plaque-forming units (PFU) (tumour incidence, 32/60); small-plaque variant, $10^{5.2}$ PFU (tumour incidence, 20/70); and minute-plaque variant, $10^{5.8}$ PFU (tumour incidence, 5/60). These differences in the values of doses causing tumours in 50% of the animals were statistically significant ($P = 0.01$).

[Defendi & Jensen \(1967\)](#) used a subcutaneous inoculum of 10^7 PFU into NB hamsters to test the

Table 3.1 Carcinogenicity studies in hamsters^a given SV40 by subcutaneous inoculation

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Controls Animals/group at start	For each target organ: tumours (%); time to tumours	Significance ^b	Comments
Hamster (NB, < 1–3 d) (NR) Up to 500 d Eddy et al. (1961)	SV40 strain, 0.2 mL primary RMKC extracts, subcutaneous (<i>n</i> = 154) Controls: extracts of human and feline tumours (<i>n</i> = 65)	Subcutaneous tumours (70%), lung (2%), kidney (1%), all undifferentiated sarcomas; 3.5–9 mo Controls (0%)	NR	Oncogenic agent subsequently identified as SV40 (Eddy et al., 1962). First report of oncogenicity by SV40 in animals.
Hamster (NB, 1–3 d) (NR) Up to 391 d Eddy et al. (1962)	SV40 strain 776, 0.2 mL, subcutaneous <i>n</i> = 13 SV40 rescued from tumours (Eddy et al., 1961), 0.2 mL, subcutaneous (<i>n</i> = 54) Controls: extracts of AGMK, SV40 + anti-SV40 serum (<i>n</i> = 17)	Strain 776: subcutaneous tumours (85%), lung (33%), all sarcomas; 7–9 mo Rescued virus: subcutaneous tumours (70%); 5 mo Controls (0%)	NR	
Hamster (NB, < 1 d) (M, F) Up to 10.5 mo Girardi et al. (1962)	SV40 strain VA45-54, 0.2 mL, subcutaneous (<i>n</i> = 94) Controls: uninfected GMK cell fluid, medium, or uninoculated (<i>n</i> = 409)	Subcutaneous tumours (45%), fibrosarcomas, peritoneal mesothelioma with lung metastases (1%); 4–10 mo Controls (0%)	NR	Tumours were serially transplanted into hamsters (age, 4–6 wk).
Hamster (different ages) (NR) Up to 17 mo Girardi et al. (1963)	SV40 strain VA45-54, 0.2 mL, subcutaneous Ages, NB to 3 mo; doses, 10 ¹ TCID ₅₀ to 3 × 10 ⁵ TCID ₅₀ ; groups ranged from <i>n</i> = 54 to <i>n</i> = 11 Virus treated with pre-immune serum (<i>n</i> = 23) and anti-SV40 rabbit serum (<i>n</i> = 24) Controls: uninfected GMK cell fluid, medium, or uninoculated (<i>n</i> = 371)	Subcutaneous tumours. Age at inoculation of high dose: NB (52/54, 96%), 7 d (24/40, 60%), 1 mo (6/26, 23%), 3 mo (0/35, 0%); 3.8–16 mo, shorter times to tumours in younger animals. Varying virus dose in NB: 3 × 10 ⁵ TCID ₅₀ (96%), 3 × 10 ³ TCID ₅₀ (18%), 3 × 10 ¹ TCID ₅₀ (0%); 3.8–14 mo Virus + normal (pre-immune) serum (96%), virus + anti-SV40 serum (0%); 10 mo Controls (0%)	NR	Subcutaneous route of inoculation (66/77, 86%) in NB hamsters more tumorigenic than intraperitoneal (2/44, 4%) or intrapulmonary (2/18, 11%).
Hamster (NB, < 1 d) (NR) Up to 8 mo Ashkenazi & Melnick (1963)	SV40 purified by terminal dilution, 0.05 mL (10 ⁶ TCID ₅₀), subcutaneous	Subcutaneous tumours (79%), undifferentiated sarcomas; 4–8 mo	NR	Tumours were serially transplanted into weanling hamsters (cheek pouch). Hamster embryo fibroblasts were transformed <i>in vitro</i> by SV40 and were tumorigenic in weanling hamsters (cheek pouch).

Table 3.1 (continued)

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Controls Animals/group at start	For each target organ: tumours (%); time to tumours	Significance ^b	Comments
Hamster (NB, 2 d) (NR) Up to 259 d Black & Rowe (1964)	SV40 strain 777, 0.1 mL ($10^{6.5}$ TCID ₅₀), subcutaneous ($n = 56$) Controls: uninoculated ($n = 24$)	Subcutaneous tumours (63%), sarcomas Controls (0%)	NR	
Hamster (NB, < 1 d) (NR) Up to 325 d Girardi (1965)	SV40 strain Rh911, 0.2 mL (10^7 – 10^8 TCID ₅₀), subcutaneous ($n = 46$) Then, 35 d later, inoculated intraperitoneally with 7×10^6 control human WI-26 cells ($n = 24$) or SV40- transformed WI-26 cells ($n = 22$)	Subcutaneous tumours in animals treated with control human cells (92%); SV40-transformed human cells prevented tumours (4%)	NR	
Hamster (NB) (NR) 11 mo Takemoto et al. (1966)	SV40 virus from Dr Bernice Eddy. Recovered plaque variants: SV40-L (large), SV40-S (small), SV40-M (minute). Serial dilutions, subcutaneous. Doses tested: 3.7, 37, and 370×10^4 PFU. ($n = 17$ –23); SV40-L ($n = 60$), SV40-S ($n = 70$), SV40-M ($n = 60$)	Dose of virus to cause tumours in 50% of animals: SV40-L: $10^{4.2}$ PFU SV40-S: $10^{5.2}$ PFU SV40-M: $10^{5.8}$ PFU	$P = 0.01$	
Hamster (NB, 1–2 d) (NR) 240 d Defendi & Jensen (1967)	SV40, 10^7 PFU/0.1 mL, subcutaneous Some aliquots of virus were irradiated with cobalt-60 source or ultraviolet radiation, which reduced infectious titres by 2–3 logs, before inoculation ($n = 13$ –39)	Although irradiation reduced infectivity, tumorigenicity was not inactivated but actually increased: ultraviolet radiation, 8/13 (62%) to 15/17 (88%); cobalt-60 source, 18/27 (67%) to 21/25 (84%)	NR	Histological features of tumours induced by untreated and irradiated virus were similar.
Hamster (NB, 1 d; adult, 2.5–3 mo) (NR) 18 mo Deichman et al. (1978)	SV40, $10^{6.5}$ – $10^{8.25}$ TCID ₅₀ /mL, WT SV40 and ts mutants, subcutaneous, 0.2 mL/ NB and 1 mL/adult $n = 73$ NB and $n = 64$ adult	Tumour incidence – WT SV40: NB (13/16, 81%), adult (0/10, 0%). tsA, tsB, tsBC mutants: NB (42%), adult (9%). Latency in NB: 103–314 d (WT SV40), 120–495 d (ts SV40)	NR	Relatively fewer tumours induced in NB animals by SV40 ts mutants A209 and A239 = 8/30 (26%).
Hamster, strain LSH (NB, < 1 d) (NR) 1 yr Lewis & Martin (1979)	WT SV40, strain 776, and derivative sT gene dl mutants, dl883, dl885, dl886, and dl890. Subcutaneous, $10^{6.0}$ – $10^{7.9}$ PFU (depending on virus); groups ranged from $n = 9$ to $n = 20$.	WT SV40, 90% tumours; dl mutants, 21% to 92%. Tumours were fibrosarcomas. WT SV40 tumours appeared more quickly.	Time to tumour appearance, WT vs dl886, $P < 0.01$.	sT not essential for SV40 oncogenicity. Tumours induced by both WT and mutant viruses contained LT.

Table 3.1 (continued)

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Controls Animals/group at start	For each target organ: tumours (%); time to tumours	Significance ^b	Comments
Hamster, strain LHC (NB, < 1 d) (NR) 1.5 yr Topp et al. (1981)	WT SV40, strain 776 ($n = 1$ group), and sT gene dl mutants ($n = 9$ groups). Subcutaneous, $\sim 10^7$ PFU in 0.2 mL. WT SV40 DNA, subcutaneous, 1 μ g in 0.1 mL. Groups ranged from $n = 4$ to $n = 14$.	WT SV40 DNA, 8/9 (89%) tumours; average latency, 6.5 mo. WT SV40, 14/14 (100%) tumours; average latency, 7.5 mo. dl mutant viruses, from 4/4 (100%) to 0/4 (0%) tumours; average latencies, 10–14 mo. Tumours were fibrosarcomas. WT SV40-induced tumours appeared more quickly.	NR	
Hamster (NB, < 2 d) (NR) 80 wk Matthews et al. (1987)	WT SV40 (strain WT 830), subcutaneous. sT dl mutants, dl884, dl883, dl890, subcutaneous, 2×10^8 PFU WT SV40, ($n = 130$); dl mutants, $n = 106$, ($n = 71$, and $n = 65$, respectively).	WT: subcutaneous fibrosarcomas (69%), abdominal lymphomas (0.8%). Mean latency: fibrosarcomas, 34 wk; lymphomas, 68 wk. dl mutants: fibrosarcomas (49%), lymphomas (15%) [dl883, 31%; dl884, 12%; dl890, 17%]. Mean latency: fibrosarcomas, 59 wk; lymphomas, 34 wk	NR	Lymphomas induced by dl mutants appeared more quickly than those induced by WT virus. Lung metastases occurred in ~5% of animals with fibrosarcomas and in almost all with lymphomas. Suggested that, in the absence of sT, the mutant virus preferentially transforms proliferating lymphoid cells.

^a Hamsters are outbred unless otherwise indicated.

^b Statistical significance is listed if reported in the study.

d, day; dl, deletion; F, female; GMK/AGMK, African green monkey kidney; LT, large T-antigen; M, male; mo, month; NB, newborn; NR, not reported; PFU, plaque-forming units; RMKC, rhesus monkey kidney cells; sT, small t-antigen; SV40, simian virus 40; TCID₅₀, 50% tissue culture infective dose; ts, temperature-sensitive; vs, versus; wk, week; WT, wild-type; yr, year

effect of irradiation (cobalt-60 source or ultraviolet radiation) on the tumorigenic potential of SV40. Group sizes ranged from $n = 13$ to $n = 39$; animals were observed for 240 days. Irradiation reduced viral infectivity by 2–3 logs before inoculation but did not inhibit tumorigenicity. Tumour frequency not only was not reduced but even appeared to be increased. Before ultraviolet irradiation, the virus induced tumours in 62% of animals, and after irradiation in 88%. Similarly, before cobalt-60 irradiation, the virus produced tumours in 67% of hamsters, and after irradiation in 84%.

Numerous studies have examined the effect of SV40 genetic variation and mutations on tumorigenic potential *in vivo*. [Deichman et al. \(1978\)](#) compared wild-type (WT) SV40 and temperature-sensitive (ts) mutants (tsA, tsB, and tsBC) in NB hamsters by subcutaneous inoculation of 0.2 mL of $10^{6.5}$ – $10^{8.25}$ TCID₅₀/mL (total $n = 73$). Animals were observed for 18 months. WT virus induced tumours in 13/16 (81%) of hamsters, whereas the ts mutants induced tumours in an average of 42% of animals. The latency period of the tumours induced by the ts mutants was extended compared with that of tumours induced by WT SV40 (120–495 days versus 103–314 days). Two mutants in late genes, tsB201 and tsBC210, induced tumours at a high frequency (15/26, 58%), whereas two early mutants, tsA209 and tsA239, induced relatively fewer tumours (8/30, 26%). Adult hamsters (age, 2.5–3 months) were inoculated subcutaneously with $10^{6.5}$ – $10^{8.25}$ TCID₅₀ of virus ($n = 64$). None of the adult animals exposed to WT SV40 developed tumours, whereas 9% (5/54) of those injected with mutant viruses did (tsA30, 2/12; tsA239, 2/8; tsBC210, 1/14).

WT SV40 strain 776 and derivative small t-antigen (sT) gene deletion mutants dl883, dl885, dl886, and dl890 were tested in strain LSH NB hamsters ([Lewis & Martin, 1979](#)). Subcutaneous inoculations of $10^{6.0}$ – $10^{7.9}$ PFU of virus were given, with groups ranging in size from $n = 9$ to $n = 20$. WT SV40 yielded tumours in 90% of animals,

whereas tumour frequencies for the deletion mutants ranged from 21% to 92%. Tumours were fibrosarcomas. WT SV40-induced tumours appeared more quickly, with a significant difference in the time to tumour appearance between WT SV40 and dl886 ($P < 0.01$). Tumours induced by both WT and mutant viruses contained LT. [This study showed that the viral sT was not essential for SV40 tumorigenicity after subcutaneous inoculation.]

After subcutaneous inoculation of NB LHC hamsters with 10^7 PFU of SV40 WT strain 776 virus, 100% of recipients ($n = 14$) developed tumours (fibrosarcomas) with an average latency of 7.5 months. sT gene deletion mutants ($n = 9$, different mutants) of the same strain were also injected subcutaneously, and tumour frequencies ranged from 4/4 (100%) to 0/4 (0%); tumour latencies varied from 10 months to 14 months ([Topp et al., 1981](#)). [This study suggested that SV40 sT might function as a tumour promoter.]

A later study showed that deletion of the SV40 sT gene altered the tissue specificity of tumour induction ([Matthews et al., 1987](#)). WT SV40 strain 830 and mutants dl884, dl883, and dl890 were inoculated subcutaneously into NB hamsters (2×10^8 PFU per injection), and the animals were observed for 80 weeks. Group sizes for different viruses were: WT SV40 ($n = 130$), dl884 ($n = 106$), dl883 ($n = 71$), and dl890 ($n = 65$). WT virus induced subcutaneous fibrosarcomas (in 69% of animals) and a single abdominal lymphoma (0.8%). In contrast, the sT deletion mutants led to abdominal lymphomas in ~15% of recipients (dl883, 31%; dl884, 12%; dl890, 17%), as well as subcutaneous fibrosarcomas (49%). Lung metastases were very common in animals with lymphomas. Tumours were LT-positive. The mean latency of fibrosarcomas induced by WT virus and by deletion mutants was 34 weeks and 59 weeks, respectively. In contrast, lymphomas induced by the mutants appeared on average by 34 weeks and the single case induced by WT virus at 68 weeks. [This study suggests that in the

absence of sT, thought to have cell growth-promoting activities, the mutant virus preferentially transforms proliferating lymphoid cells.]

(b) *Intracerebral inoculation*

See [Table 3.2](#)

Intracerebral inoculation of NB hamsters with SV40 can produce ependymomas, as first reported by [Kirschstein & Gerber \(1962\)](#). In a small study, 4 of 9 NB hamsters inoculated intracerebrally with SV40 strain 777 developed brain tumours (44%), identified as ependymomas, over a period of 3–4 months. Control animals inoculated with medium 199 ($n = 7$) did not develop tumours. In a larger study also using NB animals, SV40 strain 777, and the intracerebral route of inoculation, [Gerber & Kirschstein \(1962\)](#) showed a virus dose dependence for induction of ependymomas. Among a total of 49 inoculated animals, 100% developed brain tumours after inoculation with 10^8 TCID₅₀ of virus, 44% with 10^7 TCID₅₀, 38% with $10^{6.5}$ TCID₅₀, and 0% with 10^6 or 10^5 TCID₅₀. The tumours appeared after about 3 months. Controls ($n = 10$) inoculated with medium 199 were tumour-free at 7 months. SV40 strain PML-1, inoculated into the temporal bone of the skull of NB hamsters ($n = 27$) at 2×10^5 PFU, resulted in a tumour incidence of 85% ([Davis et al., 1979](#)). Four choroid plexus papillomas and 21 sarcomas in the region of the temporal bone developed with a latency period of about 4 months. Two tumours were cultured and were found to contain SV40 LT.

[Wilkins & Odom \(1965\)](#) confirmed SV40 induction of ependymomas in NB hamsters while surveying other species for susceptibility to SV40 brain tumours. Using the strain of SV40 from Dr A. Rabson, 10^7 TCID₅₀ inoculated intracerebrally into NB hamsters ($n = 30$) induced ependymomas in 23%, fibrosarcomas of the scalp in 27%, and cerebral cysts in 10% of hamsters. The brain tumours appeared 3.3–8 months after injection and fibrosarcomas after 5.5–12 months.

(c) *Intravenous or intracardiac inoculation*

See [Table 3.3](#)

Intravenous or intracardiac inoculation of SV40 in weanling/young adult hamsters induces a spectrum of tumours, especially lymphomas, mesotheliomas, and osteosarcomas. [Diamandopoulos \(1972, 1973\)](#) inoculated SV40 strain VA45–54 ($10^{8.5}$ TCID₅₀) intravenously using the femoral vein into weanling (age, 21–22 days) male hamsters ($n = 100$), as well as SV40 mixed with normal rabbit serum ($n = 50$). Controls included animals inoculated with SV40 mixed with anti-SV40 serum ($n = 50$) or medium ($n = 50$), or uninoculated ($n = 50$). Hamsters exposed to SV40 developed tumours (84%), as did those injected with SV40 mixed with normal rabbit serum (94%). All other groups did not develop any tumours during the 6-month observation period. The peak tumour latency was 5 months. Tumour types observed included reticulum cell sarcomas (72%), lymphosarcomas (4%), lymphocytic leukaemias (1%), and osteosarcomas (55%). It was noted that up to one third of tumour-positive animals had more than one type of malignancy. All tumours tested expressed SV40 LT. [This study showed that a different spectrum of tumour types arises after intravascular exposure to SV40 and provided the first evidence that SV40 can induce lymphomas and leukaemias.]

[Diamandopoulos & McLane \(1975\)](#) showed the effect of both host age and virus dose on tumour induction after intravenous inoculation of virus ($10^{0.5}$ – $10^{8.5}$ TCID₅₀, $n = 8$ –10 animals per group). The tumour latencies and the morphological types of tumours induced were the same as those described in [Diamandopoulos \(1972, 1973\)](#). Controls received medium or cell lysate, or were uninoculated ($n = 70$). SV40 strain VA45–54 ($10^{8.5}$ TCID₅₀) induced tumours in most male hamsters exposed at age 3 weeks (90%). Tumour frequency decreased with age at time of exposure, as follows: 1 month, 78%; 2 months, 50%; 3 months, 20%;

Table 3.2 Carcinogenicity studies of SV40 in hamsters^a by intracerebral inoculation

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Controls Animals/group at start	For each target organ: tumours (%); time to tumours	Comments
Hamster (NB, < 1 d) (NR) Up to 156 d Kirschstein & Gerber (1962)	SV40 strain 777, 0.02 mL, intracerebral <i>n</i> = 9 Controls: 0.02 mL medium 199 <i>n</i> = 7	Brain tumours (44%), ependymomas; 3–4 mo Controls (0%)	First report of induction of true glioma by a virus.
Hamster (NB, < 1 d) (NR) Up to 220 d Gerber & Kirschstein (1962)	SV40 strain 777, 0.02 mL, intracerebral, 10 ⁵ –10 ⁸ TCID ₅₀ <i>n</i> = 49 Controls: 0.02 mL medium 199 from uninfected GMK <i>n</i> = 10	Brain tumours, ependymomas, dose- dependent: 10 ⁸ TCID ₅₀ (100%), 10 ⁷ TCID ₅₀ (44%), 10 ^{6.5} TCID ₅₀ (38%); 3–3.5 mo 10 ⁶ and 10 ⁵ TCID ₅₀ (0%). Controls (0%) after 7 mo	Virus was concentrated by centrifugation.
Hamster (1 d) (NR) Up to 1 yr Wilkins & Odom (1965)	SV40 from Dr A. Rabson, titre 10 ⁷ TCID ₅₀ /0.1 mL, intracerebral inoculation of ~0.01 mL <i>n</i> = 30 Controls NR	Brain tumours: ependymomas (23%); fibrosarcomas of the scalp (27%); cerebral cysts or hydrocephalus (10%) Latency: 3.3–8 mo (brain tumours), 5.5–12 mo (fibrosarcomas)	Poor survival (71%) reduced the numbers of experimental animals for observation.
Hamster (NB) (NR) 5 mo Davis et al. (1979)	SV40 strain PML-1, 2 × 10 ⁵ PFU/0.02 mL, intracerebral (temporal) <i>n</i> = 27	Tumour incidence (85%); ~4 mo 21 sarcomas in the region of the temporal bone, 4 choroid plexus papillomas	Two tumours explanted, contained SV40 LT. Two animals had both tumour types.

^a Hamsters are outbred unless otherwise indicated.

d, day; dl, deletion; GMK/AGMK, African green monkey kidney; LT, large T-antigen; mo, month; NB, newborn; NR, not reported; PFU, plaque-forming units; SV40, simian virus 40; TCID₅₀, 50% tissue culture infective dose; yr, year

Table 3.3 Carcinogenicity studies of SV40 in hamsters^a by intravenous or intracardiac inoculation

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Controls Animals/group at start	For each target organ: tumours (%); time to tumours	Significance ^b	Comments
Hamster (weanling, 21–22 d) (M) 6 mo Diamandopoulos (1972, 1973)	SV40 strain VA45-54, 10 ^{8.5} TCID ₅₀ /mL, intravenous (femoral vein) <i>n</i> = 100 SV40 + NRS <i>n</i> = 50 Controls, SV40 + anti-SV40 serum, or culture medium, or uninoculated <i>n</i> = 50 each	Tumour incidence: SV40 (84%), SV40 + NRS (94%), all other groups (0%) Peak tumour latency: 5 mo Tumour types: reticulum cell sarcomas (72%), lymphosarcomas (4%), lymphocytic leukaemias (1%), osteosarcomas (55%)		Up to one third of tumour- positive animals had more than one type of neoplasm. First report that SV40 could induce lymphomas and leukaemias. All tumours tested contained SV40 LT.
Hamster (3 wk to 12 mo) (M) 12 mo Diamandopoulos & McLane (1975)	SV40 strain VA45-54, 10 ^{8.5} –10 ^{0.5} TCID ₅₀ /mL, intravenous (femoral vein) <i>n</i> = 8–10 per group Controls: cell lysate, culture medium, uninoculated <i>n</i> = 70	Tumour incidence decreased with age at time of exposure, dropping sharply for animals older than 2 mo: 3 wk, 90%; 1 mo, 78%; 2 mo, 50%; 3 mo, 20%; 6 mo, 12%; 9 and 12 mo, 0%; average latency increased from 7 mo to 11 mo. Only animals exposed to 10 ^{8.5} TCID ₅₀ of SV40 at 3 wk developed tumours; 10 ^{7.5} to 10 ^{0.5} TCID ₅₀ induced no tumours.		Morphological types of tumour were the same as those described in Diamandopoulos (1972, 1973) .
Hamster, 1 outbred, 3 inbred strains (weanling, 3 wk) (M) 12 mo Diamandopoulos (1978)	SV40 strain VA45-54, 10 ^{7.5} TCID ₅₀ /mL, intravenous (femoral vein) <i>n</i> = 12 per group Controls: uninoculated <i>n</i> = 12	Tumour incidence: outbred (9%), inbred strains (58–90%), controls (0%); latency = 8–11 mo		Inbred strains: LSH/SsLak, LHC/Lak, and MHA/SsLak. Morphological types of tumour were the same as those described in (Diamandopoulos, 1972, 1973) . Permanent cell lines were established from two lymphocytic leukaemias.
Hamster (weanling, 21 d) (NR) 9 mo Cicala et al. (1992)	WT SV40 strain 830, <i>n</i> = 21; and strain 776, <i>n</i> = 5, 10 ^{8.5} PFU, intracardiac SV40 sT gene dl mutants dl2006, <i>n</i> = 22; dl883, <i>n</i> = 12 Controls: culture medium, <i>n</i> = 20	WT: mesotheliomas (40–62%), abdominal lymphomas (33–40%), and osteosarcomas (10–40%); 17–22 wk sT dl mutants: abdominal lymphomas (100%); 26 wk Controls (0%)		Both B-cell lymphomas and true histiocytic lymphomas were induced. Suggested that sT is required for SV40 transformation of non-dividing cells.

Table 3.3 (continued)

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Controls Animals/group at start	For each target organ: tumours (%); time to tumours	Significance ^b	Comments
Hamster (weanling, 21 d) (M, F) 9 mo Cicala et al. (1993)	WT SV40 strain 830 and strain 776, 10 ^{8.5} PFU in 0.2 mL sT dl mutants dl883 and dl2006 Intracardiac (WT 776, <i>n</i> = 5; WT 830, <i>n</i> = 21; dl883, <i>n</i> = 12; dl2006, <i>n</i> = 22); intrapleural (WT 830, <i>n</i> = 6; WT 776, <i>n</i> = 5); intraperitoneal (WT 776, <i>n</i> = 6) Controls: culture medium <i>n</i> = 20 (intracardiac) <i>n</i> = 4 (intraperitoneal, intrapleural)	Animals injected with WT SV40 (<i>n</i> = 43): all developed tumours (100%). Of these, 70% (100%, 58% and 67% after intrapleural, intracardiac, and intraperitoneal injection, respectively) had mesotheliomas; others had lymphomas (23%) and osteosarcomas (9%). Latency: 3–6 mo. Animals injected with sT gene dl mutants (intracardiac) (<i>n</i> = 34): all developed abdominal lymphomas (100%); 1 (3%) had mesothelioma. Latency: ~6 mo Controls (0%)		First report of virus-induced mesotheliomas in mammals. SV40 LT expressed in mesothelioma tumour cell lines.
Hamster (weanling, 21 d) (M, F) 8 mo McNees et al. (2009)	WT SV40 strains [VA45-54(2E), VA45- 54(1E), SVCPC(1E)] with different regulatory region; SV40 recombinants [776-CPC(2E), 776-VA(2E)]. Intravenous, 1 × 10 ⁷ PFU in 0.2 mL <i>n</i> = 71 (total) [<i>n</i> = 70 (total)] <i>n</i> = 28 (controls: cell lysate)	Tumour incidences by different strains ranged from 82% [VA45-54(2E)] to 15% [776-CPC(2E)] (overall, 54%); median tumour latencies, 24–34 wk tumour types: mesothelioma, lymphoma, sarcoma, osteosarcoma. Controls (0%)	Tumour incidences differed for SV40 recombinants: 776-VA(2E) (10/16, 62%) <i>vs</i> 776-CPC(2E) (2/13, 15%), <i>P</i> = 0.01	Lymphoma cell lines expressed SV40 LT. Most animals were necropsied.

^a Hamsters are outbred unless otherwise indicated.

^b Statistical significance is listed if reported in the study.

1E, simple regulatory region, SV40; 2E, complex regulatory region, SV40; d, day; dl, deletion; F, female; LT, large T-antigen; M, male; mo, month; NB, newborn; NR, not reported; NRS, normal rabbit serum; PFU, plaque-forming units; sT, small t-antigen; SV40, simian virus 40; TCID₅₀, 50% tissue culture infective dose; *vs*, *versus*; wk, week; WT, wild-type

6 months, 12%; and 9 or 12 months, 0%. Average tumour latency increased from 7 months to 11 months as older animals were inoculated. Only those exposed to $10^{8.5}$ TCID₅₀ of SV40 developed tumours; lower doses of virus resulted in no tumours during the 12-month observation period. [Diamandopoulos \(1978\)](#) also found that certain inbred strains of hamsters were more susceptible than outbred hamsters to tumour induction by SV40. Tumours were the same as those described in [Diamandopoulos \(1972, 1973\)](#). An inoculum of $10^{7.5}$ TCID₅₀ intravenously (in the femoral vein) was used in weanling (age, 3 weeks) male hamsters ($n = 12$ per group). The tumour incidence was 9% in the outbred animals and 58–90% in three inbred strains, based on a 12-month observation period. Uninoculated controls did not develop tumours.

[Cicala et al. \(1992\)](#) used the intracardiac route to inoculate weanling (age, 21 days) hamsters with WT SV40 strain 830 ($n = 21$), WT SV40 strain 776 ($n = 5$), or sT deletion mutants dl2006 ($n = 22$) and dl883 ($n = 12$). The WT viruses (an inoculum of $10^{8.5}$ PFU) induced mesotheliomas (40–62%), abdominal lymphomas (33–40%), and osteosarcomas (10–40%) with tumour latencies of 17–22 weeks. In contrast, the sT deletion mutants induced abdominal lymphomas (B-cell lymphomas or true histiocytic lymphomas) in 100% of recipients, with a latency of 26 weeks. [This observation suggests a requirement for sT for SV40 transformation of non-dividing cells.] In a follow-up report, [Cicala et al. \(1993\)](#) expanded the intracardiac group and added groups that were intrapleurally or intraperitoneally inoculated with the WT SV40 830 or WT SV40 776 strains. Of those inoculated by the intrapleural route, 11 (100%) of 11 developed mesotheliomas, compared with 15 (58%) of 26 by the intracardiac route and 4 (67%) of 6 by the intraperitoneal route. All control animals injected with medium by the intracardiac ($n = 20$), intrapleural ($n = 4$), or intraperitoneal ($n = 4$) route were tumour-free after the 9-month observation period. SV40

LT was expressed in mesothelioma tumour cell lines. [This was the first description of virus-induced mesotheliomas in mammals.]

[McNeese et al. \(2009\)](#) used the intravenous route to expose weanling hamsters to WT SV40 strains VA45–54(2E) ($n = 17$), VA45–54(1E) ($n = 15$), and SVCPC(1E) ($n = 9$), and two recombinant strains, 776-CPC(2E) ($n = 13$) and 776-VA(2E) ($n = 16$). Tumour frequencies ranged from 82% for VA45–54(2E) to 15% for 776-CPC(2E), with an overall incidence of 54%. Median tumour latencies were 24–34 weeks. There was a significant difference ($P = 0.01$) between tumour frequencies after exposure to two different SV40 recombinant strains: 776-VA(2E) (62%) and 776-CPC(2E) (15%). Control animals inoculated with cell lysate ($n = 28$) did not develop tumours during the 8-month observation period. Tumour types identified histologically included mesotheliomas, lymphomas, sarcomas, and osteosarcomas. Lymphoma-derived cell lines were shown to express SV40 LT. [This study suggests that different SV40 strains can differ in tumorigenic potential *in vivo* after intravenous inoculation.]

(d) Intraperitoneal or intrapleural inoculation

See [Table 3.4](#)

Introduction of SV40 into pregnant hamsters resulted in the appearance of SV40-positive tumours in their offspring ([Rachlin et al., 1988](#)). SV40 strain 776 was inoculated intraperitoneally at doses ranging from 10^7 PFU to 10^3 PFU into pregnant hamsters on day 4–12 of gestation. Tumours developed in the offspring of mothers inoculated with 10^7 PFU of virus on day 8 (13/24, 54%) and day 12 (13/30, 43%) of gestation. Litters from mothers exposed to other doses of virus remained tumour-free, as did all the inoculated mother hamsters. The subcutaneous tumours were classified as sarcomas, and all expressed SV40 LT. Of the mothers inoculated on day 4 of gestation, 63% (12/19) of their progeny died within the first 4 weeks after birth. [This study showed that vertical transmission of SV40 can

Table 3.4 Carcinogenicity studies of SV40 in hamsters^a by intrapleural or intraperitoneal inoculation

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Controls Animals/group at start	For each target organ: tumours (%); time to tumours	Significance ^b	Comments
Hamster (offspring) Up to 1 yr Rachlin et al. (1988)	SV40 strain 776, 10 ³ –10 ⁷ PFU, intraperitoneal (dams), d 4–12 of gestation; <i>n</i> = 16–30/group Controls: medium only <i>n</i> = 23–31/group	Subcutaneous sarcomas in offspring of dams inoculated with 10 ⁷ PFU on d 8 (13/24, 54%) and d 12 (13/30, 43%). Lower doses, no tumours.		First report that vertical transmission of SV40 can lead to tumour formation in progeny. No tumours in dams. Effect of age of fetus: 63% (12/19) of progeny of mothers inoculated on day 4 of gestation died in first 4 wk after birth. All tumours contained SV40 LT.
Hamster (weanling, 21 d) (M, F) 9 mo Cicala et al. (1993)	See Table 3.3			
Hamster (weanling, 21 d) (M, F) 8 mo and 12 mo Vilchez et al. (2004)	SV40 strains VA45-54(2E), SVCPC(1E), 10 ⁷ PFU in 0.5 mL, intraperitoneal, <i>n</i> = 33 and <i>n</i> = 37 Controls: cell lysate, <i>n</i> = 78	VA45-54(2E), 21%; SVCPC(1E), 49%. Similar types of abdominal tumour in both strains (sarcomas, mesotheliomas, osteosarcomas); mean, 6–8 mo Controls: 0% tumours		Metastases found in lung and lymph nodes. SV40 LT expressed in tumour cells.
Hamster (weanling, 21 d) (M, F) 12 mo Sroller et al. (2008)	Eight SV40 WT strains and eight SV40 recombinants consisting of different regulatory regions and LT with variable T-ag–C domains, 1 × 10 ⁷ PFU in 0.5 mL, intraperitoneal, <i>n</i> = 307 (total) Controls, <i>n</i> = 187	Tumour frequencies by different WT strains: 83% (Baylor 1E), 54% (SVCPC), 31% (776 1E), 26% (777), 23% (VA45-54), 10% (776 2E), 6% (Baylor 2E), 0% (SVPML). Tumour frequencies by different recombinants: 58% (CPC-PML 1E), 37% (CPC-Baylor 1E), 35% (CPC-776 1E), 33% (776-Baylor 2E), 24% (CPC-VA 1E), 13% (776- VA 2E), 7% (776-CPC 2E), 6% (776-PML 2E)	SV40-exposed (84/307, 27%) <i>vs</i> controls (0/187, 0%), <i>P</i> = 0.0001. Tumour induction higher by viruses with simple 1E regulatory regions (38/80, 48%) <i>vs</i> viruses with complex 2E regulatory regions (7/76, 9%), <i>P</i> = 0.0001	Tumour type unspecified. LT variable domain (T-ag–C) showed little influence on SV40 tumorigenicity. Non-tumorigenic/weakly tumorigenic strains and highly tumorigenic strains transformed mouse cells <i>in vitro</i> with similar frequencies; differences in tumorigenicity <i>in vivo</i> must reflect host responses to viral infection. First report of influence of SV40 regulatory region on tumorigenic potential <i>in vivo</i> .

^a Hamsters are outbred unless otherwise indicated.^b Statistical significance is listed if reported in the study.

1E, simple viral regulatory region, single enhancer; 2E, complex, rearranged viral regulatory region; d, day; F, female; LT, large T-antigen; mo, month; M, male; NB, newborn; NR: not reported; PFU, plaque-forming units; T-ag–C, SV40 large LT C-terminal variable domain; TCID₅₀, 50% tissue culture infective dose; ts, temperature-sensitive; *vs*, *versus*; wk, week; WT, wild-type; yr, year

occur, followed by expression of viral oncogenicity in progeny animals.]

Hamsters were intrapleurally or intraperitoneally inoculated with the WT SV40 830 or WT SV40 776 strains by [Cicala et al. \(1993\)](#). This study is described in Section 3.1.1(c) and [Table 3.3](#).

[Vilchez et al. \(2004\)](#) compared the tumorigenicity of two SV40 strains by intraperitoneal inoculation of weanling hamsters with a uniform inoculum of 1×10^7 PFU. Strain VA45–54(2E) contains a complex regulatory region, whereas strain SVCPC(1E) contains a simple regulatory region with a single enhancer. The numbers of animals per virus group were $n = 33$ [VA45–54(2E)] and $n = 37$ [SVCPC(1E)]. Controls ($n = 78$) were inoculated with cell lysate. Tumour frequencies were 21% for VA45–54(2E), 49% for SVCPC(1E), and 0% in controls after observation periods of 8–12 months. The two viruses induced similar types of abdominal tumours (sarcomas, mesotheliomas, and osteosarcomas) with similar mean latencies of 6–8 months. Metastases were observed in lung and lymph nodes.

It has been demonstrated that the viral regulatory region is a genetic determinant of SV40 oncogenic potential, whereas the LT C-terminal variable domain (T-ag–C) exerts negligible influence ([Sroller et al., 2008](#)). These two genomic regions are sites of variation among SV40 isolates. Weanling hamsters were inoculated by the intraperitoneal route using 1×10^7 PFU per injection. Eight different strains of WT SV40 and eight recombinants consisting of different viral regulatory regions linked to LT with variable T-ag–C domains were included. The total numbers of experimental animals were $n = 307$ (SV40-inoculated) and $n = 187$ (controls). Tumour frequencies by different SV40 strains after a 12-month observation period ranged from 83% (Baylor 1E) to 0% (SVPML) [tumour type unspecified]. Tumour incidence was significantly increased ($P = 0.0001$) in SV40-exposed hamsters (84/307, 27%) *versus* controls (0/187, 0%). Tumour incidence was significantly

increased ($P = 0.0001$) by viruses with simple 1E regulatory regions (38/80, 48%) *versus* viruses with complex 2E regulatory regions (7/76, 9%). However, there was no difference in the median time to tumours by 1E and 2E viruses (32.5 weeks *versus* 27 weeks). The LT variable domain showed little influence on SV40 tumorigenicity. The highly tumorigenic and non-tumorigenic/weakly tumorigenic strains of SV40 transformed mouse cells *in vitro* with similar frequencies, suggesting that the differences in tumorigenicity observed *in vivo* reflected host responses to viral infection. [This was the first report of the influence of the SV40 regulatory region on oncogenic potential *in vivo*.]

3.1.2 Mouse

See [Table 3.5](#)

In the original study by [Eddy et al. \(1962\)](#) that demonstrated the tumorigenicity of SV40 in NB hamsters, NB NIH white mice were also inoculated subcutaneously with SV40 ($n = 10$) or rhesus monkey kidney cell oncogenic extracts ($n = 17$). No tumours were observed. [The Working Group noted the absence of a control group.]

The effect of co-infection with murine malaria parasites, *Plasmodium berghei yoelii*, on SV40 tumorigenicity in NB mice was examined. NB CFW mice were inoculated intravenously via the anterior facial vein with 10^4 TCID₅₀ of SV40 strain VA45–54 ([Hargis & Malkiel, 1979](#)). After 3 months, some virus-infected animals ($n = 11$) were given 5×10^6 malaria parasite-infected mouse red blood cells, whereas others were not co-infected ($n = 10$). Control animals were injected with parasites only ($n = 12$). Mice exposed to SV40 developed sarcomas of the liver and/or spleen, including 70% of those infected with SV40 only and 100% of those co-infected with virus and parasites. Tumours contained SV40 LT. Tumour latency was shorter in the co-infected animals compared with virus alone (9 months *versus* 11 months). Control animals

did not develop tumours. Adult CFW mice ($n = 31$) were treated in parallel with the virus, with or without parasites, but no tumours were observed.

3.1.3 Other species

See [Table 3.6](#)

Subcutaneous inoculation of SV40 into NB *Mastomys natalensis*, an African rodent, induced brain tumours ([Rabson et al., 1962](#)). SV40 strain 777 at 10^7 TCID₅₀ produced brain tumours (papillary ependymomas and maybe some choroid plexus papillomas) after 3.7–7.5 months in 8 of 10 animals (80%). Controls ($n = 29$) inoculated with medium or uninfected African green monkey kidney cell fluid did not develop tumours. [This was the first report of SV40 oncogenicity in species other than hamsters and of subcutaneous inoculation of SV40 producing brain tumours in animals in the absence of other tumours.]

[Wilkins & Odom \(1965\)](#) gave intracerebral inoculations of SV40 strain from Dr A. Rabson at 10^7 TCID₅₀ to Fischer rats ($n = 27$; age, 26 days and 7 weeks), rabbits ($n = 8$; age, 3 days), kittens ($n = 5$; age, 1 day), and puppies ($n = 22$; age, 1 day). Some rats and rabbits were exposed to total body irradiation (175 röntgen [45.15 mC/kg]) before virus inoculation. A group of puppies was uninoculated ($n = 9$). None of the animals developed brain tumours. [The Working Group noted that poor survival reduced the numbers of experimental animals for observation, so this study cannot be considered informative.]

Adult female dogs were injected with 10^9 TCID₅₀ of SV40 into the bladder submucosa, either alone ($n = 1$) or mixed with Freund's complete adjuvant ($n = 4$) ([Shah & Pond, 1972](#)). No neoplastic changes were observed in the bladder after observation periods of 31–57 weeks.

3.2 Infection with SV40 DNA

Hamster

See [Table 3.7](#)

Induction of tumour formation after infection with SV40 DNA was demonstrated by [Sol & Noordaa \(1977\)](#). They introduced DNA (1–2 µg) from SV40 strain VA45–54 with an infectivity titre of 1.5×10^6 PFU/µg DNA into NB hamsters ($n = 33$) by subcutaneous inoculation. Tumours (sarcomas) [type not further specified] were produced in 33% of the animals. Linearization of the viral DNA by the restriction enzyme *EcoRI* before inoculation ($n = 8$) resulted in tumours in 50% of hamsters. Control DNase-treated SV40 DNA was non-tumorigenic ($n = 32$ animals).

When NB LHC hamsters ($n = 9$) were injected subcutaneously with WT SV40 DNA (1 µg), 8 of the 9 animals developed tumours, with an average latency of 6.5 months ([Topp et al., 1981](#)).

DNAs from WT SV40 strain 777 and from deletion mutant dl2005 lacking the *sT* gene were tested in parallel ([Bouchard et al., 1984](#)). Subcutaneous injection of 2 µg of WT DNA that was linearized by the enzyme *SalI* [with or without carrier DNA (10 µg)] into NB hamsters ($n = 7$) produced fibrosarcomas at the site of inoculation in 57% of animals, with latency periods of 6–7 months. The mutant viral DNA ($n = 22$) produced tumours in 23% of hamsters, with latencies of 11–12 months.

3.3 Transgenic mouse models

3.3.1 SV40 DNA with natural viral promoter

See [Table 3.8](#) and [Table 3.9](#)

The development of transgenic mouse technology added a new dimension to studies of viral oncogenes as it became possible to analyse tumour virus proteins in host animals. SV40 LT under the control of its natural regulatory region was the first viral oncoprotein expressed in transgenic mice ([Brinster et al., 1984](#)). Since that

Table 3.5 Carcinogenicity studies of SV40 in mice

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Controls Animals/group at start	For each target organ: Tumours (%); time to tumours	Comments
Mouse, NIH white (NB) (NR) Up to 344 d Eddy et al. (1962)	SV40 strain 776, 0.1 mL, subcutaneous <i>n</i> = 10 RMKC oncogenic extracts (Eddy et al., 1961), 0.1 mL, subcutaneous <i>n</i> = 17	0%	No controls.
Mouse, strain CFW (NB and adult) (NR) Up to 12 mo Hargis & Malkiel (1979)	SV40 strain VA45-54, 10 ⁴ TCID ₅₀ /0.1 mL, intravenous, anterior facial vein NB, <i>n</i> = 10 NB injected with 5 × 10 ⁶ malaria parasite-infected mouse red blood cells (on d 90), <i>n</i> = 11 Controls: parasites only, <i>n</i> = 12 Adult, <i>n</i> = 31, some plus parasites	NB mice Sarcomas of liver and/or spleen: SV40 + parasites (100%)*; 9 mo SV40 only (70%); 11 mo Controls: parasites only (0%) Adult (0%)	Murine malaria parasites: <i>Plasmodium berghei yoelii</i> . *Four animals had lung metastases. Tumours expressed SV40 LT.

d, day; LT, large T-antigen; mo, month; NB, newborn; NR, not reported, RMKC, rhesus monkey kidney cells; SV40, simian virus 40; TCID₅₀, 50% tissue culture infective dose

beginning, SV40 LT expression has been directed to many different tissues and cell types by the use of tissue-specific promoters in transgenic mice. Those studies have established that SV40 LT is highly oncogenic. The transgenic models have contributed to fundamental principles related to SV40 LT transformation and tumour development ([Lednický & Butel, 1999](#); [Butel, 2000a, 2012](#); [Ahuja *et al.*, 2005](#); [Pipas, 2009](#); [Sáenz Robles & Pipas, 2009](#); [Gjoerup & Chang, 2010](#)). Only studies involving the use of SV40 with its natural promoter are reviewed here.

[Brinster *et al.* \(1984\)](#) created the first SV40 transgenic mice using an SV40 plasmid (pSV3) with the natural viral promoter. Of the transgenic founder pups ($n = 25$), most developed brain tumours (choroid plexus papilloma or carcinoma). Some animals also displayed thymus and kidney lesions [not further specified]. The transgenic pups died within 1–5 months. [Palmiter *et al.* \(1985\)](#) followed up with a study of transgenic mice carrying not only WT SV40 but also SV40 plasmid constructs that expressed LT only (pSV11) or sT and truncated LT (pSV8). All viral constructs contained the natural viral promoter. The number of transgenic founder pups per group ranged from $n = 4$ to $n = 9$. With WT SV40, 7 (87%) of 8 transgenic pups developed choroid plexus papillomas, as did 5 (71%) of 7 pSV11 transgenic pups. Tumours expressed SV40 LT. In contrast, none of 9 pSV8 transgenic pups developed tumours, showing the requirement for LT in SV40 tumorigenicity. Thymic hyperplasia was present in some of the mice with brain tumours. [Chen & Van Dyke \(1991\)](#) extended the studies with pSV11 (the construct that expressed LT only). Transgenic founders ($n = 13$) were created, and 9 (69%) developed choroid plexus tumours, with latency periods of 65–210 days. Some animals also exhibited thymic hyperplasia and polycystic kidneys. An SV11 transgenic line was derived; these transgenic mice developed brain tumours at approximately day 100. In SLT transgenic mice, the LT gene of

lymphotropic papovavirus, under the control of the SV40 promoter, could substitute for SV40 LT to induce brain tumours (4/9).

The nuclear-transport-defective mutant of SV40, pSV40(cT), was examined in transgenic mice by [Pinkert *et al.* \(1987\)](#). Seven (88%) of 8 transgenic founder mice died with choroid plexus papillomas and/or with kidney and thymus lesions (including tumours). Mean time to death was 52 days. Non-transgenic pups ($n = 36$) did not develop tumours. A transgenic line, 269, was established; those animals ($n = 68$) developed brain tumours within a mean time of 81 days. SV40 LT was retained in the cytoplasm of the brain tumours.

3.3.2 LT with hybrid SV40 and JCV sequences

See [Table 3.9](#)

Studies using the promoter of SV40 or JCV Mad-1 strain, driving expression of either SV40 LT or JCV LT, have revealed important information about the tropism of these viruses. [Feigenbaum *et al.* \(1992\)](#) reported that 3 of 5 founders carrying the SV40 early sequence under the control of the JCV Mad-1 promoter developed tumours, including neuroblastoma (60%), myenteric plexus tumours (60%), and choroid plexus carcinoma (40%), within 0.5–4 months, whereas 100% of 7 founders carrying the JCV early sequence under the control of the SV40 promoter developed tumours, including choroid plexus papilloma (86%), within 1–3 months. On a C57/CBA background, [Ressetar *et al.* \(1993\)](#) reported two founders with the JCV Mad-1 promoter driving SV40 LT gene expression that developed abdominal B-cell lymphoma, adrenal neuroblastoma, osteosarcoma, and astrocytoma within 1–2.5 months. In parallel, one founder with the SV40 776 promoter driving JCV LT gene expression developed choroid plexus papilloma and thymoma by 1.5 months. [Similar to the studies of JCV infection in hamsters and JCV LT transgenic mice, the animals frequently

Table 3.6 Carcinogenicity studies of SV40 in other species

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Controls Animals/group at start	For each target organ: Tumours (%); time to tumours	Comments
<i>Mastomys natalensis</i> (African rodent) (NB, < 1 d) (NR) Up to 7.5 mo Rabson et al. (1962)	SV40 strain 777, 0.05 mL (10^7 TCID ₅₀), subcutaneous <i>n</i> = 10 Controls: uninfected GMK cell fluid or medium <i>n</i> = 29	Brain tumours (80%): papillary ependymomas, maybe some choroid plexus tumours; 3.7–7.5 mo Controls (0%)	First report that SV40 oncogenicity not limited to hamsters. Finding that subcutaneous inoculation of virus can produce brain tumours without any subcutaneous or visceral tumours.
Rat, Fischer (26 d, 7 wk), rabbit (3 d), kitten (1 d), puppy (1 d) (sex NR), rat (26 mo), kitten (6 mo), rabbit (10 mo), puppy (1 yr) Wilkins & Odom (1965)	SV40 from Dr A. Rabson, titre 10^7 TCID ₅₀ /0.1 mL, intracerebral Some rats and rabbits exposed to total body irradiation (41.15 mC/kg) before inoculation Inoculation 0.1–0.2 mL rats (<i>n</i> = 27), rabbits (<i>n</i> = 8), kittens (<i>n</i> = 5), puppies (<i>n</i> = 22), Uninoculated controls: puppies only (<i>n</i> = 9)	Rats, rabbits, kittens, puppies: no lesions, except 1 rat had an incidental fibrosarcoma in the brain found at 18 mo.	Poor survival reduced the numbers of experimental animals for observation
Dog (adult) (F) 31–57 wk Shah & Pond (1972)	SV40, 10^9 TCID ₅₀ in 5 mL, injected into submucosa of bladder, <i>n</i> = 1 SV40, $10^{9.0}$ TCID ₅₀ in 1 mL mixed with 1 mL Freund's complete adjuvant, submucosa of bladder, <i>n</i> = 4	No neoplastic changes in bladder; no virus detected in blood, urine, or kidney biopsies; no antibodies to LT.	

^a Hamsters are outbred unless otherwise indicated.

d, day; F, female; GMK/AGMK, African green monkey kidney; LT, large T-antigen; mo, month; NB, newborn; NR, not reported; SV40, simian virus 40; TCID₅₀, 50% tissue culture infective dose; ts, temperature-sensitive; wk, week; yr, year

Table 3.7 Carcinogenicity studies of SV40 DNA in hamsters^a

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Controls Animals/group at start	For each target organ: Tumours (%); time to tumours	Significance ^b	Comments
Hamster (NB) (NR) 6 mo Sol & Noordaa (1977)	SV40 DNA, strain VA45-54, 1.5 × 10 ⁶ PFU/μg SV40 DNA, 1–2 μg in 70 μL/animal, subcutaneous <i>n</i> = 33 EcoRI-linearized SV40 DNA <i>n</i> = 8 DNase-treated SV40 DNA <i>n</i> = 32	SV40 DNA (33%), sarcoma Linearized SV40 DNA (50%), sarcoma DNase-treated SV40 DNA (0%)	NR	One tumour was cultured, and those cells expressed SV40 LT. Tumour type not further specified. Addition of calf thymus DNA or poly- L-ornithine to injection mixture had no effect on oncogenic potential.
Hamster, strain LHC (NB, < 1 d) (NR) 1.5 yr Topp <i>et al.</i> (1981)	WT SV40 DNA, 1 μg in 0.1 mL, ~10 ⁷ PFU in 0.2 mL, subcutaneous <i>n</i> = 9	Fibrosarcomas, 8/9 (89%); 6.5 mo	NR	
Hamster (NB, 1 d) (NR) Up to 12 mo Bouchard <i>et al.</i> (1984)	SV40 DNA, strain 777 2 μg plasmid DNA ± 10 μg carrier DNA in 10–100 μL PBS (linearized by <i>SalI</i>), subcutaneous <i>n</i> = 7 dl mutant dl2005 DNA lacking sT gene <i>n</i> = 22	Tumours at site of inoculation, fibrosarcomas WT SV40 DNA (57%); 6–7 mo sT dl mutant dl2005 DNA (23%); 11–12 mo	NR	

^a Hamsters are outbred unless otherwise indicated.

d, day; dl, deletion; LT, large T-antigen; mo, month; NB, newborn; NR, not reported; PBS, phosphate-buffered saline; PFU, plaque-forming units; sT, small t-antigen; SV40, simian virus 40; WT, wild-type; yr, year

Table 3.8 Carcinogenicity studies in transgenic mice involving SV40 with natural viral promoter^a

Species, strain (sex) Duration of observation Reference	Virus strain, dose, route Controls Animals/group at start	For each target organ: Tumours (%); time to tumours	Significance ^b	Comments
Mouse (C57BL6; C57 × SJL) (NR) Up to 5 mo Brinster <i>et al.</i> (1984)	SV40 plasmid pSV3, transgenic founder pups, <i>n</i> = 25	Most transgenic animals developed brain tumours (choroid plexus papilloma or carcinoma) and occasional thymus or kidney lesions. Latency (death), 1–5 mo	NR	First SV40 transgenic mice. Thymus and kidney lesions not further specified.
Mouse (C57 × SJL) (NR) Up to 12 mo Palmiter <i>et al.</i> (1985)	WT SV40; SV40 plasmid pSV11 (LT only); SV40 plasmid pSV8 (sT + truncated LT) Transgenic founder pups, <i>n</i> = 4–9/group	WT SV40, 7/8 (87%) CPP; pSV11, 5/7 (71%) CPP; pSV8, 0/9 (0%) CPP. Thymic hyperplasia present in some mice with CPP.	NR	Tumours expressed SV40 LT.
Mouse (C57BL/6 × SJL) (NR) Up to 18 mo Pinkert <i>et al.</i> (1987)	pSV40(cT), cytoplasmic LT mutant. Transgenic founder pups, <i>n</i> = 8; non-transgenic pups, <i>n</i> = 36 Transgenic line 269, <i>n</i> = 68	7/8 (88%) founders died, CPP and kidney and thymus lesions, including tumours. Time to death: founders, 40–134 d (mean, 52 d); transgenic 269 line, 81 d. No tumours in non-transgenic pups.	NR	SV40(cT): mutation in nuclear transport signal on LT results in retention of LT in the cytoplasm. SV40 LT was retained in the cytoplasm of CPP.
Mouse (B6D2F1) (NR) Up to 18 mo Chen & Van Dyke (1991)	SV40 plasmid pSV11 Founders, <i>n</i> = 13 Recombinant: SV40 regulatory region plus LT of lymphotropic papovavirus (SLT) Founders, <i>n</i> = 9 SV11, SV40 transgenic line	SV40 founders, 9/13 (69%), choroid plexus tumours; some also had thymic hyperplasia and polycystic kidneys; latency, 65–210 d. SLT founders, 8/9 (89%), total tumours; 4/9, choroid plexus tumours; some also had thymic hyperplasia and polycystic kidneys, same diseases as for WT SV40. Latency, 70–380 d. SV11 line, ~100 d.	NR	LT of lymphotropic papovavirus could substitute for SV40 LT to induce brain tumours.

^a SV40 LT expressed under the control of tissue-specific foreign promoters in transgenic mice can induce pre-neoplastic/neoplastic changes in many different tissues and cell types. Those systems are not reviewed here.

^b Statistical significance is listed if reported in the study.

CPP, choroid plexus papillomas; cT, cytoplasmic LT mutant; d, day; mo, month; LT, large T-antigen; NB, newborn; NR, not reported; sT, small t-antigen; SV40, simian virus 40; WT, wild-type

Table 3.9 Carcinogenicity studies of transgenic mice involving hybrid SV40 and JCV sequences

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	For each target organ: Incidence (%); time to tumours
Mouse, NR (NR) Lifetime Feigenbaum <i>et al.</i> (1992)	JCV Mad-1 promoter × SV40 LT gene <i>n</i> = 5	Tumours (60%), including neuroblastoma (60%), myenteric plexus tumours (60%), choroid plexus carcinoma (40%); 0.5–4 mo
	SV40 promoter × JCV LT gene <i>n</i> = 7	Tumours (100%), including choroid plexus papilloma (86%), renal hyperplasia (71%), thyroid hyperplasia (43%), thymic hyperplasia (29%); 1–3 mo
Mouse, C57BL/6 × CBA (M, F) Up to 75 d Ressetar <i>et al.</i> (1993)	JCV Mad-1 promoter × SV40 LT gene <i>n</i> = 2	Tumours (100%), including abdominal B-cell lymphoma (50%), adrenal neuroblastoma (50%), osteosarcoma (50%), astrocytoma (50%); 1–2.5 mo
	SV40 776 promoter × JCV LT gene <i>n</i> = 1	Tumours (100%), including choroid plexus papilloma (100%), thymic hyperplasia (100%), thymoma (100%); 1.5 mo

d, day; F, female; JCV, JC polyomavirus; LT, large T-antigen; M, male; mo, month; NR, not reported; SV40, simian virus 40

Table 3.10 Carcinogenicity studies of SV40 in combination with asbestos in experimental animals

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Controls Animals/group at start	For each target organ: tumours (%); time to tumours	Significance ^a	Comments
Hamster, Syrian golden (21 d) (F) 2 yr Kroczyńska et al. (2006)	SV40 dl883 (sT dl mutant), 10 ^{8.5} PFU, intracardiac, <i>n</i> = 28 – Crocidolite asbestos, 0.4 mg intrapleural space, plus 4 mg intraperitoneal (0.4 mg every 2 wk) <i>n</i> = 29 Virus + asbestos, <i>n</i> = 30 Controls: medium only, <i>n</i> = 30	Virus only, 0/28 (0%) Asbestos alone (6/29, 20%), peritoneal mesotheliomas; 37.6 wk Virus + asbestos (27/30, 90%), pleural and peritoneal mesotheliomas; 30.2 wk Controls (0%)	Decrease in survival: Asbestos + SV40 <i>vs</i> SV40, <i>P</i> < 0.001 Asbestos + SV40 <i>vs</i> asbestos, <i>P</i> = 0.003	SV40 and asbestos acted as co-carcinogens to induce mesotheliomas.
Mouse (transgenic), C57/BL6 (NB) (NR) 24 mo Robinson et al. (2006)	SV40 strain 776 LT under mesothelin promoter, generated high-copy-number (299 h, ~100 transgene copies) and single- copy (266 s) transgenic lines Crocidolite asbestos, intraperitoneal, 0 (untreated) or 3 mg Controls: WT mice <i>n</i> = 8–15/group	Tumours (lifetime incidence) Asbestos-treated: all developed mesotheliomas; latency: high-copy- number line, 35 wk; single-copy line, 63 wk; WT mice, 55 wk Untreated mice: high-copy-number line, 5% sarcomas; single-copy line and WT mice, 0%.	Survival time after asbestos: high-copy- number line <i>vs</i> single- copy line or WT mice (<i>P</i> < 0.0001, decrease) Direct relationship between SV40 transgene copy number and survival after asbestos, <i>r</i> ² = 0.89	<i>In vivo</i> demonstration in transgenic mice of co- carcinogenesis between SV40 and asbestos.

d, day; dl, deletion; F, female; LT, large T-antigen; mo, month; NB, newborn; NR, not reported; PFU, plaque-forming units; sT, small T-antigen; SV40, simian virus 40; *vs*, *versus*; wk, week; WT, wild-type; yr, year

^a Statistical significance is listed if reported in the study.

developed multiple tumours or lesions in a single animal. These studies suggest that promoter tropism is linked to tumorigenesis since JCV LT sequences under the control of the SV40 promoter demonstrated a similar tumour profile to transgenic mice expressing SV40 LT under the control of its own promoter, i.e. choroid plexus papilloma. The JCV promoter driving SV40 LT expression exhibited the tropism seen in JCV experimental animals, including neuroblastoma and astrocytoma. However, those animals also developed tumour types induced by SV40, including osteosarcoma and a much higher frequency of tumours of the choroid plexus, suggesting that the LT sequences may also play a role in viral tropism.]

3.4 Carcinogenicity of SV40 in combination with asbestos

See [Table 3.10](#)

Studies in hamsters and transgenic mice have revealed co-carcinogenic effects of SV40 and asbestos.

[Kroczyńska et al. \(2006\)](#) inoculated SV40 dl883 at $10^{8.5}$ PFU by the intracardiac route into female hamsters ($n = 28$; age, 21 days), which resulted in no mesotheliomas (0%). Crocidolite asbestos given as 0.4 mg in the intrapleural space plus 4 mg intraperitoneally ($n = 29$) produced peritoneal mesotheliomas in 6 recipients (20%), with a mean latency of 37.6 weeks. When given together, virus and asbestos produced pleural and/or peritoneal mesotheliomas in 27 (90%) of 30 animals, with a mean latency of 30.2 weeks. Controls inoculated with medium only ($n = 30$) developed no tumours. The following significant differences in survival between groups were observed: asbestos + SV40 *versus* SV40, $P < 0.001$; and asbestos *versus* asbestos + SV40, $P = 0.003$. [This study demonstrated that SV40 and asbestos act as co-carcinogens *in vivo* in hamsters for induction of mesotheliomas.]

[Robinson et al. \(2006\)](#) produced transgenic mice, using SV40 strain 776 LT under control of the mesothelin promoter, and generated high-copy-number (about 100 transgene copies) and single-copy SV40 transgenic mouse lines. Non-transgenic (WT) mice served as controls. Crocidolite asbestos (3 mg) was injected intraperitoneally. Group sizes ranged from $n = 8$ to $n = 15$. The lifetime tumour incidence was 0% for the untreated WT mice and the single-copy line and 5% (sarcomas) for the high-copy-number line. After treatment with asbestos, all mice developed mesotheliomas, with latency periods of 55 weeks for the WT mice, 63 weeks for the single-copy line, and 35 weeks for the high-copy-number line. There was a significant difference in survival time after asbestos treatment when the high-copy-number line was compared with either the single-copy line or the WT mice ($P < 0.0001$). There was a direct relationship between SV40 transgene copy number and survival after asbestos ($r^2 = 0.89$). [This study showed that SV40 and asbestos act as co-carcinogens *in vivo* in transgenic mice.]

4. Mechanistic and Other Relevant Data

4.1 Transforming capacity of SV40

The transforming capacity of SV40 is reflected by the induction of tumours in NB hamsters ([Eddy et al., 1961](#); [Ashkenazi & Melnick, 1963](#); [Black & Rowe, 1964](#)). SV40 induces, for example, undifferentiated sarcomas or ependymomas in NB hamsters upon inoculation of SV40 ([Eddy et al., 1961](#); [Gerber & Kirschstein, 1962](#)). Depending on the site of injection, hamsters developed leukaemias, lymphomas, or osteosarcomas after intravenous injection ([Diamandopoulos, 1972](#)), mesotheliomas after intrapleural injection ([Cicala et al., 1993](#)), and

diverse brain tumours after intracranial injection ([Gerber & Kirschstein, 1962](#)). The presence of SV40 LT in the tumour cells was proven by indirect immunofluorescence staining in lymphosarcoma cells ([Diamandopoulos, 1972](#)), or the presence of SV40 itself was assessed by testing the effect of brain tumour homogenates on fresh kidney cell cultures ([Gerber & Kirschstein, 1962](#)). Besides SV40 LT expression as tested by immunofluorescence and immunohistochemistry in SV40-induced mesotheliomas, Southern blotting revealed the presence and integration of SV40 sequences in these tumours ([Cicala et al., 1993](#)). However, it is noteworthy that the SV40 virus itself appears to be relatively harmless to whole animals since only NB hamsters were reported to be susceptible to tumour formation (see Section 3). SV40 has been shown to transform hamster kidney cells *in vitro* ([Black & Rowe, 1963](#); [Black et al., 1963](#)). SV40 also transformed other rodent cell lines, for example, mice, rats, guinea-pigs, and cattle, which are non-permissive for viral replication (reviewed in [Arrington & Butel, 2001](#); [Atkin et al., 2009](#); [Pipas, 2009](#); [Gjoerup & Chang, 2010](#)). These transformed cell lines demonstrated cytological, chromosomal, and growth abnormalities. One of the earlier SV40 transforming studies was conducted in an established immortalized mouse fibroblast cell line, NIH3T3 ([Todaro et al., 1964](#)).

Human tissues were also shown to be transformed by SV40: primary human renal cells ([Shein & Enders, 1962b](#)), primary human epithelial cells derived from buccal mucosa and skin cells ([Koprowski et al., 1962](#); [Ponten et al., 1963](#); [Jensen et al., 1964](#)), and human fetal lung tissue ([Moyer et al., 1964](#)).

The transforming capacity of SV40 resides in the early region of the viral genome, which encodes two oncogenes, LT and sT. Expression of LT, alone or in combination with sT, will oncogenically transform most rodent cell types. Expression of LT alone often allows cells to become immortalized and to grow in reduced

serum and to high saturation density. Moreover, these transformed cells escape contact inhibition, as shown in focus formation assays. LT alone, or more frequently together with sT, induces anchorage – independent growth in soft-agar and tumours in nude mice (reviewed in [Manfredi & Prives, 1994](#); [Pipas, 2009](#); [Gjoerup & Chang, 2010](#)).

In human cells, SV40 DNA was shown to induce a morphologically transformed phenotype (focus formation); however, the transformed cells are not tumorigenic in nude mice ([Sager et al., 1983](#)). The tumorigenic transformation of normal human fibroblast, kidney epithelial, and mammary epithelial cells was demonstrated to require co-expression of the SV40 early region with the gene encoding the telomerase catalytic subunit (*hTERT*), and an oncogenic allele of the *H-ras* gene ([Hahn et al., 2002](#)).

Interestingly, it has been demonstrated that transformation of primary human fibroblasts by transfection of origin-defective SV40 is significantly enhanced, indicating that viral replication might hamper the transformation ([Small et al., 1982](#); [Gjoerup & Chang, 2010](#)).

Mice transgenic for SV40 LT developed characteristic brain tumours in which SV40 LT copy numbers and transcripts were significantly elevated, indicating in this animal model a role for SV40 LT in the tumorigenesis process ([Brinster et al., 1984](#); [Messing et al., 1985](#)).

4.2 Biological properties and regulation of SV40 viral proteins relevant to carcinogenesis

The SV40 LTs are some of the most intensively studied and best understood oncoproteins. Mutants of SV40 with temperature-sensitive mutations in LT showed that this oncoprotein was required for both the initiation and the maintenance of the transformed state ([Brugge & Butel, 1975](#); [Martin & Chou, 1975](#); [Tegtmeier, 1975](#)).

This finding established that a single viral protein was capable of mediating cell transformation.

LT is a 708 amino acid nuclear phosphoprotein with biochemically separable functions important in promoting viral DNA replication in the natural life-cycle of the virus. LT is produced early in infection and initiates replication by binding to the viral origin and recruiting cellular replication factors (reviewed in [Cheng et al., 2009](#); [Fanning & Zhao, 2009](#)).

The transforming capability of SV40 LT has been mapped, in part, to its ability to directly interact with the family of retinoblastoma proteins, comprising pRb, p107, and p130 ([Goodrich et al., 1991](#)). This family of transcriptional cofactors can bind to and either antagonize or potentiate the function of numerous transcription factors ([Burkhart & Sage, 2008](#)). The pRb family members are also referred to as pocket proteins, based on the structural pocket formed from conserved A and B domains necessary for their tumour suppression functions. *RB* mutations in human malignancies map to this pocket region, and several viral oncoproteins (e.g. LT, E1A, HPV E7) interact with this same region via an LXCXE motif. Through an N-terminally positioned LXCXE motif (residues 103–107), SV40 LT interacts with all three pRb family members to disrupt their repressive complexes with E2F family members ([Zalvide et al., 1998, 2001](#); [Sullivan et al., 2000, 2004](#)). As a consequence, many E2F target genes are activated or de-repressed. These downstream targets of E2F are diverse, including genes involved in DNA replication, mitosis, DNA repair, differentiation, development, and apoptosis ([Burkhart & Sage, 2008](#)).

For LT to functionally inactivate pRb and affect transformation, the N-terminal DnaJ region containing the HPDK residues is also required. Deletion of the DnaJ domain does not prevent physical binding of LT to pRb family members; however, many functions attributed to the inactivation of pRb by LT are lost. DnaJ proteins are

molecular co-chaperones that recruit members of the family heat shock proteins for the regulation of various cellular processes, including protein folding, protein transport, and remodelling of protein complexes. The LT DnaJ domain binds cellular hsc70 and brings this powerful regulatory system in proximity to LT–pRb complexes. The energy released from hsc70 ATPase activity is postulated to be necessary for the dissociation of pRb family proteins from E2F transcription factors ([Sullivan et al., 2000](#); [Sullivan & Pipas, 2001](#)).

SV40 LT interacts with a second tumour suppressor protein, p53, to affect transformation. The p53 protein was first discovered as an SV40 LT binding protein in 1979 ([Lane & Crawford, 1979](#); [Kress et al., 1979](#); [Linzer & Levine, 1979](#)). Subsequent investigations have revealed that expression of viral genes capable of inactivating p53 is a common feature of known tumour viruses. LT binds p53 within its core DNA binding domain, thus impairing the ability of p53 to activate the transcription of its target genes. The region of LT required for p53 binding is bipartite and comprises residues 351–450 and 533–626. The p53 binding site is critical for rodent embryo fibroblast immortalization and for lifespan extension in human diploid fibroblasts ([Lin & Simmons, 1991](#); [Zhu et al., 1992](#); [Kierstead & Tevethia, 1993](#)). However, in transformation assays using established cell lines, p53 binding is often not strictly required ([Manfredi & Prives, 1994](#)). It is also noteworthy that in transgenic mice expressing mutant LT, p53 binding is not required for *in vivo* dysplasia or tumour formation in some organs, including the choroid plexus ([Chen et al., 1992](#)), pancreatic acinar cells ([Tevethia et al., 1997](#)), and intestinal enterocytes ([Markovics et al., 2005](#)). Details of the functional effects and mechanistic consequences of the interaction between LT and p53 are still not fully defined, but it is known that LT stabilizes p53 by preventing Mdm2-mediated proteasomal degradation of p53 ([Henning et al., 1997](#)). LT has

not been found to interact with the p53 family members p63 or p73 ([Marin et al., 1998](#)).

Whereas inhibition of the tumour suppressors pRb and p53 by LT is critical for LT-mediated transformation, recent work has revealed other LT targets that may also contribute to transformation. Investigations are in progress to understand the role of LT interaction with Cul7, Bub1, IRS1, NBS1, Fbw7, p300/CBP, and p400 ([Cotsiki et al., 2004](#); [Poulin et al., 2004](#); [Wu et al., 2004](#); [Kasper et al., 2005](#); [Welcker & Clurman, 2005](#); [DeAngelis et al., 2006](#); and reviewed in [Gjoerup & Chang, 2010](#)).

LT is required for initiation of viral DNA replication, and much is known through crystallographic and biophysical studies about the temporal and spatial events that occur. Currently, much more is known about the functions of SV40 LT in replication than about those of other polyomavirus LT. This *Monograph* does not deal with the specifics of LT loading, architecture, and cellular interactions at the viral origin (for reviews, see [Fanning & Knippers, 1992](#); [Fanning & Zhao, 2009](#)).

All polyomaviruses have sT. SV40 sT alone cannot transform cells; however, it can potentiate the transforming activity of LT ([Noda et al., 1986](#); [Hahn et al., 2002](#)). Two major motifs have been identified on sT: the DnaJ HPDK interaction domain, shared with LT, and a PP2A binding domain. PP2A is a family of heterotrimeric phosphatases abundantly expressed in cells. These phosphatases have diverse cellular functions dictated by modular combinations of various A scaffold, B regulatory, and C catalytic subunits. It was shown that sT binds to PP2A A and C complexes and displaces specific B subunits or prevents them from binding to the AC core complex. This interaction inhibits PP2A enzyme activity ([Pallas et al., 1990](#); [Yang et al., 1991](#)); however, there are also examples where sT delivers PP2A to the substrate, thus mediating dephosphorylation rather than inhibiting it (e.g. histone H₁, 4E-BP1) (for a review, see [Gjoerup &](#)

[Chang, 2010](#)). A microarray analysis has identified many genes involved in cell proliferation, apoptosis, integrin signalling, and immune responses whose expression is altered by sT. The altered expression of many of these genes occurs via interaction of sT with PP2A, but for several other genes the alteration appears to be independent of sT binding to PP2A. This suggests that other cellular targets of sT remain to be identified ([Moreno et al., 2004](#)).

MicroRNAs (miRNAs) are a class of ~22-nucleotide-long non-coding RNAs that function in the post-transcriptional regulation of gene expression. In eukaryotes, they are involved in processes including cell development, cell-cycle control, immunity, and oncogenesis ([Garzon et al., 2009](#)). Viral miRNAs have also been found to be encoded by the genomes of nuclear double-stranded DNA viruses, specifically viruses of the herpesvirus, adenovirus, and polyomavirus families ([Gottwein & Cullen, 2008](#); [Grundhoff & Sullivan, 2011](#)). The first miRNAs derived from polyomaviruses were identified in SV40 ([Sullivan et al., 2005](#)). Two miRNAs from a single miRNA stem-loop precursor are expressed from the late viral transcript and accumulate at late times in infection. Both miRNAs have exact binding site sequences in the early region and have been validated to direct the cleavage of early LT transcripts, resulting in decreased expression of LT and sT. Thus, these miRNA products of late gene expression exert a negative regulatory role on early gene expression. Surprisingly, this does not appear to affect the replicative fitness of SV40 in cultured cells. Although molecular clones of the virus with mutations that disrupt the pre-miRNA stem-loop structure express higher levels of LT and sT, they do not differ from WT virus in the generation of infectious virus. Cells infected with miRNA mutant SV40 were more susceptible to cytotoxic T-cell-mediated lysis *in vitro*, suggesting that the miRNA may play a role in immune evasion *in vivo* ([Sullivan et al., 2005](#)).

4.3 *In vivo* and *in vitro* evidence for a role for SV40 in human malignancies

4.3.1 *Major concerns about the methods used for the detection of SV40*

Studies suggesting a role for SV40 in human cancers include a large number of case series reporting detection of SV40 DNA by PCR in cancer tissue. However, the contribution of these studies to mechanistic evidence for the carcinogenic role of SV40 in human cancers is problematic because major concerns have been raised about the methods used for the detection of SV40 in human malignancies (see also Section 1.2 and Section 2.4 in this *Monograph*).

(a) *Detection of viral DNA by PCR*

It has been compellingly demonstrated that it is possible for commercial plasmids containing SV40 sequences to contaminate tissue samples ([López-Ríos *et al.*, 2004](#)). López-Ríos *et al.* initially found SV40 DNA in 62% of mesothelioma tissue samples using a widely used PCR targeting the LT gene. However, they observed occasional positive results in the no-DNA negative control reactions, which led them to explore the reasons for this observation. Analysis of the expected amplicon sequence showed that it was within a region of the SV40 genome included in many common laboratory plasmids. Using primers designed to amplify an intronic fragment of the SV40 genome not encompassed by the SV40 genomic region found in commercial plasmids, they failed to detect SV40 in the majority of samples and in a minority of instances (6%) observed only weak positive bands that could not be consistently amplified in repeated experiments. Control samples were consistently negative using the newly designed PCR. Although this represents the experience of a single laboratory, the source of the contamination they documented could affect results from many other laboratories. In addition, it is worth

noting that some primer sets designed to amplify the regulatory region of SV40 can also amplify BKV and JCV ([Lednicky & Butel, 1997](#)). The use of these primer sets could again lead to false-positive results for SV40 if appropriate controls are not performed. Only a few published studies after that of [López-Ríos *et al.* \(2004\)](#) have forthrightly addressed these concerns in the methodological design of their studies ([Manfredi *et al.*, 2005](#); [Schüler *et al.*, 2006](#)). The value of PCR data as evidence for a mechanistic role of SV40 in human cancers is also problematic because of the considerable inconsistency among studies in detection of viral DNA and the lack of a clear-cut and widely accepted explanation for the discrepancies across laboratories.

(b) *Detection of viral protein by immunohistochemistry*

The most common method used to demonstrate the presence of viral protein in cells has been immunohistochemistry. Immunohistochemistry is potentially very sensitive and specific, but it depends on the quality of the immunological reagents that are used. Of note, the specificity of the Pab101 monoclonal antibody raised against SV40 transformed cells of mouse origin and the Ab-1 (or Pab419) monoclonal antibody raised against full-length SV40 LT, which have been widely used for studies of the association of SV40 with tumours, has been called into question by [Pilatte *et al.* \(2000\)](#), who found that the antibody preparations are contaminated with a protein of similar size (90 kD) to SV40 LT and that the contaminating protein reacts with various secondary horseradish peroxidase-conjugated anti-mouse immunoglobulin G commercial reagents. The 90 kD protein was detected in different lots of the Ab-1 and Pab101 reagents, reacted with secondary antibodies from several commercial suppliers, and was found in murine monoclonal antibodies directed against other antigens but of the same isotype. Although the researchers showed that the problem was not

restricted to a single lot, no firm conclusions can be drawn about the implications of this finding for other studies. No studies published after the appearance of this report in 2000 have explicitly addressed this potential concern in the description of the methods used. In addition, the interpretation of immunohistochemical staining is inherently subjective, but most of the findings described below for particular cell lines and tumour tissue samples have not been confirmed by multiple independent laboratories and by the use of multiple validated immunological reagents.

In light of the above considerations, the Working Group elected to review only those studies of human cancer cell lines and human cancer tissues that included detection of SV40 proteins and associated changes in proto-oncogenes and host chromosomal structure as the evidential basis for establishing a mechanistic role for the virus. Although demonstration of the presence of viral protein in tumour tissue is not definitive evidence for an etiological role of the virus in carcinogenesis, such evidence indicates that the viral genome is transcriptionally active and supports the possibility that the viral oncogene is functional. Since all published studies of detection of viral protein also sought to detect viral DNA, these studies are inherently more robust than those relying solely on DNA detection. A potential loss in sensitivity is inevitable, but specificity is judged to be the more important criterion for addressing mechanistic questions.

4.3.2 Tumours of the central nervous system

[Bergsagel et al. \(1992\)](#) reported the detection of SV40 viral DNA in 10 of 20 choroid plexus tumours and 10 of 11 ependymomas. Immunohistochemical analysis using a polyclonal antiserum against SV40 LT revealed intense staining of 5–15% of the nuclei of tumour cells from 4 of 5 choroid plexus tumours and 3 of 6 ependymomas.

[Zhen et al. \(1999\)](#) examined 65 frozen tumour specimens of diverse histological type for SV40 LT by immunoprecipitation with the Pab101 monoclonal antibody directed against the C terminus of SV40 LT. SV40 LT was detected in 43 (66%) of 65 specimens, whereas 8 normal brain tissue samples were negative for SV40 LT. SV40 LT-positive tumours included ependymoma, choroid plexus papilloma, pituitary adenoma, astrocytoma, meningioma, glioblastoma multiforme, and medulloblastoma, with frequencies of LT-positive tumours ranging from 33% to 100%. In a subset of SV40-positive tumours, complexes between LT and p53 (18 of 18 tumours) and LT and pRb (15 of 15 tumours) were also detected. [The Working Group noted that the highly positive results reported in this study were based only on the detection of SV40 LT with the Pab101 monoclonal antibody, the specificity of which was called into question ([Pilatte et al., 2000](#); see also 4.3.1).]

[Weggen et al. \(2000\)](#) failed to detect SV40 in most brain tumour samples. Using a PCR assay targeting LT gene sequences, they found SV40 DNA in only 1 of 131 meningiomas, 2 of 116 medulloblastomas, 1 of 25 ependymomas, and 1 of 2 subependymomas. Only a single case of ependymoma contained SV40 VP1 gene sequences. Sufficient tissue was available for immunohistochemical studies of one medulloblastoma and one meningioma, and both were negative for LT protein staining using Pab101.

There are only a few studies reported about the detection of SV40 protein in human brain cancer cell lines.

[Weiss et al. \(1975\)](#) detected LT in 2 of 7 meningiomas by indirect immunofluorescence using polyclonal hamster anti-SV40 LT antiserum. Fusion of the LT-positive tumour cells with the SV40 permissive African green monkey kidney cell line, MA 134, gave rise to a very small proportion (0.01–0.05%) of cells that stained for SV40 capsid antigen using a rabbit antiserum against SV40 viral capsid.

[Martini et al. \(1996\)](#) detected SV40 LT by indirect immunohistochemical analysis with the Pab101 monoclonal antibody in 3 of 7 glioblastoma cell lines. Positive cells in the immunofluorescence assay, estimated to be 1 out of 1000, showed nuclear staining typical of LT.

[Kim et al. \(2002\)](#) detected SV40 LT in the nuclei of the D283 medulloblastoma cell line by immunohistochemistry using the Pab419 antibody directed against the N-terminal domain of SV40 LT. The Daoy medulloblastoma cell line and 4 primary medulloblastoma tumours were negative for SV40 LT staining.

[Lednický et al. \(1995\)](#) performed studies to establish whether the entire SV40 genome was present in SV40-positive paediatric brain tumours. By introducing tumour DNA into permissive monkey kidney cells by lipofection, they were able to rescue infectious SV40 virus from one SV40 DNA-positive tumour. Neither the state nor the site of integration of the viral DNA could be determined.

4.3.3 Mesothelioma

[Carbone et al. \(1994\)](#) first reported the detection of SV40 DNA sequences in human mesotheliomas. They found SV40 DNA in 29 (60%) of 48 mesotheliomas and demonstrated SV40 LT nuclear staining in 11 of 14 specimens using the Pab419 monoclonal antibody.

Results from the multi-institutional study reported by [Testa et al. \(1998\)](#) included immunohistochemical analysis of 12 mesothelioma tissues by a single laboratory. Two anti-LT monoclonal antibodies were used (Pab101 and Pab419); in this laboratory, both antibodies yielded similar results. Ten of the 12 mesotheliomas contained malignant cells positive for LT staining, which was localized to the nucleus.

[De Luca et al. \(1997\)](#) demonstrated SV40 LT in 4 mesothelioma tissues by immunoprecipitation followed by western blotting.

In contrast to these findings, there are several publications reporting failure to detect expression of LT in mesothelioma tissues.

[Galateau-Salle et al. \(1998\)](#) were able to detect SV40 DNA sequences in mesotheliomas and bronchopulmonary carcinomas, as well as in non-malignant lung samples, but immunohistochemistry performed on 15 mesotheliomas and 16 bronchopulmonary carcinomas did not show nuclear staining for SV40 LT, although nonspecific cytoplasmic staining was observed.

[Jin et al. \(2004\)](#) detected SV40 DNA sequences in 8 (44%) of 18 mesothelioma samples collected from patients in Japan. However, no immunohistochemical staining for SV40 LT was found in the 18 tumour samples. Analyses were done with both Pab101 and Pab416 (Ab-2) monoclonal antibodies, directed against the C-terminal and the N-terminal domain of SV40 LT, respectively.

[Dhaene et al. \(1999\)](#) detected SV40 DNA sequences in 13 (46%) of 28 mesotheliomas. Cytoplasmic staining but no nuclear staining was found in 10 of 13 cases by immunohistochemical analysis using Pab101 and Pab419.

4.3.4 Lymphoma and other human tumours

There are isolated reports of detection of SV40 LT expression in lymphoma and other human tumours. [Vilchez et al. \(2005\)](#) examined 45 NHLs from HIV-infected patients hospitalized in Houston, Texas, and 10 lymphomas obtained from a HIV/AIDS programme in New Jersey for the presence of SV40 DNA and LT oncoprotein expression. SV40 DNA sequences were identified in 12 (22%) of the 55 cases. Immunohistochemical analyses were carried out with the monoclonal antibodies Pab416 and Pab101. Pab101 revealed positive staining in all 12 SV40 DNA-positive samples, whereas Pab416 gave detectable staining in only 5 of those samples. None of the 68 SV40 DNA-negative samples reacted with either monoclonal antibody. [The Working Group noted that unlike Pab101, the Pab416 monoclonal antibody

was not shown to be contaminated with the 90 kD protein reported to give false-positive reactions.]

[Meneses et al. \(2005\)](#) detected SV40 virus DNA in 28 (26%) of 106 NHLs and 2 (10%) of 19 Hodgkin lymphomas obtained from HIV-negative, chemotherapy-naïve patients in Costa Rica. None of 51 reactive lymph nodes and tonsil samples or 40 gastric and hepatic carcinoma samples were positive for SV40 DNA. Expression of SV40 LT was detected by immunohistochemistry using the monoclonal antibodies Pab416 and Pab101 in 18 (64%) of 28 NHLs that contained SV40 virus DNA. None of the lymphomas or control samples that tested negative for viral DNA was positive by immunohistochemical analysis for SV40 LT. In general, few malignant cells were immunopositive, and staining was of low intensity.

[Martini et al. \(1998\)](#), in a study of NHL and Hodgkin lymphoma in HIV-positive and HIV-negative subjects, observed no significant difference in the detection rate for SV40 DNA in the two populations: 3 (10.7%) of 28 *versus* 18 (14.7%) of 122, respectively. Using semiquantitative PCR, the investigators estimated that SV40 DNA was present at 10^{-4} to 10^{-2} genome equivalents (geq) per cell, which is not consistent with the presence of the virus within all lymphoma cells. Expression of LT was recorded in 5 of 18 SV40 DNA-positive samples, and < 1% of tumour cells displayed positivity by immunohistochemical analysis with the Pab419 reagent.

[Went et al. \(2008\)](#) failed to detect expression of SV40 LT in tissue microarrays constructed with archived samples from 655 NHLs and 337 Hodgkin lymphomas diagnosed between 1974 and 2001 in patients in Italy, Switzerland, and Austria. [Of note, these investigators used the Ab-2 (Pab416) reagent, which has not been shown to potentially give false-positive reactions.]

[Martinelli et al. \(2002\)](#) detected SV40 DNA in 28 (62%) of 45 parotid gland adenomas and none of 11 normal salivary gland tissue samples. SV40 LT expression was detected

by immunohistochemistry with the Pab101 monoclonal antibody in 26 (93%) of 28 SV40 DNA-positive tumour specimens.

[Vivaldi et al. \(2003\)](#) investigated human thyroid tumours for SV40 DNA sequences by PCR and a subset of the tumours for LT expression by reverse transcriptase PCR (RT-PCR) of total tumour RNA and immunohistochemistry. LT gene transcripts were found in 9 of 13 SV40 DNA-positive papillary thyroid cancers and 8 of 11 anaplastic thyroid cancers. Transcripts were not detected in 30 SV40 DNA-negative tumour specimens. RT-PCR positive samples were further analysed by immunohistochemistry using the Pab101 monoclonal antibody. Strong cytoplasmic staining but no nuclear staining was detected in 3 of 8 papillary thyroid tumours and 8 of 8 anaplastic thyroid tumours.

Animals with SV40-induced tumours frequently produce high-level antibodies against LT oncoprotein. This observation prompted [Engels et al. \(2005\)](#) to assess whether SV40 LT antibodies are associated with NHL in humans. LT antibodies were measured by enzyme immunoassay. Hamsters with SV40-induced tumours all produced robust SV40 LT antibody levels, whereas SV40-uninfected hamsters and macaques, as well as macaques naturally infected with SV40, had much lower levels. Low levels of LT antibody response were observed in NHL cases and controls. Overall, only 5 cases (6%) and 5 controls (5%) had LT antibody responses classified as seropositive, demonstrating the lack of association between the presence of SV40 LT antibody and NHL.

4.3.5 Mechanistic studies of SV40 LT expression in human mesothelioma cells

Several publications from the Carbone laboratory have explored potential mechanisms for enhanced susceptibility of mesothelial cells to the oncogenic effects of SV40 virus. [Carbone et](#)

[al. \(1997\)](#) reported that expression of SV40 LT was associated with high levels of p53 in mesotheliomas. They also showed that LT and p53 were co-precipitated by either the Pab419 anti-SV40 LT monoclonal antibody or a monoclonal antibody to p53, suggesting that LT is physically associated with p53. SV40 LT from lysates of mesothelioma cells was also able to co-precipitate *in vitro* translated p53 protein. No p21 was detected by immunohistochemistry in mesothelioma cells co-expressing SV40 LT and p53, suggesting that the p53 was functionally inactive. The LT precipitate also contained proteins of the expected size of SV40 sT and 17K T viral proteins. In another report from the Carbone laboratory, [De Luca et al. \(1997\)](#) showed that SV40 LT in mesothelioma cells could co-precipitate retinoblastoma family proteins, pRb, p107, and pRb2/p130. [Bocchetta et al. \(2000\)](#) reported that primary human mesothelial cells infected with SV40 exhibit a unique semi-permissive infection phenotype. The cells were not lysed by infection; all expressed LT and high levels of p53 and generated transformed foci. [Foddìs et al. \(2002\)](#) found that mesothelioma tissues and mesothelioma cell lines were telomerase-positive and that *in vitro* infection with SV40 induced telomerase activity of cultured human mesothelial cells.

[Waheed et al. \(1999\)](#) detected SV40 DNA and LT and sT mRNA in 3 of 5 mesothelioma cell lines derived from primary tumour specimens. LT protein could not be detected by western blotting using a monoclonal antibody to the NH₂ terminus of LT and sT (Pab 108), indicating that the levels of LT and sT expression were quite low. Transduction of one SV40-positive cell line with an adenoviral vector expressing antisense RNA to the first 550 bp of the LT and sT coding sequence resulted in marked inhibition of cell proliferation; this suggests that LT would have a significant contribution to the ability of these cells to grow in culture. [The Working Group noted, however, that the specificity of this inhibition is unclear because long double-stranded

RNAs can also activate interferon pathways that would have the same effect. No *in vitro* molecular studies on other putatively SV40-associated cancers were described because it has not been possible to propagate a SV40-positive tumour cell line from these other cancers.]

4.4 Interaction between SV40 and potential cofactors

Several reports have suggested a role of SV40 in mesothelioma, a cancer associated with asbestos exposure (see Section 2). As described above, SV40 LT has been reported to promote a transformed phenotype in primary human mesothelial cells ([Bocchetta et al., 2000](#)). [Bocchetta et al. \(2000\)](#) found that when mesothelial cells surviving exposure to asbestos were transfected with SV40 LT and sT, they were more prone to cell transformation, but the effect could not be quantified because these cells are highly susceptible to asbestos (crocidolite)-induced cell death.

4.5 Transgenic models for cancers associated with SV40 infection

The literature on transgenic animal models of SV40 is reviewed in detail in Section 3.

4.6 Susceptible human populations

There is no evidence to support an association between SV40 infection and immunocompromised status or between SV40 infection and birth cohorts at risk for exposure to SV40 due to vaccination with potentially contaminated vaccines. [Jafar et al. \(1998\)](#) reported no difference in SV40 seroprevalence by plaque reduction neutralization assay in HIV-positive patients (16.1%) compared with HIV-negative subjects (12.0%). Furthermore, there was no significant difference in seroprevalence when subjects were stratified by year of birth as born before 1941,

between 1941 and 1962, and after 1962. In a study of lymphomas diagnosed in HIV-positive and HIV-negative patients, [Martini et al. \(1998\)](#) observed no significant difference in the prevalence of SV40 DNA in tumour tissue between the two populations (10.7% *versus* 14.8%). In a meta-analysis of cancer incidence in HIV/AIDS patients and immunosuppressed transplant recipients, brain cancers did not occur at an increased rate ([Grulich et al., 2007](#)). No data were reported for mesothelioma, most likely because this is a rare cancer. The incidence of lymphomas was significantly increased, but the known association of Epstein–Barr virus and these cancers may explain the entire effect.

4.7 Mechanistic considerations in relation to carcinogenesis

SV40 is the best-studied example for experimental transformation of a small DNA tumour virus in cell culture and experimental animals. There is broad evidence from many research groups and experimental systems that in specific experimental environments SV40 has transforming capacity. SV40 in these experimental systems follows a transformation mechanism in principle similar to that identified for oncogenic human papillomaviruses (HPV) ([IARC, 2007, 2012](#)) in human cells. In its natural host, rhesus macaques, even under conditions of immune suppression, SV40 does not appear to induce tumours (for a review, see [Butel & Lednický, 1999](#)). In humans the molecular evidence for presence and transforming activity of SV40 is scarce and controversial (see review in [Garcea & Imperiale, 2003](#)).

The prevailing model for polyomavirus- and papillomavirus-induced carcinogenesis is that at least one viral genome is persistently present and biologically active in each transformed cell and the lytic viral life-cycle is interrupted. Viral persistence can be mediated through integration

or through maintenance as viral episome. The presence of one integration site per tumour present in different parts of the tumour and in primary tumour as well as in tumour metastasis indicates that viral integration occurred before clonal tumour expansion. Viral genes encoding regulatory proteins, i.e. the LTs in polyomaviruses and the viral oncoproteins E6 and E7 in HPV, are consistently expressed through transcription and translation. Viral oncoproteins, among other functions, interact directly or indirectly with cellular tumour suppressor proteins such as pRb and p53, leading to cell-cycle and apoptosis deregulation. The presence of viral oncoproteins is nearly always necessary to maintain the transformed phenotype and in the tumour-bearing animal or human host can lead to the induction of antibodies to the viral oncoproteins. Such antibodies are rarely induced during the natural course of infection. An additional essential feature of virally induced cell transformation is the interruption of the lytic viral life-cycle. This can be due to lack of host factors essential for viral replication or lack of viral protein functions or *cis* elements on the viral genome necessary for viral replication ([IARC, 2007; Butel, 2012](#)).

SV40, like other small DNA viruses, needs the host-cell DNA replication machinery to drive viral replication. As described above in detail, SV40 LT protein can directly interact with the family of retinoblastoma proteins ([Goodrich et al., 1991](#)) and disrupt their repressive complexes with E2F family members ([Zalvide et al., 1998, 2001; Sullivan et al., 2000, 2004](#)).

SV40 LT also interacts with p53 and impairs the ability of p53 to activate the transcription of its target genes ([Kress et al., 1979; Lane & Crawford, 1979; Linzer & Levine, 1979](#)).

SV40 sT contains the DnaJ HPDK interaction domain, in common with LT, and a PP2A binding domain. SV40 sT alone cannot transform cells; however, it can potentiate the cell proliferation activity of LT ([Noda et al., 1986; Hahn et al., 2002](#)). The transforming ability of

SV40 LT appears to be much stronger than that of the human polyomaviruses BKV and JCV ([Bollag *et al.*, 1989](#)).

4.7.1 *In vitro studies*

As described in detail above, stable expression of the experimentally introduced SV40 early genome region encoding sT and LT can lead to transformation of rodent cells and also primary human cells ([Noda *et al.*, 1986](#); [Hahn *et al.*, 2002](#)). Viral DNA usually persists through integration into the host chromosome. However, episomal polyomavirus DNA has also been detected in various types of tumours and transformed cells. Whether integration is required for the transformed state is unclear. Virtually all stably SV40-transformed cells have detectable nuclear LT expression ([Butel & Lednický, 1999](#)).

[Bocchetta *et al.* \(2000\)](#) reported that human mesothelial cells infected with SV40 exhibit a unique semi-permissive infection phenotype. The cells were not lysed by infection, showed stabilization of p53, and generated transformed foci. Human fetal lung fibroblasts can be immortalized by persistent SV40 infection and can also continuously produce low amounts of infectious SV40 virions ([Morelli *et al.*, 2004](#); [Mazzoni *et al.*, 2012](#)).

4.7.2 *Studies in experimental animals*

Inoculation of SV40 virions but also DNA alone by various routes into rodents, especially hamsters, can result in a broad variety of tumours (described in Section 3). In the tumours, the SV40 genome stably persists and viral LT is consistently expressed, and tumour-bearing animals frequently develop high-titre antibodies against SV40 LT.

Studies of transgenic mice stably containing and expressing the SV40 complete early gene region under the control of the native SV40 early promoter/enhancer element have contributed to

the understanding of fundamental principles related to SV40 LT transformation and tumour development. These studies have demonstrated that continuous expression of SV40 LT in the context of transformation-permissive cells is a potent oncoprotein able to induce neoplasms in a variety of tissues, can stimulate resting cells to enter the cell cycle and proliferate, and can inhibit the pRb and p53 tumour suppressor pathways. However, in some examples inhibition of the p53 pathway was found to not be necessary for tumour formation. The transgenic SV40 mouse models further demonstrated that additional genetic changes beyond viral oncogene expression are necessary for tumour formation ([Lednický & Butel, 1999](#); [Butel, 2000b, 2012](#); [Ahuja *et al.*, 2005](#); [Pipas, 2009](#); [Sáenz Robles & Pipas, 2009](#); [Gjoerup & Chang, 2010](#)).

4.7.3 *Human tumour studies*

SV40 DNA by PCR analysis has been found in a broad variety of human tumours, including central nervous system tumours, mesothelioma, lymphoma, parotid gland tumours, and papillary and anaplastic thyroid cancers (see above for detailed study descriptions). In addition, several studies reported also the detection of SV40 LT in the nuclei of tumour cells by immunohistochemistry, or in the tumour tissues by immunoprecipitation or western blot analysis. Frequently, in immunofluorescence-positive tumour tissues only a few tumour cells stained positive, and with weak signals. The presence of LT in complex with p53 or pRb has been reported, and in a few cases also the presence of SV40 early region RNA. However, several groups did not detect SV40 DNA sequences in the same tumour types, and others reported that they could not detect LT in mesothelioma that they had identified as positive for SV40 DNA or found the SV40 LT monoclonal antibodies to stain in the cytoplasm.

Detailed studies in substantial numbers of SV40 PCR DNA-positive human tumour tissues

analysing viral load, status of the viral DNA, mutational status of the LT encoding region, involvement of the pRb pathway, and status of the pRb and p53 gene sequences are completely missing. In analogy to the occurrence of antibodies to the viral oncoproteins specifically in patients with HPV- or Merkel cell polyomavirus (MCV)-induced tumours or in experimental animals bearing SV40-induced tumours, similar antibody response to SV40 LT could be expected in patients with SV40-induced tumours. However, no such antibody data have been reported for any patient with SV40 DNA PCR positive tumours because no such studies have been done yet.

5. Summary of Data Reported

5.1 Exposure data

The natural host of SV40 is the rhesus macaque, in which the virus is maintained as a silent infection. Human exposure to SV40 is considered a rare event and is restricted to people in contact with monkeys. In the past, millions of people have been exposed through vaccination with SV40-contaminated vaccines, primarily poliovirus vaccine. It is unclear whether human-to-human transmission occurs. Exposure to SV40 is established both by detection of DNA sequences by PCR techniques and by detection of specific antibodies by ELISA or neutralization tests. Substantial doubts have been raised that laboratory contamination from plasmids or cell lines commonly used may have contributed to positive PCR results. Half of the published studies since 1995 have failed to detect SV40 DNA in healthy individuals. Similarly, serological cross-reactivity between SV40 and BKV and JCV can explain many of the discrepancies observed between the serological studies. In contrast to human polyomaviruses, well-controlled serological tests with pre-incubation of sera with BKV

and JCV capsids provide little or no evidence that SV40 does infect human populations.

5.2 Human carcinogenicity data

Research on the oncogenic effects of SV40 in humans has focused on mesothelioma, non-Hodgkin lymphoma (NHL), and brain tumours. Cohort studies have followed people who received SV40-contaminated inactivated poliovirus vaccine in the 1950s and early 1960s. Although these studies have limitations, including lack of individual exposure assessment and adjustment for potential confounding factors, the majority have not demonstrated an elevated risk of cancer related to this exposure. In addition, several case-control studies have evaluated associations of serological markers of SV40 infection with mesothelioma, NHL, brain tumours, or other cancers. Most of these studies have demonstrated a similarly low prevalence of antibodies to SV40 in cancer cases and cancer-free control subjects.

In the context of these largely negative studies, several other studies have suggested an association between SV40 and cancer. For example, in a retrospective cohort study in the United Kingdom, some birth cohorts exposed to SV40-contaminated inactivated poliovirus vaccine had an elevated incidence of mesothelioma. In a case-control study of NHL, a higher prevalence of serum SV40 antibodies was observed in cases than in controls, although the majority of SV40 antibodies appeared to be cross-reactive with BKV or JCV. Finally, an elevated incidence of haematological malignancies and neural tumours was observed in a cohort of children whose mothers received potentially contaminated poliovirus vaccines during pregnancy. However, few of the mothers developed antibodies to SV40, so it was not possible to link the cancers in those children to SV40. Limitations in these studies include uncertainties in the assessment of SV40 infection status, residual confounding by other causes

of cancer (e.g. asbestos), and small numbers of cancer cases.

In addition, many case series have reported detection of SV40 DNA in a high proportion of human tumours, notably from mesothelioma, NHL, and brain tumours, whereas others have not. The reasons for discrepancies among case series are uncertain, but they may relate to differences in patient populations or laboratory methods. Contamination of tumour specimens by low levels of SV40 DNA (present in plasmids, cell lines, or other sources) is of special concern for SV40.

5.3 Animal carcinogenicity data

The tumorigenic potential of SV40 in experimental animals has been studied extensively. Different routes of inoculation, different viral strains, and variation in host animals and experimental conditions have been used.

In 13 studies involving newborn (NB) outbred hamsters, the virus was inoculated subcutaneously. Fibrosarcomas were reproducibly induced at very high incidence (> 60%). Tumour induction was dose-dependent, with an increased tumour incidence related to higher levels of infectious SV40. Host age was found to be a factor, with the incidence of tumours decreasing with increasing age of the recipient animal at time of exposure.

In four studies, intracerebral inoculation of SV40 into NB outbred hamsters resulted in a high incidence of brain tumours classified as either ependymomas or choroid plexus tumours. One study showed a virus dose dependence for brain tumour induction.

Weanling hamsters were exposed to SV40 via the intravenous or intracardiac route in six studies. A variety of tumour types were reproducibly observed; the most frequent were lymphomas, osteosarcomas, and mesotheliomas. It was demonstrated that tumour incidence decreased with decreasing virus dose and with increasing age of the recipient animal. It was also

observed that SV40 small T-antigen (sT) deletion mutants preferentially gave rise to lymphomas.

When weanling hamsters were inoculated with SV40 by the intraperitoneal or intrapleural routes in four studies, the predominant tumour types induced were again mesothelioma, lymphoma, and osteosarcoma. In one study, intrapleural inoculation of SV40 yielded mesotheliomas in all recipients. Another study found that after SV40 inoculation of pregnant hamsters, tumours developed in the offspring. Other observations from these studies included a statistically significant difference in tumour incidence between SV40 virus strains.

In three studies, SV40 DNA was inoculated subcutaneously into NB hamsters. The viral DNA was tumorigenic; tumours were induced with similar tumour incidences and tumour types to those observed after exposure to infectious virus.

In two studies, SV40 was inoculated into neonatal mice. One study reported the development of sarcomas of the liver and/or spleen, the incidence of which was increased by co-infection of mice with murine malaria parasites. The other study was negative.

A single study in the rodent *Mastomys natalensis* reported a high incidence of brain tumours (ependymomas and maybe some choroid plexus tumours) after subcutaneous inoculation of SV40.

Four studies in transgenic mice involved the use of SV40 with its natural viral promoter. In all studies, choroid plexus tumours were observed at a high incidence. Thymic hyperplasia and renal lesions were also sometimes seen. In one study, the large T-antigen (LT) of lymphotropic papovavirus could substitute for SV40 LT to induce brain tumours.

In two studies comparing SV40 and JCV promoter and the LT gene in transgenic mice, the tumour types observed (e.g. choroid plexus papilloma) strongly correlated with the SV40 promoter tropism. Taken together, the evidence suggested that LT may contribute to tropism.

Co-injection of weanling hamsters with SV40 and crocidolite asbestos revealed a co-carcinogenic effect since virus plus asbestos yielded a significantly higher incidence of mesotheliomas. Synergy of SV40 with asbestos was also observed when transgenic mice carrying a high copy number of a transgene encoding only SV40 LT were exposed to asbestos: mesotheliomas developed with a shorter latent period than in the single-copy line or wild-type mice.

5.4 Mechanistic and other relevant data

There is strong and consistent evidence from animal and cell-culture studies that SV40 can be directly oncogenic and transforming through its oncoproteins, i.e. the LTs encoded in the early region of its genome. The mechanisms involve immortalization, transformation, and inhibition of apoptosis. There is only weak and controversial evidence for such mechanisms being active in human tumours.

- The presence of SV40 DNA, based on analysis by PCR, has been reported in a broad variety of human tumours by several groups but not by others. Experimentally substantiated doubts have been raised that laboratory contaminations with commercial plasmids may have contributed to some positive PCR results. Other differences in experimental methods may also contribute to discrepancies across laboratories.
- In the few studies that have quantified the amount of SV40 DNA, SV40 DNA, if found in human tumours, appears to be present mostly in low copy numbers. This indicates that the majority of cells within a tumour potentially associated with SV40 do not contain the viral genome. Thus, novel mechanisms for SV40-induced carcinogenesis would need to be invoked, for which there is currently little evidence.

- Studies that demonstrate viral oncogene expression in cells of SV40 DNA-positive human tumours are few, and the specificity of the two most widely used monoclonal antibodies has been questioned, based on experimental results from one study.
- Studies that demonstrate evidence for cell-cycle and apoptosis regulation by SV40 through pathways involving pRb family proteins and p53 in human tumours are few, and the evidence is weak.

6. Evaluation

6.1 Cancer in humans

There is *inadequate evidence* in humans for the carcinogenicity of SV40.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of SV40.

6.3 Overall evaluation

SV40 is *not classifiable as to its carcinogenicity to humans* (Group 3).

6.4 Rationale

- Compared with human polyomaviruses, seroresponsiveness to SV40 is very low.
- Well-conducted sero-epidemiological studies, using SV40 antibody tests that exclude antibodies that react to BKV and JCV, do not provide evidence that SV40 infects humans.
- Studies of sewage using different techniques in several developing and developed countries did not reveal the presence of SV40,

while the presence of other polyomaviruses and papillomaviruses was shown.

- Millions of people were exposed to SV40-contaminated vaccines, but multiple follow-up studies of recipients of contaminated inactivated poliovirus vaccines have not revealed them to be at increased risk of cancer compared with unexposed cohorts.
- Studies of cancer patients have not revealed increased prevalence of antibodies to SV40 compared with control populations.
- Case series that evaluated human tumours for SV40 DNA produced conflicting results, and there is concern that PCR contamination is responsible for some positive results.
- The mechanism of transformation in rodents has been very well established, but despite extensive studies, there is no persuasive evidence that this mechanism is operative in humans. (In addition, some members of the Working Group considered that the effects of viruses can be species-specific.)

In view of the strong evidence presented in Sections 1, 2, and 4, the Working Group decided to give less weight to the data from experimental animals when considering cancer in humans.

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BK POLYOMAVIRUS

1. Exposure Data

1.1 Cell and tissue tropism

The primary site of productive BK polyomavirus (BKV) replication is the epithelium of the human urinary tract. BKV has also been inconsistently detected in other tissues. Studies of cadaveric samples and biopsies from immunosuppressed individuals, using cytology, immunohistochemistry (IHC), electron microscopy, in situ hybridization (ISH), and polymerase chain reaction (PCR), have shown that foci of BKV infection can be found in a variety of locations in the urinary tract, including the simple epithelium of the kidney tubules and the stratified epithelium of the ureters, bladder lining, and urethra ([Hogan et al., 1980](#); [Boldorini et al., 2005a, b](#); [Singh et al., 2006](#)).

BKV antigens have also been detected inconsistently in a wide variety of other tissues and cell types, including the skin, brain, bone, colon (and faeces), prostate, female genital tract, and blood (reviewed in [Abend et al., 2009](#)).

1.2 Methods for the detection of BKV

The presence of BKV can be examined in several ways. Some techniques were available in 1971 ([Gardner et al., 1971](#)), and others were developed later. Originally, electron microscopy, virus isolation, and cytology were more frequently

used, whereas today PCR-based techniques are more often used for detection of BKV.

1.2.1 Histopathological examination

Inclusion bodies can be observed in polyomavirus-infected cells and have also been observed in epithelial cells from the urinary tract after BKV infection ([Gardner et al., 1971](#)).

Electron microscopy is useful for visualizing the morphology and size of the virus particles and thus for initial identification of viruses as polyomaviruses.

Indirect immunofluorescence can be used to detect BKV infection by using rabbit anti-BKV capsid viral protein 1 (VP1) to stain the infected cells on coverslips fixed with acetone and using a fluorescein isothiocyanate (FITC)-conjugated secondary antibody ([Takemoto & Mullarkey, 1973](#); [Gardner et al., 1971](#); [Knowles et al., 1989a](#)).

1.2.2 Virus isolation

BKV in clinical samples, for example the urine of immunosuppressed patients, can reproduce and give a cytopathic effect in Vero cells derived from African green monkey kidney ([Gardner et al., 1971](#)). Attempts to passage BKV in primary rhesus monkey kidney cells and human embryonic lung fibroblasts were unsuccessful ([Gardner et al., 1971](#)). Virus isolation is useful to identify many but not all strains of BKV, and is rarely used for clinical purposes. Attempts to isolate additional BKV strains have been made by using

other cell lines, such as primary human renal tubular epithelial cells and 293TT human embryonic kidney cells ([Olsen et al., 2009](#); [Broekema & Imperiale, 2012](#)).

1.2.3 Serology

Serological testing of BKV by different assays is based on detection of specific BKV immunoglobulin (Ig) G antibodies after past infection, and of IgM antibodies after primary infection. BKV isolates have been classified into four distinct genotypes based on their VP1 sequences; antibodies elicited by immunizing animals with virions of one type were less effective for neutralization of the other three types *in vitro*, indicating the presence of at least four BKV serotypes ([Knowles et al., 1989b](#); [Pastrana et al., 2012](#)). Serology is useful to identify past BKV infection, but most assays, some of which are described here, are not designed to distinguish among the four serotypes of BKV.

(a) Haemagglutination and haemagglutination inhibition assays

Some polyomaviruses haemagglutinate erythrocytes. BKV can haemagglutinate human blood group O erythrocytes. Thus, antibodies against BKV induce haemagglutination inhibition (HAI). Antibodies titres against BKV are obtained by incubating different dilutions of serum with a fixed haemagglutination unit (HAU) of BKV and then adding the blood group O erythrocytes. The highest dilution resulting in HAI is the HAI titre of the serum. HAI is not completely specific for BKV since there is serological cross-reactivity between BKV and, for example, JC polyomavirus (JCV) ([Gardner et al., 1971](#); [Takemoto & Mullarkey, 1973](#); [Taguchi et al., 1982](#); [Flaegstad et al., 1988](#)). Non-specific inhibitors present in some sera can be removed by treating the serum with neuraminidase ([Gardner et al., 1971](#); [Flaegstad & Traavik, 1985a](#)).

(b) BKV IgG- and IgM-class specific ELISA

Standard enzyme-linked immunosorbent assay (ELISA) can be used to test for IgG and IgM antibodies against BKV ([Flaegstad & Traavik, 1985a, b](#); [Kean et al., 2009](#)). Virus antigen, for example derived from extracts of BKV-infected Vero cells or other sources, such as virus-like particles (VLPs), can be used. For IgG titres, a solid-phase ELISA can be used and the BKV antigen absorbed to microtitre plates, then incubated with bovine serum albumin to inhibit later non-specific binding of antibodies. Subsequently, the relevant antiserum is added, substrate is added, and after stopping of the reaction and relevant washing procedures, the plates are read in a spectrophotometer. For IgM, the microplates are first coated with goat anti-human IgM overnight to bind human IgM from the serum, and then the procedure is similar to that of the IgG test ([Flaegstad & Traavik, 1985a, b](#); [Kean et al., 2009](#)).

(c) Luminex-based multiplex serological assays

Detection of BKV antibodies has been facilitated by the introduction of multiplex bead-based array serology [i.e. Luminex]. [Antonsson et al. \(2010\)](#) described the use of a Luminex-based multiplex serological assay for detection of antibodies to the VP1 capsid proteins of BKV. As antigen, full-length viral proteins of BKV can be expressed in bacteria in fusion with an N-terminal glutathione S-transferase (GST) domain. VLPs of different polyomaviruses can also be used as antigens. Absorbing out the activity from different cross-reacting polyomaviruses allows the assay to be more specific for the polyomavirus examined.

1.2.4 PCR-based methods

In 1989, a PCR method for detection of BKV and JCV was designed, and this assay was specific, with a sensitivity of 10 copies for

BKV and 100 copies for JCV ([Arthur et al., 1989](#)). Later, a nested PCR method with better sensitivity was developed for detection of BKV and JCV ([Bogdanovic et al., 1994](#)). With this assay, 10 copies of the genomic DNA of each of the two viruses could be detected. The primers were chosen within the early conserved regions of BKV encoding the large T-antigen (LT) and small T-antigen (sT), where JCV shares 84% homology and simian virus 40 (SV40) shares 76% homology, and the amplified products were very similar in size. To distinguish between the viruses, restriction enzyme cleavage was used or oligoprobes of the different viruses were used ([Bogdanovic et al., 1994](#); [De Mattei et al., 1995](#)). Since then, additional PCR-based methods have been developed, namely real-time quantitative PCR techniques ([Azzi et al., 1999](#); [Priftakis et al., 2003a](#)). [Many of the primers first developed for detection of BKV did not detect all subtypes ([Randhawa et al., 2011](#)).]

Multiplex/Luminex-based PCR methods have now been developed for the detection of BKV and other human polyomaviruses; these are generally very specific and sensitive and can detect copy numbers down to 10 copies/sample ([Schmitt et al., 2011](#)).

Because PCR-based methods are extremely sensitive, they pose a significant risk of false-positive detection of laboratory-derived sequences ([Cohen & Enserink, 2011](#)). Conversely, PCR primers that target portions of the BKV genome that are not fully conserved among all genotypes may fail to detect a subset of BKV isolates ([Hoffman et al., 2008](#); [Randhawa et al., 2011](#)). It is thus important that PCR results be confirmed using other DNA analysis methods. Confirmatory approaches include sequencing of PCR products to allow viral genotyping and detection of sequence variations specific to individual specimens, Southern blotting of DNA extracted from the sample, and ISH.

1.2.5 Diagnosis of BKV infection

(a) Indirect methods

Serology, for example ELISA or HAI assays, is the best way to diagnose past BKV exposure ([Flaegstad & Traavik, 1985a, b](#); [Flaegstad et al., 1986, 1988](#); [Bogdanovic et al., 1998](#); [Abend et al., 2009](#)). Serology is also superior for large extensive epidemiological studies but is more rarely used to detect primary infection since most primary infections occur asymptomatically in early childhood. Upon reactivation, not all individuals exhibit increases in anti-BKV IgG titres. Here, other tests are better, such as those based on PCR for detection of BKV.

(b) Direct methods

For diagnosis, for example of BK viraemia or BK viruria, detection of BKV DNA by nested PCR or quantitative real-time PCR is sensitive and more useful, although it is possible to use electron microscopy and virus isolation ([Gardner et al., 1971](#); [Azzi et al., 1999](#); [Bogdanovic et al., 1994, 2004](#)).

1.3 Epidemiology of BKV infection

1.3.1 Natural history, latency, persistence, and prevalence

(a) Prevalence and sero-epidemiology

BKV infection is extremely common worldwide. Sero-epidemiological studies show that overall, > 90% of the general adult population have anti-BKV IgG antibodies (see [Table 1.1](#); and additional data presented by [Gardner, 1973](#); [Shah et al., 1973](#); [Flaegstad et al., 1986](#); [Carter et al., 2003](#); [Rollison et al., 2003, 2006](#)). Furthermore, acquisition of antibodies begins in early childhood, and > 50% of all children have antibodies to BKV at age 3 years or earlier ([Gardner, 1973](#); [Shah et al., 1973](#); [Flaegstad et al., 1986](#)). The sero-prevalence of BKV is reported to be 60–90% in young adults and can reach up to 99% in the

middle-aged population, with slowly declining prevalence in very old people ([Table 1.1](#); [Walker & Padgett, 1983](#); [Lundstig & Dillner, 2006](#); [Antonsson *et al.*, 2010](#)).

(b) *Primary infection and transmission of BKV*

The route by which primary BKV infection is transmitted is still unknown. Primary infection is not correlated with any symptoms or clinical illness, but seroconversion indicates that primary infection occurs early in childhood and is horizontal ([Bofill-Mas *et al.*, 2000](#); [Bofill-Mas *et al.*, 2001](#); [Yogo *et al.*, 2007](#); [Abend *et al.*, 2009](#)). A respiratory transmission route has been suggested, and serological studies have shown examples of BKV seroconversion during mild respiratory infections, but these infections have never truly been identified as due to BKV infection ([Goudsmit *et al.*, 1982](#); [Sundsford *et al.*, 1994a](#); [Abend *et al.*, 2009](#)). Alimentary transmission has also been proposed, as a result of contamination of food and water with urine and faeces in sewage, where these viruses are frequently excreted asymptotically in the urine of healthy individuals ([Bofill-Mas *et al.*, 2001](#)). In addition, [Pietropaolo *et al.* \(2003\)](#) hypothesized that sexual transmission of BKV may be possible since BKV DNA was found in the genital organs (uterine cervix and prostatic urethra) of HIV-infected individuals at autopsy.

(c) *Persistence of BKV*

After primary infection, BKV remains latent in the kidneys, and possibly in the brain and peripheral blood ([Dörries, 2006](#)).

DNA–DNA hybridization (DNA extracted from normal renal tissue and hybridized with ³²P-labelled cloned BKV DNA) detected BKV DNA from ~30% to > 50% of the tested cadaver kidneys of patients without renal diseases, and showed that the viral genome was generally not integrated ([Heritage *et al.*, 1981](#); [Chesters *et al.*, 1983](#)). With more sensitive techniques, the detection rate increased, and the kidney is

regarded as the major site for persistent BKV infection ([Hirsch & Randhawa, 2009](#)). BKV is distributed in small foci throughout the cortex and medullary regions of the kidney, this is why it has been proposed that several renal regions should be examined, especially in case of disease ([Heritage *et al.*, 1981](#); [Chesters *et al.*, 1983](#); [Hirsch & Randhawa, 2009](#)). The specific cell types where the virus remains latent have not yet been identified, but viral inclusions have been detected in transitional renal tubules as well as in transitional epithelial cells of the urinary tract ([Hogan *et al.*, 1980](#); [Shinohara *et al.*, 1993](#); [Boldorini *et al.*, 2005a](#); [Singh *et al.*, 2006](#)).

In immunocompetent individuals, BKV DNA has occasionally been detected in peripheral blood and the central nervous system, and the frequency of detection of BKV at these sites is increased in immunocompromised individuals or in patients with other diseases ([Elsner & Dörries, 1992](#); [Schneider & Dörries, 1993](#); [Dörries *et al.*, 1994](#); [Sundsford *et al.*, 1994b](#); [Bogdanovic *et al.*, 1996](#); [Vago *et al.*, 1996](#); [Behzad-Behbahani *et al.*, 2003](#); [Bialasiewicz *et al.*, 2009](#)).

As a manifestation of persistence and reactivation in healthy individuals, BK viruria can be observed without any clinical symptoms or disease, whereas BK viraemia or the presence of BKV in cerebrospinal fluid occurs mainly in immunosuppressed patients ([Chesters *et al.*, 1983](#); [Sundsford *et al.*, 1994b](#); [Jin *et al.*, 1995](#)).

1.3.2 Pathologies other than cancer associated with BKV

Only a limited number of clinical diseases are currently known to be associated with BKV reactivation, and they are detected in immunosuppressed individuals. Two such BKV-associated diseases are haemorrhagic cystitis (HC) in allogeneic haematopoietic stem cell transplant (HSCT) recipients ([Dalianis & Ljungman, 2011](#)) and polyomavirus-associated nephropathy (PVAN) in renal transplant recipients ([Hirsch](#)

Table 1.1 Detection of BKV antibodies using different methods in healthy individuals of different ages from different populations

Reference	Study population and age	Study location	Method	No. of subjects	Prevalence of anti-BKV antibodies (%)
Brown <i>et al.</i> (1975)	Adults and children	28 isolated populations	HAI	1544	0–90% (depending on the population)
Knowles <i>et al.</i> (2003)	Adults and children	England	HAI	2435	Overall, 81%; 1–4 yr, 64%; 5–9 yr and 10–59 yr, 91%; 60–69 yr, 68%
Stolt <i>et al.</i> (2003)	Children and pregnant women	Sweden (children) Finland (women)	VLP-based ELISA blocked SV40/BKV/JCV	290 children, 1656 women	1–3 yr, 20%; 3–13 yr, 63–98%; Pregnant women 14–31 yr, 80–96%
Egli <i>et al.</i> (2009)	Blood donors	Switzerland	VLP-based ELISA	400	Overall, 82%; 20–29 yr, 87%; 50–59 yr, 71%
Kean <i>et al.</i> (2009)	Blood donors: adults (> 21 yr) and group aged 1–21 yr	Denver, Colorado, USA	VP1 capsomer-based ELISA	1501 721	> 21 yr, 82%; 1–21 yr, 73%
Antonsson <i>et al.</i> (2010)	Adults	Australia	VP1-based ELISA/ Luminex	458	> 4 yr, 97%; 25–60 yr, 99%; > 60 yr, 94%
Viscidi <i>et al.</i> (2011)	Healthy adults (1–93 yr)	Italy	VLP-based ELISA	947 total: 568 men, 374 women, 5 unknown	< 10 yr, 62%; 10–39 yr, 75–79%; 40–69 yr, 60–64%; > 70 yr, 55%

BKV, BK polyomavirus; ELISA, enzyme-linked immunosorbent assay; HAI, haemagglutination inhibition; JCV, JC polyomavirus; SV40, simian virus 40; VLP, virus-like particle; VP1, capsid viral protein 1; yr, year

& Randhawa, 2009); these are described in more detail below. BKV can also, in very rare cases, be associated with encephalitis or other diseases of the central nervous system ([Vallbracht et al., 1993](#); [Behzad-Behbahani et al., 2003](#); [Jørgensen et al., 2003](#)).

(a) *Haemorrhagic cystitis and its management*

HC associated with BKV presents with symptoms of increasing severity and is graded accordingly by different, but similar, systems ([Arthur et al., 1986](#); [Hassan et al., 2009](#)). BKV-associated HC often presents as microscopic haematuria (Grade I), macroscopic haematuria (Grade II), macroscopic haematuria with clots (Grade III), or macroscopic haematuria with clots and impaired renal function secondary to urinary tract obstruction (Grade IV), as graded, for example, by [Bedi et al. \(1995\)](#). Dysuria and lower abdominal pain are additional criteria. Most HC cases resolve, but sometimes the patients may need blood transfusions due to intensive bleeding, and together with other complications, HC may be lethal. Thus, BKV-associated HC causes significant suffering and mortality and prolongs hospital care; therefore, identifying patients at risk could help prevent, or enable better management of, the disease ([Bogdanovic et al., 2006](#)).

BKV-associated HC usually occurs 1 week to 6 months after HSCT and is classified as late-onset HC, to separate it from early-onset HC, caused 1–2 days after HSCT by the toxicity of the conditioning regimen (containing cyclophosphamide and/or busulfan) ([Brugieres et al., 1989](#); [Bogdanovic et al., 2006](#); [Hassan et al., 2009](#); [Dalianis & Ljungman, 2011](#)). BK viruria occurs in 50–100% of HSCT patients and is associated with an increased risk of HC, but it is not diagnostic for HC since only 4–25% of HSCT patients eventually develop HC, although in some reports the prevalence of HC has been suggested to be higher (50–70%) ([Arthur et al., 1986](#); [Apperley et al., 1987](#); [Azzi et al., 1994](#); [Vögeli et al., 1999](#); [Leung et al., 2002](#); [Bogdanovic et al., 2006](#); [Hassan et al.,](#)

[2009](#); [Dalianis & Ljungman, 2011](#)). Nevertheless, substantial accumulated evidence has shown that BKV is connected to HC, and several studies have been initiated to better predict the development of BKV-related HC.

A high BKV load of $> 10^6$ BKV copies/ μ L urine was suggested to be indicative for a risk of HC, but not diagnostic for HC, and neither was the quantity of BKV DNA in peripheral blood plasma and serum ([Biel et al., 2000](#); [Priftakis et al., 2003a](#); [Bogdanovic et al., 2004](#); [Erard et al., 2005](#)). Primary BKV infection was dismissed as a major cause of HC ([Bogdanovic et al., 1998](#)), and although BKV variants with mutations in the non-coding control region (NCCR) were more common in HC, the data were not conclusive ([Priftakis et al., 2001](#)). Acute graft-versus-host disease (GVHD) was also suggested as a cofactor for the development of HC but was not confirmed as such in later studies ([Ost et al., 1987](#); [Leung et al., 2002](#); [El-Zimaity et al., 2004](#); [Giraud et al., 2006](#)).

More recently, it was shown that patients receiving full myeloablative conditioning before HSCT have a higher risk of developing HC compared with patients receiving a reduced-intensity conditioning regimen ([Giraud et al., 2006](#)). Moreover, having an unrelated donor with a human leukocyte antigen (HLA) mismatch and myeloablative conditioning presented a special risk ([Yamamoto et al., 2003](#); [El-Zimaity et al., 2004](#); [Giraud et al., 2006, 2008](#)).

Treatment is focused on managing patient symptoms since there is no specific treatment for BKV-associated HC ([Atkinson et al., 1991](#); [Laszlo et al., 1995](#); [Turkeri et al., 1995](#); [Hassan et al., 2009](#)). Analgesia, hyperhydration, and forced diuresis are used, and when necessary blood transfusions are given and urological intervention performed ([Brugieres et al., 1989](#)). Antiviral agents, for example low-dose cidofovir, are also used but have not been compared in formal randomized trials ([Savona et al., 2007](#); [Cesaro et al., 2009](#)). Transfusion of mesenchymal stem cells

has been attempted to abrogate HC, but there is limited evidence of their efficacy ([Hassan et al., 2009](#)).

(b) *Polyomavirus-associated nephropathy and its management*

Polyomavirus-associated nephropathy (PVAN) emerged as a disease after the substitution of cyclosporine A with tacrolimus for immunosuppression and is found in 1–10% of renal transplant patients ([Randhawa et al., 1999](#); [Hirsch & Randhawa, 2009](#); [Ramos et al., 2009](#)). PVAN is caused by BKV in > 95% of the cases, while < 5% are attributed to JCV ([Ramos et al., 2009](#)). In general, patients were clinically asymptomatic, although high urine BKV DNA loads of > 7 log₁₀ genome equivalents (geq)/ml were observed. The high levels of BK viruria preceded the onset of detectable plasma BKV loads and histological evidence of nephropathy ([Hirsch, 2002](#); [Hirsch et al., 2002, 2005](#)).

The diagnosis of PVAN can be confirmed by demonstrating polyomavirus-specific changes in the renal allograft tissue ([Hirsch & Randhawa, 2009](#)). These changes should be confirmed by IHC for BKV. Shedding in the urine of BKV-infected “decoy cells,” of which the altered nuclei and the irregular shape often mimic changes observed in neoplastic cells, is also characteristic for PVAN.

In patients with persistent high-level BKV replication, viral variants with rearranged NCCR emerged *in vivo*, associated with 20-fold higher plasma BKV loads and more advanced BKV nephropathy in the renal allograft ([Gosert et al., 2008](#)). The NCCR variants differed widely and consisted of deletions, duplications, and complex combinations in the promoter/enhancer elements. *In vitro* studies demonstrated that rearranged NCCR variants increased BKV early gene expression and altered replication capacity *in vitro* compared with the naturally occurring archetype virus ([Gosert et al., 2008](#); [Olsen et al., 2009](#); [Myhre et al., 2010](#)).

So far, there are no effective antivirals against PVAN, and therefore the treatment of choice in many centres has been reduction of immunosuppression ([Hirsch & Randhawa, 2009](#)).

(c) *Other disease associations*

The role of BKV in systemic lupus erythematosus (SLE) has been studied, but the available data do not support a causal role of BKV for SLE-associated nephropathy ([Bendiksen et al., 2000](#); [Colla et al., 2007](#); [Lu et al., 2009](#)).

BK viraemia and BK viruria have been reported in patients with non-renal solid organ transplants, for example patients with heart and liver transplants; so far, there is no evidence for high risk of renal disease in these patients ([Puliyanda et al., 2006](#); [Amir et al., 2011](#)).

2. Cancer in Humans

Methodological considerations: case–control versus case-series study designs

Numerous studies have reported the prevalence of markers of infection by polyomaviruses in tumour tissues or blood obtained from humans with cancer. Many of these studies included specimens from individuals without cancer as “controls,” but such studies were not generally considered by the Working Group as case–control studies, given the convenience sampling strategies used or the lack of comparability of exposure measurement between comparison groups. Specifically, convenience sampling of controls led to the possibility that the control subjects were not representative of the source population. Also, the comparison of tumour tissues in cases with normal tissues (such as blood, urine, or biopsies of normal tissues) in controls may also be biased, because it is uncertain whether polyomaviruses are uniformly present in these normal tissues or can be reliably detected by the assays used. However, because

these studies contribute information on cancer sites not investigated by the case–control studies, included comparisons with both normal and pre-malignant control tissues, compared tumour tissue with a convenience sample of controls, compared different tissues in cases or controls, and/or presented findings for susceptible populations (i.e. transplant patients), they are considered here as case series.

2.1 Background

Interest in research on BKV infection in human cancer was spurred by studies of carcinogenicity in experimental animals and subsequent case series of BKV DNA detection in human cancers. Older case series have reported BKV DNA in a variety of tumours, notably in brain, pancreas, lung, liver, rhabdomyosarcoma, Kaposi sarcoma, and urinary tract cancers ([Abend *et al.*, 2009](#)). A variety of detection methods have been used, including ISH, immunohistochemistry for LT expression, serology for the viral capsid antigen VP1, or neutralization assays, as well as detection of the viral genome by PCR and characterization by sequencing ([Abend *et al.*, 2009](#)). The high sensitivity of the PCR technology is also associated with a risk of false-positive results caused by contamination.

Epidemiological studies have to a large extent used serology based on the BKV viral capsid antigen. These antibodies are induced by BKV infection early in life and are stable over time ([Stolt *et al.*, 2003](#)). BKV capsid antibodies as a marker of exposure to the virus have been validated by correlation of the presence and level of these antibodies with shedding of BKV DNA in urine ([Engels *et al.*, 2005](#)).

BKV isolates can be broadly categorized into four genotypes. Whereas more recent PCR-based studies have used primers targeting portions of the BKV genome that are conserved among all genotypes, older studies used primers designed solely based on BKV genotype I isolates. As a

result of mismatches between the primers and BKV genotypes II, III, and IV, some of these older primer sets detect non-BKV-I genotypes less efficiently ([Hoffman *et al.*, 2008](#); [Randhawa *et al.*, 2011](#)).

The four BKV genotypes are also distinct from one another in serological assays ([Knowles *et al.*, 1989b](#); [Pastrana *et al.*, 2012](#)). This raises the possibility that past serological studies, which have typically focused on BKV genotype I, may have underestimated the prevalence or serological titres for BKV genotypes II, III, and IV.

Whereas several isolates of BKV appeared to be serologically distinct (with little cross-reactivity to other BKV isolates), two BKV isolates (SB and AS strains) are broadly cross-reactive among different BKV serotypes ([Knowles *et al.*, 1989b](#)) but were reported to be serologically distinct from each other. Initial validation studies of BKV serology used VLPs of both BKV SB and AS strains. The serological responses to both these isolates were found to be highly correlated, but with somewhat higher antibody titres when using BKV SB strain as antigen ([Stolt *et al.*, 2003](#)). Although the most common BKV subtype circulating in human populations is subtype I ([Tremolada *et al.*, 2010b](#)), BKV SB strain (belonging to subtype II) and BKV AS strain (belonging to subtype III) appear to provide a broad detection of BKV antibodies, and most subsequent sero-epidemiological studies have used BKV SB strain as antigen, as far as possible, to obtain a broad detection of BKV antibodies ([Lundstig & Dillner, 2006](#)).

Recently, there has been evidence to indicate that the different BKV subtypes may have different biological characteristics. For example, subtype III appeared to be less viable when propagated in cell culture ([Tremolada *et al.*, 2010a](#)), and isolates from patients with BKV-associated nephropathy appeared to less commonly belong to subtype I than isolates from healthy subjects ([Tremolada *et al.*, 2010b](#)). Rare subtypes of BKV are viable and frequently detected in renal transplant recipients

with BKV-associated nephropathy ([Tremolada et al., 2010a](#)). Thus, it is possible that different BKV subtypes may have different associations with human disease. It would certainly be possible to design subtype-specific BKV serology, by selection of isolates from non-cross-reactive strains as antigens, but such studies have not yet been performed.

Several serology-based case-control studies have looked at the prevalence of antibodies close to the date of diagnosis and several prospective studies have assessed antibody status many years before the diagnosis of cancer. An advantage of serology-based methods is that they measure exposure of the body as a whole; so they are not susceptible to the risk of non-representative samples as it could happen when looking for viral DNA in specific tissues.

Other studies have focused on detecting BKV DNA or virus products in tumour tissue (or urine), either in case series or with comparison of “control” tissue (defined as either tissue from the same organ site in subjects without cancer or adjacent healthy tissue from cases). Here, the hypothesis is that if BKV plays a role in causing these cancers, the virus will be found more frequently in tumour tissue than in control tissue. However, such associations do not necessarily imply causation – particularly in the absence of other epidemiological evidence for an association between infection and an increased risk.

The results of prospective studies and formal case-control studies are summarized below, by cancer site. In addition, the Working Group also describes selected case series, including more recent publications not cited in previous reviews.

Many studies have investigated BKV concomitantly with JCV, using comparisons of the different polyomaviruses as a marker for the specificity of association. Some of the studies that have investigated the role of SV40 in human cancer have also investigated BKV, either as a result of cross-reactions or to be able to control

for such cross-reactions. [In the case series that evaluated tumours for the presence of SV40 DNA, BKV would have been detected had it been present, because the PCR primers used in those studies also amplify sequences from BKV and JCV.]

2.2 Cancer of the prostate

2.2.1 Cohort studies

See [Table 2.1](#)

[Newton et al. \(2005\)](#) studied the presence of BKV antibodies at baseline in a cohort study in the United Kingdom and followed up for cancer outcomes. There were no statistically significant differences in either the prevalence or the level of antibodies against BKV at baseline between cases with cancer of the prostate ($n = 31$), kidney ($n = 5$), or bladder ($n = 9$) and the controls ($n = 45$). There was no effect of age. [Although a strength of this study is the design, a case-control study nested in a prospectively followed cohort, the small size of the study is a limitation.]

2.2.2 Case-control studies

[Carter et al. \(2003\)](#) studied prostate cancer cases identified through the Seattle-Puget Sound Surveillance, Epidemiology and End Results (SEER) cancer registry between January 1993 and December 1996. Controls of similar age were selected from the same population as the cases by random-digit dialling ([Carter et al., 2001](#)). For this study, a subset of 90 cases and 72 control subjects was available and was used to examine the prevalence of SV40, BKV, and JCV antibodies in sera detected by ELISA using VLPs; 33 (37%) of the 90 case subjects were seropositive for BKV, and 42 (58%) of the 72 controls. There was a significant inverse association with prostate cancer [crude odds ratio (OR), 0.41; 95% confidence interval (CI): 0.21–0.81]. Other polyomaviruses (JCV and

Table 2.1 Cohort and nested case–control studies of BKV and cancer in humans

Reference, period, study location	Cohort description	Detection method	Organ site (ICD code)	Exposure categories	No. of cases	RR/OR (95% CI)	Covariates	Comments
Rollison <i>et al.</i> (2003) , Follow-up: 1974–2000 USA	> 45000 cohort members; 44 brain tumour cases	BKV IgG by VLP-based ELISA pre-adsorbed with JCV VLPs	Brain (ICD-9191); meninges (ICD-9192)	BKV-positive titres < 640 640 or 2560 ≥ 640 ≥ 10 240	8 17 36 19	Ref. 0.61 (0.19–1.94) 0.66 (0.22–1.95) 0.72 (0.23–2.27)	Controls matched on age, sex, and race.	Case–control study nested within prospective cohort (individually matched controls). BKV seroprevalence: 86.4% in controls, 81.8% in cases. Overlaps with Rollison <i>et al.</i> (2006) , listed above.
Newton <i>et al.</i> (2005) , 1993–99 United Kingdom	EPIC-Oxford cohort: 58000 individuals; 45 cases (31 prostate, 5 kidney, and 9 bladder cancers) and 45 matched controls	BKV antibodies in serum by VLP-based ELISA	Prostate Kidney Bladder	Doubling of anti-BKV antibody titre		0.9 (0.6–1.2) 1.8 (0.5–7.0) 0.8 (0.4–1.6)	Cases and controls matched on age, sex, geographical location, length of follow-up, and date of blood matching ($\pm$ 1 mo).	Case–control study nested within EPIC-Oxford cohort; Prevalence of anti-BKV antibodies: 91% (41/45) controls; 94% (29/31) prostate cancer; 100% (5/5) kidney cancer; 78% (7/9) bladder cancer
Stolt <i>et al.</i> (2005) , 1983 onwards Finland	1.2 million serum samples from pregnant women; 115 neuroblastoma cases of the offspring	Capsid IgG and IgM by ELISA pre-adsorbed with SV40 and JCV VLPs; BKV DNA by PCR	Neuroblastoma	BKV IgG positive BKV IgM positive	92 3	0.8 (0.5–1.3) 0.6 (0.2–1.9)	Adjusted for age at serum sampling; control women matched on sampling date, residence.	Case–control study nested within a cohort of pregnant women (12–14 wk of gestation) in Finland (115 pregnant mothers who gave birth to children with neuroblastoma, and 8 control women per case). BKV IgG/IgM seroprevalence: IgG: 80% in cases, 83% in controls; IgM: 3% in cases, 4% in controls.

Table 2.1 (continued)

Reference, period, study location	Cohort description	Detection method	Organ site (ICD code)	Exposure categories	No. of cases	RR/OR (95% CI)	Covariates	Comments
Rollison <i>et al.</i> (2006) , 1974, 1989 USA Follow-up: 1974–2002	> 45000 cohort members; 170 NHL cases	BKV IgG by VLP-based ELISA	NHL	BKV IgG positive	145	0.98 (0.64–1.48)	Controls ($n = 340$) matched 2:1 on age (± 1 yr), sex, race, freeze/thaw status of the serum or plasma, and participation in CLUE I, CLUE II, or both and blood draw date. Stratified by NHL subtype/induction period. Adjusted for EBV seropositivity.	Nested case–control study. BKV seroprevalence: 67% in cases, 67% in controls. Overlaps with Rollison <i>et al.</i> (2003) , listed below.
Lundstig <i>et al.</i> (2007) , 1973–30 yr follow-up, Norway	Cohort of 333000 individuals; 386 randomly selected male cases with no previous malignancy, who had baseline serum samples taken > 3 mo before diagnosis	BKV IgG Antibodies in serum by VLP-based ELISA	Colorectum	BKV IgG positive	273	1.1 (0.8–1.5)	Controls matched on sex, age (± 1 yr), date of blood sampling (± 2 mo), and county of residence.	Case–control study nested in a prospectively cohort. BKV IgG seroprevalence: 71% in cases, 69% in controls. Determining seropositivity using alternative cut-offs also found no evidence of excess risk.

Table 2.1 (continued)

Reference, period, study location	Cohort description	Detection method	Organ site (ICD code)	Exposure categories	No. of cases	RR/OR (95% CI)	Covariates	Comments
Chen <i>et al.</i> (2010) , Follow-up: 1983–2007 Taiwan, China	Cohort of 864 or 990 kidney transplant patients (number not clear)	Diagnosis of PVAN based on biopsy and further confirmed by IHC for BKV or JCV	Any cancer among PVAN patients	Prevalence of PVAN				Cohort of kidney transplant patients. PVAN diagnosis as a proxy of exposure to polyomaviruses (including BKV); 83% (5/6) of PVAN patients developed cancer, but only 11.5% of all recipients ($P < 0.0001$): Transitional cell carcinoma ($n = 2$), renal cell cancer ($n = 1$), squamous cell carcinoma of skin ($n = 1$), Kaposi sarcoma ($n = 1$)

BKV, BK polyomavirus; CI, confidence interval; EBV, Epstein–Barr virus; ELISA, enzyme-linked immunosorbent assay; EPIC, European Prospective Investigation into Cancer and Nutrition; Ig, immunoglobulin; IHC, immunohistochemistry; JCV, JC polyomavirus; mo, month; NHL, non-Hodgkin lymphoma; OR, odds ratio; PCR, polymerase chain reaction; PVAN, polyomavirus-associated nephropathy; Ref., reference; RR, relative risk; SV40, simian virus 40; VLPs, virus-like particles; wk, week; yr, year

SV40) had similar seroprevalences among cases and controls.

2.2.3 Case series

A study by [Martinez-Fierro *et al.* \(2010\)](#) examined the association between the presence of DNA from various viruses, including BKV, in prostate cancer, and a gene variant reported to occur more frequently in cases of prostate cancer than in healthy individuals. A total of 55 cases and 75 controls were enrolled from individuals who underwent transrectal biopsy or transurethral resection at a hospital in Mexico for various reasons (including prostate cancer). Positive and negative controls were included as part of quality control for viral sequence detection. BKV DNA testing by PCR found that all cases and controls were negative for BKV DNA.

[Das *et al.* \(2008\)](#) conducted a small study comparing the presence of BKV DNA in prostate tissue specimens from prostate cancer cases and bladder cancer cases with no prostate cancer histology. ISH techniques identified BKV DNA in a lower proportion of normal prostates (4/15; 27%) than cancerous ones (11/14; 79%). BKV LT expression was detected by IHC in a lower proportion of normal specimens (4/29) than cancerous ones (13/28), and where detected was associated with proliferative inflammatory atrophy. Case-control differences were statistically significant. [A major limitation was the small study size. The use of patients with bladder cancer as controls to which the cases were compared is also a limitation.]

[Newton *et al.* \(2006\)](#) studied a case series of 821 patients with different tumour types in Uganda. The subjects were tested for BKV antibodies by ELISA and seroprevalences analysed by cancer site, using all other cancer sites as controls. The mean optical density was ~17% lower among patients with oral cancer ($P = 0.01$, based on 30 cases) and ~20% higher among those with prostate cancer ($P = 0.01$, based on 11 cases). Almost

all subjects were seropositive, and measures of association were therefore restricted to comparison of antibody levels. [Multiple comparison testing is a possible explanation for the positive and negative associations seen.]

[Balis *et al.* \(2007\)](#) studied a prostate cancer case series of 44 patients; 19% were BKV DNA-positive by PCR. [Lau *et al.* \(2007\)](#) described a case series of 30 prostatic adenocarcinomas tested by PCR; 2 of 30 were BKV DNA-positive.

2.3 Cancer of the colon

2.3.1 Cohort study

See [Table 2.1](#)

[Lundstig *et al.* \(2007\)](#) conducted a nested case-control study of JCV and colorectal cancer (CRC) among male participants in a large cohort study in Norway. Blood samples were obtained from 330 000 individuals in 1973 as part of a health screening programme, mainly for cardiovascular disease. The national, population-based cancer registry was used to identify incident cases of CRC that subsequently developed among male cohort participants ($n = 1105$). The study was originally designed to assess leptin levels and CRC, an association reported as being specific to males. From this pool of CRC cases, 400 men who had no history of cancer at the time of cohort enrolment and were diagnosed with CRC at least 3 months after blood draw were randomly selected, of whom 386 had blood samples that could be located. Time between blood sampling and diagnosis ranged from 4 months to 28 years. An equal number of controls were matched to the CRC cases on age (within 1 year), date of blood draw (within 2 months), and county. Controls were male cohort participants who had not developed cancer as of the date of diagnosis of the case. [It was not stated whether the controls were confirmed to be alive as of the same date.] IgG antibodies to JCV and BKV were measured from the archived blood samples using VLP-based

ELISA. BKV seropositivity had a relative risk of 1.1 (95% CI, 0.8–1.5). [The study design, a case–control study nested in a prospectively followed cohort, was a strength.]

2.3.2 Case–control study

[Campello *et al.* \(2010\)](#) compared the prevalence of BKV DNA detected with PCR in tissue and blood from 94 patients with colon cancer (age, 37–90 years) and from 91 controls (age, 32–70 years), who were relatives of the case subjects. From all cases and controls, blood samples were tested for BKV DNA; from the cases, the colon cancer tumour was tested, and from controls, normal colon tissue was tested. In addition, urine samples were collected and tested from 5 patients, 32 of the family controls, and 30 healthy pregnant women living in the same area. Sensitive techniques were used, including positive and negative controls. No BKV was detected in case or control specimens, including colon tumours, colon tissue, blood, and urine. [Using relatives as controls may overmatch on determinants of infection.]

2.4 Haematological malignancies

2.4.1 Cohort studies

See [Table 2.1](#)

[Rollison *et al.* \(2006\)](#) performed a prospective study nested in a serum biobank cohort established in Washington County, Maryland, USA, in 1974 ($n \approx 24\,000$) and 1989 ($n \approx 25\,000$), with ~8000 individuals participating in both cohorts ([Rollison *et al.*, 2009](#)). Participants completed a baseline questionnaire and donated a blood sample for research. Follow-up questionnaires were administered to participants of the 1989 cohort in 1996, 1998, 2000, and 2002. Cohort participants were followed over time for cancer diagnoses using the Washington County and Maryland State cancer registries. Among cohort

participants, 170 incident cases of NHL with available blood samples were identified. Two controls were matched to each case on sex, race, age (within 1 year), and date of blood draw (within 2 weeks). Controls had to have been alive and not have developed cancer as of the date of diagnosis of the matched case. Archived baseline serum (from the 1974 cohort) or plasma (from the 1989 cohort) was obtained for all NHL cases and controls and analysed by serology for BKV. The odds ratio was 0.98 (95% CI, 0.64–1.48). [A strength of this study is that the presence of antibodies up to 30 years earlier than the case date were considered, rather than at the time of case diagnosis.]

2.4.2 Case–control studies

[Priftakis *et al.* \(2003b\)](#) conducted a case–control study of BKV or JCV infection and childhood acute lymphoblastic leukaemia (ALL) in Sweden using Guthrie card samples, which are obtained from 99.8% of newborn (NB) infants in Sweden within 3–5 days of birth for screening of metabolic disorders at a central national laboratory. Children being treated for ALL were identified from four hospitals in Sweden in 1980–2001, and their Guthrie cards were obtained ($n = 54$). Their ages ranged from 9 months to 17 years. The investigators sought to obtain Guthrie cards for children who did not develop ALL, matched to the cases on age and birthplace, but were able to obtain consent for participation from only 37 of the 54 identified controls. DNA was extracted from the blood spots on the Guthrie cards, and nested PCR was used to detect JCV and BKV sequences. To exclude the possibility of false negatives, all samples amplified the gene (*HLA-DQ*) as a control; none amplified DNA from either JCV or BKV, and thus no measures of associations were calculated.

[Engels *et al.* \(2005\)](#) performed a population-based case–control study of antibodies to JCV and BKV among patients with NHL in the

USA (724 cases, 622 controls). BKV antibody levels or the presence of BKV antibodies did not differ between the cases and controls (OR, 0.96; 95% CI, 0.77–1.21).

2.5 Neuroblastoma

2.5.1 Cohort studies

See [Table 2.1](#)

[Stolt *et al.* \(2005\)](#) conducted a population-based nested case–control study of BKV or JCV infection and childhood neuroblastoma in Finland, where > 98% of all pregnant women are screened for rubella immunity in their first trimester of pregnancy, with the leftover blood samples archived for use in research. The study identified 115 cases of childhood neuroblastoma diagnosed in 1983 and later from the population-based Finnish national cancer registry, which achieves close to 100% coverage in reporting. Serum samples were identified from 121 mothers of the 125 case subjects. Eight controls were selected for each case by locating the blood sample for the mother of the case subject and choosing the next eight samples located in the specimen box, corresponding to women whose children did not develop neuroblastoma. The blood samples were arranged in the storage boxes according to date of blood collection and geographical region, and therefore the controls were matched to the cases on these factors. The odds ratio for BKV IgG seropositivity was 0.8 (95% CI, 0.5–1.3). Likewise, BKV IgM was not associated with neuroblastoma risk (OR, 0.6; 95% CI, 0.2–1.9). The serum samples and 10 neuroblastoma cell lines were also tested for BKV DNA, but all were negative.

2.5.2 Case series

Interest in the study of BKV in neuroblastoma was started by a case series that found BKV DNA by PCR in 18 of 18 neuroblastomas

but in none of 5 healthy adrenal tissues. The BKV detection was confirmed by similar data using ISH, T-antigen staining, and immunoprecipitation of the BKV T-antigen and the p53 tumour suppressor protein ([Flaegstad *et al.*, 1999](#)). There are no other independent confirmatory studies.

2.6 Cancer of the brain

2.6.1 Cohort studies

See [Table 2.1](#)

[Rollison *et al.* \(2003\)](#) performed a prospective study nested in a serum biobank cohort in Maryland, USA. Incident diagnoses of primary malignant tumours of the brain or meninges occurring through 2000 were identified using county and state cancer registries ($n = 44$). Two controls were matched to each case on sex, race, age (within 2 years), and date of blood draw (within 45 days). Controls had to have been alive and not have developed cancer as of the date of diagnosis of the matched case. Archived baseline serum or plasma was obtained for all brain cancer cases and controls. A total of 44 brain tumour cases and 88 controls were analysed by serology for BKV, which yielded an odds ratio of 0.66 (95% CI, 0.22–1.95) for antibody titres ≥ 640 .

2.6.2 Case series

[Caldarelli-Stefano *et al.* \(2000\)](#) analysed a case series of 10 astrocytomas, 5 ependymomas, 5 oligodendrogliomas, and 5 glioblastomas with PCR. None of the tumours contained BKV DNA.

2.7 Cancer of the head and neck

Case series

[Palmieri *et al.* \(2010\)](#) analysed 294 paraffin-embedded tumour tissue samples (squamous cell carcinoma of the oral cavity) and 237 matched control tissues from the same patient and anatomical region. A single case was

BKV-positive, and none of the controls were. The study is classified as a case series since the control samples came from the same subjects as the case samples. The cancer risk associated with BKV exposure was not investigated.

2.8 Cancer of the bladder

2.8.1 Case-control studies

[Polesel *et al.* \(2012\)](#) conducted a hospital-based case-control study of human polyomavirus and human papillomavirus (HPV) infection in patients with transitional cell carcinoma (TCC) of the bladder in Italy. Patients being treated for TCC were identified through multiple hospitals in 2004–07 ($n = 114$) and compared with a control group of patients being treated for orthopaedic and other non-malignant, non-smoking-related conditions ($n = 140$). DNA was extracted from urine samples that were obtained before treatment and tested for the presence of several polyomaviruses, including BKV, and HPV DNA using PCR-based methods. Although polyomavirus detection was common, there was no significant difference between cases and controls for any of the polyomaviruses (OR for BKV, 1.45; 95% CI, 0.32–6.55).

2.8.2 Case series

[Rollison *et al.* \(2007\)](#) studied 76 bladder cancers and 46 adjacent normal tissues from the same patient; 4 of the 76 cases and 1 of the 46 controls were positive for BKV DNA. [Roberts *et al.* \(2008\)](#) studied a series of 10 urothelial carcinoma cases that occurred among renal transplant recipients, of whom 2 appeared to be positive for BKV-LT immunohistochemistry staining.

2.9 Cancer of the ovary

Case series

[Idahl *et al.* \(2010\)](#) analysed ovarian tissues from 186 women with benign conditions, borderline tumours, and epithelial ovarian cancer, and 126 control tissues from the contralateral ovary of these women. All specimens were negative for BKV DNA. The study is classified as a case series since the control samples came from the same subjects as the case samples. The cancer risk associated with BKV exposure was not investigated.

2.10 Precursors to cancer of the cervix

Case-control study

[Comar *et al.* \(2011a\)](#) reported a case-control study of high-grade cervical intraepithelial neoplasia (CIN), where 34 of 93 cases of high-grade CIN were positive for BKV DNA, but none of the 80 cases of low-grade CIN and none of the 100 healthy control subjects were. In another paper by the same author ([Comar *et al.*, 2011b](#)), 31 of 93 patients with high-grade CIN were BKV DNA-positive, compared with none of the 80 cases of low-grade CIN and none of the 105 healthy control subjects. [The numbers of positives and numbers of tested subjects are similar but not identical. The extent of overlap of study populations of these publications is not clear.]

2.11 Cancers of the renal pelvis and kidneys

[Knöll *et al.* \(2003\)](#) reported on testing for BKV DNA in 55 patients with renal pelvic urothelial carcinomas and 83 patients with renal cell carcinomas of all histological subtypes. Tumour tissue specimens were used as case specimens, whereas normal tissue specimens from the same subjects were used as controls. Seven of the renal pelvis

tumour patients and none of the kidney cancer patients were positive for BKV DNA. Among the positive patients, 4 were positive only in tumour tissue, 2 only in normal tissue, and 1 in both tumour and normal tissue.

2.12 Cancer risk among patients with polyomavirus-associated nephropathy (PVAN)

Cohort studies

See [Table 2.1](#)

[Chen et al. \(2010\)](#) followed a cohort of kidney transplant patients for up to 25 years. Inconsistent data were provided on the exact number of patients in the cohort [it is unclear whether there were 864 or 990 patients]. A total of 114 patients in the cohort developed cancer. Six transplant patients were diagnosed with PVAN (5 cases with BKV and 1 case with JCV). Five of the 6 transplant patients with PVAN developed cancer, which was significantly different from the incidence among the transplant patients without PVAN ($P < 0.0001$). These 5 cases were diagnosed with: both bladder and kidney cancer ($n = 2$), kidney cancer ($n = 1$), skin cancer ($n = 1$), and Kaposi sarcoma ($n = 1$). [The Working Group noted that this positive association could be due to confounding as heavy immunosuppressive medication could cause both PVAN and an increased risk of cancer.]

3. Cancer in Experimental Animals

The tumorigenicity of BKV has been tested by subcutaneous, intracerebral, intravenous, or intraperitoneal injection of the virus into NB hamsters, mice, and rats as well as weanling hamsters. All pertinent studies are summarized below (see [Table 3.1](#), [Table 3.2](#), and [Table 3.3](#)).

3.1 Hamster

See [Table 3.1](#)

3.1.1 Newborn hamsters

The first evidence of BKV oncogenicity was reported by [Shah et al. \(1975\)](#), in which 1 of 52 NB hamsters injected subcutaneously with BKV developed a fibrosarcoma within 12 months. Similar findings were subsequently reported by [Näse et al. \(1975\)](#), in which subcutaneous injection resulted in fibrosarcoma in 11% of 18 animals by 6 and 9 months; by [Dougherty \(1976\)](#), in which subcutaneous injection induced fibrosarcoma in 50% of 22 animals within 48 weeks; and by [van der Noordaa \(1976\)](#), in which subcutaneous injection resulted in fibrosarcoma in 5% of 155 animals within 12 months.

Additional studies in NB hamsters detected other tumour types. [Costa et al. \(1976\)](#) observed that intracerebral injection of the MMV strain resulted in ependymoma in 27% of 11 animals within 5–6 months. [Uchida et al. \(1976\)](#) reported that intracerebral injection led to ependymoma and insulinoma in 47% of 19 animals within 6 months. [Greenlee et al. \(1977\)](#) detected choroid plexus papilloma in 4% of 45 animals upon intracerebral injection of BKV in combination with anti-thymocyte serum, whereas 33 animals injected with BKV alone did not develop tumours. Consistent with these findings, [Corallini et al. \(1977\)](#) detected fibrosarcoma in 1 (2%) of 43 animals injected subcutaneously and ependymoma in 88% of 50 animals injected intracerebrally with purified virus. [Uchida et al. \(1979\)](#) used different passages of viral stocks and noted that the incidence of tumours varied by viral passage. Tumours were observed after 3–9 months. Tumours occurring in 86% of 84 animals injected intracerebrally included choroid plexus papilloma and ependymoma (30%), insulinoma (26%), osteosarcoma (33%), and other rare tumours. Tumours occurring in 79% of 14

Table 3.1 Carcinogenicity studies of BKV or BKV DNA in experimental animals

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Animals/group at start ^a	For each target organ: tumour incidence; tumour latency ^{b,c}	Significance ^d	Comments
Hamster ^e (NB, < 1 d; or 5 wk) (NR) Up to 12 mo Shah et al. (1975)	BKV 0.1 mL, 6.5 log ₁₀ TCID ₅₀ units, ^f Subcutaneous NB, <i>n</i> = 52 0.5 mL, 8.8 log ₁₀ TCID ₅₀ units, Intravenous Age 5 wk, <i>n</i> = 28	Fibrosarcoma (1/52); up to 12 mo 0/28		First report of BKV tumorigenicity. Half of weanling hamsters were thymectomized. Virus for intravenous injection concentrated by centrifugation.
Hamster (NB, < 1 d; or 5 wk) (NR) Up to 15 mo Näse et al. (1975)	BKV, 10 ⁷ TCID ₅₀ units Subcutaneous <i>n</i> = 18	Poorly differentiated fibrosarcoma (2/18; 11%); 6 and 9 mo		Virus concentrated by centrifugation. Injected volume NR.
Hamster (NB, < 1 d) (NR) Up to 12 mo Dougherty (1976)	BKV RF strain, 0.05 mL, 10 ⁶ –10 ⁷ PFU, Subcutaneous <i>n</i> = 22	Poorly differentiated fibrosarcoma (50%); 18–48 wk		Virus concentrated by centrifugation.
Hamster (NB) (NR) Up to 12 mo van der Noordaa (1976)	BKV, 10 ⁶ PFU, Subcutaneous <i>n</i> = 155	Fibrosarcoma (5%); up to 12 mo		Only 1 of 8 tumours classified histologically.
Hamster (NB, < 1 d) (NR) Up to 6 mo Costa et al. (1976)	BKV MMV strain, 0.02 mL, 10 HAU, Intracerebral <i>n</i> = 11	Ependymoma (27%); 5–6 mo		MMV papovavirus subsequently identified as BKV.
Hamster (NB, < 1 d) (NR) Up to 6 mo Uchida et al. (1976)	BKV, 0.02 mL, 2.4 × 10 ¹¹ particles, Intracerebral <i>n</i> = 19 Controls: PBS <i>n</i> = 8	Ependymoma (47%); 3–6 mo Insulinoma (47%); 5–6 mo 0%		One animal developed both tumours. One ependymoma was later reported as a pinealocytoma (see Uchida et al., 1979).
Hamster (NB, < 1 d) (NR) Up to 6 mo Greenlee et al. (1977)	BKV, 0.02 mL, 2000 HAU, Intracerebral with anti-thymocyte serum <i>n</i> = 45 Without anti-thymocyte serum <i>n</i> = 33	Choroid plexus papilloma (2/45, 4%); 5 and 6 mo 0%		Anti-thymocyte serum given at 0, 4, 8, and 12 d.

Table 3.1 (continued)

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Animals/group at start ^a	For each target organ: tumour incidence; tumour latency ^{b,c}	Significance ^d	Comments
Hamster (18–22 d) (NR) Up to 9 mo Corallini et al. (1978)	BKV purified virus, 0.4 mL, 10 ^{9.4} –10 ^{11.2} FFU, intravenous <i>n</i> = 73	Tumours (82%), including ependymoma (64%), insulinoma (11%), osteosarcoma (8%), and various abdominal tumours (1%); 2.5–9 mo		BKV-specific antiserum was mixed with virus 1 h before intravenous inoculation. Multiple tumours detected in some animals.
	Controls: BKV purified virus, 0.4 mL, 10 ^{9.4} –10 ^{11.2} FFU plus neutralizing serum, intravenous <i>n</i> = 73	0%		
	Controls: Tris-HCl buffer <i>n</i> = 48	0%		
	Controls: no treatment <i>n</i> = 140	0.7%		
Hamster (NB, < 1 d) (NR) Up to 9 mo Uchida et al. (1979)	BKV (various passages), 0.02 mL, 0.3–2.5 × 10 ⁷ PFU	Intracerebral route: tumours (86%), including choroid plexus papilloma or ependymoma (30%), insulinoma (26%), osteosarcoma (33%), and rare central neuroblastoma, pinealocytoma, peritoneal tumours, and haemangioendothelioma.		Incidence of tumours varied by passage of viral stocks.
	Intracerebral, <i>n</i> = 84			
	Subcutaneous, <i>n</i> = 14	Subcutaneous route: tumours (79%), including osteosarcoma (79%) and insulinoma (14%).		
	Intraperitoneal, <i>n</i> = 14	Intraperitoneal route: tumours (64%), including osteosarcoma (50%) and insulinoma (7%).		
	Controls: PBS or HEK extract, <i>n</i> = 44	Tumour latency: 3–9 mo		

Table 3.1 (continued)

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Animals/group at start ^a	For each target organ: tumour incidence; tumour latency ^{b,c}	Significance ^d	Comments
Hamster (NB, < 1 d) (NR) Up to 8 mo Watanabe et al. (1979)	BKV (different strains), 0.02 mL, 0.7–1.8 × 10 ⁷ PFU, intracerebral BKV (uncloned) <i>n</i> = 38 BKV wt-501 (WT) <i>n</i> = 33 BKV pm-522 (mutant) <i>n</i> = 71	Tumours (38%), including ventricular tumours (38%), pinealocytoma (15%), insulinoma (54%), and osteosarcoma (15%); 8 mo Tumours (67%), including ventricular tumours (33%) and osteosarcoma (33%); 8 mo Tumours (100%), including ventricular tumours (71%), pinealocytoma (7%), and insulinoma (50%); 4 mo		Mutant pm-522 strain induced tumours earlier on average (4 mo) than the WT and uncloned isolates (8 mo). Ventricular tumours were not characterized histologically.
Hamster (NB, < 1 d) (NR) Up to 9 mo Watanabe et al. (1982)	BKV (WT and mutants) 0.02 mL, 0.7–1.4 PFU, intracerebral BKV wt-500 (S) P33, <i>n</i> = 18 BKV wt-502 (S) P32, <i>n</i> = 17 BKV wt-501 (L) P60, <i>n</i> = 13/11 BKV pm-522 (S) P61, <i>n</i> = 12/15/15 BKV pm-522 (L) P39, <i>n</i> = 9 BKV pm-525 (L) P40, <i>n</i> = 11	Tumours (78%) Tumours (65%) Tumours (92%/36%) Tumours (92%/100%/7%) Tumours (100%) Tumours (100%)		Tumours were categorized as ventricular tumours, insulinoma, or osteosarcoma. Follow-up study of Watanabe et al. (1979) confirmed tumour profile but failed to demonstrate differences due to passage or mutant.
Hamster (3 wk) (NR) Up to 11 mo Yamaguchi et al. (1980)	BKV, 0.25 mL, 10 ⁸ FFU, intravenous <i>n</i> = 9	Osteosarcoma (22%); 8 and 11 mo		

Table 3.1 (continued)

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Animals/group at start ^a	For each target organ: tumour incidence; tumour latency ^{b,c}	Significance ^d	Comments
Hamster (18–22 d; or NB, < 1 d) (NR) Up to 18 mo Corallini <i>et al.</i> (1982)	BKV 0.2 mL, 10 ^{8.7} FFU, intravenous Age 18–22 d BKV and ALS <i>n</i> = 75	Tumours (73%), including ependymoma (53%), insulinoma (15%), lymphoma (14%), osteosarcoma (9%), sarcoma (5%), renal cell carcinoma (3%), and liver haemangiosarcoma (2%)		Animals immunosuppressed by intravenous inoculation with rabbit ALS, MP, or gamma irradiation (cobalt-60 source, 300 rad) plus MP.
	BKV and MP <i>n</i> = 34	Tumours (65%), including ependymoma (50%), insulinoma (14%), osteosarcoma (18%), and renal cell carcinoma (5%)		
	BKV and gamma irradiation plus MP <i>n</i> = 37	Tumours (46%), including ependymoma (65%), insulinoma (18%), lymphoma (12%), and sarcoma (6%)		
	BKV DNA 2 µg, intracerebral Age < 1 d <i>n</i> = 39	Ependymoma (5%)		Two other experiments failed to induce tumours after subcutaneous injection in NB hamsters or intravenous injection in weanling hamsters.
	Controls: MP, <i>n</i> = 30; MRP alone, <i>n</i> = 30	MP (0%), MRP (0%)		

Table 3.1 (continued)

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Animals/group at start ^a	For each target organ: tumour incidence; tumour latency ^{b,c}	Significance ^d	Comments
Hamster (NB; or 3–4 wk) (NR) Mouse, Swiss (NB) (NR) Up to 15 mo Corallini et al. (1977)	Hamster (3–4 wk), BKV crude virus, 1 mL, 6.4 log ₁₀ FFU, intravenous <i>n</i> = 89	Reticular cell sarcoma (2/89, 2%) ^g		Purified virus preparation was concentrated by centrifugation.
	Hamster (NB), BKV crude virus, 0.2 mL, 4.7–5.7 log ₁₀ FFU, subcutaneous (<i>n</i> = 45), 0.2 mL, intraperitoneal (<i>n</i> = 31), or 0.02 mL, intracerebral (<i>n</i> = 56)	0%, 0%, 0%		
	Hamster (NB), BKV purified virus, 0.05 mL, 7.7 log ₁₀ FFU, subcutaneous <i>n</i> = 43	Fibrosarcoma (1/43, 2%)		
	Hamster (NB), BKV purified virus, 0.02 mL, 7.3 log ₁₀ FFU, intracerebral <i>n</i> = 50	Ependymoma (44/50, 88%)		
	Hamster (NB), BKV purified virus, 0.05 mL, 7.7 log ₁₀ FFU, intraperitoneal <i>n</i> = 34	0%		
	Hamster (NB), BKV DNA, 0.02 mL, 5.4 log ₁₀ FFU, intracerebral <i>n</i> = 39	0%		
	Mouse (NB), BKV purified virus, 0.02 mL, 7.3 log ₁₀ FFU, intracerebral <i>n</i> = 31	Ependymoma (9/31, 29%)		
	Mouse (NB), BKV purified virus, 0.05 mL, 7.7 log ₁₀ FFU, subcutaneous <i>n</i> = 39	0%		
	Mouse (NB), BKV purified virus, 0.05 mL, 7.7 log ₁₀ FFU, intraperitoneal <i>n</i> = 27	0%		
	Mouse (NB), BKV DNA, 0.02 mL, 5.4 log ₁₀ FFU, intracerebral <i>n</i> = 9	0%		

Table 3.1 (continued)

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Animals/group at start ^a	For each target organ: tumour incidence; tumour latency ^{b,c}	Significance ^d	Comments
Rat, Wistar (NB) (NR) Up to 18 mo Noss et al. (1981)	BKV, 0.3 mL, 10 ^{7.8} TCID ₅₀ units, Subcutaneous <i>n</i> = 37	Tumours (35%)*, including fibrosarcoma (16%), osteosarcoma (8%), liposarcoma (5%), choroid plexus papilloma (3%), and nephroblastoma (3%); 18 mo	* <i>P</i> < 0.01	Three BKV-inoculated animals developed tumours (adenomas) considered to be spontaneous (cell lines from the tumours negative for T-antigen, and animal sera negative for anti-T-antigen antibody).
	BKV, 0.02 mL, 10 ^{6.6} TCID ₅₀ units, Intracerebral <i>n</i> = 40	Tumours (18%)**, including fibrosarcoma (5%), osteosarcoma (5%), choroid plexus papilloma (5%), and glioblastoma (3%); 6 mo	** <i>P</i> < 0.05	
	Controls: 0.9% NaCl Subcutaneous, <i>n</i> = 20; or Intracerebral, <i>n</i> = 20	Controls (0%, 0%)		
Rat, six different strains (NB, < 1 d) (NR) Up to 12 mo Noss & Stauch (1984)	BKV, 10 ^{7.3} TCID ₅₀ units, subcutaneous			Some animals were thymectomized. Tumours were mainly fibrosarcomas and osteosarcomas.
	ACI strain, <i>n</i> = 21	Tumours (24%)		
	DA strain, <i>n</i> = 30	Tumours (0%)		
	AS strain, <i>n</i> = 38	Tumours (82%)		
	Lewis strain, <i>n</i> = 28	Tumours (75%)		
	BH strain, <i>n</i> = 22	Tumours (91%)		
	BS strain, <i>n</i> = 22	Tumours (95%)		

^a Animals/group at start. Due to the long latency for BKV induction of tumours, the animals that died during the period immediately after the inoculation are largely absent from published studies and therefore have been excluded from the numbers listed. All animals/group at the start of the study are included in the table where possible.

^b Pathology nomenclature. Fibrosarcoma is indicated when specified. Insulinoma includes malignant insulinoma and pancreatic islet tumours. Ependymoma also includes papillary ependymoma and choroid plexus papilloma.

^c All tumours occurred at the site of injection unless otherwise noted.

^d Statistical significance is listed if it was reported in the study.

^e Hamsters are Syrian outbred unless otherwise indicated.

^f Various methods of virus titration for BKV are reported in the literature and are included where noted: HAU, haemagglutination units; TCID₅₀ units, 50% tissue culture infective dose units; PFU, plaque-forming units; FAFFU, focus assay fluorescence focus units; and viral particles.

^g Abdominal reticular cell sarcomas did not occur at the site of injection.

ALS, anti-hamster lymphocyte serum; BKV, BK polyomavirus; d, day; h, hour; HEK, human embryonic kidney; mo, month; MP, methylprednisolone; MRP, methylprednisolone acetate + γ -radiation; NB, newborn; NR, not reported; PBS, phosphate-buffered saline; WT, wild-type; wk, week

Table 3.2 Carcinogenicity studies of BKV DNA and c-Ha-ras DNA in hamsters

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Animals/group at start	For each target organ: tumour incidence ^a ; tumour latency	Significance	Comments
Hamster, Syrian golden (NB, < 1 d) (NR) Up to 8 wk Corallini et al. (1987a)	BKV DNA and c-Ha-ras ^b DNA, 2 µg, 50 µL, subcutaneous BKV-c-Ha-ras (oncogenic) plasmid <i>n</i> = 15 Controls: BKV alone, c-Ha-ras (oncogenic) alone, BKV plus c-Ha-ras (proto-oncogene) <i>n</i> = 15 per group	Undifferentiated sarcoma (73%); 2–8 wk Controls (0%)	NR	BKV-c-Ha-ras (oncogenic) plasmid contains the BKV early region and c-Ha- ras DNA sequences on the same plasmid. Short incubation period.
Hamsters, Syrian golden (NB, < 1 d) (NR) Up to 8 wk Corallini et al. (1988)	BKV DNA and c-Ha-ras ^b DNA, 1–2 µg, 20 µL, intracerebral BKV-c-Ha-ras (oncogenic) plasmid <i>n</i> = 33 Controls: BKV alone, c-Ha-ras (oncogenic) alone, BKV plus c-Ha-ras (proto-oncogene) <i>n</i> = 21 per group	Undifferentiated sarcoma of the brain (76%); 2–8 wk Controls (0%)	NR	BKV-c-Ha-ras (oncogenic) plasmid contains the BKV early region and c-Ha- ras DNA sequences on the same plasmid. Short incubation period.

^a All tumours occurred at the site of injection unless otherwise noted.

^b c-Ha-ras = HRas. DNA encoding human cellular HRas in the oncogenic or proto-oncogenic forms.

BKV, BK polyomavirus; d, day; NB, newborn; NR, not reported, wk, week

Table 3.3 Carcinogenicity studies in transgenic mice involving BKV with natural viral promoter

Species Duration of observation Reference	Virus strain Animals/group at start	For each target organ: tumour incidence; tumour latency	Significance	Comments
Mouse (transgenic) Up to 10 mo Small et al. (1986) , Dalrymple & Beemon (1990)	BKV Dunlop LT and sT <i>n</i> = 3 (founders) BKV Dunlop LT and sT <i>n</i> = 78 (progeny)	Hepatocellular carcinoma and renal cell carcinoma (2/3); 8–10 mo Renal cell carcinoma (40%) and thymoproliferative disorder (74%); 8–10 mo	NR	Transgenic mice characterized in Small et al. (1986) . Thymoproliferative disorders ranging from hyperplasia to thymoma and lymphoma. No T-antigen expressed in thymocytes; therefore, causation of thymoproliferative disorder by BKV unclear.

BKV, BK polyomavirus; LT, large T-antigen; mo, month; NR, not reported; sT, small T-antigen

animals injected subcutaneously included osteosarcoma (79%) and insulinoma (14%). Tumours occurring in 64% of 14 animals injected intraperitoneally included osteosarcoma (50%) and insulinoma (7%). [Watanabe et al. \(1979\)](#) studied tumorigenesis by intracerebral injection with different strains of BKV and observed that uncloned BKV induced tumours in 38% of 38 animals, including ventricular tumours (38%), pinealocytoma (15%), insulinoma (54%), and osteosarcoma (15%). In parallel, injection of BKV wt-501 wild-type (WT) strain induced tumours in 67% of 33 animals, including ventricular tumours (33%) and osteosarcoma (33%), whereas BKV pm-522 mutant strain induced tumours in 100% of 71 animals, including ventricular tumours (71%), pinealocytoma (7%), and insulinoma (50%). Extended studies by the same group ([Watanabe et al., 1982](#)) reported similar results in that different WT isolates or mutants of BKV led to varied tumour incidences, although all were tumorigenic within a 9-month observation period.

[The varied tumour incidences in NB hamsters may be explained by differences in viral stocks.]

3.1.2 Weanling hamsters

[Corallini et al. \(1977\)](#) observed reticular cell sarcoma in 2 of 89 weanling hamsters during a 15-month observation period after intravenous injection at age 3–4 weeks with crude BKV preparation. In another study, [Corallini et al. \(1978\)](#) detected tumours in 82% of 73 weanling hamsters injected intravenously with purified virus. Tumours were observed within 2.5–9 months and included ependymoma (64%), insulinoma (11%), osteosarcoma (8%), and various abdominal tumours (1%), whereas 73 animals co-injected with BKV plus neutralizing serum did not develop tumours. Similar results were seen by [Yamaguchi et al. \(1980\)](#), who injected animals intravenously at age 3 weeks and observed

osteogenic sarcoma [osteosarcoma] in 22% of 9 animals. In the most extensive study ([Corallini et al., 1982](#)), groups of weanling hamsters were inoculated intravenously in conjunction with immunosuppression using anti-hamster lymphocyte serum, methylprednisolone, or gamma irradiation plus methylprednisolone. In 55 (73%) of 75 animals that received BKV and anti-hamster lymphocyte serum, tumours were observed, including ependymoma (53%), insulinoma (15%), lymphoma (14%), osteosarcoma (9%), sarcoma (5%), renal [cell] carcinoma (3%), and liver haemangiosarcoma (2%). In 22 (65%) of 34 animals that received BKV and methylprednisolone, tumours developed, including ependymoma (50%), insulinoma (14%), osteosarcoma (18%), and renal [cell] carcinoma (5%), whereas 46% of 37 animals that received BKV and methylprednisolone plus gamma irradiation (cobalt-60 source) developed tumours, including ependymoma (65%), insulinoma (18%), lymphoma (12%), and sarcoma (6%).

[Overall, these studies demonstrated a similar range of tumours in weanling animals as observed in hamsters injected as newborns, although longer latency periods of up to 18 months were described.]

3.2 Mouse

See [Table 3.1](#)

[Corallini et al. \(1977\)](#) infected NB Swiss mice intracerebrally with BKV, and 29% of 31 injected animals developed ependymoma within 15 months. In the same study, mice injected subcutaneously ($n = 39$) or intraperitoneally ($n = 27$) with BKV or intracerebrally with BKV DNA ($n = 9$) did not develop tumours.

3.3 Rat

See [Table 3.1](#)

[Noss et al. \(1981\)](#) performed subcutaneous and intracerebral injections of BKV in NB Wistar rats. Of 40 animals injected intracerebrally, 18% developed tumours, including fibrosarcoma (5%), osteosarcoma (5%), [choroid] plexus papilloma (5%), and glioblastoma (3%), whereas 35% of 37 animals injected subcutaneously developed similar tumours, including fibrosarcoma (16%), osteosarcoma (8%), and [choroid] plexus papilloma (3%), as well as liposarcoma (5%) and nephroblastoma (3%), by 18 months. In a follow-up study, [Noss & Stauch \(1984\)](#) inoculated six different strains of NB rats (some of which were thymectomized) subcutaneously with BKV, which resulted in a broad range of tumorigenicity by 12 months. [Tumours were mainly fibrosarcomas and osteosarcomas.] Percentage tumour formation by strain, in order of frequency, was: BS strain, 95% of 22 animals; BH strain, 91% of 22 animals; AS strain, 82% of 38 animals; Lewis strain, 75% of 28 animals; ACI strain, 24% of 21 animals; and DA strain, 0% of 30 animals. [The genetic background of the animals may have contributed to oncogenic susceptibility.]

3.4 Hamsters injected with BKV DNA

See [Table 3.1](#)

In NB hamsters injected intracerebrally with 2 µg of BKV DNA, ependymoma developed in 2 (5%) of 39 injected animals. Two experiments did not report tumours after subcutaneous injection in NB hamsters or intravenous injection in weanling hamsters ([Corallini et al., 1982](#)).

3.5 Hamsters injected with BKV and c-Ha-*ras* DNA

See [Table 3.2](#)

[Corallini et al. \(1987a\)](#) inoculated NB Syrian hamsters subcutaneously with recombinant DNA containing the BKV early region genes and the human c-Ha-*ras* activated oncogene.

Eleven (73%) of 15 animals developed sarcomas. In a second study with a similar design, [Corallini et al. \(1988\)](#) showed that intracerebral inoculation produced brain sarcomas in 25 (76%) of 33 animals. [The Working Group noted the short observation period (8 weeks) used in both studies.]

3.6 Transgenic mouse models of BKV T-antigen with natural viral promoter

See [Table 3.3](#)

[Small et al. \(1986\)](#) were the first to produce transgenic mice containing the genes of BKV early region (i.e. the T-antigens, LT and sT). They used the Dunlop strain of the virus and observed hepatocellular carcinoma and renal tumours [renal cell carcinoma] in 2 of 3 founder animals by age 8–10 months. A follow-up study of 78 progeny animals ([Dalrymple & Beemon, 1990](#)) found that 40% of the animals developed renal adenocarcinoma [renal cell carcinoma] and 74% developed thymoproliferative disorders (ranging from hyperplasia to thymoma and lymphoma) by age 8–10 months. Of note, no expression of T-antigen was detected in the thymocytes.

4. Mechanistic and Other Relevant Data

4.1 Transforming capacity of BKV

Young or NB mice, rats, and hamsters as well as weanling hamsters developed tumours after inoculation of BKV via different routes. The frequency of tumour induction in hamsters is dependent on the route of injection and the titre of the inoculum. BKV is weakly oncogenic when inoculated subcutaneously, inducing fibrosarcomas, and induces mainly ependymoma tumours when inoculated intracerebrally or

intravenously (see Section 3). The spectrum of tumours in experimental animals may also vary due to the stock or strain of the viral inoculum ([Uchida *et al.*, 1979](#); [Watanabe *et al.*, 1982](#)).

NB hamsters inoculated subcutaneously with a plasmid recombinant containing the BKV early region and the c-Ha-*ras* human activated cellular oncogene (pBK/c-*rasA*) developed tumours within a few weeks. Tumours developed at the site of injection and consisted of undifferentiated sarcomas expressing both BKV large T-antigen (LT) and c-Ha-*ras* p21. Neither BKV DNA nor c-Ha-*ras*, inoculated independently, was tumorigenic ([Corallini *et al.*, 1987a](#)). The same recombinant pBK/c-*rasA*, inoculated intracerebrally, induced rapidly growing undifferentiated brain tumours in NB hamsters ([Corallini *et al.*, 1988](#)). These data suggest a synergistic interaction of BKV transforming functions with human cellular oncogenes. No tumours were obtained in primates inoculated with BKV ([London *et al.*, 1978](#)). The transforming capacity of BKV is based on random DNA integration into the host-cell genome, which guarantees the perpetuation of the viral genome and its replication along with the host chromosomes and the possibility of viral protein expression ([Chenciner *et al.*, 1980](#); [Moens & Johannessen, 2008](#)).

The transforming capacity of BKV has also been demonstrated in rodent cells. BKV, BKV complete genomic DNA, or BKV genomic fragments that include the early region can transform embryonic fibroblasts and kidney or brain cells of mouse, rat, hamster, and rabbit, cells that all are non-permissive for replication of the virus (reviewed in [Imperiale, 2001b](#); [Tognon *et al.*, 2003](#); [White & Khalili, 2004](#)). Baby hamster kidney (BHK21) cells and mouse fibroblasts (NIH3T3) transfected with the early region of the BKV genome that encodes LT and sT viral proteins are transformed to anchorage-independence, and expression of LT was shown to be essential for the transformation. In addition, when these transformed cells are transfected

with an antisense LT RNA, their ability to grow in soft agar is lost, demonstrating the need for the continued expression of LT for the maintenance of the transformed phenotype ([Nakshatri *et al.*, 1988](#)). Rodent tumour cells as well as cells transformed in culture express BKV LT in their nuclei, and the viral DNA is integrated into the cell genome. Infectious virus can be recovered from a subset of tumour cells when fused with permissive cells of human or monkey origin ([Corallini *et al.*, 1977, 1978](#)).

Transformation of human cells by BKV is inefficient and often abortive ([Portolani & Borgatti, 1978](#)). BKV-infected or -transfected human cells generally do not display a completely transformed phenotype, although they show morphological alterations and an increased lifespan ([Purchio & Fareed, 1979](#); [Grossi *et al.*, 1982](#)). Sometimes transformation is transient and cells revert to the original phenotype after a few passages in culture ([Rinaldo *et al.*, 2003](#)). However, a fully transformed phenotype was obtained for human embryonic fibroblasts and kidney cells transfected *in vitro* with plasmid recombinants containing the BKV early region and the c-Ha-*ras* or the c-MYC human activated cellular oncogene ([Pater & Pater, 1986](#); [Corallini *et al.*, 1991](#)) or with the Ad12EA1A viral oncogenes ([Vasavada *et al.*, 1986](#)). Cell lines were established from BKV-transformed human fetal brain cells, which could persistently shed BKV virions. These fetal brain cell lines had all the characteristics of transformed cells (growth in soft agar, tumorigenic in nude mice). Integrated viral DNA was not detected in any of the clones, which all retained viral genome as episomes, sometimes in large numbers ([Takemoto *et al.*, 1979](#)).

4.2 Relevant biological properties of BKV viral proteins

BKV encodes early and late genes, of which the early gene products, LT and sT, have been identified as oncoproteins. BKV LT interferes with pRb and p53 to drive infected cells into the S phase to use the cellular replication machinery. BKV LT binds to pRb family proteins pRb, p107, and p130, thus dislocating E2F and thereby enabling cell-cycle progression ([Harris et al., 1996](#)). However, since only low levels of pRb–LT complexes were detectable at in BKV LT-transduced African green monkey kidney (BSC-1) cells, E2F transcriptional activation by LT could involve other modes of E2F regulation ([Harris et al., 1998](#)).

BKV LT also binds to the tumour suppressor gene product p53, which is altered in > 50% of human cancers ([Bollag et al., 1989](#)). Upon binding, p53 is inactivated, which leads to loss of cell-cycle inhibition and prevention of apoptosis ([White & Khalili, 2004](#)). BKV LT readily binds the p53 protein available in BSC-1 cells and induces serum-independence, but is unable to allow anchorage-independent growth in semi-solid medium ([Harris et al., 1996](#)). These data support the notion that BKV LT can affect cellular growth control mechanisms, but additional events are required for full transformation of primate cells by BKV. It is possible that the inefficiency in transformation of human cells by BKV depends on the inability of BKV LT to block completely the effect of the human tumour suppressor proteins pRb and p53 ([Harris et al., 1996, 1998](#)).

It has also been reported that BKV LT itself is mutagenic. Up to 100-fold increases of spontaneous mutation frequencies have been observed in cultured baby hamster kidney cells and human peripheral blood lymphocytes upon BKV infection ([Theile & Grabowski, 1990](#)). [Trabanelli et al. \(1998\)](#) reported that transfection of BKV LT in human fibroblasts leads to cytogenetic damage, including deletions and translocations. The

chromosomal damage was evident before the appearance of immortalization and the morphologically transformed phenotype, suggesting that it is a cause rather than a consequence of transformation. Similar alterations were observed in cell lines from human glioblastoma multiforme, harbouring the LT-coding sequences of both BKV and SV40 ([Tognon et al., 1996](#)).

For SV40, interaction of sT with PP2A and subsequent interference with its function have been well established. Likewise, the same interaction of JCV sT has also been demonstrated, and the predicted homology between the PP2A binding sites suggests that BKV sT may exhibit a similar function.

The NCCR of BKV strains may have important implications for the replication, transcriptional efficacy, and transforming potential of BKV as well as the host-cell tropism and permissivity ([Moens et al., 1995](#)). Genetic variants in the NCCR of BKV have been shown to affect the transforming capacity of BKV in embryonic hamster cells and in the rat 3Y1 cell line ([Watanabe & Yoshiike, 1982](#)). WT BKV, which has three 68 bp elements within its NCCR, induces foci formation in human embryonic kidney cell cultures, but rarely in hamster or rat cells. Deletion mutants containing only one 68 bp element still induced foci formation in cultured human embryonic kidney cells, but also in rat cells, efficiently ([Watanabe & Yoshiike, 1985](#)).

4.3 Association of BKV with human tumours

Detection of BKV DNA sequences has been reported in several human tumours. Initial studies mainly used Southern blot hybridization (SBH) for the detection of viral genomes. More recently, tumour biopsies, tumour cell lines, and normal human tissues were investigated by PCR using specific primers for the early region of BKV DNA. In most of these studies, the amount of

viral DNA detected was low, generally less than one genome copy per cell. The results of the studies conducted by SBH and PCR are summarized in [Table 4.1](#). In addition, expression of the BKV early region was detected by northern blot analysis or reverse transcriptase PCR (RT-PCR) in several tumours, tumour cell lines, and normal tissues ([De Mattei et al., 1995](#); [Table 4.1](#)).

BKV DNA was detected in an episomal state and generally at a low copy number (0.2–1 genome copy per cell) in 19 (26%) of 74 human brain tumours and in 4 (44%) of 9 human pancreatic islet tumours ([Corallini et al., 1987b](#)).

BKV-specific RNA was detected in several tumours by dot-blot hybridization. In a small number of tumours (5/7), BKV LT was detected by means of a cell-lysate ELISA. Of the 5 tumours positive for BKV LT, 4 were glioblastomas also positive for BKV DNA and RNA. BKV was rescued from 8 of 22 tumours – 6 brain tumours and 2 pancreatic islet tumours – after transfection of total tumour DNA into human embryonic fibroblasts, which are permissive for BKV lytic infection. The rescued BKV DNA differed from WT BKV but was similar to BKV-IR, a virus previously isolated from a human insulinoma ([Caputo et al., 1983](#)). It was suggested that the BKV-IR variant, which bears a 253 bp deletion and an 80 bp insertion in the early region of the genome, could form a stem-loop structure that can act as a mutagen by random integration and excision ([Pagnani et al., 1986](#)). [Negrini et al. \(1990\)](#) confirmed this hypothesis and showed increased mutation frequency in cells transduced with the BKV-IR sequence.

In another study, BKV DNA was detected by SBH in 46% of brain tumours of the most common histotypes ([Dörries et al., 1987](#)). The size of hybridization products suggested that the virus may be integrated into the host chromosomal DNA; however, defined virus bands could not be visualized after digestion with restriction enzymes.

In one study, both SV40 and BKV sequences were searched for in human brain tumours. All the tumours harbouring SV40 sequences were reported to be co-infected by BKV ([Martini et al., 1996](#)). BKV DNA was detected by PCR in all of 18 neuroblastomas and in none of 5 normal adrenal gland samples. The presence of BKV DNA was confirmed by ISH in the tumour cells of 17 of the same neuroblastomas. Finally, BKV LT and p53 were co-immunoprecipitated and co-localized in tumour cells by double immunostaining, suggesting that BKV LT blocks p53 functions ([Flaegstad et al., 1999](#)).

PCR amplification of DNA sequences from BKV early and regulatory regions was carried out in urinary tract tumours; 31 (60%) of 52 samples were positive, with a range of 50–67% in different tumour types ([Monini et al., 1995](#)). The presence of BKV and JCV DNA in urothelial carcinomas of the renal pelvis and in renal cell carcinomas was confirmed by [Knöll et al. \(2003\)](#). The percentage of positive samples in the neoplastic tissues of the urinary tract was similar to that detected in the corresponding normal tissues: 60% and 56%, respectively ([Monini et al., 1995](#)) and 16% and 15%, respectively ([Knöll et al., 2003](#)). However, unlike normal kidney parenchyma, which harboured episomal BKV DNA, tumours of the urinary bladder and prostate contained either a single integration of BKV DNA or both integrated and episomal viral sequences, as shown by two-dimensional gel electrophoresis and SBH analysis ([Monini et al., 1995](#)). In both the integrated and extrachromosomal viral sequences, the late region was disrupted. Viral episomes consisted of rearranged oligomers containing cellular DNA sequences, whose size was apparently incompatible with encapsidation within a viral particle. Attempts to rescue these viral sequences by transfection of tumour DNA into permissive cells failed, suggesting that in these tumours the process of integration and formation of episomal oligomers produced a rearrangement of viral sequences responsible for the

Table 4.1 Presence and expression of BKV DNA in human tumours, tumour cell lines, and normal tissues

Tissues and cell lines	BKV DNA-positive/ samples analysed (%)	BKV RNA-positive/ samples analysed	Method	Reference
<i>Primary tumours</i>				
Brain	19/74 (26%)	11/26	SBH, RNA-DBH	Corallini et al. (1987b)
Brain	11/24 (46%)		SBH	Dörries et al. (1987)
Brain	0/75		PCR	Arthur et al. (1994)
Brain	50/58 (86%)		PCR	De Mattei et al. (1995)
Brain	74/83 (89%)		PCR	Martini et al. (1996)
Brain	0/10		PCR	Völter et al. (1997)
Neuroblastoma	18/18 (100%)		PCR	Flaegstad et al. (1999)
MED and PNET	0/20		PCR	Kim et al. (2002)
Bone	11/25 (44%)	8/11	PCR, RT-PCR	De Mattei et al. (1995)
Insulinoma	4/9 (44%)	3/7	SBH	Corallini et al. (1987b)
Prostate	11/14 (79%)		PCR, ISH	Das et al. (2008)
Prostate	8/42 (19%)		PCR	Balis et al. (2007)
Hodgkin disease	0/5		PCR	Völter et al. (1997)
ALL	0/15		PCR	MacKenzie et al. (1999)
Kaposi sarcoma	4/20 (20%)		SBH	Barbanti-Brodano et al. (1987)
Kaposi sarcoma	38/38 (100%)		PCR	Monini et al. (1996)
Kaposi sarcoma	0/2		PCR	Völter et al. (1997)
Urinary tract	31/52 (60%)		PCR	Monini et al. (1995)
Urinary tract	0/15		PCR	Völter et al. (1997)
Genital tract	32/42 (76%)		PCR	Monini et al. (1996)
<i>Cell lines from</i>				
Brain tumours	8/10 (80%)	5/5	PCR, RT-PCR	De Mattei et al. (1995)
Brain tumours	21/26 (81%)		PCR	Martini et al. (1996)
MED and PNET	0/2		PCR	Kim et al. (2002)
Bone tumours	20/20 (100%)	6/8	PCR, RT-PCR	De Mattei et al. (1995)
Kaposi sarcoma	6/8 (75%)		PCR	Monini et al. (1996)
Kaposi sarcoma	0/14		PCR	Völter et al. (1997)
<i>Normal tissues</i>				
Brain	13/13 (100%)		PCR	De Mattei et al. (1995)
Bone	2/5 (40%)	2/2	PCR, RT-PCR	De Mattei et al. (1995)
Urinary tract	10/18 (56%)		PCR	Monini et al. (1995)
Adrenal gland	0/5		PCR	Flaegstad et al. (1999)
Genital tract	22/29 (76%)		PCR	Monini et al. (1996)
PBMC	25/35 (71%)	8/8	PCR, RT-PCR	De Mattei et al. (1995)
PBMC	53/70 (76%)		PCR	Martini et al. (1996)
Lymph nodes	4/4 (100%)		PCR	Monini et al. (1996)
Skin	25/33 (76%)		PCR	Monini et al. (1996)
Sperm	18/20 (90%)		PCR	Monini et al. (1996)
Sperm	18/19 (95%)		PCR	Monini et al. (1996)
Prostate tissues	4/15 (26%)		PCR, ISH	Das et al. (2008)

ALL, acute lymphoblastic leukaemia; BKV, BK polyomavirus; ISH, *in situ* hybridization; MED, medulloblastoma; PBMC, peripheral blood mononuclear cells from healthy donors; PCR, polymerase chain reaction; PNET, primitive neuroectodermal tumour; RNA-DBH, dot-blot hybridization of total or poly A⁺ tumour RNA; RT-PCR, reverse transcriptase PCR; SBH, Southern blot hybridization

elimination of viral infectivity and potentially leading to stable expression of BKV transforming functions ([Monini et al., 1995](#)). In a metastatic bladder carcinoma, arising in an immunosuppressed transplant recipient, high-level expression of BKV LT was detected by IHC with Pab416 antibody in the primary and metastatic tumours but not in the neighbouring normal urothelium ([Geetha et al., 2002](#)).

BKV DNA was detected by PCR and ISH in epithelial cells in atrophic lesions from prostate cancer resections ([Das et al., 2004](#)). [Das et al. \(2008\)](#) demonstrated that BKV was present at a much lower frequency in non-cancerous prostates. In addition, in normal prostates, LT expression, detected by IHC with Pab416, was observed only in specimens harbouring proliferative inflammatory atrophy and prostatic intraepithelial neoplasia. [Balis et al. \(2007\)](#) also detected BKV DNA in 8 of 42 prostate cancers by PCR. Sequencing of all the positive specimens revealed that 5 samples contained WT BKV sequences and 3 harboured sequences belonging to different BKV strains: MT, TW-2, and CAP-h₂. Successful reconstitution of the virus could be achieved from some of the positive prostate cancer samples. [Zambrano et al. \(2002\)](#) detected BKV and JCV DNA in both benign and cancerous prostate samples (adenocarcinoma) by PCR and ISH analysis, suggesting that mixed infections may exist in prostate tissues.

The presence of BKV DNA has also been reported in genital papillomas and carcinomas of the uterine cervix and vulva as well as in normal genital tissues, and in Kaposi sarcomas ([Monini et al., 1996](#)).

In other reports, however, the presence of BKV DNA sequences in human brain tumours, mostly malignant glioma, medulloblastoma, and primitive neuroectodermal tumours, and in urinary tract tumours, Kaposi sarcoma, lymphoma, and ALL, analysed by PCR, was not confirmed ([Arthur et al., 1994](#); [Völter et al., 1997](#); [MacKenzie et al., 1999](#); [Kim et al., 2002](#)).

[The Working Group noted that caution should be exercised in the interpretation of studies reporting IHC staining for the detection of BKV LT, because of the cross-reactivity of the two antibodies used most often. The Pab416 monoclonal antibody raised against SV40 LT was shown to cross-react with both JCV LT and BKV LT ([Mann & Carroll, 1984](#)). The BK-T1 antibody directed specifically to BKV LT does not cross-react with SV40 LT or JCV LT ([Marshall et al., 1991](#)); however, it was shown to also recognize the cellular KU86 protein subunit present in large amounts in many human cells ([Zambrano & Villarreal, 2002](#)).]

4.4 Transgenic models for cancers associated with BKV infection

The oncogenic potential of BKV was confirmed by the generation of transgenic mice in which BKV LT expression is regulated by the native viral early promoter/enhancer. Transgenic mice expressing BKV LT develop hepatocellular carcinoma, renal tumours, and lymphoproliferative disease ([Small et al., 1986](#); [Dalrymple & Beemon, 1990](#)). Therefore, the experiments with transgenic mice confirmed that BKV displays oncogenic potential in experimental animals (see Section 3).

4.5 Susceptible human populations

Latent BKV infection can be reactivated by chronic immunosuppression. This reactivation, which is well known and is a major challenge in transplant recipients, can lead to lethal diseases such as HC, PVAN, and encephalitis (see Section 1.2). Reactivation of BKV was also reported in HIV-1-infected patients ([Gorrill et al., 2006](#)). However, there is no clear evidence to support a link between immunosuppression and cancers potentially associated with BKV. In a meta-analysis comparing the cancer

incidence in HIV/AIDS patients and immuno-suppressed transplant recipients, cancers of the brain, bladder, and prostate did not occur at an increased rate in both populations. The incidence of brain cancers was increased only in HIV/AIDS patients, that of bladder cancers was increased only in transplant recipients, and that of prostate cancers did not show any increase ([Grulich et al., 2007](#)).

A recent study suggested a putative role of mannose-binding lectin 2 (*MBL-2*) gene polymorphisms in susceptibility to BKV infection ([Comar et al., 2011b](#)).

4.6 Mechanistic considerations

There is broad evidence from many research groups and experimental systems that in specific experimental environments BKV has transforming capacity. In the experimental models, BKV follows a transformation mechanism that is, in principle, similar to that identified in experimental models with other oncogenic polyomaviruses and also for oncogenic human papillomaviruses (HPV) ([IARC, 2007, 2012](#)) in human tumours. However, direct mechanistic evidence for such activity in human tumours is scarce and controversial.

Like for HPV, polyomavirus-induced carcinogenesis in experimental systems was shown to require at least one viral genome persistently present and biologically active in each transformed cell and interruption of the lytic viral life-cycle. Viral persistence can be mediated through integration or through maintenance as viral episome. The presence of one integration site per tumour, in different parts of the tumour and in primary tumour as well as in tumour metastases, indicates that viral integration occurred before clonal tumour expansion. Viral genes encoding regulatory proteins, i.e. the T-antigens in polyomaviruses and the viral oncoproteins E6 and E7 in HPV, are consistently expressed through transcription and translation. Viral oncoproteins,

among other functions, interact directly or indirectly with cellular tumour suppressor proteins such as pRb and p53, leading to cell-cycle and apoptosis deregulation. Expression of viral oncoproteins is necessary to maintain the transformed phenotype and in the tumour-bearing animal can lead to the induction of antibodies to the viral oncoproteins. Such antibodies are rarely induced during the natural course of infection.

An additional essential feature of virally induced cell transformation is the interruption of the lytic viral life-cycle. This can be due to a lack of host factors essential for viral replication (e.g. non-permissivity of rodent cells for BKV) or a lack of viral protein functions or of *cis* elements on the viral genome necessary for viral replication.

BKV, like other small DNA viruses, needs the host-cell DNA replication machinery to drive viral replication. As described above in detail (Sections 4.1–4.3), BKV LT protein binds to the retinoblastoma family of tumour suppressors ([Harris et al., 1996](#)) and activates E2F to facilitate the transition from G1 into S phase, which can result in serum-independent cell growth ([Harris et al., 1996](#)). BKV LT also binds to the tumour suppressor protein p53 ([Bollag et al., 1989](#); [Harris et al., 1996](#); [Shivakumar & Das, 1996](#)), leading to p53 inactivation, which results in loss of cell-cycle inhibition and also interferes with apoptosis induction. The transforming ability of BKV LT appears to be weaker than that of primate polyomavirus SV40 ([Bollag et al., 1989](#); [Harris et al., 1996](#)).

4.6.1 Cell line studies

The BKV early genome region encoding LT and sT can transform rodent cells, which are non-permissive for replication of the virus. Viral DNA persists through integration into the host chromosome or as episome in a few copies per cell ([Howley & Martin, 1977](#); [Chenciner et al., 1980](#); [Beth et al., 1981](#)). Virtually all cells have

detectable nuclear LT expression ([Beth et al., 1981](#)).

In contrast, the ability of BKV to transform human cells, which are permissive and support lytic infection, is inefficient and often abortive, and the cells do not show the fully transformed phenotype with immortalization, anchorage-independent growth, and nude mouse tumorigenicity (reviewed in [Corallini et al., 2001](#)). However, in cooperation with adenovirus 12 E1A, *c-rasA*, or *c-MYC* gene products, the transfected BKV early genome region could fully transform human embryonic kidney cells ([Pater & Pater, 1986](#); [Vasavada et al., 1986](#); [Corallini et al., 1991](#)). It is possible, although very rare, to isolate cells transformed by BKV alone from permissive cultures (e.g. human fetal brain cells), indicating that the ability of the virus to transform cells is not restricted to non-permissive cells ([Takemoto et al., 1979](#); [Grossi et al., 1982](#)). In human cells, viral DNA is mostly present as free episome ([Takemoto et al., 1979](#); [Grossi et al., 1982](#)). Transformation appears to be a characteristic of a failed lytic infection, associated either with the properties of the host cell or with the defective nature of the virus used ([Atkin et al., 2009](#)).

4.6.2 Studies in animals

Inoculation of BKV by various routes into rodents can result in a broad variety of tumours, described in detail in Section 3. In the tumours, the BKV genome stably persists through random integration into the host chromosome, and early region genes are expressed. However, free episomal complete viral genomes may be present, and upon fusion of tumour cells with permissive monkey or human cells, infectious virus could be rescued ([Corallini et al., 1977, 1978](#)). This suggests that BKV-induced rodent tumour cells lack host factors necessary for the BKV lytic cycle, resulting in abrogation of viral replication.

Transgenic mice stably containing and expressing the BKV complete early gene region under the control of the native BKV early promoter/enhancer element developed renal and hepatocellular tumours and lymphoproliferative disease ([Small et al., 1986](#); [Dalrymple & Beemon, 1990](#)), supporting the concept that stable LT expression in rodent cells is a key feature of BKV transformation.

4.6.3 Human tumour studies

PCR analyses, as well as earlier studies by the less-sensitive SBH, have detected BKV DNA sequences in a broad variety of human tumours (see above for detailed study descriptions). No study analysed viral load by quantitative PCR, and only a few studies estimated viral load from SBH. BKV DNA was detected by SBH as free episomes at copy numbers estimated to range from 0.2 to 1 viral genome copy per cell in 26% of human brain tumours and 44% of human insulinomas ([Corallini et al., 1987b](#)). Only a few studies searched for LT expression by IHC or immunofluorescence, with widely contradictory results even within the same tumour type (reviewed in [Abend et al., 2009](#)).

Very few studies of human tumours found to be BKV DNA-positive have analysed cellular surrogate markers for cellular pathways expected to be affected by BKV LT-induced transformation, such as downregulation or sequestration of p53 or pR, potential upregulation of the cyclin kinase inhibitor p16^{Ink4a}, or absence of somatic mutations in the *TP53* gene.

4.6.4 “Hit-and-run” hypothesis and paracrine mechanisms

The low viral load and heterogeneous LT expression in human BKV DNA-positive tumour tissues, frequently restricted to only a few of the tumour cells, are not compatible with the direct viral transformation model, which requires viral

oncoprotein activity in each proliferating tumour cell. This led [Tognon *et al.* \(2003\)](#) to discuss the possibility of a “hit-and-run” mechanism as well as the possibility of secretion of paracrine factors by BKV LT-positive cells, which would recruit neighbouring and distant cells into proliferation. Although some experimental evidence is available for mutagenic and chromosome-damaging effects of BKV LT, evidence is lacking for the paracrine model, and at present it is purely hypothetical.

BKV LT has clastogenic effects, i.e. it can induce mutations in both rodent and human cells ([Theile & Grabowski, 1990](#)) and chromosomal damage in human cells ([Trabanelli *et al.*, 1998](#)), characterized by numerical and structural chromosomal alterations. Chromosomal damage in human cells transfected with the BKV early region was evident before the appearance of immortalization and the morphologically transformed phenotype. Similar alterations were observed in cell lines from human glioblastoma multiforme, found to harbour LT coding sequences of both BKV and SV40 ([Tognon *et al.*, 1996](#)). Since LT binds the p53 protein and inactivates its functions ([Harris *et al.*, 1996](#)), the direct clastogenic effect of the viral oncoprotein may be enhanced because it inhibits p53-induced apoptosis and allows DNA-damaged cells to survive, increasing their probability to transform and acquire immortality.

It is extremely difficult to verify a viral hit-and-run mechanism in the pathogenesis of specific human tumours. The best experimental mechanistic evidence would be to identify tumour precursors and very early tumour stages in which the virus is present and biologically active and to demonstrate that these cells have accumulated somatic mutations and chromosomal damage and later lose the viral sequences. Such evidence does not exist at present. Furthermore, a cellular molecular mechanism that potentially could allow cells to selectively lose DNA sequences is currently unknown.

5. Summary of Data Reported

5.1 Exposure data

Infection with BK polyomavirus (BKV) is highly prevalent globally; > 90% of the adult population have anti-IgG antibodies. This prevalence was independent of the serological assay that was used and whether the sera were adsorbed or blocked with virus-like particles (VLPs) of the closely related polyomaviruses JCV or SV40. Primary infection occurs early in childhood and is regarded to be asymptomatic. After primary infection, BKV establishes a persistent infection in the kidneys and is occasionally shed in the urine and is found in sewage. The transmission route for BKV is unknown, but the respiratory and oral routes have been suggested. Under conditions of immunosuppression, BKV is associated with two diseases: haemorrhagic cystitis in haematopoietic stem cell transplantation recipients and BKV-associated nephropathy in renal transplant recipients. There is consistent evidence that BKV infects humans.

5.2 Human carcinogenicity data

Most studies on BKV infection and cancer are case series or studies that compare tissues from cases with tissues from subjects without the disease but that have not been formally designed as case-control studies to ensure that the controls are drawn from the same source population as the cases.

The most studied cancer is that of the prostate. The evidence on prostate cancer associated with BKV infection is inconsistent, ranging from a high proportion of BKV DNA positivity in prostate cancer case series to studies that do not find BKV DNA at all. Possible confounding, selection biases, and differential misclassification of exposure are limitations. A small case-control study nested in a prospectively followed cohort

found no association, and a single case–control study found an inverse association between seropositivity and prostate cancer.

BKV exposure was generally not associated with cancer at other sites in a large number of investigations (e.g. colorectum, bladder, brain, non-Hodgkin lymphoma (NHL), childhood neuroblastoma, acute lymphoblastic leukaemia (ALL)). There were usually only one or a few epidemiological studies per cancer site. A case–control study of cervical cancer precursors reported an association with BKV. A single cohort study of renal transplant recipients found an increased overall cancer risk among patients with polyomavirus-associated nephropathy, although this result was based on few cancer cases.

In summary, there is inconsistent and insufficient evidence from case series and from a few case–control studies. Several prospective studies have been performed, and these have uniformly not found any association.

5.3 Animal carcinogenicity data

The tumorigenicity of BKV in experimental animals has been studied extensively in 14 studies in outbred hamsters. Subcutaneous inoculation of BKV in newborn (NB) hamsters resulted consistently in fibrosarcoma at the site of injection, and intracerebral inoculation resulted in ventricular tumours (ependymoma and choroid plexus tumours), insulinoma, and osteosarcoma. Intravenous inoculation of BKV in weanling hamsters resulted in ependymoma, insulinoma, and osteosarcoma, as well as lymphoma and renal cell carcinoma at lower incidences.

BKV also induced ependymoma in mice after intracerebral inoculation of NB animals in one study.

In one study in rats, tumours (predominantly fibrosarcoma, osteosarcoma, and brain tumours) were induced after subcutaneous or intracerebral inoculation in NB animals. In a follow-up study, the incidence of tumours (mainly fibrosarcoma

and osteosarcoma) varied from 0% to 95% in six different rat strains.

In one study, BKV DNA induced ependymoma in NB hamsters injected intracerebrally, albeit at a low incidence.

In one study of BKV in transgenic mice, the founder animals developed hepatocellular carcinoma and renal cell carcinoma. In a follow-up study, the transgenic lines (progeny animals) developed renal cell carcinoma.

BKV DNA and oncogenic *c-Ha-ras* DNA, contained on the same plasmid, produced a high incidence in NB hamsters of brain sarcoma when inoculated intracerebrally and of sarcoma when injected subcutaneously. No tumours were induced after inoculation of BKV DNA alone or the oncogenic form of *c-Ha-ras* DNA alone, or after co-injection of BKV DNA and proto-oncogenic *c-Ha-ras* DNA.

5.4 Mechanistic and other relevant data

There is consistent evidence from animal and cell-culture studies that BKV can be directly oncogenic and transforming through its oncoproteins, i.e. the T-antigens encoded in the early region of its genome. The mechanisms involve immortalization, transformation, and enhancement of cell survival. There is only weak and controversial evidence for such mechanisms being active in human tumours.

- The presence of BKV DNA, based on analysis by polymerase chain reaction (PCR), has been reported in a broad variety of human tumours by several groups but not by others. In view of the ubiquity of BKV in the human population and its ability to circulate in the body, the tumour specificity of the PCR findings has in most cases not been established convincingly.
- BKV DNA, if found in human tumours, appears to be present mostly in low copy

numbers. This indicates that the majority of cells within a tumour potentially associated with BKV do not contain the viral genome. Thus, novel mechanisms for BKV-induced carcinogenesis would need to be invoked, for which there is currently little evidence.

- Studies that demonstrate evidence for cell-cycle and apoptosis regulation by BKV through pathways involving pRb family proteins and p53 in human tumours are few, and the evidence is weak.

6. Evaluation

6.1 Cancer in humans

There is *inadequate evidence* in humans for the carcinogenicity of BKV.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of BKV.

6.3 Overall evaluation

BKV is *possibly carcinogenic to humans* (Group 2B).

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JC POLYOMAVIRUS

1. Exposure Data

1.1 Cell and tissue tropism

The primary site of productive JC polyomavirus (JCV) replication in healthy subjects appears to be the epithelium of the urinary tract, similar to BK polyomavirus (BKV). In the brain of immunocompromised individuals, JCV can infect oligodendrocytes, astrocytes, cortical neurons, and cerebellar granule cell neurons. In addition to the clear tropism of JCV for the urinary epithelium and the central nervous system (CNS), the virus can be cultured in primary human fetal glial (PHFG) cells ([Knowles & Sasnauskas, 2003](#)).

1.2 Methods for the detection of JCV

The presence of JCV can be examined by several techniques. Some techniques were already available when the virus was discovered ([Padgett et al., 1971](#)), and others were developed later.

1.2.1 Pathological examination

Inclusion bodies can be observed in polyomavirus-infected oligodendrocytes. Electron microscopy is useful for visualizing the morphology and size of the virus particles and thus for initial identification of the virus as a polyomavirus ([Padgett et al., 1971](#)).

1.2.2 Serology

Serological testing of JCV is based on detection of specific JCV immunoglobulin (Ig) G antibodies after past infection, and of IgM antibodies after primary infection ([Stolt et al., 2003, 2005](#)). Moreover, similar to other infections, an increase in IgG titres ([Hogan et al., 1980](#)), and most likely also in transient IgM antibodies, may be detected during reactivation.

(a) *Haemagglutination and haemagglutination inhibition assays*

Like other polyomaviruses (e.g. BKV), JCV can haemagglutinate human blood group O erythrocytes, and antibodies against JCV induce haemagglutination inhibition (HAI). Antibody titres against JCV are obtained by incubating different dilutions of serum with a fixed haemagglutination unit (HAU) of JCV and then adding the blood group O erythrocytes. The highest dilution resulting in HAI is the HAI titre of the serum. JCV capsid antigen can be derived from extracts of JCV-infected PHFG cells, JCV capsid viral protein 1 (VP1) virus-like particles (VLPs), or JCV VP1-glutathione S-transferase (GST) grown in *Escherichia coli* or baculovirus. The obtained HAI is independent of antigen source, but there is cross-reactivity between JCV and BKV ([Knowles & Sasnauskas, 2003](#); [Viscidi et al., 2003](#)). This activity can be absorbed out or blocked with different VLPs ([Carter et al., 2003](#); [Rollison et al., 2003](#); [Stolt et al., 2003](#)).

(b) JCV IgM- and IgG-class specific ELISA

Standard enzyme-linked immunosorbent assay (ELISA) can be used to test for IgG and IgM antibodies against JCV ([Lundstig & Dillner, 2006](#); [Kean et al., 2009](#); [Antonsson et al., 2010](#); [Boldorini et al., 2011](#)). Virus antigen is more easily derived from JCV VP1 VLPs or JCV VP1-GST grown in *Escherichia coli* or baculovirus, than from JCV obtained by virus isolation in PHFG cells (for more details, see Section 1 of the *Monograph* on BKV in this Volume).

(c) Luminex-based multiplex serological assays

Serology has been refined by the possibility of now producing large quantities of antigens. [Antonsson et al. \(2010\)](#) described the use of a Luminex-based multiplex serological assay for detection of antibodies to JCV and BKV VP1 capsid proteins as antigens, and full-length VP1 of JCV and BKV are expressed in bacteria in fusion with the N-terminal GST domain (for further details, see Section 1 of the *Monograph* on BKV in this Volume).

1.2.3 PCR-based methods

In 1989, a polymerase chain reaction (PCR) method for detection of JCV and BKV was designed, with a sensitivity of 10 copies for BKV and 100 copies for JCV ([Arthur et al., 1989](#)). Later, a nested PCR method with a sensitivity of 10 copies for JCV was developed. This method cross-detects JCV, BKV, and simian virus 40 (SV40) ([Bogdanovic et al., 1994](#)). To distinguish between the amplified products, restriction enzyme cleavage was used (for more details, see Section 1 of the *Monograph* on BKV in this Volume). Recently, multiplex/Luminex-based PCR methods have been developed for the detection of JCV and other human polyomaviruses; these are generally sensitive and specific ([Schmitt et al., 2011](#)).

*1.2.4 Diagnosis of JCV infection and reactivation**(a) Indirect methods*

Serology is useful for epidemiological studies but is more rarely used to detect primary infection or reactivation, since most primary infections and reactivations occur asymptotically ([Lundstig & Dillner, 2006](#); [Kean et al., 2009](#); [Antonsson et al., 2010](#)). Many different assays are used, as described above and in [Table 1.1](#), but there is no gold standard.

(b) Direct methods

For diagnosis of JCV reactivation, manifested for example as JC viruria or JCV in the cerebrospinal fluid, detection of JCV DNA by PCR or multiplex Luminex is more sensitive and useful, although it is possible to use electron microscopy and virus isolation ([Padgett et al., 1971](#); [Bogdanovic et al., 1994](#); [Schmitt et al., 2011](#)).

*1.3 Epidemiology of JCV infection**1.3.1 Natural history, latency, persistence, and prevalence**(a) Prevalence and sero-epidemiology*

JCV infection is common worldwide. Sero-epidemiological studies show that about 70% of all adults have anti-JCV IgG antibodies. Seroconversion to JCV in children occurs later compared with BKV, and seroprevalence rates in adults continue to increase with age ([Table 1.1](#)).

(b) Primary infection and transmission

Primary JCV infection is thought to be asymptomatic ([Taguchi et al., 1982](#); [Maginnis & Atwood, 2009](#)). The transmission route is not known; the respiratory tract and the alimentary tract (faecal-oral route) have been proposed as possible routes. JCV is frequently excreted in the urine of adults, and shedding increases with age,

Table 1.1 Detection of JCV antibodies using different methods in healthy individuals of different ages from different populations

Reference	Study population and age	Study location	Method	No. of subjects	Prevalence of anti-JCV antibodies (%)
Brown <i>et al.</i> (1975)	Adults and children	9 isolated populations	HAI	393	0–75% (depending on the population)
Taguchi <i>et al.</i> (1982)	Adults and children	Japan	HAI	136	Overall, 71%; 1–5 yr, 45%; 6–20 yr, 65–70%; 20–50 yr, 70–90%; > 51 yr, 75%
Knowles <i>et al.</i> (2003)	Adults and children	England	HAI	2435	Overall, 35%; 0–9 yr, 11–14%, then increasing; 60–69 yr, 50%
Stolt <i>et al.</i> (2003)	Children and pregnant women	Sweden (children) Finland (women)	VLP-based ELISA blocked SV40/BKV/ JCV	290 children, 1656 women	1–3 yr, 16%; 3–13 yr, 27–51%; pregnant women 14–31 yr, 62–72%
Carter <i>et al.</i> (2003)	Adults	USA	VLP-based ELISA	415	19–78 yr, 44%
Rollison <i>et al.</i> (2003)	Adults	Maryland, USA	VLP-based ELISA	88	53 ± 19 yr, 77%
Rollison <i>et al.</i> (2009)	Adults	Maryland, USA	VLP-based ELISA	607	56 ± 12 yr, 71%
Egli <i>et al.</i> (2009)	Blood donors	Switzerland	VLP-based ELISA	400	58% (increasing from 50% in donors aged 20–29 yr to 68% in donors aged 50–59 yr)
Kean <i>et al.</i> (2009)	Blood donors: adults (> 21 yr) and group aged 1–21 yr	Denver, Colorado, USA	VP1 capsomer-based ELISA	1501 721	> 21 yr, 39% 1–21 yr, 21%
Antonsson <i>et al.</i> (2010)	Adults	Australia	VP1 capsomer-based ELISA/Luminex	458	> 4 yr, 63%; < 50 yr, 60%; 50–70 yr, 68%; > 70 yr, 64%
Matos <i>et al.</i> (2010)	Blood donors	Portugal	VLP-based ELISA	21	91%
Viscidi <i>et al.</i> (2011)	Healthy adults (1–93 yr)	Italy	VLP-based ELISA	947 total: 568 men, 374 women, 5 unknown	< 10 yr, 10%; 10–39 yr, 50–60%; 40–69 yr, 69–74%; > 70 yr, 81%

BKV, BK polyomavirus; ELISA, enzyme-linked immunosorbent assay; JCV, JC polyomavirus; HAI, haemagglutination inhibition; SV40, simian virus 40; VLP, virus-like particle; VP1, capsid viral protein 1; yr, year

which could lead to the contamination of food and water with urine in sewage ([Kitamura et al., 1990, 1997](#); [Kunitake et al., 1995](#); [Bofill-Mas et al., 2000, 2001](#); [Maginnis & Atwood, 2009](#)).

(c) *Persistence of JCV*

JCV DNA can be detected in the tonsils, kidneys, lymphocytes, and brain in immunocompetent healthy individuals ([Chesters et al., 1983](#); [Tornatore et al., 1992](#); [White et al., 1992](#); [Dörries et al., 1994](#); [Monaco et al., 1996](#); [Doerries, 2006](#); [Bialasiewicz et al., 2009](#); [Maginnis & Atwood, 2009](#)).

JCV DNA and capsid proteins coding sequences have been detected, for example by *in situ* hybridization (ISH), Southern blot, and PCR, occasionally in patients without progressive multifocal leukoencephalopathy (PML), whereas JCV is consistently found in the brain of patients with PML, a disease associated with JCV ([Elsner & Dörries, 1992](#); [Ferrante et al., 1995](#); [Vago et al., 1996](#)). However, not all studies could clearly indicate the presence of JCV in the brain ([Henson et al., 1991](#); [Bogdanovic et al., 1995](#); [Perrons et al., 1996](#)). Later, it was suggested that JCV could cross the blood–brain barrier by infecting B cells and then establishing latency there ([Monaco et al., 1996](#); [Monaco et al., 1998a](#); [Bag et al., 2010](#)), and accumulated data definitely suggest that JCV can be found in the brain of immunosuppressed individuals ([Maginnis & Atwood, 2009](#); [Bag et al., 2010](#)).

JCV is also detected in sera and in white blood cells of immunosuppressed and immunocompetent patients ([Dörries et al., 1994](#); [Dubois et al., 1997](#)).

(d) *Reactivation of JCV*

JC viruria is observed in healthy individuals without any clinical disease ([Chesters et al., 1983](#); [Kitamura et al., 1997](#); [Maginnis & Atwood, 2009](#)). JC viraemia or the presence of JCV in cerebrospinal fluid is very rare in immunocompetent individuals and is reported

mainly in immunosuppressed patients, and the findings are less concordant in healthy individuals ([Tornatore et al., 1992](#); [Dörries et al., 1994](#); [Koralnik et al., 1999](#); [Delbue et al., 2007](#); [Focosi et al., 2008](#); [Bag et al., 2010](#)).

In immunosuppressed individuals, for example people with HIV/AIDS, haematopoietic stem cell transplant recipients, organ transplant recipients, or immunosuppressed multiple sclerosis patients, uncontrolled JCV replication can lead to PML ([Major et al., 1992](#)). Very rarely, JCV can also be observed in haemorrhagic cystitis, mainly associated with BKV ([Dalianis & Ljungman, 2011](#)).

1.3.2 Diseases associated with JCV

(a) *Progressive multifocal leukoencephalopathy and its management*

PML is a severe subacute demyelinating disease of the CNS caused by JCV infection ([Major et al., 1992](#); [Whiteman et al., 1993](#); [Kleinschmidt-DeMasters & Tyler, 2005](#); [Maginnis & Atwood, 2009](#); [Bag et al., 2010](#); [Hellwig & Gold, 2011](#)). PML is characterized by mental deterioration, impairment of speech and vision, and motor weakness ([Astrom et al., 1958](#); [Khalili & White, 2006](#); [Bag et al., 2010](#)). Gradually, paralysis of limbs and sensory dysfunction develop, and cortical blindness, dementia, and coma follow. Prognosis in PML is poor, and the disease is fatal within 6–12 months of its onset (Fong & Toma, 1995; [Kleinschmidt-DeMasters & Tyler, 2005](#)).

How JCV enters the brain has been debated, and three scenarios have been proposed. JCV can enter the brain by haematogenous spread after reactivation in the kidneys or in blood cells, hence its multifocal distribution ([Tornatore et al., 1992](#); [Maginnis & Atwood, 2009](#)). Alternatively, JCV is already latent in the brain and is reactivated locally ([Elsner & Dörries, 1992](#)). Finally, a third, more unlikely, proposed scenario is a late primary infection in immunocompromised children ([Stoner et al., 1988a](#)).

JCV causes lytic infection of the myelin-producing oligodendrocytes and subsequent demyelination. PML is one of the rare demyelinating diseases where a direct viral infection of oligodendrocytes has been demonstrated ([Ludwin, 1997](#)). Macroscopically the foci are presented as grey or brownish regions in the white matter, especially in conjunction with grey matter, and the lesions occur in the cerebral hemispheres but also sometimes in the cerebellum, the brain stem, and the spinal cord ([Whiteman et al., 1993](#); [Bag et al., 2010](#)). Microscopically, it is possible to observe changes in oligodendrocytes and astrocytes, with enlarged oligodendrocytes with abnormal inclusions within large swollen nuclei. The astrocytes are also infected, enlarged with deformed and bizarre-shaped nuclei, often multinucleated, and they resemble transformed cells in malignant glioblastoma tumours ([Astrom et al., 1958](#); [Richardson, 1961](#)).

Recently, another type of PML-like disease has been described in relation to immune reconstitution syndrome. It is associated with a marked inflammatory reaction, where the lesions are characterized by either diffuse or focal perivascular infiltrates of T cells, monocytes, macrophages, B cells, and plasma cells. This variant usually develops in immunosuppressed individuals where the treatment is abrogated or in people with HIV/AIDS who receive antiviral therapy, and where the reconstitution of the immune system recognizes and reacts against JCV in the brain, thus enhancing neurological symptoms ([Bag et al., 2010](#); [Kappos et al., 2011](#)).

(b) Granule cell neuropathy

In addition, in PML, or separately in granule cell neuropathy (GCN), JCV variants have been detected with small deletions in the C terminus of the VP1 region, and named JCV GCN ([Dang et al., 2012](#)). This variant is associated with lytic infection of cerebellar granule cell neurons and can cause symptoms connected to the function of the cerebellum ([Koralnik et al., 2005](#)).

There is no known established antiviral therapy for PML (for reviews, see [Cinque et al., 2009](#); [Bag et al., 2010](#); [Kappos et al., 2011](#)).

(c) JCV in polyomavirus-associated nephropathy

Polyomavirus-associated nephropathy (PVAN) emerged as a disease after the substitution of cyclosporine A with tacrolimus for immunosuppression and is found in 1–10% of renal transplant patients ([Randhawa et al., 1999](#); Hirsch & Randhawa, 2009; [Ramos et al., 2009](#)). PVAN is caused by BKV in > 95% of the cases, while < 5% are attributed to JCV ([Ramos et al., 2009](#)). So far, there are no effective antivirals against PVAN, and therefore the treatment of choice in many centres has been reduction of immunosuppression (Hirsch & Randhawa, 2009).

2. Cancer in Humans

Methodological considerations: case–control versus case series studies

Numerous studies have reported the prevalence of markers of infection by polyomaviruses in tumour tissues or blood obtained from humans with cancer. Many of these studies included specimens from individuals without cancer as “controls,” but such studies were not generally considered by the Working Group as case–control studies, given the convenience sampling strategies used or the lack of comparability of exposure measurement between comparison groups. Specifically, convenience sampling of controls led to the possibility that the control subjects were not representative of the source population. Also, the comparison of tumour tissues in cases with normal tissues (such as blood, urine, or biopsies of normal tissues) in controls may also be biased because it is uncertain whether polyomaviruses are uniformly present in these normal tissues

or can be reliably detected by the assays used. However, because these studies contribute information on cancer sites not investigated by the case–control studies, included comparisons with both normal and pre-malignant control tissues, compared tumour tissue with a convenience sample of controls, compared different tissues in cases or controls, and/or presented findings for susceptible populations (i.e. transplant patients), they are considered here as case series.

2.1 Background

No cohort studies have been conducted examining the association between JCV infection and human cancers. However, five case–control studies nested within three distinct cohorts have been conducted to investigate the association between JCV seropositivity and colorectal cancer (CRC) ([Lundstig et al., 2007](#); [Rollison et al., 2009](#)), non-Hodgkin lymphoma (NHL) ([Rollison et al., 2006](#)), brain tumours ([Rollison et al., 2003](#)), and childhood neuroblastomas ([Stolt et al., 2005](#)). These studies are similar to cohort studies in that they establish the temporal relationship between exposure and disease (i.e. JCV exposure occurred before the onset of cancer) and are summarized as prospective studies in [Table 2.1](#). In addition, a case–control study of childhood leukaemia measured JCV antibodies based on blood samples obtained at birth ([Priftakis et al., 2003](#)). Although not nested within a cohort, this study also assessed exposure status of the cases before disease onset. Case–control studies of JCV and cancer in humans that assessed exposure retrospectively are presented in [Table 2.2](#) and include studies of NHL ([Engels et al., 2005b](#)), colon cancer ([Campello et al., 2010](#)), bladder cancer ([Polesel et al., 2012](#)), and prostate cancer ([Carter et al., 2003](#)). [These studies are limited in their interpretation as they cannot rule out reverse causality (i.e. antibody levels are affected by the state of disease).]

Case–control studies incorporated several different biomarkers of JCV infection and/or exposure, each with different strengths and limitations. Among these studies, six of them ([Carter et al., 2003](#); [Rollison et al., 2003, 2006, 2009](#); [Engels et al., 2005b](#); [Lundstig et al., 2007](#)) used serological markers of JCV exposure among adults, all of which measured IgG to JCV capsid proteins or VLPs using ELISA. Circulating IgG antibodies to JCV capsid proteins persist decades after initial JCV infection in adolescence and therefore are useful for establishing past exposure to infection. However, capsid antibodies are not a specific marker for reactivation of latent JCV infection [which may be more relevant for cancer etiology than initial infection, given that JCV reactivation theoretically occurs closer to the time of cancer initiation]. JC viruria, a marker of active JCV replication, was observed among bladder and colon cancer cases ([Campello et al., 2010](#); [Polesel et al., 2012](#)). JCV viremia is another marker of active infection, although it has only been incorporated into one case–control study of childhood leukaemia, in which the presence of JCV was assessed in blood spots obtained at birth ([Priftakis et al., 2003](#)). None of the blood spots were JCV DNA-positive in either the cases or controls. Serological markers of exposure to the T-antigen oncoproteins would also be of interest. However, no published epidemiological studies of cancer in humans have investigated seroreactivity to JCV T-antigens.

2.2 Cancer of the colorectum

2.2.1 Prospective studies

[Lundstig et al. \(2007\)](#) conducted a nested case–control study of JCV and CRC among male participants in a large cohort study in Norway. Blood samples were obtained from 330 000 individuals enrolled in the Janus biobank in Norway. The national, population-based cancer registry was used to identify incident cases of CRC that

Table 2.1 Prospective studies of JCV and cancer in humans

Reference, study location	Study design	Cases	Controls	Measure of JCV	Findings	Confounders assessed?	OR (95%CI)	Comments
Priftakis et al. (2003) , Sweden	Case-control	54 children who developed ALL	37 children who did not develop ALL	DNA by PCR; measured in Guthrie card samples from birth	No JCV DNA was detected in any of the case or control samples	No (matching was unsuccessful; age distribution of controls not stated)	None calculated (none of the cases or controls were JCV-positive)	Exposure measured in samples collected before diagnosis.
Rollison et al. (2003) , USA	Nested case-control	44 brain cancer cases	88 controls Matched on age, sex, race, date of blood draw	Capsid IgG by VLP-based ELISA	Seroprevalence: Cases: 82% Controls: 75%	Yes (matching factors)	OR _{all} = 1.46 (0.61–3.50) OR _{GBM} = 2.38 (0.64–8.86) OR _{astro.} = 0.81 (0.17–3.78) OR _{others} = 1.00 (0.013–8.00)	Cases and controls selected from an underlying cohort of ~40000 individuals in Maryland, USA. Overlaps with Rollison et al. (2006, 2009) .
Stolt et al. (2005) , Finland	Nested case-control	115 mothers of childhood neuroblastoma cases	918 mothers of children who did not develop neuroblastoma (matched on age and region)	Capsid IgG and IgM by VLP-based ELISA	Seroprevalence (IgG/IgM): Cases: 71%/8% Controls: 74%/9%	Yes (matching factors; sex distributions not presented)	IgG: 0.9 (0.6–1.4) IgM: 0.9 (0.4–1.9)	Cases and controls were selected from a population-based cohort of pregnant women undergoing rubella screening.
Rollison et al. (2006) , USA	Nested case-control	170 NHL	340 Matched on age, sex, date of blood draw	Capsid IgG by VLP-based ELISA	Seroprevalence: Cases: 65% Controls: 68%	Yes (matching factors, education and smoking status; BKV and EBV seroreactivity also assessed)	OR _{all} = 0.83 (0.56–1.23) OR _{DLBCL} = 0.88 (0.49–1.56) OR _{follicular} = 1.18 (0.49–2.85) OR _{other} = 0.63 (0.33–1.22)	Cases and controls selected from an underlying cohort of ~40000 individuals in Maryland, USA. Overlaps with Rollison et al. (2003, 2009) .
Lundstig et al. (2007) , Norway	Nested case-control	386 CRC (men only)	386 Matched on age, date of blood draw, county	Capsid IgG by VLP-based ELISA	Seroprevalence: Cases: 72% Controls: 74%	Yes (matching factors)	OR _M = 0.91 (0.65–1.27)	Cases and controls selected from an underlying cohort of 330000 individuals from the Janus biobank in Norway.

Table 2.1 (continued)

Reference, study location	Study design	Cases	Controls	Measure of JCV	Findings	Confounders assessed?	OR (95%CI)	Comments
Rollison et al. (2009) , USA	Nested case-control	611 CRC 123 adenomas	611 123 Matched on age, sex, race, date of blood draw	Capsid IgG by VLP-based ELISA	Seroprevalence: CRC cases: 72% CRC controls: 75%, Adenoma cases: 47% Adenoma controls: 51%	Yes (matching factors, smoking status, BMI; also assessed parity, HRT, C-reactive protein levels)	CRC: OR _{all} = 0.90 (0.79–1.03) OR _M = 1.00 (0.81–1.24) OR _F = 0.84 (0.71–0.99) Adenoma: OR _{all} = 0.84 (0.56–1.27) OR _M = 2.31 (1.20–4.46) OR _F = 0.31 (0.14–0.67)	Cases and controls selected from an underlying cohort of ~40000 individuals in Maryland, USA. Overlaps with Rollison et al. (2003, 2006) .

ALL, acute lymphoblastic leukaemia; astro, astrocytoma; BKV, BK polyomavirus; BMI, body mass index; CI, confidence interval; CRC, colorectal cancer; DLBCL, diffuse large B-cell lymphoma; EBV, Epstein–Barr virus; ELISA, enzyme-linked immunosorbent assay; F, female; follicular, follicular lymphoma; GBM, glioblastoma; HRT, hormone replacement therapy; Ig, immunoglobulin; JCV, JC polyomavirus; M, male; NHL, non-Hodgkin lymphoma; OR, odds ratio; PCR, polymerase chain reaction; VLP, virus-like particle.

Table 2.2 Case–control studies of JCV and cancer in humans

Reference, study location	Cases	Controls	Measure of JCV	Findings	Confounders assessed?	OR (95%CI)	Comments
Carter <i>et al.</i> (2003) USA	90 prostate cancer cases from Seattle population-based case–control study	72 population-based controls selected by RDD	VLP-based ELISA and neutralization assays for antibodies	% JCV seropositive: Prostate cases: 52% Controls: 64%	No	[crude OR, 0.62; 95%CI: 0.31–1.22] (Stated in text no significant difference for prostate patients <i>versus</i> controls).	Study was designed to investigate prevalence of SV40 antibodies in human populations; prostate cancer cases and controls were obtained from a larger population-based case–control study; cases and controls are stated to be between the ages of 40 and 64 yr, although it is unclear whether there was an age difference between cases and controls.
Engels <i>et al.</i> (2005b) USA	724 NHL	622 Matched on age, sex, ethnicity, study site	Capsid IgG by VLP-based ELISA	Seroprevalence: Cases: 49% Controls: 59%	Yes (matching factors)	0.70 (0.56–0.87)	Population-based selection of cases and controls
Campello <i>et al.</i> (2010) Italy	94 patients with colon cancer	91 relatives of the cases who underwent screening colonoscopy, including: 57 normal colon, 20 adenoma, 10 hyperplastic polyp, 4 other colon conditions	JCV DNA by real-time PCR in colon tissues and in some urine samples	No JCV DNA was detected in any of the samples JCV DNA detected in 5 (100%) of 5 urine samples obtained from cases and 14 (44%) of 32 controls	No	No OR calculated for JCV DNA in colon tissues since none was found $P < 0.05$ for the comparison of JCV DNA in urine	Urine samples were only available for 5 of 94 cases and 32 of 91 controls; it is unclear whether these groups are representative of the overall study population.

Table 2.2 (continued)

Reference, study location	Cases	Controls	Measure of JCV	Findings	Confounders assessed?	OR (95%CI)	Comments
Polesel <i>et al.</i> (2012) Italy	114 patients with TCC of the bladder	140 patients with orthopaedic disorders and other illnesses unrelated to smoking	DNA by PCR; measured in urine samples	Prevalence of JCV DNA in urine: Cases: 66% Controls: 71%	Yes (sex, age, education, smoking, alcohol)	1.19 (0.62–2.28)	Hospital-based selection of cases and controls

ALL, acute lymphoblastic leukaemia; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; JCV, JC polyomavirus; NHL, non-Hodgkin lymphoma; OR, odds ratio; OS, osteosarcoma; PCR, polymerase chain reaction; RDD, random-digit dialling; SV40, simian virus 40; TCC, transitional cell carcinoma; VLP, virus-like particle; yr, year

subsequently developed among male cohort participants ($n = 1105$). These men were selected for a separate case-control study of leptin levels and CRC. From this pool of CRC cases, 400 men who had no history of cancer at the time of cohort enrolment and were diagnosed with CRC at least 3 months after blood draw were randomly selected, of whom 386 had blood samples that could be located. Time between blood sampling and diagnosis ranged from 4 months to 28 years. An equal number of controls were matched to the CRC cases on age (within 1 year), date of blood draw (within 2 months), and county. Controls were male cohort participants who had not developed cancer as of the date of diagnosis of the case. [It was not stated whether the controls were confirmed to be alive as of the same date.] IgG antibodies to JCV and BKV were measured from the archived blood samples using VLP-based ELISA.

Baseline JCV seroprevalence was similar among men who subsequently developed CRC (72%) and those who did not (74%) (odds ratio [OR], 0.91; 95% confidence interval [CI]: 0.65–1.27). Two alternative cut-offs (based on optical density values) were considered in the definition of JCV seropositivity that distinguished healthy children who shed JCV in their urine from those who did not. These alternative cut-offs resulted in odds ratios of 0.82 (95% CI, 0.61–1.12) and 0.69 (95% CI, 0.51–0.95) for the association between JCV seropositivity and CRC.

[Strengths of the study include its prospective design and long duration of follow-up (up to 28 years). Other than the matching factors of age and date of blood draw, no other potential confounders were considered. Furthermore, only men were included in the study, and thus results cannot necessarily be generalized to women.]

[Rollison *et al.* \(2009\)](#) conducted a nested case-control study of JCV seroreactivity and CRC and colorectal adenoma among participants in two cohort studies established in Washington County, Maryland, USA, in 1974 ($n \approx 24\,000$) and 1989 (n

$\approx 25\,000$), with ~ 8000 individuals participating in both cohorts. Participants completed a baseline questionnaire and donated a blood sample for research. Follow-up questionnaires were administered to participants of the 1989 cohort in 1996, 1998, 2000, and 2002. Cohort participants were followed over time for cancer diagnoses using the Washington County and Maryland State cancer registries, and 611 incident cases of CRC were identified. One control was matched to each case on sex, race, age (within 1 year), and date of blood draw (within 2 weeks). Controls had to have been alive and not have developed cancer as of the date of diagnosis of the matched case. In addition to the CRC cases and controls, 123 colorectal adenoma cases were identified based on self-report of adenoma diagnosis in the follow-up questionnaires and subsequent verification through retrieval of pathology reports. These 123 adenoma cases were matched on sex, race, age, and date of blood draw to controls who reported they had undergone a colonoscopy but were not diagnosed with an adenoma based on the same follow-up questionnaire to which the case responded. Archived baseline serum (from the 1974 cohort) or plasma (from the 1989 cohort) was obtained for all CRC cases, adenoma cases, and controls for the measurement of IgG antibodies to JCV using a VLP-based ELISA. In addition to the matching factors, other confounders adjusted for in the analysis included baseline smoking status and body mass index (BMI). Levels of C-reactive protein, parity, and use of hormone replacement therapy were also evaluated as potential confounders but were not included in the final models.

Overall, baseline JCV seroprevalence was observed to be 72% in CRC cases and 75% in controls (OR, 0.90; 95% CI, 0.79–1.03). Among men, the association was null (OR, 1.00; 95% CI, 0.81–1.24), whereas a statistically significant inverse association was observed among women (OR, 0.84; 95% CI, 0.71–0.99). When analyses were restricted to the 1989 cohort and adjusted

for baseline smoking status and BMI, the associations between JCV seropositivity and CRC for both men and women were null. No clear differences were observed in JCV-associated CRC across categories of anatomical location, stage at diagnosis, or time between blood draw and diagnosis.

No association was observed between JCV seropositivity and colorectal adenoma overall (OR, 0.84; 95% CI, 0.56–1.27). However, when analyses were stratified by sex, two different patterns emerged, with JCV seropositivity associated with a > 2-fold increased risk of adenoma among men (OR, 2.31; 95% CI, 1.20–4.46), and a decreased risk of adenoma among women (OR, 0.31; 95% CI, 0.14–0.67; $P_{\text{interaction}} < 0.001$). Among men, the positive association was particularly pronounced for those diagnosed with multiple adenomas (OR, 6.71; 95% CI, 1.34–33.60) and those with larger adenomas (OR, 3.83; 95% CI, 1.20–12.25). No clear differences in JCV-associated adenoma risk were observed by adenoma number or size among women, or by anatomical location or histology among men or women.

[Strengths of the study include the prospective design, long duration of follow-up, inclusion of both men and women, and information available on confounders. Limitations include the reliance on self-report of no diagnosis of adenomas for the controls who were matched to the adenoma cases, and the differences in JCV seroprevalence observed between the two cohorts (75% vs 56% in the 1974 and 1989 cohorts, respectively), suggesting that length of storage may have affected the measurement of JCV antibody levels. However, given that cases and controls were matched on cohort participation and date of blood draw, it is unlikely that any underestimation of seroprevalence resulting from storage effects would have biased the observed results. Reasons for the sex difference in observed JCV-associated adenoma risk are unclear.]

2.2.2 Case-control studies

[Campello *et al.* \(2010\)](#) obtained colon tumour tissues from a series of 94 patients with colon cancer and normal colon tissues from 91 of their relatives who underwent screening colonoscopies. In addition, blood samples were obtained from all study participants, and urine samples were obtained from a subset of 5 colon cancer patients and 32 relative controls. Quantitative PCR was used to measure JCV DNA in colon tissues, blood samples, and urine samples. None of the blood samples or colon tissues were JCV DNA-positive. JCV DNA was detected in the urine of 5 (100%) colon cancer patients and 14 (44%) controls [crude OR, ∞ ; 95% CI, 1.5– ∞]. [Although there is limited evidence that a greater proportion of cases than controls were shedding JCV DNA, it is unclear how representative the cases and controls, for whom urine samples were available, were of the larger study population. In addition, the lack of JCV DNA in the colon tumour tissues of the cases suggests that JCV DNA detected in the urine of these patients was not involved with the development or maintenance of the tumour.]

2.3 Non-Hodgkin lymphoma (NHL)

2.3.1 Prospective studies

[Rollison *et al.* \(2006\)](#) conducted a nested case-control study of JCV seroreactivity and NHL among participants of the same Maryland cohorts described above for the CRC case-control study in Section 2.2.1. Among cohort participants, 170 incident cases of NHL with available blood samples were identified. Two controls were matched to each case on sex, race, age (within 1 year), and date of blood draw (within 2 weeks). Controls had to have been alive and not have developed cancer as of the date of diagnosis of the matched case. Archived baseline serum (from the 1974 cohort) or plasma (from the

1989 cohort) was obtained for all NHL cases and controls for the measurement of IgG antibodies to JCV using a VLP-based ELISA. In addition to the matching factors, other factors assessed as potential confounders included education, smoking status, and seroreactivity for BKV and Epstein–Barr virus (EBV).

Of NHL cases, 65% were determined to be JCV seropositive at baseline, compared with 68% of controls (OR, 0.83; 95% CI, 0.56–1.23). In the subset of cases and controls for whom EBV antibody levels had been measured as part of another study, adjustment for antibodies to EBV early and viral capsid antigens did not change the association between baseline JCV seropositivity and NHL development. Baseline seropositivity was not associated with diffuse large B-cell lymphoma, follicular lymphoma, or other types of lymphoma. When time between baseline blood draw and NHL diagnosis was considered (< 10, 10–19, or 20–29 years), JCV was not significantly associated with NHL development.

[Strengths of the study include the prospective design, the long duration of follow-up, and the availability of BKV and EBV antibody levels for the same study participants. Limitations include limited sample size for stratified analyses and potential storage effects on the measurement of antibody levels.]

2.3.2 Case–control studies

[Engels et al. \(2005b\)](#) conducted a population-based case–control study of antibodies to JCV among patients with NHL in four areas covered by the Surveillance, Epidemiology and End Results (SEER) registry programme: the state of Iowa and the metropolitan areas of Detroit, Los Angeles, and Seattle. Cases of NHL diagnosed in 1998–2000 were identified through the SEER registries and were limited to those aged 20–74 years at diagnosis ($n = 1321$). Controls were recruited from the general population and frequency-matched to the NHL cases on age, sex,

and ethnic origin. Controls were identified using random-digit dialling for those aged 20–64 years and through Medicare eligibility files for those aged 64–74 years (total number of controls, 1057). The measurement of JCV and BKV IgG antibodies by VLP-based ELISA was limited to 724 NHL cases and 622 controls for whom blood samples were available.

JCV seroprevalence was observed to be 49% among NHL cases and 59% among controls (OR, 0.70; 95% CI, 0.56–0.87). Using an alternate cut-off point that best distinguished JCV shedders from non-shedders in a separate study population, a statistically significant inverse association was observed between JCV seropositivity and NHL (OR, 0.54; 95% CI, 0.43–0.68). No clear differences in the inverse association between JCV seropositivity and NHL were observed when results were stratified by NHL histology, site (nodal vs extranodal), or prior treatment received for NHL.

[Strengths of the study include the large sample size, diversity of the study population, and availability of information on BKV seroreactivity. Limitations of the study include potential selection bias introduced by non-participation in the case and/or control groups (participation rates are not provided in the manuscript) and/or the unavailability of samples on a subset of participants, and the retrospective design (i.e. JCV antibodies were measured after NHL diagnosis).]

2.4 Cancer of the brain

2.4.1 Prospective studies

[Rollison et al. \(2003\)](#) conducted a nested case–control study of JCV seroreactivity and incident brain tumours among participants of the same Maryland cohort studies described above in Sections 2.2.1 ([Rollison et al., 2009](#)) and 2.3.1 ([Rollison et al., 2006](#)). Incident diagnoses of primary malignant tumours of the brain or

meninges occurring through 2000 were identified using county and state cancer registries ($n = 44$). Two controls were matched to each case on sex, race, age (within 2 years), and date of blood draw (within 45 days). Controls had to have been alive and not have developed cancer as of the date of diagnosis of the matched case. Archived baseline serum (from the 1974 cohort) or plasma (from the 1989 cohort) was obtained for all brain cancer cases and controls for the measurement of IgG antibodies to JCV by an ELISA using JCV virion particles as antigen.

Baseline seropositivity for JCV was observed to be 82% for brain tumour cases and 75% for controls (OR, 1.46; 95% CI, 0.61–3.50). When seropositive individuals were further divided into those with lower versus higher titres, no difference in brain tumour risk was observed. Baseline JCV seropositivity was associated with an increased risk of subsequent glioblastoma ($n = 28$), although this association was not statistically significant (OR, 2.38; 95% CI, 0.64–8.86). No associations were observed between JCV seropositivity and astrocytoma ($n = 9$) or other types of brain tumours ($n = 7$). No difference in JCV-associated brain tumour risk was observed by age at diagnosis (< 65 years vs ≥ 65 years). The association between JCV seropositivity and brain tumours was greater for those diagnosed with brain tumours > 7.8 years after blood draw (the median value; OR, 2.95) than for those diagnosed closer to blood draw (OR, 0.89), although neither association was statistically significant. In the same study, baseline BKV and SV40 antibodies were not associated with brain tumour development. [Study strengths include the prospective design and the availability of antibody levels to BKV and SV40. The major limitation of the study was its small sample size.]

2.4.2 Case–control studies

No data were available to the Working Group.

2.5 Cancer of the bladder

2.5.1 Prospective studies

No data were available to the Working Group.

2.5.2 Case–control studies

[Polesel *et al.* \(2012\)](#) conducted a hospital-based case–control study of human polyomavirus and human papillomavirus (HPV) infection in transitional cell carcinoma (TCC) of the bladder in Italy. Patients being treated for TCC were identified through multiple hospitals in 2004–07 ($n = 114$) and compared with a control group of patients being treated for orthopaedic and other non-malignant, non-smoking-related conditions ($n = 140$). DNA was extracted from urine samples that were obtained before treatment and tested for the presence of polyomavirus and HPV DNA using PCR-based methods. JCV DNA was detected in urine samples from 66% of TCC cases and 71% of controls, corresponding to an odds ratio of 1.19 (95% CI, 0.62–2.28) after adjustment for age, sex, education, tobacco smoking, and alcohol consumption. No statistically significant associations were observed for BKV or HPV. [JCV shedding in the urine is a marker of active viral replication, not past infection. Therefore, it is unclear whether JC viruria is an appropriate biomarker for use in retrospective case–control studies, particularly when the cancer (or other conditions for which the controls are being treated), or hospitalization itself, could be associated with JCV reactivation and viral shedding.]

2.6 Childhood malignancies

In this section, two case–control studies are described that investigated the association between JCV infection possibly acquired *in utero* and subsequent development of childhood cancer. One nested case–control study measured JCV IgG and IgM antibodies in blood samples

obtained from mothers during pregnancy ([Stolt et al., 2005](#)), and another case-control study assessed the presence of JCV DNA prospectively in blood samples obtained from newborn (NB) infants at the time of birth ([Priftakis et al., 2003](#)). Neither assessed exposure to or acquisition of JCV infection by the child after birth. [It is unclear whether vertical transmission of JCV occurs.]

2.6.1 Prospective studies

[Stolt et al. \(2005\)](#) conducted a population-based nested case-control study of JCV infection and childhood neuroblastoma in Finland, where > 98% of all pregnant women are screened for rubella immunity in their first trimester of pregnancy, with the leftover blood samples archived for use in research. The study identified 115 cases of childhood neuroblastoma diagnosed in 1983 and later from the Finnish national cancer registry. Serum samples were identified from the corresponding mothers. Eight controls were selected for each case by locating the blood sample for the mother of the case subject and choosing the next eight samples located in the specimen box, corresponding to women whose children did not develop neuroblastoma. The blood samples were arranged in the storage boxes according to date of blood collection and geographical region, and therefore the controls were matched to the cases on these factors.

Using the archived blood, IgG and IgM antibodies to JCV and BKV were assessed with a VLP-based ELISA. The seroprevalence of JCV IgG was 71% in mothers of children who developed neuroblastoma and 74% in mothers of children who did not develop neuroblastoma (OR, 0.9; 95% CI, 0.6–1.4). Similarly, no case-control difference was observed for JCV IgM seroprevalence (8% in mothers of cases and 9% in mothers of controls; OR, 0.9; 95% CI, 0.4–1.9). Similar associations were observed when cases diagnosed

at age younger than 1 year were analysed separately from those diagnosed at older ages.

[The population-based selection of cases reduced the potential for selection bias, and the selection of eight control samples from the same box of specimens in effect matched the controls to the cases on age and geographical region. The measurement of both IgG and IgM is a strength of this study, although they were measured in blood samples obtained from the mothers. Therefore, the presence of the infection in the fetus could not be definitively established. It is unclear whether mother-to-child transmission of JCV occurs.]

[Priftakis et al. \(2003\)](#) conducted a case-control study of JCV infection and childhood acute lymphoblastic leukaemia (ALL) in Sweden using Guthrie card samples, which are obtained from 99.8% of NB infants in Sweden within 3–5 days of birth for screening of metabolic disorders at a central national laboratory. Children being treated for ALL were identified from four hospitals in Sweden in 1980–2001, and their Guthrie cards were obtained ($n = 54$). Their ages ranged from 9 months to 17 years. The investigators sought to obtain Guthrie cards for children who did not develop ALL, matched to the cases on age and birthplace, but were able to obtain consent from only 37 of the 54 identified controls. DNA was extracted from the blood spots on the Guthrie cards, and nested PCR was used to detect JCV and BKV sequences. All samples amplified the control gene (*HLA-DQ*), but none amplified DNA from either JCV or BKV, and thus no measures of association were calculated.

[The primary strength of the study is that the use of archived Guthrie cards does establish a temporal relationship between exposure and disease. Limitations include questionable internal and external validity resulting from the methods of case and control selection. For example, it is unclear how representative the case group was of all childhood ALL cases in Sweden, as only 54 cases diagnosed over a period of 21 years were

included in the study. If demographic factors of children treated for ALL outside of the four hospitals included in the study were somehow associated with JCV and/or BKV infection, then it is possible that the results are not generalizable to all cases of ALL. The age and sex distributions of the cases and controls were not presented; therefore, it is unclear whether there could have been selection bias resulting from the fact that only 69% of the selected controls were ultimately included in the analysis.

In terms of assay sensitivity, the PCR assay used was highly sensitive and reported the ability to detect JCV DNA in other specimens obtained from a separate group of transplant patients. There is a possibility that *in utero* infection of JCV might not result in JCV DNA-positive peripheral blood leukocytes (PBLs); the authors cite their previous studies of congenital cytomegalovirus and human T-cell lymphotropic virus, in which Guthrie cards were used to detect viral DNA in PBLs.]

2.6.2 Case-control studies

No data were available to the Working Group.

2.7 Other cancers

2.7.1 Prospective studies

No data were available to the Working Group.

2.7.2 Case-control studies

[Carter et al. \(2003\)](#) measured JCV antibodies in blood samples obtained from 90 prostate cancer cases and 72 controls selected from a larger population-based case-control study of prostate cancer conducted in Seattle, Washington, USA. JCV antibodies were detected in samples from 52% of prostate cancer cases and 64% of controls [crude OR, 0.62; 95% CI, 0.31–1.22]. [The study was designed to assess the prevalence of SV40 antibodies in different human populations and

measured JCV and BKV antibodies to assess potential cross-reactivities with antibodies to SV40. No odds ratio was calculated to estimate the magnitude of associations for JCV antibodies and prostate cancer, and it is unclear whether the association could have been confounded by demographic factors such as age. Although it is stated in the methods that both cases and controls were aged 40–62 years, it is unclear whether there were age differences between the prostate cancer cases and controls.]

2.7.3 Case series

Two studies investigated JCV antibodies across series of patients with different diagnoses ([Hogan et al., 1983](#); [Niv et al., 2010b](#)). Antibodies were detected in a variable proportion of cases [but it is unclear whether there were any statistically significant differences in [Hogan et al. \(1983\)](#)]. [Niv et al. \(2010b\)](#) measured JCV antibodies by ELISA in serum samples obtained from a series of patients undergoing colonoscopy and reported significantly higher mean titres for 25 patients with CRC, 12 with advanced adenomas, and 19 with simple adenomas compared with 41 with no colon disease. A third study detected JCV DNA in PBLs of 57% of 74 of patients with Hodgkin lymphoma ([M'kacher et al., 2010](#)).

There have been numerous published studies reporting the prevalence of JCV DNA and/or T-antigen (LT) expression in tumour tissues or blood obtained from humans with cancer. Many of these studies included non-tumour tissues obtained from individuals without cancer as “controls” but did not meet the definition of a case-control study, given the convenience sampling strategies used and the lack of comparability of exposure measurement between comparison groups ([Del Valle et al., 2001a, 2005](#); [Hori et al., 2005](#); [Theodoropoulos et al., 2005](#); [Muñoz-Mármol et al., 2006](#); [Abdel-Aziz et al., 2007](#); [Murai et al., 2007](#); [Zheng et al., 2007a, b](#); [Kutsuna et al., 2008](#); [Selgrad et al., 2008](#); [Del Valle](#)

& Khalili, 2010; Ksiaz et al., 2010; Martinez-Fierro et al., 2010; Niv et al., 2010b). However, because these studies contribute information on cancer sites not investigated by the case–control studies (i.e. prostate, lung, oesophagus, tongue, stomach, and oral cavity), included comparisons with both normal and pre-malignant control tissues, and/or presented findings for susceptible populations (i.e. transplant patients), they are presented in a separate table (Table 2.3).

Results of these tumour case series vary greatly. Based on the case series presented in Table 2.3, the proportion of human tumour tissues that tested positive for JCV DNA by PCR was 26–61% for CRC, 5–50% for colorectal adenoma, 53–68% for lung cancer, 26–86% for gastric cancer, and 0–100% for brain tumours. In individual studies evaluating single cancer types, JCV DNA prevalence ranged from 0% in prostate cancer cases (Martinez-Fierro et al., 2010) to 100% of oesophageal cancer cases (Del Valle et al., 2005). Factors possibly contributing to variations in study findings include differences in sample size (ranging from < 10 to > 100 cases), preparation of tumour tissues (fresh frozen vs paraffin-embedded), age of archived samples, demographic characteristics of the study populations, and sensitivity and specificity of the laboratory methods used to detect JCV. Of those studies that measured both JCV DNA and LT expression, the proportion of tumours that were JCV DNA-positive tended to be greater than the proportion that expressed LT. These markers were often assessed for different subgroups of samples, and the concordance between DNA and LT expression results was not always stated.

An even greater number of tumour case series have been published in which no “control” tissues were included (Rencic et al., 1996; Aoki et al., 1999; Krynska et al., 1999a; Laghi et al., 1999; MacKenzie et al., 1999; Smith et al., 1999; Caldarelli-Stefano et al., 2000; Weggen et al., 2000; Del Valle et al., 2001a, b, 2002a, 2004; Enam et al., 2002; Kim et al., 2002; Zambrano

et al., 2002; Boldorini et al., 2003; Knöll et al., 2003; Newcomb et al., 2004; Casini et al., 2005; Okamoto et al., 2005; Rollison et al., 2005; Goel et al., 2006; Shin et al., 2006; Balis et al., 2007; Giuliani et al., 2007, 2008; Giraud et al., 2008; Jung et al., 2008; Lin et al., 2008; Noshio et al., 2008, 2009; Vasishta et al., 2009; Yamaoka et al., 2009; Niv et al., 2010a; Palmieri et al., 2010; Karpinski et al., 2011; Ramamoorthy et al., 2011; Shen et al., 2011; Vilkin et al., 2012). Some of these studies included normal tissues adjacent to the tumour tissues, but there were no tissues from individuals without cancer. Many of these studies are investigations of the association between the presence of JCV and other non-viral tumour markers (Del Valle et al., 2001a, b; Enam et al., 2002; Goel et al., 2006; Jung et al., 2008; Noshio et al., 2008, 2009; Yamaoka et al., 2009; Niv et al., 2010a; Karpinski et al., 2011; Vilkin et al., 2012). Cancer sites investigated in the context of these case series but not in the case–control studies include the anus, prostate, stomach, kidney, and melanoma. Results of these case series studies are not included in this review due to the limitations of their study design.

[Some of the case series that evaluated tumours for the presence of SV40 DNA would have detected other polyomaviruses (JCV or BKV) had they been present, because the PCR primers used in those studies also amplify sequences from BKV and JCV. Examples of such studies, which failed to detect JCV or BKV DNA, include Vilchez et al. (2002, 2005) for NHL, Bergsagel et al. (1992) and Martini et al. (1996) for brain tumours, and Carbone et al. (1994) for mesothelioma.]

2.8 Intervention studies

No data were available to the Working Group.

Table 2.3 Case series of JCV in human cancer: studies that compared the prevalence of JCV DNA and/or LT expression in tumour tissues from cancer cases with that in control tissues obtained from individuals without the cancer of interest

Reference, study location	Cases	Controls	Measure of JCV	Findings	Confounders assessed?	Measure of association calculated?	Comments
Del Valle <i>et al.</i> (2001a) , USA	8 medulloblastoma	10 normal brain	DNA by PCR IHC for LT	% JCV DNA+ by PCR: Medullo: 100% of 4 % LT + by IHC: Medullo: 38% of 8 Normal brain: 0%	No (ages of cases: 4–15 yr; ages of controls: 0–70 yr)	No	Convenience sample of cases
Del Valle <i>et al.</i> (2005) , USA	19 oesophageal cancer	10 Barrett's oesophagus with dysplasia 10 Barrett's oesophagus 9 oesophagitis 12 achalasia 10 normal oesophagus	IHC for LT in all samples; PCR for JCV DNA in only a small subset	0% LT + by IHC: Oesophageal cancer: 53% All others: 0% % JCV DNA+ by PCR: 100% of 5 oesophageal cancer 85% of 13 non-cancer	No	No	
Hori <i>et al.</i> (2005) , Japan	23 CRC 21 adenoma	20 normal colon mucosa (from pathology archives)	DNA by PCR IHC for LT	% JCV DNA+ by PCR: CRC: 26% Adenoma: 5% Normal colon: 0% % LT + by IHC: CRC: 0% Adenoma: 0% Normal colon: 0%	No	$P = 0.008$ No	Convenience sample of cases
Theodoropoulos <i>et al.</i> (2005) , Greece	80 CRC	25 adenoma 20 normal colon tissue	DNA by PCR	% JCV DNA+ by PCR: CRC: 61% Adenoma: 60% Normal: 30%	No	No	Convenience sample of cases
Muñoz-Mármol <i>et al.</i> (2006) , Spain	55 gliomas 5 medulloblastoma (patients from hospital) 109 CNS tumours (TMA)	15 “reactive gliosis” tissues, some obtained from patients with brain tumours (patients from hospital) 21 “reactive gliosis” tissues (TMA)	DNA by PCR IHC for LT	% JCV DNA+ by PCR: Glioma patients: 5% Medullo patients: 0% Gliosis patients: 13% % LT + by IHC: CNS on TMA: 0% Gliosis on TMA: 0%	No	No	Convenience sample of cases in addition to the use of TMA (possibly commercial)

Table 2.3 (continued)

Reference, study location	Cases	Controls	Measure of JCV	Findings	Confounders assessed?	Measure of association calculated?	Comments
Abdel-Aziz et al. (2007) , Japan	62 lung cancer	23 normal lung tissues	DNA by nested PCR, IHC	% JCV DNA+ by PCR: Lung cancer: 53% Normal lung: 44%	No (groups appear to differ by age)	$P = 0.048$	Convenience sample of cases
Murai et al. (2007) , Japan	22 gastric cancer	22 adjacent non-cancerous tissue 10 normal gastric mucosa	DNA by PCR IHC for LT	% JCV DNA+ by PCR: Gastric cancer: 86% Adjacent non-cancerous tissue: 86% Normal gastric mucosa: 100% LT + by IHC: Gastric cancer: 5% Adjacent non-cancerous tissue: 0% Normal gastric mucosa: 0%	No (similar sex and age distribution among gastric cancer cases and controls)	No	Convenience sample of cases
Zheng et al. (2007a) , Japan	103 lung cancer	18 normal lung (autopsies)	DNA by PCR	% JCV DNA+ by PCR: Lung cancer: 68% Normal lung: 11%	No	$P < 0.05$	Convenience sample of cases
Zheng et al. (2007b) , Japan	50 lung cancer 20 gastric cancer	20 normal lung tissues 10 normal gastric mucosa	DNA by nested PCR; IHC, ISH	% JCV DNA+ by PCR: Lung cancer: 58% Normal lung: 10% Mean viral load (copies/ μ g) Gastric carcinoma: 5000 Normal gastric mucosa: 1000	No	$P < 0.001$ $P < 0.001$	Convenience sample of cases
Kutsuna et al. (2008) , Japan	56 tongue cancer	60 tongue dysplasia 68 glossitis tissues	DNA by real-time PCR	Mean viral load (copies/ μ g): Tongue cancer: 982 Tongue dysplasia: 659 Glossitis: 411	No (groups appear to differ by age)	$P < 0.05$	

Table 2.3 (continued)

Reference, study location	Cases	Controls	Measure of JCV	Findings	Confounders assessed?	Measure of association calculated?	Comments
Selgrad <i>et al.</i> (2008) , the Netherlands	26 adenoma _{LTR} 40 adenoma _{IC}	15 normal colon _{LTR} 21 normal colon _{IC}	DNA by PCR IHC for LT	% JCV DNA+ by PCR: Adenoma _{LTR} : 62% Adenoma _{IC} : 50% Normal _{LTR} : 67% Normal _{IC} : 24% % LT + by IHC: Adenoma _{LTR} : 50% Adenoma _{IC} : 5%	No (groups appear to differ by age)	$P < 0.001$ $P = 0.025$ $P = 0.0002$	Consecutive LTRs recruited from one hospital
Del Valle & Khalili (2010) , USA	50 colon cancer 30 brain tumour	2 normal colon 2 normal brain	IHC for LT	% LT + by IHC: Colon cancer: 34% Brain cancer: 3% Normal colon: 0% Normal brain: 0%	No	No	A commercial TMA was the source of samples
Ksiai <i>et al.</i> (2010) , Tunisia	61 gastric cancer	53 paired adjacent normal 23 controls with normal gastric tissue	JCV DNA by PCR	% JCV DNA+ by PCR: Gastric cancer: 26% Paired adjacent normal: 6% Normal gastric: 9%	No	No	
Martinez-Fierro <i>et al.</i> (2010) , Mexico	55 prostate cancer	75 non-malignant prostate tissues (only 13% “normal”)	DNA by PCR	% JCV DNA+ by PCR: Tumour: 0% Non-cancer tissues: 0%	No (significant difference in age reported between comparison groups)	No	Cross-sectional study of patients undergoing prostate biopsy; case-control status determined by pathology review
Niv <i>et al.</i> (2010b) , Israel	11 CRC	7 advanced adenoma	DNA by PCR	% JCV DNA+ by PCR: CRC: 36% Adenoma: 29%	No	No	Cross-sectional design: recruitment of consecutive patients undergoing colonoscopy

CNS, central nervous system; CRC, colorectal cancer; DNA+, DNA-positive; IC, immunocompetent; IHC, immunohistochemistry; ISH, *in situ* hybridization; JCV, JC polyomavirus; LT, JCV T-antigen; LTRs, liver transplant recipients; medullo, medulloblastoma; P, P value; PCR, polymerase chain reaction; TMA, tissue microarray; yr, year

2.9 Susceptible populations

Immunosuppressed patients are known to have an increased risk of cancer. JCV is known to be associated with PML and renal disease in transplant patients. No studies have investigated JCV and cancer in immunosuppressed patients. A single case series that compared the prevalence of JCV DNA in colorectal adenomas that developed among liver transplant recipients (LTRs) with the prevalence in colorectal adenomas that developed among immunocompetent individuals observed a JCV DNA prevalence of 63% in adenomas among LTRs *versus* 5% in adenomas among immunocompetent individuals ($P < 0.001$) ([Selgrad et al., 2008](#)). However, there was no difference in JCV DNA prevalence between adenoma tissues and normal colon tissues among LTRs.

3. Cancer in Experimental Animals

3.1 Monkey

See [Table 3.1](#)

Several experiments using owl monkeys or squirrel monkeys inoculated with purified JCV have been described. Most of the monkeys were inoculated intracerebrally as adults. The virus was often delivered to many animals by multiple routes, and a small subset received immunosuppressive drugs before and after inoculation in attempts to aid in tumour induction.

[London et al. \(1978\)](#) injected JCV intracerebrally into both hemispheres simultaneously via subcutaneous and intravenous routes for a total of 588 800 HAU or 5.89×10^9 plaque-forming units (PFU) per animal. In 2 of 4 adult owl monkeys, brain tumours were observed, including grade III–IV astrocytoma within 16 months and malignant mixed glial/neuronal tumours within 25 months of inoculation. [This was the first report of neuro-oncogenicity of JCV

in non-human primates.] In a follow-up study, 4 of 6 adult squirrel monkeys injected intracerebrally with the JCV Mad-1 strain developed brain tumours, including glioblastoma in 3 animals and grade III astrocytoma in 1 animal, within 14–29 months ([Houff et al., 1983](#); [London et al., 1983](#)).

[Rieth et al. \(1980\)](#) reported similar tumours induced by JCV in owl and squirrel monkeys, but at lower frequencies. In adult owl monkeys injected with JCV Mad-1 intracerebrally in both hemispheres as well as intravenously, brain tumours developed within 12–15 months in 15% of 26 animals, including 11% characterized as glioblastoma and 4% as grade III astrocytoma. In the same study, in adult squirrel monkeys injected with JCV Mad-1 intracerebrally in both hemispheres, glioblastoma developed in 1 (13%) of 8 animals within 15 months.

Similarly, [Major et al. \(1987\)](#) observed glioblastoma in 1 (25%) of 4 juvenile owl monkeys injected intracerebrally with an inoculum including the Mad-1 strain of JCV plus $1\text{--}5 \times 10^6$ glioblastoma cells harvested from another owl monkey. Tumours developed after 28 months in 1 of the 3 animals surviving to 46 months. Of note, cell lines were cultured from the glioblastoma cells, and infectious virions were isolated from these. Further characterization of the virus, termed JCV-586, revealed a Mad-4-like promoter sequence. Antibody cross-reactivation studies revealed differences in the T-antigen encoded by JCV-586 compared with the Mad-1 and Mad-4 strains. [This study is the only report of infectious virus recovered from the tissues or tumours of any experimental animals inoculated with JCV.]

3.2 Hamster

See [Table 3.1](#)

Inoculation of JCV into newborn (NB) Syrian golden hamsters has been performed in many studies by various routes, including the

Table 3.1 Carcinogenicity studies of JCV in experimental animals

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Animals/group at start ^a	For each target organ: tumour incidence; tumour latency ^b	Comments
Monkey, owl monkey (adult) (NR) Up to 25 mo London et al. (1978)	JCV, 0.15 mL (intracerebral), 38400 HAU ^d per hemisphere; plus 1 mL (subcutaneous), 256000 HAU; plus 1 mL (intravenous), 256000 HAU <i>n</i> = 4 Controls: uninfected PHFG cells ^e , <i>n</i> = 8; untreated, <i>n</i> = 2	Brain tumours (2/4) grade III–IV astrocytoma ; 16 mo malignant mixed glial/neuronal tumours ; 25 mo Controls (0%)	First report of neuro-oncogenicity of JCV in non-human primates. Likely the JCV Mad-1 strain; see Rieth et al. (1980) .
Monkey, owl monkey and squirrel monkey (adult) (NR) Up to 15 mo Rieth et al. (1980)	Owl monkeys: JCV Mad-1 strain, 0.15 mL (intracerebral), 38400 HAU per hemisphere with (<i>n</i> = 20) or without (<i>n</i> = 6) 0.4 mL (intravenous), 102400 HAU <i>n</i> = 26 Controls: uninfected PHFG cells, <i>n</i> = 8 Squirrel monkeys: JCV Mad-1 strain, 0.2 mL, 64000 HAU per hemisphere, intracerebral <i>n</i> = 8 Controls: Flow 7000 supernatant, <i>n</i> = 3	Brain tumours (15%): glioblastoma (11%), grade III astrocytoma (4%); 12–15 mo Controls (0%) Glioblastoma (1/8); 15 mo Controls (0%)	
Monkey, squirrel monkey (adult) (M, F) Up to 29 mo London et al. (1983) , Houff et al. (1983)	JCV Mad-1 strain, 0.1 mL, 2000 HAU, intracerebral <i>n</i> = 6 Controls: uninfected PHFG cells <i>n</i> = 3	Brain tumours (4/6): glioblastoma (3/6), grade III astrocytoma (1/6); 14 –29 mo Controls (0%)	
Monkey, owl monkey (juvenile) (NR) Up to 46 mo Major et al. (1987)	JCV Mad-1 strain, 0.3 mL plus 1–5 × 10 ⁶ tumour cells from owl monkey glioblastoma, intracerebral <i>n</i> = 4	Glioblastoma (1/3); 28 mo	Infectious virions were isolated from cell lines cultured from cells of the inoculated glioblastoma. Virus was termed JCV-586. One animal died 2 wk after inoculation.

Table 3.1 (continued)

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Animals/group at start ^a	For each target organ: tumour incidence; tumour latency ^b	Comments
Hamster ^f (NB, < 1 d) (NR) Up to 6 mo Walker et al. (1973) , Zu Rhein & Varakis (1979)	JCV Mad-4 strain, 0.02 mL, 10 ⁶ TCID ₅₀ units (2048 HAU) for each injection site, subcutaneous and intracerebral <i>n</i> = 63 Controls: extract of PHFG cells <i>n</i> = 39	Brain tumours (83%): medulloblastoma (95% of tumour- bearing animals), PNET, and papillary ependymoma; 4–6 mo Controls (0%); after 6 mo	First report of neuro-oncogenicity of JCV. Inoculum partially purified and concentrated by centrifugation. Gliomatosis noted in some animals.
Hamster (NB, < 1 d) (NR) 6.5 mo Padgett et al. (1977)	All strains, 0.02 mL, 2560 HAU, intracerebral JCV Mad-2 strain <i>n</i> = 20 JCV Mad-3 strain <i>n</i> = 2 JCV Mad-4 strain <i>n</i> = 22 Controls: uninfected PHFG cells <i>n</i> = 6	All tumours observed after 6.5 mo Brain tumours (100%): medulloblastoma (95%), glioblastoma, ependymoma, and abdominal neuroblastoma (5%) Brain tumours (2/2, 100%) Brain tumours (91%): pinealocytoma (45%), medulloblastoma (45%), and abdominal neuroblastoma (5%) Controls (0%); after 8 mo	First report of strain tropism. First report of extracranial tumours induced by intracranial injection. Inoculum partially purified and concentrated by centrifugation. Multiple brain tumours or lesions detected in many animals.
Hamster (NB, < 1 d) (M, F) Up to 6 mo Quay et al. (1977)	JCV Mad-4 strain, 0.02 mL, intracerebral <i>n</i> = 22	Pinealocytoma (45%); after 6 mo	Inoculum partially purified and concentrated by centrifugation.
Hamster (NB, < 1 d) (NR) Up to 11 mo Varakis et al. (1978)	JCV Mad-1 strain, 0.02 mL, 5120 HAU, intraocular <i>n</i> = 31 Controls: semi-purified extract of PHFG cells <i>n</i> = 7	Peripheral neuroblastoma (32%), including adrenal neuroblastoma (1/31, 3%); 6–11 mo Controls (0%); after 11 mo	Inoculum partially purified. Leakage of inoculum and vitreous fluid during injection; therefore, there was a variation in the amount of retained inoculum.

Table 3.1 (continued)

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Animals/group at start ^a	For each target organ: tumour incidence; tumour latency ^b	Comments
Hamster (NB, 1–3 d) (NR) Up to 7.5 mo Zu Rhein (1983)	JCV (various strains), 0.02 mL, 2560 HAU, intracerebral for most experiments <i>n</i> = ~650 Controls: uninfected PHFG cells <i>n</i> = ~250	Tumours in order of frequency: medulloblastoma, glioblastoma and astrocytic tumours, PNET and other central neuroblastoma, pinealocytoma, pituitary adenocarcinoma, retinoblastoma, peripheral neuroblastoma, ependymoma, choroid plexus papillomas, malignant peripheral nerve sheath tumours, and meningioma. Controls (0%)	Review of all published hamster studies to date, including new information on neuropathology of tumours not previously published. Strain associations noted: medulloblastomas observed with Mad-1 and Mad-2; peripheral neuroblastomas with Mad-1; pinealocytomas with Mad-4. Animals inoculated younger more prone to developing primitive neuronal tumours. Gliomatosis and angiomatous lesions were noted in some animals.
Hamster (NB < 1 d) (NR) Up to 5.5 mo Nagashima et al. (1984)	JCV Tokyo-1 strain, 0.025 mL, 64 HAU, intracerebral <i>n</i> = 21 Controls: uninfected PHFG cells, <i>n</i> = 5	Brain tumours (100%): medulloblastoma (95%) and ependymoma (10%); 3–5.5 mo Controls (0%)	One animal developed medulloblastoma and ependymoma. Gliomatosis noted in some animals.
Hamster (NB, ~24 h) (NR) Up to 2 yr Brun & Jonsson (1984)	PML patient brain tissue homogenate, 0.1 mL, subcutaneous <i>n</i> = 11	Angiosarcoma (2/11, 18%)	
Rat, Sprague Dawley (NB < 24 h) (NR) Up to 63 wk Ohsumi et al. (1985)	JCV Tokyo-1 strain, 0.02 mL, 10 × 2 ⁹ HAU, intracerebral <i>n</i> = 27 Controls NR	PNET (74%); 21–61 wk	No tumours detected in cerebellum or brainstem.
Rat, Sprague Dawley (NB, < 24 h) (NR) 104 wk Ohsumi et al. (1986)	JCV Tokyo-1 strain, 0.02 mL, 10 × 2 ⁹ HAU, intracerebral <i>n</i> = 27 Controls NR	PNET (78%); 21–70 wk	No tumours detected in cerebellum or brainstem.

Table 3.1 (continued)

Species, strain (age) (sex) Duration of observation Reference	Virus strain, dose, route Animals/group at start ^a	For each target organ: tumour incidence; tumour latency ^b	Comments
Rat, Sprague Dawley (NB, < 24 h; or 7 d) (NR) Up to 40 wk Horie et al. (1989)	JCV Tokyo-1 strain, 0.02 mL, 10 × 2 ⁹ HAU, intracerebral NB, < 24 h, <i>n</i> = 30 7 d, <i>n</i> = 27 Controls: 0.02 mL saline, intracerebral, NB, < 24 h <i>n</i> = 5	< 24 h: PNET (73%); 12–26 wk 7 d: PNET (63%); 12–40 wk Controls (0%)	Multiple brain tumours or lesions detected in many animals. Average of 2–2.2 tumours per animal. No tumours detected in cerebellum or brainstem.

^a Animals/group at start. Due to the long latency for JCV induction of tumours, the animals that died during the period immediately after the inoculation are largely absent from published studies and therefore have been excluded from the numbers listed. All animals/group at the start of the study are included in the table where possible.

^b Pathology nomenclature. Medulloblastoma includes cerebellar medulloblastoma. PNET, primitive neuroectodermal tumours, include unspecified neuroectodermal tumours not arising from the cerebellum. Glioblastoma refers to grade IV glioma and also includes glioma, malignant glioma, and glioblastoma multiforme. Malignant peripheral nerve sheath tumours include malignant schwannoma.

^c HAU, haemagglutination units. This is the accepted nomenclature for titration of JCV as JCV is not amenable to plaque assay and does not cause substantial cytopathic effects in infected cell lines. 1 HAU is equivalent to ~10⁴ plaque-forming units (PFU). TCID₅₀ (50% tissue culture infective dose) units are included where noted.

^d PHFG cells, primary human fetal glial cells. Cultures of human fetal brain cells, in which JCV undergoes productive infection, were used for initial isolation of the virus from tissue of brain from patients with progressive multifocal leukoencephalopathy and for propagation of the isolates.

^e Hamsters are Syrian outbred unless otherwise indicated.

d, day; F, female; JCV, JC polyomavirus; h, hour; M, male; mo, month; NB, newborn; NR, not reported; PML, progressive multifocal leukoencephalopathy; wk, week; yr, year

intracerebral, intraocular, and subcutaneous routes.

The tumorigenic potential of JCV was first reported by [Walker *et al.* \(1973\)](#) and further described by [Zu Rhein & Varakis \(1979\)](#), in which NB Syrian golden hamsters were inoculated both subcutaneously and intracerebrally with the Mad-4 strain of JCV. Of 63 injected animals, 52 (83%) developed brain tumours after a latency period of 4–6 months; 95% of the tumours detected were glioblastomas, and the remaining tumours were reported as primitive neuroectodermal tumours (PNETs) and papillary ependymomas.

[Quay *et al.* \(1977\)](#) reported induction of pinealocytoma after 6 months in 45% of 22 NB hamsters inoculated intracerebrally with the Mad-4 strain of JCV.

In addition to the Mad-4 strain of JCV, [Padgett *et al.* \(1977\)](#) evaluated the Mad-2 and Mad-3 strains; all three of these strains induced brain tumours after 6.5 months in 91–100% of intracerebrally injected NB hamsters. Tumours included medulloblastoma, glioblastoma, pinealocytoma, ependymoma, and abdominal neuroblastoma. [This study was the first to report both extracranial tumours (abdominal neuroblastoma) induced by intracerebral inoculation with the Mad-2 and Mad-4 strains as well as the observation that multiple tumour types were often found to arise in individual animals.]

Intraocular inoculation of the Mad-1 strain of JCV was shown to induce peripheral neuroblastoma after 6–11 months in 32% of 31 NB hamsters ([Varakis *et al.*, 1978](#)).

A study using the Tokyo-1 strain at low titres (64 HAU) reported the induction of brain tumours in 100% of 21 inoculated animals (including medulloblastoma in 95% and ependymoma in 10% of tumour-bearing animals) after 3–5.5 months ([Nagashima *et al.*, 1984](#)). [This study suggests that low viral titres can be tumorigenic and do not result in longer latent periods.]

Detailed pathological analysis by [Zu Rhein \(1983\)](#) of ~650 tumours reported in some of the studies described above outlined several tumours that occurred with lower frequency. In addition to the tumours most commonly observed, including (in order of frequency) medulloblastoma, glioblastoma and astrocytic tumours, PNET and other central neuroblastoma, pinealocytoma, ependymoma, and peripheral neuroblastoma, [Zu Rhein \(1983\)](#) described pituitary adenocarcinoma, retinoblastoma, choroid plexus papilloma, malignant peripheral nerve sheath tumours, and meningioma as also being induced by JCV.

A small study by [Brun & Jonsson \(1984\)](#) reported angiosarcoma induction within 2 years in 2 of 11 NB hamsters injected subcutaneously with homogenates of brain tissue from a patient with PML.

3.3 Rat

See [Table 3.1](#)

Similar studies performed using NB Sprague Dawley rats resulted in the induction of PNETs. This series of studies was performed with the Tokyo-1 strain of JVC, whose promoter structure shares high similarity with one of the Mad-4 strain.

Two separate studies by [Ohsumi *et al.* \(1985, 1986\)](#) reported that 74% of 27 animals and 78% of 27 animals injected intracerebrally with the Tokyo-1 strain developed PNETs after 21–61 weeks and 21–70 weeks, respectively.

To determine whether the age of the animals at the time of inoculation affected tumorigenesis, [Horie *et al.* \(1989\)](#) injected the JCV Tokyo-1 strain into rats as newborns or 7 days after birth. PNETs developed in 73% of 30 animals injected as newborns and 63% of 27 animals injected at age 7 days, demonstrating similar susceptibility in these age groups. A maximum latency of 26 weeks was reported for animals injected as newborns versus 40 weeks for animals injected

Table 3.2 Carcinogenicity studies in transgenic mice involving JCV with natural viral promoter

Species, strain (sex) Reference	Virus strain Animals/group at start	For each target organ: tumour incidence; tumour latency	Comments
Mouse, transgenic (M, F) Small et al. (1986)	JCV Mad-1 LT & sT <i>n</i> = 5 founders	Peripheral neuroblastoma (4/5); 14–16 wk to 10 mo	No offspring of successfully bred founders developed tumours, but developed dysmyelination; transgene may have been germline mosaic.
Mouse, FVB/N (NR) Franks et al. (1996)	JCV Mad-4 LT & sT <i>n</i> = 4 founders	Peripheral neuroblastoma (2/4); 4–6 mo	
Mouse, FVB/N (NR) Krynska et al. (1999b)	JCV(CY) archetype LT & sT	Medulloblastoma/PNET (35 of 79 founders and progeny, 44%); 9–13 mo	First report of oncogenicity of archetype strain.
Mouse, C57 x SJL (NR) Gordon et al. (2000) , Shollar et al. (2004)	JCV Mad-4 LT & sT <i>n</i> = 10 founders	Tumours (80%), including: pituitary adenoma (30%), peripheral neuroblastoma (20%), PNET (10%), and undetermined (20%); 4–5 mo	Multiple tumours or lesions detected in many animals. Malignant peripheral nerve sheath tumours seen in progeny (not founders) after 9 mo.

F, female; JCV, JC polyomavirus; LT, large T-antigen; M, male; mo, month; NR, not reported; PNET, primitive neuroectodermal tumours; sT, small T-antigen; wk, week

at age 7 days. These ranges of latency fall well within the periods of 61 and 70 weeks observed by the same group using similar virus stock and titres ([Ohsumi et al., 1985, 1986](#)). [Therefore, the age of inoculation up to 7 days did not appear to have any impact on tumour latency.]

3.4 Transgenic mouse models

3.4.1 JCV T-antigen with natural viral promoter

See [Table 3.2](#)

Through the use of transgenic mice containing the genes of JCV early region (i.e. the T-antigens) under the control of the viral early promoter, the role of T-antigens can be studied in the absence of sequences encoding the capsid proteins. Of note, the JCV early region also encodes JCV sT and other, alternatively spliced forms of large T-antigen. To date, several independent lines of transgenic mice have been generated, which exhibit distinct phenotypes. Intracellular

expression of JCV T-antigens has typically been detected in tumour tissues but is not observed in non-tumour tissue.

[Small et al. \(1986\)](#) were the first to use JCV T-antigen transgenic mice with the JCV Mad-1 promoter. Peripheral neuroblastoma was observed in 4 of 5 founder animals, of which 3 died within 14–16 weeks and the remaining animal developed tumours by age 10 months. Offspring of successfully bred founders did not develop tumours but exhibited dysmyelination, which may be due in part to transmission of the transgene to offspring at a low frequency, suggesting germline mosaicism.

JCV T-antigen transgenic mice under the control of the Mad-4 promoter were generated using both an FVB/N and a C57 x SJL background. On the FVB/N background, 2 of 4 founders developed peripheral neuroblastoma within 4–6 months ([Franks et al., 1996](#)), whereas on the C57 x SJL background 80% of 10 founder animals developed tumours, including pituitary adenoma (30%), peripheral neuroblastoma

Table 3.3 Carcinogenicity studies of JCV and SV40 in transgenic mice involving hybrid JCV and SV40 sequences

Species, strain (sex) Duration of observation Reference	Virus strain Animals/group at start	For each target organ: tumour incidence; tumour latency	Comments
Mouse, transgenic (M, F) Feigenbaum et al. (1992)	JCV Mad-1 promoter × SV40 LT and sT genes <i>n</i> = 5	Tumours (3/5), including: neuroblastoma (3/5), myenteric plexus tumours (3/5), and choroid plexus carcinoma (2/5); 0.5–4 mo	Multiple tumours or lesions detected in many animals. Two JCV–SV40 chimeric founders developed dysmyelination.
	SV40 776 promoter × JCV LT and sT genes <i>n</i> = 7	Tumours (100%), including: choroid plexus papillomas (6/7), renal hyperplasia (5/7), thyroid hyperplasia (3/7), and thymic hyperplasia (2/7); 1–3 mo	
Mouse, C57BL/6 × CBA (M, F) Ressetar et al. (1993)	JCV Mad-1 promoter × SV40 LT and sT genes <i>n</i> = 2 (M, F)	Tumours (2/2), including: abdominal B-cell lymphoma (1/2), adrenal neuroblastoma (1/2), osteosarcoma (1/2), and astrocytoma (1/2); 1–2.5 mo	Multiple tumours or lesions detected in many animals.
	SV40 776 promoter × JCV LT and sT genes <i>n</i> = 1 (F)	Tumours (1/1), including: choroid plexus papillomas, thymic hyperplasia, and thymoma; 1.5 mo	

JCV, JC polyomavirus; F, female; M, male; LT, large T-antigen; mo, month; sT, small T-antigen; SV40, simian virus 40.

(20%), and PNET (10%), at age 4–5 months. Similar to the hamster studies described in Section 3.2, multiple brain tumours or lesions could be detected in many animals. Malignant peripheral nerve sheath tumours were observed in the progeny at age > 9 months ([Gordon et al., 2000](#); [Shollar et al., 2004](#)).

In JCV T-antigen transgenic mice using the JCV(CY) or archetype strain of the virus on an FVB/N background, medulloblastoma or PNET developed in 35 (44%) of 79 founder animals and their progeny by age 9–13 months ([Krynska et al., 1999b](#)). [This is the first study reporting the oncogenicity of the archetype strain of JCV, which is believed to be the naturally circulating strain of the virus. Also noteworthy from the transgenic studies described above are the propensity of the archetype strain for inducing medulloblastoma and the possible contribution

of genetic background towards the spectrum of tumours induced by JCV T-antigen.]

3.4.2 T-antigen with hybrid JCV and SV40 sequences

See [Table 3.3](#)

Studies in transgenic mice using the promoter of SV40 776 strain or JCV Mad-1 strain, driving expression of either SV40 T-antigen or JCV T-antigen, have revealed important information about the tropism of these viruses.

[Feigenbaum et al. \(1992\)](#) reported that 3 of 5 founders carrying the SV40 early sequence under the control of the JCV Mad-1 promoter developed tumours, including neuroblastoma (3/5), myenteric plexus tumours (3/5), and choroid plexus carcinoma (2/5), within 0.5–4 months, whereas 100% of 7 founders carrying the JCV

early sequence under the control of the SV40 776 promoter developed tumours, including choroid plexus papilloma (6/7), within 1–3 months.

On a C57/CBA background, [Ressetar et al. \(1993\)](#) reported that 2 founders with the JCV Mad-1 promoter driving SV40 T-antigen gene expression developed tumours, including abdominal B-cell lymphoma (1/2), adrenal neuroblastoma (1/2), osteosarcoma (1/2), and astrocytoma (1/2), within 1–2.5 months. In parallel, 1 founder with the SV40 776 promoter driving JCV T-antigen gene expression developed choroid plexus papillomas, thymic hyperplasia and thymoma by 1.5 months. Similar to the studies of JCV infection in hamsters (see Section 3.2) and JCV T-antigen transgenic mice (see Section 3.4), the animals frequently developed multiple tumours or lesions in a single animal.

[These studies suggest that promoter tropism is linked to tumorigenesis, as JCV T-antigen sequences under the control of the SV40 promoter demonstrated a tumour profile similar to that of transgenic mice expressing SV40 T-antigen under the control of its own promoter, i.e. observation of choroid plexus papilloma. The JCV promoter driving SV40 T-antigen expression exhibited the tropism seen in JCV experimental animals, i.e. observation of neuroblastoma and astrocytoma. However, those animals also developed tumour types induced by SV40, including osteosarcoma and a much higher frequency of tumours of the choroid plexus, suggesting that the T-antigen sequences may also play a role in viral tropism.]

4. Mechanistic and Other Relevant Data

4.1 Transforming capacity of JCV

When inoculated into small rodent models and non-human primates, which are non-permissive for productive JCV infection, JCV

leads mostly to formation of tumours of neural origin (see Section 3). JCV is to date the only human virus known to induce solid tumours in non-human primates. The JCV genome was found to be integrated in all astrocytomas that developed in adult owl monkeys 16–36 months after intracranial inoculation with the virus. Integration occurred at a limited number of sites in the cellular DNA, indicating a clonal origin of the tumours. No JCV was found in normal brain tissue from the same animals ([Miller et al., 1984](#)). The JCV large T-antigen (LT) viral protein was detected in these owl monkey tumours and in cells derived from them ([Major et al., 1984, 1987](#)).

Despite its strong oncogenic potential *in vivo*, JCV transforms, rather inefficiently, only a few cultured cell types (e.g. rodent fibroblasts and PHFG cells). The low efficiency of JCV to convert cells to a transformed state has been shown to be due to both reduced expression and reduced activity of the JCV T-antigens ([Mandl et al., 1987](#); [Bollag et al., 1989](#); [Haggerty et al., 1989](#); [Trowbridge & Frisque, 1993](#)).

4.2 Relevant biological properties of JCV viral proteins

JCV encodes five T-antigen proteins derived from alternative splicing of a viral early precursor mRNA. Large T-antigen (LT), T'₁₃₅, T'₁₃₆, T'₁₆₅, and small t-antigen (sT) all share the initial N-terminal 82 residues ([Trowbridge & Frisque, 1995](#)). At their C termini, JCV LT and T'₁₆₅ have 33 residues in common, but sT and the remaining T' antigens encode for different sequences. These variations in the five T-antigen isoforms of JCV increase the functional repertoire of its early region.

The DnaJ, LXCXE, and bipartite p53 binding domains critical for interactions with Hsc70, pRB, and p53, respectively, in SV40 LT (see the *Monograph* on SV40 in this Volume) are conserved in synteny with JCV LT. As a result

of additional splicing events in the genesis of the JCV T' antigens, only the DnaJ and LXCXE domains are shared in common with JCV LT. JCV LT and the three T' isoforms have been shown to interact *in vitro* and *in vivo* with pRb, p107, and p130 (Bollag *et al.*, 2000, 2006). Despite this, overexpression of LT and T' proteins individually and in combination is unable to induce Rat-2 cells to form dense foci (Trowbridge & Frisque, 1993). However, co-expression of the H-*ras* oncogene complements JCV T-antigens to form dense foci and to immortalize rat embryo fibroblasts (Bollag *et al.*, 2006). This cooperative effect with H-*ras* is best seen when the products of the entire early region are expressed, as opposed to the expression of single JCV LT isoforms, and may implicate the importance of sT contributions to the transformed phenotype. JCV sT has a conserved PP2A binding motif that interacts with its cognate cellular partner, similar to other polyomaviruses; in addition, two LXCXE domains have been defined that bind to members of the Rb family (Bollag *et al.*, 2010). The functional effects of Rb family interactions found uniquely in JCV sT are currently unknown.

Furthermore, it has been shown that JCV LT directly interacts with β -catenin, leading to increased protein stability (Gan *et al.*, 2001; Enam *et al.*, 2002). In JCV LT-expressing cells, an increased nuclear β -catenin expression has been described, possibly pointing to a regulation of c-myc and cyclin D1 (reviewed in White & Khalili, 2004). SV40 LT regulation of insulin receptor substrate 1 (IRS-1) is involved in SV40-related transformation (Fei *et al.*, 1995). In analogy to SV40, the interaction of JCV LT with IRS-1 has been shown, and may also contribute to JCV malignant transformation (Lassak *et al.*, 2002).

JCV LT has also been implicated in chromosomal instability and aneuploidy. LT expression significantly increases the spontaneous mutation frequencies (Theile & Grabowski, 1990) and is able to induce chromosomal instability

in lymphocytes (Neel *et al.*, 1996) and human colonic cells (Ricciardiello *et al.*, 2001).

In addition, agnoprotein, an 8 kDa JCV protein produced late in the infection cycle, was shown to inhibit cell growth by deregulating progression through the cell cycle. Agnoprotein can physically interact with p53, and the interaction can lead to increased expression of the cell-cycle inhibitor p21 (Darbinyan *et al.*, 2002).

4.3 *In vivo* and *in vitro* evidence for a role of JCV in human malignancies

In vitro studies have demonstrated the transforming potential of JCV and the ability of JCV T-antigens to interact with and functionally inactivate the tumour suppressor proteins p53 and pRb and to interact with other molecular pathways broadly incriminated in carcinogenesis (see Section 4.2). In addition, studies in experimental animals have demonstrated the ability of JCV to induce formation of tumours (see Section 3). Many studies reporting an association between JCV and human cancers are case series describing detection of JCV DNA by PCR in cancer tissue.

[The value of these studies to elucidate a mechanistic role for the virus in human cancers is problematic because PCR signals are weak and it is difficult to entirely exclude the possibility of contamination. Furthermore, most of the reports come from a single group of investigators, Khalili and colleagues for brain tumours and Boland and colleagues for gastrointestinal cancers. Where investigations have been undertaken by multiple laboratories, there is considerable inconsistency among studies in detection of viral DNA and there is no clear-cut and widely accepted explanation for these discrepancies across laboratories. In light of the above considerations, we elected to review only those studies of human cancer cell lines and human cancer

tissues that included detection of JCV proteins and associated changes in proto-oncogenes and host chromosomal structure as the evidentiary basis for establishing a mechanistic role for the virus. Although demonstration of the presence of viral protein in tumour tissue is not definitive evidence for an etiological role of the virus in carcinogenesis, such evidence indicates that the viral genome is transcriptionally active and supports the possibility that the viral oncogene is functional. Since all published studies of viral protein detection also sought to detect viral DNA, these studies are inherently more robust than those relying solely on DNA detection. A potential loss in sensitivity is inevitable, but specificity is judged to be the more important criterion for addressing mechanistic questions.]

The most common method used to demonstrate the presence of viral protein in cells has been immunohistochemistry (IHC). This method, although potentially very sensitive and specific, depends on the quality of the immunological reagents that are used. The most commonly used reagent has been a monoclonal antibody (Pab416) directed against SV40-transformed cells of mouse origin. The specificity of the antibody for JCV LT was demonstrated by nuclear staining of a JCV-transformed hamster glial cell, by detection of a nuclear antigen in PML human brain tissue, and by the reduction of staining after pre-incubation with polyclonal hamster anti-JCV tumour serum ([Stoner et al., 1988b](#)).

[An inherent limitation of IHC is that the interpretation of test results is subjective. In this regard, the general statement can be made that the methods sections of many published studies do not explicitly state that more than one person reviewed slides and that the reviewer was blinded to the source of the sample. Many of the findings described below for particular cell lines and tumour tissue samples have not been confirmed by multiple independent laboratories and by the use of multiple validated immunological reagents. No endeavour has been made

to organize a multiinstitutional study to analyse a common set of samples under a standard protocol.]

4.3.1 *Medulloblastoma, oligodendroglioma, and other tumours of the CNS*

JCV induces formation of tumours of neural origin in experimental animals. Intracerebral inoculation of the virus into Syrian hamsters resulted in cerebellar medulloblastomas, glioblastomas, malignant astrocytomas, and PNETs. Injection of JCV into the brains of NB rats caused undifferentiated tumours of neuroectodermal origin in the brain of 75% of animals. Intracranial inoculation of virus into owl monkeys led to development of brain tumours, primarily malignant astrocytomas (see Section 3). These animal experiments prompted investigators to seek evidence for a role of JCV in human tumours of neural origin. The reports summarized below are those in which viral DNA was detected by PCR and the viral proteins LT and/or agnoprotein of JCV were detected by IHC analysis.

(a) *Medulloblastoma*

[Krynska et al. \(1999a\)](#) reported detection of JCV DNA in 22 of 23 medulloblastomas. Of note, SV40 DNA was also detected in 5 tumours (22%). By IHC using Pab416, 4 (25%) of 16 samples were positive for LT expression. The number of nuclei that were immunopositive ranged from 5% to 20% of the total tumour cells per high-power ($\times 400$) microscopic field. The same group of investigators subsequently extended their analysis to expression of agnoprotein in medulloblastoma tissues. Using a polyclonal antibody that recognizes agnoprotein produced by immunization of rabbits with three synthetic peptides derived from the JCV agnoprotein open reading frame, [Del Valle et al. \(2002a\)](#) reported detection of agnoprotein by IHC in 11 (69%) of 16 medulloblastomas. Nine of the 11 agnoprotein-positive tumour samples also showed immunoreactivity

when tested with Pab416. Double labelling of tumour samples with antibodies to LT and agnoprotein showed differential localization, with LT staining in nuclei and the agnoprotein in the cytoplasmic perinuclear compartment within single neoplastic cells. In contradiction to these findings, [Vasishta et al. \(2009\)](#) failed to detect JCV LT by IHC using Pab416 in medulloblastoma tissue samples from 22 Indian children.

(b) *Lymphoma of the CNS*

Reports of detection of JCV in peripheral blood leukocytes (PBLs) and lymphocytes led [Del Valle et al. \(2004\)](#) to examine 27 primary CNS lymphomas for the presence of JCV. Twelve of the patients were HIV-1-infected. When primers for LT sequences were used, JCV DNA was found in 22 (81%) of 27 cases, and the same 22 cases were also positive for sequences encoding agnoprotein. DNA for the late viral capsid protein VP1 was detected in 8 (30%) of 27 samples. PCR for the EBV LMP-1 gene was positive in 16 (70%) of 23 samples. JCV LT was detected by IHC with Pab416 in the nuclei of tumour cells in 6 cases (22%). Staining for JCV agnoprotein in the cytoplasm was observed in 6 cases; 3 samples were positive for both JCV proteins. Expression of EBV LMP-1 protein was detected in 16 (59%) of 27 samples, 4 of which were also positive for JCV LT. Since EBV is a well recognized etiological agent in CNS lymphomas, the role of JCV in the development of these EVB-containing tumours and perhaps by extension to other CNS lymphomas is uncertain.

(c) *Diverse tumours of the CNS*

[Del Valle et al. \(2001b\)](#) examined 85 brain tumours of diverse histology, obtained from patients living in the United Kingdom, Greece, and the USA, for the presence of JCV DNA and expression of LT. LT-positive nuclei were found by IHC analysis using Pab416 in 2 (18%) of 11 oligodendrogliomas, 14 (35%) of 40 astrocytomas, 6 (22%) of 27 glioblastomas, 5 (83%) of

6 ependymomas, and 1 of 1 gliomatosis cerebri. In a separate report, the presence of JCV LT protein was observed in the nuclei of 8 (44%) of 18 oligodendrogliomas and in 2 of 2 anaplastic oligodendrogliomas by IHC using Pab416 ([Del Valle et al., 2002b](#)). Agnoprotein was found in 10 (56%) of 18 oligodendrogliomas and in 1 of 2 anaplastic oligodendrogliomas. [Rencic et al. \(1996\)](#) reported the detection of JCV DNA and expression of LT in tissue from the oligoastrocytoma of an immunocompetent HIV-negative man aged 61 years. They were able to demonstrate the presence of several transcripts of the expected size for LT by primer extension assays. In addition, LT was detected by co-immunoprecipitation and western blot using Pab416. Low levels of LT were also detected in tumour tissue by IHC. Two case reports detected JCV LT expression in glioblastoma multiforme tumours ([Del Valle et al., 2002c](#); [Piña-Oviedo et al., 2006](#)).

(d) *Negative studies of JCV LT expression in tumours of the CNS*

Two publications report on the failure to detect LT expression in human brain tumours. [Hayashi et al. \(2001\)](#) failed to detect JCV genome by PCR, LT mRNA by ISH, or LT protein by IHC using Pab108, a monoclonal antibody directed against an NH₂-terminal epitope of SV40 LT, in 8 human medulloblastoma tissue samples. [The Pab108 reagent is described as cross-reacting with BKV LT, but its reactivity with JCV LT is uncertain ([Gurney et al., 1986](#)).] [Muñoz-Mármol et al. \(2006\)](#) tested formalin-fixed, paraffin-embedded tissue samples from 14 astrocytomas, 18 glioblastomas, 9 oligodendrogliomas, 8 oligoastrocytomas, 6 ependymomas, 5 medulloblastomas, and, as controls, 15 reactive gliosis samples for the presence of JCV DNA sequences and expression of JCV LT. Only 2 samples, a subependymoma and a reactive gliosis, gave consistent JCV PCR amplification results, and all of the tumour cases and reactive gliosis cases were negative by IHC analysis for LT staining. IHC was performed with

Pab2024, a monoclonal antibody that recognizes the NH₂-terminal region common to JCV early proteins, and Pab416, the above-mentioned SV40 LT monoclonal antibody that is known to cross-react with JCV LT.

4.3.2 Tumours of the gastrointestinal tract

(a) Cancer of the colorectum

Although tumour formation in experimental animals suggests possible involvement of JCV in tumours of neural origin, the search for JCV-associated tumours in humans has been more wide-ranging. The presence of JCV DNA sequences in human colon and colorectal cancers was first described by [Laghi et al. \(1999\)](#). Subsequent studies included analyses for JCV LT expression in colorectal cancers (CRCs). [Enam et al. \(2002\)](#) examined 27 adenocarcinomas of the colon for JCV DNA sequences and viral onco-gene protein expression. JCV LT DNA sequences were detected in 22 (82%) of the 27 samples. IHC analysis using Pab416 revealed the presence of LT protein in 17 samples (63%) and agnoprotein in 15 samples (56%). [Hori et al. \(2005\)](#) detected JCV LT DNA sequences in 6 (26%) of 23 CRCs from Japanese patients, 1 (5%) of 21 colonic adenomas, and 0 of 20 normal colonic mucosa samples. However, they failed to detect staining for LT using the Pab416 immunoreagent. JCV DNA was detected in 77 (77%) of 100 CRCs and in 18 (72%) of 25 matched corresponding normal tissues. When Pab416 was used, 43 of 100 colon cancer specimens demonstrated positive nuclear LT expression, including 56% of cancers that harboured JCV DNA. LT expression was not observed in any normal colonic tissues ([Goel et al., 2006](#)). [Lin et al. \(2008\)](#) examined CRC tissues from 22 patients living in Taiwan, China, for the presence of JCV. JCV DNA was detected in 19 samples (86%), and LT expression was demonstrated in 14 samples (64%).

Detection of JCV LT expression in adenomatous polyps was also reported ([Jung et al., 2008](#)).

Among 74 adenomatous polyps of the colon, 61 (82%) harboured JCV LT DNA sequences and 12 (16%) demonstrated the presence of LT by IHC analysis using a mouse monoclonal antibody specific for JCV LT (clone Pab2003) and the Pab416 monoclonal antibody. No LT staining was seen in adjacent normal epithelial cells. In another study of adenomatous polyps, the investigators observed positive nuclear staining for JCV LT in 13 (50%) of 26 polyps from liver transplant recipients (LTRs) compared with the detection of LT in only 2 (5%) of 40 adenomas from control, non-transplant patients ([Selgrad et al., 2008](#)). All the LT-positive samples harboured JCV DNA, and no LT expression was seen in non-neoplastic cells.

(b) Other tumours of the gastrointestinal tract

JCV DNA and LT expression have also been described in other cancers of the gastrointestinal tract. [Shin et al. \(2006\)](#) reported detection of JCV LT DNA sequences in 21 (57%) of 37 gastric cancers and expression of LT protein in 9 (39%) of 23 gastric cancers, using Pab416. [Murai et al. \(2007\)](#) detected JCV LT DNA sequences in fresh frozen tissue from 19 (86%) of 22 gastric cancers, 19 (86%) of 22 adjacent non-cancerous tissues, and 10 of 10 normal gastric mucosa samples. Quantitation of JCV load by real-time PCR demonstrated equivalent copy numbers (~5 copies/100 cells) in all three types of tissue. LT expression was detected by IHC analysis using Pab101 in 1 (5%) of 22 gastric cancer samples and in none of the normal tissues. [Yamaoka et al. \(2009\)](#) detected JCV LT protein expression by IHC using Pab416 in 44 (49%) of 90 gastric cancers from Japanese patients. LT expression was detected in a similar percentage in EBV-positive and EBV-negative cancers. LT expression was negatively associated with microsatellite instability and p53 mutations and positively associated with nuclear/cytoplasmic β -catenin localization, allelic loss, and aberrant methylation. [Del Valle et al. \(2005\)](#) detected JCV DNA in 11 (85%) of 13

samples from normal or benign oesophagus and in 5 of 5 oesophageal cancers. JCV LT protein was detected by IHC using Pab416 in 10 (53%) of 19 carcinoma tissue samples, and agnoprotein was detected in 8 samples (42%). In contrast, none of the samples of normal or benign oesophagus were positive for viral protein.

4.3.3 *Tissue microarrays with diverse tumour types*

[Del Valle & Khalili \(2010\)](#) tested commercial tissue arrays of human tumours of various origins for the presence of JCV LT and agnoprotein by IHC analysis. LT was detected with Pab416 and agnoprotein with a polyclonal anti-peptide antibody produced in the investigators' laboratory. The arrays contained 27 types of diverse human cancers, 50 adenocarcinomas of the colon, 2 normal colon samples, 30 human brain tumours of glial origin, and 2 normal brain samples. LT was detected in 11 (37%) of the 30 brain tumours. All histological types of glial tumours showed reactivity, except pilocytic astrocytomas. Ten of the LT-positive tumours were also positive for agnoprotein. The 2 normal brain samples were immunonegative for both proteins. LT was detected in 17 (34%) of 50 adenocarcinomas of the colon, and agnoprotein was detected in 15 (30%) of these samples. Both normal colon samples were negative for LT and agnoprotein. JCV proteins were detected in one or both samples of glioblastoma multiforme, oesophageal carcinoma, thyroid papillary and follicular carcinomas, breast lobular carcinoma, lung squamous cell carcinoma, Wilms' tumour, and ovarian serous carcinoma. Tumours negative for JCV proteins included larynx squamous cell carcinoma, breast ductal carcinoma, gastric carcinomas, lung adenocarcinoma, hepatic carcinomas, renal cell carcinoma, ovary mucinous carcinoma, endometrial carcinomas, colon adenocarcinomas, rectal maltoma, dermatofibrosarcoma protuberans, and skin squamous cell

carcinoma. All immunoreactive tumours were positive for both LT and agnoprotein.

[Zheng et al. \(2007a\)](#) reported the detection of JCV DNA by nested PCR in 70 (68%) of 103 lung carcinomas from Japanese patients compared with a detection rate of 11% (2/18) in normal lung tissue. By real-time PCR, significantly higher copy numbers were detected in lung carcinomas compared with normal lung tissue and in squamous cell lung carcinoma compared with adenocarcinoma. An unspecified number of tumour samples with high JCV copy number showed expression of LT by IHC using Pab416.

4.3.4 *Mechanistic studies of JCV LT expression*

In addition to demonstrating the expression of T-antigen in human tumour tissues, several studies have examined interactions of JCV LT with cell-cycle regulatory proteins and proteins of signalling pathways implicated in carcinogenesis, and have demonstrated associations of LT expression with chromosomal instability and phenotypic properties of cancer cells.

(a) *Interactions with cell-cycle regulatory proteins*

[Del Valle et al. \(2001a\)](#) observed p53 staining in 3 medulloblastoma samples that were also JCV LT-immunoreactive; no p53 staining was seen in 5 LT-negative samples. Although both LT-negative and LT-positive tumours were positive for pRb staining, the frequency of pRb-positive cells was higher in LT-positive cases (range, 26–47%) than in LT-negative cases (5–11%). All tumour samples positive for LT and none of those negative for LT were immunoreactive for Rb2/p130. Double-label IHC showed that p53 and pRb positivity often occurred in the same cells that were LT-positive. [Enam et al. \(2002\)](#) investigated expression of p53 and β -catenin, a key component of the wnt signalling pathway, in colon cancer samples. In a collection of 27 tumour tissue samples, p53 was detected in 19 (70%), of which 13 were also

positive for LT, with frequent nuclear co-localization of the two proteins, suggesting a possible interaction between the two proteins. β -catenin was detected in 5 tumour samples that were positive for LT staining; co-localization of the two proteins in nuclei was shown by double labelling. To demonstrate the interaction between LT and β -catenin, the HCT116 colon cancer cell line was transfected with a plasmid expressing JCV LT; the two proteins were shown to be present in immune complexes pulled down by anti-LT antibody and not by control pre-immune serum. In a subsequent study, [Enam et al. \(2006\)](#) transfected the SW480 colon cancer cell line with plasmids expressing LT and TCF-4, another regulator of the wnt pathway, and demonstrated a physical interaction between these proteins by immunoprecipitation using antibody against LT followed by western blot with antibody against TCF-4. [Ricciardiello et al. \(2003\)](#) performed similar transfection experiments using the RKO colon cancer cell line. Transfection with JCV genome resulted in LT expression in RKO cells, accumulation of β -catenin in nuclei in association with LT, and an increase in p53 expression. Dual immunostaining and co-immunoprecipitation demonstrated an interaction between LT and p53, and between LT and β -catenin in JCV-transfected cells. [The Working Group noted that the above experiments using transfection of cells with expression vectors, while demonstrating the ability of JCV LT to interact with β -catenin, do not prove that the same interaction occurs *in vivo*, and expression levels may be higher than would occur *in vivo* so the physiological relevance of the experimental system is uncertain.]

(b) *Chromosomal instability and DNA methylation*

[Ricciardiello et al. \(2003\)](#) observed evidence of chromosomal instability, characterized by chromosomal breakage, dicentric chromosomes, and an increased number of chromosomes, in

whole JCV-transfected RKO colon cancer cells. [Nosho et al. \(2009\)](#) examined the association between LT expression in CRCs and a variety of molecular alterations. JCV LT expression was detected by IHC in 271 (35%) of 766 CRCs. LT expression was significantly associated with chromosomal instability, defined as the number of chromosomal segments with loss of heterozygosity (LOH). JCV LT expression was also associated with p53 expression, β -catenin expression, COX-2 expression, and loss of p21 expression. LT expression was inversely associated with DNA methylation at 8 well validated CpG island methylator phenotype loci. However, in multivariate logistic regression analysis, only the associations with p53 expression and chromosomal instability remained significant. [Goelet al. \(2006\)](#) also characterized chromosomal instability and DNA methylation of CRC tissues. They observed a significant association between LOH as a measure of chromosomal instability and LT expression; 27 (56%) of 48 CRCs that were positive for LT protein had LOH, whereas 16 (31%) of 52 LOH-negative tumours were LT-positive. They also found a significant increase in the methylation of 9 tumour suppressor genes in the LT-positive tumours compared with the tumours that were positive for only JCV DNA or were negative for both JCV DNA and protein.

(c) *Insulin-like growth factor 1 receptor signalling pathway*

Del Valle and Khalili and co-workers have reported evidence for involvement of the insulin-like growth factor 1 receptor signalling pathway in JCV LT-positive medulloblastomas ([Del Valle et al., 2002d](#); [Khalili et al., 2003](#)). IRS-1, a major signalling molecule in this pathway, is translocated to the nucleus in the presence of LT in both medulloblastoma cell lines and biopsies from patients. Molecular characterization of the interaction of these two proteins indicated that the N-terminal portion of IRS-1 binds to the C-terminal region of JCV

LT. Furthermore, competitive blocking of this interaction attenuated anchorage-independent growth of LT-positive medulloblastoma cells in culture.

(d) *Role of the non-coding regulatory region*

JCV genotypes can be broadly classified into archetype and rearranged variants based on the structure of the non-coding control region (NCCR). Rearranged variants are exclusively found in PML lesions, suggesting that these strains may be uniquely pathogenic for this lesion, although the precise mechanism for CNS pathogenesis has not been elucidated. The role of NCCR variants in JCV-associated cancers has received limited attention. [Rencic et al. \(1996\)](#) identified a 19 bp nucleotide deletion in the 98 bp repeat in an isolate from an oligoastrocytoma. [Ricciardiello et al. \(2001\)](#) found that the only JCV strain present in the human colon (colon cancer tissue and non-neoplastic gastrointestinal tissues) is Mad-1, a classic rearranged strain, and that a variant with a single 98 bp sequence is found exclusively in cancer tissue. [This finding may indicate that rearranged strains are involved in JCV-associated cancers; however, Mad-1 is also the most common laboratory strain of JCV.]

4.4 Transgenic models for cancers associated with JCV infection

Although JCV is not oncogenic in normal mice and does not transform mouse cells *in vitro*, transgenic mice expressing JCV early genes under the control of the JCV promoter have been shown to develop similar tumours of neuroectodermal origin, i.e. adrenal neuroblastomas, medulloblastomas or PNETs, and pituitary tumours (see Section 3).

4.5 Susceptible human populations

Chronic immunosuppression can reactivate latent JCV infection. In immunosuppressed individuals, for example people with HIV/AIDS or transplant recipients, uncontrolled JCV replication can lead to PML (see Section 1.3).

Nonetheless, there is no clear evidence to support an association between immunosuppression and human tumours potentially associated with JCV. In a meta-analysis of cancer incidence in HIV/AIDS patients and immunosuppressed transplant recipients, the incidence of brain cancers was increased only in HIV/AIDS patients, and the incidence of colon cancers was increased only in transplant recipients ([Grulich et al., 2007](#)).

4.6 Mechanistic considerations

There is evidence from several research groups and experimental systems that in specific experimental environments JCV has transforming capacity, following a mechanism that is, in general, similar to that identified for Merkel cell polyomavirus (MCV) (see the *Monograph* on MCV in this Volume) and also for oncogenic human papillomaviruses (HPV) ([IARC, 2007, 2012](#)) in human tumours. However, direct mechanistic evidence for such activity in human tumours is scarce and controversial.

Like for HPV, polyomavirus-induced carcinogenesis in experimental systems was shown to require at least one viral genome persistently present and biologically active in each transformed cell and interruption of the lytic viral life-cycle. Viral persistence can be mediated through integration or through maintenance as viral episome. The presence of one integration site per tumour, in different parts of the tumour and in primary tumour as well as in tumour metastases, indicates that viral integration occurred before clonal tumour expansion. Viral genes encoding regulatory proteins, i.e.

the T-antigens in polyomaviruses and the viral oncoproteins E6 and E7 in oncogenic HPV, are consistently expressed through transcription and translation. Viral oncoproteins, among other functions, interact directly or indirectly with cellular tumour suppressor proteins such as pRb and p53, leading to cell-cycle and apoptosis deregulation ([Ludlow & Skuse, 1995](#)). Expression of viral oncoproteins is necessary to maintain the transformed phenotype and in the tumour-bearing animal can lead to the induction of antibodies to the viral oncoproteins. Such antibodies are rarely induced during the natural course of infection.

An additional essential feature of virally induced cell transformation is the interruption of the lytic viral life-cycle. This can be due to a lack of host factors essential for viral replication or a lack of viral protein functions or of *cis* elements on the viral genome necessary for viral replication.

JCV, like other small DNA viruses, needs the host-cell DNA replication machinery to drive viral replication. As described above in detail (Sections 4.1–4.3), JCV LT protein binds to the retinoblastoma family of tumour suppressors ([Dyson *et al.*, 1989, 1990](#); [Bollag *et al.*, 2006](#)), leading to sequestration of these proteins and thus facilitating the transition from G1 into S phase. JCV LT also binds to p53, leading to p53 sequestration, and thus interferes with apoptosis induction ([Bollag *et al.*, 1989](#)). The transforming ability of JCV LT appears to be much weaker than that of primate polyomavirus SV40 ([Haggerty *et al.*, 1989](#); [Trowbridge & Frisque, 1993](#)).

4.6.1 Cell line studies

The early region of the JCV genome when stably introduced and expressed in cultured rodent cells such as rat fibroblasts and baby hamster kidney cells can lead, although inefficiently, to cell transformation characterized by cell growth in anchorage-dependent conditions

([Bollag *et al.*, 1989](#); [Haggerty *et al.*, 1989](#); [Hayashi *et al.*, 2001](#)).

4.6.2 Studies in animals

Apart from humans, no other host appears to be permissive for JCV replication. Non-permissivity of target cells is a prerequisite for cellular transformation by fully infectious virions or by experimental introduction of full viral genomes.

Intracerebral but also subcutaneous JCV inoculation in several mammalian species, including hamsters, rats, and non-human primates (i.e. owl monkeys and squirrel monkeys) resulted in various nervous system tumours (see Section 3). Tumour tissues from owl monkey tumours and cell lines established from explanted tumours consistently expressed LT and showed evidence of viral genome persistence and chromosomal integration of the JCV genome ([London *et al.*, 1978](#); [Miller *et al.*, 1983, 1984](#)).

Transgenic mice stably containing and expressing the JCV complete early gene region developed brain tumours within 6–8 months after birth ([Small *et al.*, 1986](#)). LT expression was detectable in all tumours in ~25–75% of the tumour cell nuclei. Variations in the promoter/enhancer regions controlling early genome region expression may account for differences in tumour type and tumour characteristics ([Feigenbaum *et al.*, 1992](#); [Ressetar *et al.*, 1993](#); [Krynska *et al.*, 1999b](#); [Gordon *et al.*, 2000](#)).

4.6.3 Human tumour studies

Data associating JCV with various human tumours have been provided by PCR-based detection of JCV DNA sequences. In view of the rather ubiquitous presence of JCV in the human population, great care has to be applied to the interpretation of the PCR results. Mostly only case series were analysed, and only a few studies reported on JCV PCR analyses in adjacent

non-tumour tissues from the same patient and in control tissues from non-tumour patients.

As described in detail above, JCV DNA PCR positivity was reported for glioblastomas, oligoastrocytomas, oligodendrogliomas, and medulloblastomas, for colorectal, gastric, and oesophageal cancers, and finally for lung cancer. At the same time, other groups did not find JCV DNA in series of some of these tumours.

In the two studies analysing viral load, copy numbers significantly less than one viral genome copy per cell were reported. [Murai *et al.* \(2007\)](#) found ~5000 JCV copies/ μ g of cellular DNA extracted from gastric cancer (corresponding to ~1 viral genome per 33 cells) and ~10-fold fewer copies in normal mucosa. [Zheng *et al.* \(2007a\)](#) reported 3000 copies of JCV/ μ g of lung cancer DNA (corresponding to ~1 genome copy per 50 cells) and ~10-fold fewer copies in normal lung tissue.

No studies demonstrating viral integration or JCV LT mutations or partial genome deletion resulting in loss of replication function have been reported. Further, data demonstrating the presence of JCV in tumour cells directly by ISH and microdissection, or in cell lines derived from such tumours are not available.

A few research groups analysed JCV DNA PCR-positive human tumour tissues by IHC and reported expression of JCV LT, whereas other groups did not find LT staining.

One research group reported the expression of agnoprotein (which can bind to p53 and can disrupt the cell cycle) in several human tumours of the CNS ([Darbinyan *et al.*, 2002](#)).

Even fewer studies analysed pRb and p53 expression in cells in which JCV LT and/or agnoprotein were detected. No clear, consistent data on involvement of pRb and p53 pathways in JCV DNA-positive tumours have been reported so far. No studies analysing p16^{Ink4a}, which in HPV-transformed human tumour cells is almost always found upregulated as a consequence of pRb downregulation, have been reported. In organs

in which both HPV-induced and HPV-negative tumours are found, p53 is frequently mutated in HPV-negative tumours, whereas in HPV-driven tumours, p53 is almost always not mutated. Such studies comparing JCV DNA-positive and JCV DNA-negative tumours have not yet been reported.

5. Summary of Data Reported

5.1 Exposure data

Infection with JC polyomavirus (JCV) is highly prevalent globally; ~70% of the adult population have anti-JCV IgG antibodies. This prevalence was independent of the serological assay that was used and whether the sera were adsorbed or blocked with virus-like particles (VLPs) of the closely related polyomaviruses, BKV or SV40. Primary infection occurs in childhood and is regarded to be asymptomatic. After primary infection, JCV establishes a persistent infection in the kidneys and is occasionally shed in the urine and is found in sewage. JCV has also been suggested, especially in immunosuppressed individuals, to establish a silent infection in the brain. The transmission route for JCV is unknown, but the respiratory and oral routes have been suggested. In some immunosuppressed individuals, such as individuals with HIV/AIDS or patients receiving immunosuppressive therapy, uncontrolled JCV replication in the brain can lead to progressive multifocal leukoencephalopathy (PML). There is consistent evidence that JCV infects humans.

5.2 Human carcinogenicity data

The potential role for JCV infection in cancer etiology has been investigated in 6 prospective studies, 4 case-control studies, and 15 tumour case series that included non-cancer tissues from

individuals without cancer. For CRC, there have been 3 case–control studies, including 2 nested within cohorts with prospective exposure assessment, that observed no significant association with JCV exposure. Two case–control studies of NHL, including one with prospective exposure assessment, observed no significant association with JCV exposure. One case–control study of brain tumours with prospective exposure assessment observed no significant association with JCV exposure. A case–control study of childhood neuroblastoma and a case–control study of ALL with prospective exposure assessment observed no associations with JCV exposure. Neither bladder cancer nor prostate cancer was associated with JCV exposure, each in a single case–control study.

Findings were inconsistent across case series of colon, prostate, lung, stomach, tongue, and oesophagus cancer tissues. All formal prospective and case–control studies have uniformly not found any association. Based on this overall evidence, there is no clear association between JCV and human cancer.

5.3 Animal carcinogenicity data

Five studies were performed in adult owl monkeys and squirrel monkeys in which JCV was inoculated intracerebrally, sometimes in combination with intravenous and subcutaneous injection of the virus. The tumours consistently induced were astrocytoma and glioblastoma (grade III and IV glial tumours).

Seven studies were performed in outbred newborn (NB) hamsters inoculated intracerebrally with JCV. The most frequently induced tumours were medulloblastoma, glioblastoma and astrocytic tumours, primitive neuroectodermal tumours (PNETs), pituitary adenoma, peripheral neuroblastoma, ependymoma, and choroid plexus papilloma.

In three studies in NB rats inoculated intracerebrally with JCV, PNETs were induced at a very high incidence.

In six studies using JCV T-antigen transgenic mice, the most common tumours observed were peripheral neuroblastoma, medulloblastoma, PNETs, and pituitary adenoma. In a comparison of JCV and SV40 regulatory regions and T-antigen genes in transgenic mice, the tumour type observed strongly correlated with the tropism of the promoter used, although the T-antigen sequences may also contribute to tropism.

5.4 Mechanistic and other relevant data

There is consistent evidence from animal and cell-culture studies that JCV can be directly oncogenic and transforming through its oncoproteins, i.e. the T-antigens encoded in the early region of its genome. The mechanisms involve immortalization, transformation, and enhancement of cell survival. There is only weak and controversial evidence for such mechanisms being active in human tumours.

- The presence of JCV DNA, based on analysis by PCR, has been reported in a broad variety of human tumours by several groups but not by others. In view of the ubiquity of JCV in the human population and its ability to spread throughout the body, the tumour specificity of the PCR findings has not been established convincingly.
- JCV DNA, if found in human tumours, appears to be present mostly in low copy numbers. This indicates that the majority of cells within a tumour potentially associated with JCV do not contain the viral genome. Thus, novel mechanisms for JCV-induced carcinogenesis would need to be invoked, for which there is currently little evidence.

- Demonstration of viral oncogene expression in cells of JCV DNA-positive human tumours is limited.
- Studies that demonstrate evidence for cell-cycle and apoptosis regulation by JCV through pathways involving pRb family proteins and p53 in human tumours are few, and the evidence is weak.

6. Evaluation

6.1 Cancer in humans

There is *inadequate evidence* in humans for the carcinogenicity of JCV.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of JCV.

6.3 Overall evaluation

JCV is *possibly carcinogenic to humans* (Group 2B).

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MERKEL CELL POLYOMAVIRUS

1. Exposure Data

1.1 Cell and tissue tropism

Merkel cell polyomavirus (MCV) was first discovered clonally integrated in Merkel cell carcinoma (MCC) ([Feng et al., 2008](#)). Merkel cells reside in the basal layers of the skin and express dual epithelial/neuroendocrine phenotypic markers. In conjunction with sensory afferent neurites, Merkel cells normally function as mechanoreceptors. A comprehensive initial study by Loyo and colleagues showed that MCV DNA can be detected, in varying amounts, in specimens from a wide range of different tissues ([Loyo et al., 2010](#)). The highest amounts of MCV DNA are detected in oral samples and are shed from apparently healthy skin surfaces in the form of assembled virions ([Wieland et al., 2009](#); [Loyo et al., 2010](#); [Schowalter et al., 2010](#)). Kidney and bladder specimens carry a much lower level of MCV DNA, indicating that the urinary tract is unlikely to be a major site of productive MCV infection, as it is for BK polyomavirus (BKV) and JC polyomavirus (JCV). It appears that MCV DNA can be readily detected in environments occupied by humans ([Foulongne et al., 2011](#)). The possibility of environmental contamination should be considered a very serious concern for polymerase chain reaction (PCR)-based studies of MCV ([Cohen & Enserink, 2011](#)).

1.2 Methods for the detection of MCV

Detection of current MCV infection is based on the detection of viral DNA by PCR amplification, and current and past infection can be detected by the detection of specific antibodies. The sequence diversity of known wild-type (WT) MCV isolates is very low, with > 98.5% nucleotide identity across the viral genome.

1.2.1 Detection of MCV DNA

Viral DNA is detected by PCR, nested PCR, real-time PCR, quantitative PCR (qPCR), and rolling circle amplification (RCA), using different primers specific to the genes encoding MCV large T-antigen (LT), small T-antigen (sT), and capsid viral protein 1 (VP1) antigen (for references, see [Table 1.1](#)). Methodological differences across studies may explain some of the variations observed in MCV DNA prevalence. Samples positive for one set of primers only could be attributed to the failure of PCR to detect low levels of viral DNA, or to disruption of the virus during integration (for MCC samples). For PCR amplification from formalin-fixed, paraffin-embedded (FFPE) samples, as used in many studies, it has been reported that formalin fixation may fragment DNA and results in differences in amplification according to the size of the amplicons ([Kassem et al., 2008](#)). As reported by [Pastrana et al. \(2012\)](#), this is particularly important for

Table 1.1 Detection of MCV DNA in healthy skin or inflammatory skin lesions

Reference	Study location	Samples tested	Methods	IC patients	No. of subjects	Detection of MCV DNA n (%)
<i>PCR and nPCR</i>						
Feng et al. (2008)	USA	Skin/inflammatory skin lesions	PCR	–	18	1 (5.6%)
Dworkin et al. (2009)	USA	Normal skin adjacent (SCC patients)	PCR	–	32	9 (28.1%)
Wieland et al. (2009)	Germany	Perilesional healthy skin	nPCR	–	34	8 (23.5%)
Andres et al. (2010a)	Germany	Inflammatory skin lesions	PCR + SB	–	23	2 (8.7%)
Andres et al. (2010b)	Germany	Sun-exposed skin with seborrhoeic keratosis	PCR + SB	–	12	2 (16.6%)
Mogha et al. (2010)	France	Skin	PCR	–	20	2 (10.0%)
Mangana et al. (2010)	Switzerland	Skin	PCR	–	11	0 (0.0%)
Mertz et al. (2010a)	Switzerland	Common wart	PCR	–	30	2 (6.7%)
Mertz et al. (2010a)	Switzerland	Common wart	PCR	+	16	1 (6.3%)
<i>RT-qPCR and RCA</i>						
Garneski et al. (2009)	North America	Skin	RT-qPCR	–	15	0 (0.0%)
Foulongne et al. (2010b)	France	Skin swabs	RT-qPCR	–	25	25 (100.0%)
Schowalter et al. (2010)	USA	Skin swabs	RCA	–	35	14 (40.0%)
Faust et al. (2011)	Sweden, Austria	Benign skin lesions	RT-qPCR	–	119	22 (18.5%)
Wieland et al. (2011)	Germany	Forehead swabs	RT-qPCR	–	239	118 (49.4%)
Wieland et al. (2011)	Germany	Forehead swabs	RT-qPCR	+	210	124 (59.0%)

IC, immunocompromised; MCV, Merkel cell polyomavirus; nPCR, nested PCR; RCA, rolling circle amplification; PCR, polymerase chain reaction; RT-qPCR, real-time quantitative PCR; SB, Southern blot hybridization; SCC, squamous cell carcinoma

samples containing very low copy numbers per cell.

Comparisons of the results of MCV DNA prevalence and viral load must be undertaken with caution.

1.2.2 Detection of MCV antibodies

Most of the studies investigating anti-MCV antibodies use VP1, or VP1 plus VP2 virus-like particles (VLPs) as antigens, and they are produced in insect cells, human embryonic kidney 293TT cells, or glutathione S-transferase (GST)-VP1 recombinant protein (capsomeres) ([Kean et al., 2009](#); [Tolstov et al., 2009](#); [Touzé et al., 2010](#); [Chen et al., 2011](#); [Viscidi et al., 2011](#)). Immunological tests using VLPs or capsomeres derived from strain MCC350 were found to be less sensitive than tests using capsomeres derived from strain MCC339 ([Kean et al., 2009](#)). However, the VP1 protein of strain MCC350 did not assemble into VLPs or capsomeres ([Touzé et al., 2010](#)). In addition to VLPs and capsomere enzyme-linked immunosorbent assay (ELISA)-based assays, neutralization assays using MCV pseudovirions produced in human embryonic kidney 293TT cells and Luminex-based multiplex serological assays (for further details, see Section 1 of the *Monograph* on BKV in this Volume) have been used ([Pastrana et al., 2009](#)).

Pre-incubation of serum samples with JCV, BKV, and lymphotropic papovavirus (LPV) has shown that there is limited cross-reactivity between MCV and these other human polyomaviruses ([Kean et al., 2009](#); [Tolstov et al., 2009](#); [Touzé et al., 2010](#); [Viscidi et al., 2011](#)). Pseudovirion neutralization assays also support the specificity of MCV reactivity ([Pastrana et al., 2009](#)).

Detection of antibodies against MCV LT and sT is useful to assess the presence of MCV in MCC and to monitor tumour progression in MCC patients ([Paulson et al., 2010](#)). However, they are rarely detected in the general population

and are therefore not used for detecting exposure to the virus.

1.2.3 Detection of MCV proteins

Detection of the MCV LT (using the monoclonal antibody CM2B4) is a marker commonly used to examine the presence of MCV in MCC cells. This antibody recognizes the MCV LT and 57kT isoforms but will not detect MCV sT ([Busam et al., 2009](#)).

1.3 Epidemiology of infection

1.3.1 Seroprevalence of MCV

MCV serology studies show that a high proportion of adults have been exposed to MCV and that infection is acquired early in life ([Carter et al., 2009](#); [Kean et al., 2009](#); [Pastrana et al., 2009](#); [Tolstov et al., 2009, 2011](#); [Sadeghi et al., 2010](#); [Touzé et al., 2010](#); [Chen et al., 2011](#); [Faust et al., 2011](#); [Touzé et al., 2011](#); [Viscidi et al., 2011](#); [Table 1.2](#)). The age-specific seroprevalence of MCV indicates widespread exposure early in life, with a seroprevalence of 20% in children aged 1–5 years ([Kean et al., 2009](#)) and of 35–50% in children aged < 10–15 years ([Tolstov et al., 2009](#); [Chen et al., 2011](#); [Viscidi et al., 2011](#)).

Seroprevalences of 46–87.5% have been reported in adults ([Carter et al., 2009](#); [Kean et al., 2009](#); [Pastrana et al., 2009](#); [Tolstov et al., 2009, 2011](#); [Touzé et al., 2010, 2011](#); [Viscidi et al., 2011](#)). Among newly infected individuals, a transient increase in immunoglobulin (Ig) M antibodies that declines over 1–2 years is observed, and the majority of individuals (65%) demonstrate a robust IgG seroconversion, with stable levels over time ([Tolstov et al., 2011](#)). An increase in MCV antibodies ELISA optical density (OD) values with age has also been reported in adults ([Viscidi et al., 2011](#)), suggesting that antibody titres increase with age.

Table 1.2 Detection of MCV antibodies in healthy subjects and in patients with cancer other than MCC or in patients with skin diseases

Reference	Study location	MCV antigen used	Age (years)	No. of subjects	Anti-MCV antibodies <i>n</i> (%)
<i>Children</i>					
Kean <i>et al.</i> (2009)	USA	GST-VP1	1–4	112	23 (20.5%)
			5–17	517	182 (35.2%)
Tolstov <i>et al.</i> (2009)	USA	VP1 + VP2 VLPs	2–15	81 ^a	38 (46.9%)
Chen <i>et al.</i> (2011)	Finland	VP1 VLPs	1–4	298 ^b	19 (6.4%)
			5–13	26 ^c	9 (34.6%)
			1–4	158 ^d	6 (4%)
Viscidi <i>et al.</i> (2011)	Italy	VP1 VLPs	1–9	42	19 (45.2%)
			10–19	38	23 (60.5%)
<i>Adults</i>					
Carter <i>et al.</i> (2009)	USA	GST-VP1	42–86	76	40 (53%)
			24–77	451	268 (59.4%)
Kean <i>et al.</i> (2009)	USA	GST-VP1	18–70s	1593	734 (46.1%)
Pastrana <i>et al.</i> (2009)	USA	VP1 + VP2 pseudovirions	47–75	48	42 (87.5%)
Tolstov <i>et al.</i> (2009)	USA	VP1 + VP2 VLPs	> 18	166	107 (64.5%)
Sadeghi <i>et al.</i> (2010)	Finland	VP1 VLPs	18–45	462	212 (45.9%)
Touzé <i>et al.</i> (2010)	Belgium	VP1 VLPs	18–25	101	78 (77%)
Touzé <i>et al.</i> (2010)	Italy	VP1 VLPs	18–85	194	150 (77.5%)
Faust <i>et al.</i> (2011)	Sweden, Austria	VP1 + VP2 VLPs	50–94	434 ^e	283 (65.2%)
Tolstov <i>et al.</i> (2011)	USA	VP1 + VP2 VLPs	18–69	564	447 (79.3%)
Touzé <i>et al.</i> (2011)	France, Italy	VP1 VLPs	22–90	82	70 (85.4%)
Viscidi <i>et al.</i> (2011)	Italy	VP1 VLPs	20–70s	865	640 (74%)

^a The subjects were children with Langerhans cell histiocytosis (LCH).

^b The subjects were children with otitis media (*n* = 158) or acute lower respiratory infection (*n* = 140).

^c The subjects were children with acute lower respiratory infection.

^d The subjects were children with otitis media.

^e This population included subjects with squamous cell carcinoma (*n* = 67), basal cell carcinoma (*n* = 159), actinic keratoses (*n* = 785), seborrhoeic keratosis (*n* = 1), and other benign lesion (*n* = 1).

GST, glutathione S-transferase; MCV, Merkel cell polyomavirus; VLPs, virus-like particles; VP1, capsid viral protein 1; VP2, capsid viral protein 2

MCV seropositivity was not found to be associated with other chronic viral infections (HIV, hepatitis B virus, hepatitis C virus) or with sexual activity, and no specific symptoms or signs were associated with MCV seroconversion at adult age ([Tolstov et al., 2011](#)).

1.3.2 Prevalence of MCV DNA

Using PCR or nested PCR, MCV DNA has been detected in 0–28.1% of samples taken from a variety of skin samples ([Table 1.1](#); [Feng et al.,](#)

[2008](#); [Dworkin et al., 2009](#); [Andres et al., 2010a, b](#); [Mangana et al., 2010](#); [Mertz et al., 2010a, b](#); [Mogha et al., 2010](#); [Wieland et al., 2011](#)). With more sensitive techniques such as real-time PCR, qPCR, or RCA, MCV sequences have been amplified in up to 100% of skin samples examined ([Garneski et al., 2009](#); [Foulongne et al., 2010a](#), [Schowalter et al., 2010](#); [Faust et al., 2011](#); [Wieland et al., 2011](#)). Furthermore, [Wieland et al. \(2009\)](#) reported the detection of MCV DNA in 6 (30%) of 20 anal swabs and in 3 (50%) of 6 penile swabs. In addition to the detection of MCV on

the skin, high levels of MCV DNA detection have also been reported in the oral cavity, with detection rates ranging from 8.3% to as high as 39–60% ([Dworkin et al., 2009](#); [Wieland et al., 2009](#); [Loyo et al., 2010](#)). The quantity of MCV DNA has been reported to be lower in the skin than in the oral cavity mucosa ([Loyo et al., 2010](#)), although [Foulongne et al. \(2010b\)](#) observed that MCV DNA is less frequently detected in buccal mucosa swabs than in skin swabs. The variation observed in the detection of MCV may be due in part to the sampling methods, including biopsies and surface swaps.

Low levels of MCV can be amplified from many human tissues ([Feng et al., 2008](#); [Kantola et al., 2009](#); [Bergallo et al., 2010](#); [Loyo et al., 2010](#)). This low level of viral DNA may indicate that MCV is systemically distributed, but not pathogenic, in most tissues, where it may undergo low-level replication, persistence, or latency.

1.3.3 Primary infection and transmission

Infection with MCV occurs early in childhood and is widespread among adults ([Kean et al., 2009](#); [Tolstov et al., 2009, 2011](#); [Sadeghi et al., 2010](#); [Touzé et al., 2010](#); [Chen et al., 2011](#); [Viscidi et al., 2011](#)). The virus is present on all skin surfaces and is part of the normal skin flora. The detection of the same DNA sequence at different skin surfaces from the same individual ([Schowalter et al., 2010](#)) and the absence of variation in prevalence of DNA detection at different skin locations ([Foulongne et al., 2010b](#)) suggest that MCV is often established as a persistent infection of the skin in healthy subjects. The MCV viral load on the skin surface varies from as little as 1 copy per 1000 cells to 1000 copies per cell ([Katano et al., 2009](#); [Loyo et al., 2010](#); [Mogha et al., 2010](#); [Faust et al., 2011](#); [Pastrana et al., 2012](#)). In addition, [Pastrana et al. \(2012\)](#) did not observe significant variation in viral load according to age or sex of subjects or between different skin locations in the same subject. Furthermore, MCV

antibody titres are highly correlated with DNA viral load on the skin ([Faust et al., 2011](#); [Pastrana et al., 2012](#)), indicating that individuals with very high anti-MCV titres are those with persistent and active skin shedding of MCV virions.

The exact mode(s) of MCV transmission, the site(s) of initial infection, and the existence of a latent phase have not yet been characterized ([Foulongne et al., 2010a, b](#); [Loyo et al., 2010](#); [Schowalter et al., 2010](#)). MCV has not been detected in fetal autopsy samples, and thus vertical transmission from mother to infant does not seem to occur ([Sadeghi et al., 2010](#)). However, this does not exclude the possibility of perinatal transmission at time of delivery.

MCV DNA was detected in 0.6–1.3% of nasopharyngeal aspirates of children and in 2.1–8.5% of adults ([Bialasiewicz et al., 2009](#); [Goh et al., 2009](#); [Kantola et al., 2009](#)). MCV was also detected in 3.5% of tonsils, 6.7% of lung tissues, and 17.2% of bronchoalveolar and bronchoaspirates ([Kantola et al., 2009](#); [Babakir-Mina et al., 2010](#); [Loyo et al., 2010](#)). The presence of MCV in the upper and lower respiratory tract thus raises questions about its potential aerodigestive transmission ([Bialasiewicz et al., 2009](#); [Goh et al., 2009](#); [Kantola et al., 2009](#); [Babakir-Mina et al., 2010](#); [Abedi Kiasari et al., 2011](#)), as suggested for other polyomaviruses.

MCV was not detected in cerebrospinal fluid, peripheral blood mononuclear cells, urine, plasma/blood, brain or prostate tissue samples, or frozen autopsy samples from both immunocompetent and immunosuppressed subjects ([Kassem et al., 2008](#); [Bluemn et al., 2009](#); [Duncavage et al., 2009a](#); [Giraud et al., 2009](#); [Katano et al., 2009](#); [Lam et al., 2010](#); [Sadeghi et al., 2010](#); [Dang et al., 2011](#)). However, others have reported detection of MCV DNA sequences in 15–25% of urine samples and in 0.1–12% of serum samples ([Kantola et al., 2009](#); [Bofill-Mas et al., 2010](#); [Husseiny et al., 2010](#)). Although observed in only a fraction of the studies, the detection of MCV in serum raised the possibility of blood transmission; however,

this route of transmission is not expected to have a subsequent role since most adults have antibodies against MCV. The low copy number of MCV in the urine could be due to contamination by skin when passing urine.

MCV is present in urban sewage and river water and is detected in 85% of environmental surface samples, suggesting that viral DNA is present on all objects in contact with humans ([Bofill-Mas et al., 2010](#); [Foulongne et al., 2011](#)). Viral MCV DNA from environmental surface samples remains detectable after DNase treatment before nucleic acid extraction ([Foulongne et al., 2011](#)), indicating the presence of encapsidated DNA belonging to potentially infectious viruses. This was confirmed by [Schowalter et al. \(2010\)](#), and thus transmission of MCV from environmental sources to humans is a possible mode of transmission.

1.4 Diseases associated with MCV

MCV is associated with MCC ([Feng et al., 2008](#)). This is discussed in detail in Sections 2 and 4 of this *Monograph*.

MCV has not been associated with any other specific primary disease or symptoms to date.

2. Cancer in Humans

Methodological considerations: case-control versus case-series study designs

Numerous studies have reported the prevalence of markers of infection by polyomaviruses in tumour tissues or blood obtained from humans with cancer. Many of these studies included specimens from individuals without cancer as “controls,” but such studies were not generally considered by the Working Group as case-control studies, given the convenience sampling strategies used or the lack of comparability of exposure measurement between comparison groups.

Specifically, convenience sampling of controls led to the possibility that the control subjects were not representative of the source population. Also, the comparison of tumour tissues in cases with normal tissues (such as blood, urine, or biopsies of normal tissues) in controls may also be biased because it is uncertain whether polyomaviruses are uniformly present in these normal tissues or can be reliably detected by the assays used. However, because these studies contribute information on cancer sites not investigated by the case-control studies, included comparisons with both normal and pre-malignant control tissues, compared tumour tissue with a convenience sample of controls, compared different tissues in cases or controls, and/or presented findings for susceptible populations (i.e. transplant patients), they are considered here as case series.

2.1 Background

There are few epidemiological studies (no cohort studies, few case-control studies) evaluating the association between MCV and human cancer. However, multiple case series describing detection of MCV DNA in a large fraction of MCC tumours are consistent and provide some evidence of the association between MCV and MCC. Furthermore, some studies also explore the joint association between MCV and other cancer sites, as a first or subsequent primary after MCC.

2.2 Case-control studies

2.2.1 Merkel cell carcinoma

MCC is a neuroectodermal tumour that originates from Merkel cells. MCC is a rare tumour (approximate incidence, 4 cases per million), but in some regions of the world, its incidence is on the rise ([Hodgson, 2005](#)). In the USA, there are about 1500 cases per year and the reported incidence has significantly increased between 1986

and 2001 ([Hodgson, 2005](#)). [It is unclear why there is such an increase, but better registration of MCC and changes in the tumour classification cannot be ruled out.] MCC is more common in white men, and the average age at presentation is 70 years ([Agelli & Clegg, 2003](#)).

MCC tends to recur locally and gives rise to regional as well as distant metastases. It is an aggressive skin tumour with a disease-specific survival rate of 60–70% at 5 years after diagnosis ([Reichgelt & Visser, 2011](#)). MCC occurs more often among immunosuppressed subjects, such as organ transplant recipients or HIV-positive persons, suggesting a possible infectious etiology. It has been postulated that exposure to ultraviolet (UV) radiation could also play a role. MCC is often diagnosed in sun-exposed areas of the skin ([Mogha *et al.*, 2010](#)). [However, Merkel cells are unevenly distributed in skin ([Lacour *et al.*, 1991](#)), and it is unclear whether this has an impact on tumour site distribution.] Special stains are used to distinguish MCC from other forms of cancer, such as small cell lung cancer (SCLC), lymphoma, and small cell melanoma ([Smith & Patterson, 2001](#)).

Few case–control studies have been published analysing the association between MCV and MCC, and these are described in [Table 2.1](#) and in the following text.

[Carter *et al.* \(2009\)](#) and [Paulson *et al.* \(2010\)](#) carried out a case–control study with 205 MCC cases and 530 population controls. Cases included 139 subjects matched on age and sex to controls from the repository at the Fred Hutchinson Cancer Research Center and 66 individuals who were unmatched. Case subjects were identified from January 1, 2008, to May 1, 2010. Multiplex serology antibody was used to specifically recognize antibodies against VP1, LT, and sT antigens of MCV and BKV. MCC cases were more likely than control subjects to have antibody reactivity to MCV capsid (odds ratio [OR], 5.5; 95% confidence interval [CI]: 2.9–11.2). However, the association with antibodies to MCV sT was the

strongest (OR, 63.2; 95% CI, 24.4–164.0). The association with antibodies to MCV LT was also strong (OR, 16.9; 95% CI, 7.8–36.7). These associations were not observed for antibodies against BKV. [The study populations of [Carter *et al.* \(2009\)](#) and [Paulson *et al.* \(2010\)](#) overlap, although the extent of overlap is not clear.]

[Viscidi *et al.* \(2011\)](#) from the USA evaluated MCV capsid antibodies in 33 MCC cases that had been diagnosed at Moffitt Cancer Center in Tampa, Florida, between 2006 and 2008 and compared them with 37 controls who were patients undergoing skin cancer screening and were free of skin cancer. Seroprevalence of antibodies to MCV VP1 was 90.9% (30/33) in the cases and 67.6% (25/36) in the controls [OR, 4.4; 95% CI, 0.99–26.7], and mean antibody levels were higher in the cases than in the controls (1876.0 vs 1521.5; $P_{\text{age-adjusted}} = 0.04$).

2.2.2 Other cancers

The role of MCV has been reported so far in only one case–control study each for squamous cell carcinoma (SCC) of the skin, transitional cell carcinoma of the bladder, and oesophageal cancer ([Table 2.2](#)).

[Rollison *et al.* \(2012\)](#) compared seroresponse against MCV capsid in 173 SCC cases and in 300 controls screened negative for skin cancer. Seroresponse was evaluated through fluorescence-based multiplex serology. MCV DNA was evaluated in the cases through multiplex PCR targeting a conserved region of the LT gene. MCV DNA was present in 55 (38%) of 145 cases. MCV antibodies were significantly higher in SCC cases compared with levels observed in controls. Overall seropositivity was higher in SCC cases (OR, 1.58; 95% CI, 0.96–2.6). MCV titres were significantly higher among MCV DNA-positive cases (OR, 2.49; 95% CI, 1.03–6.04), with increasing risk with increasing antibody levels (fourth quartile vs first quartile, OR, 3.93; 95% CI, 1.43–10.76). When transplant recipients ($n = 5$) were excluded,

Table 2.1 Case-control studies of Merkel cell carcinoma and MCV

Reference, study location	Total no. of cases	Total no. of controls	Control source (hospital, population)	Detection method	Organ site	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Covariates	Comments
Carter <i>et al.</i> (2009) , USA	41	Group 1 (<i>n</i> = 76), matched; group 2 (<i>n</i> = 451), women	Hospital-based (group 1); population-based (group 2)	Multiplex antibody-binding VP1-GST fusion proteins; Seropositivity: MFI > 5000 units for strains MCVw162 and MCC350; qPCR	MCC	Antibodies to: MCVw162 VP1	36 (88%)	6.6 (2.3–18.8)	Age, sex	No cross-reactivity between MCVw162 VP1 and VP1 of other polyomaviruses (BKV, JCV, WUV, or KIV); MCV DNA detected in 24 (77%) of the 31 MCC tumours available, with 22 (92%) of these 24 patients also carrying antibodies against MCV.
Paulson <i>et al.</i> (2010) , USA, Germany	205	530	Random-digit dialling, frequency matched by age and gender	MCC multiplex antibody: binding to antibodies against VP1, LT, and sT of MCV and BKV; Seropositivity: MFI > 5000 units;	MCC	Antibodies to: MCV sT MCV LT MCV VP1	51 31	63.2 (24.4–164.0) 16.9 (7.8–36.7) 5.5 (2.9–11.2)		This study overlaps with the study by Carter <i>et al.</i> (2009) . Cases included 139 subjects, matched on age and sex to controls and 66 individuals not matched.
Viscidi <i>et al.</i> (2011) , USA	33	37	Cancer-free	VLP-based ELISA	MCC	Overall positivity; mean antibody	30	[4.4 (0.99–26.7)]	Age	

BKV, BK polyomavirus; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; GST, glutathione S-transferase; JCV, JC polyomavirus; KIV, KI polyomavirus; LT, large T-antigen; MCC, Merkel cell carcinoma; MCV, Merkel cell polyomavirus; MFI, median fluorescence intensity; qPCR, quantitative polymerase chain reaction; sT, small T-antigen; VLP, virus-like particles; VP1, capsid viral protein; WUV, WU polyomavirus.

Table 2.2 Case-control studies of other cancers and MCV

Reference, study location	Total no. of cases	Total no. of controls	Control source (hospital, population)	Detection method	Organ site	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Covariates	Comments
Rollison <i>et al.</i> (2012) USA	173	300	Screened negative for skin cancer	Multiplex serology to MCV capsid; multiplex PCR	Skin SCC	MCV seropositive cases			Age, sex	All analyses with JCV were negative for an association.
						All	140	1.58 (0.96–2.60)		
						MCV DNA-positive	48	2.49 (1.03–6.04)		
						Low	6	1.0 (ref)		
						High quartile	26	3.93 (1.43–10.76)		$P_{\text{trend}} < 0.01$
						High quartile in DNA-positive vs DNA-negative	26	5.76 (1.82–18.28)		$P_{\text{trend}} = 0.0004$
Polesel <i>et al.</i> (2012) Italy	114	140	Hospital	Multiplex PCR in urine	Bladder TCC	MCV DNA	3	0.51 (0.12–2.13)	Age, sex, education, tobacco smoking, alcohol drinking	
Sitas <i>et al.</i> (2012) Australia, China, South Africa, eastern Europe, Islamic Republic of Iran, and Brazil	1561	2502	Mixed hospital- and population-based	Multiplex serology to MCV VP1	Oesophageal cancer	MCV VP1	1261	0.88 (0.73–1.06)	Full adjustment based on individual case-control analysis	

CI, confidence interval; JCV, JC polyomavirus; MCV, Merkel cell polyomavirus; PCR, polymerase chain reaction; SCC, squamous cell carcinoma; TCC, transitional cell carcinoma, VP1, capsid viral protein 1

the risk persisted (OR, 3.45; 95% CI, 1.25–9.52). The case–case comparison for the presence of high titres of antibodies to MCV comparing DNA-positive with DNA-negative cases showed that the odds ratio was 5.76 when subjects in the fourth quartile were compared with those in the first quartile (95% CI, 1.82–18.28; $P_{\text{trend}} = 0.0004$). Among MCV DNA-negative subjects, no association was observed with antibody response. No association was observed between JCV sero-response and SCC irrespective of MCV status.

[Polesel *et al.* \(2012\)](#) from Italy reported on 114 cases with transitional cell carcinoma of the bladder and 140 hospital controls. Urine samples were evaluated for the presence of MCV DNA using multiplex PCR to detect LT sequences. The presence of MCV was detected in 3 (2.7%) cases and 8 (5.8%) controls (adjusted OR, 0.51; 95% CI, 0.12–2.13). Lower detection of polyomavirus was observed among women compared with men and among current or former smokers compared with never-smokers.

[Sitas *et al.* \(2012\)](#) explored the presence of antibodies to MCV in 1561 oesophageal cancer cases and 2502 controls originated from 6 case–control studies in Australia, China, South Africa, central and eastern Europe, Brazil, and the Islamic Republic of Iran. Using a multiplex approach to measure seroresponse to MCV, VP1 antibodies were detected in 80.8% of cases and in 83.4% of controls (OR, 0.88; 95% CI, 0.73–1.06).

2.3 Case series

[Table 2.3](#) describes some of the case series of MCC in which MCV was evaluated. Reports including fewer than 5 cases were excluded.

2.3.1 Merkel cell carcinoma

It was in 2008 that [Feng *et al.* \(2008\)](#) studied MCC samples by digital transcriptome subtraction and detected a fusion transcript between an undescribed LT and a human receptor tyrosine

phosphatase. This finding, together with subsequent investigations, led to the identification of MCV. Since that initial study, multiple case series have been published using different approaches to detect MCV using either formalin-fixed paraffin-embedded (FFPE) tissue or fresh tissue, with variation also in time periods of data collection but with a consistent detection of MCV DNA of > 50%.

[Pastrana *et al.* \(2009\)](#) from the USA studied 21 histologically confirmed MCC cases, all of which harboured MCV DNA. A control series of 48 was retrieved from a paid plasma donor bank. Samples were analysed using a reported vector-based neutralization assay to quantitate MCV-specific serum antibody response. All cases displayed substantial MCV-specific antibody responses. Although 88% (42/48) of controls without MCC were MCV seropositive, the geometric mean titre (GMT) of the control group was 59-fold lower than that of the MCC patient group (GMT of 21 500 in controls vs 222 000 in cases; $P = 0.0001$). Only 4% (2/48) of control subjects displayed neutralizing titres greater than the mean titre of the MCV-positive MCC patient population. MCC tumours were found not to express detectable amounts of MCV VP1 [suggesting that the strong humoral responses observed in MCC patients could be explained by an unusually immunogenic MCV infection, and not by viral antigen expressed by the MCC tumour itself.] Specific evaluation of antibodies against BKV did not correlate with that observed for MCV [suggesting that the immune response associated with MCV was unlikely to be explained by an immunodeficient status of cancer patients.]

[Tolstov *et al.* \(2009\)](#) from Pittsburgh, USA, evaluated MCV through VLP enzyme immunoassay (EIA) in 21 MCV DNA-positive MCC cases and in 6 MCV DNA-negative MCC cases. Controls included 166 blood donors, 100 commercially available blood donors, and 50 adults with systemic lupus erythematosus

Table 2.3 Case series of Merkel cell carcinoma and MCV

Reference, study location	Total no. of cases	Total no. of controls	Control source (hospital, population)	Detection method	Exposure categories	Results	Comments
Feng et al. (2008) USA	10	59	Case-case study	Digital transcriptome subtraction	Presence of vDNA	8 of 10 (80%)	6 of the 8 MCV-positive MCC had integration. No mention of integration in the control samples.
Pastrana et al. (2009) USA	21	48	Plasma donor bank	Neutralizing antibodies	Positivity GMT	100% 222000	All cases were MCV DNA-positive.
Tolstov et al. (2009) USA	27	316	Blood donors (166), commercially available blood donors (100), and patients with systemic lupus erythematosus (50)	VLP EIA	MCV antibody positivity	100% of vDNA-positive cases; 50% of vDNA-negative cases	
Garneski et al. (2009) USA, Australia	37	0	NA	PCR to detect MCV LT and sT	Presence of vDNA	62% of primary MCCs; 23% of MCC recurrences; 46.7% of nodal metastases; 100% of distant metastases	PCR modified from Feng et al. (2008) .
Paulson et al. (2009) USA	28	0	NA	PCR to detect MCV LT and sT	Presence of vDNA	59%	PCR based on Garneski et al. (2009)
Sastre-Garau et al. (2009) France	10	1241	Wide range of other human tumours	PCR and RT-PCR to detect MCV LT and sT	Presence of vDNA vDNA integration LT and sT mRNA	100% 100% 100%	Very high specificity
Shuda et al. (2009) USA, Spain, Israel	36	325 + 104	Tissue arrays available from commercial sources of haematological malignancies and subjects with HIV status known	IHC for LT, MCV DNA, and qPCR	Presence of vDNA Expression of LT protein in tumour cells Viral genome copies per cell	70% of 10 58.30% 5.2 copies/cell	2% of CLLs were DNA-positive; HIV-positive subjects had low MCV copy number per cell (range, $2.8-8.8 \times 10^{-3}$). None of the 6 CK20-negative MCC tumours had MCV DNA.

Table 2.3 (continued)

Reference, study location	Total no. of cases	Total no. of controls	Control source (hospital, population)	Detection method	Exposure categories	Results	Comments
Sihto et al. (2009) Finland	207	22	Randomly selected FFPE tissue samples from university archives, including other cancers and normal tissue	qPCR using the LT1, LT3, and VP1 primer pairs	Presence of vDNA LT1 LT3 VP1	79.80% 29.60% 49.50% 11.10%	PCR based on Feng et al. (2008) . Detailed information retrieved from Sihto et al. (2011)
Touzé et al. (2009) France	32	9	NETs of the lung, intestine, and cervix	Nested PCR with LT1 and VP1 primer pairs	Presence of vDNA in FFPE ($n = 20$) In fresh tissue ($n = 12$)	45% 100%	PCR based on Feng et al. (2008)
Nakamura et al. (2010) Japan	19	Not reported	Various normal tissues	PCR and RT-PCR, viral integration, IHC	Presence of vDNA Expression of LT in MCV DNA – positive cases	57.90% 70%	
Foulongne et al. (2010a) France	18	24	Other skin diseases ($n = 18$); healthy subjects ($n = 6$)	PCR and real-time PCR	Presence of vDNA in tumour samples In distant non-tumour samples	82% 71%	PCR based on Feng et al. (2008)
Werling et al. (2011) Germany	32	Not reported	Glabrous skin, human colon, heart tissues, and pancreatic carcinomas	Real-time PCR using primers for LT and VP1	Presence of vDNA	84%	PCR based on Becker et al. (2009)
Schrama et al. (2011) Australia, Germany	174			PCR for LT gene and viral copy	Presence of vDNA Viral copies/cell > 10	86.8% 20%	PCR based on Becker et al. (2009)
Sihto et al. (2011) Finland	93			qPCR	Presence of vDNA	96.7% MCV LT expression strongly associated with presence of MCV DNA in the tumour ($P < 0.0001$)	The cases had also been reported in Sihto et al. (2009)

Table 2.3 (continued)

Reference, study location	Total no. of cases	Total no. of controls	Control source (hospital, population)	Detection method	Exposure categories	Results	Comments
Touzé <i>et al.</i> (2011) France	68	82	Sera from 23 patients with skin cancer or other skin disease; 36 Italian blood donors; 23 patients with infectious diseases	qPCR/VLP-based ELISA	Antibody positivity GMT High antibody titres > 10 000	Cases:100% Controls: 85% Cases: 21701 Controls: 1608 Cases: 64.7% Controls 7.3%	PCR based on Laude <i>et al.</i> (2010) ; better progression-free survival was associated with high antibody titres (HR, 4.6; 95% CI: 1.7–12.2)

CI, confidence interval; CLL, chronic lymphocytic leukaemia; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; FFPE, formalin-fixed, paraffin-embedded; GMT, geometric mean titre; HR, hazard ratio; IHC, immunohistochemistry; LT, large T-antigen; MCC, Merkel cell carcinoma; MCV, Merkel cell polyomavirus; mRNA, messenger RNA; NA, not applicable; NETs, neuroendocrine tumours; PCR, polymerase chain reaction; qPCR, quantitative PCR; RT-PCR, reverse transcriptase PCR; sT, small T-antigen; vDNA, viral DNA; VLP, virus-like particle, VP1, capsid viral protein 1

(SLE). Whereas 100% of the MCV DNA-positive tumours had antibodies to MCV, only half of the MCV DNA-negative MCC tumours had detectable antibodies. Among controls, sero-response was detected in 64% of blood donors, in 63% of commercial donors, and in 74% of SLE patients, showing evidence for prior MCV exposure. [The estimated odds ratio using all cases and all controls was 1.84. A limitation of the study is the use of samples from a blood bank; the demographics of the control population are unknown, and there is no information on potential confounding factors.]

[Garneski *et al.* \(2009\)](#) and [Paulson *et al.* \(2009\)](#) are two overlapping reports from the USA studying MCV in 28 MCC specimens from 25 patients using PCR and FFPE tumours and fresh tissue. MCV was detected in 13 (59%) of 22 patients.

[Sastre-Garau *et al.* \(2009\)](#) investigated DNA, RNA, and viral integration from 10 cases of MCC and 1241 specimens of a wide range of human tumours from France. All 10 cases of MCC (100%) were MCV DNA-positive, whereas none of the 1241 specimens of other tumour types harboured MCV. Both sT and LT viral DNA sequences were found to be significantly expressed in all MCCs. MCV DNA was integrated in all the MCC cases in a single integration site in each case. [The authors interpreted this observation as indicative that integration takes place before clonal expansion.] Low copy numbers of viral genomes were present per cell, but there was always expression of both LT and sT genes. MCV LT transcripts were found to harbour mutations in the 3' part of the LT protein in the majority of cases. [This mutation is relevant as it may affect the replicative properties of the virus. The high specificity of the study is surprising in view of other case series.]

[Shuda *et al.* \(2009\)](#) explored MCV DNA in 36 MCC cases and in 325 haematological malignancies from the USA, Spain, and Israel. The study included an MCC tissue core microarray

for 36 cases and a tissue microarray for lymphoid malignancies available from commercial sources. Further additional haematolymphoid tissues were obtained from tissue banks. Samples were evaluated using the CM2B4 monoclonal antibody that recognizes endogenous and transfected MCV LT antigen. The study showed expression of MCV LT protein localized to nuclei of tumour cells in MCC cases. The average number of LT DNA sequence copies per cell was 5.2 (range, 0.8–14), and 21 (58.3%) of 36 cases were CM2B4-positive. The study also included peripheral blood mononuclear cells from 83 subjects undergoing genetic testing for Factor V Leiden deficiency and 21 HIV-positive subjects with no MCC. qPCR was used in these samples to detect and measure MCV LT or VP2 DNA sequences. Among all of these subjects, only 3 (14.3%) of the HIV-positive subjects were positive for MCV DNA at low copy number per cell (range, $2.8\text{--}8.8 \times 10^{-3}$ for either LT or VP2 sequences). [Results on haematological malignancies are shown in Section 2.3.2.]

[Sihto *et al.* \(2009\)](#) analysed FFPE MCC tissue samples from 114 of 207 patients diagnosed in Finland from the period 1979–2004 for the presence of MCV DNA. qPCR and DNA sequencing was performed following the methods and using the primers of [Feng *et al.* \(2008\)](#), with an additional probe. MCV DNA was present in 91 (79.8%) of the MCC cases.

[Touzé *et al.* \(2009\)](#) investigated the presence of MCV in 32 patients with MCC (20 FFPE and 12 fresh tissue samples) and 9 neuroendocrine tumours (NETs) from lung ($n = 5$), intestine ($n = 3$), and cervix ($n = 1$). MCV was detected by nested PCR using the LT1 and VP1 primers published by [Feng *et al.* \(2008\)](#); 20 (45%) of the FFPE MCC samples and all of the 12 fresh tissue samples were MCV-positive, and none of the NETs were MCV-positive.

[Wieland *et al.* \(2009\)](#) from Germany explored MCV DNA from a range of 355 skin lesions, including MCC, eyebrow hairs, anogenital and oral specimens, and blood samples, by means of

PCR as described by [Feng et al. \(2008\)](#) and by sequencing. MCV DNA was detected in 88% of 19 MCCs, in contrast to 16% of other skin tumours. MCV was also found in anogenital and oral samples (31%) and eyebrow hairs (50%) of HIV-positive men and in forehead swabs (62%) of healthy controls. Of 13 blood samples examined, 1 was positive for MCV DNA and corresponded to an MCC patient.

[Nakamura et al. \(2010\)](#) from Japan explored 19 MCC cases, all CK20-positive, for MCV by means of real-time PCR using the LT 77 bp primer (as in [Katano et al., 2009](#)); 11 cases (57.9%) were MCV DNA-positive. Immunohistochemical (IHC) analysis using a polyclonal antibody detected LT antigen in 7 (70%) of 10 MCV DNA-positive cases. Antibody staining was explored in several other organs, including 10 normal skin samples, with negative results.

[Foulongne et al. \(2010a\)](#) from France studied a series of patients with MCC ($n = 18$), patients with other skin diseases ($n = 18$), and healthy subjects ($n = 6$). Exposure to MCV was evaluated through detection of MCV DNA using the methods of [Feng et al. \(2008\)](#), real-time PCR, and sequencing of LT gene. MCV DNA was identified in 78% of MCC cases, in 28% of skin lesions ($P = 0.007$), and in 17% of healthy skin samples. Sequencing analysis did not identify signature mutations.

[Werling et al. \(2011\)](#) conducted a study using paraffin-embedded samples of MCCs ($n = 52$) that had been removed surgically from 32 different patients (26 primary MCC tumours, 9 recurrences, 15 lymph node metastases, 1 metastasis of the thoracic wall, 1 parotid gland metastasis) between 1995 and 2008 at the Department of Pathology of the University Medical Centre Mannheim, Germany. Samples of human glabrous skin, colon, heart tissues, and pancreatic carcinomas to be used as positive and negative controls were obtained from the University Medical Centre Mannheim and the University Hospital Heidelberg. Real-time PCR was used

to detect MCV LT and VP1 genes, following [Becker et al. \(2009\)](#). Among all cases, 84% were MCV-positive, with fully concordant results for all samples derived from the same patient.

[Schrama et al. \(2011\)](#) reported on 174 FFPE samples of MCC cases from Australia and Germany using PCR and sequencing of LT gene as described by [Becker et al. \(2009\)](#). A total of 116 (85.3%) of 136 European MCC cases and 33 (86.8%) of 38 Australian cases were both MCV-positive and MCV LT-positive. A substantial number of cases (30/149) had > 10 copies of viral genome per cell. Samples derived from the same patient were concordant in relation to MCV status. MCV-positive cases were significantly more likely to be females, with tumours located on the extremities, and less likely to report a previous history of skin cancer.

[Sihto et al. \(2011\)](#) from Finland identified 207 incident MCC cases in a population-based nationwide cohort from the period 1979–2004. Of those, 91 MCC cases with histologically confirmed MCC and with clinical information and representative tumour tissue available were included in the study. MCV DNA was detected using PCR, qPCR, and DNA sequencing. MCV LT expression was strongly associated with the presence of MCV DNA in the tumour ($P < 0.0001$).

[Touzé et al. \(2011\)](#) from France investigated MCV DNA, VP1 protein expression in tumour cells, and antibodies to MCV using ELISA in 68 MCC patients and compared them with 82 controls (patients with skin lesions, including cancer; patients with infectious diseases; and blood donors). High antibody titres $> 10\,000$ were observed to be significantly more common in cases compared with controls. The GMT was also 14 times higher in cases than controls. VP1 expression was not observed in MCV DNA-positive tumours or in surrounding normal skin, or in MCV DNA-negative cases.

2.3.2 Chronic lymphocytic leukaemia and other lymphomas

CLL is the most common leukaemia in developed countries and is characterized by the accumulation of monoclonal mature B-cells aberrantly expressing CD5. Several reports have investigated the potential role of MCV in its etiology, mainly based on the increased incidence of CLL among MCC patients observed in some reports.

Several case reports are available, with a wide range of positivity from 0% to 33%. We describe the largest cases series.

[Shuda et al. \(2009\)](#) explored MCV DNA in 36 MCC cases and in 325 haematological malignancies from the USA, Spain, and Israel. [This study is also described in Section 2.3.1.] The study included haematolymphoid tissues obtained from tissue banks. Among the 325 haematological malignancies, 2.2% showed evidence for MCV DNA by PCR. None were positive at high viral copy numbers, and none of 173 lymphoid malignancies examined on tissue microarrays expressed MCV LT protein. Only 1 of 33 CLL cases was MCV-positive. All of the 11 post-transplant patients with a lymphoproliferative disorder were negative for MCV LT protein in tumour cells.

[Toracchio et al. \(2010\)](#) from the USA and Canada analysed 353 specimens, including 152 non-Hodgkin lymphomas (NHL), 44 Hodgkin lymphomas, 110 benign lymph nodes, 27 lymph nodes from patients with metastasis, and 20 extranodal tissue samples. MCV DNA was detected by qPCR in 13 (6.6%) of 196 lymphomas, including 5 (20.8%) of 24 CLL specimens, and in 11 (10%) of 110 benign lymph nodes, including 8 (13.1%) of 61 samples of reactive hyperplasia and 3 (10.3%) of 29 normal lymph nodes. Other samples were MCV-negative. Sequence analysis of 9 virus-positive samples confirmed the presence of MCV; three viral strains were represented. IHC testing showed that among 17 lymphomas,

7 of which were positive for MCV DNA, only 1 T-cell lymphoma expressed MCV T-antigen.

[Pantulu et al. \(2010\)](#) from Germany studied 70 CLL cases and investigated the presence of MCV DNA in blood. PCR to detect MCV was performed using primers for LT3, M1/M2, and VP1. PCR products were sequenced. MCV DNA was detected in 19 patients (27.1%) and in only 13.4% of the healthy blood donors. Mutations (246 bp LT antigen deletion) were observed in 6 of 19 MCV-positive cases. [No information was provided for the control group apart from noting that they were healthy blood donors and were younger compared with the CLL patients.]

[Andres et al. \(2010a\)](#) from Germany explored a series of 19 subjects with cutaneous lymphomas, 12 cases of pseudolymphomas, and 23 subjects with inflammatory skin disease. MCV was evaluated through PCR, followed by Southern blotting. MCV was detected in 17% of the lymphoma cases, in 15.4% of the pseudolymphoma cases, and in 8% of the inflammatory skin disease cases.

[Tolstov et al. \(2010\)](#) evaluated 18 CLL and 18 acute lymphoblastic leukaemia (ALL) patients for antibody response to MCV and showed no statistically significant differences in seroresponse between CLL and ALL patients (median values, 0.645 vs 0.521 OD units, respectively).

[Teman et al. \(2011\)](#) from the USA examined 18 consecutive CLL cases and 17 low-grade follicular lymphomas as a control group from the period 2002–10. FFPE samples were used to look for MCV DNA using PCR and real-time PCR. When PCR was used, all subjects tested negative, but when real-time PCR was used, 33% of CLL cases showed a low level of MCV amplification. All control subjects were negative for MCV.

2.3.3 Merkel cell carcinoma and other cancers

There exist in the literature many case reports of concomitant MCC cases and CLL. Pooling the available data, there are about 50 patients with

MCC and CLL; in 12 cases MCC was the primary tumour [for a review of case reports, see [Tadmor et al., 2011](#)].

[Tolstov et al. \(2010\)](#) reported on 2 cases with concurrent CLL and MCC from the USA. The IHC evaluation of tumour cells with CM2B4 and CD20 showed MCV LT protein expression in metastatic MCC cells but not in the CD20-positive CLL cells.

Several large studies had observed a stronger than expected association between MCC and other cancers using record linkage data. [Howard et al. \(2006\)](#) from the USA explored incident primary cancers in 1306 patients with MCC from 11 population-based cancer registries from the Surveillance, Epidemiology and End Results (SEER) programme. Patients with first primary MCC were at significantly increased risk of developing a subsequent cancer (standardized incidence ratio [SIR], 1.22; 95% CI, 1.01–1.45; $n = 122$), with significant excesses restricted to the first year after diagnosis (SIR, 1.71; 95% CI, 1.21–2.33; $n = 39$). Significantly elevated site-specific risks were observed for salivary gland cancers (SIR, 11.55; 95% CI, 2.32–33.76; $n = 3$), biliary sites other than liver and gallbladder (SIR, 7.24; 95% CI, 1.46–21.16; $n = 3$), and NHL (SIR, 2.56; 95% CI, 1.23–4.71; $n = 10$). Non-significantly increased risks of 2-fold or higher were seen for CLL and cancers of the small intestine and brain. A SIR of 1.36 (95% CI, 1.19–1.55; $n = 221$) for MCC as a second primary malignancy was observed among patients with all other first primary cancers taken together. In particular, significant 3–7-fold excesses of MCC followed CLL (SIR, 6.89; 95% CI, 3.77–11.57; $n = 14$), multiple myeloma (SIR, 3.70; 95% CI, 1.01–9.47; $n = 4$), NHL (SIR, 3.37; 95% CI, 1.93–5.47; $n = 16$), and malignant melanoma (SIR, 3.05; 95% CI, 1.74–4.95; $n = 16$). [The lack of specificity of the associations including several distinctive haematological malignancies argues against, but does not exclude, that MCV could play an etiological role.]

[Koljonen et al. \(2009, 2010\)](#) identified in a retrospective study 4164 CLL patients and 172 MCC cases based on the Finnish Cancer Registry from 1979 to 2006. In [Koljonen et al. \(2009\)](#), 6 cases had both CLL and MCC and were selected for MCV analysis. FFPE tissue was available in 5 cases, in which morphological and IHC diagnosis was confirmed for MCC. MCV DNA was tested by means of real-time PCR (LT3 region; [Sihto et al., 2009](#)). In the 5 cases (100%) MCV DNA was identified, with a median copy number of 5.49 relative to the control gene (*PTPRG*) DNA copy number. [A limitation of this study was that CLL samples were not tested, and therefore this study was not informative.]

Of the 172 MCC patients identified by [Koljonen et al. \(2010\)](#), a total of 34 second primary cancers were detected in 30 individuals after the diagnosis of MCC. Female MCC patients were diagnosed with 25 subsequent cancers (SIR, 2.35; 95% CI, 1.52–3.47; $P < 0.001$) and male patients with 9 cancers (SIR, 2.32, 95% CI, 1.06–4.40; $P < 0.05$). The MCC patients had an increased risk for a subsequent cancer (any site) compared with age-, gender- and calendar period-matched members of the general population (SIR, 2.34; 95% CI, 1.62–3.27). The risks for basal cell carcinoma (BCC) of the skin ($n = 11$; SIR, 3.48; 95% CI, 1.74–6.22) and CLL ($n = 2$; SIR, 17.9; 95% CI, 2.16–64.6) were significantly elevated.

[Kaae et al. \(2010\)](#) used data from the Danish national health and population registers on MCC diagnoses, deaths, and population counts during the study period (1978–2006) to calculate MCC incidence rates, cumulative risks of MCC at age 100 years, and MCC mortality rates by tumour stage. A total of 185 persons were diagnosed with MCC. MCC incidence between 1995 and 2006 was 2.2 cases per million person-years. MCC incidence was statistically significantly increased > 1 year after a diagnosis of SCC of the skin (SIR, 14.6; 95% CI, 8.4–25.6), BCC (SIR, 4.3; 95% CI, 2.7–6.6), malignant melanoma (SIR, 3.3; 95% CI, 1.1–10.3), Hodgkin lymphoma (SIR, 17.6; 95%

CI, 2.5–126), CLL (SIR, 12.0; 95% CI, 3.8–37.8), and NHL (SIR, 5.6; 95% CI, 1.4–22.4). [The Working Group considered that these high SIRs could be affected by an increased surveillance of cancer patients.]

[Bzhalava et al. \(2011\)](#) investigated the risk of secondary cancers after the diagnosis of MCC using the national cancer registries in Denmark, Norway, and Sweden. The overall cancer incidence was increased among patients diagnosed with MCC compared with the general population in these countries (79 secondary cancers total, SIR, 1.38; 95% CI, 1.10–1.72; 49 secondary cancer in females, SIR, 1.7; 95% CI, 1.29–2.25; 30 secondary cancers in males, SIR, 1.05; 95% CI, 0.73–1.5). There were significantly increased incidence ratios for non-melanoma skin cancers (NMSCs) (34 secondary cancers, SIR 8.35; 95% CI, 5.97–11.68), melanoma of skin (6 secondary cancers, SIR, 4.29; 95% CI, 1.93–9.56), and laryngeal cancer (2 secondary cancers, SIR, 9.51; 95% CI, 2.38–38). The SIRs for these three cancer sites were also elevated on restricting the follow-up to cancers occurring ≥ 1 year after MCC diagnosis. [The Working Group considered that these high SIRs could be affected by an increased surveillance of cancer patients.]

2.3.4 Other cancers of the skin

Several reports based on collection of skin cancers other than MCC have been published exploring the presence of MCV in tumour lesions or skin lesions. The range of MCV DNA detection varied across the series, with a range from 0% to 19.2% for both SCC and BCC.

[Wieland et al. \(2009\)](#) from Germany explored MCV DNA from a range of 355 skin lesions, including MCC, eyebrow hairs, anogenital and oral specimens, and blood samples, by means of PCR as described by [Feng et al. \(2008\)](#) and by sequencing [see Section 2.3.1]. MCV DNA was detected in 16% of skin tumours other than MCC.

[Kassem et al. \(2010\)](#) explored the presence of MCV in Germany with PCR using the LT3 and VP1 primer pairs as described by [Feng et al. \(2008\)](#). Included were 26 BCC samples from 6 patients with naevoid basal cell carcinoma syndrome (NBCCS) and 42 trichoblastoma samples from 41 patients. Of 26 BCC samples, 5 (19.2%) were MCV-positive with LT3 primers, 1 of them was also positive using VP1 primers. No PCR amplification was obtained, using any of the two MCV primer pairs LT3 and VP1, for the 3 BCC samples from 1 of the 6 NBCCS patients. Of 42 trichoblastoma samples, 12 samples (from 11 subjects) were positive using MCV LT3. None of them was positive for MCV VP1 DNA. MCV viral loads in BCC and in trichoblastoma samples were 2–10-fold lower compared with MCC positive controls.

[Reisinger et al. \(2010\)](#) from the USA evaluated the expression of MCV LT protein through detection with CM2B4 in 20 patients with MCC with and without secondary SCC or BCC tumours. MCV LT was detected in 15 (75%) of 20 MCC tumours, including 11 MCC tumours from patients with secondary SCC or BCC. In contrast to MCC, none of these secondary BCC or SCC was positive for MCV LT.

2.3.5 Other cancer sites

A few studies have explored the presence of MCV in a variety of cancer sites. MCV was not detected in melanoma ([Giraud et al., 2008](#); [Koburger et al., 2011](#)), in colon cancer ([Militello et al., 2009](#); [Campello et al., 2011](#)), in mesothelioma ([Bhatia et al., 2010a](#)), in prostate cancer ([Bluemn et al., 2009](#)), in lung cancer ([Andres et al., 2009a](#); [Joh et al., 2010](#); [Stebbing et al., 2010](#)), in central nervous system tumours ([Giraud et al., 2009](#)), in NETs ([Duncavage et al., 2009b](#); [Chernock et al., 2011](#)), and in fibroxanthoma ([Andres et al., 2010c](#)).

2.4 Susceptible populations

Few studies are available exploring specifically the association between MCV and human cancer in immunosuppressed populations. AIDS patients had a 13-fold increased age- and sex-adjusted risk of developing MCC compared with the general population ([Engels et al., 2002](#)). Likewise, there was a 16-fold over-representation of MCC in chronically immunosuppressed patients ([Heath et al., 2008](#)). [Ridd et al. \(2009\)](#) examined the presence of polyomavirus DNA in 156 NMSCs among organ transplant recipients from the USA, which included 85 SCCs, 37 keratoacanthomas, 28 Bowen disease (BD), and 6 actinic keratoses. MCV was detected using the approach of [Feng et al. \(2008\)](#). Polyomavirus sequences were detected in only 1 case of actinic keratoses (2.7%), with fainter bands compared with those seen for a control series of MCC (7 of 13 MCC cases were positive).

[Kassem et al. \(2009\)](#) from Germany explored the presence of MCV in a group of 56 NMSCs from 11 immunosuppressed patients, and 147 NMSC lesions from 125 immunocompetent patients were tested for MCV by PCR, targeting the LT and VP1 DNA sequences. NMSC included SCC, BCC, and BD. In addition, normal skin and 89 colorectal cancers were tested. MCV-specific sequences were found significantly more frequently in NMSC of immunosuppressed patients compared with immunocompetent patients (positivity to LT3 or VP1, 62.5% vs 32%, respectively, $P < 0.001$). In particular, BD and BCC revealed a significant increased association with MCV in immunosuppressed patients (positivity to LT3 or VP1, 69% in BD and 72.3% in BCC in immunosuppressed patients and 17.4% in BD and 37.5% in BCC in immunocompetent patients; all comparisons were statistically significant with $P < 0.01$). No MCV was detected within normal skin, and only 3 of 89 additionally tested colorectal cancers were MCV-positive.

3. Cancer in Experimental Animals

No data were available to the Working Group.

4. Mechanistic and Other Relevant Data

4.1 Transforming capacity of MCV

Only limited experimental data on the mechanisms related to a direct transforming capacity of MCV are available. However, current findings suggest unique features for this virus, which could distinguish it from other known human polyomaviruses. For example, expression of the sT, but not the LT, induces transformation of rodent fibroblasts and serum-independent growth of human fibroblasts, independent of PP2A and Hsc70 binding ([Shuda et al., 2011](#)).

4.2 Relevant biological properties of MCV-encoded viral proteins

The MCV genome encompasses a non-coding region and two major transcription units with early and late genes. The early region encodes LT, sT, and a 57kT protein, all of which represent alternatively spliced variants that share a 78 amino acid N-terminal ([Shuda et al., 2008](#)). The MCV T-antigens have several functions in common with the simian virus 40 (SV40)-related viruses. The shared leader sequence of sT and LT encompasses a functional DnaJ domain that binds Hsp70 and is conserved among all MCV T-antigen isoforms ([Kwun et al., 2009](#)). The late region, encoding the viral structural proteins VP1, VP2, and VP3, is expressed after the onset of viral DNA replication. In addition, MCV encodes a microRNA that is expressed from the late region and is oriented antisense to the early transcripts ([Seo et al., 2009](#); [Lee et al., 2011](#)).

The MCV LT antigen is a nuclear protein with a predicted mass of 92 kDa (Shuda *et al.*, 2008, 2009; Nakamura *et al.*, 2010). LT retains most of the features of the SV40 LT protein, including a DnaJ domain, pRb interaction domain, origin-binding domain (OBD), and helicase/ATPase domain (Feng *et al.*, 2008; Shuda *et al.*, 2008). No evidence for a direct interaction between MCV LT and p53 has thus far been found. Thus, like SV40 LT, MCV LT has dual capacities, i.e. to target at least one major class of tumour suppressor protein and to initiate viral DNA replication.

MCV LT exon 2 encodes pRb binding (Shuda *et al.*, 2008) and viral origin replication (Kwun *et al.*, 2009) functions. pRb binding is mediated by a classic LXCXE (LFCDE) motif common to tumorigenic adenoviruses, polyomaviruses, and papillomaviruses (Chemes *et al.*, 2011). It is unknown which of the proteins of the pRb family (pRb, p107, p130) are the principal targets for the virus in infected cells, but preliminary evidence suggests that MCV LT binds all of these proteins (Shuda *et al.*, 2008). As with SV40 LT binding to pRb, MCV LT binding to pRb may inhibit E2F repression and activate transcription of E2F-responsive genes active at the G1/S cell-cycle transition. It is unknown whether the DnaJ domain of LT participates in this inhibition, as has been seen with SV40 LT (Ahuja *et al.*, 2005).

The 5' portion of LT exon 2 encodes a 200 amino acid domain, termed the Merkel unique region (MUR) due to its relative lack of homology with the LT proteins of other polyomaviruses (Liu *et al.*, 2011). The MUR extends to the LXCXE motif and contains a binding site for the cytoplasmic vacuolar sorting protein Vam6p, which is then translocated to the nucleus with LT (Liu *et al.*, 2011). While the function of this interaction remains obscure, MCV LT antagonizes lysosomal clustering induced by Vam6p overexpression (Liu *et al.*, 2011) and, surprisingly, mutation of the interaction site enhances the replication of MCV genomic DNA in otherwise

poorly MCV-permissive cultured cell lines (Feng *et al.*, 2011).

Intact LT is also required for MCV genome replication and is found in replicating virus from asymptomatic skin infections (Schowalter *et al.*, 2010). The OBD has been crystallized partially bound to MCV origin DNA sequences (Harrison *et al.*, 2011), revealing similar features to those of SV40 LT. Replication can be initiated *in vitro* with a ≥ 71 bp central region of the origin (Kwun *et al.*, 2009).

sT antigen is expressed from an unspliced open reading frame that encompasses LT exon 1 and most of the LT intron. The sT open reading frame encodes a protein with a predicted size of 22 kDa. Unlike SV40, MCV sT is sufficient to induce serum-independent growth of immortalized human cells and to fully transform immortalized rodent fibroblasts (NIH3T3 and Rat-1) into independently growing cancer cell lines; this is not enhanced by co-expression of either wild-type (WT) or tumour-derived forms of MCV LT. MCV sT antigen binds to the serine/threonine protein phosphatase 2 (PP2A) A and C subunits. However, this does not appear to be critical for *in vitro* transformation of rodent cells and for serum-independent growth of human cells induced by MCV sT antigen. Mutations in the MCV sT PP2A interaction domain abolish its ability to enhance LT-mediated origin replication, yet do not have major effects on MCV sT as an oncoprotein, suggesting that PP2A targeting is not the primary function of the protein during cell transformation (Shuda *et al.*, 2011). Instead, MCV sT activates cap-dependent translation by maintaining hyperphosphorylation of the 4E-BP1 regulatory protein in a PP2A-independent manner (Shuda *et al.*, 2011). 4E-BP1 is a downstream target of the Akt-mTOR signalling pathway. When 4E-BP1 is hyperphosphorylated, it releases the protein translation initiation factor eIF4E, which then binds to 7-methylguanosine-capped mRNA, thereby recruiting the ribosomal machinery required for

translation. Accordingly, enhancement of cell transformation may result from dysregulated cap-dependent translation promoted via hyperphosphorylation of 4E-BP1 by sT ([Dowling et al., 2010](#); [Shuda et al., 2011](#)).

The MCV 57kT transcript is similar to LT but has a second intronic splice that deletes portions of the OBD and the helicase function. 57kT currently has no known biological activity. Under *in vitro* replication conditions, the LT cDNA alone is not sufficient for maximum efficiency of virus replication, but co-expression of LT with sT promotes replication of the viral genome with an efficiency that is comparable to that of the WT virus. Co-expression of LT with 57kT does not increase replication over that seen with LT alone, and neither MCV sT nor 57kT individually have replication capacity ([Kwun et al., 2009](#); [Feng et al., 2011](#)). Knockdown of all T-antigen transcripts or the sT transcript by small interfering RNA (siRNA) results in inhibition of origin replication, confirming the functional importance of sT expression to MCV origin replication ([Kwun et al., 2009](#)).

4.3 *In vivo* and *in vitro* evidence for a role of MCV in human malignancies

4.3.1 *Effects of ectopically expressed MCV genes on cell growth in vitro*

WT LT protein does not initiate transformation of immortalized rodent fibroblasts (NIH3T3 and Rat-1), as measured by colony formation in soft agar, focus formation, or cell growth in low-serum conditions ([Shuda et al., 2011](#)). Still, tumour-derived truncated MCV LT proteins induce tenuous multicellular aggregates in soft agar that do not develop into full colonies, suggesting that truncated LT proteins do not transform cells but have an increased cell proliferation capacity compared with the WT

protein ([Shuda et al., 2011](#)). It remains to be seen whether this is due to increased protein stability or acquisition of particular features. However, these findings do not imply that MCV LT does not play a role in MCV-induced tumorigenesis. Nearly all tumour mutations found so far preserve the pRb-binding domain, i.e. a function that may contribute to the development of MCC. Consistent with this hypothesis, survival of tumour cells from established MCC-derived cell lines was found to depend on a functional pRb interaction domain ([Houben et al., 2010a](#)).

Unlike MCV LT, sT acts as a transforming oncoprotein that is sufficient to fully transform rodent fibroblasts (NIH3T3 and Rat-1) into independently growing cancer cell lines characterized by loss of contact inhibition, anchorage independence, and serum-independent growth ([Shuda et al., 2011](#)). sT also accelerates the proliferation of human immortalized fibroblasts. This characteristic appears not to be shared with the SV40 sT antigen. Cell transformation by sT is not enhanced by co-expression of either WT or tumour-derived forms of MCV LT. MCV sT-induced transformation is not dependent on its interaction with PP2A or with heat shock protein, and mutated sT recombinant proteins that fail to bind PP2A or Hsc70 still retain the capacity to transform rodent cells as efficiently as does the WT protein. However, expression of a constitutively active 4E-BP1 protein, which cannot be phosphorylated, antagonizes sT-induced Rat-1 cell transformation, implying that sT maintenance of hyperphosphorylated 4E-BP1 is required for sT antigen-mediated cell transformation. In addition, MCV sT increases the steady-state phosphorylation of other mTOR targets, including pp70 S6K and 4E-BP2, but the significance of these functions is unknown ([Shuda et al., 2011](#)).

4.3.2 MCV in Merkel cell carcinoma

(a) MCV genome copies

The detection of MCV genome by PCR in most MCC tumours reported in several case series suggests a close association between MCV and this rare cancer ([Table 4.1](#); see also Section 2.3.1). A review of > 1000 tumours from MCC patients that were reported in several studies conducted through May 2011 revealed that about 75% of tumours are positive for MCV DNA (see [Table 4.1](#)).

In the studies for which quantitative analysis has been done, > 90% of the MCV-positive MCCs bear > 0.6 genome copies per cell. [Shuda et al. \(2009\)](#) first reported an average of at least 1 copy (range, 0.8–14.3 copies) of the MCV genome per tumour cell in the 8 MCV-positive MCCs analysed ([Shuda et al., 2009](#)). Other groups reported numbers of genome copies varying between 3×10^{-4} and 4×10^3 per MCC tumour cell, and between 0.02 and 10 for MCC cell lines ([Katano et al., 2009](#); [Koljonen et al., 2009](#); [Sastre-Garau et al., 2009](#); [Sihto et al., 2009](#); [Bhatia et al., 2010b](#); [Fischer et al., 2010](#); [Foulongne et al., 2010a](#); [Foulongne et al., 2010b](#); [Houben et al., 2010a](#); [Laude et al., 2010](#); [Waltari et al., 2011](#)).

Infection with MCV is common in the general population, and most adults shed MCV from the surface of their skin (see Section 1). This is why MCV is also found in non-MCC skin biopsies and in skin swabs, but usually in much smaller copy numbers. Despite extensive variation in virus abundance, which could be due to the detection method used, to differences in tissue quality and the proportion of tumour versus non-tumour cells, and to other technical differences between studies, non-MCC tissues almost always contain fewer genome copies per cell than do MCC samples in the same study. MCV DNA has been detected in cutaneous swabs from the face, trunk, and upper and lower limbs. MCV DNA levels were higher in swabs obtained from patients with MCC (median, 861 copies/ng DNA,

corresponding to 5 copies/cell) than in those from patients with other skin diseases (median, 45 copies/ng DNA, corresponding to 0.27 copies/cell; $P < 0.001$) or from clinically healthy volunteers (median, 43 copies/ng DNA, corresponding to 0.26 copies/cell; $P < 0.001$) ([Foulongne et al., 2010b](#)). In another study, MCV DNA was found in 14 (40%) of 34 swabs drawn across the skin of the forehead ([Schowalter et al., 2010](#)). However, certain non-MCC skin samples were reported to have > 1 copy of MCV per cell ([Faust et al., 2011](#)), and skin-swab specimens from some subjects were found to contain thousands of copies of MCV per copy of β -globin ([Pastrana et al., 2012](#)). A possible explanation for this discrepancy is that MCV is produced in higher levels in the outermost layers of the skin, while wiping of the skin with ethanol or iodine solution, for example when skin punch biopsies are taken, may remove potential viral DNA from the epidermis. Thus, cutaneous swabbing may produce a more thorough sample for testing of skin viruses ([Forslund et al., 2004](#)).

MCV DNA was also detected in low copy numbers in several other human tissues. The relative viral copy numbers in different tissues were estimated by arbitrarily assuming that MCC had an average of 10 MCV genome copies per cell (range, 0.05–173). Based on this assumption, saliva had an average of 0.13 genome copies per cell (range, 0.01–5); oral cavity, liver, and skin samples had an average of 0.026, 0.015, and 0.007 copies, respectively; and lung, kidney, bladder, and prostate cancer had < 0.001 genome copies per cell ([Loyo et al., 2010](#)). The copy numbers in lymphoid cells from different groups of patients without MCC were found to range between 0.001 and 0.01 genome copies per cell ([Shuda et al., 2009](#); [Pancaldi et al., 2011](#)).

(b) Antibody response to MCV

Human seroresponsiveness against MCV LT and sT was specifically associated with MCC. LT- or sT-specific antibodies were present in only

Table 4.1 Detection of MCV DNA in biopsies from MCC patients

Total patients (%)	Male patients (%)	Female patients (%)	Study location	Reference
8/10 (80%)			Pennsylvania, USA	Feng et al. (2008)
8/9 (88.9%)			France	Foulongne et al. (2008)
30/39 (77%)	13/20 (65%)	17/19 (89%)	Germany	Kassem et al. (2008)
21/31 (68%)	8/13 (61.5%)	13/18 (72%)	Germany	Andres et al. (2009b)
45/53 (84.9%)			Germany	Becker et al. (2009)
15/17 (88%)	12/12 (100%)	3/5 (60%)	New York, USA	Busam et al. (2009)
22/29 (76%)			Missouri, USA	Duncavage et al. (2009a)
16/37 (43%)			North America, Australia	Garneski et al. (2009)
90/98 (92%)			Germany	Helmbold et al. (2009)
11/14 (78.6%)	4/5 (80%)	7/9 (77.7%)	Japan	Nakajima et al. (2009)
10/10 (100%)			France	Sastre-Garau et al. (2009)
91/114 (79.8%)	67/80 (83.7%)	24/34 (70.5%)	Finland	Sihto et al. (2009)
21/32 (65.6%)			France	Touzé et al. (2009)
7/8 (87.5%)			Hungary	Varga et al. (2009)
30/34 (88%)			Germany	Wieland et al. (2009)
21/33 (64%)	8/14 (57%)	14/19 (73.6%)	Germany	Andres et al. (2010b)
60/70 (86%)			Italy	Asioli et al. (2011)
17/23 (74%)	13/17 (76%)	4/6 (75%)	Ohio, USA	Bhatia et al. (2010b)
14/18 (78%)			France	Foulongne et al. (2010a)
34/59 (57.6%)	15/28 (53.5%)	19/31 (61.3%)	Germany	Handsichel et al. (2010)
31/33 (94%)			France	Laude et al. (2010)
20/30 (66.7%)			Switzerland	Mangana et al. (2010)
67/87 (77%)			Finland	Waltari et al. (2011)
20/26 (77%)			Japan	Kuwamoto et al. (2011)
19/104 (18.3%)	9/58 (15.5%)	10/46 (21.7%)	Australia	Paik et al. (2011)
149/174 (85.6%)			Australia, Germany	Schrama et al. (2011)
Total				
877/1192 (73.6%)	149/247 (60.3%)	11/187 (59.3%)		

Compiled by the Working Group from case series conducted through May 2011.
MCC, Merkel cell carcinoma; MCV, Merkel cell polyomavirus

0.9% of 530 control serum samples tested, and in 40.5% of 205 samples from MCC cases ([Paulson et al., 2010](#)). In addition, the GMT of 2900 (95% CI, 1500–5800) for antibodies recognizing LT in the 56 initial MCC patients was > 10-fold greater than that in the 36 matched controls: 200 (95% CI, 100–300). The difference in sT reactivity was even greater: GMTs of 2100 (95% CI, 800–1500) for MCC cases versus 5 (95% CI, 1–30) for controls. Furthermore, these authors found that titres of LT and sT antibodies dropped rapidly (about 8-fold per year) in 8 of 10 treated patients whose cancer did not progress, whereas they increased in 6 of 6 cases with progressive disease. The results suggest that ongoing expression of T-antigens within the MCC tumour is sometimes reflected by humoral responses. A similar trend to high antibody titres, albeit with VP1 VLPs as an antigen, was reported by [Touzé et al. \(2011\)](#), who detected antibodies against MCV in all patients with MCC and in 85% of controls. High antibody titres were rarely observed in controls (7.3%), but they were detected in 64.7% of patients with MCC.

Although a few exceptions have been identified, a strong positive correlation between the overall viral load and the average serological titre has been reported ([Faust et al., 2011](#); [Pastrana et al., 2012](#)). Given the correlation between MCV DNA loads and antibody titre, together with the higher titres of MCV-specific antibodies in MCC patients, it is likely that MCC patients have increased MCV viral load.

(c) *Viral gene expression in Merkel cell carcinoma*

If MCV is a causal agent of MCC, it is likely that MCC cells will express MCV proteins to initiate and maintain the tumorigenic phenotype. Yet a transient expression of potentially oncogenic viral proteins could act as a carcinogen that promotes the accumulation of genetic lesions and the formation of cancer. In this scenario, MCV may only be necessary for

tumour initiation, whereas a gradual loss of MCV may take place during tumour progression and perhaps explain the MCCs with low viral load and even with no MCV. This situation has been recently supported by T-antigen knockdown experiments that showed a lack of T-antigen dependence of an MCV-infected MCC cell line (LoKe) for cell growth ([Houben et al., 2012b](#)). However, this finding has been reported in one cell line, while incomplete knockdown of T-antigens may account for the sustained growth of these cells under the given experimental conditions. Finally, MCV-negative tumours may represent cells that have never been infected by the virus and therefore may have a distinct nature.

Transcripts corresponding to LT, sT, and 57kT mRNAs have been detected by Northern blot hybridization and mapped from four MCC tumours ([Shuda et al., 2008](#)).

LT protein expression in MCC was demonstrated using a monoclonal antibody (CM2B4) to a conserved peptide epitope in exon 2 of LT that can detect a truncated LT in protein extracts from MKL-1 cells, which is consistent with premature termination ([Shuda et al., 2009](#)). As expected, a diffuse nuclear and granular localization was evident from staining cells that ectopically express LT with this antibody. A similar pattern of strong reactivity among most tumour cells, but not in surrounding tissue, was evident from analysis by IHC of MCV DNA-positive MCC biopsies. Of 30 MCCs, 21 (70%) were positive for LT protein, and this finding was concordant with DNA detection. Nevertheless, one MCV DNA-positive MCC scored negative for LT expression ([Shuda et al., 2009](#)). A similar trend of positive staining in the majority of MCV PCR-positive MCCs was reported subsequently ([Busam et al., 2009](#), 37/51, 72.5%; [Reisinger et al., 2010](#), 15/20, 75%; [Wieland et al., 2012](#), 3/3, 100%; [Erovic et al., 2013](#), 29/30, 97%). CM2B4 did not show immunoreactivity with other skin tumours (see Section 4.3.3). Similar results were obtained

with polyclonal rabbit antibody against MCV LT, which detected 8 of 10 MCV PCR-positive MCC cases by IHC analysis but did not produce any signal in various organs, including lymph nodes, liver, spleen, brain, salivary glands, lung, pancreas, muscle, heart, and uterus ([Nakamura et al., 2010](#)).

In addition to LT, sT protein expression is detected in MCV-infected cell lines and in MCCs ([Shuda et al., 2011](#)). MCV sT is expressed more commonly in MCCs than is MCV LT ([Shuda et al., 2011](#)). Increased sensitivity of PCR-based DNA detection compared with IHC detection of a protein could account for the discrepancy of MCV detection obtained for certain MCCs. Yet some MCV DNA-positive MCCs lack LT expression but express robust sT, thus suggesting that in certain cases MCV sT plays a critical tumorigenic role ([Shuda et al., 2011](#)).

By using IHC staining, Pastrana and co-workers failed to detect expression of the VP1 capsid protein in each of 10 MCV LT-positive MCCs, suggesting that the virus does not replicate in MCCs ([Pastrana et al., 2009](#)).

Low-level expression of MCV microRNA has been reported in 19 (50%) of 38 MCV-positive MCCs. The amount of viral DNA positivity correlates with copies of MCV microRNA. This virus-encoded microRNA is predicted to target LT but also to target genes that may play a role in promoting immune evasion and regulating viral DNA replication ([Lee et al., 2011](#)).

(d) *Mutations in MCV LT and other viral genes*

MCV is clonally integrated in the tumour cell DNA, while mutations at the C-terminal end of the LT antigen that abrogate its OBD and helicase activity have been documented ([Feng et al., 2008](#)). Therefore, the heterogeneity of the viral genome present in tumours could result in loss of function but may also generate new distinctive features.

Nearly all MCV genomes analysed so far from tumours are truncated either through

point mutations or via integration into the human chromosome ([Feng et al., 2008](#); [Shuda et al., 2008](#); [Sastre-Garau et al., 2009](#); [Laude et al., 2010](#)). These tumour-specific mutations are C-terminal to the LXCXE motif and do not affect pRb binding but disrupt the replicative functions (OBD or helicase). The sequence and expression of the sT protein are unaffected by these mutations as well. The tumour-specific LT mutations prevent virus DNA replication and are incompatible with a transmissible agent. This presumably has the effect of preventing DNA replication from integrated viral origins that would generate onion-skinning and DNA fragmentation. Due to truncating mutations, the molecular size of the MCV LT protein varies among cell lines and tumours.

Mutations or deletions were identified that prematurely truncate exon 2 encoding the LT helicase in all sequenced LT genes from 9 MCV-positive MCCs ([Shuda et al., 2008](#)). In line with this, most LT mutations reported in another study ([Laude et al., 2010](#)) were located downstream of the pRB-binding domain, while LT exon 2 interruption resulting from genomic integration has also been found. Mutations with a stop codon were detected downstream of the LT nuclear localization signal (NLS), within the upstream OBD and the helicase domain, of 5 of 11 LT gene sequences from MCV-positive MCCs. In addition, the conservation of the LT NLS motif in all 11 MCV-positive MCCs analysed suggested the necessity for tumorigenesis of nuclear targeting of the mutated LT ([Nakamura et al., 2010](#)); yet a few sequences of MCV LT had a stop-codon mutation before or within the NLS, while a few others encoded a non-truncated protein ([Shuda et al., 2008](#); [Laude et al., 2010](#)). The latter cases could potentially display mutations at other sites critical for MCV replication. It is likely that a truncating mutation that prevents expression of both the OBD and the helicase domain is found in the classic MCV-infected cell line MKL-1 ([Shuda et al., 2008](#)). In contrast,

full-length LT coding sequences with no truncating mutations were obtained in control tissues and skin swabs from non-MCC patients who were found to harbour MCV ([Shuda et al., 2008](#); [Mogha et al., 2010](#); [Schowalter et al., 2010](#)). Only one truncation of the C-terminal 15 amino acids of the sT protein was identified in one skin swab of a healthy volunteer ([Schowalter et al., 2010](#)). Substitution, deletion, and insertion mutations were identified in 7 of 10 DNA sequences of buffy coats. These mutations could have an effect on the function of sT, but they are not expected to alter LT function as they are situated on its intronic sequence ([Pancaldi et al., 2011](#)). Since high mutation rates have not generally been observed in cellular cDNA from MCCs ([Shuda et al., 2008](#)), a mutator phenotype was ruled out, whereas a selection for certain LT mutations in the tumours has been suggested.

Unlike WT MCV LT, two different mutated LTs derived from MCC failed to support replication from the MCV origin, as evidenced by replication assays via two experimental approaches: a plasmid containing the MCV replication origin and the tumour cell line MKL-1 ([Shuda et al., 2008](#)). When these findings are taken together, it appears that the LT-truncating mutations result from selective pressure to avoid virus DNA replication and/or completion of the viral life cycle, and are in line with the perception that polyomavirus transformation does not require free replicating virus.

Despite the expression of replication-deficient LT in MCC, viral particles were detected in the cell cytoplasm and the nucleus of one primary MCC by electron microscopy, suggesting the expression of a functional LT and the occurrence of active viral replication. Although these findings may suggest that not all MCV-positive MCCs merely express truncated LT, they require careful consideration as they lack PCR examination for the presence of other cutaneous polyomaviruses, while the nature of the observed virus

particles and the virus-producing cells has not been evaluated ([Wetzels et al., 2009](#)).

Limited polymorphisms in both sT and LT sequences, without signature mutations, have been detected in some MCC biopsies ([Foulongne et al., 2010a](#); [Laude et al., 2010](#)). Furthermore, coexistence of integrated LT-truncated viral sequences and integrated concatemers or episomes has been documented in different body compartments ([Sastre-Garau et al., 2009](#); [Laude et al., 2010](#)). This situation might explain the case studied by [Wetzels et al. \(2009\)](#), hence explaining the presence of viral particles in MCV-positive MCC cells. Yet another group reported sequences encoding full-length LT in MCCs, casting doubt on the role of truncated LT in the development of MCC ([Schrama et al., 2010](#)). In conclusion, MCV-positive MCCs may express truncated LT and/or full-length LT from an integrated MCV and/or episomal genome. Of note, the discrepancy in the findings of viral sequences encoding truncated LT or full-length LT may be due to limitations of the PCR method that selectively amplifies the most abundant template. It may also stem from a mixed population of infected cells. Hence, further studies are required to resolve the significance of LT mutations in MCC.

In addition to LT truncation, 90 bp and 200 bp deletions of the VP1 gene that alter the open reading frame have been reported in MCC ([Kassem et al., 2008](#); [Feng et al., 2011](#)). The extent to which this alteration applies to the function of VP1 has not been evaluated. However, point mutations in the VP1 gene of at least one MCC-derived isolate, MCV350 (also termed MCC350), have been shown to exhibit defective assembly ([Pastrana et al., 2009](#)). A possible implication of this finding is that ongoing expression of assembly-competent VP1 might be detrimental to the developing MCC.

Another viral mutation has been identified during sequencing of the MCV replication origin from one non-tumour tissue, eight MCCs, and two cell lines: a single point mutation in a

tumour-derived MCV strain at a critical pentanucleotide sequence outside of the canonical LT-binding region. This natural mutation abrogates MCV origin replication ([Kwun et al., 2009](#)).

(e) *Genomic integration of MCV*

Like high-risk human papillomaviruses (HPVs), tumour-derived MCV genomes are integrated into the MCC cellular genome. The integration pattern of MCV DNA is clonal, and the primary tumours as well as metastatic cells carry the same integration signature. Southern blot hybridization and PCR-based analyses of MCC and cell lines are consistent with the virus integrating at a single insertion site, sometimes as head-to-tail concatemer, in each particular tumour ([Feng et al., 2008](#); [Sastre-Garau et al., 2009](#); [Fischer et al., 2010](#); [Laude et al., 2010](#)). The monoclonal pattern of MCV integration into MCC suggests that virus integration precedes tumour-cell expansion and thus argues for a causative role of MCV infection in MCC development. The clonal finding fulfils Hill's criterion of temporal relationship, which is an important epidemiological causality criterion. Viral DNA could be episomal in certain MCV-positive cells, but this may represent coexistent episomally replicating virus ([Sastre-Garau et al., 2009](#); [Fischer et al., 2010](#)).

Integration of MCV on chromosome 3p14.2, disrupting the receptor tyrosine phosphatase type G (*PTPRG*) gene, was identified during the initial discovery of the virus ([Feng et al., 2008](#)). *PTPRG* has been identified as a putative tumour suppressor gene that is hypermethylated in certain malignancies. Sastre-Garau and co-workers mapped random integrations of MCV DNA at distinct chromosomal loci, including those in the vicinity of *MYC* and *IL20RA* ([Sastre-Garau et al., 2009](#)). In another study, integration sites were found close to the *PARVA* and *DENND1A* genes, involved in cell junction and in formation of clathrin-coated vesicles or cell adhesion and cytoskeleton, respectively ([Laude et al., 2010](#)). An

additional integration site was mapped adjacent to the transcriptional activator TEAD1 encoding gene and another, which led to a complex rearrangement, within intronic sequences of the GDP-mannose 4,6-dehydratase (*GMD5*) gene.

Duncavage and colleagues used next-generation sequencing to reveal unique integration sites of MCV in four MCCs, with the exception of a paired primary tumour and metastasis samples, which showed identical sites ([Duncavage et al., 2011](#)). Alteration of the expression of cellular genes located in the vicinity of the integration sites could also participate in the induction of the tumorigenic phenotype, yet no evidence of deregulation of putative adjacent cellular genes has been detected, favouring an alternative mechanism of tumorigenesis ([Sastre-Garau et al., 2009](#)).

(f) *Merkel cell carcinoma cell lines*

Fully transformed and tumorigenic MCV-positive and MCV-negative MCC lines have been established ([Shuda et al., 2008](#); [Fischer et al., 2010](#); [Houben et al., 2010b](#)). Based on neuroendocrine markers and the characteristics of their tumour-derived cell lines, MCC can be categorized into classic and variant types that form suspension or adherent cell cultures, respectively ([Leonard et al., 1993](#)). Three variant lines and one classic MCC cell line were tested for the presence of MCV DNA; the virus, in a clonal concatemeric integration form, was found only in the classic variant MKL-1 cell line, which expresses both LT and sT mRNAs and lacks the 57kT mRNA due to the elimination of a putative splice-donor junction. A truncating mutation (stop codon at nucleotide 1452) that prevents expression of the OBD and the helicase domain has been documented in this cell line as well ([Shuda et al., 2008](#)).

Seven MCC cell lines, including four classic ones, one variant, and two intermediate phenotype subtypes, were analysed for the presence of MCV. The virus was detected in two classic and

two intermediate phenotype lines. Of note, levels of MCV genome varied, with three cell lines strongly positive for MCV DNA, whereas two contained minute amounts of the virus, detectable only by means of nested PCR, suggesting that only a minority of these cells contain the virus ([Fischer et al., 2010](#)). Like [Shuda et al. \(2008\)](#), this group did not identify the virus in the variant subtype. Therefore, as not all classic-type cultures were MCV-positive, no clear correlation could be drawn between the phenotypic characteristic of the cells and the presence of the virus. Also in accordance with [Shuda et al. \(2008\)](#) were the findings of clonal integration of head-to-tail concatemers of the virus and premature stop-codon mutations that truncate LT while preserving the pRB-binding domain ([Fischer et al., 2010](#)). Taken together, these findings suggest the importance of MCV infection in a subtype of MCC cell lines and show that neutralizing pRb activity by LT, along with pausing of virus replication, is important for the malignant process.

Knockdown of the viral LT locus (pan T-antigen) with short-hairpin RNA (shRNA) interferes with the proliferation and survival of most established MCC cell lines harbouring the MCV genome ([Houben et al., 2010b, 2012a](#)). Yet since one MCV-infected MCC cell line did not show dependence on T-antigen expression for survival, it seems that there may be a stage where expression of the viral proteins is not essential ([Houben et al., 2012b](#)). [However, it cannot be completely excluded that the LT knockdown in this experiment might not be different enough.] Neither caspase activation nor alterations in the expression levels of several pro- and anti-apoptotic proteins, including p53, have been associated with the decreased survival of the MCV-infected cells after LT repression. As predicted, MCV-negative cell lines are unaffected by MCV LT knockdown ([Houben et al., 2010b](#)).

Interestingly, knockdown of sT alone retarded MCV-positive cell growth to a similar

extent as did shRNA targeting pan T-antigen, but did not induce MCC cell death. Knockdown of pan T-antigen was more efficient in inhibiting cell-cycle entry, measured by BrdU incorporation, than was sT knockdown alone. Even though no cooperation has been observed in rodent cell transformation assays between MCV sT and LT proteins, there is evidence to suggest that this process does occur. Expression of MCV sT does not rescue MCC cells in which the T-antigen locus has been repressed, indicating that other T-antigen isoforms also play a role in MCV-induced oncogenesis ([Shuda et al., 2011](#)).

The requirement of the T-antigen locus for tumorigenesis has been substantiated by means of an inducible shRNA system that produced regression of MCC in NOD/SCID mice upon knockdown of LT and sT. The growth-inhibitory effect of LT shRNA was almost completely restored by ectopic expression of shRNA-resistant LT, whereas mutant LT protein that does not interact with pRb failed to restore knockdown of LT ([Houben et al., 2010b](#)).

Taken together, these studies suggest that LT and sT are important for the survival of MCV-infected cells, and that the virus acts as a key factor in signalling events that maintain the tumour phenotype in MCV-positive cells.

(g) *Alteration of specific proto-oncogenes and chromosomal abnormalities*

Little is known about relevant genetic alterations in MCC and the different pathways involved in the pathogenesis of MCV-negative and MCV-positive MCCs. Katano *et al.* reported that MCV-positive cases showed round and vesicular nuclei with a fine granular chromatin and small nucleoli, whereas MCV-negative cases showed polygonal nuclei with diffusely distributed chromatin ([Katano et al., 2009](#)). In line with this, multiobserver morphometric analyses to determine differences in tumour-cell morphology between MCV-positive and MCV-negative MCCs identified more irregular nuclear shapes and

abundant cytoplasm in MCV-negative tumours, which may reflect biological differences between these subgroups ([Kuwamoto et al., 2011](#)).

Several studies published before the discovery of MCV examined the expression and genomic organization of different cellular genes in MCC. For example, mutations of *p53* and the related tumour protein 73 (TP73) have been reported ([Van Gele et al., 2000](#)). Deletion of the fragile histidine triad (*FHIT*) gene at 3p14.2 and its aberrant expression have also been reported in MCC ([Leonard et al., 1996](#); [Sozzi et al., 1996](#); [Popp et al., 2002](#)). Furthermore, hypermethylation of the cellular genes *p14*, cyclin-dependent kinase inhibitor 2A (*CDKN2a*) and Ras association domain family 1A (*RASSF1A*) has been reported in MCC ([Lassacher et al., 2008](#); [Helmbold et al., 2009](#)). However, since not all MCCs are MCV-positive, both virus-dependent and virus-independent oncogenic pathways may contribute to the development of MCC.

Discrepancies in the molecular and potentially oncogenic pathways between MCV-positive and MCV-negative MCCs have been analysed in several recent studies. By using comparative genomic hybridization (CGH), Paulson and co-workers identified several regions of relatively frequent genomic alterations in MCC, including a deletion of 5q12-21, a deletion of 13q14-21, which contains the gene of the tumour suppressor pRB, and focal amplification at 1p34 that was centred on the *L-MYC* (*MYCL1*) gene. When virus-positive MCC was compared with MCC without detectable virus, there was a trend, which was not significant, towards more genomic aberrations in virus-negative tumours, with a notable exception (of unknown significance) of a deletion of chromosome arm 19q in the majority of virus-positive MCC but rarely in virus-negative MCC ([Paulson et al., 2009](#)). Interestingly, no significant correlation between *RASSF1A* methylation and MCV infection was identified when MCCs were analysed for the combined presence of hypermethylation and virus infection, suggesting that

virus infection does not contribute to this type of hypermethylation ([Helmbold et al., 2009](#)). CGH analysis of 10 MCV-positive MCCs did not show any imbalance in 3 tumours, while recurrent imbalances were gains of 1q (2 cases), 6p (3 cases), and 11 (2 cases), and loss of 17p (2 cases) ([Sastre-Garau et al., 2009](#)).

IHC was used to show that MCV-positive MCC with relatively high MCV DNA abundance (i.e. 0.06–1.2 viral copies/cell) had significantly higher pRb and terminal deoxynucleotide transferase ([Bhatia et al., 2010b, c](#)). A similar correlation between MCV DNA positivity, LT expression, and pRb detection in MCC has been reported by [Sihto et al. \(2011\)](#). In contrast, Houben and co-workers did not find a correlation between virus abundance in MCC tissues and pRb expression ([Houben et al., 2010a](#)). The function of p53-regulated pathways in MCC has not yet been evaluated. Significantly higher p53 protein levels correlated with low viral copy numbers (i.e. 0.0005–0.0035 genome copies/cell) or with the absence of MCV ([Bhatia et al., 2010b](#)). In line with this, p53-positive MCCs were reported to contain a lower copy number of MCV than p53-negative tumours, with the mean number of MCV DNA copies decreasing as the percentage of p53-positive nuclei in the sample increased. This group also found a similar trend of inverse association for the receptor tyrosine kinase KIT ([Waltari et al., 2011](#)). This may indicate that MCC arises through sequential multiple oncogenic pathways and that accumulation of mutations in host genes, enhanced by virus infection, is important for the onset of MCC. In accord with this suggestion is the notion that there is a long time interval between the primary exposure to MCV and the development of MCC.

(h) *Intermediate working model of MCV tumorigenesis*

The LT-truncating mutations as well as the common and exclusive integration of the MCV genome in MCC render it less likely that MCV

is simply a coincidental passenger infection in these tumours. This notion is sustained by the recent finding that expression of the T-antigens is vital for the survival and growth of MCV-positive MCC cell lines and xenograft tumours ([Houben et al., 2010a](#)). Because virus integration and LT mutation are presumed to be uncommon, the requirement for both events may explain why MCC is rare, despite widespread infection with MCV.

4.3.3 MCV in other tumours

Several studies described in Section 2 reported weak associations of MCV DNA positivity for tumours other than MCC. With one exception ([Pantulu et al., 2010](#)), reporting on CLL and discussed below, none of the studies showed viral loads approaching 1 viral genome copy per cell or evidence for biological activity of the virus in the tumour tissues.

(a) Non-MCC skin tumours

MCV DNA was detected significantly more often in MCC than in biopsy samples of non-MCC skin tumours, in samples of the perilesional area and in histologically healthy skin samples from immunocompetent patients. Since MCV DNA has been detected only by nested PCR in non-MCC samples, relatively smaller viral loads in these samples have been suggested ([Wieland et al., 2009](#)). Similarly, Becker and colleagues found MCV DNA in 12.5% of BCC samples, and the viral load was 4 orders of magnitude lower than in MCC ([Becker et al., 2009](#)). Likewise, the viral load in NMSC samples was reported to be 3–4 orders of magnitude lower than that in MCV-positive MCCs ([Kassem et al., 2009](#)). MCV DNA was detected in 12 (28.6%) of 42 keratoacanthoma and in 14 (26.9%) of 52 SCC samples, in which MCV DNA loads were > 2 orders of magnitude lower than those in MCC ([Wieland et al., 2011](#)).

(b) Chronic lymphocytic leukaemia

A link between CLL and MCC has been suggested, and various studies have reported the presence of the MCV genome in CLL ([Quaglino et al., 1997](#); [Brenner et al., 2001](#); [Vlad & Woodlock, 2003](#); [Agnew et al., 2004](#); [Howard et al., 2006](#); see also Section 2.3.2). The physiological relevance of these findings is supported by the detection of MCV DNA by PCR in lymphocyte and inflammatory monocyte populations from MCC patients and in blood samples from healthy individuals, demonstrating that MCV can be lymphotropic ([Shuda et al., 2009](#); [Laude et al., 2010](#); [Mertz et al., 2010b](#); [Pancaldi et al., 2011](#)). Of note, circulating Merkel cells have also been detected in the peripheral blood of an elderly man with MCC and a history of CLL and multiple myeloma ([Hartley et al., 2010](#)). Patients with MCV-positive MCC tumours have an elevated risk of CLL ([Koljonen et al., 2009](#)), but direct examination of CLL tumours does not always reveal MCV genome positivity in most of the samples examined, and when detected, MCV DNA is only present at very low levels (see also Section 2.3.2). [Teman et al. \(2011\)](#), for example, reported MCV DNA in 6 (33%) of 18 CLL cases, and only 1 case presented positive MCV LT immunostaining with CM2B4 antibody. In contrast, a study of two patients with concurrent MCC and CLL demonstrated that the MCV LT protein is expressed only in the MCC-affected cells and not in the CLL-affected cells ([Tolstov et al., 2010](#)). Comparison between MCV antibody positivity in CLL patients and in a leukaemia control group also did not reveal differences in the rates of MCV prevalence and in antibody titres ([Tolstov et al., 2010](#)).

Striking, however, is the detection of MCV genome in 4 of 6 cases of CLL by fluorescence in situ hybridization (FISH), revealing patterns compatible with viral integration ([Haugg et al., 2011](#)). In this study, MCV genome and protein expression was identified from a subset of CLL tumours, and the viral genome presented a

246 bp deletion within the LT helicase domain, which is reminiscent of MCV infection in MCC; however, since all mutations were identical in the different patients, conclusions should be drawn with caution. In addition, extremely low viral copy numbers were observed, corresponding to < 1 viral genome copy per 1000 cells (3–4 orders of magnitude lower than in MCC tissues) ([Pantulu et al., 2010](#)).

4.4 Interaction between MCV and other potential cofactors

MCC is a relatively rare disease. The high prevalence of MCV infection in the general population and the low rate of MCC indicate that environmental and host genetic factors may influence the progression from infection to cancer. To evaluate the cofactors of MCV, it is important to note the complex and multifactorial origin of cancer. To date, evidence for cofactors of MCV comes from case–control studies in which exposure is determined after the diagnosis of disease, while many potential cofactors have not been evaluated rigorously in epidemiological studies. Assuming that ~80% of MCCs involve MCV infection, one may gain some information on potential cofactors from the literature published before the discovery of MCV. Obviously, these studies did not measure MCV, which is a potential confounder.

Old age, immune deficiency related to viral infections, organ transplantation, CLL (see Section 4.5), and exposure to UV radiation have been proposed as predisposing factors to MCC ([Rockville Merkel Cell Carcinoma Group, 2009](#)).

The importance of sunlight in the development of MCC is suggested by the fact that these tumours occur almost exclusively on sites exposed to the sun. The risk of MCC is possibly increased by exposure to UV radiation. Furthermore, MCC develops more frequently in individuals with light-coloured skin who live at more equatorial

latitudes and is rare in individuals with darker skin ([Miller & Rabkin, 1999](#)). In a study of 1380 psoriasis patients receiving UVA phototherapy, 3 patients developed MCC, a much higher rate than expected from the incidence rate of the general population ([Lunder & Stern, 1998](#)). Exposure to sunlight may induce DNA mutations in the MCV genome. Besides this direct role, UV exposure may also contribute to MCC development through immunosuppression. To understand the mechanism underlying this observation, MCV transcripts were examined after exposure of healthy volunteers to normal solar radiation; an increase in the expression of sT mRNA was observed ([Mogha et al., 2010](#)). *In vitro* studies confirmed induction of the sT transcript, which was attributed to activation of the early MCV promoter shortly after UV irradiation ([Mogha et al., 2010](#)).

4.5 Susceptible populations

Primary evidence to support the concept that MCC might be caused by an infectious agent came from reports of increased risk of MCC in immunocompromised subjects. Immunosuppression was recognized as a risk factor for MCC in organ transplant recipients ([Formica et al., 1994](#); [Douds et al., 1995](#)) and in AIDS patients ([Catlett et al., 1992](#)). (See also more detailed studies in Section 2.4.) Furthermore, MCC incidence sharply increases in elderly populations, and MCC is extremely uncommon before the age of 50. Together with the increased risk among the elderly, which may reflect an age-related decline in cellular immune surveillance, these clinical findings are consistent with MCC having an infectious etiology.

Immunosuppression related to lymphohaematopoietic cancers may also increase the risk of MCV-associated MCC. Statistically significant increased risks of MCC occurring 1 year after NHL, multiple myeloma, and CLL were reported, with SIR of 3.28, 4.91, and 7.43, respectively

(Howard *et al.*, 2006). A stronger inter-relationship was reported between MCV-positive MCC and CLL, with highly elevated SIR, 15.7 (95% CI, 3.2–46.0; $P < 0.01$) and also for CLL diagnosed after MCC, with SIR of 17.9 (95% CI, 2.2–64.6; $P < 0.001$) (Koljonen *et al.*, 2009).

Epidermodysplasia verruciformis (EV) is a very rare, autosomal recessive inherited skin condition associated with diminished cell-mediated immunity and high susceptibility to infection with specific HPVs. It is characterized by the development of multiple skin lesions, in particular BCC and SCC, predominantly at sites that are exposed to the sun. A case report describing a Brazilian EV patient who developed MCV-associated MCC could suggest similar host genetic susceptibility factors for HPV and MCV (Oliveira *et al.*, 2010). EV patients appear to have higher MCV viral loads, and MCV DNA was detected in carcinoma in situ, in invasive SCC, and in common warts of all 6 EV patients analysed (Mertz *et al.*, 2011). In addition, another case report described the occurrence of MCC in a young woman with no apparent risk factors but with a family history for a variant of EV, suggesting an incomplete EV with a distinct phenotype characterized by immunological abnormalities, further highlighting an association between immune disruption and susceptibility to MCC (Halvorson *et al.*, 2011).

4.6 Transgenic models for cancers associated with MCV infection

No studies with transgenic mice carrying MCV-derived genes and resulting in tumour formation have been reported.

4.7 Mechanisms of carcinogenesis

There is strong evidence that cell transformation of MCV in human MCC follows a mechanism generally similar to that used by other

transforming polyomaviruses in experimental systems and by oncogenic HPV. This mechanism is characterized by the persistent presence of at least one biologically active viral genome in each transformed cell. Viral persistence can be mediated through integration or through maintenance as a viral episome. The presence of the same integration site per tumour present in primary tumours, in recurrent tumours, and in tumour metastases is a marker of clonal tumour expansion and indicates that viral integration occurred before tumour expansion. Viral genes encoding regulatory proteins, i.e. the T-antigens in polyomaviruses and E6 and E7 in HPV, are consistently expressed. Apart from their other functions, viral oncoproteins interact directly or indirectly with cellular tumour suppressor proteins such as pRb and p53, leading to deregulation of the cell cycle and apoptosis. Presence of viral oncoproteins is necessary to maintain the transformed phenotype, which can lead – in the tumour-bearing animal or in the human patient – to the induction of antibodies to the viral oncoproteins. In the absence of tumours, such antibodies are rarely induced during the natural course of infection.

4.7.1 Studies in Merkel cell carcinoma tissues

According to the current literature (see Section 4.3 for references), with a total of > 1000 MCC cases analysed by many different research groups, about 75% of tumours were found to contain MCV DNA. MCV DNA has also been detected in recurrent tumours as well as in nodal and distant metastases (Feng *et al.*, 2008; Garneski *et al.*, 2009; Sastre-Garau *et al.*, 2009).

However, the detection of viral DNA alone in tumour tissues is not sufficient proof of a causal association. MCV is an ubiquitous virus, and MCV DNA can be detected, in varying amounts, in specimens from a wide range of different tissues (Loyo *et al.*, 2010) and even in the human environment (Foulongne *et al.*, 2011), raising the

possibility of contamination from neighbouring non-tumour tissues. Therefore, environmental contamination should be considered a very serious concern with PCR-based studies ([Cohen & Enserink, 2011](#)).

MCV integration at a single site is frequently present in MCV DNA-positive primary MCCs and metastases. There appears to be no specificity with respect to the cellular integration sites.

Full-length intact MCV LT is required for MCV genome replication and is encoded in replicating virus from asymptomatic skin infections ([Schowalter et al., 2010](#)). MCV LT binds to probably all pRb family proteins ([Shuda et al., 2008](#)), whereas no evidence for a direct interaction between MCV LT and the tumour suppressor protein p53 is available.

In contrast, most LT sequences isolated from MCC tissues are truncated as a result of point mutation or have deleted C-terminal LT sequences downstream of the pRb-binding domains ([Feng et al., 2008](#); [Shuda et al., 2008](#); [Sastre-Garau et al., 2009](#); [Foulongne et al., 2010a](#); [Laude et al., 2010](#)). These tumour-specific LT alterations result in loss of replication function ([Shuda et al., 2008](#)) and thus may contribute to the stability of the integrated viral genomes that still contain the viral origin of DNA replication.

In MCC, the MCV T-antigen gene region is transcribed, giving rise to sT, LT, and 57kT. The 57kT transcript is similar to LT but has a second intronic splice site that deletes portions of the OBD and the helicase function ([Feng et al., 2008](#)). No specific function has been attributed to 57kT. [Sastre-Garau et al. \(2009\)](#) found both sT and LT viral sequences to be significantly expressed in MCCs that all had > 0.6 viral genome copies per cell.

Monoclonal antibody CM2B4 targets an epitope on LT and 57kT upstream of the pRb-binding domain and can selectively stain nuclei of MCV DNA-positive MCC tissues ([Busam et al., 2009](#); [Shuda et al., 2009](#); [Reisinger et al., 2010](#); [Wieland et al., 2012](#); [Erovic et al.,](#)

[2013](#)). Using this antibody, [Busam et al. \(2009\)](#) reported that 27 (75%) of 36 MCCs stained positive for MCV LT. In the majority of the tumours, the staining of tumour cell nuclei was strong and homogeneous, labelling > 75% and up to 100% of the tumour cell population. In a subgroup for which data on viral load were available, 5 of 7 tumours with > 0.8 viral genome copies per cell and none of 3 tumours with < 0.001 viral genome copies per cell stained positive with CM2B4 ([Shuda et al., 2009](#)).

Using the monoclonal antibody CM5E1, which recognizes an epitope specific to MCV sT, [Shuda et al. \(2011\)](#) detected sT expression in 47 (92%) of 51 MCC tumours versus only 38 (75%) positive for MCV LT. Interestingly, 10 tumours were sT-positive only, versus 1 tumour staining for LT only.

In HPV-induced carcinogenesis, pRb is targeted for degradation by the viral oncoprotein E7, resulting in concomitant upregulation of cyclin kinase inhibitor p16^{Ink4a}, while the viral oncoprotein E6 inactivates p53 through ubiquitination and degradation, and the TP53 sequence is not mutated ([IARC, 2007, 2012](#)). [Sihto et al. \(2011\)](#) found 61 (67%) of 91 MCC tissues to express MCV LT. LT expression was strongly associated with pRb expression, and viral load was > 0.1 copies per cell. LT expression was also associated with downregulation of p53 and absolute absence of TP53 mutations, whereas TP53 mutations were found in 52% of the LT-negative MCCs. However, p16^{Ink4a} was upregulated irrespective of the MCV LT status. Similar observations were made in a smaller MCC series by [Bhatia et al. \(2010b\)](#).

[Paulson et al. \(2010\)](#) demonstrated that antibodies to MCV sT and LT are strongly associated with MCC. Among 530 population control subjects, these antibodies were present in only 0.9% and were of low titre. In contrast, among 205 MCC cases, 40.5% had serum IgG antibodies that recognize a portion of T-antigen shared between LT and sT. After tumour removal, titres of T-antigen antibodies fell (> 8-fold per year)

in patients whose cancer did not recur, whereas they increased rapidly in those with progressive and metastatic disease preceding clinical detection of disease spread. These results suggest that antibodies recognizing T-antigen are induced in response to T-antigen expression in the tumour, reflect tumour burden, and could serve as surrogate markers for MCV-induced carcinogenesis.

[Touzé *et al.* \(2011\)](#) used VLP ELISA in MCC patients and found 14-fold higher GMTs of serum antibodies to MCV major capsid protein VP1 compared with controls. VP1 expression was absent in MCV DNA-positive tumours. In MCV antibody-positive MCC patients, [Pastrana *et al.* \(2009\)](#) found GMTs of MCV-neutralizing antibodies to be about 60-fold higher than those in MCV antibody-positive controls. The higher MCV capsid antibody levels may indicate higher viral load in MCC patients before tumour diagnosis.

4.7.2 Studies in animals and in cell lines

[Shuda *et al.* \(2008\)](#) demonstrated the presence of MCV DNA in one of four MCC cell lines. The MCV genome was integrated and encoded a truncated T-antigen. [Fischer *et al.* \(2010\)](#) analysed 7 other MCC cell lines and found 5 to be MCV DNA-positive, but only 3 with > 1 viral genome copy per cell, and demonstrated MCV integration for one of these 3 lines.

shRNAi targeting exon 1 shared by all three MCV T-antigen proteins knocked down all T-antigen expression and triggered growth arrest and/or cell death in three MCV-positive MCC cell lines, but not in one MCV-negative cell line ([Houben *et al.*, 2010b](#)), demonstrating that T-antigen expression is necessary for maintenance of the transformed phenotype.

Rescue experiments in a xeno-transplantation model expressing various forms of LT during lentivirus-mediated drug-inducible (sh) RNAi T-antigen knockdown demonstrated that LT expression is also essential for tumour growth

in vivo. LT with a mutated pRb-binding domain failed to rescue targeting of pRb family proteins, suggesting that pRb binding by LT is essential for proliferation of MCC cells ([Houben *et al.*, 2012a](#)).

Lentiviral shRNA targeting the intron 1 sequence can selectively knock down sT and inhibit growth of the MCC cell line MKL-1 to a similar extent as shRNA targeting all T-antigen isoforms, but it did not cause cell death, suggesting that both sT and LT separately contribute to MCV-associated tumorigenesis ([Shuda *et al.*, 2011](#)). Furthermore, sT but not LT results in rodent fibroblast transformation and human fibroblast serum-independent growth, independent of PP2A and Hsc70 binding. sT appears to act downstream in the mTOR signalling pathway to preserve eukaryotic translation initiation factor 4E-BP1 hyperphosphorylation, resulting in dysregulated cap-dependent translation ([Shuda *et al.*, 2011](#)). These data suggest an oncoprotein function for sT.

Taken together, there is strong evidence generated by biochemical and biological studies performed in animal cells, in human MCC tissues, and in MCC-derived cell lines that MCV encodes oncoproteins that directly contribute to the development and maintenance of MCC.

4.7.3 MCV in chronic lymphocytic leukaemia

[Pantulu *et al.* \(2010\)](#) detected the presence of MCV DNA in highly purified leukaemic cells from 27% of 70 CLL patients. Viral copy numbers were 3–4 orders of magnitude lower than those in MCC tissues (corresponding to < 1 viral genome copy per 1000 cells). In 6 of 19 MCV DNA-positive CLL samples, the MCV LT sequences contained the same novel 246 bp deletion in the C-terminal helicase domain. This deletion was absent in MCV DNA-positive CLL samples. In addition, FISH with MCV probes revealed hybridization patterns compatible with viral integration in 4 of the 6 CLL samples ([Haugg *et al.*, 2011](#)). In two bone marrow samples containing nodular CLL

infiltrates, a faint staining with CM2B4 indicated LT expression in a significant number of CLL cells. The detection of MCV, including LT deletions and LT expression in CLL cells, could argue for a potential role of MCV in a significant subset of CLL cases. However, the extremely low viral copy number observed is not compatible with the requirement for viral persistence and viral oncogene expression in each tumour cell. Although the sequences adjacent to the deletions revealed some sequence heterogeneity, the deletion boundaries in all 6 patients were identical at the nucleotide level. This is in contrast to the observations with MCC, in which no identical deletions were found in different patients.

5. Summary of Data Reported

5.1 Exposure data

Merkel cell virus (MCV) was first discovered in human Merkel cell carcinoma (MCC), a rare and highly aggressive skin cancer. MCV infection in humans has been investigated by a variety of methods, including serology and DNA detection. Collectively, these studies show that a high proportion of adults are infected with MCV and that infection is acquired early in life and is evident in all age groups. Detection of MCV in the skin of most adults suggests that MCV is common in human skin. MCV has not been associated with any human disease other than MCC. The mode of transmission, route of dissemination, cellular tropism, and latency characteristics remain to be elucidated. There is consistent evidence that MCV infects humans.

5.2 Human carcinogenicity data

5.2.1 *Merkel cell carcinoma*

An etiological role of MCV in MCC is supported by a few case-control studies and several case series. There are no prospective cohort studies. Case-control studies show odds ratios ranging from 4.4 to 6.6 for serological markers of MCV infection and from 16.9 to 63.2 for serological markers of viral early gene expression. Little is known about other risk factors for MCV infection and their role in MCC, and therefore potential confounding could not be ruled out. However, there is consistency across a large number of case series in different populations and regions, in which MCV DNA is detected in tumour tissue of most MCC cases (prevalence ranges from 59% to 100%).

5.2.2 *Other cancers*

Overall, there is no clear association between MCV and other cancers.

5.3 Animal carcinogenicity data

No data were available to the Working Group.

5.4 Mechanistic and other relevant data

There is strong mechanistic evidence that MCV can directly contribute to the development of a large proportion of MCCs. There is weak and inconsistent evidence for a direct contribution of MCV to other human cancers.

- Multiple independent laboratories worldwide have reported the presence of MCV DNA in about 75% of > 1000 MCCs that have been analysed by PCR.
- In most MCV DNA-positive MCCs for which the viral DNA load has been analysed, MCV

genome copy numbers close to or more than 1 copy per cell have been demonstrated.

- In the majority of MCV-positive MCCs that have been studied for clonality, viral DNA is clonally integrated in the cellular genome. Integration appears to precede clonal expansion.
- In the majority of MCV-positive MCCs that have been analysed for the status of the MCV early genome region, the LT gene sequences contained truncating mutations at the C terminus that were associated with replication deficiency and possibly other functions, while the sT gene sequences were not mutated.
- Expression of the sT and LT antigens has been repeatedly demonstrated at the transcriptional and translational levels in cells of MCC.
- MCC patients have measurable antibodies to MCV-specific sT and LT, but MCV-infected individuals without MCC rarely do.
- Expression of the T-antigens is essential for the survival of MCV-positive MCC-derived cell lines and for the propagation of MCC engrafted into NOD/SCID mice.
- The intact sT and LT antigens of MCV interact with key cellular regulatory proteins to subvert cellular control pathways.
- sT of MCV transforms immortalized rodent fibroblasts and converts immortalized human fibroblasts to serum-independent growth.

6. Evaluation

6.1 Cancer in humans

There is *limited evidence* in humans for the carcinogenicity of MCV. A positive association has been observed with MCC.

6.2 Cancer in experimental animals

There is *inadequate evidence* in experimental animals for the carcinogenicity of MCV.

6.3 Overall evaluation

MCV is *probably carcinogenic to humans* (Group 2A).

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LIST OF ABBREVIATIONS

AGMK	African green monkey kidney
AID	activation-induced deaminase
ALL	acute lymphoblastic leukaemia
APC	antigen-presenting cell
BCC	basal cell carcinoma
BD	Bowen disease
BKV	BK polyomavirus
bp	base pair
CGH	comparative genomic hybridization
CI	confidence interval
CIDR	cysteine-rich interdomain region
CIN	cervical intraepithelial neoplasia
CLL	chronic lymphocytic leukaemia
CNS	central nervous system
CRC	colorectal cancer
EBV	Epstein–Barr virus
EIA	enzyme immunoassay
EIR	entomological inoculation rate
ELISA	enzyme-linked immunosorbent assay
EV	epidermodysplasia verruciformis
FFPE	formalin-fixed, paraffin-embedded
FFU	fluorescence focus units
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
GCN	granule cell neuropathy
geq	genome equivalents
GMK	green monkey kidney
GMT	geometric mean titre
GST	glutathione S-transferase
GVHD	graft-versus-host disease
HAI	haemagglutination inhibition
HAU	haemagglutination unit
HC	haemorrhagic cystitis
HEK	human embryonic kidney
HIV	human immunodeficiency virus

HIV-1	human immunodeficiency virus type 1
HLA	human leukocyte antigen
HPV	human papillomavirus
HPyV	human polyomavirus
HSCT	haematopoietic stem cell transplantation
HSV-1	herpes simplex virus type 1
Ig	immunoglobulin
IHC	immunohistochemistry
IPV	inactivated poliovirus vaccine
IRS-1	insulin receptor substrate 1
ISH	in situ hybridization
JCV	JC polyomavirus
LOH	loss of heterozygosity
LT	large T-antigen
LTR	liver transplant recipient
MCC	Merkel cell carcinoma
MCV	Merkel cell polyomavirus
miRNA	microRNA
MLV	Moloney leukaemogenic virus
MP	methylprednisolone
MUR	Merkel unique region
NB	newborn
NBCCS	naevoid basal cell carcinoma syndrome
NCCR	non-coding control region
NCRR	non-coding regulatory region
NET	neuroendocrine tumour
NHL	non-Hodgkin lymphoma
NLS	nuclear localization signal
NMSC	non-melanoma skin cancer
OB	origin-binding domain
OD	optical density
OPV	oral poliovirus vaccine
OR	odds ratio
ORF	open reading frame
PBL	peripheral blood leukocyte
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PfEMP1	parasite erythrocyte membrane protein 1
PFU	plaque-forming units
PHFG	primary human fetal glial
PML	progressive multifocal leukoencephalopathy
PNET	primitive neuroectodermal tumour
PVAN	polyomavirus-associated nephropathy
qPCR	quantitative polymerase chain reaction
RCA	rolling circle amplification
RR	relative risk
RSV	respiratory syncytial virus
RT-PCR	reverse transcriptase polymerase chain reaction

SBH	Southern blot hybridization
SCC	squamous cell carcinoma
SCLC	small cell lung cancer
SEER	Surveillance, Epidemiology and End Results
shRNA	short-hairpin RNA
SIR	standardized incidence ratio
siRNA	small interfering RNA
SIV	simian immunodeficiency virus
SLE	systemic lupus erythematosus
SNP	single-nucleotide polymorphism
sT	small T-antigen
SV40	simian virus 40
TCC	transitional cell carcinoma
TCID ₅₀	50% tissue culture infective dose
Th	T-helper
TLR	toll-like receptor
TNF	tumour necrosis factor
UV	ultraviolet
VCA	viral capsid antigen
VLP	virus-like particle
VP1	capsid viral protein 1
VZV	varicella zoster virus
WT	wild-type



This Volume of the *IARC Monographs* provides evaluations of malaria (a disease caused by infection with *Plasmodium* parasites) and four polyomaviruses – simian virus 40 (SV40), and BK, JC and Merkel cell viruses.

The global burden of malaria is enormous, with about 50% of the world's population at risk. In regions where malaria is highly endemic (holoendemic), such as sub-Saharan Africa and Papua New Guinea, a role for malaria has long been suspected in the etiology of endemic Burkitt lymphoma, which in these regions represents up to 70% of childhood cancers.

In the 1950s and early 1960s, millions of people worldwide received vaccines against poliovirus that were contaminated with SV40, a polyomavirus whose natural host is the rhesus macaque.

Infection with human polyomaviruses is widespread in the general population, with the proportion of adults infected ranging from 50% to more than 90% worldwide. BK and JC viruses, first isolated in 1971, are naturally human-tropic polyomaviruses that are responsible for rare, lethal, nonmalignant diseases in immunosuppressed people. Merkel cell virus was discovered in 2008 in a rare skin cancer in humans.

An *IARC Monographs* Working Group reviewed epidemiological evidence, animal bioassays, and mechanistic and other relevant data to reach conclusions as to the carcinogenic hazard to humans of these infections.

