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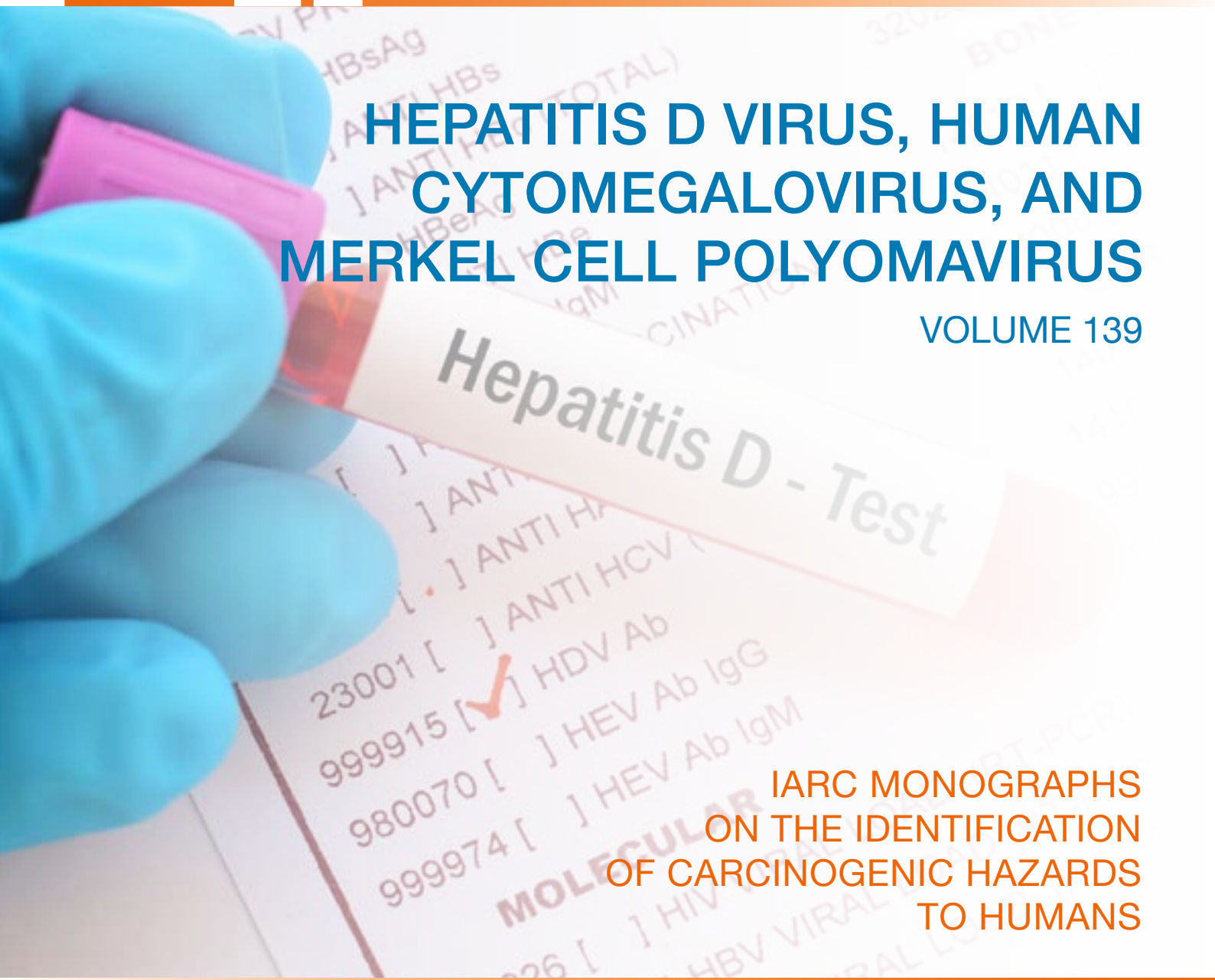

HEPATITIS D VIRUS, HUMAN CYTOMEGALOVIRUS, AND MERKEL CELL POLYOMAVIRUS

VOLUME 139

IARC MONOGRAPHS
ON THE IDENTIFICATION
OF CARCINOGENIC HAZARDS
TO HUMANS

International Agency for Research on Cancer
World Health Organization

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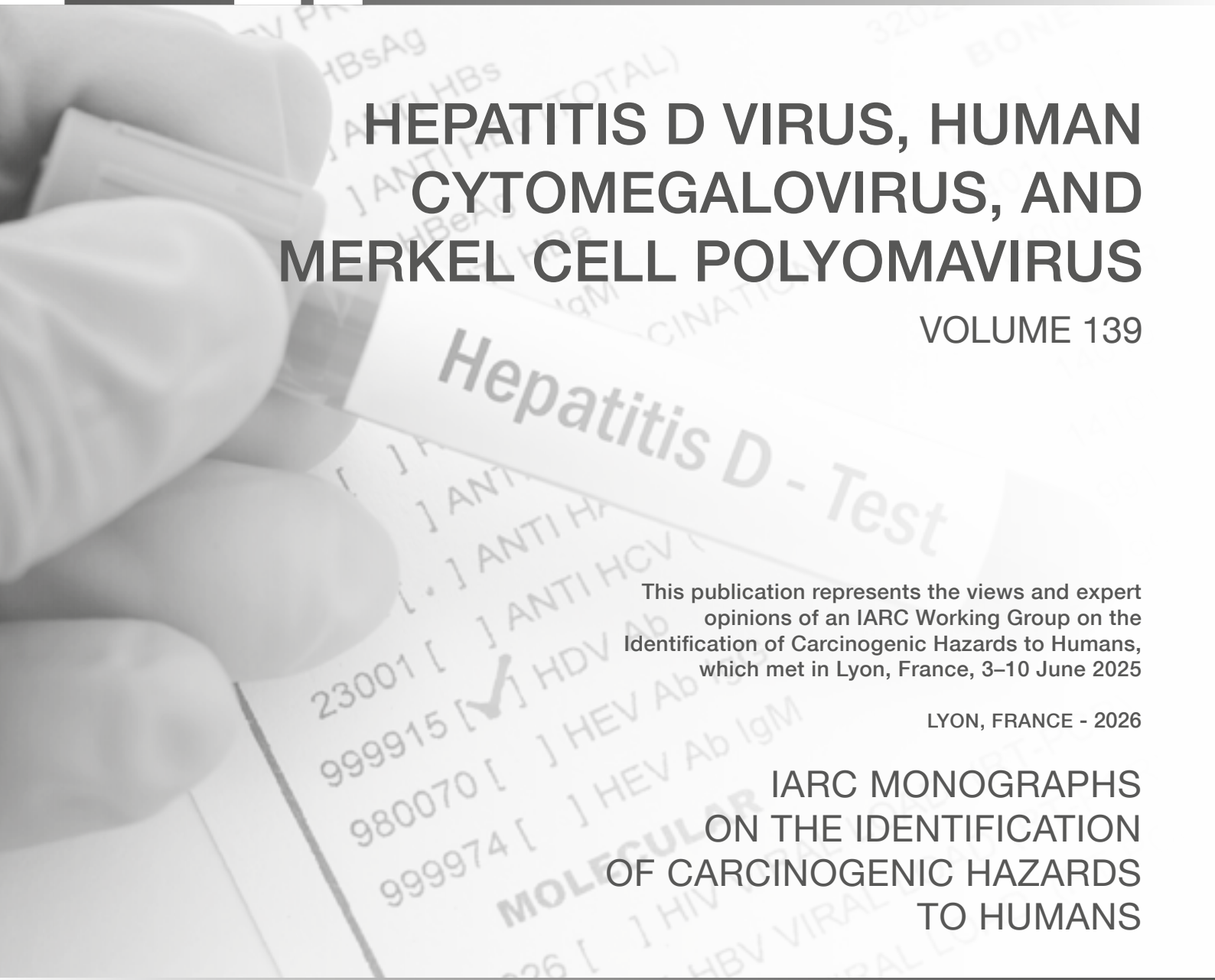

HEPATITIS D VIRUS, HUMAN CYTOMEGALOVIRUS, AND MERKEL CELL POLYOMAVIRUS

VOLUME 139

This publication represents the views and expert opinions of an IARC Working Group on the Identification of Carcinogenic Hazards to Humans, which met in Lyon, France, 3–10 June 2025

LYON, FRANCE - 2026

IARC MONOGRAPHS ON THE IDENTIFICATION OF CARCINOGENIC HAZARDS TO HUMANS

International Agency for Research on Cancer

World Health Organization

IARC MONOGRAPHS

In 1969, the International Agency for Research on Cancer (IARC) initiated a programme on the evaluation of the carcinogenic hazard of chemicals to humans, involving the production of critically evaluated monographs on individual chemicals. The programme was subsequently expanded to include evaluations of carcinogenic hazards 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 cancer hazard to humans with the help of international working groups of experts in carcinogenesis and related fields; and to identify gaps in evidence. The lists of IARC evaluations are regularly updated and are available on the internet at https://monographs.iarc.who.int/.

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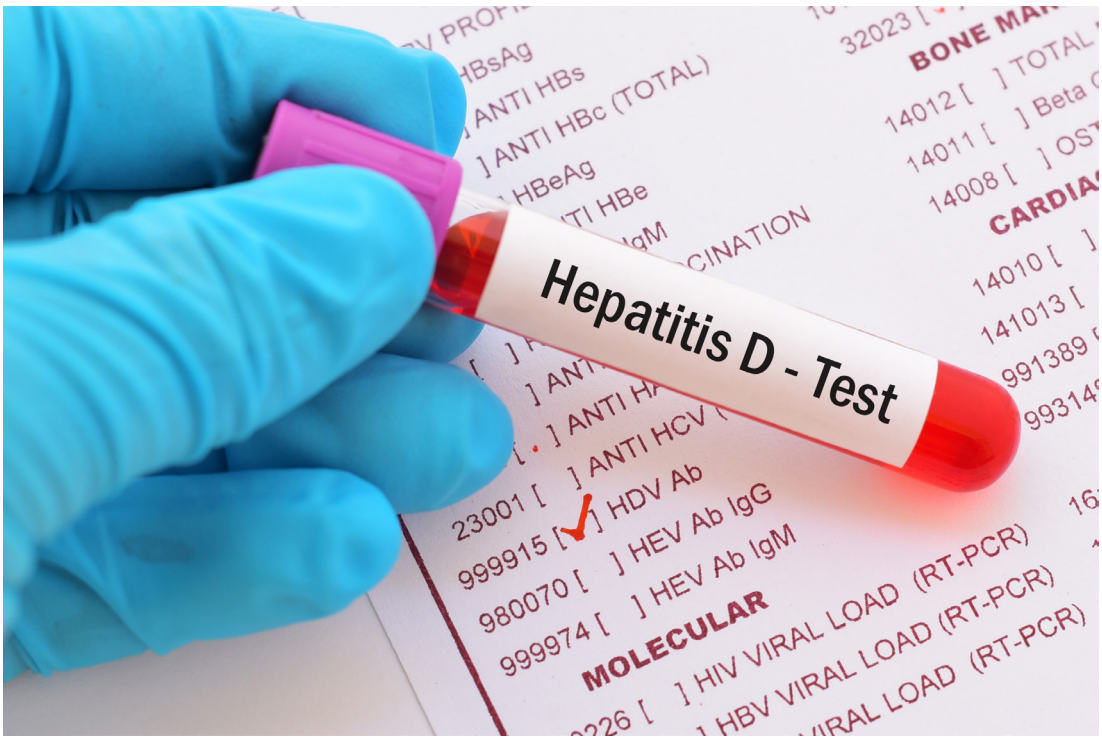

About the cover: The photo shows a requisition form and blood test for serum antibodies to hepatitis D virus antigen.

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NOTE TO THE READER

The evaluations of carcinogenic hazard in the *IARC Monographs on the Identification of Carcinogenic Hazards to Humans* series are made by international working groups of independent scientists. The *IARC Monographs* classifications do not indicate the level of risk associated with a given level or circumstance of exposure. The *IARC Monographs* do not make recommendations for regulation or legislation.

Anyone who is aware of published data that may alter the evaluation of the carcinogenic hazard of an agent to humans is encouraged to make this information available to the *IARC Monographs* programme, International Agency for Research on Cancer, 25 avenue Tony Garnier, CS 90627, 69366 Lyon Cedex 07, or via email at imo@iarc.who.int, 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 *IARC Monographs* programme. Corrigenda are published online on the relevant webpage for the volume concerned (IARC Publications: https://publications.iarc.who.int/).

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PREAMBLE

The Preamble to the *IARC Monographs* describes the objective and scope of the programme, general principles and procedures, and scientific review and evaluations. The *IARC Monographs* embody principles of scientific rigour, impartial evaluation, transparency, and consistency. The Preamble should be consulted when reading a *Monograph* or a summary of a *Monograph*'s evaluations. Separate Instructions for Authors describe the operational procedures for the preparation and publication of a volume of the *Monographs*.

A. GENERAL PRINCIPLES AND PROCEDURES

1. Background

Soon after the International Agency for Research on Cancer (IARC) was established in 1965, it started to receive frequent requests for advice on the carcinogenicity of chemicals, including requests for lists of established and suspected human carcinogens. In 1970, an 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 next year, the IARC Governing Council adopted a resolution that IARC should prepare "monographs on the evaluation of carcinogenic risk of chemicals to man", which became the initial title of the series.

In succeeding years, the scope of the programme broadened as *Monographs* were developed for complex mixtures, occupational exposures, physical agents, biological organisms, pharmaceuticals, and other exposures. In 1988, "of chemicals" was dropped from the title, and in 2019, "evaluation of carcinogenic risks" became "identification of carcinogenic hazards", in line with the objective of the programme.

Identifying the causes of human cancer is the first step in cancer prevention. The identification of a cancer hazard may have broad and profound implications. National and international authorities and organizations can and do use information on causes of cancer in support of actions to reduce exposure to carcinogens in the workplace, in the environment, and elsewhere. Cancer prevention is needed as much today as it was when IARC was established, because the global burden of cancer is high and continues to increase as a result of population growth and ageing and upward trends in some exposures, especially in low- and middle-income countries (https://publications.iarc.who.int/Non-Series-Publications/World-Cancer-Reports).

IARC's process for developing *Monographs*, which has evolved over several decades, involves

the engagement of international, interdisciplinary Working Groups of expert scientists, the transparent synthesis of different streams of evidence (exposure characterization, cancer in humans, cancer in experimental animals, and mechanisms of carcinogenesis), and the integration of these streams of evidence into an overall evaluation and classification according to criteria developed and refined by IARC. Since the *Monographs* programme was established, the understanding of carcinogenesis has greatly deepened. Scientific advances are incorporated into the evaluation methodology. In particular, strong mechanistic evidence has had an increasing role in the overall evaluations since 1991.

The Preamble is primarily a statement of the general principles and procedures used in developing a *Monograph*, to promote transparency and consistency across *Monographs* evaluations. In addition, IARC provides Instructions for Authors (https://monographs.iarc.who.int/preamble-instructions-for-authors/), which specify more detailed working procedures. IARC routinely updates these Instructions for Authors to reflect advances in methods for cancer hazard identification and accumulated experience, including input from experts.

2. Objective and scope

The objective of the programme is to prepare, with the engagement of international, interdisciplinary Working Groups of experts, scientific reviews and evaluations of evidence on the carcinogenicity of a wide range of agents.

The *Monographs* assess the strength of the available evidence that an agent can cause cancer in humans, based on three streams of evidence: on cancer in humans (see Part B, Section 2), on cancer in experimental animals (see Part B, Section 3), and on mechanistic evidence (see Part B, Section 4). In addition, the exposure to each agent is characterized (see Part B, Section 1). In this Preamble, the term "agent" refers to any chemical, physical, or biological entity or exposure circumstance (e.g. occupation as a painter) for which evidence on the carcinogenicity is evaluated.

A cancer *hazard* is an agent that is capable of causing cancer, whereas a cancer *risk* is an estimate of the probability that cancer will occur given some level of exposure to a cancer hazard. The *Monographs* assess the strength of evidence that an agent is a cancer hazard. The distinction between hazard and risk is fundamental. The *Monographs* identify cancer hazards even when risks appear to be low in some exposure scenarios. This is because the exposure may be widespread at low levels, and because exposure levels in many populations are not known or documented.

Although the *Monographs* programme has focused on hazard identification, some epidemiological studies used to identify a cancer hazard are also used to estimate an exposure–response relationship within the range of the available data. However, extrapolating exposure–response relationships beyond the available data (e.g. to lower exposures, or from experimental animals to humans) is outside the scope of *Monographs* Working Groups (IARC, 2014). In addition, the *Monographs* programme does not review quantitative risk characterizations developed by other health agencies.

The identification of a cancer hazard should trigger some action to protect public health, either directly as a result of the hazard identification or through the conduct of a risk assessment. Although such actions are outside the scope of the programme, the *Monographs* are used by national and international authorities and organizations to inform risk assessments, formulate decisions about preventive measures, motivate effective cancer control programmes, and choose among options for public health decisions. *Monographs* evaluations are only one part of the body of information on which decisions to control exposure to carcinogens may be based.

Options to prevent cancer vary from one situation to another and across geographical regions and take many factors into account, including different national priorities. Therefore, no recommendations are given in the *Monographs* with regard to regulation, legislation, or other policy approaches, which are the responsibility of individual governments or organizations. The *Monographs* programme also does not make research recommendations. However, it is important to note that *Monographs* contribute significantly to the science of carcinogenesis by synthesizing and integrating streams of evidence about carcinogenicity and pointing to critical gaps in knowledge.

3. Selection of agents for review

Since 1984, about every five years IARC convenes an international, interdisciplinary Advisory Group to recommend agents for review by the *Monographs* programme. IARC selects Advisory Group members who are knowledgeable about current research on carcinogens and public health priorities. Before an Advisory Group meets, IARC solicits nominations of agents from scientists and government agencies worldwide. Since 2003, IARC also invites nominations from the public. IARC charges each Advisory Group with reviewing nominations, evaluating exposure and hazard potential, and preparing a report that documents the Advisory Group's process for these activities and its rationale for the recommendations.

For each new volume of the *Monographs*, IARC selects the agents for review from those recommended by the most recent Advisory Group, considering the availability of pertinent research studies and current public health priorities. On occasion, IARC may select other agents if there is a need to rapidly evaluate an emerging carcinogenic hazard or an urgent need to re-evaluate a previous classification. All evaluations consider the full body of available evidence, not just information published after a previous review.

A *Monograph* may review:

(a) An agent not reviewed in a previous *Monograph*, if there is potential human exposure and there is evidence for assessing its carcinogenicity. A group of related agents (e.g. metal compounds) may be reviewed together if there is evidence for assessing carcinogenicity for one or more members of the group.

(b) An agent reviewed in a previous *Monograph*, if there is new evidence of cancer in humans or in experimental animals, or mechanistic evidence to warrant re-evaluation of the classification. In the interests of efficiency, the literature searches may build on previous comprehensive searches.

(c) An agent that has been established to be carcinogenic to humans and has been reviewed in a previous *Monograph*, if there is new evidence of cancer in humans that indicates new tumour sites where there might be a causal association. In the interests of efficiency, the review may focus on these new tumour sites.

4. The Working Group and other meeting participants

Five categories of participants can be present at *Monographs* meetings:

(i) *Working Group* members are responsible for all scientific reviews and evaluations developed in the volume of the *Monographs*. The Working Group is interdisciplinary and comprises subgroups of experts in the fields of (a) exposure characterization, (b) cancer in humans, (c) cancer in experimental animals, and (d) mechanistic evidence. IARC selects Working Group members on the basis of expertise related to the subject matter and relevant methodologies, and absence

of conflicts of interest. Consideration is also given to diversity in scientific approaches and views, as well as demographic composition. Working Group members generally have published research related to the exposure or carcinogenicity of the agents being reviewed, and IARC uses literature searches to identify most experts. Since 2006, IARC also has encouraged public nominations through its Call for Experts. IARC's reliance on experts with knowledge of the subject matter and/or expertise in methodological assessment is confirmed by decades of experience documenting that there is value in specialized expertise and that the overwhelming majority of Working Group members are committed to the objective evaluation of scientific evidence and not to the narrow advancement of their own research results or a pre-determined outcome (Wild and Cogliano, 2011). Working Group members are expected to serve the public health mission of IARC, and should refrain from consulting and other activities for financial gain that are related to the agents under review, or the use of inside information from the meeting, until the full volume of the *Monographs* is published.

IARC identifies, from among Working Group members, individuals to serve as Meeting Chair and Subgroup Chairs. At the opening of the meeting, the Working Group is asked to endorse the selection of the Meeting Chair, with the opportunity to propose alternatives. The Meeting Chair and Subgroup Chairs take a leading role at all stages of the review process (see Part A, Section 7), promote open scientific discussions that involve all Working Group members in accordance with normal committee procedures, and ensure adherence to the Preamble.

(ii) *Invited Specialists* are experts who have critical knowledge and experience but who also have a conflict of interest that warrants exclusion from developing or influencing the evaluations of carcinogenicity. Invited Specialists do not draft any section of the *Monograph* that pertains to the description or interpretation of cancer data, and they do not participate in the evaluations. These experts are invited in limited numbers when necessary to assist the Working Group by contributing their unique knowledge and experience to the discussions.

(iii) *Representatives of national and international health agencies* may attend because their agencies are interested in the subject of the meeting. They do not draft any section of the *Monograph* or participate in the evaluations.

(iv) *Observers* with relevant scientific credentials may be admitted in limited numbers. Attention is given to the balance of Observers from constituencies with differing perspectives. Observers are invited to observe the meeting and should not attempt to influence it, and they agree to respect the Guidelines for Observers at *IARC Monographs* meetings. Observers do not draft any section of the *Monograph* or participate in the evaluations.

(v) The *IARC Secretariat* consists of scientists who are designated by IARC and who have relevant expertise. The IARC Secretariat coordinates and facilitates all aspects of the evaluation and ensures adherence to the Preamble throughout development of the scientific reviews and classifications (see Part A, Sections 5 and 6). The IARC Secretariat organizes and announces the meeting, identifies and recruits the Working Group members, and assesses the declared interests of all meeting participants. The IARC Secretariat supports the activities of the Working Group (see Part A, Section 7) by searching the literature and performing title and abstract screening, organizing conference calls to coordinate the development of pre-meeting

Table 1 Roles of participants at *IARC Monographs* meetings

Category of participant	Role			
	Prepare text, tables, and analyses	Participate in discussions	Participate in evaluations	Eligible to serve as Chair
Working Group members	✓	✓	✓	✓
Invited Specialists	✓[a]	✓		
Representatives of health agencies		✓[b]		
Observers		✓[b]		
IARC Secretariat	✓[c]	✓	✓[d]	

[a] Only for the section on exposure characterization.
[b] Only at times designated by the Meeting Chair and Subgroup Chairs.
[c] When needed or requested by the Meeting Chair and Subgroup Chairs.
[d] Only for clarifying or interpreting the Preamble.

drafts and discuss cross-cutting issues, and reviewing drafts before and during the meeting. Members of the IARC Secretariat serve as meeting rapporteurs, assist the Meeting Chair and Subgroup Chairs in facilitating all discussions, and may draft text or tables when designated by the Meeting Chair and Subgroup Chairs. Their participation in the evaluations is restricted to the role of clarifying or interpreting the Preamble.

All participants are listed, with their principal affiliations, in the front matter of the published volume of the *Monographs*. Working Group members and Invited Specialists serve as individual scientists and not as representatives of any organization, government, or industry (Cogliano et al., 2004).

The roles of the meeting participants are summarized in Table 1.

5. Working procedures

A separate Working Group is responsible for developing each volume of the *Monographs*. A volume contains one or more *Monographs*, which can cover either a single agent or several related agents. Approximately one year before the meeting of a Working Group, a preliminary list of agents to be reviewed, together with a Call for Data and a Call for Experts, is announced on the *Monographs* programme website (https://monographs.iarc.who.int/).

Before a meeting invitation is extended, each potential participant, including the IARC Secretariat, completes the WHO Declaration of Interests form to report financial interests, employment and consulting (including remuneration for serving as an expert witness), individual and institutional research support, and non-financial interests such as public statements and positions related to the subject of the meeting. IARC assesses the declared interests to determine whether there is a conflict that warrants any limitation on participation (see Table 2).

Approximately two months before a *Monographs* meeting, IARC publishes the names and affiliations of all meeting participants together with a summary of declared interests, in the interests of transparency and to provide an opportunity for undeclared conflicts of interest to be brought to IARC's attention. 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).

The Working Group meets at IARC for approximately eight days to discuss and finalize the scientific review and to develop summaries

Table 2 Public engagement during *Monographs* development

Approximate timeframe	Engagement
Every 5 years	IARC convenes an Advisory Group to recommend high-priority agents for future review
~1 year before a *Monographs* meeting	IARC selects agents for review in a new volume of the *Monographs* IARC posts on its website: Preliminary List of Agents to be reviewed Call for Data and Call for Experts Request for Observer Status WHO Declaration of Interests form
~8 months before a *Monographs* meeting	Call for Experts closes
~4 months before a *Monographs* meeting	Request for Observer Status closes
~2 months before a *Monographs* meeting	IARC posts the names of all meeting participants together with a summary of declared interests, and a statement discouraging contact of the Working Group by interested parties
~1 month before a *Monographs* meeting	Call for Data closes
~2–4 weeks after a *Monographs* meeting	IARC publishes a summary of evaluations and key supporting evidence
~9 months after a *Monographs* meeting	IARC Secretariat publishes the verified and edited master copy of plenary drafts as a *Monographs* volume

and evaluations. At the opening of the meeting, all participants update their Declaration of Interests forms, which are then reviewed by IARC. Declared interests related to the subject of the meeting are disclosed to the meeting participants during the meeting and in the published volume (Cogliano et al., 2004). The objectives of the meeting are peer review and consensus. During the first part of the meeting, subgroup sessions (covering exposure characterization, cancer in humans, cancer in experimental animals, and mechanistic evidence) review the pre-meeting drafts, develop a joint subgroup draft, and draft subgroup summaries. During the last part of the meeting, the Working Group meets in plenary session to review the subgroup drafts and summaries and to develop the consensus evaluations. As a result, the entire volume is the joint product of the Working Group, and there are no individually authored sections. After the meeting, the master copy is verified by the IARC Secretariat and is then edited and prepared for publication. The aim is to publish the volume within approximately nine months of the Working Group meeting. A summary of the evaluations and key supporting evidence is prepared for publication in a scientific journal or is made available on the *Monographs* programme website soon after the meeting.

In the interests of transparency, IARC engages with the public throughout the process, as summarized in Table 2.

6. Overview of the scientific review and evaluation process

The Working Group considers all pertinent epidemiological studies, cancer bioassays in experimental animals, and mechanistic evidence, as well as pertinent information on exposure in humans. In general, for cancer in humans, cancer in experimental animals, and mechanistic evidence, only studies that have been published or accepted for publication in the openly available scientific literature are reviewed. Under some circumstances, materials that are publicly available and whose content is final may be reviewed if there is sufficient information to permit an evaluation of the quality of the methods and results of the studies (see Step 1,

below). Such materials may include reports and databases publicly available from government agencies, as well as doctoral theses. The reliance on published and publicly available studies promotes transparency and protects against citation of premature information.

The principles of systematic review are applied to the identification, screening, synthesis, and evaluation of the evidence related to cancer in humans, cancer in experimental animals, and mechanistic evidence (as described in Part B, Sections 2–4 and as detailed in the Instructions for Authors). Each *Monograph* specifies or references information on the conduct of the literature searches, including search terms and inclusion/exclusion criteria that were used for each stream of evidence.

In brief, the steps of the review process are as follows:

Step 1. Comprehensive and transparent identification of the relevant information: The IARC Secretariat identifies relevant studies through initial comprehensive searches of literature contained in authoritative biomedical databases (e.g. PubMed, PubChem) and through a Call for Data. These literature searches, designed in consultation with a librarian and other technical experts, address whether the agent causes cancer in humans, causes cancer in experimental systems, and/or exhibits key characteristics of established human carcinogens (in humans or in experimental systems). The Working Group provides input and advice to IARC to refine the search strategies, and identifies literature through other searches (e.g. from reference lists of past *Monographs*, retrieved articles, and other authoritative reviews).

For certain types of agents (e.g. regulated pesticides and pharmaceuticals), IARC also provides an opportunity to relevant regulatory authorities, and regulated parties through such authorities, to make pertinent unpublished studies publicly available by the date specified in the Call for Data. Consideration of such studies by the Working Group is dependent on the public availability of sufficient information to permit an independent evaluation of (a) whether there has been selective reporting (e.g. on outcomes, or from a larger set of conducted studies); (b) study quality (e.g. design, methodology, and reporting of results), and (c) study results.

Step 2. Screening, selection, and organization of the studies: The IARC Secretariat screens the retrieved literature for inclusion based on title and abstract review, according to pre-defined exclusion criteria. For instance, studies may be excluded if they were not about the agent (or a metabolite of the agent), or if they reported no original data on epidemiological or toxicological end-points (e.g. review articles). The Working Group reviews the title and abstract screening done by IARC, and performs full-text review. Any reasons for exclusion are recorded, and included studies are organized according to factors pertinent to the considerations described in Part B, Sections 2–4 (e.g. design, species, and endpoint). Inclusion of a study does not imply acceptance of the adequacy of the study design or of the analysis and interpretation of the results.

Step 3. Evaluation of study quality: The Working Group evaluates the quality of the included studies based on the considerations (e.g. design, methodology, and reporting of results) described in Part B, Sections 2–4. Based on these considerations, the Working Group may accord greater weight to some of the included studies. Interpretation of the results and the strengths and limitations of a study are clearly outlined in square brackets at the end of study descriptions (see Part B).

Step 4: *Report characteristics of included studies, including assessment of study*

quality: Pertinent characteristics and results of included studies are reviewed and succinctly described, as detailed in Part B, Sections 1–4. Tabulation of data may facilitate this reporting. This step may be iterative with Step 3.

Step 5: *Synthesis and evaluation of strength of evidence*: The Working Group summarizes the overall strengths and limitations of the evidence from the individual streams of evidence (cancer in humans, cancer in experimental animals, and mechanistic evidence; see Part B, Section 5). The Working Group then evaluates the strength of evidence from each stream of evidence by using the transparent methods and defined descriptive terms given in Part B, Sections 6a–c. The Working Group then develops, and describes the rationale for, the consensus classification of carcinogenicity that integrates the conclusions about the strength of evidence from studies of cancer in humans, studies of cancer in experimental animals, and mechanistic evidence (see Part B, Section 6d).

7. Responsibilities of the Working Group

The Working Group is responsible for identifying and evaluating the relevant studies and developing the scientific reviews and evaluations for a volume of the *Monographs*. The IARC Secretariat supports these activities of the Working Group (see Part A, Section 4). Briefly, the Working Group's tasks in developing the evaluation are, in sequence:

(i) Before the meeting, the Working Group ascertains that all appropriate studies have been identified and selected, and assesses the methods and quality of each individual study, as outlined above (see Part A, Section 6). The Working Group members prepare pre-meeting working drafts that present accurate tabular or textual summaries of informative studies by extracting key elements of the study design and results, and highlighting notable strengths and limitations. They participate in conference calls organized by IARC to coordinate the development of working drafts and to discuss cross-cutting issues. Pre-meeting reviews of all working drafts are generally performed by two or more subgroup members who did not participate in study identification, data extraction, or study review for the draft. Each study summary is written or reviewed by someone who is not associated with the study.

(ii) At the meeting, within subgroups, the Working Group members critically review, discuss, and revise the pre-meeting drafts and adopt the revised versions as consensus subgroup drafts. Subgroup Chairs ensure that someone who is not associated with the study leads the discussion of each study summary. A proposed classification of the strength of the evidence reviewed in the subgroup using the *IARC Monographs* criteria (see Part B, Sections 6a–c) is then developed from the consensus subgroup drafts of the evidence summaries (see Part B, Section 5).

(iii) During the plenary session, each subgroup presents its drafts for scientific review and discussion to the other Working Group members, who did not participate in study identification, data extraction, or study review for the drafts. Subgroup Chairs ensure that someone who is not associated with the study leads the discussion of each study summary. After review, discussion, and revisions as needed, the subgroup drafts are adopted as a consensus Working Group product. The summaries and classifications of the strength of the evidence, developed in the subgroup in line with the *IARC Monographs* criteria

(see Part B, Sections 6a–c), are considered, revised as needed, and adopted by the full Working Group. The Meeting Chair proposes an overall evaluation using the guidance provided in Part B, Section 6d.

The Working Group strives to achieve consensus evaluations. Consensus reflects broad agreement among the Working Group, but not necessarily unanimity. The Meeting Chair may poll the Working Group to determine the diversity of scientific opinion on issues where consensus is not apparent.

Only the final product of the plenary session represents the views and expert opinions of the Working Group. The entire *Monographs* volume is the joint product of the Working Group and represents an extensive and thorough peer review of the body of evidence (individual studies, synthesis, and evaluation) by an interdisciplinary expert group. Initial working papers and subsequent revisions are not released, because they would give an incomplete and possibly misleading impression of the consensus developed by the Working Group over a full week of deliberation.

B. SCIENTIFIC REVIEW AND EVALUATION

This part of the Preamble discusses the types of evidence that are considered and summarized in each section of a *Monograph*, followed by the scientific criteria that guide the evaluations. In addition, a section of General Remarks at the front of the volume discusses the reasons the agents were scheduled for evaluation and any key issues encountered during the meeting.

1. Exposure characterization

This section identifies the agent and describes its occurrence, main uses, and production locations and volumes, where relevant. It also summarizes the prevalence, concentrations in relevant studies, and relevant routes of exposure in humans worldwide. Methods of exposure measurement and analysis are described, and methods of exposure assessment used in key epidemiological studies reviewed by the Working Group are described and evaluated.

Over the course of the *Monographs* programme, concepts of exposure and dose have evolved substantially with deepening understanding of the interactions of agents and biological systems. The concept of exposure has broadened and become more holistic, extending beyond chemical, physical, and biological agents to stressors as construed generally, including psychosocial stressors (National Research Council, 2012; National Academies of Sciences, Engineering, and Medicine, 2017). Overall, this broader conceptualization supports greater integration between exposure characterization and other sections of the *Monographs*. Concepts of absorption, distribution, metabolism, and excretion are considered in the first subsection of mechanistic evidence (see Part B, Section 4a), whereas validated biomarkers of internal exposure or metabolites that are routinely used for exposure assessment are reported on in this section (see Part B, Section 1b).

(a) Identification of the agent

The agent being evaluated is unambiguously identified. Details will vary depending on the type of agent but will generally include physical and chemical properties relevant to the agent's identification, occurrence, and biological activity. If the material that has been tested in experimental animals or in vitro systems is different from that to which humans are exposed, these differences are noted.

For chemical agents, the Chemical Abstracts Service Registry Number is provided, as well as the latest primary name and other names in common use, including important trade names,

along with available information on the composition of common mixtures or products containing the agent, and potentially toxic and/or carcinogenic impurities. Physical properties relevant to understanding the potential for human exposure and measures of exposure used in studies in humans are summarized. These might include physical state, volatility, aqueous and fat solubility, and half-life in the environment and/or in human tissues.

For biological agents, taxonomy and structure are described. Mode of replication, life-cycle, target cells, persistence, latency, and host responses, including morbidity and mortality through pathologies other than cancer, are also presented.

For foreign bodies, fibres and particles, composition, size range, relative dimensions, and accumulation, persistence, and clearance in target organs are summarized. Physical agents that are forms of radiation are described in terms of frequency spectrum and energy transmission.

Exposures may result from, or be influenced by, a diverse range of social and environmental factors, including components of diet, sleep, and physical activity patterns. In these instances, this section will include a description of the agent, its variability across human populations, and its composition or characteristics relevant to understanding its potential carcinogenic hazard to humans and to evaluating exposure assessments in epidemiological studies.

(b) Detection and analysis

Key methods of detection and quantification of the agent are presented, with an emphasis on those used most widely in surveillance, regulation, and epidemiological studies. Measurement methods for sample matrices that are deemed important sources of human exposure (e.g. air, drinking-water, food, residential dust) and for validated exposure biomarkers (e.g. the agent or its metabolites in human blood, urine, or saliva) are described. Information on detection and quantification limits is provided when it is available and is useful for interpreting studies in humans and in experimental animals. This is not an exhaustive treatise but is meant to help readers understand the strengths and limitations of the available exposure data and of the epidemiological studies that rely on these measurements.

(c) Production and use

Historical and geographical patterns and trends in production and use are included when they are available, to help readers understand the contexts in which exposures may occur, both within key epidemiological studies reviewed by the Working Group and in human populations generally. Industries that produce, use, or dispose of the agent are described, including their global distribution, when available. National or international listing as a high-production-volume chemical or similar classification may be included. Production processes with significant potential for occupational exposure or environmental pollution are indicated. Trends in global production volumes, technologies, and other data relevant to understanding exposure potential are summarized. Minor or historical uses with significant exposure potential or with particular relevance to key epidemiological studies are included. Particular effort may be directed towards finding data on production in low- and middle-income countries, where rapid economic development may lead to higher exposures than those in high-income countries.

(d) Exposure

A concise overview of quantitative information on sources, prevalence, and levels of exposure in humans is provided. Representative data from research studies, government reports and websites, online databases, and other citable, publicly available sources are tabulated. Data

from low- and middle-income countries are sought and included to the extent feasible; information gaps for key regions are noted. Naturally occurring sources of exposure, if any, are noted. Primary exposure routes (e.g. inhalation, ingestion, skin uptake) and other considerations relevant to understanding the potential for cancer hazard from exposure to the agent are reported.

For occupational settings, information on exposure prevalence and levels (e.g. in air or human tissues) is reported by industry, occupation, region, and other characteristics (e.g. process, task) where feasible. Information on historical exposure trends, protection measures to limit exposure, and potential co-exposures to other carcinogenic agents in workplaces is provided when available.

For non-occupational settings, the occurrence of the agent is described with environmental monitoring or surveillance data. Information on exposure prevalence and levels (e.g. concentrations in human tissues) as well as exposure from and/or concentrations in food and beverages, consumer products, consumption practices, and personal microenvironments is reported by region and other relevant characteristics. Particular importance is placed on describing exposures in life stages or in states of disease or nutrition that may involve greater exposure or susceptibility.

Current exposures are of primary interest; however, information on historical exposure trends is provided when available. Historical exposures may be relevant for interpreting epidemiological studies, and when agents are persistent or have long-term effects. Information gaps for important time periods are noted. Exposure data that are not deemed to have high relevance to human exposure are generally not considered.

(e) Regulations and guidelines

Regulations or guidelines that have been established for the agent (e.g. occupational exposure limits, maximum permitted levels in foods and water, pesticide registrations) are described in brief to provide context about government efforts to limit exposure; these may be tabulated if they are informative for the interpretation of existing or historical exposure levels. Information on applicable populations, specific agents concerned, basis for regulation (e.g. human health risk, environmental considerations), and timing of implementation may be noted. National and international bans on production, use, and trade are also indicated.

This section aims to include major or illustrative regulations and may not be comprehensive, because of the complexity and range of regulatory processes worldwide. An absence of information on regulatory status should not be taken to imply that a given country or region lacks exposure to, or regulations on exposure to, the agent.

(f) Critical review of exposure assessment in key epidemiological studies

Epidemiological studies evaluate cancer hazard by comparing outcomes across differently exposed groups. Therefore, the type and quality of the exposure assessment methods used are key considerations when interpreting study findings for hazard identification. This section summarizes and critically reviews the exposure assessment methods used in the individual epidemiological studies that contribute data relevant to the *Monographs* evaluation.

Although there is no standard set of criteria for evaluating the quality of exposure assessment methods across all possible agents, some concepts are universally relevant. Regardless of the agent, all exposures have two principal dimensions: intensity (sometimes defined as concentration or dose) and time. Time considerations include

duration (time from first to last exposure), pattern or frequency (whether continuous or intermittent), and windows of susceptibility. This section considers how each of the key epidemiological studies characterizes these dimensions. Interpretation of exposure information may also be informed by consideration of mechanistic evidence (e.g. as described in Part B, Section 4a), including the processes of absorption, distribution, metabolism, and excretion.

Exposure intensity and time in epidemiological studies can be characterized by using environmental or biological monitoring data, records from workplaces or other sources, expert assessments, modelled exposures, job-exposure matrices, and subject or proxy reports via questionnaires or interviews. Investigators use these data sources and methods individually or in combination to assign levels or values of an exposure metric (which may be quantitative, semi-quantitative, or qualitative) to members of the population under study.

In collaboration with the Working Group members reviewing human studies (of cancer and of mechanisms), key epidemiological studies are identified. For each selected study, the exposure assessment approach, along with its strengths and limitations, is summarized using text and tables. Working Group members identify concerns about exposure assessment methods and their impacts on overall quality for each study reviewed (see Part B, Sections 2d and 4d). In situations where the information provided in the study is inadequate to properly consider the exposure assessment, this is indicated. When adequate information is available, the likely direction of bias due to error in exposure measurement, including misclassification (overestimated effects, underestimated effects, or unknown) is discussed.

2. Studies of cancer in humans

This section includes all pertinent epidemiological studies (see Part B, Section 2b) that include cancer as an outcome. These studies encompass certain types of biomarker studies, for example, studies with biomarkers as exposure metrics (see Part B, Section 2) or those evaluating histological or tumour subtypes and molecular signatures in tumours consistent with a given exposure (Alexandrov et al., 2016). Studies that evaluate early biological effect biomarkers are reviewed in Part B, Section 4.

(a) Types of study considered

Several types of epidemiological studies contribute to the assessment of carcinogenicity in humans; they typically include cohort studies (including variants such as case–cohort and nested case–control studies), case–control studies, ecological studies, and intervention studies. Rarely, results from randomized trials may be available. Exceptionally, case reports and case series of cancer in humans may also be reviewed. In addition to these designs, innovations in epidemiology allow for many other variants that may be considered in any given *Monographs* evaluation.

Cohort and case–control studies typically have the capacity to 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. Well-conducted cohort and case–control studies provide most of the evidence of cancer in humans evaluated by Working Groups. Intervention studies are much less common, but when available can provide strong evidence for making causal inferences.

In ecological 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 in the population under study. In ecological studies, data on individual exposure and outcome are not available, which renders this type of study more prone to confounding and exposure misclassification. In some circumstances, however, ecological studies may be informative, especially when the unit of exposure is most accurately measured at the population level (see, for example, the *Monograph* on arsenic in drinking-water; IARC, 2004).

Exceptionally, case reports and case series may provide compelling evidence about the carcinogenicity of an agent. In fact, many of the early discoveries of occupational cancer hazards came about because of observations by workers and their clinicians, who noted a high frequency of cancer in workers who share a common occupation or exposure. Such observations may be the starting point for more structured investigations, but in exceptional circumstances, when the risk is high enough, the case series may in itself provide compelling evidence. This would be especially warranted in situations where the exposure circumstance is fairly unusual, as it was in the example of plants containing aristolochic acid (IARC, 2012a).

The uncertainties that surround the interpretation of case reports, case series, and ecological studies typically make them inadequate, except in rare instances as described above, to form the sole basis for inferring a causal relationship. However, when considered together with cohort and case–control studies, these types of study may support the judgement that a causal relationship exists.

Epidemiological studies of benign neoplasms, pre-neoplastic lesions, malignant precursors, and other end-points are also reviewed when they relate to the agents reviewed. On occasion they can strengthen inferences drawn from studies of cancer itself. For example, benign brain tumours may share common risk factors with those that are malignant, and benign neoplasms (or those of uncertain behaviour) may be part of the causal path to malignancies (e.g. myelodysplastic syndromes, which may progress to acute myeloid leukaemia).

(b) Identification of eligible studies of cancer in humans

Relevant studies of cancer in humans are identified by using systematic review principles as described in Part A, further elaborated in the Instructions for Authors, and as detailed below. Eligible studies include all studies in humans of exposure to the agent of interest with cancer as an outcome. Multiple publications on the same study population are identified so that the number of independent studies is accurately represented. Multiple publications may result, for example, from successive follow-ups of a single cohort, from analyses focused on different aspects of an exposure–disease association, or from inclusion of overlapping populations. Usually in such situations, only the most recent, most comprehensive, or most informative report is reviewed in detail.

(c) Assessment of study quality and informativeness

Epidemiological studies are potentially susceptible to several different sources of error, summarized briefly below. Qualities of individual studies that address these issues are also described below.

Study quality is assessed as part of the structured expert review process undertaken by the Working Group. A key aspect of quality assessment is consideration of the possible roles of chance and bias in the interpretation of epidemiological studies. Chance, which is also called random variation, can produce misleading study results. This variability in study results is strongly influenced by the sample size: smaller studies are more likely than larger studies to have effect estimates that are imprecise. Confidence intervals

around a study's point estimate of effect are used routinely to indicate the range of values of the estimate that could easily be produced by chance alone.

Bias is the effect of factors in study design or conduct that lead an association to erroneously appear stronger or weaker than the association that really exists between the agent and the disease. Biases that require consideration are varied but are usually categorized as selection bias, information bias (e.g. error in measurement of exposure and diseases), and confounding (or confounding bias) (Rothman et al., 2008). Selection bias in an epidemiological study occurs when inclusion of participants from the eligible population or their follow-up in the study is influenced by their exposure or their outcome (usually disease occurrence). Under these conditions, the measure of association found in the study will not accurately reflect the association that would otherwise have been found in the eligible population (Hernán et al., 2004). Information bias results from inaccuracy in exposure or outcome measurement. Both can cause an association between hypothesized cause and effect to appear stronger or weaker than it really is. Confounding is a mixing of extraneous effects with the effects of interest (Rothman et al., 2008). An association between the purported causal factor and another factor that is associated with an increase or decrease in incidence of disease can lead to a spurious association or absence of a real association of the presumed causal factor with the disease. When either of these occurs, confounding is present.

In assessing study quality, the Working Group consistently considers the following aspects:

- **Study description:** Clarity in describing the study design and its implementation, and the completeness of reporting of all other key information about the study and its results.
- **Study population:** Whether the study population was appropriate for evaluating the association between the agent and cancer. Whether the study was designed and carried out to minimize selection bias. Cancer cases in the study population must have been identified in a way that was independent of the exposure of interest, and exposure assessed in a way that was not related to disease (outcome) status. In these respects, completeness of recruitment into the study from the population of interest and completeness of follow-up for the outcome are essential measures.
- **Outcome measurement:** The appropriateness of the cancer outcome measure (e.g. mortality vs incidence) for the agent and cancer type under consideration, outcome ascertainment methodology, and the extent to which outcome misclassification may have led to bias in the measure(s) of association.
- **Exposure measurement:** The adequacy of the methods used to assess exposure to the agent, and the likelihood (and direction) of bias in the measure(s) of association due to error in exposure measurement, including misclassification (as described in Part B, Section 1f).
- **Assessment of potential confounding:** To what extent the authors took into account in the study design and analysis other variables (including co-exposures, as described in Part B, Section 1d) that can influence the risk of disease and may have been related to the exposure of interest. Important sources of potential confounding by such variables should have been addressed either in the design of the study, such as by matching or restriction, or in the analysis, by statistical adjustment. In some instances, where direct information on confounders is unavailable, use of indirect methods to evaluate the potential impact of confounding on exposure–disease associations is appropriate (e.g. Axelson and Steenland, 1988; Richardson et al., 2014).

- **Other potential sources of bias:** Each epidemiological study is unique in its study population, its design, its data collection, and, consequently, its potential biases. All possible sources of bias are considered for their possible impact on the results. The possibility of reporting bias (i.e. selective reporting of some results and the suppression of others) should be explored.
- **Statistical methodology:** Adequacy of the statistical methods used and their ability to obtain unbiased estimates of exposure–outcome associations, confidence intervals, and test statistics for the significance of measures of association. Appropriateness of methods used to investigate confounding, including adjusting for matching when necessary and avoiding treatment of probable mediating variables as confounders. Detailed analyses of cancer risks in relation to summary measures of exposure such as cumulative exposure, or temporal variables such as age at first exposure or time since first exposure, are reviewed and summarized when available.

For the sake of economy and simplicity, in this Preamble the list of possible sources of error is referred to with the phrase "chance, bias, and confounding", but it should be recognized that this phrase encompasses a comprehensive set of concerns pertaining to study quality.

These sources of error do not constitute and should not be used as a formal checklist of indicators of study quality. The judgement of experienced experts is critical in determining how much weight to assign to different issues in considering how all of these potential sources of error should be integrated and how to rate the potential for error related to each of these considerations.

The informativeness of a study is its ability to show a true association, if there is one, between the agent and cancer, and the lack of an association, if no association exists. Key determinants of informativeness include: having a study population of sufficient size to obtain precise estimates of effect; sufficient elapsed time from exposure to measurement of outcome for an effect, if present, to be observable; presence of an adequate exposure contrast (intensity, frequency, and/or duration); biologically relevant definitions of exposure; and relevant and well-defined time windows for exposure and outcome.

(d) Meta-analyses and pooled analyses

Independent epidemiological studies of the same agent may lead to inconsistent results that are difficult to interpret or reconcile. Combined analyses of data from multiple studies may be conducted as a means to address this ambiguity. 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 and O'Rourke, 2008).

The strengths of combined analyses are increased precision because of increased sample size and, in the case of pooled analyses, the opportunity to better control for potential confounders and to explore in more detail interactions and modifying effects that may explain heterogeneity among studies. A disadvantage of combined analyses is the possible lack of comparability of data from various studies, because of differences in population characteristics, subject recruitment, procedures of data collection, methods of measurement, and effects of unmeasured covariates that may differ among studies. These differences in study methods and quality can influence results of either meta-analyses or pooled analyses. If published meta-analyses are to be considered by the Working Group, their adequacy needs to be carefully evaluated, including the methods used to identify eligible studies

and the accuracy of data extracted from the individual studies.

The Working Group may conduct ad hoc meta-analyses during the course of a *Monographs* meeting, when there are sufficient studies of an exposure–outcome association to contribute to the Working Group's assessment of the association. The results of such unpublished 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, the following key considerations apply: the same criteria for data quality must be applied as for individual studies; sources of heterogeneity among studies must be carefully considered; and the possibility of publication bias should be explored.

(e) Considerations in assessing the body of epidemiological evidence

The ability of the body of epidemiological evidence to inform the Working Group about the carcinogenicity of the agent is related to both the quantity and the quality of the evidence. There is no formulaic answer to the question of how many studies of cancer in humans are needed from which to draw inferences about causality, although more than a single study in a single population will almost always be needed. The number will depend on the considerations relating to evidence described below.

After the quality of individual epidemiological studies of cancer has been assessed and the informativeness of the various studies on the association between the agent and cancer has been evaluated, a judgement is made about the strength of evidence that the agent in question is carcinogenic to humans. In making its judgement, the Working Group considers several aspects of the body of evidence (e.g. Hill, 1965; Rothman et al., 2008; Vandenbroucke et al., 2016).

A strong association (e.g. a large relative risk) is more likely to indicate causality than is a weak association, because it is more difficult for confounding to falsely create a strong association. However, it is recognized that estimates of effect of small magnitude do not imply lack of causality and may have impact on public health if the disease or exposure is common. Estimates of effect of small magnitude could also contribute useful information to the assessment of causality if level of risk is commensurate with level of exposure when compared with risk estimates from populations with higher exposure (e.g. as seen in residential radon studies compared with studies of radon from uranium mining).

Associations that are consistently observed in several studies of the same design, or in studies that use different epidemiological approaches, or under different circumstances of exposure are more likely to indicate a causal relationship than are isolated observations from single studies. If there are inconsistent results among investigations, possible reasons are sought (e.g. differences in study informativeness because of latency, exposure levels, or assessment methods). Results of studies that are judged to be of high quality and informativeness are given more weight than those of studies judged to be methodologically less sound or less informative.

Temporality of the association is an essential consideration: that is, the exposure must precede the outcome.

An observation that cancer risk increases with increasing exposure is considered to be a strong indication of causality, although the absence of a graded response is not necessarily evidence against a causal relationship, and there are several reasons why the shape of the exposure–response association may be non-monotonic (e.g. Stayner et al., 2003). 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.

Confidence in a causal interpretation of the evidence from studies of cancer in humans is enhanced if it is coherent with physiological and biological knowledge, including information about exposure to the target organ, latency and timing of the exposure, and characteristics of tumour subtypes.

The Working Group considers whether there are subpopulations with increased susceptibility to cancer from the agent. For example, molecular epidemiology studies that identify associations between genetic polymorphisms and inter-individual differences in cancer susceptibility to the agent(s) being evaluated may contribute to the identification of carcinogenic hazards to humans. Such studies may be particularly informative if polymorphisms are found to be modifiers of the exposure–response association, because evaluation of polymorphisms may increase the ability to detect an effect in susceptible subpopulations.

When, in the process of evaluating the studies of cancer in humans, the Working Group identifies several high-quality, informative epidemiological studies that clearly show either no positive association or an inverse association between an exposure and a specific type of cancer, a judgement may be made that, in the aggregate, they suggest evidence of lack of carcinogenicity for that cancer type. Such a judgement requires, first, that the studies strictly meet 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 ruled out with reasonable confidence. In addition, all studies that are judged to be methodologically sound should (a) be consistent with an estimate of relative effect of unity (or below unity) for any observed level of exposure, (b) when considered together, provide a combined estimate of relative risk that is at or below unity, and (c) have a narrow confidence interval. Moreover, neither any individual well-designed and well-conducted 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 must be noted that evidence of lack of carcinogenicity obtained from several epidemiological studies can apply only to the type(s) of cancer studied, to the exposure levels reported and the timing and route of exposure studied, to the intervals between first exposure and disease onset observed in these studies, and to the general population(s) studied (i.e. there may be susceptible subpopulations or life stages). Experience from studies of cancer in humans indicates that the period from first exposure to the development of clinical cancer is sometimes longer than 20 years; therefore, latency periods substantially shorter than about 30 years cannot provide evidence of lack of carcinogenicity. Furthermore, there may be critical windows of exposure, for example, as with diethylstilboestrol and clear cell adenocarcinoma of the cervix and vagina (IARC, 2012a).

3. Studies of cancer in experimental animals

Most human carcinogens that have been studied adequately for carcinogenicity in experimental animals have produced positive results in one or more animal species. For some agents, carcinogenicity in experimental animals was demonstrated before epidemiological studies identified their carcinogenicity in humans. Although this observation 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) present a carcinogenic hazard to humans. Accordingly, in the absence of additional scientific information, such as strong evidence that a given agent causes cancer in

experimental animals through a species-specific mechanism that does not operate in humans (see Part B, Sections 4 and 6; Capen et al., 1999; IARC, 2003), these agents are considered to pose a potential carcinogenic hazard to humans. The inference of potential carcinogenic hazard to humans does not imply tumour site concordance across species (Baan et al., 2019).

(a) Types of studies considered

Relevant studies of cancer in experimental animals are identified by using systematic review principles as described in Part A, further elaborated in the Instructions for Authors, and as detailed below. Consideration is given to all available long-term studies of cancer in experimental animals with the agent under review (or possibly metabolites or derivatives of the agent) (see Part A, Section 7) after a thorough evaluation of the study features (see Part B, Section 3b). Those studies 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, 2018).

In addition to conventional long-term bioassays, alternative studies (e.g. in genetically engineered mouse models) may be considered in assessing carcinogenicity in experimental animals, also after a critical evaluation of the study features. For studies of certain exposures, such as viruses that typically only infect humans, use of such specialized experimental animal models may be particularly important; models include genetically engineered mice with targeted expression of viral genes to tissues from which human cancers arise, as well as humanized mice implanted with the human cells usually infected by the virus.

Other types of studies can provide supportive evidence. These include: experiments in which the agent was administered in the presence of factors that modify carcinogenic effects (e.g. initiation–promotion studies); studies in which the end-point was not cancer but a defined precancerous lesion; and studies of cancer in non-laboratory animals (e.g. companion animals) exposed to the agent.

(b) Study evaluation

Considerations of importance in the interpretation and evaluation of a particular study include: (i) whether the agent was clearly characterized, including the nature and extent of impurities and contaminants and the stability of the agent, and, in the case of mixtures, whether the sample characterization was adequately reported; (ii) whether the dose was monitored adequately, particularly in inhalation experiments; (iii) whether the doses, duration and frequency of treatment, duration of observation, and route of exposure were appropriate; (iv) whether appropriate experimental animal species and strains were evaluated; (v) whether there were adequate numbers of animals per group; (vi) whether animals were allocated randomly to groups; (vii) whether the body weight, food and water consumption, and survival of treated animals were affected by any factors other than the test agent; (viii) whether the histopathology review was adequate; and (ix) whether the data were reported and analysed adequately.

(c) Outcomes and statistical analyses

An assessment of findings of carcinogenicity in experimental animals involves consideration of (i) study features such as route, doses, schedule and duration of exposure, species, strain (including genetic background where applicable), sex, age, and duration of follow-up; (ii) the spectrum of neoplastic response, from pre-neoplastic lesions and benign tumours to malignant neoplasms; (iii) the incidence, latency, severity, and multiplicity of neoplasms and pre-neoplastic

lesions; (iv) the consistency of the results for a specific target organ or organs across studies of similar design; and (v) the possible role of modifying factors (e.g. diet, infection, stress).

Key factors for statistical analysis include: (i) number of animals studied and number examined histologically, (ii) number of animals with a given tumour type or lesion, and (iii) duration of survival.

Benign tumours may be combined with malignant tumours in the assessment of tumour incidence when (a) they occur together with and originate from the same cell type as malignant tumours in an organ or tissue in a particular study and (b) they appear to represent a stage in the progression to malignancy (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.

Evidence of an increased incidence of neoplasms with increasing level of exposure strengthens the inference of a causal association between the exposure and the development of neoplasms. The form of the dose–response relationship can vary widely, including non-linearity, depending on the particular agent under study and the target organ. The dose–response relationship can also be affected by differences in survival among the treatment groups.

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 and Bailer, 1989; Bieler and Williams, 1993). The choice of the most appropriate statistical method requires consideration of whether 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 and a survival-adjusted analysis would be warranted. 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 that 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–Haenszel 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).

The concurrent control group is generally the most appropriate comparison group for statistical analysis; however, for uncommon tumours, 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, sex, 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). 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.

Meta-analyses and pooled analyses may be appropriate when the experimental protocols are sufficiently similar.

4. Mechanistic evidence

Mechanistic data may provide evidence of carcinogenicity and may also help in assessing the relevance and importance of findings of cancer in experimental animals and in humans (Guyton et al., 2009; Parkkinen et al., 2018) (see Part B, Section 6). Mechanistic studies have gained in prominence, increasing in their volume, diversity, and relevance to cancer hazard evaluation, whereas studies pertinent to other streams of evidence evaluated in the *Monographs* (i.e. studies of cancer in humans and lifetime cancer bioassays in rodents) may only be available for a fraction of agents to which humans are currently exposed (Guyton et al., 2009, 2018). Mechanistic studies and data are identified, screened, and evaluated for quality and importance to the evaluation by using systematic review principles as described in Part A, further elaborated in the Instructions for Authors, and as detailed below.

The Working Group's synthesis reflects the extent of available evidence, summarizing groups of included studies with an emphasis on characterizing consistencies or differences in results within and across experimental designs. Greater emphasis is given to informative mechanistic evidence from human-related studies than to that from other experimental test systems, and gaps are identified. Tabulation of data may facilitate this review. The specific topics addressed in the evidence synthesis are described below.

(a) Absorption, distribution, metabolism, and excretion

Studies of absorption, distribution, metabolism, and excretion in mammalian species are addressed in a summary fashion; exposure characterization is addressed in Part B, Section 1. The Working Group describes the metabolic fate of the agent in mammalian species, noting the metabolites that have been identified and their chemical reactivity. A metabolic schema may indicate the relevant metabolic pathways and products and whether supporting evidence is from studies in humans and/or studies in experimental animals. Evidence on other adverse effects that indirectly confirm absorption, distribution, and/or metabolism at tumour sites is briefly summarized when direct evidence is sparse.

(b) Evidence relevant to key characteristics of carcinogens

A review of Group 1 human carcinogens classified up to and including *IARC Monographs* Volume 100 revealed several issues relevant to improving the evaluation of mechanistic evidence for cancer hazard identification (Smith et al., 2016). First, it was noted that human carcinogens often share one or more characteristics that are related to the multiple mechanisms by which agents cause cancer. Second, different human carcinogens may exhibit a different spectrum of these key characteristics and operate through distinct mechanisms. Third, for many carcinogens evaluated before Volume 100, few data were available on some mechanisms of recognized importance in carcinogenesis, such as epigenetic alterations (Herceg et al., 2013). Fourth, there was no widely accepted method to search systematically for relevant mechanistic evidence, resulting in a lack of uniformity in the scope of mechanistic topics addressed across *IARC Monographs* evaluations.

To address these challenges, the key characteristics of human carcinogens were introduced to facilitate systematic consideration of mechanistic evidence in *IARC Monographs* evaluations (Smith et al., 2016; Guyton et al., 2018). The key characteristics described by Smith et al. (2016) (see Table 3), such as "is genotoxic", "is immunosuppressive", or "modulates receptor-mediated effects", are based on empirical observations of the chemical and biological properties associated with the human carcinogens identified by

Table 3 The key characteristics of carcinogens

	Ten key characteristics of carcinogens
1.	Is electrophilic or can be metabolically activated to an electrophile
2.	Is genotoxic
3.	Alters DNA repair or causes genomic instability
4.	Induces epigenetic alterations
5.	Induces oxidative stress
6.	Induces chronic inflammation
7.	Is immunosuppressive
8.	Modulates receptor-mediated effects
9.	Causes immortalization
10.	Alters cell proliferation, cell death, or nutrient supply

From Smith et al. (2016).

the *IARC Monographs* programme up to and including Volume 100. The list of key characteristics and associated end-points may evolve, based on the experience of their application and as new human carcinogens are identified. Key characteristics are distinct from the "hallmarks of cancer", which relate to the properties of cancer cells (Hanahan and Weinberg, 2000, 2011). Key characteristics are also distinct from hypothesized mechanistic pathways, which describe a sequence of biological events postulated to occur during carcinogenesis. As such, the evaluation approach based on key characteristics, outlined below, "avoids a narrow focus on specific pathways and hypotheses and provides for a broad, holistic consideration of the mechanistic evidence" (National Academies of Sciences, Engineering, and Medicine, 2017).

Studies in exposed humans and in human primary cells or tissues that incorporate end-points relevant to key characteristics of carcinogens are emphasized when available. For each key characteristic with adequate evidence for evaluation, studies are grouped according to whether they involve (a) humans or human primary cells or tissues or (b) experimental systems; further organization (as appropriate) is by end-point (e.g. DNA damage), duration, species, sex, strain, and target organ as well as strength of study design. Studies investigating susceptibility related to key characteristics of carcinogens (e.g. of genetic polymorphisms, or in genetically engineered animals) can be highlighted and may provide additional support for conclusions on the strength of evidence. Findings relevant to a specific tumour type may be noted.

(c) Other relevant evidence

Other informative evidence may be described when it is judged by the Working Group to be relevant to an evaluation of carcinogenicity and to be of sufficient importance to affect the overall evaluation. Quantitative structure–activity information, such as on specific chemical and/or biological features or activities (e.g. electrophilicity, molecular docking with receptors), may be informative. In addition, evidence that falls outside of the recognized key characteristics of carcinogens, reflecting emerging knowledge or important novel scientific developments on carcinogen mechanisms, may also be included. Available evidence relevant to criteria provided in authoritative publications (e.g. Capen et al., 1999; IARC, 2003) on thyroid, kidney, urinary bladder, or other tumours in experimental animals induced by mechanisms that do not operate in humans is also described.

(d) Study quality and importance to the evaluation

Based on formal considerations of the quality of the studies (e.g. design, methodology, and reporting of results), the Working Group may give greater weight to some included studies.

For observational and other studies in humans, the quality of study design, exposure assessment, and assay accuracy and precision are considered, in collaboration with the Working Group members reviewing exposure characterization and studies of cancer in humans, as are other important factors, including those described above for evaluation of epidemiological evidence (García-Closas et al., 2006, 2011; Vermeulen et al., 2018) (Part B, Sections 1 and 2).

In general, in experimental systems, studies of repeated doses and of chronic exposures are accorded greater importance than are studies of a single dose or time-point. Consideration is also given to factors such as the suitability of the dosing range, the extent of concurrent toxicity observed, and the completeness of reporting of the study (e.g. the source and purity of the agent, the analytical methods, and the results). Route of exposure is generally considered to be a less important factor in the evaluation of experimental studies, recognizing that the exposures and target tissues may vary across experimental models and in exposed human populations. Non-mammalian studies can be synthetically summarized when they are considered to be supportive of evidence in humans or higher organisms.

In vitro test systems can provide mechanistic insights, but important considerations include the limitations of the test system (e.g. in metabolic capabilities) as well as the suitability of a particular test article (i.e. because of physical and chemical characteristics) (Hopkins et al., 2004). For studies on some end-points, such as for traditional studies of mutations in bacteria and in mammalian cells, formal guidelines, including those from the Organisation for Economic Co-operation and Development, may be informative in conducting the quality review (OECD, 1997, 2016a, b). However, existing guidelines will not generally cover all relevant assays, even for genotoxicity. Possible considerations when evaluating the quality of in vitro studies encompass the methodology and design (e.g. the end-point and test method, the number of replicate samples, the suitability of the concentration range, the inclusion of positive and negative controls, and the assessment of cytotoxicity) as well as reporting (e.g. of the source and purity of the agent, and of the analytical methods and results). High-content and high-throughput in vitro data can serve as an additional or supportive source of mechanistic evidence (Chiu et al., 2018; Guyton et al., 2018), although large-scale screening programmes measuring a variety of end-points were designed to evaluate large chemical libraries in order to prioritize chemicals for additional toxicity testing rather than to identify the hazard of a specific chemical or chemical group.

The synthesis is focused on the evidence that is most informative for the overall evaluation. In this regard, it is of note that some human carcinogens exhibit a single or primary key characteristic, evidence of which has been influential in their cancer hazard classifications. For instance, ethylene oxide is genotoxic (IARC, 1994), 2,3,7,8-tetrachlorodibenzo-*para*-dioxin modulates receptor-mediated effects (IARC, 1997), and etoposide alters DNA repair (IARC, 2012a). Similarly, oncogenic viruses cause immortalization, and certain drugs are, by design, immunosuppressive (IARC, 2012a, b). Because non-carcinogens can also induce oxidative stress, this key characteristic should be interpreted with caution unless it is found in combination with other key characteristics (Guyton et al., 2018). Evidence for a group of key characteristics can strengthen mechanistic conclusions (e.g. "induces oxidative stress" together with "is electrophilic or can be metabolically activated to an

electrophile", "induces chronic inflammation", and "is immunosuppressive"); see, for example, 1-bromopropane (IARC, 2018).

5. Summary of data reported

(a) Exposure characterization

Exposure data are summarized to identify the agent and describe its production, use, and occurrence. Information on exposure prevalence and intensity in different settings, including geographical patterns and time trends, may be included. Exposure assessment methods used in key epidemiological studies reviewed by the Working Group are described and evaluated.

(b) Cancer in humans

Results of epidemiological studies pertinent to an evaluation of carcinogenicity in humans are summarized. The overall strengths and limitations of the epidemiological evidence base are highlighted to indicate how the evaluation was reached. The target organ(s) or tissue(s) in which a positive association between the agent and cancer was observed are identified. Exposure–response and other quantitative data may be summarized when available. When the available epidemiological studies pertain to a mixed exposure, process, occupation, or industry, the Working Group seeks to identify the specific agent considered to be most likely to be responsible for any excess risk. The evaluation is focused as narrowly as the available data permit.

(c) Cancer in experimental animals

Results pertinent to an evaluation of carcinogenicity in experimental animals are summarized to indicate how the evaluation was reached. For each animal species, study design, and route of administration, there is a statement about whether an increased incidence, reduced latency, or increased severity or multiplicity of neoplasms or pre-neoplastic lesions was observed, and the tumour sites are indicated. Special conditions resulting in tumours, such as prenatal exposure or single-dose experiments, are mentioned. Negative findings, inverse relationships, dose–response patterns, and other quantitative data are also summarized.

(d) Mechanistic evidence

Results pertinent to an evaluation of the mechanistic evidence on carcinogenicity are summarized to indicate how the evaluation was reached. The summary encompasses the informative studies on absorption, distribution, metabolism, and excretion; on the key characteristics with adequate evidence for evaluation; and on any other aspects of sufficient importance to affect the overall evaluation, including on whether the agent belongs to a class of agents for which one or more members have been classified as carcinogenic or probably carcinogenic to humans, and on criteria with respect to tumours in experimental animals induced by mechanisms that do not operate in humans. For each topic addressed, the main supporting findings are highlighted from exposed humans, human cells or tissues, experimental animals, or in vitro systems. When mechanistic studies are available in exposed humans, the tumour type or target tissue studied may be specified. Gaps in the evidence are indicated (i.e. if no studies were available in exposed humans, in in vivo systems, etc.). Consistency or differences of effects across different experimental systems are emphasized.

6. Evaluation and rationale

Consensus evaluations of the strength of the evidence of cancer in humans, the evidence of cancer in experimental animals, and the mechanistic evidence are made using transparent criteria and defined descriptive terms. The Working Group then develops a consensus overall evaluation of the strength of the evidence of carcinogenicity for each agent under review.

An evaluation of the strength of the evidence is limited to the agents under review. When multiple agents being evaluated are considered by the Working Group to be sufficiently closely related, they may be grouped together for the purpose of a single and unified evaluation of the strength of the evidence.

The framework for these evaluations, described below, may not encompass all factors relevant to a particular evaluation of carcinogenicity. After considering all relevant scientific findings, the Working Group may exceptionally assign the agent to a different category than a strict application of the framework would indicate, while providing a clear rationale for the overall evaluation.

When there are substantial differences of scientific interpretation among the Working Group members, the overall evaluation will be based on the consensus of the Working Group. A summary of the alternative interpretations may be provided, together with their scientific rationale and an indication of the relative degree of support for each alternative.

The categories of the classification refer to the strength of the evidence that an exposure is carcinogenic and not to the risk of cancer from particular exposures. The terms *probably carcinogenic* and *possibly carcinogenic* have no quantitative significance and are used as descriptors of different strengths of evidence of carcinogenicity in humans; *probably carcinogenic* signifies a greater strength of evidence than *possibly carcinogenic*.

(a) Carcinogenicity in humans

Based on the principles outlined in Part B, Section 2, the evidence relevant to carcinogenicity from studies in humans is classified into one of the following categories:

***Sufficient evidence of carcinogenicity*:** A causal association between exposure to the agent and human cancer has been established. That is, a positive association has been observed in the body of evidence on exposure to the agent and cancer in studies in which chance, bias, and confounding were ruled out with reasonable confidence.

***Limited evidence of carcinogenicity*:** A causal interpretation of the positive association observed in the body of evidence on exposure to the agent and cancer is credible, but chance, bias, or confounding could not be ruled out with reasonable confidence.

***Inadequate evidence regarding carcinogenicity*:** The available studies are of insufficient quality, consistency, or statistical precision to permit a conclusion to be drawn about the presence or the absence of a causal association between exposure and cancer, or no data on cancer in humans are available. Common findings that lead to a determination of inadequate evidence of carcinogenicity include: (a) there are no data available in humans; (b) there are data available in humans, but they are of poor quality or informativeness; and (c) there are studies of sufficient quality available in humans, but their results are inconsistent or otherwise inconclusive.

***Evidence suggesting lack of carcinogenicity*:** There are several high-quality 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 the studied cancers at any observed level of exposure. The results from these studies

alone or combined should have narrow confidence intervals with an upper limit below or close to the null value (e.g. a relative risk of unity). Bias and confounding were ruled out with reasonable confidence, and the studies were considered informative. A conclusion of *evidence suggesting lack of carcinogenicity* is limited to the cancer sites, populations and life stages, 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.

When there is *sufficient evidence*, a separate sentence identifies the target organ(s) or tissue(s) for which a causal interpretation has been established. When there is *limited evidence*, a separate sentence identifies the target organ(s) or tissue(s) for which a positive association between exposure to the agent and the cancer(s) was observed in humans. When there is *evidence suggesting lack of carcinogenicity*, a separate sentence identifies the target organ(s) or tissue(s) where evidence of lack of carcinogenicity was observed in humans. Identification of a specific target organ or tissue as having *sufficient evidence* or *limited evidence* or *evidence suggesting lack of carcinogenicity* does not preclude the possibility that the agent may cause cancer at other sites.

(b) Carcinogenicity in experimental animals

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

***Sufficient evidence of carcinogenicity*:** A causal relationship has been established between exposure to the agent and cancer in experimental animals based on 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 and/or under different protocols. An increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practices (GLP), can also provide *sufficient evidence*.

Exceptionally, a single study in one species and sex may 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 marked 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, for example, (a) the evidence of carcinogenicity is restricted to a single experiment and does not meet the criteria for *sufficient evidence*; (b) the agent increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential; (c) the agent increases tumour multiplicity or decreases tumour latency but does not increase tumour incidence; (d) the evidence of carcinogenicity is restricted to initiation–promotion studies; (e) the evidence of carcinogenicity is restricted to observational studies in non-laboratory animals (e.g. companion animals); or (f) there are unresolved questions about the adequacy of the design, conduct, or interpretation of the available studies.

***Inadequate evidence regarding carcinogenicity*:** The studies cannot be interpreted as showing either the presence or the absence of a carcinogenic effect because of major

qualitative or quantitative limitations, or no data are available on cancer in experimental animals.

***Evidence suggesting lack of carcinogenicity*:** Well-conducted studies (e.g. conducted under GLP) involving both sexes of at least two species are available showing that, within the limits of the tests used, the agent was not carcinogenic. The conclusion of *evidence suggesting lack of carcinogenicity* is limited to the species, tumour sites, age at exposure, and conditions and levels of exposure covered by the available studies.

(c) Mechanistic evidence

Based on the principles outlined in Part B, Section 4, the mechanistic evidence is classified into one of the following categories:

***Strong mechanistic evidence*:** Results in several different experimental systems are consistent, and the overall mechanistic database is coherent. Further support can be provided by studies that demonstrate experimentally that the suppression of key mechanistic processes leads to the suppression of tumour development. Typically, a substantial number of studies on a range of relevant end-points are available in one or more mammalian species. Quantitative structure–activity considerations, in vitro tests in non-human mammalian cells, and experiments in non-mammalian species may provide corroborating evidence but typically do not in themselves provide strong evidence. However, consistent findings across a number of different test systems in different species may provide strong evidence.

Of note, "strong" relates not to potency but to strength of evidence. The classification applies to three distinct topics:

(a) Strong evidence that the agent belongs, based on mechanistic considerations, to a class of agents for which one or more members have been classified as carcinogenic or probably carcinogenic to humans. The considerations can go beyond quantitative structure–activity relationships to incorporate similarities in biological activity relevant to common key characteristics across dissimilar chemicals (e.g. based on molecular docking, –omics data).

(b) Strong evidence that the agent exhibits key characteristics of carcinogens. In this case, three descriptors are possible:

1. The strong evidence is in exposed humans. Findings relevant to a specific tumour type may be informative in this determination.
2. The strong evidence is in human primary cells or tissues. Specifically, the strong findings are from biological specimens obtained from humans (e.g. ex vivo exposure), from human primary cells, and/or, in some cases, from other humanized systems (e.g. a human receptor or enzyme).
3. The strong evidence is in experimental systems. This may include one or a few studies in human primary cells and tissues.

(c) Strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans. Certain results in experimental animals (see Part B, Section 6b) would be discounted, according to relevant criteria and considerations in authoritative publications (e.g. Capen et al., 1999; IARC, 2003). Typically, this classification would not apply when there is strong mechanistic evidence that the agent exhibits key characteristics of carcinogens.

***Limited mechanistic evidence*:** The evidence is suggestive, but, for example, (a) the studies cover a narrow range of experiments, relevant end-points, and/or species; (b) there are unexplained inconsistencies in the studies of similar design; and/or (c) there is unexplained incoherence across studies of different end-points or in different experimental systems.

***Inadequate mechanistic evidence*:** Common findings that lead to a determination of inadequate mechanistic evidence include: (a) few or no data are available; (b) there are unresolved questions about the adequacy of the design, conduct, or interpretation of the studies; (c) the available results are negative.

(d) Overall evaluation

Finally, the bodies of evidence included within each stream of evidence are considered as a whole, in order to reach an overall evaluation of the carcinogenicity of the agent to humans. The three streams of evidence are integrated and the agent is classified into one of the following categories (see Table 4), indicating that the Working Group has established that:

The agent is carcinogenic to humans (Group 1)

This category applies whenever there is *sufficient evidence of carcinogenicity* in humans.

In addition, this category may apply when there is both *strong evidence in exposed humans that the agent exhibits key characteristics of carcinogens* and *sufficient evidence of carcinogenicity* in experimental animals.

The agent is probably carcinogenic to humans (Group 2A)

This category generally applies when the Working Group has made at least *two of the following* evaluations, *including at least one* that involves either exposed humans or human cells or tissues:

- *Limited evidence of carcinogenicity* in humans,
- *Sufficient evidence of carcinogenicity* in experimental animals,
- *Strong evidence that the agent exhibits key characteristics of carcinogens.*

If there is *inadequate evidence regarding carcinogenicity* in humans, there should be *strong evidence in human cells or tissues that the agent exhibits key characteristics of carcinogens*. If there is *limited evidence of carcinogenicity in humans*, then the second individual evaluation may be from experimental systems (i.e. *sufficient evidence of carcinogenicity* in experimental animals or *strong evidence in experimental systems that the agent exhibits key characteristics of carcinogens).*

Additional considerations apply when there is *strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans* for one or more tumour sites. Specifically, the remaining tumour sites should still support an evaluation of *sufficient evidence in experimental animals* in order for this evaluation to be used to support an overall classification in Group 2A.

Separately, this category generally applies if there is *strong evidence that the agent 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*.

The agent is possibly carcinogenic to humans (Group 2B)

This category generally applies when only one of the following evaluations has been made by the Working Group:

- *Limited evidence of carcinogenicity* in humans,
- *Sufficient evidence of carcinogenicity* in experimental animals,

Table 4 Integration of streams of evidence in reaching overall classifications (the evidence in *bold italic* represents the basis of the overall evaluation)

Stream of evidence			Classification based on strength of evidence
Evidence of cancer in humans[a]	**Evidence of cancer in experimental animals**	**Mechanistic evidence**	
Sufficient	Not necessary	Not necessary	**Carcinogenic to humans (Group 1)**
Limited or Inadequate	***Sufficient***	***Strong (b)(1) (exposed humans)***	
Limited	***Sufficient***	Strong (b)(2–3), Limited, or Inadequate	**Probably carcinogenic to humans (Group 2A)**
Inadequate	***Sufficient***	***Strong (b)(2) (human cells or tissues)***	
Limited	Less than Sufficient	***Strong (b)(1–3)***	
Limited or Inadequate	Not necessary	***Strong (a) (mechanistic class)***	
Limited	Less than Sufficient	Limited or Inadequate	**Possibly carcinogenic to humans (Group 2B)**
Inadequate	***Sufficient***	Strong (b)(3), Limited, or Inadequate	
Inadequate	Less than Sufficient	***Strong (b)(1–3)***	
Limited	***Sufficient***	***Strong (c) (does not operate in humans)***[b]	
Inadequate	***Sufficient***	***Strong (c) (does not operate in humans)***[b]	**Not classifiable as to its carcinogenicity to humans (Group 3)**
All other situations not listed above			

[a] Human cancer(s) with highest evaluation.

[b] The *strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans* must specifically be for the tumour sites supporting the classification of *sufficient evidence in experimental animals*.

• *Strong evidence that the agent exhibits key characteristics of carcinogens.*

Because this category can be based on evidence from studies in experimental animals alone, there is **no** requirement that the strong mechanistic evidence be in exposed humans or in human cells or tissues. This category may be based on *strong evidence in experimental systems that the agent exhibits key characteristics of carcinogens.*

As with Group 2A, additional considerations apply when there is *strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans* for one or more tumour sites. Specifically, the remaining tumour sites should still support an evaluation of *sufficient evidence in experimental animals* in order for this evaluation to be used to support an overall classification in Group 2B.

The agent is not classifiable as to its carcinogenicity to humans (Group 3)

Agents that do not fall into any other group are generally placed in this category.

This includes the case when there is *strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans* for one or more tumour sites in experimental animals, the remaining tumour sites do not support an evaluation of *sufficient evidence in experimental animals*, and other categories are not supported by data from studies in humans and mechanistic studies.

An evaluation in Group 3 is not a determination of non-carcinogenicity or overall safety. It often means that the agent is of unknown carcinogenic potential and that there are significant gaps in research.

If the evidence suggests that the agent exhibits no carcinogenic activity, either through *evidence suggesting lack of carcinogenicity* in both humans and experimental animals, or through *evidence suggesting lack of carcinogenicity* in

experimental animals complemented by strong negative mechanistic evidence in assays relevant to human cancer, then the Working Group may add a sentence to the evaluation to characterize the agent as well-studied and without evidence of carcinogenic activity.

(e) Rationale

The reasoning that the Working Group used to reach its evaluation is summarized so that the basis for the evaluation offered is transparent. This section integrates the major findings from studies of cancer in humans, cancer in experimental animals, and mechanistic evidence. It includes concise statements of the principal line(s) of argument that emerged in the deliberations of the Working Group, the conclusions of the Working Group on the strength of the evidence for each stream of evidence, an indication of the body of evidence that was pivotal to these conclusions, and an explanation of the reasoning of the Working Group in making its evaluation.

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GENERAL REMARKS

This one-hundred-and-thirty-ninth volume of the *IARC Monographs* contains evaluations of the carcinogenic hazard to humans of hepatitis D virus (HDV), human cytomegalovirus (HCMV), and Merkel cell polyomavirus (MCPyV). The Advisory Group to Recommend Priorities for the *IARC Monographs* during 2025–2029 recommended that all three agents be evaluated with high priority (IARC, 2024).

HDV was previously evaluated by the *IARC Monographs* programme in 1993 as *not classifiable as to its carcinogenicity to humans* (Group 3) on the basis of *inadequate* evidence for cancer both in humans and in experimental animals (Volume 59; IARC, 1994). The Advisory Group recommendation to re-evaluate HDV was based on the availability of new evidence on cancer in humans.

MCPyV was previously evaluated by the *IARC Monographs* programme in 2012, when it was classified as *probably carcinogenic to humans* (Group 2A) on the basis of *limited* evidence for cancer in humans and *inadequate* evidence for cancer in experimental animals (Volume 104; IARC, 2013). The Advisory Group recommendation to re-evaluate MCPyV was based on the availability of new mechanistic studies and evidence on cancer in humans, including for the first time a prospective study, which supported a temporal association between MCPyV infection and Merkel cell carcinoma (MCC).

HCMV has not been previously evaluated by the *IARC Monographs* programme. The Advisory Group recommendation for evaluation was based on the availability of relevant evidence on cancer in humans and mechanistic studies.

A summary of the findings of the present volume appears in *The Lancet Oncology* (Karagas et al., 2025).

Considerations in evaluating mechanistic evidence for viruses

The deliberations of Volume 139 highlighted some methodological challenges inherent in determining the carcinogenicity of infectious agents. No animal cancer models were available for HDV and HCMV, so there was a particular reliance on data from studies of human cancer, alongside an evaluation of the mechanistic evidence for these viruses, applying the key characteristics of carcinogens (KCs).

Since the most recent evaluations of viruses by the *IARC Monographs* programme, in Volumes 100B and 104 (IARC, 2012, 2013), the present meeting for Volume 139 was the first at which a Working Group of experts approached the evaluation of the mechanistic evidence for viruses by applying the KCs framework. Evidence for the

KC "is electrophilic or can be metabolically activated to an electrophile" (KC1) was considered not to be applicable to these viruses. The Working Group noted that evidence for the KC "induces oxidative stress" (KC5), specifically, production of reactive oxygen species (ROS), is a common outcome of infections by many different viruses (influenza, coronavirus, hepatitis C virus, etc.). Therefore, evidence related to this KC, as indicated in the Preamble to the *IARC Monographs*, was considered with caution. The Working Group noted that the applicability of in vivo non-human mammalian experimental systems when assessing each of the specific KCs in the context of a virus infection should be determined to be adequate or not on a case-by-case basis, especially when considering the strength of evidence from mammalian models in rodents or in non-human primates. Humanized models were considered to be not relevant because they typically only reflect the specific behaviour of the human virus in specific humanized tissues and/or cell types.

Challenges in the evaluation of HCMV

Viruses normally induce alterations in the host cell, because they depend on host cells for survival, metabolic processes, and final replication. These alterations may include changes to the host DNA, by incorporation of viral DNA (or a portion of it) into the host genome or by breaking the host genome, or simply by directing protein transduction. Alterations to DNA repair mechanisms or epigenetic effects are essential components of the virus replication machinery. Moreover, all viruses interfere with the immune system and need to evade certain immune responses to replicate. Based on the above, the Working Group recognized the challenges involved in distinguishing the KC-associated end-points that were relevant to carcinogenesis from the several end-points that were relevant to virus survival.

The Working Group noted that evaluating the mechanistic evidence for HCMV was particularly challenging for several reasons, including the nature of the HCMV replication cycle, and gaps in the understanding of its complex biology and pathogenesis. First, there was an extensive body of literature comprising thousands of potentially relevant publications, which made a comprehensive analysis difficult. A large number of the retrieved references were beyond the scope of the present evaluation, as HCMV-derived DNA sequences are widely used in molecular biology; for example, the HCMV major immediate-early promoter (MIEP) is incorporated into numerous plasmid constructs. Second, HCMV is a complex virus with many genes and potential genes that encode hundreds of proteins, microRNAs (miRNAs), and other non-coding RNAs. Owing to this complexity and the often contradictory findings regarding the effects exerted by the virus and its many products, it was difficult to perform a comprehensive analysis of its overall impact on the host cell. The Working Group noted that most information stemmed from studies in lytically infected cells, which die at the end of a productive replication cycle. It was questioned whether lytic-phase cells are a useful model to identify relevant KC-associated end-points, for example, "causes immortalization" (KC9). It was argued that the virus establishes latent (and chronically active) infections that persist for the lifetime of the host. It seems likely that any cancer caused by HCMV will result from latently or abortively infected cells, as has been shown for other oncogenic viruses. Nevertheless, lytically infected cells may also contribute to the development of cancer via the release of proinflammatory and immunosuppressive cytokines. According to the published literature (a limited number of studies from few research groups), abortive

or chronic HCMV infections can lead to cell immortalization in vitro. However, there is a lack of full understanding regarding latent HCMV infection. Long-term latency seems to occur in haematopoietic stem cells (CD34+). These cells may differentiate and become fully permissive to HCMV infection, resulting in a lytic replication cycle and cell death. Alternatively, they may become latently infected and express viral oncoproteins. Finally, under certain conditions, some cell types infected with HCMV may undergo abortive infection cycles; these cells do not undergo a full lytic replication cycle, and thus do not maintain the HCMV genome. Very little is known about such abortive infection cycles. Although abortive infections have been shown to exist, both in vitro and in vivo, the Working Group noted that there was far less information available about abortive HCMV infections than about lytic and latent infections.

For the above-mentioned reasons, the mechanistic evidence for HCMV was considered to be *limited*. A small minority of the Working Group concluded that the evidence for inducing epigenetic alterations and causing immortalization was consistent and coherent, and therefore that the mechanistic evidence for HCMV was *strong* and supported a Group 2A classification for HCMV, rather than the Group 2B classification reached by the Working Group overall.

Methodological issues in determining exposure to infectious agents

A reliable exposure assessment, which is crucial in human cancer studies, can pose particular challenges in studies on infectious agents. The three viruses evaluated in Volume 139 all cause chronic infections, the presence of which can be detected accurately by immunological and molecular assays. However, the timing of infection in relation to cancer occurrence is important, particularly for an agent such as HCMV, which attains high infection prevalences in most populations by early adulthood.

The timing of infection can only be definitively determined through cohort studies, either retrospective or prospective, with serial specimen collection to pinpoint the time window of infection. However, a key issue was determining whether the infection preceded the cancer. For MCPyV, the availability of data from nested case–control studies in which infection was assessed before cancer development provided key information to support the present evaluation; such information was absent from the previous evaluation in Volume 104 (IARC, 2013). Similarly for HDV, the availability of multiple prospective studies in which HDV was determined to be present or absent before cancer development assisted the present evaluation. For HCMV, however, there were very few studies in which samples collected before cancer diagnosis were analysed, and for those studies in which they were, generally small numbers of individuals were recorded as transitioning from negative to positive infection status. In some studies, the timing of infection was based on the clinical record or personal recollection of a past illness consistent with acute acquisition of the infection, but this approach also has major limitations because of the low specificity of illness syndromes and low sensitivity for less severe infections. In practice, infection status in most human cancer studies, including for HDV, was determined via testing of a biological specimen at a single time point. For case–control and cross-sectional studies, this approach prompted the further question as to whether the presence of cancer had an impact on the ability of the assay to detect infection, raising the possibility of ascertainment bias.

The relevant exposure window for HCMV in the potential causal pathway for childhood acute lymphoblastic leukaemia (ALL) is unclear. In

most studies, exposure to HCMV was assessed in perinatally derived samples, but in at least one study (Wiemels et al., 2022), maternal exposure may have been detected. In the available studies on childhood ALL, it has not yet been assessed whether infection after birth can play a role, and much of the current evidence came from neonatal blood spots. It has also been hypothesized that HCMV infection may be more closely related to specific subtypes of ALL, but the available evidence did not allow the Working Group to examine this in detail. The availability of more data on the relevance of certain biomarkers of HCMV infection, such as HCMV protein levels (HCMV-viral interleukin 10, vIL-10), would have aided the Working Group in its evaluation.

Use of case series in the present evaluation

The evaluation of human cancer evidence by the Working Group often relies heavily on studies that use comparative designs, such as cohort studies or case–control studies. In the present volume, the Working Group considered case-series data to be of complementary value in some instances. For MCPyV, this included a review of case series on clonal integration, to determine whether there was evidence that integration occurred early on during the development of the tumour, before the expansion of tumour cells. This information was seen as complementary evidence of a biologically plausible temporal association, when combined with evidence from nested case–control studies. For HCMV, case series were also of utility in the evaluation of evidence on a relationship with brain cancer (a cancer site for which it can be particularly challenging to obtain control tissue), in particular, by showing that there was high variability across studies in the detection of HCMV in tumour cells. As both false negatives and false positives are a concern, this variability reduced confidence in the validity of the exposure assessment in studies involving various types of control series.

Within-person comparisons of the presence of the virus in tumour and non-tumour (often adjacent) tissue from cases were present for some cancer sites, including the colorectum. These studies were considered to offer modest complementary evidence, for triangulation purposes, to that provided by traditional case–control studies, as they would control for interpersonal confounding and could demonstrate specificity of the infections to the tumour cells themselves rather than the surrounding area. However, the Working Group considered that within-person comparison studies may be vulnerable to ascertainment bias because of the possibility of differential detectability of the infection marker in the tumour tissue compared with the surrounding tissue, and possibly bias from reverse causation, whereby tumour cells are more prone to infection. Additionally, for many cancer types, including colorectal cancer, the evidence, both from studies using between-person comparisons and from within-person comparisons was too sparse to triangulate the findings.

Merkel cell carcinoma

The Working Group concluded that there is *sufficient* evidence that MCPyV causes MCC in humans and noted that there is little evidence for other causes of MCC besides MCPyV. An *IARC Monographs* Working Group recently concluded that there is *limited* evidence that hydrochlorothiazide (a photosensitizing drug) causes MCC (IARC, 2026). MCC is a rare and aggressive neuroendocrine carcinoma (Busam et al., 2025) with the second highest mortality rate after melanoma among skin cancers (Yaghi et al., 2022). MCC is predominantly cutaneous (in more than 90% of cases) and typically occurs

on sun-exposed body sites (Yaghi et al., 2022; Busam et al., 2025). Very rarely, it occurs on the mucosa of the genitourinary or gastrointestinal tract (Yaghi et al., 2022). Cases of primary MCC occurring in the salivary glands have also been described (Choi et al., 2025). However, in some cases, metastases are detected in people without a detectable primary tumour or history of MCC, which reduces certainty that MCCs identified at sites other than skin reflect true primary sites. MCC is more common in White men, and it is rare in people younger than age 50 years, with a median age at diagnosis of 75 years (Busam et al., 2025). The incidence of MCC is increasing worldwide and appears to be higher in countries in the southern hemisphere, with highest incidence reported in Australia (2.5 per 100 000 person-years) (Mohsen et al., 2024). In immunocompromised individuals, MCC incidence is 5–20-fold that in the general population (Clarke et al., 2015; Busam et al., 2025).

Scope of the systematic review

Standardized searches of the PubMed database (NCBI, 2025) were conducted for HDV, HCMV, and MCPyV for each outcome (cancer in humans; cancer in experimental animals; and mechanistic evidence, including the KCs). The literature trees for HDV, HCMV, and MCPyV, including the full set of search terms for each agent name and each outcome type, are available online.[a]

As described in the Preamble to the *IARC Monographs* (last revised in 2019; IARC, 2019), the Working Group reviews publicly available scientific data, such as peer-reviewed papers in the scientific literature, and may also review unpublished reports, if made available in their final form by governmental agencies and if they contain enough detail for critical review. A public Call for Data was opened on the IARC website 1 year ahead of the meeting for Volume 139. Eligible studies were only those published or accepted for publication in the openly available scientific literature by the time of the Working Group meeting.

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[a] The literature trees for the monographs in the present volume are available at: https://hawcproject.iarc.who.int/assessment/765/ (HDV) and https://hawcproject.iarc.who.int/assessment/713/ (HCMV), and https://hawcproject.iarc.who.int/assessment/773/ (MCPyV).

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HEPATITIS D VIRUS

1. Exposure Characterization

1.1 Identification

1.1.1 Structure, genome, and classification

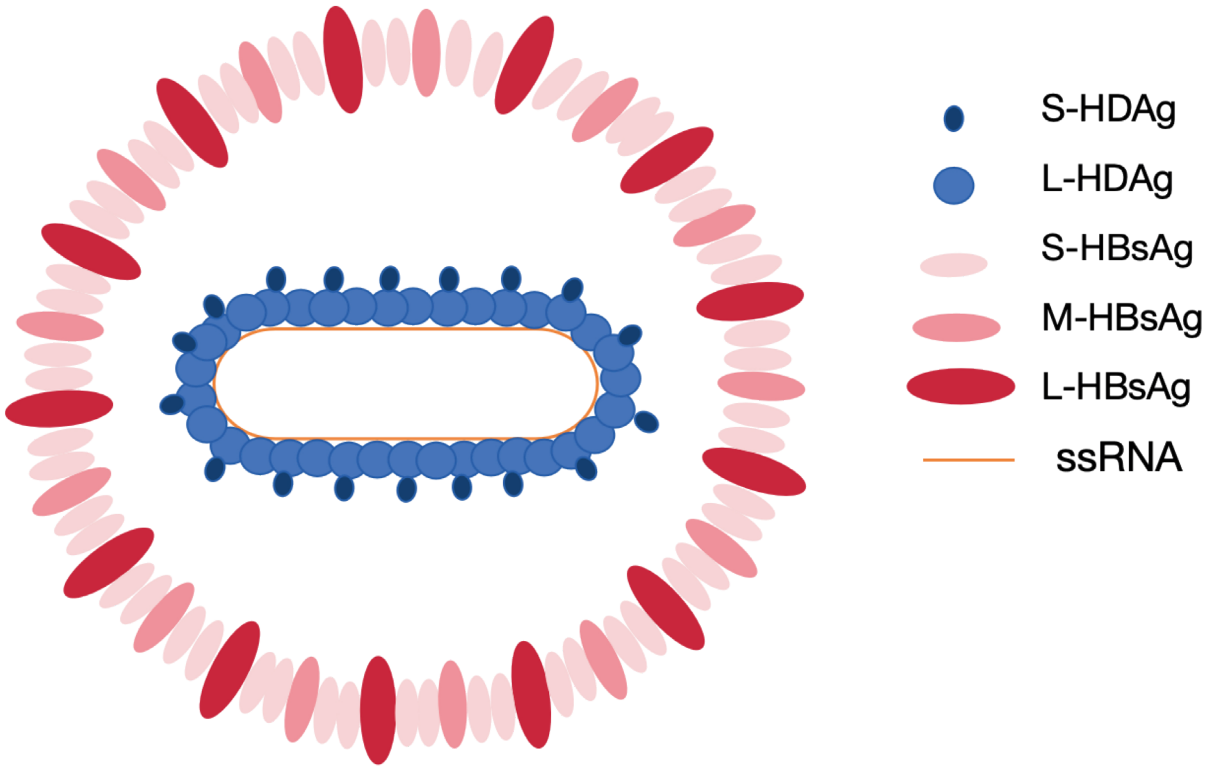
Hepatitis D virus (HDV), also known as hepatitis delta virus, was discovered in 1977 by Rizzetto and colleagues when examining the hepatitis B core antigen (HBcAg) in human liver tissue (Rizzetto et al., 1977). HDV is an enveloped virus with a circular, negative-sense, single-stranded RNA genome that must be transcribed into positive-sense RNA before translation (Fig. 1.1). HDV is a defective satellite virus of hepatitis B virus (HBV). HDV's mechanism of replication is independent of HBV (and relies on the host cell's RNA polymerases), but it uses the hepatitis B virus surface antigen (HBsAg) to assemble its viral particles and is hence dependent on HBV for its propagation (Zuccola et al., 1998; Sureau and Negro, 2016). HDV is currently classified in the genus *Deltavirus* within the family *Kolmioviridae* (Kuhn et al., 2024).

HDV is a small virus with an average diameter of 36–43 nm that is without visible surface projections (ICTV, 2011). Its structure has two main components – the HDV genome and HDV antigen (HDAg) (Fig. 1.1). The core of the virus contains a negative-sense, single-stranded circular RNA genome, which is about 1.7 kb in size. HDAg is the only known protein encoded by the HDV genome (Chen et al., 1986) and plays a crucial role in HDV replication and infectivity.

There are two forms of HDAg: the 24 kDa small HDAg (S-HDAg) is involved in virus replication, whereas the 27 kDa large HDAg (L-HDAg) is involved in virus particle assembly (Caviglia et al., 2022). S-HDAg binds to HDV RNA, specifically recognizing the rod-shaped structure of the viral genome. The assembly domain of S-HDAg is the only rigid component of the protein, whereas other regions remain dynamic, suggesting that structural flexibility is important for their function (Yang et al., 2024). L-HDAg shares structural similarities with S-HDAg but has additional functions attributable to its C-terminal region, which contains a prenylation motif that is crucial for the regulation of replication and assembly (Guo et al., 2024). Both S-HDAg and L-HDAg form HDV ribonucleoprotein (RNP) complexes with the single-stranded RNA genome of HDV.

HDV is enveloped by three forms of HBsAg – large (L), medium (M), and small (S) – together with host-derived lipids. HDV is therefore dependent on concurrent infection with HBV for particle assembly. Both S-HBsAg and L-HBsAg are crucial for HDV viral assembly (Bruss and Ganem, 1991), virion packaging, and virus infectivity (Rizzetto et al., 1980; Sureau et al., 1993; Zuccola et al., 1998) (see Section 4.1.5).

Fig. 1.1 Structure of HDV



HBsAg, hepatitis B surface antigen; HDAg, hepatitis D antigen; HDV, hepatitis D virus; L-HBsAg, large hepatitis B surface antigen; L-HDAg, large hepatitis D antigen; M-HBsAg, middle hepatitis B surface antigen; S-HBsAg, small hepatitis B surface antigen; S-HDAg, small hepatitis D antigen; ssRNA, single-stranded RNA.

The structure of HDV includes a circular RNA genome, two forms of HDAg proteins (L and S), and an envelope formed by HBsAg (L, M, and S). Created by the Working Group.

The HDV genome exhibits a significant degree of genetic diversity, with up to 16% divergence within the same genotype and 20–40% divergence between HDV genotypes. Eight different HDV genotypes have been described (Magnius et al., 2018), and different genotypes may exhibit distinct geographical distributions and clinical outcomes (see Section 1.3.2 for the geographical distribution of the different genotypes). The high variability of the HDV genome is largely caused by the absence of dedicated proofreading ability of host RNA polymerases (Krushkal and Li, 1995; Pacin-Ruiz et al., 2022), which results in frequent mutations and sequence variations. Unlike DNA polymerases, which possess exonuclease activity to correct errors during replication, RNA polymerases lack this proofreading function. As a result, the replication of the HDV genome, which is composed of RNA, is prone to errors. These errors accumulate over time, contributing to the genetic diversity of the virus.

1.1.2 Host range and tissue tropism

HDV infects hepatocytes (Chen et al., 1986; Gudima et al., 2007; Brunetto et al., 2023). Like HBV, HDV has a narrow host species range. Current evidence indicates that natural HDV infection occurs only in humans, but the virus may infect other species under laboratory conditions. HDV enters liver cells via a sodium/taurocholate cotransporting polypeptide (NTCP) receptor that recognizes HBsAg. The pre-S1 region of the HBV envelope protein (amino acids

21–47), present only in L-HBsAg, contains a domain critical for the binding of HDV (Neurath et al., 1986). This epitope is highly specific to liver cells, particularly those in the human liver, which explains why HDV infection is primarily restricted to humans. Hepatocytes derived from most non-hominoid animals (including macaques, mice, rats and pigs) are insensitive to HBV and HDV. For in vivo studies, liver-humanized mice (e.g. mice engrafted with human hepatocytes or transgenic mice expressing human receptor NTCP) can be used to study HDV infection (Lempp et al., 2017; Müller et al., 2018; Takeuchi et al., 2019).

1.2 Methods of detection

The diagnosis of HDV infection initially depended on detecting HDAg in the liver tissue of HBsAg-positive patients through immunohistochemical staining, but this invasive method is now rarely used because of the availability of more advanced and less invasive tests. After the discovery of HDV, serological testing evolved from enzyme-linked immunosorbent assay (ELISA) for antibodies to HDV (anti-HDV) in the 1970s and 1980s to qualitative HDV RNA polymerase chain reaction (PCR) in the 1990s and quantitative PCR in the 2000s. Early tests lacked standardization until the World Health Organization (WHO) implemented quality control measures in 2013, significantly improving reliability. Today, diagnosis of HDV primarily involves the detection of antibodies to HDV (anti-HDV) and HDV RNA, whereas levels of the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and HBV markers (HBsAg, HBV DNA) aid in clinical evaluation and monitoring (Majeed et al., 2023).

1.2.1 Serum and plasma

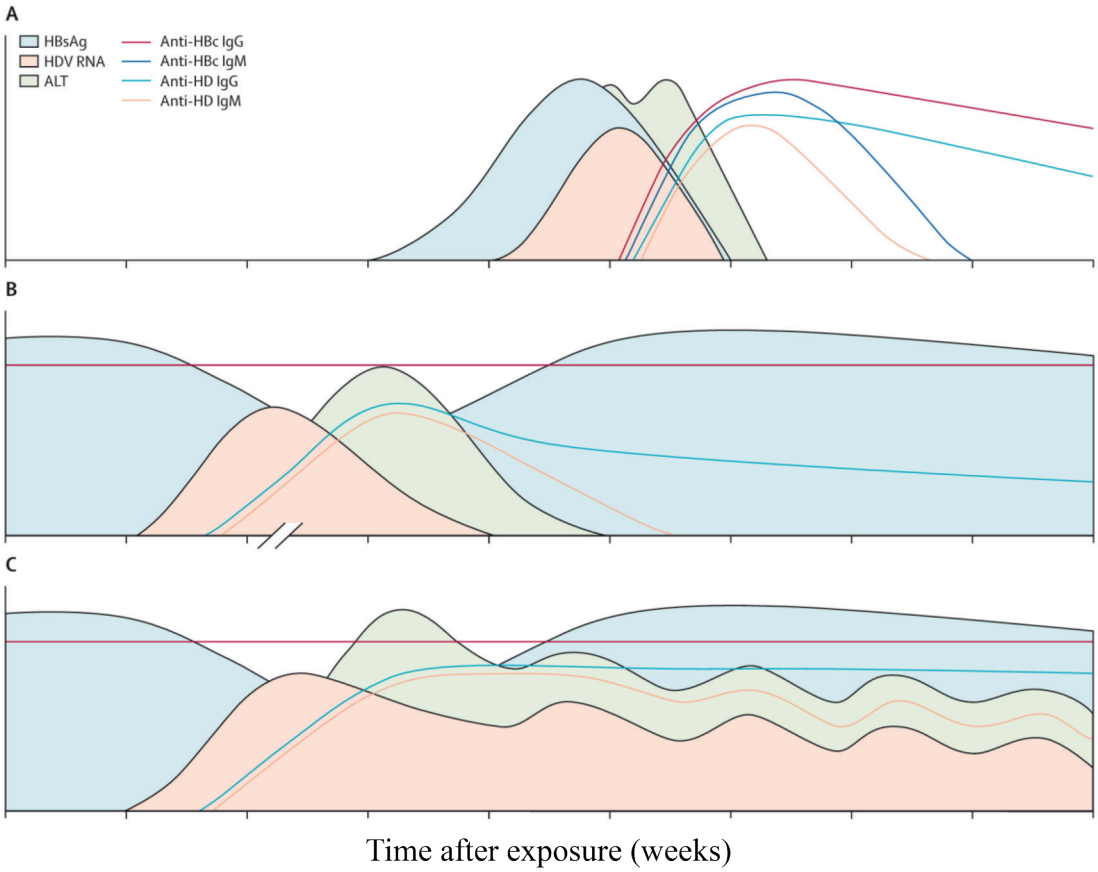
The diagnosis of HBV infection is based on the detection of HBsAg. To confirm HDV infection in HBsAg-positive patients (HBV-infected), screening can be performed for HDV antibodies and testing for HDV RNA in the serum of HDV antibody–positive patients. The typical evolution of serological and virological markers after infection is shown in Fig. 1.2 (Hughes et al., 2011).

(a) Antibodies to HDV

Anti-HDV IgM antibodies are detectable within 2–3 weeks of symptom onset and disappear by 2 months after acute infection, although anti-HDV IgM is also elevated in patients with chronic HDV infection during disease flares (Fig. 1.2), whereas anti-HDV IgG antibodies persist in serum (Koh et al., 2019a). [Therefore, IgM is not commonly used to detect primary HDV infection, which is determined using HDV RNA measurement. The Working Group noted that measuring anti-HDV IgM is not an adequate means of measuring prior HDV infection. Anti-HDV IgG is an adequate long-term marker for the detection of past HDV infection.]

Anti-HDV – immunoglobulins IgM and IgG, or total anti-HDV – are detected by enzyme immunoassay or radioimmunoassay (RIA) (Liu et al., 2025) in serum or plasma. A more sensitive quantitative microarray antibody capture (Q-MAC) assay was developed to detect anti-HDV IgG in human sera and could predict HDV RNA positivity quite well in patient samples. (Chen et al., 2017). The European Association for the Study of the Liver (EASL) guidelines recommend that all HBsAg-positive patients should be tested for anti-HDV antibodies with a validated assay (Brunetto et al., 2023). Meta-analyses have shown that the sensitivity and specificity of anti-HDV IgG were both greater than 95% (Pan et al., 2023a; Akuffo et al., 2024). [The Working Group noted that HDV serological

Fig. 1.2 Typical evolution of serological and virological markers during HDV infection



ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HDV, hepatitis D virus; Ig, immunoglobulin.

(A) Simultaneous coinfection with HBV and HDV, resulting in clearance of both viruses in almost all patients. (B) HDV superinfection of an HBV carrier with self-limited outcome. The spontaneous clearance of HDV RNA might take years to occur (indicated by a break on the *x*-axis) and, in few cases, can herald the loss of HBsAg (not depicted). (C) HDV superinfection of an HBV carrier with chronic persistent viral replication; the more common outcome after superinfection.

From Hughes et al. (2011). Reprinted from *The Lancet*, Volume 378, issue 9785, Hughes SA et al., Hepatitis delta virus, pp. 73–85, Copyright (2011), with permission from Elsevier.

tests, particularly those for IgG, are effective screening tools for HDV.]

(b) HDV RNA

HDV infection is confirmed by the detection of HDV RNA in serum. HDV RNA can be detected by dot blot hybridization (Jardi et al., 1995) or reverse transcription-polymerase chain reaction (RT-PCR). PCR also allows cloning and sequencing of HDV to determine the viral genomic sequence (Juang et al., 2024; Wedemeyer et al., 2025). A systematic review and meta-analysis, including 10 studies, were conducted to assess the diagnostic accuracy of testing for HDV RNA. It was shown that the sensitivity of assays to detect HDV RNA (hybridization and PCR assays) for the diagnosis of active hepatitis D was 92% and the specificity was 90% (Chen et al., 2023). [The Working Group noted that this review included hybridization assays and PCR-based assays. Sensitivity may vary but is much higher in PCR-based methods.] With the continuous development of PCR technology, the emergence of techniques such as nested reverse

transcription PCR (nested RT-PCR), reverse transcription-quantitative polymerase chain reaction (RT-qPCR), one-step RT-qPCR (da Silva Queiroz et al., 2023; Liu et al., 2025), and digital droplet PCR (ddPCR) (Xu et al., 2022) has led to continuous improvements in the sensitivity and specificity of HDV RNA detection. Sequential quantification of HDV RNA is used to determine the response to antiviral treatment (Anolli et al., 2025).

A summary of diagnostic tests for the evaluation of patients with HDV infection is presented in Table 1.1.

(c) HDV antigen

Several commercial assay systems are available for the detection of HDAg; however, these are of limited use in serum specimens. Immunocompetent patients exposed to HDV rapidly develop anti-HDV antibodies, which bind and mask the serum antigen released from the virion, rendering it undetectable in these assays. Serum HDAg is detectable only transiently in blood specimens collected early at the onset of HDV infection, before the rise in antibodies within the first weeks, and its finding is therefore diagnostic of primary HDV infection (Olivero and Smedile, 2012).

1.2.2 Serological markers of HBV-HDV coinfection and superinfection

HDV infection can be acquired either simultaneously with HBV (coinfection), leading to acute hepatitis B and acute hepatitis D, or subsequently, as a secondary infection in individuals with chronic HBV infection (HDV superinfection) (Fig. 1.2). The diagnosis of acute HBV-HDV coinfection relies on the concurrent detection of markers for acute HBV infection (HBsAg, anti-HBcAg IgM, and anti-HBcAg IgG) and acute HDV infection (anti-HDV IgM and IgG, and serum HDV RNA). The most specific marker of HBV-HDV coinfection is the identification of anti-HDV IgM together with elevated levels of anti-HBcAg IgM (Brunetto et al., 2023). Acute HBV-HDV coinfection is followed by clearance of both viruses in approximately 95% of patients, whereas more than 90% of HDV superinfections result in chronic HBV-HDV infection (Negro and Lok, 2023). Additionally, in individuals with a known history of chronic hepatitis B (CHB), HDV superinfection may cause exacerbation of symptoms and elevated levels of hepatic enzymes. The absence of or low levels of anti-HBcAg IgM can help differentiate superinfection from coinfection, with the latter typically characterized by high levels of anti-HBcAg IgM. In the context of HDV superinfection, HBV replication may be decreased (Koh et al., 2019b; Brunetto et al., 2023).

As it is often difficult to distinguish between coinfection and superinfection in studies on cancer in humans, hereafter, for simplicity, "HBV-HDV coinfection" is used to refer to individuals who are currently infected with both HBV and HDV, regardless of the relative timing of each initial infection.

1.2.3 Liver tissue

HDAg in the liver can be tested for by staining of frozen or formalin-fixed paraffin-embedded sections with immunofluorescence- or immunoperoxidase-labelled antibody to HDAg (Rizzetto, 1989). The reported sensitivities of this test for the identification of HDV vary and, because of the availability of serological assays, liver immunohistochemistry analyses are not commonly used (Koh et al., 2019b).

Table 1.1 Summary of diagnostic tests for the evaluation of patients with HDV infection

Test (technique)[a]	Purpose[a]	Remarks[a]	Sensitivity[b]	Specificity[b]	Reference
Anti-HDV IgG antibody (ELISA)	Detects IgG antibodies against HDV, indicates previous or ongoing HDV infection	Should be the first diagnostic screening test employed and should be performed in all HBsAg-positive patients	97.4%[b]	95.3%[b]	Akuffo et al. (2024)
Anti-HDV IgM antibody (ELISA)	Detects IgM antibodies against HDV; indicates ongoing acute or chronic HDV infection	Can be used to determine disease activity in patients who test positive for anti-HDV IgG antibodies Available tests are not standardized	62.0%[b]	85.0%	Akuffo et al. (2024)
HDV RNA, qualitative (RT-PCR)	Detects HDV RNA; indicates HDV replication and active infection	Gold standard to determine HDV infection Test result can be a false negative if primers are not optimized for all HDV genotypes	92%[b]	90%[b]	Chen et al. (2023)
HDV RNA, quantitative (RT-qPCR)	Determines the level of HDV RNA in the blood	Can be useful in the context of antiviral treatment to monitor treatment response There is no association between serum HDV RNA level and the grade or stage of liver disease	NR	NR	Anolli et al. (2025)
HDV genotyping (direct sequencing or RFLP analysis of RT products)	Determines the HDV genotype	Different HDV genotypes may be associated with distinct clinical outcome	NR	NR	Altuğlu et al. (2007)

HBsAG, hepatitis B virus surface antigen; HDV, hepatitis D virus; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; NR, not reported; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; RFLP, restriction fragment length polymorphism; RT, reverse transcription.

[a] Adapted from Wedemeyer and Manns (2010).

[b] The sensitivity and specificity values mentioned above are averages described in meta-analyses.

1.3 Epidemiology of HDV infection

1.3.1 Transmission of infection to humans and risk factors

As HDV is dependent on HBV for propagation, individuals infected with HBV are also at risk of contracting HDV. HDV spreads primarily through three main routes: bloodborne transmission, sexual contact, and familial transmission (Lanini et al., 2019; Mentha et al., 2019; WHO, 2025b). Bloodborne transmission occurs through sharing needles, transfusion of contaminated blood products, tattooing, and haemodialysis. Sometimes blood donors who are unaware of their HBV and HDV infections may cause transmission through blood transfusion (Mansour et al., 2012).

Sexual transmission is possible through unprotected sexual contact, especially among individuals with multiple partners. Mother-to-child transmission has been reported during childbirth if the mother is coinfected with HBV and HDV. Importantly, since HDV requires HBV for propagation, the vaccine against HBV (containing HBsAg) can effectively prevent HDV infection in individuals not infected with HBV. [The Working Group was not aware of information in the literature that would allow conclusions to be drawn on the proportion of HBV-HDV coinfections and superinfections among the total number of HDV infections. In addition, since most HBV-HDV coinfections are cleared, it is more difficult to estimate the prevalence of coinfection than that of superinfection, which is usually not cleared.]

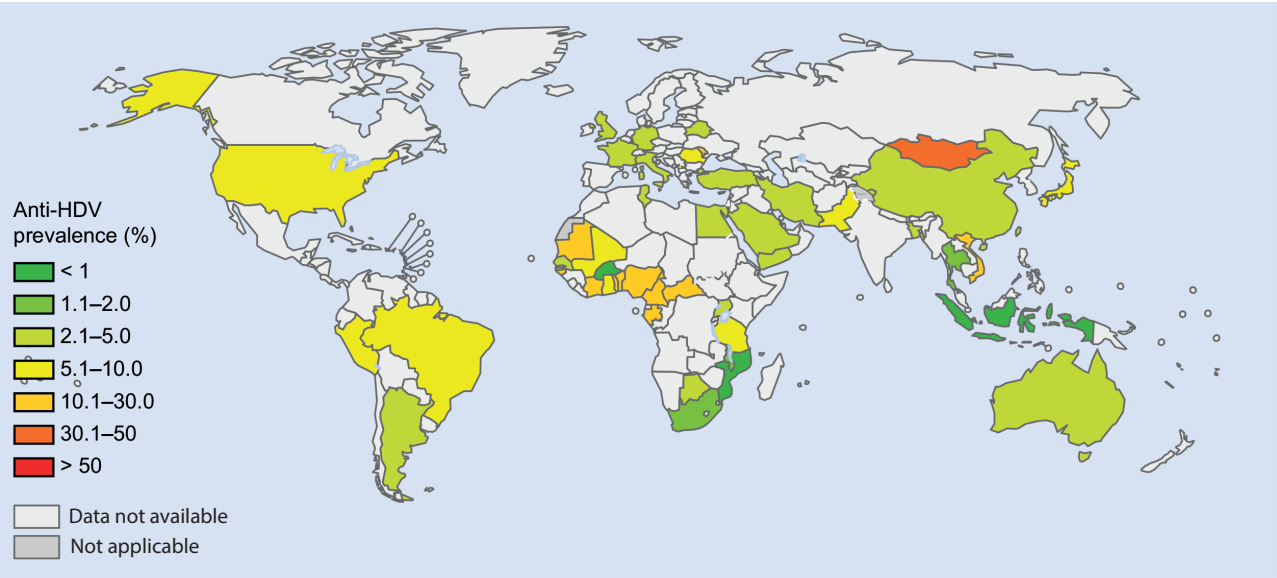
Interpreting the epidemiology of HDV requires the consideration of several key factors. HDV infection depends on the presence of HBV, and most studies test only HBsAg-positive individuals for HDV. HDV infection can reduce HBV DNA levels (Mahale et al., 2018; Lucifora et al., 2023; Negro and Lok, 2023). It is not known whether it could also lead to a decrease in HBsAg, which would complicate detection and diagnosis. [The Working Group noted that data in humans do not support complete suppression of HBsAg in HDV-infected individuals.] Superinfection with HDV and HBV can significantly aggravate damage to liver function (see Section 4), increasing mortality and potentially influencing prevalence data. Furthermore, geographical variation in HDV prevalence reflects the distribution of the virus as well as exposure to HBV and infection patterns in the tested populations. Epidemiological studies often classify HDV prevalence data on the basis of HBV infection levels, emphasizing the interconnected nature of these two viruses in understanding their global impact (Negro and Lok, 2023). In general, HDV is found in areas with a high prevalence of HBV infection in Central and West Africa, Central and North Asia, some Pacific Islands, the Middle East, eastern Europe, and parts of South America (Stockdale et al., 2020, see Fig. 1.3). Given these considerations, the Working Group summarized the epidemiology of HDV infection according to the endemicity of hepatitis B.

1.3.2 Prevalence of HDV infection in the general population

Except for South America and south-eastern Asia, the main genotype of HDV that is prevalent globally is type 1 (see Section 4.1.2). Type 4 is more prevalent in south-eastern Asia, whereas type 3 is the main genotype prevalent in South America (Stockdale et al., 2020). Owing to the particularity of the dependence of HDV on HBV infection for replication, the calculation of the prevalence of HDV is based on HBsAg-positive patients; all prevalence values in the present monograph, unless stated otherwise, are reported as the percentage within HBsAg-positive individuals by region (see also Table 1.2).

[Owing to the natural history of HDV infection, which requires prior or simultaneous infection with HBV, most studies on the

Fig. 1.3 Seropositivity for HDV in people who are positive for HBV in the general population worldwide



HBV, hepatitis B virus; HDV, hepatitis D virus.

From Stockdale et al. (2020). ©2020 European Association for the Study of the Liver. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/). *J Hepatol.* 2020, 73, 523–532.

prevalence of HDV infection are performed among HBV-positive individuals. The Working Group noted that this could make it difficult to determine the prevalence of HDV in the general population and its impact on global health. To better perceive these numbers, hereafter the global variation in HBV prevalence is described.] The Polaris Observatory study reported a global prevalence of 3.2% or 258 million people on the basis of a country- and region-level modelling study of 170 countries in 2022 (Razavi-Shearer et al., 2023), and in a review from 2023, Hsu et al. (2023) described varying prevalence across Europe: below 1% in western and northern regions of Europe, and 4–8% in several eastern European countries and regions. The prevalence of HBV in parts of Asia in the Western Pacific Region varied among countries: 0.9% in Australia, 1% in Japan, 4.4% in the Republic of Korea, 6.3% in China, 11% in Mongolia, and 18.8% in the Solomon Islands (WHO, 2025a). In the USA, the prevalence of HBV is estimated as 0.71% (Lim et al., 2020). Up to 9.4% of people in native communities in the Peruvian Amazon were classified as chronic carriers of HBsAg (Cabezas et al., 2006).

A few studies have estimated the overall global prevalence of HDV in the general population, reporting values that ranged from 0.11% to 0.98% (Chen et al., 2019a; Miao et al., 2020; Stockdale et al., 2020). Miao et al. (2020) estimated regional prevalence to vary from as low as 0% (southern sub-Saharan Africa), 0.09% (central Europe), 0.20% (North America, high income), or 0.25% (western Europe) to as high as 4.04% (Oceania) or 8.31% (central Asia).

(a) Europe

In Europe, the prevalence of HDV infection in HBV-positive individuals varies widely across different countries (Table 1.2). For instance, a prevalence of 9.28% (for anti-HDV–positive) has been reported from Italy for the period 2019–2021 (Brancaccio et al., 2023). In France, a lower

Table 1.2 Prevalence of positivity for HDV antibody and HDV RNA in various populations that are seropositive for HBsAg, by country or region

Country or region	Period	Group studied	Population studied	Availability of sample	No. of HBsAg+ individuals tested for HDV	Prevalence (%) of positivity for anti-HDV or HDV RNA	Reference
Europe							
Italy	2019–2021	HBsAg+ for ≥ 6 mo	Nationwide study	Clinical laboratory	4583	9.28% (anti-HDV+)	Brancaccio et al. (2023)
France	1997–2011	HBsAg+	Blood donors	National bloodbank	4492	[1.98%] (HDV RNA+)	Servant-Delmas et al. (2014)
Belgium	2014–2021	HBsAg+ population	Follow-up every 6–12 mo	Liver clinic	534	[12.5%] (anti-HDV+); [47%] (HDV RNA+)	Weichselbaum et al. (2024)
Scotland	2011–2016	Population with chronic HBV	Newly diagnosed HBV	NHS specialist virology laboratory	1133	2.0% (HDV RNA+)	Jackson et al. (2018)
Greece	2016–2021	HBsAg+ population	Liver clinic patients	RNA in central laboratory; Ag in different laboratories	2696	5.8% (anti-HDV+); [63%] (HDV RNA+)	Papatheodoridis et al. (2023)
Germany	2000–2011	HBsAg+ population	Chronic HBV	University hospital laboratory	2844	[7.4%] (anti-HDV+); [64.5%] (HDV RNA+)	Reinheimer et al. (2012)
Poland, south-east	2017	HBsAg+ population		Clinical laboratory	138	[2.29%] (anti-HDV+)	Pleśniak and Wawrzynowicz-Syczewska (2019)
Poland, Silesia	2020–2022			Clinical laboratory	177	3.4% (anti-HDV+)	Włosowicz et al. (2024)
Romania	2011	HBsAg+ population	New and chronic HBV, national study	Clinical laboratory	2761	23.1% (anti-HDV+); 16.4% (HDV RNA+)	Gheorghe et al. (2015)
Switzerland	2002–2013	Population with chronic HBV	HBV-infected Swiss residents	Clinical laboratory (retrospective study)	338	4.4% (anti-HDV+)	Hirzel et al. (2015)
Spain	2000–2017	HBsAg+ population	Patients in regular follow-up, HDV testing every 5 yr	Clinical laboratory	478	4.0% (anti-HDV+)	Aguilera et al. (2018a)
Kingdom of the Netherlands	2017–2019	HBsAg+ population	Outpatients were tested for HDV every 6–12 mo	University medical centre laboratory	925	3.7% (anti-HDV+); [2.0%] (HDV RNA+)	Beudeker et al. (2021)
Asia							
Japan, Hokkaido	2006–2022	HBsAg+ population	University hospital patients	University hospital laboratory	601	1.7% (anti-HDV+)	Sasaki et al. (2023)

Table 1.2 (continued)

Country or region	Period	Group studied	Population studied	Availability of sample	No. of HBsAg+ individuals tested for HDV	Prevalence (%) of positivity for anti-HDV or HDV RNA	Reference
Republic of Korea	2013–2019	Population with chronic HBV	Patients from national database	National health insurance database	444 203	0.3% (anti-HDV+)	Cho et al. (2023)
Kazakhstan	2017–2019	Population with chronic HBV	Patients treated in hepatology centres in 13 cities	Regional central laboratories	834	31.29% (anti-HDV+); 30.70% (HDV RNA+)	Ilyassova et al. (2023)
Mongolia	NR	HBsAg+ population	Rural population with self-reported liver disease	Clinical laboratory	431	65.20% (anti-HDV+); [73.7%] (HDV RNA+)	Magvan et al. (2023)
China, Jilin province	2021–2022	Population with chronic HBV	University hospital outpatients	Clinical laboratory	5594	3.6% (anti-HDV+); 1.2% (HDV RNA+)	Zi et al. (2023)
Qatar	2010–2019	HBsAg+ population	Patients in 12 hospitals	Electronic laboratory system (database)	2348	5.3% (anti-HDV+)	Abdallah et al. (2022)
India	2018–2019	HBsAg+ population	Patients with acute hepatitis	Clinical laboratory	372	2.1% (anti-HDV+)	Sonkar et al. (2022)
Viet Nam	2018–2019	Population with chronic HBV	Chronic HBV infection, liver cirrhosis, HCC, military hospital	Clinical laboratory	205	16.1% (anti-HDV+)	Hoan et al. (2021)
Taiwan, China	2018–2019	Chronic HBV population	District hospital	Hospital laboratory	346	1.2% (anti-HDV+)	Lee et al. (2021)
Uzbekistan	2016–2018	HBsAg+ population	Cirrhosis patients, national centre	Institute of virology	3694	80.8% (anti-HDV+)	Khodjaeva et al. (2020)
Islamic Republic of Iran, western provinces	2004–2016	HBsAg+ population	Hepatitis screening, general population		1749	1.7% (anti-HDV+)	Sayad et al. (2018)
Israel	1998–2016	HBsAg+ population	Adults	Laboratory database	613	[5.4%] (anti-HDV+)	Eilat-Tsanani et al. (2022)
Türkiye	2010	HBsAg+ population	HBV-infected patients	Clinical laboratory	35 013	5.5% (anti-HDV+)	Uraz et al. (2023)
Afghanistan	2015	HBsAg+ population	Randomly selected adult population	Clinical laboratory	234	2.1% (anti-HDV+)	Husseini et al. (2019)
Pakistan	2016–2017	HBsAg+ population	Pregnant women	Clinical laboratory	63	20.6% (anti-HDV+); [30.8%] (HDV RNA+)	Aftab et al. (2019)
Saudi Arabia	1996–1997	HBsAg+ population	Primary screening in the general population	Clinical laboratory	780	8.6% (anti-HDV+)	Al-Traif et al. (2004)

Table 1.2 (continued)

Country or region	Period	Group studied	Population studied	Availability of sample	No. of HBsAg+ individuals tested for HDV	Prevalence (%) of positivity for anti-HDV or HDV RNA	Reference
North America							
USA	2014–2020	HBV+ patients	Adult patients diagnosed with HBV infection or HBV-HDV coinfection	Medical records, nationwide	144 975	4.6% (anti-HDV+)	Gish et al. (2024a)
Canada	2012–2019	HBsAg+ population	Patients from tertiary care-based clinical cohort	Hospital samples	7080	4.8% (anti-HDV+); 3.1% (HDV RNA+)	Osiowy et al. (2022)
Mexico	NR	HBsAg+ population	Hospitalized patients with cirrhosis	Hospital samples	116 000	0.2% (adjusted anti-HDV+); 69.9% (HDV RNA+)	Razavi-Shearer et al. (2024)
South America							
Brazil	2012–2016	HBsAg+ population	Randomly selected general population (for representability)	General population samples (home visits)	92	8.7% (anti-HDV+)	Nunes et al. (2021)
Colombia	NR	HBsAg+ population	Voluntary participation of general population adults	Collected through home visits and meetings	23	43.5% (anti-HDV+); 30.4% (HDV RNA+)	di Filippo Villa et al. (2015)
Africa							
Ethiopia	2015	Population with chronic HBV	Hospitalized patients without HIV or HCC	Hospital samples	1267	1.5% (anti-HDV+); 0.9% (HDV RNA+)	Aberra et al. (2018)
Egypt	2016–2018	HBsAg+ population	HBV patients in outpatient clinics	Hospital samples	631	3.5% (anti-HDV+)	Elzefzafy et al. (2022)
Cameroon	NR	HBsAg+ population	Randomly selected general population (for representability)	General population samples (home visits)	1621	13.8% (anti-HDV+)	Besombes et al. (2020)
Nigeria	NR	HBsAg+ population	Patients hospitalized in gastroenterology unit of the hospital with chronic HBV	Hospital samples	180	18.9% (anti-HDV+)	Abdulkareem et al. (2021)
Libya	2015–2018	HBsAg+ population	National screening in Libya – blood donors, preoperative patients, pregnant women, patients with kidney and liver disease, pre-marriage screening	Screening samples	1873	1.7% (anti-HDV+)	Daw et al. (2020)

Table 1.2 (continued)

Country or region	Period	Group studied	Population studied	Availability of sample	No. of HBsAg+ individuals tested for HDV	Prevalence (%) of positivity for anti-HDV or HDV RNA	Reference
Algeria	2007–2008	HBsAg+ population	Patients with chronic HBV followed at hospital's hepatology department	Hospital samples	134	5.4% (anti-HDV+)	Gourari et al. (2019)
Gabon	2005–2008	HBsAg+ population	Randomly selected general population (for representability)	General population samples	303	27.7% (anti-HDV+)	Groc et al. (2019)
Central African Republic	2010	HBsAg+ population	Volunteer students and pregnant women (in maternity wards)	General population and hospital samples	179	10.6% (anti-HDV+)	Komas et al. (2018)
Burkina Faso	2013–2014	HBsAg+ population	Blood donors, pregnant women, outpatients	Routine samples	117	3.4% (anti-HDV+)	Sanou et al. (2018)
Tunisia	2002–2012	HBsAg+ population	HBsAg+ patients from gastroenterology hospital departments	Hospital samples	1615	2.0% (anti-HDV+)	Yacoubi et al. (2015)
Mauritania	2008–2009	HBsAg+ population	Pregnant women and patients having routine checkups	Routine samples	155	58.1% (anti-HDV+)	Mansour et al. (2012)
Oceania							
Kiribati	2017	HBsAg+ population	Voluntary patients who recalled having previously tested positive for HBsAg	[It was not clear to the Working Group whether these were general population samples or hospital-based.]	201	55.7% (anti-HDV+); 40.8% (HDV RNA+)	Jackson et al. (2020)
Australia	1997–2016	HBsAg+ population	Individuals tested for HDV in the public health-care system	Public health database	4407	4.1% (anti-HDV+)	Coghill et al. (2018)

Ag, antigen; anti-HDV, antibody to HDV; anti-HDV+, positive for antibody to HDV; HBsAg, hepatitis B surface antigen; HBsAg+, positive for HBsAg; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HDV, hepatitis D virus; HDV RNA+, positive for HDV RNA; HIV, human immunodeficiency virus; mo, month(s); NHS, National Health Service; NR, not reported; USA, United States of America; yr, year(s).

prevalence of 1.98% (for HDV RNA positivity) was reported for the period 1997–2011 (Servant-Delmas et al., 2014). In Belgium, over the period 2014–2021, a prevalence of 10.98% for anti-HDV positivity and 4.59% for HDV RNA positivity was reported (Weichselbaum et al., 2024). In studies in other countries, such as Greece and Germany, moderate prevalence rates of 5.8% and 7.0% for anti-HDV positivity were reported (Reinheimer et al., 2012; Papatheodoridis et al., 2023). In a study in Romania, high prevalences of 23.1% for anti-HDV positivity and 16.4% for HDV RNA positivity were reported (Gheorghe et al., 2015), whereas in studies in Switzerland and Spain relatively lower figures were reported, namely 4.4% in Switzerland and 4.0% in Spain (Hirzel et al., 2015; Aguilera et al., 2018a). This variability highlighted regional differences and underscored the complex geographical distribution of HDV in Europe. [The Working Group noted that some of these differences between countries might reflect differences in the representativeness of the study population for estimates of national prevalence of HDV.]

(b) Asia

Asia exhibited significant geographical variations in HDV prevalence. In a study from Japan, Sasaki et al. (2023) reported a low prevalence of 1.7% for anti-HDV positivity among HBsAg-positive individuals, and data from China showed a moderate prevalence of 3.6% for anti-HDV positivity (Zi et al., 2023). In countries such as the Islamic Republic of Iran and India, HDV prevalence was relatively low, ranging from 1.7% to 2.1% for anti-HDV positivity among HBsAg-positive individuals, respectively (Sayad et al., 2018; Sonkar et al., 2022). Mongolia was noteworthy for its extremely high prevalence of HDV among HBV-positive individuals, with 65.2% of individuals testing positive for anti-HDV and 48.03% testing positive for HDV RNA (Magvan et al., 2023). The variations across Asia underscored the diverse burden of HDV infection in different regions of the continent. [The Working Group noted that differences in the predominant mode of HDV transmission are likely to play a role in these differences between countries, with higher HDV prevalence in areas where blood-borne transmission is more common.]

(c) North America

In North America, the prevalence of HDV among individuals with HBV infection is moderate. In the USA, there is a prevalence of 4.6% for anti-HDV positivity in patients with hepatitis B (Gish et al., 2024a), whereas in Canada there is a similar rate of 4.8% for anti-HDV positivity and 3.1% for HDV RNA positivity among the HBsAg-positive population (Osiowy et al., 2022). In Mexico, the adjusted prevalence of anti-HDV–positive individuals among HBsAg-positive individuals was reported as 0.2% (Razavi-Shearer et al., 2024).

(d) South America

In South America, the prevalence of HDV infection varies significantly across countries. In studies in Brazil, an 8.7% prevalence of HDV was reported in HBsAg-positive individuals (Nunes et al., 2021), whereas in studies in Colombia there was an extremely high prevalence of 43.5% for anti-HDV positivity and 30.4% for HDV RNA positivity among HBsAg-positive individuals (di Filippo Villa et al., 2015). [This marked difference reflects the geographical and ethnic variability within South American populations, with some areas experiencing much higher levels of HDV infection.]

(e) Africa

In Africa, HDV prevalence was generally high. For example, in Gabon, the prevalence of HDV infection among HBsAg-positive individuals was 27.7% (Groc et al., 2019), and in Nigeria, it reached 18.9% (Abdulkareem et al., 2021). In other countries, such as Ethiopia and Libya,

lower prevalence rates of 1.5% and 1.7%, respectively, were reported (Aberra et al., 2018; Daw et al., 2020). [The prevalence of HDV in sub-Saharan Africa is influenced by ethnic factors and local variations in HBV endemicity.]

(f) Oceania

In Oceania, the prevalence of HDV infection is particularly high in some regions, especially in Kiribati, where 55.7% of the tested HBsAg-positive population was infected with HDV, and 40.8% tested positive for HDV RNA (Jackson et al., 2020). In a study in Australia, a relatively lower prevalence of 4.1% was reported for anti-HDV positivity among HBsAg-positive individuals (Coghill et al., 2018). [The Working Group noted that the stark contrast in Kiribati highlights the varying burden of HDV infection within the region.]

(g) Specific populations

The prevalence of HDV is often high in people who inject drugs. In the USA, a study conducted between 1998 and 2000 among HBsAg-positive individuals who were injecting drug users (IDUs) within the past 30 days found that 35.6% were also infected with HDV (Mahale et al., 2018). In Taiwan, China, a study involving 1697 IDUs found that among 155 HBsAg-positive participants, 39 (25.2%) were seropositive for anti-HDV (Lu et al., 2021). A study in IDUs in Spain indicated that when comparing data from 1993–1996 and 2011–2014, of 209 enrolled participants in 1993–1996, 62 (29.7%) were anti-HDV–positive, whereas in the second phase, only 2 (4.2%) of the 47 enrolled participants were found to be anti-HDV–positive (Aguilera et al., 2018b).

Sexual transmission of HDV has been described in a survey conducted in California, USA, in 1986–1987. It included 1368 female sex workers, and results suggested that among HBV-carrying IDUs, 21% had HDV infections; in HBV-carrier non-IDUs, 6% had HDV infections (Rosenblum et al., 1992). A prospective study in Taiwan, China, showed that there was a higher proportion of individuals who had engaged in high-risk sexual behaviours in a population that was coinfected with HBV and HDV (90%) than in a population that was HBsAg-positive alone (22.8%). The authors concluded that this suggested that heterosexual exposure, particularly contact with sex workers, is an important route of HDV transmission in this area where HBV infection was endemic (Liaw et al., 1990). In a study in Sydney, Australia, Bodsworth et al. (1989) found that among 204 homosexual men with acute or chronic HBV infection, total antibody to HDV was detected in eight men, and HDAg was detected in one additional man (4.4% of the total).

Familial transmission has been less well studied. A study in the Russian Federation that included 383 HBV-HDV–coinfected patients and their family members showed that 15 out of 42 patients (18 families) were HDV RNA–positive (35.7%). In two mothers, replication markers for both viruses were found, and their daughters (aged 15 and 17 years) were serum HDV RNA–positive and were diagnosed with liver cirrhosis. This showed the possibility of vertical (familial) transmission (Kozhanova et al., 2015).

Some study results showed a correlation between older age and prevalence of HDV. A study in China showed that the screening-positive rates of total HDV antibody and HDV RNA in people aged 31–50 years were lower than those in people aged 51–80 years; 87.69% of patients with hepatitis D were aged 51–70 years (Zi et al., 2023).

1.3.3 Diseases other than cancer and co-exposures associated with infection

HDV infection can lead to more severe diseases, including liver diseases and extrahepatic complications, than does HBV infection alone (see Section 4). The infection can manifest either as a coinfection (simultaneous infection

with HBV and HDV) or superinfection (HDV infection in someone already chronically infected with HBV) (Hughes et al., 2011).

HDV-infected patients may be coinfected with other viruses, such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV). HDV infection relies on the presence of HBV. The results of a meta-analysis suggested that the prevalence of HIV-HBV-HDV coinfection was 13.4% in 4079 patients with chronic hepatitis, with 560 patients being triply coinfected (Chen et al., 2022).

1.3.4 Treatment and vaccination rates

Current treatment options for chronic HDV infection (described in more detail in Section 4.1.3) are limited but evolving. The primary treatment for HDV and HBV infection is pegylated interferon alpha (peg-IFNα); however, its effectiveness is variable, and many patients experience side-effects such as flu-like symptoms, depression, and neutropenia, which can limit its use (Ye and Chen, 2021). A more recent therapeutic development is bulevirtide, which aims to prevent HDV from entering liver cells and has received regulatory approval in Europe as a treatment for chronic HDV infection (EMA, 2020). Cure of HDV infection may be more frequent with the combination of bulevirtide and peg-IFN (El Messaoudi et al., 2024). In addition, liver transplantation is an option that can be considered for patients with end-stage liver disease or hepatocellular carcinoma (HCC).

As HDV relies on HBV for its propagation, HBV vaccination serves as an indirect method for the prevention of HDV infection (WHO, 2025b). Since individuals who are immune to HBV cannot become infected with HDV, universal HBV vaccination has significantly reduced the incidence of both HBV and HDV infections. However, HBV vaccination does not protect individuals who are already chronically infected with HBV. For these patients, there are currently no vaccines specifically targeting HDV.

Global HBV vaccination programmes have been widely implemented, especially in countries with a high prevalence of HBV and HDV. In 2024, WHO estimated the vaccination coverage for a third dose of the vaccine in its regions to be 75% (African Region), 81% (Eastern Mediterranean Region), 89% (European Region), 86% (Region of the Americas), 94% (South-East Asia Region), and 89% (Western Pacific Region), ranging from 39% (Sudan) to 99% (Turkmenistan) (WHO, 2026). These programmes have proven effective in reducing the burden of both infections, with many countries reporting high vaccination rates (Goyal and Murray, 2014). Global HBV vaccination coverage among WHO Member States reached 83% by 2020, although birth dose administration varied regionally (6–84%), with a global average of 42%. In Europe, reported rates were 91% for infant three-dose vaccination, marking a 10% rise since 2009. The WHO 2030 Sustainable Development Goals aim to reduce HBV infection incidence by 90% and HBV-related mortality by 65% through enhanced immunization strategies (Zacharias et al., 2015; Gökengin et al., 2023), significantly lowering the number of new HDV infections.

1.4 Quality of exposure assessment in key epidemiological studies of cancer and mechanistic studies in humans

The Working Group reviewed exposure assessment methods used in 17 key studies on cancer in humans (see Table S1.3, Annex 1, Supplementary material for Section 1, Exposure characterization, online only, available from: https://publications.iarc.who.int/667). Across those studies, the methods of assessment for HDV infection varied substantially in design and rigour. Therefore, it was difficult to determine

the overall quality of the exposure assessments. HDV infection was generally defined by laboratory-based measures. The laboratory assays employed in these studies can be categorized as those that detect antibodies to HDV (anti-HDV in serum) and those that detect evidence of the virus itself (HDV RNA in blood or HDV antigen in liver tissue). Among these studies, several different commercial antibody assays were used or the anti-HDV assay used was not specified. Most of the assays for HDV RNA were described as "in house". Test sensitivity was reported for five of the HDV RNA assays (Romeo et al., 2009, 2014; Béguelin et al., 2017, 2023; Jang et al., 2021a).

Acute infection with HDV can result from simultaneous infection with HBV (coinfection) or HDV infection in the presence of pre-existing CHB (superinfection) (see Section 1.2.2). Most HBV-HDV coinfections result in the clearance of both viruses, whereas most cases of superinfection result in the development of chronic hepatitis D (CHD) (Negro and Lok, 2023). Among the studies selected for review, only one (Ji et al., 2012) included data on acute HDV infection. That study assessed HDV status according to International Classification of Diseases 7th revision (ICD-7) codes, which provided no information on whether HDV infection resulted from coinfection or superinfection.

In a preferred approach for defining HDV infection status (WHO, 2024), both past and current HDV infection status are assessed in HBsAg-positive individuals via testing for total anti-HDV antibodies followed by testing for HDV RNA in serum or plasma to diagnose CHD. Testing for HDV RNA is preferred over HDV antigen testing because it can be performed in blood samples, therefore not requiring a liver biopsy. Studies that used sequential testing for HBsAg, anti-HDV, and HDV RNA were categorized by the Working Group as either "good assessment of CHD based on anti-HDV and HDV RNA testing" (Romeo et al., 2009; Béguelin et al., 2017, 2023; Mahale et al., 2019; Jang et al., 2021a) or "adequate marker of CHD assessed based on anti-HDV and HDV RNA testing, but method specifics are not reported and performance is unknown" (Brancaccio et al., 2019), depending on whether the anti-HDV test used was well described and of good quality.

For other studies, assessment of HDV was limited to testing for anti-HDV with no or incomplete testing for virus detection. Exposure assessment for such studies was described either as "good assessment of past or current HDV infection" (Amougou et al., 2016) or "adequate marker of past or current HDV infection was assessed, but no specifics on method were reported and performance was unknown" (Raimondo et al., 1984; Trichopoulos et al., 1987; Fattovich et al., 2000; Kushner et al., 2015; Roulot et al., 2025; Wong et al., 2025). In some of these studies, levels of HDV RNA were measured (e.g. Roulot et al., 2025), which provides useful information to distinguish between individuals with active viral replication (RNA-positive) and those with inactive or cleared infections (RNA-negative), although this information was not used for the identification of HDV infection in all participants.

The two oldest studies under evaluation (Raimondo et al., 1984; Trichopoulos et al., 1987) employed RIAs for the detection of anti-HDV. RIAs are no longer in common use for HDV infection, and the Working Group noted that these may be less accurate than nucleic acid assays (Akuffo et al., 2024), though direct evidence to determine the appropriateness of RIA methods for detecting HDV infection was lacking.

In the two largest studies under consideration, HDV infection was defined solely on the basis of ICD diagnostic codes, and no information was provided on the laboratory methods used to assess infection (Ji et al., 2012; Mallet et al., 2017). Diagnostic practices in these populations may have varied over time and across study centres. Therefore, the Working Group was unable to evaluate the quality of these exposure definitions.

Finally, in contrast to the other 16 studies, in which the risk of HCC in people with HBV-HDV coinfection was compared with that in people with HBV infection alone, there was one study (Romeo et al., 2014) on the relationship between HDV RNA levels and risk of HCC among individuals with CHD. The HDV RNA assay used in that study appeared to be suitable for quantitative exposure analysis.

2. Cancer in Humans

Introduction

HDV has been previously evaluated by the *IARC Monographs* programme as *not classifiable as to its carcinogenicity to humans* (Group 3) (Volume 59; IARC, 1994). There was *inadequate* evidence regarding cancer in humans, because only a handful of case–control studies, mostly with limited statistical power, were available at the time.

A systematic search was conducted in the PubMed database to identify relevant studies. The search terms and the lists of retrieved studies are available from: https://hawcproject.iarc.who.int/assessment/765/.

Since the previous evaluation, the literature on HDV and cancer risk in humans has grown appreciably. A large number of cohort and case–control studies were available, as well as several published meta-analyses. All studies had HCC as the outcome. Case series were considered but judged to be not informative. Ecological studies, case reports, or studies in which HCC was combined with other liver diseases, were not considered further.

As HDV requires HBV (which is classified by IARC in Group 1, *carcinogenic to humans*; IARC, 2012) to infect hepatocytes, studies that did not include analyses restricted to HBsAg-positive patients were not considered further, because such studies would be strongly confounded by HBV infection.

Primary studies considered to be informative were studies limited to HBsAg-positive patients; cohort studies that ensured consideration of basic demographic variables (i.e. those in which crude analysis only was presented were excluded); and case–control studies that ensured some comparability between cases and controls (e.g. clear definition of the control group, presentation of basic demographic characteristics by cases and controls, matching) in the study design. Meta-analyses of studies were also included.

2.1 Hepatocellular carcinoma

2.1.1 Cohort studies

See Table 2.1.

Thirteen cohort studies on the epidemiological evidence for an association between HDV infection and subsequent development of HCC were considered to be informative and are described below (Fattovich et al., 2000; Romeo et al., 2009, 2014; Ji et al., 2012; Kushner et al., 2015; Béguelin et al., 2017, 2023; Mallet et al., 2017; Brancaccio et al., 2019; Jang et al., 2021a; Wranke et al., 2023; Roulot et al., 2025; Wong et al., 2025). A fourteenth study, although providing only descriptive statistics, was considered to be informative as it evaluated the role of HDV genotypes in HCC development (Su et al., 2006).

The other identified cohort studies were considered to be uninformative, five because of the lack of clear exposure (Ieluzzi et al., 2014) or outcome data (Caturelli et al., 2003; Senturk et al., 2008; Liao et al., 2014; d'Arminio Monforte et al., 2024); five because of low sample size (Craxì et al., 1984; Sheng et al., 2007; Abbas et al., 2014; Fernández-Montero et al., 2014; Tarhan et al., 2025); 15 because of the presentation of strictly descriptive statistics (Colombo et al., 1983; Smedile et al., 1991; Tamura et al., 1993; Fattovich et al., 1995; Liaw et al., 2004; Mumtaz et al., 2005;

Table 2.1 Epidemiological studies on liver cancer and HDV

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Fattovich et al. (2000) Europe January 1973 to December 1991 Cohort	200 HBsAg+ cohort of patients with compensated cirrhosis (EUROHEP); eligibility includes: (i) HBsAg positivity; (ii) histological diagnosis of cirrhosis according to international criteria; (iii) no history or clinical evidence at enrolment of complication of cirrhosis; (iv) no clinical evidence of HCC at entry; (v) information on serum HBeAg and anti-HDV at presentation; (vi) follow-up at the enrolment centre for ≥ 6 mo; (vii) no antiviral or steroid treatment during follow-up. *Exposure assessment method*: HDV infection assessed with anti-HDV in serum and HDV antigen in liver.	Liver (HCC), incidence	HDV status (unadjusted RR): Anti-HDV–/ HBeAg– Anti-HDV+/ HBeAg–	 19 5	 1 0.8 (0.3–2.0)	None	*Exposure assessment critique*: Adequate marker of past or current HDV infection was assessed, but no specifics on method were reported and performance was unknown. Method for anti-HDV testing was not specified. *Design and analysis:* *Strengths*: Median follow-up, 80 mo (range, 6–198 mo); good retention; data were available on HBsAg, HBeAg, HBV DNA, anti-HDV, and anti-HIV, as well as serum antibodies to HCV; stringent criteria for HCC diagnosis. *Limitations*: All participants had cirrhosis at baseline; unclear how many participants in the anti-HDV+ group had HCV or HIV (n = 36, positive for either), and these variables were not accounted for in the models; the small sample of participants with HCC lead to imprecise estimates. Alcohol use and BMI were not accounted for. *Other comments*: Supersedes Fattovich et al. (1995). Enrolment follow-up dates were assumed to be those reported in Realdi et al. (1994).
		Liver (HCC), incidence	HDV status (adjusted RR): Anti-HDV–/ HBeAg– Anti-HDV+/ HBeAg–	 19 5	 1 3.2 (1.0–10.0)	Age, sex, $\log_{10}$(bilirubin), albumin, γ-globulins, platelets, HBeAg status	

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Su et al. (2006) Taipei Veterans General Hospital, Taiwan, China 1983–2003 Cohort	194 Taiwanese patients with chronic HBV (HBsAg+) and HDV dual infections (anti-HDV+). *Exposure assessment method*: PCR, genotypes determined by RFLP, northern blot hybridization.	Liver (HCC), incidence	Incidence of HCC (%): HDV genotype 1 (n = 46) HDV genotype 2 (n = 72) *P* value, 0.008	 15 8	 32.6% 11.1%	None	*Strengths*: Exclusion of individuals with HIV, HCV, or alcohol use disorder. Results by HDV genotype. *Limitations*: Provided only unadjusted data without reporting risk estimates.
Romeo et al. (2009) Milan, Italy 1978–2006 Cohort	299 patients with HDV infection of ≥ 6 mo duration who were consecutively admitted to the Liver Centre, Maggiore Hospital, Milan; 32% had cirrhosis at baseline and an additional [27.4%] had cirrhosis during follow-up. *Exposure assessment method*: IHC for HDV antigen in liver tissue or serum HDV RNA.	Liver (HCC), incidence	HDV RNA (crude OR): Not present Present	 12 34	 1 1.43 (0.67–3.06)	NR	*Exposure assessment critique*: Good assessment of CHD according to detection of HDV antigens in tissue or of serum HDV RNA. There were concerns over sample preservation over time. *Design and analysis:* *Strengths*: The study was specifically designed to evaluate progression of HDV; HDV replication activity was assessed; long follow-up time (median, 233 mo); HCV and HIV infection were evaluated; time since cirrhosis diagnosis was taken into account for results generation; treatment modality was taken into account.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Romeo et al. (2009) (cont.)							*Limitations*: Long enrolment window; mostly a descriptive paper with only crude modelling techniques presented; HCV and HIV were not evaluated in the context of HCC development owing to small numbers (5 and 1, respectively); crude assessment of HDV (HDV RNA was reported either present or not present). Alcohol use and BMI were not accounted for. *Other comments*: Overlap with study by Romeo et al. (2014).
Romeo et al. (2014) Milan, Italy 1978–2006 Cohort	193 (105 without cirrhosis); patients with an HDV infection of ≥ 6 mo duration who were consecutively admitted at the Liver Centre, Maggiore Hospital, Milan; must have frozen baseline and at least one additional frozen serum sample. *Exposure assessment method*: Quantitative HDV RNA via real-time PCR.	Liver (HCC), incidence	HDV RNA, overall cohort, including those with cirrhosis at baseline (crude OR): Negative Positive	 NR NR	 1 1.32 (1.02–1.71)	None	*Exposure assessment critique*: Good assessment of HDV RNA levels that allowed an activity–response association to be determined. However, the extended follow-up period may have compromised the HDV exposure assessment owing to sample degradation, freeze–thaw cycles, and evolving clinical protocols over time, introducing potential misclassification or selection bias.
		Liver (HCC), incidence	HDV RNA, overall cohort, including those with cirrhosis at baseline (adjusted OR): Negative Positive	 NR NR	 1 1.42 (1.04–1.95)	HBV DNA, HBsAg, age, sex, alcohol consumption, HBeAg, IFN	
		Liver (HCC), incidence	HDV RNA, excluding those with cirrhosis at baseline (crude OR): Negative Positive	 NR NR	 1 1.66 (1.04–2.65)	None	
		Liver (HCC), incidence	HDV RNA, excluding those with cirrhosis at baseline (OR): Negative Positive	 NR NR	 1 2.64 (1.13–6.16)	HBV DNA, HBsAg, age, sex, alcohol consumption, HBeAg, IFN	

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Romeo et al. (2014) (cont.)							*Design and analysis:* *Strengths*: Analyses were conducted for the whole subcohort (including those with cirrhosis at baseline) and separately for those without cirrhosis at baseline, and adjusted models were presented. *Limitations*: Long enrolment window. Data on HCV and HIV were not presented (although the analyses were known to have been conducted). Sample sizes were small. Non-standard use of logistic regression in a cohort design. BMI was not accounted for. *Other comments*: Overlap with earlier study by Romeo et al. (2009).

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Ji et al. (2012) Sweden 1 January 1997 to 31 December 2008 Cohort	9160 patients with HBV, identified through the Swedish Hospital Discharge Register and Outpatient Registry (ICD-10 codes: B181, chronic HBV infection without HDV; B180, chronic HBV infection with HDV; and B171, acute HDV infection in HBV carriers); linkage with the Swedish Cancer Registry (first primary HCC) and Cause of Death Register (to identify the date of death); linkage with Swedish National Population and Housing Census for information on individual-level SES; linkage with Emigration Registry to identify the date of emigration. Exclusion of those with HIV or HCV. *Exposure assessment method*: Clinical record codes.	Liver (HCC), incidence	SIR (comparison with HBV cohort): HBV without HDV infection HBV with acute HDV infection HBV with chronic HDV infection	 46 9 8	 1 6.11 (2.77–11.65) 3.90 (1.61–7.22)	Age (5-yr age groups), sex, 5-yr period for the date of diagnosis, SES, residential area	*Exposure assessment critique*: Unable to evaluate testing because HDV was defined based on clinical codes. *Design and analysis:* *Strengths*: Population-based data set; models adjusted for age, sex, 5-yr period for diagnosis, SES, and residential area. Adjustment for 5-yr period for diagnosis reduced concerns over the long recruitment period. *Limitations*: Lack of quantitative data on viral load or duration of infection; diagnosis was contingent on proper clinical diagnosis across the time frame (although the 5-yr period for diagnosis was adjusted for); data on risk factors were missing (e.g. alcohol use, BMI); no information on cirrhosis status; no information regarding treatment.
		Liver (HCC), incidence	Cox proportional hazards model (HR): HBV infection alone Acute HDV infection with HBV Chronic HDV infection with HBV	 46 9 8	 1 3.85 (1.87–7.89) 2.42 (1.29–4.56)	Unspecified	

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Kushner et al. (2015) USA October 1999 to December 2013 Cohort	2008; HBsAg+ cohort of patients within the Veterans Health Administration who were tested for HDV (positive and negative were included). *Exposure assessment method*: HDV antibody testing.	Liver (HCC), incidence	HDV status (HR fully adjusted model):			Age, alcohol, cirrhosis, nucleos(t)ide, HCV	*Exposure assessment critique*: Adequate marker of past or current HDV infection, assessed on the basis of anti-HDV, but method specifics were not reported and performance was unknown. *Design and analysis:* *Strengths*: Large, well-defined medical system/ study population; there was adjustment for most risk factors (e.g. alcohol use, HCV, cirrhosis). *Limitations*: Testing for HDV was conducted on only 7.8% of HBsAg+ participants in the study (could lead to misclassification bias); had to differentiate acute from chronic infection; potential overadjustment for cirrhosis as it was unclear if this reflected cirrhosis at baseline or at any point during the course of follow-up; person-time was not defined. There was selection bias as testing was done for clinical reasons. No adjustment for BMI and HIV.
			Negative	NR	NC		
			Positive	23	1.9 (1.1–3.7)		
		Liver (HCC), incidence	HDV status (HR partially adjusted model):			Age, significant alcohol use, cirrhosis	
			Negative	NR	1		
			Positive	23	2.1 (1.1–3.9)		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Wong et al. (2025) USA 2010–2023 Cohort	4437 veterans at Veterans Affairs health-care facilities in the USA, diagnosed with CHB and tested for HDV, without cirrhosis or HCC history; 71 (HBV+ and HDV+) patients were propensity score–matched (1:2, on age, sex, race, HBeAg status, CHB antiviral therapy, HCV, HIV, diabetes mellitus, and documented alcohol use disorders) to 140 HBV+ and HDV– individuals. *Exposure assessment method*: Positive for anti-HDV or HDV RNA.	Liver (HCC), incidence	Propensity score–matched analysis of HCC incidence (incidence per 100 person-years): HBV only (n = 140) HDV plus HBV (n = 71)	NR NR	0.41 (0.13–1.27) 1.28 (0.53–3.09)	Age, sex, race, HBeAg status, CHB antiviral therapy, HCV, HIV, diabetes mellitus, and documented alcohol use disorders at time of CHB diagnosis	*Exposure assessment critique*: Assessment of past or current HDV infection was based on anti-HDV alone for some patients. Modest overlap of eligibility with Kushner et al. (2015). *Design and analysis:* *Strengths:* Comprehensive propensity score matching. Exclusion of baseline cirrhosis. *Limitations*: Imprecise CIs; limited HDV testing rates, there was selection bias as testing was done for clinical reasons. *Other comments*: The Working Group calculation assumed a typing error in the denominator reported in the paper, as the reported incidence rate per 100 000 would be impossible. An incidence rate of 1.28 per 100 was deemed plausible and formed the basis of this calculation. The Working Group assumed five events with a mean follow-up close to the median of 5.57 yr for the HDV group and three events with a mean follow-up of 5.21 yr for the HBV-only group.
		Liver (HCC), incidence	Propensity score–matched HCC incidence (IRR): HBV only (n = 140) HDV + HBV (n = 71)	NR NR	1 [3.12 (0.75–13.05)]		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Béguelin et al. (2017) Switzerland, Europe January 1988 to December 2014 Cohort	727 participants from the Swiss HIV Cohort Study: HIV-infected, HBsAg+ adults who initiated ART and had stored samples available. Unspecified if cirrhosis at baseline was included. *Exposure assessment method*: Anti-HDV+ and HDV RNA+.	Liver (HCC), incidence	HDV status (HR): Negative Positive	 NR NR	 1 9.30 (3.03–28.61)	Age, sex, CD4 count, stage of HIV infection, most likely source of HIV transmission, HCV coinfection	*Exposure assessment critique*: Good assessment of CHD according to anti-HDV (also assessed HDV RNA testing). *Design and analysis:* *Strengths*: Large, well-defined/established cohort; multivariable data analyses accounted for HIV, HCV, and other important confounders. *Limitations*: No data on cirrhosis or lifestyle factors such as alcohol use or BMI; HDV status was based on a single time point. *Other comments*: Some overlap with the study by Béguelin et al. (2023).

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Béguelin et al. (2023) Europe, Israel, Argentina January 1988 to December 2019 Cohort	1556 participants from the Swiss HIV Cohort Study and EuroSIDA with a positive HBsAg test between January 1988 and December 2019; participants consisted of people living with HIV; exclusion was on the basis of lack of data on HDV infection or stored serum for analysis. Not reported whether cirrhosis at baseline was included. *Exposure assessment method*: Anti-HDV+ in all participants; HDV RNA in a subset of those who were anti-HDV+.	Liver (HCC), incidence Liver (HCC), incidence	HDV status (adjusted HR): Anti-HDV– Anti-HDV+ HDV status (adjusted HR): HDV– and anti-HDV+ with undetectable HDV RNA Active HDV replication (HDV RNA+)	 21 14 NR NR	 1 6.3 (2.5–16.0) 1 8.3 (3.0–22.7)	Age, sex, CD4 cell count, prior AIDS infection, stage of HIV infection, most likely source of HIV transmission, geographical region	*Exposure assessment critique*: Good assessment of past or current HDV infection; CHD was not assessed in all participants. *Design and analysis:* *Strengths*: Use of two well-established, large, prospective cohort studies; robust analyses with multivariable modelling; HDV RNA subanalysis to evaluate the role of active infection. *Limitations*: Long follow-up period and likely to be changes in detection methods; lack of cirrhosis/fibrosis, viral load data, genotype, or treatment data (75% of patients were receiving antivirals); all HIV+ patients. *Other comments:* There was some overlap with the study by Béguelin et al. (2017), but this paper added the EuroSIDA cohort and expanded the time frame to 2019.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Mallet et al. (2017) France January 2008 to December 2013 Cohort	48 189 adult patients in metropolitan France who were discharged with a primary or associated diagnosis of CHB during the study period and who had not received a liver or other solid organ transplant or allograft stem cell transplant. *Exposure assessment method*: Clinical record codes.	Liver (HCC), incidence	HDV status (HR): Mono-infection (HBV) Coinfection with HDV	 NR 514	 1 1.53 (1.39–1.68)	Sex, age at baseline in 10-yr categories, residency area in five main French regions, obesity status	*Exposure assessment critique*: Unable to evaluate testing because HDV was defined based on clinical codes. *Design and analysis:* *Strengths*: Large, comprehensive data source of private and public billing claims; data were available on relevant confounders; multivariable modelling. Obesity was accounted for. *Limitations*: Analyses were conducted separately for alcohol use disorder, HCV, and HIV infection, which are not mutually exclusive conditions (i.e. models were not adjusted for alcohol use, HCV and/or HIV status, although data were available); no information on underlying cirrhosis; temporality of infection status, viral load, and genotype, etc. were not reported.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Brancaccio et al. (2019) Italy (Naples, Padua, Foggia) 2007–2014 Cohort	112 individuals; 56 HDV+ and 56 HBV-monoinfected. Patients aged 18 yr, with cirrhosis or advanced fibrosis and chronic HDV infection. Patients had to be non-eligible for peg-IFN, and patients with HIV or HCV coinfection, other causes of liver disease, alcohol abuse, active drug use, or HCC were excluded. The control cohort was selected from HBV-monoinfected patients who were ineligible, nonresponsive, or intolerant to peg-IFN and had started therapy with either entecavir or tenofovir at standard doses, as followed-up for patients with HDV; controls were matched 1:1 to the HDV-infected patients by age (≤ 2 yr), gender, platelet count, albumin level, bilirubin and INR (within a value of 5).	Liver (HCC), incidence	HDV status (unadjusted HR): Negative Positive	 6 17	 1 4.17 (1.65–10.56)	Matching factors: age (within 2 yr), gender, platelet count, albumin level, bilirubin, and INR (within a value of 5)	*Exposure assessment critique*: Adequate marker of CHD assessed on the basis of HDV RNA testing, but method specifics were not reported and performance was unknown. *Design and analysis:* *Strengths*: Well-defined/ monitored cohort with matched controls; patients with HCV and HIV were excluded. *Limitations*: Study design was akin to that of a non-randomized clinical trial; all participants had cirrhosis or advanced fibrosis at baseline; alcohol use (outside the definition of alcohol abuse, which was an exclusion criterion) was not accounted for; longitudinal design in the context of treatment response was not evaluated in regard to clinical outcomes. BMI was not accounted for.
		Liver (HCC), incidence	Adjusted HR: HDV– HDV+	 6 17	 1 2.34 (0.98–6.42)	Child–Pugh score, platelet count, matching factors	

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Brancaccio et al. (2019) (cont.)	*Exposure assessment method*: Testing for HBsAg, anti-HDV antibodies, and HDV RNA.						
Jang et al. (2021a) Taiwan, China June 2000 to July 2018 Cohort	1349 patients with CHB allocated to receive NAs, consecutively enrolled in a medical centre in Taiwan, China; exclusion was based on "alcoholism" (≥ 20 g/day), coinfection with HIV or HCV, ongoing IFN-based therapy, pre-existing HCC before the use of NAs, use of NAs for chemotherapy prophylaxis. *Exposure assessment method*: Commercial HDV ELISA or HDV RNA assay.	Liver (HCC), incidence	HDV RNA, full cohort (HR): Negative Positive	 11 2	 1 5.73 (1.35–24.29)	Age, sex, diabetes, BMI, platelet count, AST, ALT, HBV DNA, HBeAg positivity, liver cirrhosis, use of entecavir/ tenofovir disoproxil/ other nucleotide analogues	*Exposure assessment critique*: Good assessment of CHD according to HDV RNA. *Design and analysis:* *Strengths*: Well-defined cohort and robust analytics; sensitivity analysis by underlying cirrhosis; exclusion of HCV- and HIV-infected individuals. *Limitations*: All patients were receiving NAs; low sample size of HCC cases positive for HDV RNA (used as primary exposure).
		Liver (HCC), incidence	HDV RNA, cirrhotic patients (HR): Negative Positive	 3 2	 1 4.45 (1.04–19.09)	Sex, BMI, platelet count, HBeAg positivity	

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Wranke et al. (2023) Germany 1987–2013 Cohort	350 patients, all anti-HDV+, followed at Hannover Medical School. Patients were included in the study if they had detectable HBsAg and either anti-HDV IgM antibodies or HDV RNA for ≥ 6 mo. Only patients with available follow-up of ≥ 6 mo and a minimum of two visits were included. Exclusion criteria were liver transplant, non-alcoholic steatohepatitis, alcohol use, or metabolic disease, or HCC before enrolment. *Exposure assessment method*: Anti-HDV+, either anti-HDV IgM or HDV RNA for > 6 mo.	Liver (HCC), incidence	Kaplan–Meier Chronic HBV Chronic HDV *P* value, log-rank test, 0.03	 12 20	 [1] [1.45 (1.13–1.85)]	Matching variables (age, gender, region of origin, HBeAg status, and bilirubin)	*Exposure assessment critique*: Algorithm for defining CHD was appropriate, although minimal methodological details were provided. *Other comments*: Dates taken from a previous related manuscript by Wranke et al. (2017); HR was calculated based on formula 14 in the study by Tierney et al. (2007) (numbers of events were calculated from the reported 11.4% and 6.9% prevalence of HCC in the HDV and HBV groups, respectively). *Design and analysis:* *Strengths*: Matching of participants by age, sex, region of origin, HBeAg status and bilirubin; electronic health record cohort with ≥ 6 mo of follow-up. *Limitations*: Although matching was conducted, factors such as HCV, HIV, treatment, and cirrhosis were not accounted for; more robust analyses were undertaken (Cox models, propensity scoring, and inverse probability weighting), but not in a manner that would be informative for the exposure to outcome of interest.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Roulot et al. (2025) France DeltaVir, from 2000 to 2014; ANRS CO12 CirVir, March 2006 to June 2012 Cohort	413 participants with cirrhosis, from two nationwide cohorts: (i) DeltaVir, including patients with chronic HDV infection; and (ii) the ANRS CO12 CirVir cohort, including patients with viral cirrhosis. Patients with HIV or HCV were excluded. *Exposure assessment method:* HDV antibody testing, assay not reported.	Liver (HCC), incidence	HCC during follow-up (adjusted HR): HBV mono-infection (n = 271) HDV coinfection (n = 142)	 NR NR	 1 2.94 (1.19–7.25)	Age, excessive alcohol consumption, tobacco consumption, BMI, AST, GGT, albumin, bilirubin, platelets, HBV-HDV treatment (yes/no)	*Exposure assessment critique*: Adequate marker of past or current HDV infection assessed on the basis of anti-HDV, but method specifics were not reported, and performance was unknown; HDV RNA levels were used descriptively. *Design and analysis:* *Strengths*: Multivariable adjustment; all had HBV. *Limitations*: Cirrhosis-only cohort; imprecise CIs.
		Liver (HCC), incidence	HCC during follow-up (adjusted 5-yr incidence) HBV mono-infection (n = 271) HDV coinfection (n = 142) Fine–Gray test P value, < 0.001	 NR NR	 4.4% 20%	Age	

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Raimondo et al. (1984) Palermo, Italy 1977–1983 Case–control	*Cases*: 79 consecutively recruited patients with HCC (68 of whom had cirrhosis). *Controls*: 79 patients with inactive cirrhosis pair-matched to cases on sex and age (from within a larger control group of 260 patients with active or inactive cirrhosis, or healthy chronic HBsAg carriers). All patients had been HBsAg+ for ≥ 6 mo. *Exposure assessment method*: Anti-HDV+. Sera were tested by RIA.	Liver (HCC), incidence	Anti-HDV, compared with matched paired controls with inactive cirrhosis (crude unconditional OR): Anti-HDV– Anti-HDV + vs –	[71] [8]	1 [0.23 (0.08–0.58)]	Age, sex	*Exposure assessment critique*: Adequate marker of past or current HDV infection assessed on the basis of RIAs that are no longer in common use because of lower sensitivity. *Other comments*: For the Working Group meta-analysis, the calculated precise CIs were used: 0.083783–0.579171. Comparisons with unmatched controls (including healthy HBsAg carriers) were uninformative. *Design and analysis:* *Strengths*: Matching between HCC cases and controls with cirrhosis. Inclusion was restricted to patients with chronic HBsAg carriage (> 6 mo). *Limitations*: The restriction of the analysis to controls with cirrhosis may be associated with an underestimation of the contribution of HDV to HCC. Outcome misclassification was possible as it was unclear whether HCC was excluded in the controls with cirrhosis.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Trichopoulos et al. (1987) Athens, Greece April 1976 to October 1984 Case–control	*Cases*: 87 HBsAg+ cases of HCC diagnosed by either histology or a combination of clinical and laboratory features and AFP level of > 600 ng/mL recruited from eight hospitals. *Controls*: 29 HBsAg+ participants with diagnoses other than neoplasia or liver disease, hospitalized in the same hospital as the cases. No matching was mentioned, but it was said that the populations were comparable in terms of residence (urban or rural). All participants were Caucasian [White], of Greek nationality. *Exposure assessment method*: Anti-HDV+. Sera were tested by RIA.	Liver (HCC), incidence	Anti-HDV frequency in HBsAg+ (OR not calculable, 0 count in controls) (%):			None	*Exposure assessment critique*: Assessment of past or current HDV infection on the basis of RIAs that are no longer in common use as were of lower sensitivity. *Other comments*: OR calculated using 0.5 count in controls. *Design and analysis:* *Strengths:* Cases and controls were well defined. Specifically designed to assess the role of HDV in HCC. *Limitations*: No matching or adjustment for confounders. Selection criteria were not described, with potential selection bias. Outcome (HCC) was not clearly excluded in controls.
			Cases, anti-HDV+	9	10%		
			Controls, anti-HDV+	0	0%		
			Exact two-tailed test *P* value, 0.067				
		Liver (HCC), incidence	Anti-HDV frequency in HBsAg+ (crude OR):			None	
			Cases, anti-HDV–	78	1		
			Cases, anti-HDV+	9	[6.69 (0.38–119.16)]		
		Liver (HCC), incidence	Delta antigen (%):			None	
			Cases, +	0	0%		
			Controls, +	0	0%		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Amougou et al. (2016) Yaoundé, Cameroon February 2013 to January 2014 Case–control	*Cases*: 88 (58 HBsAg+) cases of HCC (defined by mass on ultrasound plus AFP level of > 400 ng/mL; histology when possible), consecutively recruited at Central Hospital, Yaoundé. *Controls*: 85 (9 HBsAg+) patients from the same centre, for whom clinical evidence of liver disease, a history of hepatitis, elevated AFP (> 15.5 ng/mL) and ultrasound evidence of a liver mass had been excluded, matched 1:1 on age and sex. *Exposure assessment method*: Anti-HDV+.	Liver (HCC), incidence	Anti-HDV positivity, among HBV+ cases (adjusted OR): Anti-HDV– Anti-HDV+	 34 24	 1 5.65 (0.66–48.16)	Age, gender	*Exposure assessment critique*: Good assessment of past or current HDV infection based on anti-HDV. *Design and analysis:* *Strengths*: Setting with high prevalence of HDV; clear case definition; controls without liver disease; consecutive enrolment. *Limitations*: Low precision of the estimate; accounted only for age and sex as confounders (HCV infection was described but not accounted for in the analysis; HIV and alcohol use were not accounted for).
Mahale et al. (2019) Gambia September 1997 to January 2001 Case–control	*Cases*: 431 including HCC ($n = 312$) and cirrhosis without HCC ($n = 119$). HCC diagnosis was made by histology or a combination of ultrasound lesions and AFP elevation (AFP > 20 ng/mL). Cirrhosis was diagnosed with ultrasound.	Liver (HCC), incidence	HCC vs controls (adjusted OR): HBsAg–/ anti-HDV– HBsAg+/ anti-HDV– HBsAg+/ anti-HDV+	 NR NR 18	 1 9.09 (6.06–13.63) 31.25 (6.81–143.36)	Age, sex, ethnicity, study site, earth floor, alcohol abuse, cigarette smoking, family history of cancer, anti-HCV, ser-249 TP53 mutation, BMI	*Exposure assessment critique*: Good assessment of CHD based on HDV RNA (specimen degradation may have reduced sensitivity for HDV RNA). Analysis of HDV RNA positivity was not performed as there were no HDV RNA+ controls.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Organ site (histopathology), incidence or mortality	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Mahale et al. (2019) (cont.)	*Controls*: 470 patients recruited from the same centres, with no clinical evidence of liver disease and normal AFP (< 5 ng/mL), matched on sex and age. *Exposure assessment method*: Sequential testing algorithm based on Q-MAC, western blot, and HDV RNA.	Liver (HCC), incidence	HCC vs controls (unconditional OR): HBsAg+/ anti-HDV– HBsAg+/ anti-HDV+	 223 27	 1 [4.72 (1.10–20.32)]	Sex, age	*Design and analysis:* *Strengths*: Well-designed study. Accounted for confounders in the primary analysis (although this included HBsAg– individuals). *Limitations*: Cases included HCC and cirrhosis – the primary analysis was deemed less informative concerning the risk of HCC. Low prevalence of HDV – no HDV RNA+ among the controls. Unable to account for confounders (HIV, HDV, BMI, alcohol use) in Working Group analyses restricted to HBsAg+.
		Liver (HCC), incidence	HCC vs controls (unconditional OR): HBsAg+/ HDV RNA– HBsAg+/ HDV RNA+	 NR 17	 1 [11.67 (0.69–196.59)]		

AFP, alpha-fetoprotein; AIDS, acquired immunodeficiency syndrome; ALT, alanine aminotransferase; ANRS, Agence nationale pour la recherche sur le SIDA et les hépatites virales; anti-HDV, antibody to HDV; anti-HDV+ or anti-HDV–, positive or negative for antibody to HDV; ART, antiretroviral therapy; AST, aspartate aminotransferase; BMI, body mass index; CD4, cluster of differentiation 4; CHB, chronic hepatitis B; CHD, chronic hepatitis D; CI, confidence interval; CirVir, viral cirrhosis; ELISA, enzyme-linked immunosorbent assay; EUROHEP, Concerted Action on Viral Hepatitis; EuroSIDA, Clinical and Virological Outcome of European Patients Infected With HIV; GGT, gamma-glutamyl transferase; HBeAg, hepatitis B e antigen; HBeAg+ or HBeAg–, positive or negative for hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBsAg+ or HBsAg–, positive or negative for HBsAg; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HDV, hepatitis D virus; HDV RNA+ or HDV RNA–, positive or negative for HDV RNA; HIV, human immunodeficiency virus; HR, hazard ratio; ICD, International Classification of Diseases; IFN, interferon; Ig, immunoglobulin; IHC, immunohistochemistry; INR, international normalized ratio; IRR, incidence rate ratio; mo, month(s); NAs, nucleotide or nucleoside analogues; NC, not calculated; NR not reported; OR, odds ratio; PCR, polymerase chain reaction; peg-IFN, pegylated interferon; Q-MAC, quantitative microarray antibody capture; RFLP, restriction fragment length polymorphism; RIA, radioimmunoassay; RR, relative risk; SES, socioeconomic status; SIR, standardized incidence ratio; USA, United States of America; VA, Veterans Affairs; vs, versus; yr, year(s).

Buti et al., 2011; Asmah et al., 2014; Lima et al., 2018; Wranke et al., 2018; Bockmann et al., 2020; Hernàndez-Èvole et al., 2020; Palom et al., 2020; Jachs et al., 2021; Ramos-Rincon et al., 2022); and five because of the exclusive presentation of crude analyses (Manesis et al., 2013; Coghill et al., 2018; Spaan et al., 2020; Huang et al., 2025; Russo et al., 2025).

The Working Group gave consideration to the potential impact of confounding, in particular for liver cirrhosis, which may be caused by HDV-dependent and HDV-independent processes and was considered to be both a confounder and a mediator for the association between HDV and HCC (see Section 2.2.3).

A common limitation included a lack of data on the timing or duration of the infection at the time of measurement or clinical diagnoses.

In a multicentre retrospective longitudinal study in western Europe (the Concerted Action on Viral Hepatitis, EUROHEP study), Fattovich et al. (2000) assessed the incidence of HCC and rates of hepatic decompensation in patients with compensated cirrhosis who were positive for HBsAg and were recruited between January 1973 and December 1991. After limiting inclusion to patients with no steroidal or antiviral treatment during follow-up, 200 participants were assessed, of whom 39 (20%) were found to be anti-HDV–positive at enrolment. After a median follow-up time of 80 months (range, 6–198 months), HCC developed in five patients (13%) who were positive for anti-HDV and negative for hepatitis B e-antigen (HBeAg; an early-phase marker of HBV infection), 19 patients (16%) who were anti-HDV–negative and HBeAg-negative, and three patients (7%) who were anti-HDV–negative and HBeAg-positive, but in none of the patients who were anti-HDV–positive and HBeAg-positive. The unadjusted risk of HCC was not increased in the anti-HDV–positive relative to anti-HDV–negative patients (relative risk, RR, 0.8; 95% confidence interval, CI, 0.3–2.0). After adjusting for age, sex, $\log_{10}$ (bilirubin), albumin, γ-globulins, platelets, and HBeAg status, the risk of HCC among anti-HDV–positive individuals was 3.2 (95% CI, 1.0–10.0). [The Working Group noted that 36 participants were positive for anti-HCV and/or anti-HIV, but these variables, as well as factors such as obesity and alcohol consumption, were not accounted for in the models, leading to the possibility of residual confounding. Given that HIV and HCV share common transmission routes with HDV – such as injected drug use and unprotected sex among high-risk populations – and are independently associated with increased risk of HCC (IARC, 2012), their presence may consequently exaggerate the observed association between HDV and HCC if not properly adjusted for. Furthermore, the restriction to cirrhotic patients may decrease the magnitude of the association between HDV and HCC. Imprecision of estimates was also noted.]

Su et al. (2006) studied a cohort of HBV-HDV–coinfected patients consecutively enrolled in Taiwan, China, between 1983 and 2003, aiming to ascertain the clinical significance of HBV and HDV genotypes for liver pathogenesis. A total of 194 HBsAg-positive, anti-HDV–positive patients were included, of whom 51 were infected by HDV genotype 1 and 74 were infected by genotype 2. At baseline, 34 patients had HCC. Over a follow-up of 135 months, HCC incidence was higher among those with genotype 1 than those with genotype 2 (32.6% versus 11.1%; $P = 0.008$). [The Working Group noted that a limitation of this study was that only non-adjusted descriptive data for HCC were provided. Furthermore, the inclusion of cirrhotic patients, without further adjustment for other cirrhosis risk factors, such as high body mass index (BMI), may be associated with residual confounding. A strength of this study was the availability of genotyping data; indeed, the fact that HDV genotypes are differentially associated with HCC development may suggest a viral contribution. Individuals with HIV, HCV, or alcohol use disorder were excluded, and mean ages were comparable between the

two genotypes, which reduced concerns over confounding.]

In a sequence of two papers from the same cohort, Romeo et al. (2009, 2014) published data on patients who were positive for anti-HDV for ≥ 6 months who were consecutively admitted to the Liver Centre, Maggiore Hospital, in Milan, Italy, between 1978 and 2006. Unlike the other studies evaluated in this section, the papers by Romeo and colleagues focused exclusively on anti-HDV–positive patients and assessed whether HDV viral load influences disease progression and HCC incidence. [The Working Group noted that this particular design offered insight into the effect of active viral replication, which gives key information on the risk of HCC associated with viral load.] The earlier manuscript (Romeo et al., 2009) included 299 patients followed over a mean of 233 months. All patients were HBsAg-positive and anti-HDV–positive at baseline, 13 were also found to be HCV RNA–positive, and 13 were anti-HIV–positive. During follow-up, 46 patients (15%) developed HCC, and the univariable OR for the association between HCC and detectable HDV RNA (by in-house nested RT and real-time PCR; positive being determined as > 500 cp/mL) was reported to be 1.43 (95% CI, 0.67–3.06). In a subsequent study, these investigators examined the relation between HDV levels and risk of HCC. After exclusion of participants without a baseline sample and at least one follow-up serum sample, the subsequent study (Romeo et al., 2014) was limited to 193 patients, of whom 28 developed HCC during follow-up. HDV RNA levels were found to be associated with HCC in both univariable (OR, 1.32; 95% CI, 1.02–1.71) and multivariable (OR, 1.42; 95% CI, 1.04–1.95) models. Furthermore, after excluding patients with cirrhosis at baseline ($n = 105$), HDV RNA levels remained associated with HCC development in both univariable (OR, 1.66; 95% CI, 1.04–2.65) and multivariable (OR, 2.64; 95% CI, 1.13–6.16) models. [The Working Group noted the key strengths of these studies, including the prospective design (with repeated measures), long follow-up, and adjustment for major confounders within well-characterized cohorts. However, the extended follow-up period may have compromised the HDV exposure assessment owing to sample degradation, freeze–thaw cycles, and evolution of clinical protocols over time, introducing potential misclassification or selection bias. Additionally, neither study fully accounted for cirrhosis caused by HCV, BMI, and alcohol use, and the absence of data on key confounders such as HCV, alcohol use, and BMI – combined with limited sample size restricting formal assessment of HCV and HIV – raised the possibility of residual confounding and overestimation of the observed associations. The Working Group noted the nonstandard use of logistic regression in a cohort design.]

Ji et al. (2012) linked data from the Swedish Hospital Discharge and Outpatient Registry to the National Cancer Registry to compare risk of HCC for individuals diagnosed with chronic HBV infection alone and for individuals with both HBV and either acute or chronic HDV infection between 1997 and 2008. After excluding those with HIV and HCV, 8510 patients presented with HBV infection alone, 323 with HBV and acute HDV infection, and 327 with HBV and chronic HDV infection; the incidence of HCC was found to be 46, 9, and 8, respectively. Standardized incidence ratios (SIRs) (adjusted for age, sex, 5-year period for diagnosis, socioeconomic status, and residential area) were reported to be 6.11 (95% CI, 2.77–11.65) for those with acute HDV infection and 3.90 (95% CI, 1.61–7.22) for those with chronic HDV infection, compared with individuals with HBV infection alone. Cox regression analyses demonstrated that the hazard ratios (HRs) for developing HCC were 3.85 (95% CI, 1.87–7.89) for acutely infected individuals and 2.42 (95% CI, 1.29–4.56) for chronically infected individuals. [The Working Group noted that, although confounders such as HIV and HCV infection, age, and sex were considered

in the analysis of this population-based data set, no data were presented on alcohol use, BMI, history of treatment, or diagnosis of cirrhosis in this study, resulting in the possibility of residual confounding. Additionally, HDV diagnosis was contingent on proper clinical assessment, which may have changed across the time frame, resulting in the possibility of exposure misclassification (although 5-year period for diagnosis was adjusted for).]

Kushner et al. (2015) used the Veterans Health Administration database to conduct a retrospective cohort study of HBsAg-positive individuals from October 1999 to December 2013. Of the 25 603 patients with a positive test for HBsAg, only 2008 (7.8%) were tested for HDV (by serology, anti-HDV); of those who were tested, 73 (3.4%) were found to be positive. HDV-positive patients were more likely to report substance and/or alcohol abuse, have HCV and/or HIV coinfection, and have cirrhosis. Among those who were tested for HDV, the incidence rate of HCC was appreciably higher in HDV-positive individuals (23.0 per 1000 person-years) compared with those who were HDV-negative (8.0 per 1000 person-years). Multivariable Cox proportional hazard models demonstrated that HDV infection remained an independent predictor of HCC, even after adjustment for age, chronic HCV infection, cirrhosis, nucleotide or nucleoside therapy, and alcohol use (HR, 1.9; 95% CI, 1.1–3.7). A later study using the Veterans Health Administration database by Wong et al. (2025) leveraged data for all veterans diagnosed with CHB between 2010 and 2023. Of the 27 548 individuals with CHB, 4437 (16.11%) were tested for HDV. After excluding patients with cirrhosis or HCC at baseline, 71 patients with CHB and concurrent HDV were identified and were 1:2 propensity score–matched to 140 patients with CHB and a negative HDV test. Age, sex, race, HBeAg status, CHB antiviral therapy, HCV, HIV, diabetes mellitus, and documented alcohol use disorders at the time of CHB diagnosis were used for propensity matching. The incidence of HCC during a mean follow-up of 4.38 years was higher in individuals with concurrent HDV infection – 1.28 (95% CI, 0.53–3.09) per [100] person-years – than in monoinfected individuals with CHB – 0.41 (95% CI, 0.13–1.27), although the difference [IRR, 3.12; 95% CI, 0.75–13.05)] was imprecise ($P = 0.12$). [The Working Group noted that Kushner et al. (2015) adjusted for several key confounders, including HCV, alcohol use, and antiviral therapy, supporting an independent association between HDV and HCC. Nevertheless, the use of administrative data and the lack of adjustment for BMI may have introduced residual confounding; additionally, adjustment for cirrhosis in this study probably induced downward bias on the estimate, as some cirrhosis may be a result of HDV infection. In contrast, Wong et al. (2025) minimized confounding through comprehensive propensity score matching and exclusion of baseline cirrhosis, although HCC risk was a secondary focus of the study, which was likely to be underpowered to adequately assess an association. The overlap of eligibility periods with Kushner et al. (2015) was considered to be minimal, and selection bias arising from low rates of HDV testing (limited primarily to individuals with advanced liver disease) within the Veterans Health Administration may have biased results in an unpredictable direction.]

Béguelin et al. (2017) conducted a study in the Swiss HIV Cohort Study (SHCS), an ongoing prospective study of HIV-infected adults in Switzerland, to evaluate the incidence of liver disease as well as overall and liver-related mortality after initiation of antiretroviral therapy (ART). HIV-positive adults with a positive test result for HBsAg between January 1988 and December 2014 were considered. After excluding those who had not initiated ART and those without available stored serum samples, 104 HDV-positive (diagnosed by serology) and 623 HDV-negative participants were included in the study. HDV-positive patients were more

likely to be people who inject drugs, have positive HCV serology, and have genotype D HBV infection. Multivariable analyses adjusted for age, sex, cluster of differentiation 4 (CD4) count, stage of HIV infection, most likely source of HIV transmission, and HCV coinfection. HDV infection was associated with the occurrence of HCC (adjusted HR, 9.30; 95% CI, 3.03–28.61). In a later study, Béguelin et al. (2023) combined the SHCS study with the EuroSIDA study (a prospective observational cohort of about 23 000 people living with HIV, enrolled since 1994, and monitored across 100 hospitals in 35 European countries, and in Israel and Argentina) to describe the epidemiological characteristics and evaluate the impact of HDV on clinical outcomes in people living with HIV. The final cohort included 1556 HBsAg-positive participants with serum available, of whom 237 (15.2%) were anti-HDV–positive. Fourteen (5.9%) of the HDV-positive and 21 (1.6%) of the HDV-negative participants developed HCC during a median follow-up of 10.8 years (interquartile range, IQR, 5.6–17.8). Anti-HDV was found to be associated with HCC in multivariable Cox models (HR, 6.3; 95% CI, 2.5–16.0), with a stronger effect size in those who were HDV RNA–positive (HR, 8.3; 95% CI, 3.0–22.7). [The Working Group noted as strengths that this was a cohort with regular clinical follow-up, and major confounders were accounted for in the analysis. The multivariable analysis was also adjusted for HIV stage and CD4 count, but it could not be excluded that HIV infection may itself have an impact on liver carcinogenesis. However, cirrhosis or fibrosis (mediated by other known risk factors for liver cancer that were not adjusted for, e.g. BMI), viral load, genotype, lifestyle factors, and treatment data (e.g. treatments against HBV replication) were not accounted for in these studies, leading to the likelihood of residual confounding that may have resulted in overestimation of the observed association. It should also be noted that [75%] of the patients were treated with antiretrovirals with anti-HBV activity. Despite some overlap in the SHCS cohort inclusion between Béguelin et al. (2017) and Béguelin et al. (2023), both studies were considered to be informative because they offered complementary strengths: the 2017 study provided stronger internal validity by focusing on ART-treated, HBsAg-positive SHCS participants from 1988 to 2014, with consistent data collection under standardized national protocols, whereas the 2023 study provided more data by including HBsAg-positive individuals from both SHCS (1988–2019) and EuroSIDA (1994–2019), spanning a broader and more recent time frame across diverse international settings.]

Mallet et al. (2017) used ICD-10 codes in the Programme de Médicalisation des Systèmes d'Information database to assess progression of liver disease (including HCC) in adult patients living in Metropolitan France who were discharged with either a primary or associated diagnosis of CHB between January 2008 and December 2013. After excluding those who had undergone liver, other solid organ, or allograft stem cell transplants, 48 189 patients were included in the study; of these patients, 5143 (10.7%) had a recorded diagnosis of HDV infection, and 514 [1.1%] of these developed HCC. After adjustment for sex, age at baseline in 10-year categories, residency area in five main regions of France, and obesity status, HDV coinfection was found to increase the risk of HCC development (HR, 1.53; 95% CI, 1.39–1.68). [The Working Group noted that, in this large population-based study, analyses were conducted separately for alcohol use disorder, diabetes, HCV, and HIV infection, which are not mutually exclusive conditions. Cirrhosis status was not excluded from the analyses, and known cirrhosis risk factors were not controlled for in the analysis, resulting in concerns regarding residual confounding or mediation leading to a possible underestimation of the magnitude of the true association between HDV and HCC.]

Brancaccio et al. (2019) recruited patients with chronic HDV infection and advanced fibrosis or cirrhosis who were ineligible for peg-IFN therapy in a study to verify clinical outcomes after long-term treatment with entecavir or tenofovir. Individuals with HCV or HIV coinfection, other causes of liver disease, "alcohol abuse", active drug use, or presence of HCC were excluded. After these exclusions, 56 patients with HDV infection and 56 HBV-monoinfected patients (controls) matched to cases on age (± 2 years), gender, platelet count, albumin level, bilirubin, and international normalized ratio were evaluated for clinical events. All patients received entecavir (0.5 mg per day) or tenofovir (245 mg per day) during the course of follow-up. After a median of 50 months of follow-up for both the HDV-infected (range, 6–160 months) and HBV-monoinfected (range, 6–168 months) groups, 23 cases of HCC were reported, equating to a rate of 3.12 and 1.12 per 1000 patient-months, respectively. After adjustment for Child–Pugh score and platelet count, HDV-infected patients were 2.34 (95% CI, 0.98–6.42) times as likely to develop HCC as were HBV-monoinfected patients. [The Working Group noted that this study accounted for most major confounders either by matching or adjustment. However, all patients in this study had advanced fibrosis/cirrhosis, and thus the true effect of HDV on HCC risk may have been underestimated. Additionally, the lack of longitudinal data integration in relation to treatment adherence and response may have resulted in residual confounding.]

Jang et al. (2021a) recruited CHB patients eligible for nucleotide or nucleoside analogues (NAs) consecutively enrolled in a medical centre in Taiwan, China between June 2000 and July 2018. The exclusion criteria were: consumption of ≥ 20 g of alcohol per day; coinfection with HIV or HCV; ongoing IFN therapy; pre-existing HCC; use of NAs for chemotherapy prophylaxis. Of the 1349 participants in the study, 31 were anti-HDV–positive, and 13 were HDV RNA–positive. In total, 76 patients developed HCC over a follow-up of 5409.5 person-years (range, 1.0–15.0 years; annual incidence, 1.4%). The cumulative incidence at 1, 3, and 5 years of follow-up was 2.3%, 5.4% and 7.5%, respectively. Cox proportional hazard models demonstrated that HDV RNA positivity was strongly associated with HCC development (HR, 5.73; 95% CI, 1.35–24.29). In a sensitivity analysis restricted to the 392 patients with cirrhosis, of whom 62 developed HCC, HDV RNA positivity was the main factor associated with HCC development (HR, 4.45; 95% CI, 1.04–19.09). [The Working Group acknowledged that this study leveraged a large, well-characterized cohort of CHB patients with extended follow-up and employed rigorous multivariable and stratified analyses, including among cirrhotic participants. However, limitations were the small number of HDV RNA–positive cases of HCC and the limited follow-up time for this subgroup, along with the retrospective design, which may introduce potential selection bias.]

Wranke et al. (2023) evaluated long-term clinical outcomes among chronically HDV-infected individuals compared with individuals who were monoinfected with HBV and treated at the Hannover Medical School in Germany. Eligibility was based on detectable HBsAg and either anti-HDV IgM or HDV RNA for a minimum of 6 months. Inclusion required at least two follow-up visits over 6 months. Patients with prior liver transplantation, HCC, or liver disease from alcohol use, metabolic causes, or non-alcoholic steatohepatitis were excluded. Participants were matched to monoinfected patients on age, gender, region of origin, HBeAg status, and bilirubin. Mean follow-up for the cohort was 6.9 years (range, 0.60–23.6 years), during which time 32 cases of HCC were diagnosed, 20 among participants with chronic HDV infection, and 12 among those with chronic HBV monoinfection. Kaplan–Meier analysis was used to evaluate potential differences in HCC incidence between

groups and indicated that the incidence of HCC was higher in the group with chronic HDV infection compared with the group with chronic HBV infection (log-rank *P* value, 0.03) [HR, 1.45; 95% CI, 1.13–1.85]. [The Working Group noted that, although matching was conducted on several relevant factors, data on treatment for HCV, HIV, and HBV were not presented, which could lead to confounding and subsequent overestimation of the effect size. Additionally, monoinfected individuals were noted to have less frequent cirrhosis, which could result from both HDV-dependent and HDV-independent processes, but relevant analyses accounting for confounding by cirrhosis were not conducted.]

Roulot et al. (2025) used data from two cohorts in France, ANRS CO12 CirVir and DeltaVir, limited to individuals with histologically confirmed cirrhosis without HCV or HIV infections; 142 HBV-HDV–coinfected and 271 HBV-monoinfected cirrhosis patients were included in the study. The cumulative age-adjusted 5-year incidence of HCC was higher in HDV-coinfected individuals (20.2%) than in HBV-monoinfected individuals (4.4%) ($P < 0.001$). After additional adjustment for tobacco use, BMI, aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), albumin, total bilirubin, platelets, and treatment group, HDV infection remained associated with HCC development during follow-up (HR, 2.94; 95% CI, 1.19–7.25). [The Working Group noted that this study was conducted within a large national cohort, had well-defined comparison groups, and used multivariable adjustment for major confounders. Nevertheless, all patients had cirrhosis, and the analysis was adjusted for liver function markers, which may overlook a key phase in HCC development for some individuals and could bias results towards the null if cirrhosis is partly caused by HDV. Additionally, the HBV-HDV–coinfected patients tended to be younger and displayed fewer non-liver comorbidities, which may have attenuated the results.]

2.1.2 *Case–control studies and meta-analyses*

See Table 2.1.

(a) Case–control studies

Among the case–control studies identified through the literature search, studies were considered to be uninformative if comparability between cases and controls could not be ascertained (e.g. poor definition of the control group, lack of matching); if cases included chronic liver disease and no separate analysis for HCC was presented; and/or if the study population was not restricted to HBsAg-positive patients or an analysis of HBsAg-positive patients was not possible (Cronberg et al., 1984; Cénac et al., 1986, 1987, 1995; Toukan et al., 1987; Arya et al., 1988; Zhang et al., 1989; Caporaso et al., 1991; Kage et al., 1992; Bile et al., 1993; Zeng et al., 1993; Oyunsuren et al., 2006; Yushchuk et al., 2020). Additionally, the Working Group considered that the publication by Tzonou et al. (1991) was uninformative for the evaluation, given that the previous publication (Trichopoulos et al., 1987) provided a more informative analysis of the association between HDV and HCC. The study by Di Bisceglie et al. (1991) was also considered to be uninformative, as neither cases nor controls were found to be HDV-positive.

The Working Group also screened the reference lists of available meta-analyses and included one additional study (Raimondo et al., 1984). The remaining references were excluded because of the lack of a formal case–control design (e.g. Gu et al., 2014) or the lack of comparability between cases and controls (e.g. Liaw et al., 1987).

Among the case–control studies retained as being informative, only one (Mahale et al., 2019) accounted for HCV infection, alcohol consumption, and BMI as potential confounders in the primary analysis, and none accounted for HIV infection. Individuals with cirrhosis were used as controls in one study (Raimondo et al., 1984),

whereas the other studies excluded controls with liver diseases or cirrhosis.

Raimondo et al. (1984) conducted a hospital-based case–control study in Italy that included HBsAg-positive patients, comparing patients with HCC ($n = 79$; based on liver histology or an alpha-fetoprotein (AFP) level of >500 ng/mL, associated with compatible liver sonography or scintiscan) with age- and sex-matched patients with inactive cirrhosis ($n = 79$) (selected from within a wider group of 260 patients with cirrhosis). The prevalence of anti-HDV antibodies was lower in the group with HCC than in the group of matched patients with inactive cirrhosis (10.1% versus 32.9%), which allowed calculation of an OR of [0.23 (95% CI, 0.08–0.58)]. [The Working Group noted that the study included three control groups: individuals with inactive cirrhosis, those with active cirrhosis, and healthy HBsAg carriers without clinical or laboratory evidence of liver disease. However, as a limitation, matching on age and sex was performed only between the patients with HCC and the patients with inactive cirrhosis, rendering the comparison with healthy carriers uninformative, as it was based on an unmatched set. The Working Group noted that the comparison with controls with cirrhosis may underestimate a possible contribution of HDV to HCC development (see Section 2.1). Outcome misclassification was a particular concern, as it could not be ensured that HCC was excluded in the controls with cirrhosis. Furthermore, exposure misclassification could not be excluded as the diagnosis of HDV infection relied upon IgG determination by RIA, which has a lower sensitivity than do the techniques used presently (Akuffo et al., 2024).]

Trichopoulos et al. (1987) conducted a multicentre hospital-based case–control study in Greece. Patients from eight major hospitals in Athens were included between April 1976 and October 1984. HCC cases ($n = 194$, of which 87 were HBsAg-positive) were diagnosed according to either histology ($n = 113$) or a combination of clinical features and an AFP level of >600 ng/mL ($n = 81$). Controls ($n = 456$, of which nine were HBsAg-positive) were hospital patients with diagnoses other than neoplasia or liver disease. Cases and controls were all Greek, Caucasian [White], and had comparable socioeconomic status and place of residence. Sera from all HBsAg-positive cases and controls were tested for anti-HDV. Of the 87 cases of HCC that were HBsAg-positive, 9 were anti-HDV–positive. All of the 29 HBsAg-positive controls were anti-HDV–negative ($P = 0.067$). Of the 9 HCC cases that were anti-HDV–positive, 4 (44%) did not have associated cirrhosis. [The Working Group noted that this study was specifically designed to evaluate a potential role for HDV in the development of HCC, using HBV monoinfection as a comparator, and suggested the presence of anti-HDV in HCC arising in non-cirrhotic patients. However, the comparability of the two groups was incompletely described, as there was no matching or adjustment for confounders and the selection criteria were not described, leading to potential outcome misclassification, although this was a minor concern given that the controls had no known liver disease. Furthermore, HDV diagnosis was based on IgG detection by RIA, which has a lower sensitivity than do the techniques used presently (Akuffo et al., 2024), leading to exposure misclassification.]

Amougou et al. (2016) conducted a hospital-based case–control study at a single centre in Yaoundé, Cameroon, between February 2013 and January 2014, with the aim of identifying the prevalence of HBV, HCV, and HDV infections among patients with and without HCC. Cases ($n = 88$) were consecutively enrolled patients with a diagnosis of HCC, as defined by a liver mass on ultrasound and elevated AFP level (AFP >400 ng/mL). Histology assessment was not systematic. The controls ($n = 85$) included patients at the same centre for whom clinical evidence of liver disease, a history of hepatitis,

elevated AFP level (AFP >15.5 ng/mL), and ultrasound evidence of a liver mass had been excluded, matched 1:1 on age and sex. Both cases and controls were tested for HBsAg, and HBsAg-positive patients were further tested for anti-HDV antibodies by ELISA. It was found that 58 cases and 9 controls were HBsAg-positive. Anti-HDV was positive for 41.4% of HBsAg-positive cases of HCC (24 of 58 cases) and 11.1% of the HBsAg-positive controls (1 of 9 cases), with an adjusted OR (adjusted on age and sex) of 5.65 (95% CI, 0.66–48.16) for anti-HDV positivity in HCC cases, relative to HBV-monoinfected HCC cases. [The Working Group considered this to be an informative, well-conducted study that was performed in a setting with a high prevalence of HDV. The analysis without restricting to HBsAg-positive patients was not deemed informative. The study provided an estimate adjusted for age and sex, but a limitation was that it did not account for other key confounders such as HCV. Imprecision in the estimates was also noted.]

Mahale et al. (2019) conducted a multicentric case–control study including patients from liver clinics in the Gambia between September 1997 and January 2001 to investigate the role of HDV infection in risk of HCC or cirrhosis and to evaluate the performance of the HDV Q-MAC assay. HCC cases ($n = 312$) were diagnosed by histology or a combination of occupying lesions on ultrasound and elevated AFP (> 20 ng/mL). Controls ($n = 470$) were patients recruited from the same centres, with no clinical evidence of liver disease and normal AFP (< 5 ng/mL), matched on sex and age. HDV exposure was evaluated by the Q-MAC assay (with thresholds previously determined for anti-HDV positivity >0.164 units, and HDV RNA positivity >1.659 units) and validated by western blot and RT-PCR, respectively. HBsAg was positive for 183 cases of HCC and 80 controls. Among the HBsAg-positive patients, anti-HDV was positive in 18/183 (11/183 were HDV RNA–positive) HCC cases and 2/80 (0 HDV RNA–positive) controls. Multivariable analyses were performed, initially using HBsAg-negative, anti-HDV–negative subjects as reference, adjusting for several factors, including BMI and HCV; compared with HBsAg-negative and anti-HDV–negative, the OR for HBsAg-positive and anti-HDV–negative was 9.09, whereas the OR for HBsAg-positive and anti-HDV–positive was 31.25. It was possible for the Working Group to calculate the unconditional OR for the risk of HCC in HBsAg-positive and anti-HDV–positive cases compared with HBsAg-positive and anti-HDV–negative controls ([OR, 4.72; 95% CI, 1.10–20.32]). [The Working Group considered that the analyses performed by the authors, using HBsAg-negative patients as a reference, introduced HBV infection as a major confounder. Therefore, the Working Group considered that the unadjusted Working Group analysis using HBsAg-positive and HDV-negative as a reference was more informative.]

(b) Meta-analyses

A meta-analysis conducted by Chang et al. (2022) was considered uninformative as it did not include further informative studies or subgroup analysis than that previously performed by Alfaiate et al. (2020a). The meta-analysis conducted by Mbaga et al. (2022) was considered uninformative because the risk estimations were calculated relative to the general population and were not restricted to HBsAg-positive patients. The study by Miao et al. (2020) was deemed uninformative because of incomplete reporting.

Four meta-analyses were considered to be informative (Alfaiate et al., 2020a; Stockdale et al., 2020; Kamal et al., 2021; Gish et al., 2024b). [Although some of these meta-analyses included studies that were not considered informative individually by the Working Group, given the criteria described above, the inclusion of a broader body of literature than could have been considered in the present monograph was considered informative. Indeed, the Working Group recognized the efforts made by the authors of

these meta-analyses towards completeness and capture of this very broad body of literature. This provided a way for the Working Group to test whether the findings under the stringent Working Group criteria were consistent with results from other authors when broader criteria were applied.]

Alfaiate et al. (2020a) conducted a systematic review and random-effects meta-analysis aiming to determine the risk of developing HCC in chronic HDV infection, in comparison with HBV monoinfection. All studies published until 2019 were considered, if enough information was provided to calculate a measure of association (odds ratio, unless otherwise reported by the study authors). Ninety-three studies were retained for qualitative and quantitative synthesis, classified by the authors as 25 cohorts and 68 case–control studies. The overall analysis showed an increase in the risk of HCC in HDV-infected patients (meta-OR, 1.28; 95% CI, 1.05–1.57), with important heterogeneity among studies ($I^2 = 67\%$). Risk estimations were particularly high in subgroup analyses considering only prospective studies (meta-OR, 2.77; 95% CI, 1.79–4.28), studies accounting for confounders by either matching or adjustment (meta-OR, 2.07; 95% CI, 1.48–2.90), and studies including only people living with HIV (meta-OR, 7.13; 95% CI, 2.83–17.92). An increased risk was also found in analyses restricted to cohort studies (but not case–control studies), studies published after 2010 (but not before), and in studies excluding patients treated by NAs and/or IFN. When the analysis was restricted to studies in patients with cirrhosis ($n = 5$; 709 patients), the meta-OR was 2.17 (95% CI, 0.96–4.9). A new analysis was reported in a later publication that included only prospective cohorts with histologically proven cirrhosis ($n = 3$), with a meta-OR of 2.36 (95% CI, 1.34–4.14) (Alfaiate et al., 2020b). [The Working Group noted that this meta-analysis was designed to evaluate a potential association between HDV infection and HCC, using HBV monoinfection as a comparator. It had broad inclusion criteria and included an important number of studies, including cross-sectional studies analysed as case–control studies, as well as studies with noncomparable groups. Subgroup analyses accounted for potential confounders. Among confounders, the presence of cirrhosis in controls may have contributed to an underestimation of the real contribution of HDV to HCC development, as it is itself a step in the transformation pathway.]

A second meta-analysis aiming to evaluate the association of HDV infection and HCC in comparison to HBV monoinfection was conducted by Kamal et al. (2021) using a random-effects model and restricting to longitudinal studies published in peer-reviewed journals, with a minimum of 20 patients per group. It included 12 cohort studies ($n = 63\,719$ patients). It identified a twofold risk of HCC in HDV-infected patients, compared with HBV-monoinfected (aggregated effect size, 2.12; 95% CI, 1.14–3.95; I^2, 72%). The risk of HCC remained increased in studies restricted to persons living with HIV, studies excluding HIV or HCV infections, prospective studies, and studies providing adjusted risk estimates. The increase was imprecise in studies in patients with cirrhosis at baseline (effect size, 1.73; 95% CI, 0.39–7.79) and in retrospective studies (effect size, 1.43; 95% CI, 0.57–4.29). [The Working Group noted that a strength of this study was that the analysis was restricted to cohort studies, which restricts to studies allowing an evaluation of temporality between HDV exposure and HCC outcome.]

Stockdale et al. (2020) estimated HDV prevalence in selected population groups, using general population or asymptomatic HBsAg-positive people as controls. In patients with HCC, the prevalence of HDV was higher than in the HBsAg comparator (asymptomatic controls) population from the same region (meta-OR, 3.13; 95% CI, 1.50–6.51). [The Working Group highlighted that this meta-analysis included a large

number of studies that were deemed uninformative for the present evaluation as they were mainly based on comparison of prevalence among patients with and without HCC. Although this body of literature is not described above, as the Working Group restricted its review to studies that provided a well-defined control group, the meta-analysis by Stockdale et al. (2020) provided a useful overview of a large body of literature on comparison of prevalence with the asymptomatic HBsAg-positive population, which collectively could provide support for the present evaluation.]

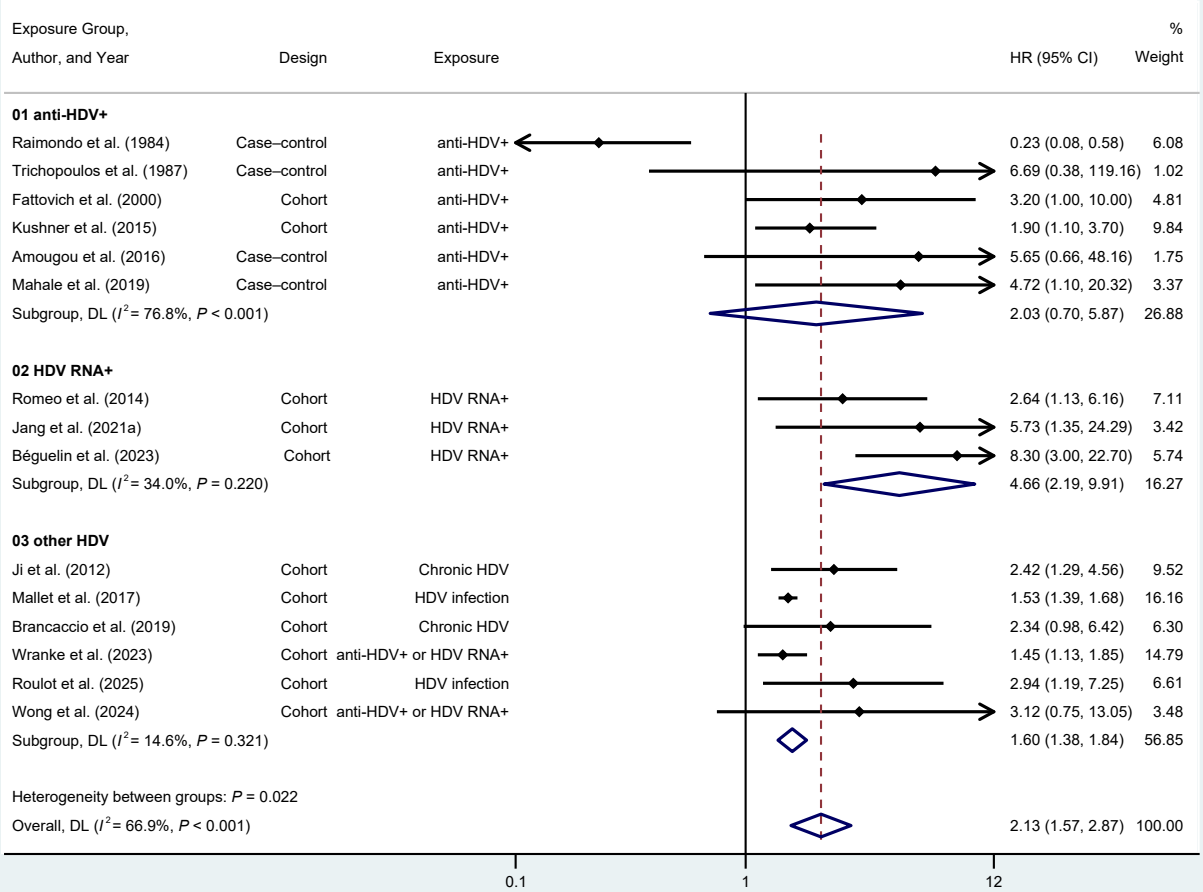
Gish et al. (2024b) conducted a random-effects meta-analysis aimed at determining a possible association between HDV RNA status and advanced liver disease in HBsAg-positive anti-HDV–positive patients; 12 studies (4876 patients) were included, six of which reported an association between HDV status and HCC. When compared with the patients with HDV RNA–negative status, patients with HDV RNA–positive status had a higher risk of progressing to HCC (meta-HR, 2.97; 95% CI, 1.87–4.7). [The Working Group noted that the results of this well-conducted meta-analysis supported an association between HDV viraemia and HCC and hence a potential oncogenic role of HDV. However, the small number of studies limited the power of the analysis.]

2.1.3 *Meta-analyses carried out by the Working Group*

The Working Group conducted a meta-analysis of epidemiological studies on the association between HDV and HCC that were considered to be informative for the present evaluation (Section 2.1.1, Section 2.1.2). Studies considered to be informative were those that limited to HBsAg-positive patients, cohort studies that ensured consideration of basic demographic variables (i.e. studies that presented crude analysis only were excluded), and case–control studies that ensured some comparability between cases and controls (e.g. definition of the control group, presentation of basic demographic characteristics by cases and controls, matching) in the study design (see Sections 2.1.1 and 2.1.2).

Random-effects meta-analysis models were used. A meta-analysis was conducted first by pooling studies using anti-HDV, HDV RNA, or other markers of HDV infection (Fig. 2.1). Additionally, several sensitivity analyses were conducted (Fig. 2.2, Fig. 2.3, Fig. 2.4; and Fig. S2.5 and Fig. S2.6, see Annex 2, Supplementary material for hepatitis D virus, Section 2, Cancer in humans, online only, available from: https://publications.iarc.who.int/667). For studies reporting risk estimations for both anti-HDV and HDV RNA, the HDV-RNA estimation was included, with the exception of the study by Mahale et al. (2019), which had no HDV RNA–positive controls. To minimize the possibility of individuals being counted twice in a meta-analysis, study results noted to be from overlapping populations were not pooled. Funnel plots were produced to allow visual inspection of potential reporting or publication biases (Fig. 2.7). [The Working Group considered the funnel plots to have skew, which could be consistent with modest publication bias among studies. However, the Working Group also noticed that publication bias was not evident in other published meta-analyses, which had much less strict criteria than the meta-analyses conducted by the Working Group and that also confirmed the positive association.] There was some indication of modest heterogeneity. For example, the I^2 was 79.6% (Fig. 2.1) for the anti-HDV group but decreased to 0% after the exclusion of the study by Raimondo et al. (Fig. S2.6; see Annex 2, Supplementary material for hepatitis D virus, Section 2, Cancer in humans, online only, available from: https://publications.iarc.who.int/667), which was considered particularly affected by outcome misclassification and for which the Working Group had concerns about exposure assessment (see Section 1.4). The Working Group meta-analysis was designed to

Fig. 2.1 Meta-analysis for hepatocellular carcinoma and antibodies to HDV, HDV RNA positivity, and HDV infection



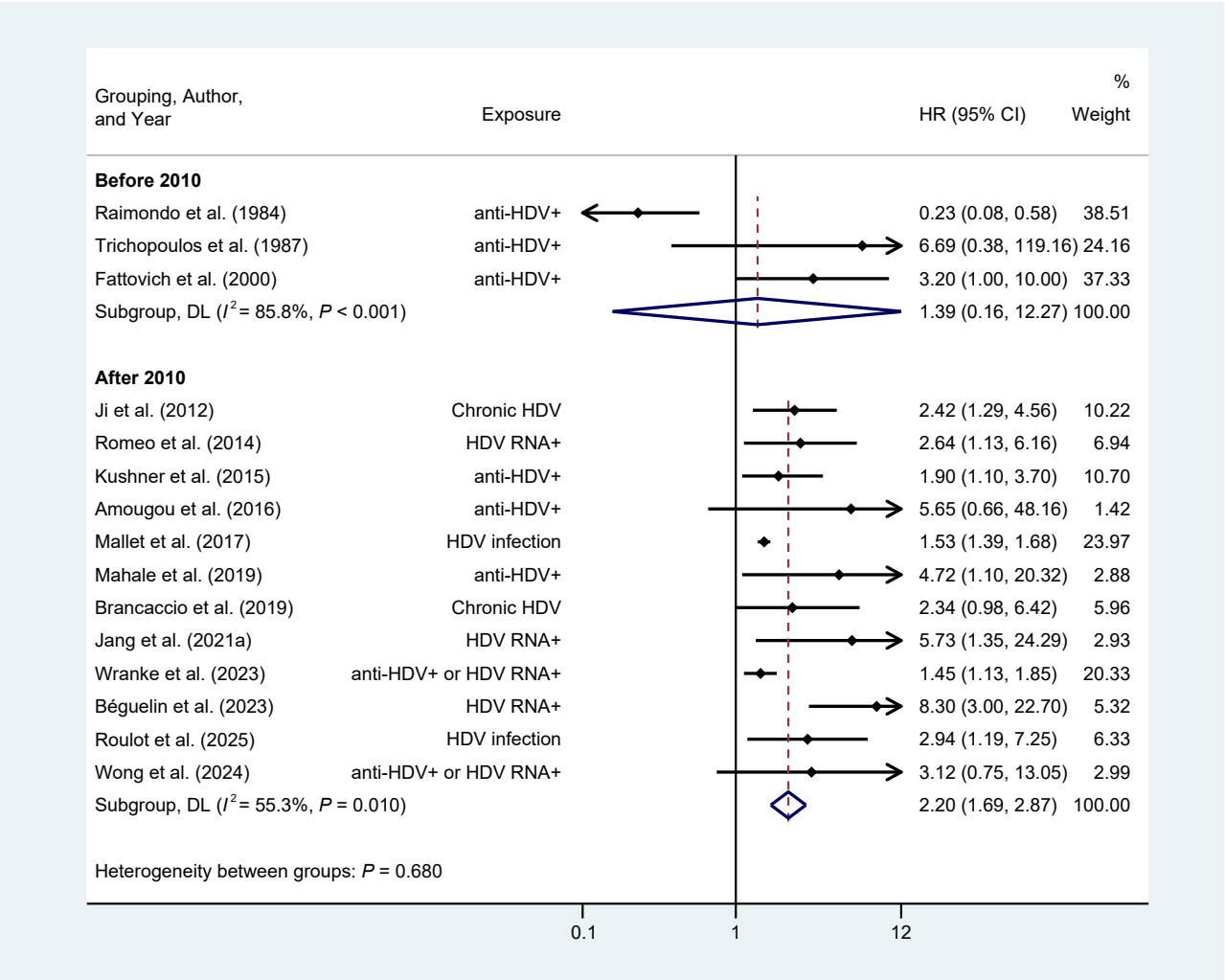
anti-HDV+, positive for antibody to HDV; CI, confidence interval; DL, DerSimonian–Laird method; HDV, hepatitis D virus; HDV RNA+, positive for HDV RNA; HR, hazard ratio.

Béguelin et al. (2017) was excluded because of large overlap with Béguelin et al. (2023).

Created by the Working Group.

facilitate additional analyses relevant to the evaluation, e.g. to stratify studies on the basis of their methodological concerns. The Working Group noted that a source of heterogeneity could be the timing of the studies, with the older studies being more subject to exposure and outcome misclassification and selection bias (see Section 2.2). Therefore, a stratified analysis by temporality (before and after 2010, when commercial tests became available) was performed (Fig. 2.2). Additionally, studies were stratified according to how cirrhosis was handled in the analysis (Fig. 2.3) or according to whether key confounders (HIV, HCV, alcohol use, BMI) were accounted for (Fig. 2.4). For the sensitivity analysis, an analysis was conducted including only cohort studies (Fig. S2.6, Annex 2) or excluding Raimondo et al. (1984), owing to concerns regarding the use of patients with cirrhosis as the comparator (Fig. S2.5, Annex 2).

Fig. 2.2 Meta-analysis for HDV and hepatocellular carcinoma, stratified by time period



anti-HDV+, positive for antibody to HDV; CI, confidence interval; DL, DerSimonian–Laird method; HDV, hepatitis D virus; HDV RNA+, positive for HDV RNA; HR, hazard ratio.

Weights and between-subgroup heterogeneity test are from random-effects model.

Created by the Working Group.

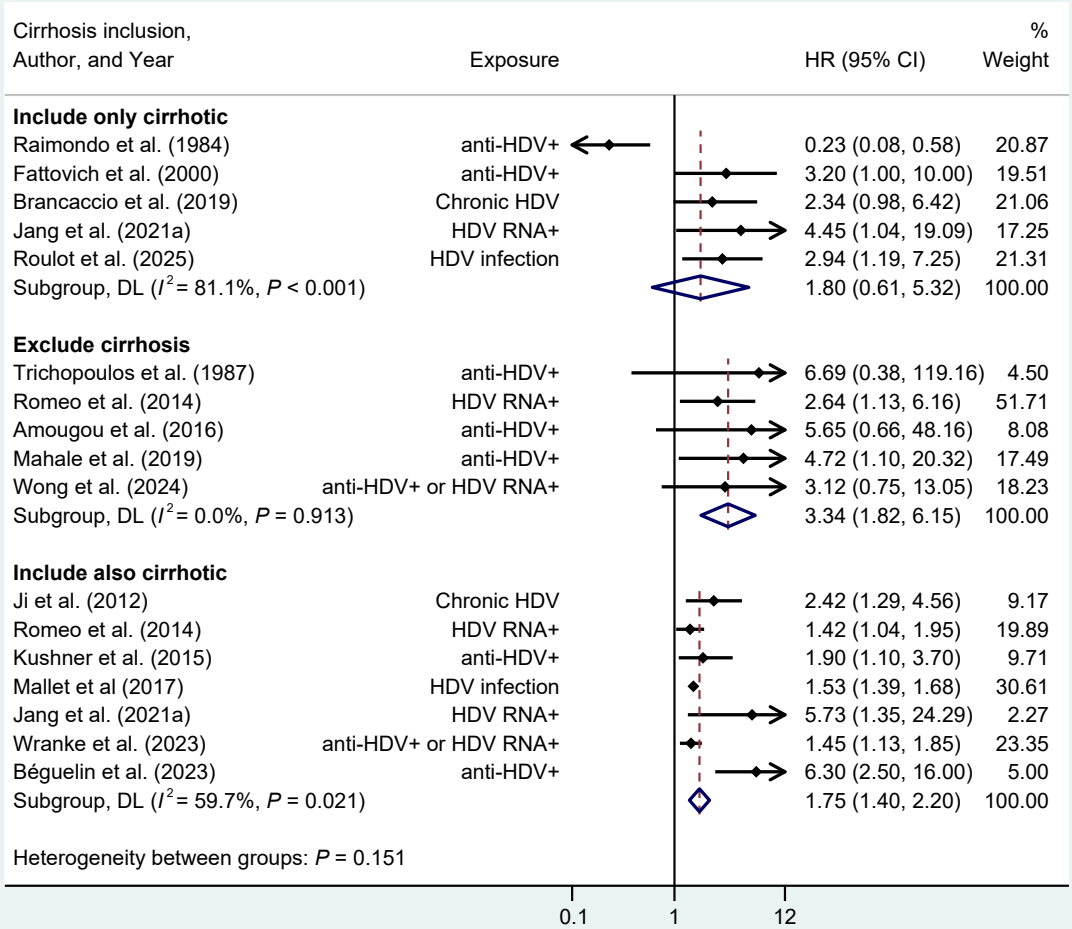
The results of the meta-analyses are reported and discussed in the evidence synthesis, Section 2.2.

2.2 Evidence synthesis for cancer in humans

This section provides a synthesis of evidence on the association between HDV infection in people with CHB and subsequent risk of cancer.

There were 13 cohort studies (Fattovich et al., 2000; Romeo et al., 2009, 2014; Ji et al., 2012; Kushner et al., 2015; Béguelin et al., 2017, 2023; Mallet et al., 2017; Brancaccio et al., 2019; Jang et al., 2021a; Wranke et al., 2023; Roulot et al., 2025; Wong et al., 2025) and four case–control studies (Raimondo et al., 1984; Trichopoulos et al., 1987; Amougou et al., 2016; Mahale et al., 2019). An additional cohort was considered as providing supportive evidence, giving insights into the contribution of different viral genotypes

Fig. 2.3 Meta-analysis for HDV and hepatocellular carcinoma, stratified by how cirrhosis was treated in the analysis



anti-HDV+, positive for antibody to HDV; CI, confidence interval; DL, DerSimonian–Laird method; HDV, hepatitis D virus; HDV RNA+, positive for HDV RNA; HR, hazard ratio.

Weights and between-subgroup heterogeneity test are from a random-effects model.

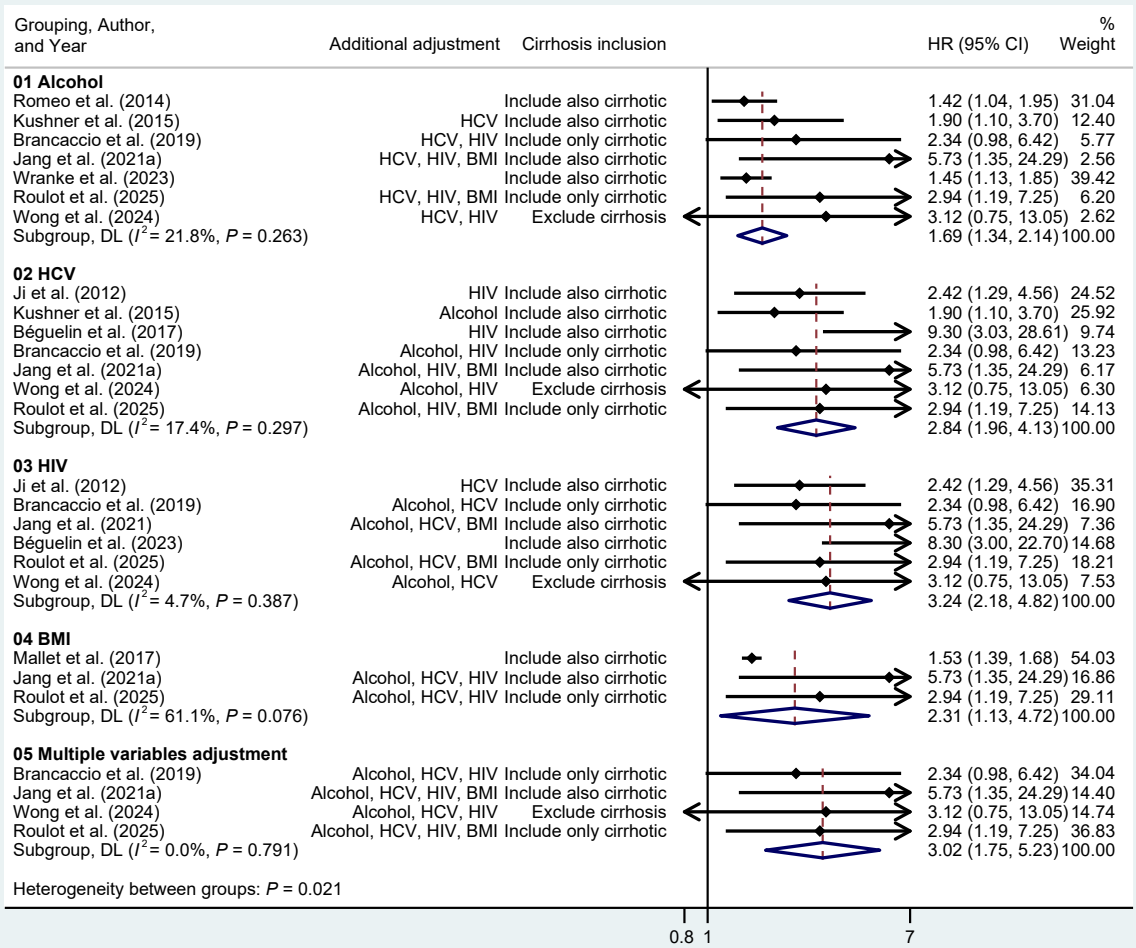
Created by the Working Group.

in the development of HCC in HDV-infected patients (Su et al., 2006). Four meta-analyses were considered (Alfaiate et al., 2020a; Stockdale et al., 2020; Kamal et al., 2021; Gish et al., 2024b). Additionally, the Working Group conducted a meta-analysis that included 11 cohort and four case–control studies (for studies with largely overlapping populations, only the largest study was included; see Section 2.1.3).

2.2.1 Studies evaluated

Informative cohort studies and case–control studies were prioritized according to methodological rigour, multivariable adjustment, and appropriate exposure and outcome definitions. The informative cohort studies were conducted in various geographical and clinical settings and included 13 studies by Fattovich et al. (2000); Romeo et al. (2009, 2014); Ji et al. (2012); Kushner

Fig. 2.4 Meta-analysis for HDV and hepatocellular carcinoma, stratified by consideration of risk factors for liver cancer



anti-HDV+, positive for antibody to HDV; BMI, body mass index; CI, confidence interval; DL, DerSimonian–Laird method; HCV, hepatitis C virus; HDV, hepatitis D virus; HDV RNA+, positive for HDV RNA; HIV, human immunodeficiency virus; HR, hazard ratio.

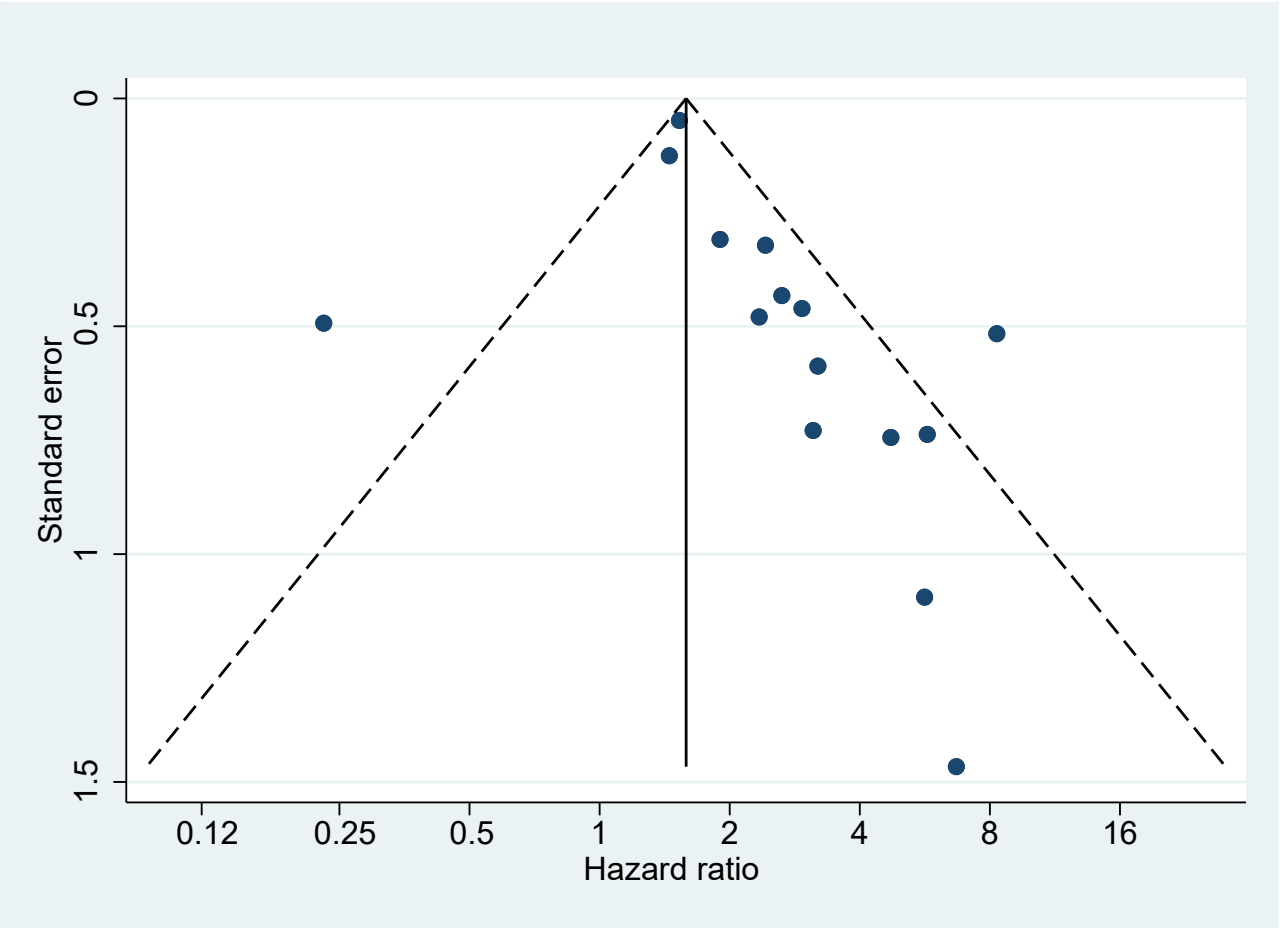
Multiple variable adjustment grouping includes studies that conditioned on three or more of the variables, HCV, HIV, BMI, and alcohol.

Created by the Working Group.

et al. (2015); Béguelin et al. (2017, 2023); Mallet et al. (2017); Brancaccio et al. (2019); Jang et al. (2021a); Wranke et al. (2023); Roulot et al. (2025); and Wong et al. (2025). As HDV is a satellite virus that can establish a productive infection only in HBV-infected individuals, these studies evaluated HCC risk in HBV-HDV–coinfected populations compared with HBV-monoinfected controls and included adjusted estimates (HR or OR) for HCC development. A study including patients who were positive for both HBsAg and anti-HDV, comparing patients who were positive or negative for HDV RNA, was also included (Romeo et al., 2009, 2014). A fourteenth cohort provided supportive evidence as it evaluated the specific contribution of HDV genotypes to HCC among HDV-positive patients (Su et al., 2006).

The cohort studies were primarily hospital-based or registry-based, enrolling HBsAg-positive patients from clinical centres or national

Fig. 2.7 Funnel plot for studies included in the meta-analysis in Fig. 2.1



The funnel plot uses a fixed-effects model. Fig. 2.1 depicted the meta-analysis for hepatocellular carcinoma and antibodies to hepatitis D virus (HDV), HDV RNA positivity, and HDV infection.

Created by the Working Group.

databases and using serological or RNA testing for HDV; several studies focused on subgroups, such as patients with cirrhosis or infected with HIV. The four case–control studies considered to be informative (Raimondo et al., 1984; Trichopoulos et al., 1987; Amougou et al., 2016; Mahale et al., 2019) used hospital-based controls who were free of liver disease, except for the study by Raimondo et al. (1984), which used patients with cirrhosis as controls.

Together, the cohort and case–control studies spanned a broad geographical range that included Europe (e.g. France, Germany, Italy, Sweden), Africa (e.g. Cameroon, Gambia), Asia (e.g. Taiwan, China), and North America (e.g. USA), and were varied in design quality and scope. This body of literature formed the primary evidence base for assessing the carcinogenicity of HDV in humans.

2.2.2 Exposure assessment quality considerations

In the included studies, HDV infection was assessed mostly through serology (for anti-HDV antibodies), which in some studies was complemented by HDV RNA quantification. In addition,

some studies were based on records of HDV infection in clinical data.

As described in Section 1.4, the ideal approach would be to assess both past and current HDV infection status among individuals who are positive for HBsAg by testing for antibodies to HDV followed by testing for HDV RNA.

Measuring HDV RNA is the gold standard for viral detection, as it allows the discrimination of active viral replication in exposed patients; therefore, studies using this method were considered particularly informative for the present evaluation.

Serology-based studies used IgG or total (IgG and IgM) antibodies that do not discriminate between current and past infections and consequently may underestimate the association between HDV and HCC. One study used IgM antibodies (Wranke et al., 2023), which are considered a surrogate measure of active infection. Studies based on medical records are subject to selection bias – as testing is prompted by clinical indication or changing guidelines – and to exposure misclassification because of potential incompleteness and lack of delineation of diagnostic methodology within the medical records.

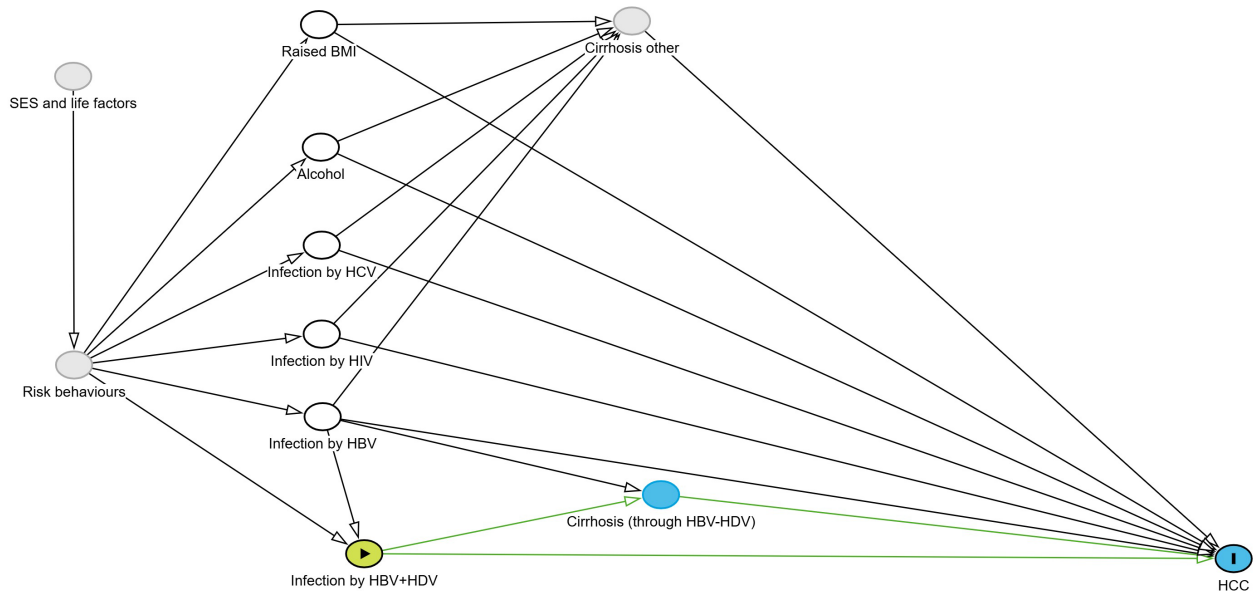
Exposure misclassification also posed a problem in studies with long-term sample storage and variable assay quality over time, potentially introducing nondifferential misclassification bias that could attenuate the observed association towards the null, particularly in the older studies. This effect was well demonstrated in the studies by Romeo et al. (2009, 2014), because the focus on HDV RNA status within an anti-HDV–positive population allowed for direct comparison between individuals with active viral replication (RNA-positive) and those with inactive or cleared infections (RNA-negative).

In older studies that were carried out before the availability of commercial nucleic acid assays (around 2010), the sensitivity of the exposure assessment was low, consequently biasing the results towards the null. Indeed, in the two oldest studies under evaluation (Raimondo et al., 1984; Trichopoulos et al., 1987), RIAs were employed for the detection of anti-HDV. RIAs are no longer in common use for HDV and may be less accurate than nucleic acid assays (Akuffo et al., 2024). Other concerns about older studies are described below.

2.2.3 Confounding and bias

Several important sources of confounding and bias were identified across the evaluated studies. A major concern was the inadequate consideration of relevant liver cancer risk factors such as coinfection with HIV and/or HCV, BMI, and alcohol consumption (IARC, 2012, 2018). Additionally, cirrhosis status, which frequently mediates the association between the various aforementioned risk factors and HCC development, was inconsistently accounted for across studies. Consequently, the consideration of these variables and the subsequent directionality of the anticipated effect on the overall effect size depends on the proposed biological pathway; directed acyclic graphs (DAGs) depicting this paradigm can be found in Fig. 2.8, Fig. 2.9, and Fig. 2.10. Per the DAG, two arrows were drawn between HDV infection and HCC development, allowing representation of the occurrence of HDV-associated HCC mediated by or independent of cirrhosis, given evidence of the presence of HCC in HDV-infected individuals across both pathways (Trichopoulos et al., 1987; Allaire et al., 2021). Lack of adjustment for factors such as HIV, HCV, alcohol use, and BMI would be likely to lead to residual confounding and therefore could overestimate the resulting effect size. Two studies (Jang et al., 2021a; Roulot et al., 2025) accounted for all these factors, either through adjustments or exclusion, and showed positive results. The Working Group conducted stratified meta-analyses to assess the effects of individual and grouped adjustment for these factors to better understand their impact on the

Fig. 2.8 Directed acyclic graph showing the effect of adjusting for other causes of cirrhosis, but not conditioning on cirrhosis mediated through HDV



BMI, body mass index; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HDV, hepatitis D virus; HIV, human immunodeficiency virus; SES, socioeconomic status.

Adjustment for other causes of cirrhosis (e.g. alcohol and HCV) but not adjusting for cirrhosis should lead to adequate adjustment for assessing the total causal effect of HDV.

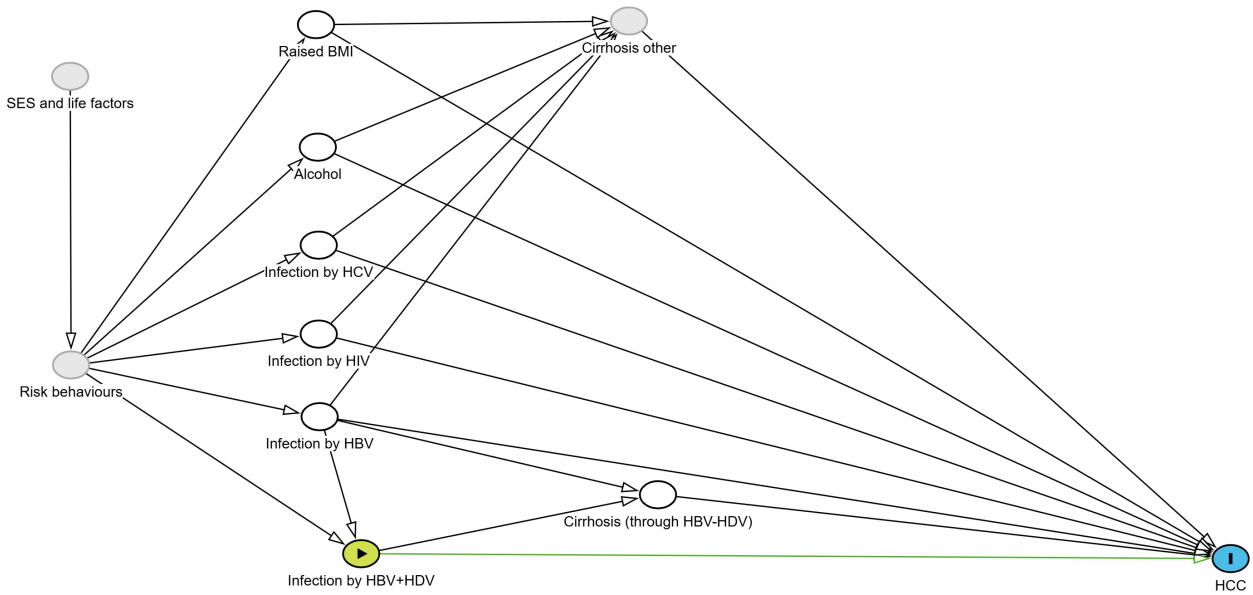
Created by the Working Group.

association between HDV and HCC (Fig. 2.4). Findings from these meta-analyses did not identify any strong confounders that could otherwise nullify the observed positive associations between HDV and HCC. Consequently, the Working Group concluded that any additional residual confounding by HIV, HCV, alcohol use, and BMI could not fully explain the observed positive association found across the studies reviewed. Conversely, selection of study participants on the basis of cirrhosis (i.e. exclusion of cirrhotic patients or only including cirrhotic patients) or adjustment for cirrhosis in multivariable models was likely to bias effect estimates towards the null, as the HDV–HCC association could be partly mediated by cirrhosis. The Working Group conducted a meta-analysis stratified by cirrhosis inclusion criteria (Fig. 2.3) to verify this anticipated effect; nevertheless, the overall results remained consistent.

Not accounting for treatment with NAs, which inhibit HBV replication but do not affect HDV infection, could bias the results upwards. However, this was not expected to be a systematic confounder, given that such treatment was not applied systematically in all cohorts.

Additionally, selection bias was introduced in retrospective studies such as those by Kushner et al. (2015) and Wong et al. (2025), in which HDV testing was not systematic, but rather driven by clinical indication, resulting in bias in an unpredictable direction. Older studies may be particularly prone to selection bias, because HDV screening guidelines were less comprehensive in the past (Pan et al., 2023b). Outcome misclassification was also possible in particular in older studies, because clinical diagnosis

Fig. 2.9 Directed acyclic graph showing adjustment for other causes of cirrhosis and conditioning on cirrhosis mediated through HDV



BMI, body mass index; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HDV, hepatitis D virus; HIV, human immunodeficiency virus; SES, socioeconomic status.

Adjustment for cirrhosis (all) will hinder assessment of the total causal effect.

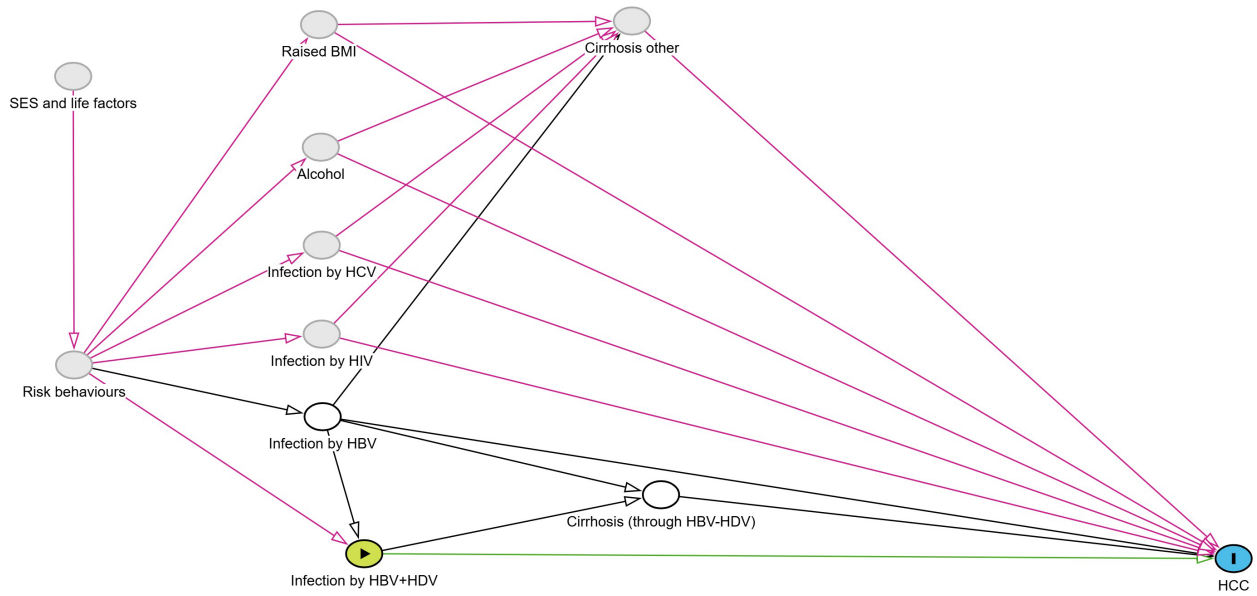
Created by the Working Group.

criteria have changed over time. Raimondo et al. (1984) used patients with cirrhosis as controls, which may underestimate the effect of HDV on HCC risk as cirrhosis is itself a precancerous condition. Furthermore, higher mortality from HDV-associated cirrhosis decompensation in early years may have limited the identification of HCC as a later outcome (Mokdad et al., 2014). These methodological concerns highlighted the need for cautious interpretation and strengthened the relevance of studies with rigorous design and adjustment methods.

2.2.4 Cancer of the liver

The Working Group conducted meta-analyses including the studies deemed informative, which confirmed a positive association between HDV infection and HCC (meta-estimate, 2.13; 95% CI, 1.57–2.87; I^2, 67%). Additionally, in multiple previously published meta-analyses, a positive association between HDV infection and HCC was consistently reported, with effect estimates indicating that risk was two to three times higher among HDV-infected individuals. Alfaiate et al. (2020a) reported a meta-OR of 1.28, with higher estimates among prospective (meta-OR, 2.77) and adjusted (meta-OR, 2.07) studies. Kamal et al. (2021) found a pooled effect size of 2.12 when restricting the analysis to longitudinal data, reinforcing evidence of the temporality of disease (HCC) after infection. Gish et al. (2024b) further highlighted that HDV RNA positivity conferred a meta-HR of 2.97 for HCC, compared with HDV RNA–negative infection and HBV monoinfection, suggesting that active viral replication may be a critical factor in disease progression, which was confirmed by the Working Group meta-analysis of studies evaluating HDV RNA (meta-estimate, 4.66; 95% CI,

Fig. 2.10 Directed acyclic graph showing conditioning on cirrhosis through other causes and conditioning on cirrhosis mediated through HDV



BMI, body mass index; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HDV, hepatitis D virus; HIV, human immunodeficiency virus; SES, socioeconomic status.

Non-adjustment for other causes of cirrhosis would be incorrect as it would hinder assessment of the total causal effect and allow confounding through other known risk factors for HCC.

Created by the Working Group.

2.19–9.91). Additionally, to test for consistency of the results, the Working Group restricted the meta-analysis to cohort studies only (Fig. S2.5, see Annex 2, Supplementary material for hepatitis D virus, Section 2, Cancer in humans, online only, available from: https://publications.iarc.who.int/667) (meta-estimate, 2.14; 95% CI, 1.66–2.77) and also excluded the study by Raimondo et al. (1984) (Fig. S2.6, Annex 2), for which there were particular concerns regarding outcome and exposure misclassification (meta-estimate, 2.26; 95% CI, 1.74–2.91). Removing the study by Raimondo et al. also drastically reduced heterogeneity. Overall, despite using diverse inclusion criteria for the studies, the results were all consistent among these different meta-analyses.

Across the 13 informative cohort studies reviewed by the Working Group, multiple analyses demonstrated significantly elevated risks of HCC among HDV-infected individuals compared with those with HBV monoinfection. For instance, Mallet et al. (2017) reported an adjusted HR of 1.53 in a large French national database, whereas Ji et al. (2012) found a markedly increased incidence in patients with chronic HDV infection (HR, 2.42), using population-level Swedish registry data. Notably, studies such as that by Béguelin et al. (2023), which pooled European HIV cohort data, observed a large increase in risk (HR, 6.3) among HDV-infected individuals, and even greater risk (HR, 8.3) in individuals who were HDV RNA–positive, indicating a possible role for viraemia in carcinogenesis. Romeo et al. (2014) demonstrated a dose–response relation between HDV RNA levels and HCC development, particularly among non-cirrhotic patients, suggesting a direct oncogenic effect of the virus. By stratifying risk

according to viral load, these studies underscored the importance of HDV replication activity in driving disease progression to HCC. This specificity strengthened the biological plausibility of a causal link.

Further support came from studies using well-defined cirrhotic populations. Roulot et al. (2025) observed a 5-year cumulative incidence of HCC of 20.2% in HBV-HDV–coinfected individuals with cirrhosis compared with 4.4% in HBV-monoinfected individuals. This substantial difference persisted after adjusting for a wide array of covariates, including liver function parameters and treatment groups. Jang et al. (2021a) found HDV RNA positivity to be one of the strongest predictors of HCC in a large cohort in Taiwan, China, even after stratifying by cirrhosis status, further reinforcing the biological plausibility of HDV-induced liver carcinogenesis.

The case–control studies also contributed to this conclusion. Mahale et al. (2019) reported data from a large case–control study in the Gambia and showed that HDV positivity, measured using a rigorous diagnostic approach, including HDV RNA quantification, was markedly higher in cases than in controls (OR, 4.72). Amougou et al. (2016) similarly reported elevated odds (aOR, 5.65) in Cameroon, although the estimate was imprecise owing to the relatively small sample size. Trichopoulos et al. (1987) found evidence of HDV in HCC patients without cirrhosis, supporting a model in which HDV may contribute to carcinogenesis independently from the cirrhosis pathway. The study by Raimondo et al. (1984) yielded an inverse association (OR, 0.23), but the use of cirrhotic controls and limited detection methods for outcome and exposure assessment complicated interpretation; as shown in the meta-analysis carried out by the Working Group (see Section 2.1.3), the exclusion of this study did not affect the results, rather, its exclusion reduced heterogeneity and led to increased confidence in the meta-estimate. The biological role of variation in HDV genotype in the development of HCC was suggested by the study by Su et al. (2006), in which genotype 1 conferred a stronger risk of HCC development than did genotype 2, although HCC risk was present in individuals with both genotypes.

The collective body of epidemiological evidence supported a positive association between HDV infection and the development of HCC. Taken together, attributes of the body of evidence included the strength and consistency of the association, temporality, dose–response relation, biological plausibility, and coherence across study types. Overall, the body of evidence indicated that a plausible association is seen between HDV infection and HCC and that chance, bias, and confounding could all be ruled out with high confidence.

3. Cancer in Experimental Animals

No data were available to the Working Group.

4. Mechanistic Evidence

4.1 Virus infection, distribution, and cell interaction

4.1.1 Infection

After transmission through broken skin or contact with infected body fluids (see Section 1.3.1), the propagation of an HDV infection depends on the presence of HBsAg to assemble new infectious particles and allow release from cells (Sureau and Negro, 2016).

The first symptoms, which appear after an incubation period of 3–7 weeks, are similar to those observed in other forms of viral hepatitis and include fever, fatigue, loss of appetite, nausea, vomiting, jaundice, dark urine, and

light-coloured stool (Farci and Niro, 2012; Lange et al., 2022).

HDV infects hepatocytes, usually causing more severe symptoms than does HBV monoinfection. Two main forms of infection occur: (i) coinfection, when a patient is simultaneously infected with both HBV and HDV; and (ii) superinfection, when a patient who is chronically monoinfected with HBV is exposed to a new infection with HDV (Lange et al., 2022) (see also Section 1.3.1).

Most patients with coinfections fully recover, with only a small percentage developing chronic infection (~2%) or fulminant infection, a severe form of hepatitis that can progress rapidly to liver failure. However, > 90% of superinfected patients develop progressive chronic hepatitis (Farci and Niro, 2012). An HDV infection, whether through coinfection or superinfection, is more likely to result in fulminant hepatitis than is infection with HBV or HCV alone (Botelho-Souza et al., 2017).

The interplay between HDV and HBV inside the cell exhibits different patterns. During chronic infection, some patients may display fluctuating HDV and HBV viraemia, whereas during a primary infection, patients may show persistently high HDV and HBV viraemia, or HDV dominance and suppression of HBV replication (Schaper et al., 2010).

4.1.2 Genotypes

Eight HDV genotypes (1–8) have been identified (Dény, 2006). Genotype 1 is the most prevalent worldwide, and is mainly found in Europe, North America, and China (see Section 1.3.2). Genotypes 2 and 4 are found in Japan and Taiwan, China, and genotype 3 was only detected in South America, namely the Amazon basin. Genotypes 5 to 8 were first identified in individuals of African origin from migrant populations in Europe (Dény, 2006). It is thought that genotype 3 HDV is responsible for the most aggressive form of the disease, when associated with genotype F HBV (Casey et al., 1996; Gomes-Gouvêa et al., 2009).

4.1.3 Treatment

Treatment options for HDV are scarce and of limited efficacy. NAs used to treat patients with HBV are not effective against HDV. Peg-IFNα remains the most frequently recommended option, but treatment is long (≥ 48 weeks), and the virus response rate is low. Moreover, treatment with peg-IFNα is associated with considerable adverse side-effects (Caviglia and Rizzetto, 2020; Pan et al., 2023b).

Identification of NTCP as the cellular receptor for HBV and HDV has allowed the development of an inhibitor of virus entry, bulevirtide, a 47–amino acid lipopeptide that competitively binds to NTCP (Ni et al., 2014; Blank et al., 2016). In a randomized phase III clinical trial with 150 patients, the percentage of virological responders increased during treatment weeks 24 to 96. After subcutaneous injection of the drug at 2 mg, the response rate increased from 53% to 79%; at 10 mg, the response varied from 70% to 87%. However, most patients were infected with HDV genotype 1, and the efficacy of the drug against other genotypes is not fully established (Wedemeyer et al., 2024). In a similar trial, with primary readout at 48 weeks, the authors reported a response to the virus in 45% of patients after a 2 mg injection of bulevirtide, in 48% of patients after a 10 mg injection, and in 2% of the control group (Wedemeyer et al., 2023). The combination of treatment with bulevirtide and peg-IFNα has shown the highest antiviral efficacy in a short-term 24-week study (Asselah et al., 2024).

Lonafarnib inhibits cellular farnesyltransferase and prevents HDV assembly (Bordier et al., 2002). A placebo-controlled phase III clinical trial in 407 people showed that lonafarnib was effective in reducing viral load when administered for 48 weeks in combination with

the protease inhibitor ritonavir and peg-IFNα (Etzion et al., 2023).

Liver transplantation is also an option for patients with end-stage liver disease or HCC.

4.1.4 The virion

HDV virions are enveloped, roughly spherical particles, with a diameter of about 36 nm. The outer coat is composed of host lipids and HBV-encoded HBsAg surrounding an inner nucleocapsid consisting of gRNA bound to ~200 copies of the two isoforms (small and large, S and L) of the only HDV-encoded protein, HDAg (Gudima et al., 2002; Sureau and Negro, 2016).

The genome consists of a negative-sense, covalently closed, circular single-stranded RNA (cccRNA) of ~1700 nucleotides. (Kos et al., 1986; Huang and Lo, 2010). In native conditions, the RNA genome folds into an unbranched rod-like structure, promoted by extensive intramolecular base pairing (Chen et al., 1986; Kos et al., 1986; Huang and Lo, 2010).

4.1.5 Virus replication

After binding to NTCP, fusion of the virion with the cell membrane releases the nucleocapsid-like RNP into the cytosol. Subsequently, the RNP is imported into the nucleus where transcription and replication occur (Taylor, 2015; Sureau and Negro, 2016). The synthesis of viral circular genomic and antigenomic RNA (gRNA and agRNA) is thought to occur via a double rolling circle mechanism. This process begins with the production of linear multimeric molecules from the circular genomic template, which are then self-cleaved by a ribozyme activity, resulting in unit-length circular agRNAs. These agRNAs subsequently undergo self-ligation to form circular agRNAs. The circular agRNAs again serve as templates for gRNA synthesis via a second rolling circle mechanism (Taylor, 2015; Sureau and Negro, 2016).

The agRNA contains a single open reading frame (ORF) encoding HDAg (Taylor, 2015; Sureau and Negro, 2016). This protein is translated from 0.8 kb messenger RNA (mRNA) that is modified by addition of a 5′-cap and a 3′-polyadenosine tail, like mRNAs synthesized by RNA polymerase II (pol II) (Hsieh et al., 1990; Taylor, 2015). It was calculated that an infected cell accumulates approximately 300 000 copies of gRNA; this corresponds to 13 000–60 000 copies of agRNA and about 600 copies of virus-specific mRNA (Chen et al., 1986; Gudima et al., 2002).

The accumulation of virus-specific RNA is inhibited by low concentrations of α-amanitin, which supports the involvement of cellular RNA pol II in this process (Chang et al., 2008). Transcription of gRNA is carried out by RNA pol II, but controversy regarding the synthesis of agRNA persists. It was suggested that RNA polymerase I or III may be involved in agRNA synthesis, because this was found to be resistant to low α-amanitin concentrations capable of inhibiting RNA pol II activity (Modahl et al., 2000).

During virus replication, some agRNAs are post-transcriptionally modified by editing at a specific site by a host adenosine deaminase (ADAR1). Consequently, an adenosine in the amber stop codon UAG is transformed into an inosine, ultimately resulting in a UIG codon, which the cellular transcription machinery ultimately recognizes as UGG, the codon for tryptophan. This change leads to the extension of the ORF by 19 amino acids, until the next stop codon is reached. Thus, two proteins are produced that differ in size and in C-terminal sequence: (i) S-HDAg, with 194–195 amino acids; and (ii) L-HDAg, with 213–214 amino acids (Polson et al., 1996; Wong and Lazinski, 2002).

L-HDAg is mostly synthesized at later phases in the replication cycle. As a consequence, replication is suppressed, and the accumulated genomes

form new RNPs that are exported to the cytoplasm in a process mediated by a nuclear export signal (NES) present in L-HDAg. Interaction of RNPs with HBsAg occurs at the endoplasmic reticulum (ER), resulting in the assembly of new virions that are secreted from the cell (Lee et al., 2001).

Synthesis of HBsAg may occur from mRNAs transcribed from episomal covalently closed circular DNA molecules (cccDNA) or from HBV DNA integrated into host chromosomes. The latter represents a stable source of HBsAg necessary to propagate HDV even in the absence of HBV replication (Freitas et al., 2014).

4.1.6 Post-translational modifications and functional domains of HDAg

S-HDAg and L-HDAg share a nuclear localization signal (NLS) at amino acids 66–75 (Alves et al., 2008), a coiled-coil domain (CCD; amino acids 12–60) (Moraleda et al., 2000), and an RNA-binding domain (RBD; within amino acids 97–146) (Lee et al., 1993). An additional functional domain, the nuclear export signal (NES), is found at amino acids 198–210 of L-HDAg (Lee et al., 2001). S-HDAg and L-HDAg undergo post-translational modification, including phosphorylation (Chang et al., 1988; Mu et al., 2001), methylation (Li et al., 2004), acetylation (Mu et al., 2004), and sumoylation (in the case of S-HDAg) (Tseng et al., 2010) by host enzymes. Sumoylation could not be detected in L-HDAg. [The Working Group noted that the functional significance of most post-translational modifications is still unclear.] An additional post-translational modification mediated by a host farnesyltransferase occurs at cysteine residue 211 of L-HDAg (Glenn et al., 1992; Otto and Casey, 1996). Isoprenylation of this amino acid is necessary for interaction with HBsAg during virion assembly (Hwang and Lai, 1993; Lee et al., 1994).

S-HDAg and L-HDAg play different roles during virus replication. S-HDAg is essential for the accumulation of HDV RNA in infected cells, whereas L-HDAg acts as a dominant-negative inhibitor of virus replication and interacts with HBsAg during virion assembly (Hwang and Lai, 1994; Lee et al., 1994). Other functions have been assigned to HDAg: transport of the genome to the nucleus after virus entry and removal of the virus capsid (decapsidation) (Chou et al., 1998), stimulation of ribozyme activity (Jeng et al., 1996), and suppression of editing (Cheng et al., 2003). [The Working Group noted that some of these functions were reported only in experiments in vitro and were not validated in vivo.]

4.1.7 HDV propagation in the absence of HBV

Results obtained in vivo using a liver-humanized mouse model and experiments in vitro in human hepatocarcinoma cell lines provided evidence suggesting that propagation of HDV RNPs may occur in the absence of HBsAg, although there is no evidence that this occurs in humans. Envelope proteins from other viruses, namely vesiculovirus, flavivirus, and hepacivirus, can package HDV RNPs, allowing the spread of newly assembled virus particles to other cells and tissues. Furthermore, HCV envelope proteins support the assembly of HDV RNPs in vivo in liver-humanized mice (Perez-Vargas et al., 2019). The spread of HDV RNA and HDAg was also reported to be mediated by exosomes in vitro, and as result of cell division (Zhang et al., 2022; Khabir et al., 2024). These findings may help to explain the detection of HDV sequences and antigens in epithelial cells of the minor salivary gland in patients with Sjögren syndrome who were HDV-positive and HBV-negative (Hesterman et al., 2023).

4.1.8 Experimental models

Primary human hepatocytes (PHHs), tree shrew (*Tupaia belangeri*) hepatocytes, and the HepaRG (human bipotent progenitor) liver cell line can replicate the HDV virus; however, only PHHs can support the entire virus replication cycle. [The Working Group noted that many different cell lines (including HeLa and HEK293), not only human hepatoma lines, have been used in stable or transient transfection experiments with plasmids coding for the HDV genome or separately for individual virus components. The Huh7-END cell line has been developed as a novel tool to study HDV (Gudima et al., 2002; Ni et al., 2019; Heuschkel et al., 2021).]

More recently, hepatocyte-like cells derived from human pluripotent stem cells were shown to be permissive to HDV infection and to support the replication and release of new virions when coinfected with HBV (Chi et al., 2024). The identification of NTCP as the cellular receptor for HDV has allowed the hepatoma cell lines to be modified to express NTCP, thus making them susceptible to the virus (Lee et al., 2023).

Initially, in vivo experiments were mostly performed in infected chimpanzees (*Pan troglodites*) and woodchucks (*Marmota monax*) (Rizzetto et al., 1980). Transgenic mice (*Mus musculus*) expressing human NTCP and liver-humanized mice were the most popular in vivo experimental models (Lütgehetmann et al., 2012; He et al., 2015).

4.2 Evidence relevant to key characteristics of carcinogens

This section reviews the mechanistic data for the key characteristics of carcinogens (Smith et al., 2016) encompassed by the agent HDV. Evidence was available for the key characteristics "alters DNA repair and causes genomic instability", "induces oxidative stress", "induces chronic inflammation", "causes immortalization", and "alters cell proliferation, cell death, or nutrient supply". No data were available for the remaining key characteristics.

4.2.1 Alters DNA repair or causes genomic instability

Humans

(i) Exposed humans

Pollicino et al. (2011) investigated the interplay between HBV and HDV in liver tissue and serum samples from 21 HBV-HDV–coinfected patients and 22 HBV-monoinfected patients with chronic liver disease. The aim of the study was to characterize the impact of HDV on HBV replication and transcriptional activity within the liver, a subject that is poorly understood at the intrahepatic level. Real-time quantitative PCR (real-time qPCR) was applied to measure intrahepatic levels of HBV DNA, cccDNA, relaxed circular DNA (rcDNA), pregenomic RNA (pgRNA), and pre-S/S RNAs, as well as HDV RNA in liver and serum. The results revealed that HDV coinfection significantly reduces HBV replication: HDV-positive patients had median levels of serum HBV DNA that were lower by ~5-logs and levels of intrahepatic HBV rcDNA and cccDNA that were lower by ~2-logs than those of HBV-monoinfected individuals. Similarly, transcriptional markers of HBV replication, pgRNA, and pre-S/S RNAs were also significantly decreased. Despite this reduction in HBV replication, serum levels of HBsAg were similar in the two groups. The amount of HBsAg produced per cccDNA molecule was significantly higher in HDV-positive individuals, suggesting a dissociation between HBV genome replication and envelope protein production. Although HDV-positive patients produced fewer HBV transcripts, the transcriptional output per cccDNA molecule did not differ significantly between the groups. However, the ratio of pgRNA:total HBV RNA was substantially lower in coinfected patients, suggesting selective

inhibition of pgRNA synthesis. This selective suppression may reflect differential regulation of HBV promoters in the context of HDV coinfection, supporting the idea that HDV exerts a "smart" inhibitory effect, reducing HBV DNA production while maintaining HBsAg synthesis, which is essential for HDV particle assembly. Moreover, five of the 21 HDV-infected patients harboured HBV genomes with large deletions in the basal core promoter/precore region, potentially impairing pgRNA transcription and contributing to reduced replication. These deletions were not observed in HBV-monoinfected patients and were associated with lower HBV viraemia. [The Working Group noted that the study provided evidence that HDV interferes with HBV biology in a selective and sophisticated manner. HDV reduced HBV replication and pgRNA transcription while preserving the expression of envelope proteins, thereby securing the resources necessary for HDV propagation. However, the number of study participants was small.]

Diaz et al. (2018) conducted a transcriptomics study to define the molecular signature and oncogenic mechanisms underlying HDV-associated HCC (see also Section 4.2.2). The study included liver tissue and serum samples from 12 patients with chronic HDV infection: five with HDV-related HCC, and seven with non-HCC HDV cirrhosis. Transcriptomic profiling was performed both on whole liver tissue and on laser capture–microdissected (LCM) malignant and non-malignant hepatocytes, allowing for high-resolution analysis of gene expression in the tumour microenvironment. Gene expression analysis revealed a distinctive molecular signature in HDV-associated HCC, which was characterized by strong upregulation and coregulation of genes involved in DNA replication, DNA damage response, DNA repair, and cell cycle control, including *BRCA1*, *BARD1*, *H2AFX*, *CDK1*, and *MSH2*. Six key pathways were enriched: GADD45 signalling, sonic hedgehog, G2/M DNA-damage checkpoint regulation, cyclins and cell cycle regulation, 14-3-3 σ pathway, and hereditary breast cancer signalling. A t-distributed stochastic neighbour embedding (t-SNE) analysis confirmed that a cluster of 37 genes, including several chromosomal instability-associated genes (e.g. *TOP2A*, *MELK*, *PRC1*, *TRIP13*, *KIF20A*), was not only overexpressed but highly coregulated in HDV malignant hepatocytes (MH).

[The Working Group noted that these findings suggested that genes involved in the maintenance of genome stability, DNA replication, and DNA repair are upregulated and coregulated to a greater degree in HDV-MH than in HBV-MH and HCV-MH.] Intrahepatic viral load analysis showed that HDV replication was lower in tumour regions than in the surrounding nontumorous tissue, whereas HBV DNA was undetectable or detected at extremely low levels in all HDV-associated cases of HCC, consistent with the suppressive effect of HDV on HBV replication. [The Working Group noted that the findings supported the hypothesis that HDV may drive the development of HCC through a unique mechanism of genome instability and dysregulation of DNA repair pathways that is distinct from that used by HBV. Despite the reliance of HDV on HBV, the study clearly distinguished HDV-associated HCC from HBV-associated HCC at the molecular level. In addition, the Working Group noted that the information provided by this study may be indicative of impairment of the DNA damage response and DNA repair pathways after HDV infection, potentially leading to carcinogenesis, i.e. *BRCA1*, *TOP2A*. However, the study was limited by the small number of study participants and samples analysed.]

(ii) Human cells in vitro

Mendes et al. (2013) used quantitative proteomics ($^{16}O/^{18}O$ labelling, liquid chromatography-tandem mass spectrometry, LC-MS/MS) in

HEK-293 cells and observed alterations in the expression of 89 different proteins involved in cell cycle disruption and G2/M checkpoint deregulation, including downregulation of p53 and 14-3-3 σ, after HDV replication. Moreover, pathway and network analysis indicated interference with the DNA damage response and control of cell proliferation. [The Working Group considered that the study provided some evidence of alteration in DNA repair pathways and potential genetic instability as a consequence of HDV RNA replication in vitro. The study relevance was limited by the experimental system used, which did not reflect the physiological conditions observed in vivo in infected patients; however, data were confirmed in a small group of patients, as reported by Diaz et al. (2018).]

Synopsis

[Overall, the Working Group noted that few studies were available that supported evidence of alterations in DNA repair and genomic instability induced by HDV.]

4.2.2 Induces epigenetic alterations

Humans

(i) Exposed humans

Zhang et al. (2016) investigated the expression of long noncoding RNAs (lncRNAs) in HCC of different viral etiologies (HBV, HCV, and HDV). Using 88 liver tissue samples from well-characterized patients, including paired tumour and adjacent non-tumour tissues from 25 patients with virus-associated HCC (11 associated with HBV, 10 with HCV, and 4 with HDV), 20 patients with cirrhosis without HCC, and 18 controls, the authors profiled 101 lncRNAs (83 from a commercial real-time qPCR panel plus 18 literature-based) to identify patterns of virus-specific dysregulation. Principal component analysis and hierarchical clustering revealed a clear distinction between tumour and non-tumour tissues, but not between viral etiologies. However, specific lncRNAs showed etiology-dependent expression. Notably, PCAT-29 was significantly downregulated in HBV-related HCC, aHIF and PAR5 in HCV-associated HCC, and Y3 in HDV-associated HCC. In contrast, some lncRNAs were consistently altered across all cases of HCC regardless of etiology, such as BC017743 and BC043430 (upregulated), and LINC01152 and TMEVPG1 (downregulated), suggesting common tumorigenic roles. Three previously reported lncRNAs – DBH-AS1, hDREH, and hPVT1 – also showed differential expression depending on viral context. For example, hPVT1 was significantly upregulated only in HBV-associated HCC, potentially via the TGFβ pathway. Similarly, DBH-AS1 and hDREH (known to be regulated by HBV-X protein) were specifically altered in HBV-associated HCC but not in HDV-associated or HCV-associated HCC, consistent with the known role of HDV in the suppression of HBV replication. [The Working Group noted that the study was well conducted; however, the number of HDV-infected patients was small, and only a single microarray analysis was performed.]

(ii) Human cells in vitro

Liao et al. (2009) investigated how HDV regulates host gene expression through epigenetic mechanisms. Using human Huh7 cells (from HCC) engineered to stably or transiently express either S-HDAg or L-HDAg, the authors identified clusterin (*CLU*) as a gene that was consistently upregulated in response to HDV antigens. Clusterin is a glycoprotein associated with cytoprotection and tumour progression. Expression of both HDAg isoforms was shown to increase levels of clusterin mRNA and protein, with a stronger effect observed for S-HDAg. HDV replication in Huh7 cells also led to enhanced expression of clusterin, correlating with the appearance of HDAg. Importantly, this effect was not mediated via the canonical

promoter response elements (e.g. CRE), but rather via histone H3 hyperacetylation, as demonstrated through chromatin immunoprecipitation (ChIP), luciferase reporter assays, and treatment with the histone deacetylase (HDAC) inhibitor trichostatin A. The upregulation of clusterin was associated with decreased sensitivity to adriamycin, a chemotherapeutic agent, suggesting a survival advantage. [The Working Group noted that these studies suggested that HDV antigens, particularly in the context of active viral replication, could epigenetically modulate host gene expression via histone acetylation. This contributed to the enhanced survival potential of liver cells, supporting the role of HDV as a cofactor in liver carcinogenesis through indirect, non-genotoxic mechanisms. However, the Working Group noted that the experimental system used in this study did not reflect the pathophysiological conditions observed in infected liver cells, thus limiting the relevance of the results for the present evaluation.]

Benegiamo et al. (2013) investigated how HDV alters DNA methylation processes in liver cancer cells using an in vitro model of Huh7 cells transfected with a plasmid expressing both S-HDAg and L-HDAg. The aim of the study was to clarify the epigenetic mechanisms through which HDV may contribute to HCC development. The authors found that HDV expression specifically upregulated the DNA methyltransferase DNMT3B (but not DNMT1), at both the mRNA and protein level. This upregulation was mediated by STAT3 phosphorylation at tyrosine 705 (Y705), as inhibition of STAT3 with NSC74859 significantly reduced DNMT3B expression. To explore functional consequences, the authors used a methylation array based on real-time qPCR and found that of the 24 HCC-related genes tested, *E2F1* was the only promoter hypermethylated in HDV-expressing cells. This hypermethylation correlated with downregulation of *E2F1* mRNA and was associated with G2/M cell cycle arrest, a phenotype observed in many viral infections. Treatment with the DNA methyltransferase inhibitor azacytidine reversed both DNMT3B upregulation and E2F1 silencing. These findings indicated that HDV antigens, via STAT3-mediated DNMT3B activation, can drive targeted DNA hypermethylation (e.g. of the tumour suppressor gene *E2F1*) and promote cell cycle arrest, supporting an epigenetic, non-genotoxic mechanism of viral carcinogenesis. [The Working Group noted that, similarly to the previous study, the experimental system was not adequate to allow a conclusion to be made regarding triggering of epigenetic alterations in HDV-infected liver cells.]

Mota et al. (2009) investigated the proteomic changes induced by HDV in a human hepatoma model using Huh7-D12 cells, which stably express HDV RNPs, including both S-HDAg and L-HDAg. These cells replicate HDV RNA and express HDV antigens constitutively, mimicking persistent infection in vitro. The authors used two-dimensional electrophoresis combined with matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry to compare the proteomes of Huh7-D12 cells with those of parental Huh7 cells. They identified 23 differentially expressed proteins: 15 were downregulated and eight were upregulated in HDV-expressing cells. Functional categories included metabolism and energy pathways, nucleic acid metabolism, cytoskeletal maintenance, protein transport, and signal transduction. [The Working Group noted that upregulated proteins included La protein and HMGB1 (involved in regulation of the metabolism of nucleobases, nucleosides, nucleotides, and nucleic acids), lamin C (involved in cell growth and maintenance), and GRP78/BiP, associated with RNA (involved in protein metabolism); downregulated proteins included triosephosphate isomerase (TPI) and pyruvate carboxylase (involved in glucose metabolism; energy pathways), histone H1-binding protein (NASP) (involved in signal transduction, cell communication) and

PABP2 (involved in regulation of the metabolism of nucleobases, nucleosides, nucleotides, and nucleic acids).] Transcriptional downregulation of NASP and TPI was confirmed by real-time PCR, whereas western blot validated upregulation of La and lamin C proteins. Additionally, protein–protein interaction network analysis revealed that NASP, TPI, and PABP2 form a subnetwork that is possibly regulated by SETDB1, a histone methyltransferase known to interact with DNMT3A/3B, linking HDV expression to epigenetic gene silencing pathways.

[The Working Group noted that the findings were indicative of the hypothesis that HDV modulates host cell proteostasis and survival pathways, potentially contributing to hepatocyte dysfunction, immune escape, and hepatocarcinogenesis via non-genotoxic, epigenetically mediated mechanisms; however, this was not confirmed by functional assays. The Working Group also noted that Huh7-D12 cells have an estimated 40 copies of the integrated HDV complementary DNA (cDNA) genome, presumably on chromosome 1. It was not clear how integration of these genomes affected the expression of cellular genes at the site of integration and the neighbouring region.]

Synopsis

[The Working Group noted that few studies were available that reported on evidence of end-points relevant to epigenetic alterations.]

4.2.3 Induces oxidative stress

Humans

(i) Exposed humans

No studies that addressed the key characteristic of carcinogens "induces oxidative stress" in exposed humans were available to the Working Group.

(ii) Human cells in vitro

Goto et al. (2000), in a series of experiments in various cell types, including HeLa cells (a human cervical carcinoma cell line), Huh-7 cells, and HepG2 cells (hepatoblastoma cell line), observed that L-HDAg was able to activate serum response factor (SRF) and the associated serum response element (SRE) transduction pathway. [The Working Group noted that SRF mediates the activation of proteins encoded by immediate-early genes such as c-fos (a proto-oncogene) and pip92. Furthermore, the Working Group noted that the authors did not observe an increase in the activation of NF-κB or AP-1 or cAMP response element (CRE) pathways. Although Goto et al. (2000) did not examine reactive oxygen species (ROS) directly, their findings demonstrated that SRF/SRE-dependent transcription can be activated independently of canonical ROS-related pathways, such as those involving Elk1 or PKA. This suggested that alternative mechanisms, unrelated to oxidative stress, may also modulate SRF/SRE activity and should be considered when interpreting the effects of ROS on these transcriptional pathways.]

Park et al. (2009) observed that the L-HDAg, but not S-HDAg, increased tumour necrosis factor alpha (TNFα)-induced NF-κB activation in Huh7 cells, using a luciferase reporter gene assay. In addition, L-HDAg was able to induce translocation of NF-κB (p65) and to promote the expression of target genes such as COX-2.

Williams et al. (2012) investigated the molecular mechanisms by which L-HDAg (also known as p27) contributes to liver pathogenesis in HDV infection. Using Huh7 cells transfected with L-HDAg or S-HDAg (p24), the authors demonstrated that L-HDAg induces high levels of ROS, leading to the activation of two key oncogenic transcription factors, STAT-3 and NF-κB. ROS production was significantly increased in cells expressing L-HDAg, compared with cell expressing S-HDAg or control cells, and was

dependent on the isoprenylation site (C211) of L-HDAg. The activation of STAT3 and NF-κB in luciferase reporter assays was dose-dependent, and it was strongly reduced when the cells were exposed to ROS scavengers (e.g. NAC, PDTC) or calcium chelators (e.g. TMB-8, BAPTA-AM), although mitochondrial calcium did not appear to be essential. This indicated that ROS induction by L-HDAg may involve calcium-dependent but non-mitochondrial mechanisms.

STAT-3 activation included phosphorylation at tyrosine 705 (Y705), nuclear translocation, and binding to DNA. In addition to luciferase reporter assays, NF-κB activation was also confirmed by purification of p65-bound DNA elements (pull-down assay). The study further showed that L-HDAg increases NADPH oxidase 4 (Nox4) promoter activity in reporter gene assays and increases Nox4 protein levels, suggesting that this oxidase was the major source of ROS. The ER stress response was only weakly induced by L-HDAg and did not appear to be the main contributor to ROS production in this model. Notably, these effects were absent with S-HDAg, which lacks the C-terminal 19 amino acids of L-HDAg, emphasizing the functional specificity of L-HDAg. The authors proposed a mechanistic model in which L-HDAg triggers oxidative stress via Nox4 activation, leading to STAT-3 and NF-κB signalling, which promotes inflammation, proliferation, survival, and other pathways associated with liver damage and hepatocarcinogenesis in HBV-HDV coinfection. [The Working Group noted that the authors confirmed the results of some previous studies describing the role of NF-κB and STAT3 for HBV and HCV involvement in HCC, as reported in a review by He and Karin (2011).]

More recently, Chen et al. (2019b) identified a novel mechanism by which HDV, via S-HDAg, contributes to liver injury and potentially to hepatocarcinogenesis. Using human fetal hepatocyte-derived L-02 cells, the authors demonstrated that S-HDAg directly and specifically binds to GSTP1 (glutathione *S*-transferase P1, a key antioxidant and detoxifying enzyme) mRNA, without affecting mRNA levels. This interaction leads to post-transcriptional repression of GSTP1 protein expression. Through a combination of SELEX (systematic evolution of ligands by exponential enrichment), pull-down assays, biolayer interferometry, and co-transfection experiments, the study showed that S-HDAg binds to the coding region of GSTP1 mRNA, inhibiting its translation. This interaction resulted in increased ROS accumulation and apoptotic cell death. Restoration of GSTP1 expression, either by small interfering RNA (siRNA) knockdown of S-HDAg or exogenous GSTP1 overexpression, rescued the cells from oxidative stress and apoptosis. [The Working Group noted that this mechanism operated independently of promoter methylation or microRNA (miRNA) action, highlighting a non-classical, epigenetic-like mechanism of post-transcriptional gene regulation. The Working Group also noted that the authors considered GSTP1 silencing to be a relevant event in measuring the potential effects of HDV in hepatocarcinogenesis, because it was frequently found in hepatitis-associated HCC.]

Smirnova et al. (2023) explored how HDV contributes to hepatocyte injury by inducing oxidative stress, activating the Nrf2/ARE (nuclear factor erythroid 2-related factor 2/antioxidant response element) defence pathway, and triggering the unfolded protein response (UPR). Using Huh7 cells, they studied overexpression of HDV antigens (S-HDAg and L-HDAg), as well as autonomous viral replication via transfection with a trimeric HDV genome plasmid. L-HDAg, and to a lesser extent S-HDAg, significantly increased the intracellular production of ROS, including superoxide anions, measured using redox-sensitive fluorescent probes such as 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), dihydroethidium (DHE), and mitochondrial superoxide indicator (MitoSOX). This oxidative stress was linked to

upregulated mRNA expression of extramitochondrial ROS-generating enzymes: NADPH oxidases (NOX1, NOX4), cytochrome P450 2E1 (CYP2E1), and ER oxidoreductin 1α (Ero1α). Chemical inhibition of NOX or CYP2E1 reduced ROS accumulation, confirming their functional role. Additionally, L-HDAg, but not S-HDAg, activated the Nrf2/ARE pathway, a key antioxidant defence mechanism. This was confirmed via luciferase reporter assays and increased mRNA or protein levels of the Nrf2 target genes HO-1 and Nqo1. Nuclear translocation of Nrf2 further validated its activation. Both S-HDAg and L-HDAg also induced ER stress, as shown by activation of the UPR signalling pathways (cyclic AMP-dependent transcription factor ATF6α, ATF6; proline-rich receptor-like protein kinase, PERK; serine/threonine-protein kinase/endoribonuclease, IRE1); by the increased expression of CHOP, Grp78, and XBP1; as well as by UPR-responsive promoters (UPRE, ERSE, AARE). [The Working Group noted that these effects were also reproduced in cells expressing the full HDV genome. However, in HDV-infected HepaRG^{NTCP} cells, the activation of the oxidative stress response or UPR pathways could not be confirmed owing to low infection efficiency (< 10%), highlighting a limitation of current models of infection for studying virus-induced intracellular changes.]

Synopsis

[The Working Group identified several studies reporting the overexpression of genes involved in pathways related to oxidative stress in hepatoma cell lines transiently transfected with plasmids coding for S-HDAg, L-HDAg, or the whole HDV cDNA. The findings regarding the potential of HDAgs and HDV RNA replication to induce oxidative stress were somewhat inconsistent. Some studies in vitro indicated a major contribution of S-HDAgs, others indicated a major contribution of L-HDAgs. However, ROS production was not directly assessed. Furthermore, one study reported the induction of ER stress by HDAgs, but this evidence was not supported by another study. No information was available on the induction of oxidative stress by HDV in experimental systems in vivo.]

4.2.4 Induces chronic inflammation and immune response

(a) Humans

(i) Exposed humans

[The Working Group noted that an inflammatory response and persistence of inflammation in the liver after infection with HDV was previously described in *IARC Monographs* Volume 59 (IARC, 1994).]

Govindarajan et al. (1984, 1986) reported that acute HDV infection could stimulate degenerative and inflammatory reactions; however, in most cases this was without specific alterations or differences when compared with the effects of HBV monoinfection. Persistence and chronicity of the inflammatory effects were reported in HBV-positive HDV-superinfected patients with progress to cirrhosis. In several studies of patients who were chronically infected with HBV, those who were also infected with HDV had more severe chronic lesions in the liver, portal inflammation, lobular inflammation, and necrosis (Kanel et al., 1984; Lok et al., 1985; Govindarajan et al., 1986).

Kanel et al. (1984) followed patients with chronic hepatitis who underwent biopsy or autopsy. Of a total of 57 patients with chronic HBV infection, as assessed by level of serum HBsAgs measured by radioimmunoassay, 18 also had chronic HDV infection. The authors observed, by histopathology, an increase in mononuclear inflammatory infiltration and hyperplasia in HDV-positive patients. Within the liver parenchyma, HDV-positive patients had a greater degree of inflammatory lymphocytic infiltration and necrosis. Additionally, a greater

degree of nuclear polyploidy and dysplasia was observed in patients who were positive for HDV compared with patients with CHB who were negative for the presence of HDAg. [The Working Group noted that the study was conducted with a limited number of patients, and that information on age, sex, or liver status of the patients was missing. In addition, only a qualitative "scoring method" was used to assess the histopathology findings.]

Lok et al. (1985) described 106 patients, mostly men, who were HBV carriers with HBsAg positivity for ≥ 6 months, who underwent liver biopsy. Of these 106 patients, 20 were also positive for HDV and tended to be younger than the rest of the group (mean age, 30.3 years versus 38 years, respectively). The authors observed that HDV-infected patients had higher mean levels of serum ALT and more severe liver damage, including cirrhosis, compared with HBV-monoinfected patients. [The Working Group noted that the "scoring method" to assess the severity of histopathology findings limited the informativeness of the study.]

In another observational study of a group of 23 patients with chronic HBV infection and HDV superinfection, Govindarajan et al. (1986, 1984) collected and analysed liver biopsy samples ($n = 62$) over a period of 18 years. Two to five biopsies from each individual were collected (mean, 2.8). Patients were grouped according to the moment biopsies were taken in relation to HDV infection onset – two patients had biopsies taken before and after HDV infection, 12 patients had initial biopsies at the time of acute HDV infection and several biopsies taken during the follow-up period (0.5–11 years), and 9 had chronic infection with both HDV and HBV at the time of the first biopsy. The HDV antigen was detected by immunostaining, and five out of 53 biopsies gave negative results, although all patients had been previously diagnosed with either acute or chronic HDV infection. The antigen positivity in the biopsy did not correlate with the duration of the infection or the degree of liver inflammation or damage. HDV superinfection in patients with chronic HBV infection induced severe necroinflammatory reactions that persisted for about 15 months and often led to cirrhosis. [The Working Group noted that the severity and progression of histopathology findings were graded using a semiquantitative scoring method, as described by Knodell et al. (1981), and biochemical parameters or specific mechanistic end-points associated with the key characteristic of carcinogens were not assessed in the study.]

In more recent studies, it was confirmed that infection with HDV and HBV induces exacerbation of liver damage and disease, when compared with a single infection with either HBV or HCV (Oliveri et al., 1991; Choi et al., 2007; Niro et al., 2010; Romeo et al., 2014).

Oliveri et al. (1991) described male and female HBV-monoinfected ($n = 14$) as well as HBV-HDV–infected ($n = 10$) patients with mean ages of 56 and 48 years, respectively. The HBV-HDV–infected patients showed early onset of cirrhosis along with the occurrence of high levels of replication for both viruses in 40% of patients ($n = 4$), mostly young. HBV and HDV were detected using commercial immunoassays, and serum HBV DNA and HDV RNA were detected by hybridization. The cirrhosis was characterized by significant "necroinflammation", with a significant increase in necrosis. [The Working Group noted that necrosis could be a promoter of cell proliferation and new mitoses. The Working Group also noted that the study was conducted in a small number of patients, and little information was provided on the assessment of liver damage and or cirrhosis.]

In a retrospective study of 188 patients with diagnosed HDV infection who were under periodic surveillance at hospital liver units in Turin, Italy, Niro et al. (2010) collected information on demographics, efficacy of treatment for liver disease, and liver complications. Of the

188 patients, 126 (67%) tested positive for serum IgM anti-HDV antibodies, 171 (91%) tested positive for anti-HBeAg, 175 (93%) tested positive for serum HDV RNA, and 61 (33%) tested positive for serum HBV DNA. Histology showed that 82 patients (43%) had chronic hepatitis with no evidence of cirrhosis, and the remaining 106 individuals had a clinical or histological diagnosis of cirrhosis. Cirrhosis was assessed by liver histology and/or platelet count, ultrasound, and endoscopic features; HCC was assessed by contrast imaging with computed tomography (CT) scan, magnetic resonance imaging, and second-generation contrast ultrasound. The results showed that 82 patients (44%) had chronic hepatitis and in 21 (25%) of these the disease evolved into cirrhosis. Hepatic decompensation and HCC were observed in 42 (33%) and 17 (13%), respectively, of the 127 cirrhotic patients. Cox regression analysis showed that the 5- and 10-year survival rates were 96.8% and 81.9%, respectively, for patients with chronic hepatitis, and 83.9% and 59.4% for patients with cirrhosis ($P < 0.01$).

Romeo et al. (2014) evaluated a cohort of patients ($n = 193$) from Maggiore Hospital, Milan, Italy, who were chronically infected with HDV; they were mostly men, with a mean age of 40.8 years. For 76% of the patients, the modality of infection was unknown, and the remaining 24% of patients were infected by transfusion (7%), intravenous drug use (12%), or sexual activity (5%). Assessment of levels of HDV RNA and HBV DNA was performed by real-time qPCR. The authors observed that high levels of HDV viraemia (> 600 000 copies/mL) in non-cirrhotic patients were associated with progression to cirrhosis in multivariable analysis (OR, 1.56; 95% CI, 1.14–2.12; $P = 0.005$). Moreover, the authors found a positive significant correlation between HDV RNA levels and HCC in the multivariable analysis (OR for cirrhosis, 1.56; 95% CI, 1.14–2.12; $P = 0.005$; OR for HCC, 2.64; 95% CI, 1.13–6.16; $P = 0.025$).

Joshi et al. (2023) reported that coinfection induced liver fibrosis, as measured by liver stiffness and serum markers. The 24 patients studied included 16 HBV-HDV–coinfected patients (nine men and seven women; median age, 42 years) and 8 HBV-monoinfected patients (four men and four women; median age, 39 years), with a median follow-up of 5 years. In the group of coinfected patients, 11 were HDV RNA–positive (five were treated with IFN plus NAs, three were treated only with NAs, and three were untreated) and five were RNA-negative (three were previously treated with IFN plus NAs, one was treated only with NAs, and one was untreated). Stimulation in vitro of peripheral blood mononuclear cells (PBMCs) with commercially available HBcAg and HDAg (5 µg each) showed weaker $CD3^+$ $CD4^+$ T-cell proliferation in HDV RNA–positive versus HDV RNA–negative HBV-HDV–positive patients ($P < 0.05$). In HDV RNA–positive patients, ALT levels correlated with TNFα expression, high IL-10 levels, and an increased proportion of $CD14^+$ $TNF\alpha^+$ cells. The observations did not depend on the treatment status of the patients. [The Working Group noted that, although the patients were well characterized with respect to serological and virological parameters, the number of study participants was small, and the possible presence of other infections was not taken into account.]

More recently, Bockmann et al. (2025) described the involvement of the cytokine C-X-C motif chemokine CXCR3/CXCL9–11 receptor/ligand axis of T cells in the HDV-triggered inflammatory immune response, and liver infiltration in liver biopsy and serum samples from HDV-infected patients. The authors analysed PBMCs in sampled plasma from 27 patients who were infected with HBV (14 men, 13 women; median age, 43 years), 20 patients infected with HBV and HDV (13 men; seven women; median age, 47 years), and 14 uninfected controls (gender and age not reported), as well as liver biopsies from 18 HBV-infected patients (10 men and eight

women; median age, 47 years), 18 HBV-HDV–infected patients (13 men, 5 women; median age, 43 years) and 14 uninfected control patients (gender and age not reported). Positivity for infection with either HBV or HDV was assessed by detection of anti-HBsAg and anti-HDsAg antibodies, respectively. Compared with HBV-infected patients, HBV-HDV–infected patients showed a significant increase in mRNA expression of chemokines CXCL9, CXCL10, CXCL11, and CXR3 in liver biopsies (by real-time PCR and by in situ visualization). In experiments analysing the plasma of HBV-infected and HBV-HDV–infected patients, the median frequency of $CXCR3^+CD4^+$ T cells was significantly higher in HBV-HDV–infected patients than in HBV-infected patients. Additionally, the percentage of $CXCR3^+CD8^+$ natural killer (NK) T cells was slightly increased in the HBV-HDV–infected group, whereas the frequency of $CXCR3^+$ NK and $CD8^+$ T cells was not significantly different between patient groups. Further subtype analysis showed that the $CXCR3^+CD4^+$ T cells were mainly effector memory T cells ($CD4^+CXCR3^+CD45RA^-CCR7^-$) in HDV patients. [The Working Group noted that the findings confirmed previous reports of stronger release of immune related chemokines and cytokines, and these correlated with liver inflammation in HBV-HDV–infected patients when compared with the HBV-monoinfected patients. However, as noted for the previous study, the possible presence of other infections was not taken into account.]

In another report, Diaz et al. (2018) (see also Section 4.2.1) studied a small cohort of male and female patients that included five patients with HDV who underwent liver transplantation for HDV-associated HCC (all men; mean age, 57 years); seven patients with HDV and non-HCC cirrhosis who underwent liver transplantation for end-stage liver disease (four men, three women; mean age, 56 years); 11 patients with HCV-associated HCC who underwent liver transplantation (10 men, one women; mean age, 56.7 years); and 11 patients with HBV-associated HCC (all men; mean age, 60 years). Liver specimens (from tumour and non-tumour areas) and serum samples were collected for gene profiling and the characterization of specific signatures by microarray analysis. Liver specimens were graded according to fibrosis status, and tumour differentiation was also determined. Titres of serological markers, by immunoassay, were used to assess the grade of the infection, and real-time qPCR was used to detect the presence of HBV DNA or HDV RNA in the liver. Tumour and non-tumour liver specimens were characterized by alterations in specific gene pathways. Among the 20 most commonly altered gene pathways identified in specimens obtained from malignant liver tissues in HDV-associated HCC, the authors identified significant downregulation of the hepatic fibrosis and hepatic stellate cell (HSC) activation pathway. In addition, deregulation of the STAT3 pathway (which is involved in normal development as well as in the regulation of cancer metastases) was observed. Significantly altered regulation of six additional pathways (sonic hedgehog, GADD45, DNA damage–induced 14-3-3 σ, cyclins, cell cycle regulation, and cell cycle) involved in DNA repair, chromosome instability, and cell cycle control were also identified. [The Working Group noted that STAT3 can mediate tumour-promoting inflammation and pro-oncogenic inflammatory pathways, including the NF-κB and IL-6 pathways.]

In an observational study, Sahin et al. (2019) retrospectively investigated data on fibrosis markers from 1216 patients with chronic liver disease in 2004–2018, and they also studied 331 patients with HBV and HBV-positive HDV-related cirrhosis. Patients (mostly men and with a mean age of 51.3 ± 9.18 years) were selected on the basis of diagnosis of cirrhosis, and for infection with each virus according to serological antigen titre. Reasons for exclusion from the study were chronic alcohol consumption (14

patients) and diabetes type 1 (16 patients). Of the 331 patients, 136 had HBV-induced cirrhosis without cancer; 92 had HBV-induced cirrhosis with cancer; 80 had HBV-HDV infection–induced cirrhosis without cancer; and 23 had infection-induced cirrhosis with cancer. Levels of serum enzymes ALT and alkaline phosphatase (ALP) were not different between patients with or without cancer. [The Working Group considered that the study was relevant in that it described some end-points related to chronic inflammation involved in the progression to cancer. Although AFP-based marker levels were significantly higher in patients with cancer, the levels of all non-AFP-based markers were higher in patients with cirrhosis. The Working Group also noted that a clear analysis to compare HBV-infected patients and HBV-HDV–infected patients was not performed. Moreover, the authors sought to describe HCC with prediction scores (qualitative), thus limiting the informativeness of the study in relation to the role of HDV infection in progression from fibrosis to cirrhosis.]

Wranke et al. (2018) studied the virological characteristics of 1576 patients with ongoing or past HDV infection from the Hepatitis Delta International Network (HDIN) registry, established in 2011. The authors identified anti-HDV IgM as a marker for hepatitis disease activity and reported a correlation between high anti-HDV IgM levels and more advanced disease activity.

In a retrospective study, Gu et al. (2014) analysed disease outcome in a cohort of 507 patients infected with HBV and tested at the WS Hospital of the Third Military Medical University, in Chongquing, China, between 1998 and 2010. Of these patients, 61 were identified as having a coinfection. The remaining were superinfected and were categorized in other groups. The data for these patients with HBV were compared with those for a group of 213 control patients who were HDV-negative and HBV-positive. The authors compared inflammatory markers in groups of patients with different grades of disease severity. They observed an elevation in levels of ALT in chronically HDV-positive patients compared with chronically HDV-negative, HBV-positive patients. For other parameters (total bilirubin, direct bilirubin, albumin and γ-globulin), significant differences were observed between patients with serious hepatitis D compared with those with serious hepatitis B, and patients with severe hepatitis D compared with those with severe hepatitis B. [The Working Group noted that this study was limited because the study participants were not matched and the presence of other infections was not assessed, thus the data were subject to potential bias from cofounding. In addition, ALT was the only parameter related to inflammation that was altered.]

In an investigation of the efficacy of long-term, high-dose peg-IFNα-2a in patients ($n = 13$) with CHD, Heller et al. (2014) analysed liver histopathology outcomes and observed a decrease in levels of liver damage parameters such as ALT in treated patients. [The Working Group noted that the authors described only predictors of hepatitis outcome, without an appropriate comparison group (patients with HBV). The study was considered to be of limited relevance to the present evaluation.]

Differences in disease severity and cytokine-related genotypes have been investigated in HDV-infected patients carrying six specific polymorphisms compared with patients with CHB. Karatayli et al. (2014) followed 67 patients with CHD (47 men, 20 women; mean age, 36 ± 11 years) and 119 with CHB (85 men, 38 women; mean age, 38 ± 12 years) from the Hepatology Clinic of the Gastroenterology Department of Ankara University Medical School, in Ankara, Türkiye. HDV-infected patients showed significantly higher grades of fibrosis and lower platelet counts than did HBV-infected patients. Expression analysis of polymorphisms for interleukin 10 (IL-10), TNFα, interferon gamma (IFNγ), vitamin D receptor FokI (VDR FokI), and VDR TaqI was

conducted using genomic DNA from peripheral blood leukocytes. The high-secretory IL-10–1082 GG genotype was significantly less frequent in patients with CHD than in patients with CHB and resolved HBV, whereas the high-secretory VDR TaqI TT genotype was significantly more frequent in patients with CHD than in patients with CHB and individuals with resolved HBV, potentially contributing to the more severe liver disease associated with CHD compared with CHB. [The Working Group noted that confounders and bias were not considered, thus limiting the relevance of the study for the present evaluation.]

In a retrospective cross-sectional study conducted in Guandong Province, China, in a cohort of all consecutively hospitalized patients with HDV infection between 2005 and 2011 ($n = 426$), Liao et al. (2014) observed an increase in the incidence of fibrosis (88.6% versus 78.3%; $P = 0.044$; stage F2 and above) and necroinflammation (67.1% and 43.8%; $P < 0.001$; stage A2 and above) in liver biopsies of 79 HBV-HDV–infected versus 240 HBV-monoinfected patients. Patients with CHD also had significantly higher ALT levels than did patients with CHB. When stratified by ALT level (ALT, 1–2 upper limit normal, ULN; 2–5 ULN; and > 5 ULN), 46.7%, 56.5%, and 80.5% of CHD patients had significant necroinflammation and 86.7%, 87.0%, and 90.3% had significant fibrosis, respectively. The frequency of end-stage liver disease was higher among HBV-HDV–infected patients than HBV-monoinfected patients, and HDV infection was found to be an independent risk factor for end-stage liver disease (OR, 1.428; 95% CI, 1.116–1.827; $P = 0.005$).

In a study in 61 patients infected with HBV and HDV genotype 3 (HDV-3), an aggressive genotype of the virus found exclusively in South America, Souza-Campos et al. (2023) identified 17 patients with advanced fibrosis and cirrhosis and 44 with no fibrosis or mild fibrosis. The authors reported a positive association between serum levels of AST and ALT enzymes and the level of necroinflammation in liver tissue from patients infected with HBV and HDV. In addition, levels of several systemic inflammatory molecules were found to be positively associated with fibrosis stage, with high correlations for cytokine C-C motif chemokine ligand CCL19 and chemokine MCP.4 (CCL13). [The Working Group noted that the study was performed without comparison with HBV-monoinfected patients or with HDV-positive patients infected with genotypes other than HDV-3.]

Increased levels of circulating markers of inflammation were also observed in the study by Townsend et al. (2020). The authors investigated increases in the incidence of systemic bacterial infections after HBV monoinfection or HBV-HDV infection in serum from 53 HBV-positive, 43 HDV-positive, and 36 healthy control individuals. They observed that infections were associated with an increase in serum microbial products such as membrane lipopolysaccharides or peptidoglycans; this increase in bacterial products was associated with higher levels of specific cytokines, in patterns that varied between HBV-positive, HDV-positive, and healthy individuals. Poly-peptidoglycan levels measured in HBV-monoinfected and HBV-HDV–infected patients were positively correlated with levels of several general inflammatory, antimicrobial (type 1), and pro-fibrosis/allergic inflammatory (type 2) cytokines. However, in contrast to HBV monoinfection, HBV-HDV infection was positively associated with a higher ratio of systemic type 1 (general inflammatory, antimicrobial) and type 2 (pro-fibrosis, allergy) immune responses.

End-points relevant to the immune response

Townsend et al. (2019) assessed serum cytokines and immune markers in 96 patients (53 with HBV, 43 with HBV-HDV infection) and 33 healthy controls. The aim of the study was to characterize microbial translocation and the associated immune response in patients with chronic HBV or HBV-HDV infection. Individuals

were divided into three groups according to the AST-to-platelet ratio index (APRI): mild (< 0.5), moderate (0.5–1.0), and severe (> 1.0). Both HBV-infected and HBV-HDV–infected patients displayed higher levels of cytokines such as TNFα, IL-12p40, CXCL9, and CXCL10 than did the healthy controls, indicating a type 1 immune response. HBV-infected patients also showed increased levels of IFNγ and IL-12p70. The HBV-infected patients also showed higher levels of IL-4, IL-13, and CCL26, which are cytokines involved in a type 2 immune response, than did healthy controls or patients infected with both HBV and HDV. Levels of chemokines CCL2 and CCL13 were lower in samples from HBV-HDV–infected patients than in those from the other two groups, indicating the absence of a type 2 immune response in HDV infections. Levels of IL-17, a key driver of a type 3 immune response, were elevated in both HBV-infected as well as HBV-HDV–infected patients, compared with controls. [The Working Group noted that the establishment of a chronic inflammation status driven by bacterial translocation could contribute to disease severity and progression in HBV-infected and HBV-HDV–infected patients.]

Jang et al. (2021b) conducted a longitudinal study of a cohort of 1185 HBV-infected patients (mean age, 47.1 years; 73.4% men) of whom 495 were HBeAg-positive and under treatment with NAs and 334 had liver cirrhosis. Seropositivity rates for patients with anti-HDV antibodies and HDV RNA were 2.0% and 0.8%, respectively. The authors reported a strong positive association between HDV seropositivity and liver damage, as measured by ALT levels, particularly in the presence of low HBV seropositivity.

Nicolete et al. (2016) studied 29 patients with HDV divided into three groups: nine untreated patients with detectable HDV RNA; eight anti-HDV–positive patients without detectable HDV RNA; and 12 patients who responded positively to 24 weeks of therapy with peg-IFNα and remained HDV RNA–negative for 6 months after treatment. Serum interleukins IL-2, IL-10, IL-12, IFNγ, and TNFα were measured by ELISA. Patients treated with peg-IFNα (responders) showed significantly higher serum levels of IL-2 ($P = 0.0008$) and IL-12 ($P = 0.02$) when compared with the other groups. These two cytokines were positively correlated with each other ($P = 0.0143$).

Landahl et al. (2019) studied the HDV-specific $CD4^+$ and $CD8^+$ T-cell responses of 32 HDV-infected patients by immunospot analysis and measurement of intracellular IFNγ production. Cells were stimulated in vitro with 21 different 20-mer HDAg peptides. No differences in response were found between RNA-positive and RNA-negative patients. [The Working Group noted the small sample size from the above studies, that limited the informativeness of the results.]

Semenov et al. (2017) examined 232 residents of Yakutia, in the Russian Federation, including 127 patients with various forms of chronic hepatitis and 105 healthy individuals. Relative ratios of mature T lymphocytes ($CD3^+$), T helpers ($CD4^+$), T suppressors ($CD8^+$), B lymphocytes ($CD72^+$), and NK cells ($CD16^+$) were assessed by flow cytometry. In patients with CHD, high activity of the infectious process correlated with increased activity of cytotoxic cells and simultaneous development of a deficiency in mature, functionally active T lymphocytes. In the group of HDV-infected patients with liver cirrhosis, the authors observed a decrease in the number of T-helper and B lymphocytes, but no modification in the concentrations of serum immunoglobulins.

[The Working Group noted shortcomings in this study, including the lack of information about the number of patients with hepatitis B, C, or D; their characteristics; and the type of treatment received.]

Aslan et al. (2006) investigated cytotoxic $CD4^+$ T lymphocytes in 76 patients with viral hepatitis. Thirty-seven out of 76 patients were chronically infected with HBV, and of these 22

were also infected with HDV. Seventeen patients were infected with HCV and 22 with HIV. PBMCs from 24 healthy individuals were used as negative controls. Liver cirrhosis was found in 47% of HDV-infected patients. None of the patients were under antiviral treatment. The highest ratios of $CD4^+$ T lymphocytes were found in HBV-HDV–infected individuals ($P = 0.04$ versus HBV and HCV patients), and the presence of $CD4^+$ T lymphocytes was associated with elevated AST levels ($P = 0.01$) and decreased platelet counts ($P = 0.03$). An expansion of perforin-positive $CD4^+$ T lymphocytes was particularly evident in HBV-HDV–infected patients (46% versus 20%, 29%, and 36% in HBV-infected, HCV-infected, and HIV-infected patients, respectively) and could potentially contribute to liver disease.

Kefalakes et al. (2021) investigated innate and adaptive immune responses in blood and liver samples from 24 patients coinfected with HBV and HDV (who were treated with NAs and were HBV DNA–negative) and 30 non-infected controls. The study was conducted using multiparameter flow cytometry, and the results were correlated with disease severity. Populations of mucosal-associated invariant T (MAIT) cells and NK cells, involved in the innate immune response, were reduced in the livers of HDV-infected patients compared with the non-infected controls. MAIT-cell degranulation, assessed by cell surface expression of CD107a, correlated negatively with HDV viraemia and with the Ishak fibrosis score. The ratio of activated NK cells in the liver correlated negatively with HDV viraemia. Activation and degranulation of MAIT and NK cells were more frequent in the liver than in the blood, and the ratio of degranulating NK cells in the blood correlated negatively with the Ishak fibrosis score. [The Working Group noted that the populations of MAIT and NK cells were more frequently activated in the liver than in the blood.]

Schirdewahn et al. (2017) analysed the immune-cell phenotypes and frequencies in samples from 49 chronically HBV-HDV–infected patients (48 with genotype 1 HDV), 25 HBV-infected patients, and 18 healthy controls. Despite high interindividual variation, total T-lymphocyte subsets ($CD3^+$, $CD4^+$, and $CD8^+$) and the ratio of regulatory T cells ($CD4^+CD25^{high}CD127^{low/-}$) were not significantly different between groups. Significant upregulation of the senescence marker CD57 was observed in $CD8^+$ T cells from HDV-infected patients. In vitro stimulation with HDV-specific peptides elicited reduced T-cell proliferation and decreased production of IFNγ and TNF in both $CD4^+$ and $CD8^+$ T cells from HDV-infected patients. The weak HDV-specific T-cell proliferation and cytokine response could only be partly restored by blocking the programmed death 1 (PD1) pathway in the presence of anti-programmed death ligand 1 (PDL-1). [The Working Group noted that the expression of CD57 was associated with replicative senescence and poor response to proliferative stimuli; the authors also hypothesized that premature ageing of immune cells and impaired T-cell functionality could contribute to the establishment of persistent HDV infection.]

Kefalakes et al. (2019) isolated PBMCs from 28 patients with chronic HDV infection, identified HDV-specific $CD8^+$ T-cell epitopes, and characterized HDV-specific $CD8^+$ T cells. An analysis of clinical parameters of patients and HDV sequence variations was performed.

The ratio of activated ($CD38^+$) HDV-specific $CD8^+$ T cells (measured in PBMCs) positively correlated with AST activity, whereas $CD8^+$ T-cell production of IFNγ, stimulated by HDV peptides, correlated inversely with HDV titre. HDV-specific $CD8^+$ T cells did not express the terminal differentiation marker CD57. HDV-specific $CD8^+$ T cells were found to be activated but not terminally differentiated. About half of the HDV-specific $CD8^+$ T cells

had a memory cell-like PD1^{+}CD127^{+}TCF1^{hi}T-bet^{low} phenotype, which associated having HDV sequence variants with reduced human leukocyte antigen (HLA) binding and reduced T-cell activation. [The Working Group noted that the activated subset of memory-like HDV-specific CD8^{+} T cells was considered functional but unable to clear HDV, owing to the presence of virus escape variants.]

Dias et al. (2019) studied the role of MAIT cells, a T-cell subset that is highly enriched in the liver, during HDV infection.

MAIT cells from 41 patients with chronic HBV-HDV coinfection, 38 patients with chronic HBV infection, and 57 healthy controls were analysed ex vivo and in vitro after stimulation. The ratio of circulating MAIT cells was substantially decreased in the peripheral blood of HBV-HDV–infected patients, with signs of decline that were also observed in the liver. The analysis of residual circulating MAIT cells in patients with chronic HDV infection revealed overexpression of several activation markers, along with functionally impaired responsiveness and reduced expression of the degranulation markers CD107a and granzyme B (GrzB). In contrast, circulating MAIT cells from patients with chronic HBV infection displayed only a slight decrease. HDV-infected patients exhibited monocyte activation and increased levels of pro-inflammatory cytokines IL-12 and IL-18, which could promote MAIT-cell death. It was suggested that IL-12 and IL-18 may contribute to MAIT-cell activation and subsequent loss during HDV infection. [The Working Group considered that the design of the study was of good quality; however, it was noted that the patients, both HBV-infected and HBV-HDV–infected, had received different antiviral treatments. Levels of MAIT cells sampled from HBV-HDV–infected patients did not differ between patients treated with peg-IFNα and nontreated patients, but a slight increase was observed in patients treated with NAs. In addition, eight out of 38 HBV-infected patients (~20%) had received treatment at the time of cell sampling. This could potentially have affected the study results.]

Lunemann et al. (2015) investigated the immune response in 31 treatment-naive patients with chronic HBV-HDV infection, 15 patients with chronic HBV infection, and 49 healthy controls. An analysis of NK cells (CD56^{+} and CD16^{+}) was performed before and during treatment with IFNα (peg-IFNα, 180 μg/week), and the results were compared with those for patients with HBV infection and for healthy controls. The ratio of total NK cells was higher in untreated patients with chronic hepatitis D (HBV-HDV–infected) than in healthy controls (13.8% versus 9.8%). Additionally, the ratio of CD56^{bright} NK cells in HBV-HDV–infected patients was higher by more than twofold than in healthy controls. Although patients with CHD (HBV-HDV–infected) and healthy controls showed a similar capacity to degranulate (CD107a upregulation) and produce IFNγ, TNFα, and MIP-1, this response was generally lower in patients with HDV. [The Working Group noted that the finding indicated that hepatitis D, if left untreated, resulted in the accumulation of CD56^{bright} cells, with a reduced capacity to respond to IFNα.]

The same study also investigated the effect of peg-IFNα treatment in 16 previously untreated patients with HDV. After 12 weeks of treatment, the number of total lymphocytes and NK cells dropped to almost half of the initial number, and a fivefold reduction was observed in the absolute numbers of CD57^{+}CD56^{dim} NK cells. In addition, the NK cell response was impaired, with significantly lower levels of degranulation (CD107a) and production of MIP-1β and TNFα. In HBV-HDV–infected patients, 12 weeks of treatment with peg-IFNα resulted in selective loss of terminally differentiated NK cells and enrichment of immature NK cells. The treatment was also linked to a switch in intracellular signalling from STAT4 to STAT1 in NK cells. Response to peg-IFNα treatment in HBV-HDV–infected

patients was positively correlated with higher initial levels of NK cells; and these patients presented high numbers of NK cells throughout treatment, compared with non-responders.

(ii) Human cells in vitro

[The Working Group reviewed some in vitro studies that suggested that HDV infection could induce an acute IFN response through the melanoma differentiation antigen 5 (MDA5) pathway.]

Gillich et al. (2023) investigated how retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (including RIG-I; MDA5; and laboratory of genetics and physiology 2, LGP2) sense viral HDV RNA and induce an antiviral IFN response. Using HDV-susceptible HepaRG^{NTCP} cells and PHHs, the authors depleted individual RLRs and reconstituted cells with different LGP2 variants, then quantified HDV replication and IFN induction over time. The results showed that both MDA5 and LGP2, but not RIG-1, are required for IFN activation and HDV suppression, since depletion of either LGP2 or MDA5 reduced the HDV-induced IFN response. Because the IFN response had previously been shown to suppress the cell division–mediated spread of HDV, the authors further tested the role of LGP2 and MDA5 in this process. In cells with intact IFN activation, the number of HDV-positive cells declined dramatically after cell division; the reduction was significantly alleviated in knockout (KO) LGP2^{KO} cells and MDA5^{KO} cells, demonstrating that the LGP2-induced IFN response restricts cell division–mediated HDV spread. [The Working Group noted that an LGP2 variant (Q425R), which is predominant in Africans who develop less-severe chronic hepatitis D, mediated a higher basal and more rapid HDV-induced IFN response, as well as stronger HDV suppression, compared with wildtype LGP2 (Gillich et al., 2023).]

In another study, Zhang et al. (2018) studied the effect of HDV and HBV infection on a broad range of interferon-stimulated genes (ISGs). The authors showed that, after 9 days of infection, only HDV, but not HBV, activated ISGs in the hepatoma cell line HepG2^{NTCP}. To investigate the pattern of ISG induction, PHHs were infected with HDV, and IFNα, β, λ1, and λ2/3 mRNAs were quantified using real-time qPCR on days 1, 3, 5, and 7 post-infection. The mRNA levels of all the IFNs except IFNα increased from day 2, peaking at day 5 to day 7. Similar results were obtained in HepG2^{NTCP} and HepaRG^{NTCP} cell lines.

However, at later time points, IFN and ISG mRNA levels decreased significantly in both cell lines. Despite this decrease, the intracellular HDV RNA level remained unchanged until day 23 in HepG2^{NTCP} cells, whereas it declined significantly in HepaRG^{NTCP} cells. [The Working Group noted that, although HDV RNA triggered an IFN response, this response declined at later time points (Zhang et al., 2018).]

The authors also investigated the production of IFN after HDV infection in two hepatic cell lines: (i) Huh7^{NTCP} cells, which have defective IFN production but intact downstream IFN signalling that can be activated upon treatment with exogenous IFN; and (ii) HepaRG^{NTCP} cells, which are competent for IFN production and signalling. Quantification of viral replication was achieved by counting HDAg-positive cells, and measuring intracellular HDV RNA at day 5 post-infection and day 5 post-passage. HDV-positive cells and intracellular HDV RNA levels slightly decreased (< 33%) after each cell passage in Huh7^{NTCP} cells but declined significantly (95.3%) after the first cell passage of HepaRG^{NTCP}. This suggested that cell division–mediated HDV spread is highly efficient in IFN production–deficient Huh7^{NTCP} cells, but impaired in IFN-competent HepaRG^{NTCP} cells. Consistent with these results, HepaRG^{NTCP} cells, but not Huh7^{NTCP} cells, showed increased levels of IFN (intracellular mRNAs of IFNβ, IFNλ1, and radical *S*-adenosyl methionine domain containing 2 (RSAD2), which is an ISG) at day 5 post-HDV infection. In addition,

blocking the HDV-induced IFN response with the JAK1/2 inhibitor ruxolitinib, or by knocking down MDA5, prevented activation of IFNs and ISGs and restored HDV spread in dividing HepaRG^{NTCP} cells (Zhang et al., 2022).

Alfaiate et al. (2016) measured inflammatory markers in differentiated HepaRG (dHepaRG) cells that were either previously infected with HBV or left uninfected, and then infected the cells with HDV 6 days later to simulate an HDV superinfection (i.e. HDV infection of cells already infected with HBV). Both HDV-infected and -superinfected dHepaRG cells supported strong intracellular replication of HDV. Whereas HBV alone did not induce any innate gene expression, both HDV infection and superinfection strongly activated a type I IFN response with the induction of ISGs such as RSAD2 and IFI78. HDV infection did not induce NF-κB–responsive genes such as IL-6, IL-8, and IL-1β, as measured by real-time qPCR. HDV infection induced a strong IFN response at the peak of RNA replication (peaking on day 6 after infection and declining by day 21), independently of HBV replication. Treatment with IFNα resulted in a significant reduction in the replication of both HBV and HDV.

Lucifora et al. (2023) analysed the effect of HDV on HBV and various experimental models, including PHHs, HepaRG cells, liver-humanized mouse models (HuHep mice), and liver specimens from patients. In this context, a transcriptomics analysis showed overexpression of ISGs in liver specimens from HBV-HDV–infected patients, in HDV- and or HBV-infected HepaRG cells, in PHHs from three different donors, as well as in liver samples from infected HuHep mice.

A subset of 73 ISGs was found to be significantly upregulated in PHHs, in HepaRG cells, in liver-humanized mice infected with both HBV and HDV, and in liver samples of HBV-HDV–infected patients, when compared with cells expressing only HBV antigens. [The Working Group noted that the report of an HDV-specific gene expression signature consisting of ISGs supports a strong role of an innate immune response to HDV infection, a feature not observed in HBV-infected patients.]

More recently, Bockmann et al. (2025) reported overexpression of chemokines CXCL9–11 in cultured HDV-infected PHHs, confirming the role of hepatocytes in producing chemokines and triggering liver inflammation after infection with HDV, and confirming results in patients and mice.

Other data showed significant reduction of HDV RNA levels, by up to 50%, after stimulation of dHepaRG cells, or PHHs treated with IL-1β for 24 hours (Delphin et al., 2021). In addition, no antiviral effects were observed in cells infected with HBV and treated with lamivudine, or in cells infected with HDV and treated with IFNα in a short treatment time (up to 72 hours). [The Working Group noted that it was not clearly described whether HDV replication was suppressed by IL-1β, which could represent the factor that mediates the immune response elicited by the virus.]

One study found higher levels of chitinase 3–like 1 (CHI3L1), a profibrogenic factor found to be overexpressed in the ageing liver, and in the livers of patients with HDV-related cirrhosis compared with those whose cirrhosis was caused by other etiological agents (such as HBV, HCV, or alcohol). In vitro studies in PHHs showed that recombinant CHI3L1 promotes the proliferation and activation of primary human HSCs, the major drivers of liver fibrosis (Nishimura et al., 2021) (see also Section 4.2.4(a)(i)).

[The Working Group noted that the available studies indicated that, in contrast to HBV, HDV induces a strong acute IFN response through the MDA5 pathway, which, however, does not persist for more than several days after infection. This cellular response, a type of innate immune response, efficiently suppresses cell division–mediated HDV spread and also, to some extent,

the early stages of HDV de novo infection, but only marginally impairs RNA replication in resting hepatocytes (Zhang and Urban, 2020).]

Other studies investigated the effects of expression of the large (L-) or small (S-) HDAg to elucidate the molecular mechanisms of HDV pathogenesis (Park et al., 2009; Pugnale et al., 2009).

Overexpression of L-HDAg increased TNFα-induced NF-κB transcriptional activation in Huh7 cells, whereas overexpression of S-HDAg did not. In addition, L-HDAg promoted TRAF2-induced NF-κB signal transduction and significantly increased COX-2 protein levels in Huh7 cells, contributing to HDV pathogenesis (Park et al., 2009).

Another study conducted in human hepatoma cells transfected with the complete HDV genome showed that some ISGs were downregulated in response to treatment with IFNα, and phosphorylation of both STAT1 and STAT2 was inhibited (Pugnale et al., 2009).

[The Working Group noted that the above-mentioned studies used cell lines transfected with specific plasmids to express HDV antigens but that the cells were not directly exposed to HDV.]

Choi et al. (2007) investigated the effects of L-HDAg and S-HDAg on signalling induced by TGFβ and c-Jun in Huh7 cells and Cos7 cells (African green monkey kidney fibroblast-like cells). Cells were transiently co-transfected with an HDAg (either L- or S-) expression plasmid and a TGFβ–luciferase reporter plasmid. Overexpression of L-HDAg, but not S-HDAg, caused an increase of 10–13-fold in TGFβ-induced transcriptional activation compared with the vector control in Huh7 cells. In addition, the authors observed downstream activation of Smad DNA binding by L-HDAg and reported evidence for a direct interaction between HDAg and the protein SMAD family member 3 (Smad3). [The Working Group noted that Smad3 is a key mediator of fibrosis; it can modulate the growth arrest induced by TGFβ by controlling the expression of c-myc and p21. In addition, it has been reported that L-HDAg can activate c-Jun–dependent AP-1 signalling, potentially influencing TGFβ activity by acting on the c-Jun–Smad3 interaction. The Working Group further noted that the signalling cascade induced by L-HDAg could contribute to the process of fibrosis and to cirrhosis.]

End-points relevant to the immune response

Using two different HBV- and HDV-permissive hepatoma cell lines – NTCP-expressing HepG2 ($HepG2^{NTCP}$) and RLR-expressing Huh7.5 ($Huh7.5^{RLR}$) cells – Altstetter et al. (2021) studied intracellular innate immunity to HDV infection and coinfection with HBV. The authors found that HDV is initially detected by the pattern recognition receptor MDA5 and, after a 7-day lag-phase, a strong type I IFN response is induced, and overexpression of ISGs (namely OAS1, CXCL10, and IFIT1) is observed. HDV infection and replication enhanced T-cell cytotoxicity and increased the sensitivity of infected cells to T-cell effector functions.

The protein adenosine deaminase acting on RNA 1 (ADAR1) is thought to be part of the innate cellular immune system. Two different forms of ADAR1 are expressed: IFNα-induced ADAR1-L and constitutively expressed ADAR1-S. Hartwig et al. (2006) investigated the activity of ADAR1-L and ADAR1-S in Huh7 cells stimulated with IFNα (1000 U/mL IFNα, every 48 hours for 7 days), and in unstimulated Huh7 cells (controls) that had been transfected with a plasmid encoding full-length replicating HDV RNA (pSVL-D3). An increase of almost twofold in RNA editing driven by ADAR1-L (assessed by real-time qPCR and followed by restriction digestion of the product) was observed in IFNα-stimulated Huh7 cells.

The innate immune response to HDV was investigated by Lange et al. (2023) in hepatocyte-like cells (HLCs) derived from human pluripotent stem cells (hPSCs). Infection of

HLCs with HDV, produced in Huh7^{NTCP} cells, resulted in induction of the IFNβ and IFNλ responses, overexpression of ISGs, and activation of the NF-κB pathway. The levels of IFNs and the ISG induction correlated with the levels of intracellular HDV RNA. Treating cells with the JAK/STAT pathway inhibitor ruxolitinib and the NF-κB pathway inhibitor ML120B showed that the innate immune response was dependent on JAK/STAT and NF-κB signalling cascades and did not inhibit HDV replication. However, pretreatment of HLCs with IFNα2b could reduce virus replication levels (~1 log), as determined by real-time qPCR.

HDV-infected human HepG2, Huh-7, and HepaRG cells overexpressing human NTCP (hNTCP) were co-cultured in vitro with human NK cells (Groth et al., 2023). The HDV-infected cells activated the NK cells, as assessed by expression of the marker CD69. Co-culture with HDV-infected HepG2^{NTCP} cells resulted in overexpression of programmed cell death protein PD-1, which is known to be upregulated by activated or exhausted CD8$^+$ T cells during viral infection, along with increased levels of IFNγ and the degranulation marker CD107a in NK cells. Additionally, upregulation of ISGs (including ADAR, ZCCHC2, SAMD9/SAMD9L, IFIT5, RIG1, MX2, ISG20, and HERC6, all known to be involved in the innate antiviral response) was observed in NK cells co-cultured with HDV-infected HepG2^{NTCP} cells. The increased production and release of IFNβ and IFNγ by infected HepG2^{NTCP} cells were shown to enhance NK cell activity and to mediate the elimination of HDV-infected cells via the TRAIL/TRAIL-R2 axis. The analysis of peripheral blood from HBV-infected and HBV-HDV–infected patients showed a decrease in the ratio of NK cells, and an increase in the activation marker CD69$^+$ phenotype in HBV-HDV–infected patients, but not in patients with HBV infection.

(b) Experimental systems

Suárez-Amarán et al. (2017) investigated liver pathology, genome editing, and activation of the innate immune response in male C57BL/6 mice (age, 8 weeks) infected with adeno-associated vectors (AAVs) carrying AAV-HDV and AAV-Luc (AAV-HDV); AAV-HBV and AAV-Luc (AAV-HBV); AAV-HDV and AAV-HBV (AAV-HBV-HDV); or AAV-Luc (×2) that was used as a control. The presence of the virus was tested on days 7, 14, 21, and 45 after AAV injection, and viral genomes were quantified using real-time qPCR and qPCR. The infectivity of serum from coinfected mice was assessed in vitro in HepG2^{NTCP} cells up to day 21. The authors observed portal and lobular inflammation, and cytoplasmic swelling, accompanied by mitosis; these were more severe after coinfection. In line with the histology data, increased expression of genes involved in the induction of liver damage, necrosis and cell death, inflammation, proliferation, fibrosis, and cirrhosis was detected by microarray analysis of liver samples from the infected mice; significance was higher in AAV-HDV-HBV–infected than in AAV-HDV– or AAV-HBV–monoinfected mice. In addition, higher levels of IFNβ gene expression and of ISGs (Mx1, ISG15, 2′-5′OAS, PKR, IP-10, and ADAR1) were observed in AAV-HDV–infected and AAV-HBV-HDV–infected groups than in AAV-HBV–monoinfected mice.

In a follow-up study (also described below), Usai et al. (2020) infected C57BL/6 mice with the same AAVs. In HBV-HDV–infected mice, they observed hepatocyte necrosis and apoptosis accompanied by elevated serum ALT levels. Transcriptomic analyses revealed strong activation of type I and II IFN pathways and TNFα signalling. In addition, the authors reported infiltration of activated CD4$^+$ and CD8$^+$ T cells, NK cells, and inflammatory macrophages. However, depletion studies and experiments in knockout mice demonstrated that T cells, NK

cells, and macrophages were not required for HDV-induced liver damage

In addition, the same group (Maestro et al., 2021) more recently described the role of HDAg expression during acute infection and its effects on the liver, both in the HDV mouse model (in C57BL/6 mice) and in vitro in human hepatoma cell lines. By constructing different mutants, they were able to block the expression of either S-HDAg (HDV-ΔS-HDAg), L-HDAg (HDV-ΔL-HDAg), or both (HDV-ΔHDAg). They reported that the expression of S-HDAg in the absence of L-HDAg resulted in increased liver damage, as shown by elevated serum ALT and AST enzyme levels, increased cell nucleus size, and number of cells positive for caspase 3, indicating increased apoptosis. The absence of L-HDAg also resulted in an increase in macrophages ($F4/80^+$ cells) and both $CD8^+$ and $CD4^+$ T cells in the mouse liver. Additionally, expression levels of the cytokines IL-1β, IFNγ, IL-6, TNFα, and TGFβ were higher in HDV-ΔL-HDAg– than in HDV-infected wild-type mice at 14 and 21 days post-infection, in line with the observed liver damage; however, only TNFα expression was significantly higher.

End-points relevant to immune response

A chimeric liver-humanized mouse model (uPA/SCID), lacking B and T cells, was used to study the IFN response to HDV infection. Experiments were performed in mice coinfected with HDV and HBV (genotypes 1 and C, respectively), and in mice that had been infected with HBV 10 weeks earlier and then superinfected with HDV (Duehren et al., 2024). After infection, mice were treated with IFNα or IFNλ (30 μg/kg, twice weekly for 12 weeks). A stronger inhibitory effect on HBV and HDV replication was observed in mice treated with IFNα than with IFNλ.

He et al. (2015) established a transgenic mouse model expressing human NTCP (hNTCP-Tg). Mice were infected with 1×10^{10} GEq of HDV at day 9 after birth and killed on day 2, 6, 10, 14, or 18 post-infection. The levels of intrahepatic HDV RNA peaked at day 6 post-infection and then declined by 3 logs in the next 12 days. RNA sequencing analyses of liver samples revealed the upregulation of several genes involved in the type I IFN response, as well as several other putative genes that could be involved in the modulation of HDV infection. It was concluded that the resolution of HDV infection may not be dependent on the adaptive immune response and might be triggered by innate immunity.

Winer et al. (2018) created an immunocompetent transgenic mouse model expressing human NTCP (hNTCP BACTg). HBV-HDV infection was shown to induce innate immune responses and overexpression of ISGs (including PKR, OAS-L, IP-10, and Mx1) when compared with HBV infection. Increased ratios of NK T cells and MAIT cells were also observed in HBV-HDV–infected mice.

Giersch et al. (2015) infected humanized uPA/SCID/beige (USB) mice with HBV, or with both HDV and HBV, and investigated the antiviral immune response. In human hepatocytes transfected into the USB mice, HBV-HDV infection was associated with a more robust antiviral state, including enhanced expression of human-specific cytokines hIP10, hTGFβ, hIFNβ, and hIFNλ, which remained lower or below the limits of detection in both non-infected, and HBV-infected mice.

Usai et al. (2020) delivered HDV and HBV replication-competent genomes and HDV antigens to mouse liver tissue in vivo, using AAVs. Histological observations showed hepatocyte necrosis and apoptosis in liver tissues infected with both HBV and HDV, which were associated with elevated serum levels of TNFα. However, the use of *Rag1*-KO mice lacking T, B, MAIT, and NK cells, macrophage depletion in C57BL/6 WT mice via intravenous administration of clodronate-loaded liposomes, and additional NK depletion through intraperitoneal injection of an anti-mouse NK1.1 antibody in *Rag1*-KO mice, indicated that T cells, NK cells, and macrophages

did not account for the observed HDV-induced liver damage, because aminotransferase levels remained stable in these mice until day 21 post-injection. Previous transcriptomic analysis of liver cells showed activation of type I and II IFN and TNFα pathways in HBV-HDV–infected mice (Suárez-Amarán et al., 2017). The authors further investigated the relevance of other cytokines, such as TNFα. Treatment with the TNFα antagonist etanercept (9 mg/kg) resulted in a significant reduction in Casp3-positive apoptotic cells and HDV-associated liver injury. On the other hand, injection of AAV-S-HDAg or AAV-L-HDAg into *Rag1*-KO mice and C57BL/6 WT mice resulted in an increase in ALT levels, suggesting that HDAgs may play an important role in HDV-induced liver damage. Indeed, Casp3 expression was observed by immunohistochemistry in liver infected with AAV-S-HDAg or AAV-L-HDAg on days 14 and 7, respectively, coinciding with the peak level of aminotransferase. Liver damage induced by S-HDAg and L-HDAg was found to be TNFα-independent, because concomitant treatment with etanercept did not affect liver damage. It was concluded that both TNFα and HDAgs play a relevant role in HDV-induced liver damage. [The Working Group noted that the results of studies in wildtype, transgenic, or liver-humanized mice did not support suppression of the immune system by HDV.]

Synopsis

[Overall, the Working Group noted that there was evidence from several studies in exposed humans that HDV infection is associated with chronic inflammation, with increases in several pro-inflammatory cytokines, and more rapid progression to cirrhosis when compared with HBV monoinfection. A similar pattern of cytokine secretion was observed in PBMC culture supernatants from infected patients. Findings were similar in retrospective and observational studies and cohorts of distinct size. In addition, a higher ratio of activated MAIT cells was found in the intrahepatic immune-cell population. Findings in human primary hepatocytes and susceptible liver cell lines infected with both HBV and HDV or with HBV alone were consistent with those in exposed humans. HDV infection was consistently associated with inflammation and induction of the innate immune response. Activation of the IFN response was reported to involve the MDA5 pathway in HDV-infected HepaRG^{NTCP} and PHHs. The Working Group noted that studies in exposed humans of end-points relevant to the immune response were still scarce and generally limited by small sample size. However, the few findings were consistent with HDV triggering a strong innate immune response associated with production of IFN and other pro-inflammatory cytokines. The observed persistence of the response, associated with necroinflammation, supported the initiating of more rapid progression to cirrhosis. Overall, the available studies addressing the immunological response in exposed humans, in human primary cells, and in experimental systems supported evidence for a role for HDV in the induction of chronic inflammation and of the innate immune response.]

4.2.5 Causes immortalization

Humans

Exposed humans

Expression of c-myc, c-myb, and c-fos proto-oncogenes was analysed by immunohistochemistry in liver biopsy specimens collected from HBsAg-positive carriers, including eight patients with CHD; six of these had detectable HDAg in both liver and serum, and two had detectable HDAg only in serum. Thirty-two liver biopsy specimens from patients with liver disorders unrelated to HDV and without evidence of HDV infection served as controls (Tappero et al., 1994). Expression of c-myc was detected in the

nuclei of cells from CHD patients who simultaneously expressed HDAg. Furthermore, c-myc could not be detected in cells from any control patients except one, or in cells from patients with acute HDV infection. Expression of c-fos and c-myb was not detected in liver tissues from CHD patients. Additionally, nuclear accumulation of c-myc was observed after transfection of HBsAg-positive (PCL/PRF/5) and HBsAg-negative (HepG2) human liver cell lines with a plasmid containing a trimer of full-length HDV cDNA.

The specificity of the antibody to c-myc was confirmed against several controls in order to rule out cross-reactivity with HDAg. Despite this, the antibody failed to detect c-myc by immunoblotting in HDAg-positive liver homogenates, although several nonspecific bands were observed. It remained possible that the monoclonal antibody used recognized unrelated nuclear epitopes. [In addition to the small number of study participants and samples, the Working Group noted that the anti–c-myc monoclonal antibody (mAb) used in this study could not detect c-myc protein by immunoblotting (RIA) of HDAg-positive liver homogenates, even though several other nonspecific bands were detected. It is possible that the mAb used in these experiments recognized other epitopes in the nucleus besides those of c-myc.]

Cavallone et al. (2023) used next-generation sequencing to investigate the miRNA profile in whole serum from 75 individuals: 25 with CHB, 25 with CHD, and 25 HBeAg-negative and HBV-infected (ENI). Thirty-nine miRNAs were found to be upregulated in individual with CHB versus ENI and 31 of them also in CHD versus ENI. The miRNA profile of patients with CHB or CHD was similar, with the single exception of miR-200a-3p, which was overexpressed in patients with CHB compared with patients with CHD.

Previously, miR-200a-3p was found to be downregulated in HBV-associated HCC, and its expression was negatively correlated with that of HBV-X protein (Shi et al., 2017; Li et al., 2022). Targets of miR-200a-3p include known tumour suppressor genes, including PCDH9, a member of the protocadherin family (Lv et al., 2017; Shi et al., 2019); A20 (Shi et al., 2021; Wan et al., 2021); and CRMP1 (collapsin response mediator protein 1) (Zang et al., 2016; Cai et al., 2017).

Lu et al. (2011) synthesized a pseudo-knotted HDV ribozyme (RZ57) targeting the RNA component of human telomerase (hTR). Transfection into liver carcinoma (7402) and colon cancer (HCT116) cells led to an ~90% reduction in telomerase activity, accompanied by growth arrest and spontaneous apoptosis.

Synopsis

[Overall, the Working Group noted that few studies were available on end-points relevant to immortalization. A limited number of samples were considered in the studies. In some studies, the miRNA profile was similar in serum from patients with CHB or CHD.]

4.2.6 Alters cell proliferation, cell death, or nutrient supply

(a) Humans

(i) Exposed humans

Yu et al. (2021) screened for genes and pathways that were potentially involved in HDV-induced carcinogenesis using three microarray data sets: (i) GSE55092, containing 39 cancer specimens and 81 para-cancerous specimens (collected at less than 2 cm away from the edge of a tumour) from 11 patients with HBV-associated HCC; (ii) GSE98383, containing 11 cancer specimens and 24 para-cancerous specimens from five patients with HDV-associated HCC); and (iii) RNA sequencing data from 371 patients with HCC for whom clinical data were available from

the Cancer Genome Atlas (TCGA). Using Gene Ontology (GO) analysis, weighted gene co-expression network analysis, and protein–protein interaction (PPI) network construction, the study identified seven key genes (*CDCA5*, *CENPH*, *MCM7*, *CDC6*, *CDC45*, *CDCA8*, and *MCM4*) associated with HDV-related HCC. These seven differentially expressed genes (DEGs) with potential involvement in HDV carcinogenesis included: (a) three already reported in previous studies: *CDCA5* (involved in double-strand break repair and in stabilizing the cohesin complex associated with chromatin during mitosis), *CENPH* (essential for kinetochore formation and sister chromatid separation), and *MCM7* (involved in initiation of DNA replication in eukaryotes); and (b) four new genes: *CDC6*, *CDC45*, *MCM4* (all involved in the initiation of DNA replication in eukaryotes), and *CDCA8*, which is required for microtubule stabilization and spindle formation during mitosis. In a univariate and multivariate Cox proportional hazards model analysis in the same cohort of patients, HCC stage and *CDCA8* gene expression were identified as independent factors associated with overall survival of patients with HDV-associated HCC. [The Working Group noted the small number of HDV patients ($n = 5$) included in the GSE98383 microarray data set, and that the comparison was with liver from cancer patients. Among the seven overexpressed DEGs identified in the HDV microarray data set, five are known to be involved in the initiation of DNA replication. However, it was difficult to interpret how HDV could trigger alterations to DNA replication and/or cell cycle regulation.]

In a preliminary study, Nishimura et al. (2021) performed a transcriptomic analysis of liver tissues from 54 adult organ donors without evidence of fibrosis. They were divided into two age groups: 18 individuals aged between 18 and 40 years, and 36 individuals older than 40 years. The study identified 13 age-dependent DEGs, six being downregulated (*SSBP2*, encoding single-stranded DNA-binding protein 2; *KCNJ3*, encoding G protein–activated inward rectifier potassium channel 1; *RBMS1*, encoding RNA-binding motif, single-stranded interacting protein 1; *ZNF518B*, encoding zinc finger protein 518B; *SERPINI1*, encoding neuroserpin; and AIF1L, encoding allograft inflammatory factor 1-like), and seven being upregulated (three tumour suppressors: *ZIC1*, encoding zinc finger protein ZIC 1; *LGALS4*, encoding galectin 4; *CSTA*, encoding peptide transporter carbon starvation (CstA); and other proteins: CHI3L1, encoding chitinase–3–like protein 1; TBX15, encoding T-box transcription factor 15; and KDSR, encoding 3-ketodihydrosphingosine reductase) in older donors. Among these genes, *CHI3L1* showed the greatest age-dependent increase in expression (3.8-fold) in older liver donors. Subsequently, Nishimura et al. (2021) investigated the expression of *CHI3L1* mRNA in liver specimens from 64 patients with cirrhosis of various etiology. In cirrhotic tissues, the highest expression of *CHI3L1* was observed patients with HDV infection, followed by HCV-, HBV-, and lastly alcohol-induced cirrhosis. In addition, *CHI3L1* was shown to be expressed in PHHs and human HSCs, but not in HepG2 or Huh7 cells. In vitro stimulation of primary HSCs with recombinant human *CHI3L1* resulted in an increased number of Ki-67–positive cells compared with unstimulated HSCs. This suggested that CHI3L1 expression may promote HSC proliferation and increase the susceptibility of ageing liver and cirrhotic tissue from HDV-infected individuals to fibrosis progression. [The Working Group noted that CHI3L1 is involved in inflammation and tissue remodelling. In addition, the protein is linked to cell migration and proliferation in cells from various organs and species.]

Diaz et al. (2018) used an integrated genomic approach to study serum and liver specimens obtained at the time of liver transplantation from patients with HDV-associated HCC ($n = 5$), non-HCC HDV-associated cirrhosis ($n = 7$), HCV-associated HCC who underwent liver

transplantation ($n = 11$), or HBV-associated HCC ($n = 11$). Tissue samples were obtained from the tumour and the corresponding adjacent nontumorous tissue. The serological HBV profile, activity grade, and stage of fibrosis of the adjacent nontumorous tissue were similar between the groups of HDV-associated HCC and non-HCC HDV-associated cirrhosis patients. Transcriptomic analysis was performed on whole liver tissues, on malignant hepatocytes (MH) and non-malignant hepatocytes from laser capture–microdissected tissue (LCM) using microarray methodology for gene expression profiling.

In tumour samples from whole liver tissue and LCM, a small number of genes were found to be differentially expressed, and the corresponding fold changes were closely correlated. The gene expression profile of adjacent nontumorous areas was similar to that of the liver of patients with HDV-associated cirrhosis without HCC. HDV-infected malignant hepatocytes (HDV-MH) were characterized by an enrichment of upregulated transcripts associated with six pathways involved in cell cycle/DNA replication, damage, and repair (sonic hedgehog; GADD45; DNA damage–induced 14-3-3 σ cyclins; cell cycle regulation; cell cycle: G2/M DNA-damage checkpoint regulation; and hereditary breast cancer). Of the six pathways that were identified in HDV-MH, none was found in HBV-MH. However, two of the six pathways, GADD45 and cell cycle, were also found in HCV-MH. The molecular pathways of HBV-MH were primarily associated with metabolic processes, retinoic acid receptor, cell remodelling, and motility functions. Furthermore, a large cluster of 37 genes associated with cell proliferation and DNA damage and repair, including *BRCA1*, *BARD1*, *HMMR*, and *MSH2*, was found to be coregulated (Diaz et al., 2018). [The Working Group noted that different molecular signatures for HDV-associated and HBV-associated HCC were described, suggesting the involvement of distinct molecular mechanisms in carcinogenesis related to HDV and HBV.]

Using the GSE107170 data set, Zhang et al. (2021) compared DEGs for HDV-associated HCC and cirrhosis, and for HBV-associated (HBV-HCC) and HDV-associated HCC (HDV-HCC). Among the DEGs analysed for HBV-HCC and HDV-HCC, a total of 364 upregulated and 214 downregulated DEGs were identified; 93 of the upregulated DEGs and 36 genes of the downregulated DEGs overlapped with those in the analysis for HDV-associated HCC and cirrhosis. The authors used the DEGs database previously published and updated by Diaz et al. (2018). It included 12 liver tumour specimens from five patients with HDV-HCC (aged 57.2 ± 3 years) and 29 cirrhotic liver specimens from seven patients with HDV non-HCC cirrhosis (aged 55.8 ± 1 years). GO analyses showed that the upregulated DEGs were significantly enriched for muscle tissue development, organophosphate catabolic process, nucleotide catabolic process, and negative regulation of target of rapamycin (TOR) signalling, as well as protein heterodimerization activity, ubiquitin–protein ligase binding, GTPase activity, and core promoter sequence-specific DNA binding. The downregulated DEGs were significantly enriched for nuclear receptor activity, steroid hormone receptor activity, β-catenin binding, regulation of lipid metabolic process, response to a steroid hormone, biological events about DNA transcription, and canonical Wnt signalling pathway. [The Working Group noted that the data set used for the analysis was obtained from a small number of samples.]

Expression of proliferating cell nuclear antigen (PCNA) and HDV RNA in neoplastic and non-neoplastic liver tissues collected from 19 patients with CHD was investigated by Negro et al. (1997). Among them were four cases of cirrhosis with superimposed HCC. Liver tissues from 16 patients with CHB and three patients with normal liver tissue were used as controls

to assess cell proliferation. The PCNA labelling index (PLI), defined as the number of PCNA-positive hepatocyte nuclei divided by the total number of hepatocytes, was calculated and found to be comparable in both CHD and CHB tissues. However, the PLI in CHD and CHB tissue samples was significantly higher than that observed in normal liver tissues. Additionally, the hepatocyte proliferation rate did not correlate with the level of HDV replication as determined by in situ hybridization.

(ii) Human cells in vitro

There were two studies with the aim of investigating the epithelial–mesenchymal transition (EMT) in the context of in vitro replication of HDV cDNA clones obtained from infected patients (Shih et al., 2012; Juang et al., 2024).

EMT is associated with enhanced cell migratory capacity, invasiveness, resistance to apoptosis, and production of extracellular matrix components (Kalluri and Neilson, 2003).

Shih et al. (2012) used Huh7 cells transfected with full-length HDV cDNA to study the replication capacity of HDV and its ability to induce EMT. The cDNAs were obtained from the serum of seven HDV-infected patients (genotypes 1, 2, and 4). Four cDNA clones originated from patients who went into remission after initial ALT elevation, and three cDNA clones were obtained from individuals who progressed to CHD, cirrhosis, or HCC. Samples were collected at two stages of infection: before and after ALT elevation. Replication capacity was assessed by quantification of HDV RNA by qPCR and PCR cloning and HDAg expression by western blot. The capacity to induce EMT was determined by levels of vimentin and E-cadherin protein expression, and of two known EMT regulators, twist and snail.

Low replication capacity and low EMT activity of cDNA clones were associated with disease remission. In contrast, high replication efficiency of cDNA clones showed a tendency to induce more robust EMT activity (increased expression of vimentin, twist, and snail; and lower expression of E-cadherin). In addition, transfection of Huh7 cells with a plasmid encoding L-HDAg resulted in increased levels of expression of intracellular vimentin, twist, and snail, and this was not observed in cells transfected with a plasmid encoding S-HDAg. [The Working Group noted that experiments aimed at determining if a dose-dependent response of EMT activity was induced by L-HDAg were performed in cells transfected with different amounts (from 15 to 30 µg) of the plasmid. However, the efficiency of transfection was also dependent on the amount of DNA applied, and doubling of the amount of DNA in the vial did not necessarily mean that twice as much DNA would be delivered into cells.]

Juang et al. (2024) analysed an HDV genotype 2 (HDV-2) cDNA clone isolated from serum sample from an individual with HBV-associated HCC. When compared with a genotype 1 strain (HDV-1), the HDV-2 cDNA clone showed lower replication capacity in transfected Huh7 cells (up to 74% at day 12 post-transfection, as determined by northern blot). Moreover, transfection of Huh7 cells with plasmids coding for HDV-2 L-HDAg and S-HDAg was found to promote cell migration, assessed by wound healing and cell invasion assays (4.6-fold and 3.4-fold, respectively, when compared with control Huh7 cells), as determined in experiments using Matrigel invasion chambers. Expression of EMT markers snail and slug was enhanced in Huh7 cells transfected with HDV-2 L-HDAg and S-HDAg, whereas expression of E-cadherin was reduced.

The mechanisms of activation of the twist promoter by L-HDAg in Huh7 cells were investigated by Liang et al. (2020). It was reported that L-HDAg induced the expression of TGFβ and of EMT markers such as twist, snail, and vimentin, but reduced the expression of E-cadherin. This effect could be partly reversed after treatment of

cells with statins, which are thought to inhibit isoprenylation of L-HDAg.

[The Working Group noticed several limitations in the study design and quality because of: (i) the use of anti-HDAg human serum of unknown origin and specificity in several experiments; (ii) the use of different methods to detect and quantify intracellular and extracellular virus RNAs; and (iii) the incorrect interpretation of and conclusions drawn from some western blot results.]

Mendes et al. (2013) performed a proteomic analysis of HEK293 cells expressing S-HDAg under the control of a tetracycline-inducible promoter and transiently transfected with mutated HDV genomic RNA that was unable to express S-HDAg. In one in vitro study, the HEK293 cell line was conditionally expressing S-HDAg and engineered to mimic HDV replication. Peptides generated by trypsin digestion were labelled either with ^{16}O or ^{18}O and analysed by LC-MS. About 3000 proteins were quantified: 49 DEGs resulted from the presence of S-HDAg, and 40 DEGs as a consequence of HDV RNA replication. All differentially expressed proteins were grouped according to their biological function using GO, and most proteins with changes in abundance related to expression of HDAg or HDV RNA were found to be involved in protein metabolism and energy pathways (relevant to the present key characteristic, KC10). Further validation of proteomic results was performed by western blot with antibodies directed to p53, ELAV, transportin 1 (TNPO1), cofilin-1, EIF3D, and 10 kDa heat-shock protein (HSPE). Among the 89 DEGs identified, *TP53* was found to be downregulated upon expression of S-HDAg, when compared with controls. Western blot analysis showed that expression of 14-3-3 σ, which is regulated by *TP53*, was also downregulated. The protein 14-3-3 σ binds to the phosphorylated CDK1–cyclin B complex to induce cell cycle arrest at the G2/M cell cycle checkpoint. Impairment of the G2/M cell cycle checkpoint may contribute to instability of the genome, accumulation of mutations, and uncontrolled cell proliferation.

[The Working Group noted that these proteins are involved in various metabolic pathways, regulation of the cell cycle, apoptosis, or DNA repair capacity; as such, they are relevant to the key characteristics "alters DNA repair and causes genomic instability" (KC3) and "alters cell proliferation, cell death, or nutrient supply" (KC10). In addition, ingenuity pathway analysis (IPA) was used to identify signalling pathways that were possibly altered during HDV replication. The most affected canonical pathways in the presence of HDAg were glycolysis/gluconeogenesis, followed by EIF2 signalling and protein ubiquitination pathways, then PI3K/AKT signalling, pyruvate metabolism, and cell cycle: G2/M DNA damage checkpoint regulation.]

[The Working Group considered that the alterations in gene expression described in HEK293 cells may not reflect the actual events occurring in human hepatocytes. Indeed, the origin of HEK293 cells is controversial, since they were shown to express several proteins typically found in neurons (Shaw et al., 2002).]

Cole et al. (1991) addressed a possible involvement of HDAgs in the induction of necrosis and/or apoptosis. The authors transfected HeLa and HepG2 cells with a plasmid coding for HDAg and observed the formation of necrotic cells that, according to the authors, morphologically resembled the apoptotic bodies seen in liver tissues from patients with HDV. It was also claimed that levels of HDAg expression in individual necrotic cells were comparable to those in hepatocytes from a chimpanzee with acute HDV infection. [The Working Group identified some limitations of the study: (i) the number of cells in extracts used in immunoblots was adjusted by determining DNA concentrations; this was not correct because of the different karyotypes and DNA contents of the various cell types used; (ii) expression levels of HDAg were compared by

counting bound ^{125}I in the absence of appropriate controls; and (iii) the morphological similarities found between in vitro cultured dead cells after HDAg expression and apoptotic bodies seen in cells from patients with HDV do not allow the conclusion to be drawn that HDAg may be specifically cytotoxic to infected hepatocytes in vivo.]

(b) Experimental systems

Non-human mammalian experimental systems in vitro

Wang et al. (2001) transfected African green monkey (*Cercopithecus aethiops*) CV1 cells with a plasmid coding for HDV cDNA and did not find evidence for a possible association between HDV replication, apoptosis (by annexin staining), and cell cycle arrest (by propidium iodide staining). In addition, HDV replication did not increase sensitivity to apoptosis inducers such as TNFα in NIH3T3 cells, Fas-mediated killing in BJAB cells, or staurosporine in various fibroblastic cell lines.

To elucidate the molecular mechanism of HDV pathogenesis, Choi et al. (2007) used Huh7 and Cos-7 cells (from *C. aethiops*) to examine the effects of S-HDAg and L-HDAg expression on TGFβ and c-Jun–induced signalling cascades. It was observed that L-HDAg, but not S-HDAg, stimulated both TGFβ and c-Jun signal activation, which required isoprenylation of L-HDAg. Moreover, the presence of L-HDAg resulted in overexpression of plasminogen activator inhibitor-1 (PAI-1), which is induced by TGFβ. [The Working Group noted that, in some of the experiments, HDAgs were expressed only separately after transfection in the two cell types.]

Tavanez et al. (2020) identified a specific interaction between the splicing factor SF3B155 and HDV genomic RNA using a yeast three-hybrid approach. Changes in several constitutive and alternative splicing events for the tumour suppressor transcript RBM5 were found in Huh7-D12a cells, which stably express HDV.

Synopsis

[Overall, the Working Group noted that there was a small number of available studies in human patients infected with HDV. In most of the studies, transcriptomics was used to identify relevant genes or pathways involved in HDV replication effects, considering malignant liver tissues compared with adjacent non-malignant tissues and with HCC tissue from patients with other etiologies, including HBV-monoinfected patients. Some studies identified a considerable number of genes involved in pathways related to DNA replication, DNA repair, cell cycle checkpoint regulation, cell proliferation, and migration. Similar findings were obtained in human cell lines in vitro. However, the studies were limited in number, and the findings were dispersed among models of different design, quality, and end-points, which made interpretation difficult.]

5. Summary of Data Reported

5.1 Exposure characterization

Hepatitis D virus (HDV) is an RNA virus discovered in 1977 and is currently classified in the genus *Deltavirus* within the *Kolmioviridae* family. It is a defective virus that requires the hepatitis B surface antigen (HBsAg) as its envelope, which means that hepatitis B virus (HBV) infection is needed to establish HDV infection in humans.

HDV has been previously evaluated by the *IARC Monographs* programme as *not classifiable as to its carcinogenicity to humans* (Group 3) (Volume 59; IARC, 1994). Hepatitis B virus was previously evaluated in Volume 100B as *carcinogenic to humans* (Group 1) (IARC, 2012).

HDV infection is highly specific to hepatocytes and, in humans, can be acquired either simultaneously with or after HBV infection, leading to coinfection or superinfection, respectively. These different acquisition patterns can lead to different clinical outcomes, with chronic infection resulting much more frequently after superinfection than coinfection.

HDV is commonly tested only within HBsAg-positive populations, first by serology (measurement of HDV immunoglobulin G, IgG) and then by confirmation of the presence of HDV RNA by real-time quantitative polymerase chain reaction (qPCR). In liver biopsy tissue, HDV infection is detected using real-time qPCR or immunohistochemistry.

The prevalence of HDV infection within HBsAg-positive populations ranges between 1% and 10% in most regions of the world; however, it was reported to reach up to 65% in one population. Prevalence has been shown to be increased in people who inject drugs and individuals with high-risk sexual behaviours. HDV positivity within the overall population has been estimated to be between 0.1% and 1%.

HBV vaccination is considered to be effective in the prevention of HDV in HBV-uninfected people. HDV antiviral treatment, such as interferon alpha (IFNα) and bulevirtide, reduces viral load and disease progression in some patients.

5.2 Cancer in humans

A total of 17 informative studies – including 13 cohort studies and four case–control studies – formed the core evidence base, supplemented by four published meta-analyses and an additional meta-analysis that was carried out by the Working Group. These studies spanned diverse geographical regions and populations and included both general population and human immunodeficiency virus (HIV)-positive cohorts. HDV exposure was typically assessed via serology and/or quantification of HDV RNA, with the latter representing a more specific measure of active viral replication and disease progression. Serological assays may underestimate risk by failing to distinguish active from past infection, whereas RNA-based measures provide clearer insight into the relationship between viral activity and carcinogenesis. Despite variability in design and methodology, findings across these studies converged on a consistent positive association between HDV infection and increased risk of hepatocellular carcinoma (HCC), as shown by the results of the meta-analysis carried out by the Working Group (meta-estimate, 2.13; 95% CI, 1.57–2.87).

The Working Group evaluated a range of potential biases and confounders, including coinfections (hepatitis C virus, HCV; and HIV), alcohol use, body mass index (BMI), and antiviral treatment. Two studies addressed all relevant factors through either statistical adjustment or exclusion criteria and demonstrated positive associations between HDV and HCC. Additionally, stratified and sensitivity analyses conducted by the Working Group showed that residual confounding was unlikely to fully account for the observed associations. Importantly, adjustment for cirrhosis status, which may lie on the causal pathway, often attenuated effect estimates, suggesting that this adjustment might lead to underestimation of the true effect of HDV. Similarly, the selective application of antiviral therapy among HBV-monoinfected individuals could bias comparisons, but this too was judged unlikely to nullify the association.

Risk estimates across cohort studies generally fell between 1.5 and 6.0 in HBV-infected populations, with some estimates as high as 8.3 in groups of individuals who were also positive for HIV and HDV RNA. Dose–response relations between HDV RNA levels and HCC risk provided further support for a causal link. The case–control studies, despite having smaller sample sizes and some methodological limitations, independently supported this conclusion

with similarly elevated odds ratios. The findings of the meta-analysis carried out by the Working Group and of meta-analyses reported in the literature reinforced the pattern of increased risk, showing consistent effect sizes across different inclusion criteria.

The collective body of epidemiological evidence supported a positive association between HDV infection and the development of HCC, above that of HBV monoinfection. The attributes of the body of evidence included strength and consistency of association, temporality, a dose–response relation, biological plausibility, and coherence across study types. Collectively, the body of evidence indicated that there is a plausible association between HDV infection and HCC, and that chance, bias, and confounding could all be ruled out with high confidence.

5.3 Cancer in experimental animals

No data were available to the Working Group.

5.4 Mechanistic evidence

HDV is a satellite virus of HBV that infects and replicates in human hepatocytes. The HDV genome (genomic RNA) is a circular single-stranded RNA molecule. The complementary RNA to gRNA molecule, called the antigenome (agRNA), contains a single open reading frame (ORF) from which two forms of the same protein, the small and large delta antigens (S-HDAg and L-HDAg), are derived by an editing mechanism. The two antigens share most of the sequence and functional domains, but their roles in the HDV replication cycle are diverse: S-HDAg enhances the accumulation of virus RNAs, and L-HDAg is essential for virion assembly and interaction with HBsAgs.

There is consistent and coherent evidence that HDV infection exhibits key characteristics of carcinogens.

HDV infection induces chronic inflammation. The evidence is consistent and coherent in exposed humans and in human primary cells transfected into tissues and is suggestive in experimental systems.

A consistent body of evidence obtained from exposed humans suggested that, when compared with HBV monoinfection, HDV infection is associated with an increase in pro-inflammatory cytokines, including interleukins IL-1β, IL-2, IL-4, IL-5, IL-10; tumour necrosis factor alpha (TNFα); IFNβ and IFNγ, and chemokines CXCL9, CXCL10, CXCL11, and CXR3. When compared with HBV-monoinfected patients, HDV-infected patients exhibit a more rapid progression through fibrosis to cirrhosis, which is of high relevance for HCC. A similar pattern of cytokine secretion was also observed in PBMC culture supernatants from infected patients. These observations seemed to be consistent in observational studies of different designs. In addition, a higher frequency of activated ($CD38^+$, $HLA\text{-}DR^+$, and $PD\text{-}1^+$) mucosal-associated invariant T (MAIT) cells were found in the intrahepatic immune-cell population. MAIT cells are known to be involved in the inflammatory response and secrete IL-17, which promotes the differentiation of macrophages into the inflammatory M2 subtype as well as vascular endothelial growth factor (VEGF)-mediated angiogenesis. The results of studies in primary human hepatocytes and susceptible liver cell lines infected with HBV and HDV or with HBV alone were consistent with those obtained in exposed humans.

In various test systems, HDV infection has also been consistently associated with induction of the innate immune response, namely with an increase in the production of IFNβ, λ1, and λ2/3, and other cytokines that can have pro-inflammatory activity. Activation of the IFN response was reported to involve the melanoma differentiation antigen 5 (MDA5) pathway in HDV-infected HepaRG cells expressing sodium taurocholate

cotransporting polypeptide (HepaRG^{NTCP}), and primary human hepatocytes.

No evidence was available for the key characteristics of carcinogens "is electrophilic or can be metabolically activated to an electrophile" and "modulates receptor-mediated effects". For the other key characteristics of carcinogens, the evidence was sparse.

6. Evaluation and Rationale

6.1 Cancer in humans

There is *sufficient* evidence in humans for the carcinogenicity of hepatitis D virus. Hepatitis D virus causes hepatocellular carcinoma.

6.2 Cancer in experimental animals

There is *inadequate* evidence in experimental animals regarding the carcinogenicity of hepatitis D virus.

6.3 Mechanistic evidence

There is *strong* mechanistic evidence that hepatitis D virus exhibits key characteristics of carcinogens in exposed humans and in human primary cells.

6.4 Overall evaluation

Hepatitis D virus is *carcinogenic to humans* (Group 1).

6.5 Rationale

The Group 1 evaluation for hepatitis D virus (HDV) is based on *sufficient* evidence for cancer in humans. In numerous cohort and case–control studies spanning diverse geographical regions, higher risk of hepatocellular carcinoma was observed among people infected with HDV who were also infected with hepatitis B virus (HBV), a known liver carcinogen. Exposure to HDV magnified the already high risk of hepatocellular carcinoma seen among those who were also exposed to HBV.

There was also *strong* mechanistic evidence in exposed humans and in primary human hepatocytes that HDV infection induces chronic inflammation. The evidence was consistent in exposed humans, in whom persistent activation of the innate immune response and exacerbated production of pro-inflammatory cytokines have been observed. Patients infected with both HDV and HBV experienced more severe hepatic lesions, including necroinflammation, leading to more rapid progression to cirrhosis, than did patients who were chronically infected with HBV alone. Evidence obtained in HDV-infected primary human hepatocytes was coherent with that obtained in exposed humans.

Evidence regarding cancer in experimental animals was *inadequate* because no data were available.

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HUMAN CYTOMEGALOVIRUS

1. Exposure Characterization

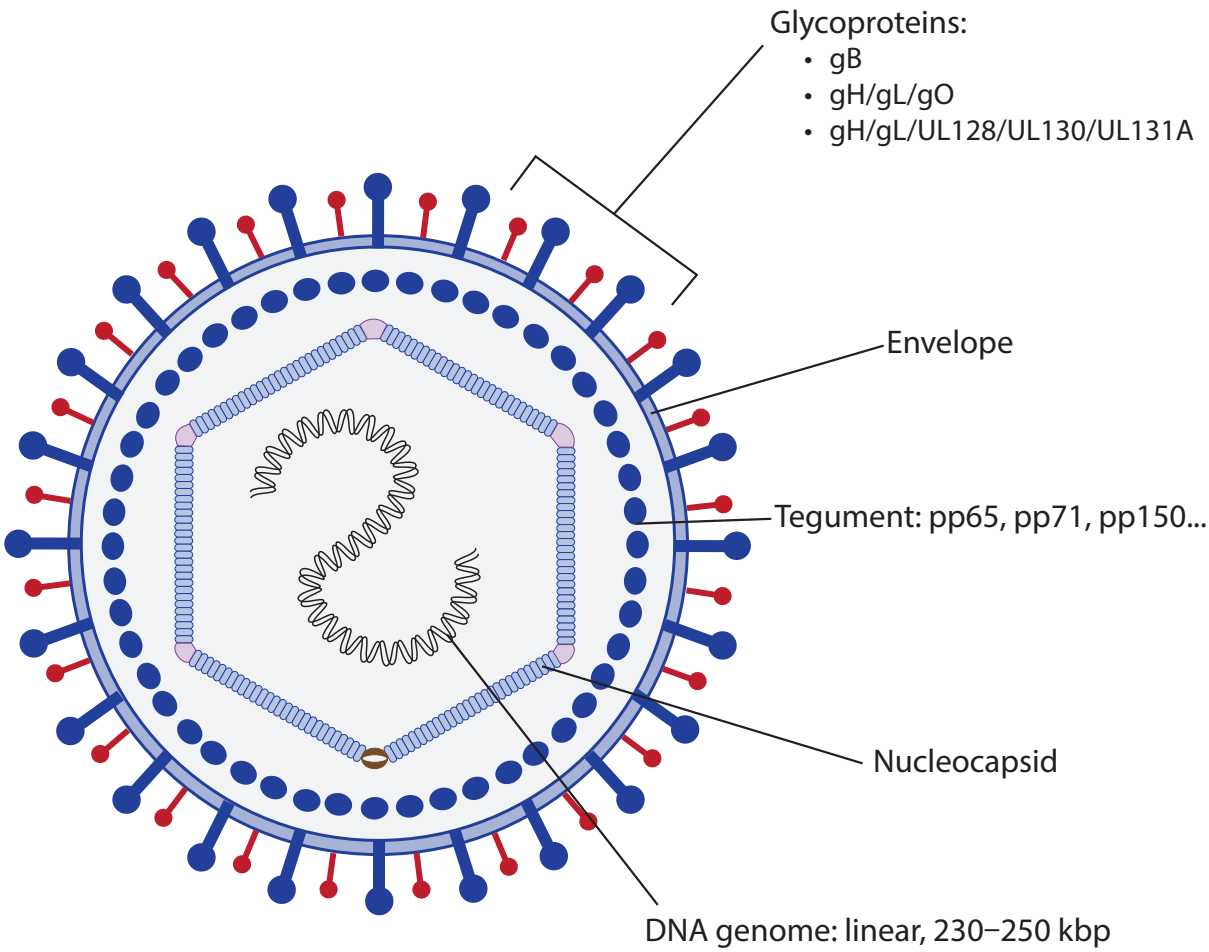
1.1 Identification

1.1.1 Structure and life cycle

Human cytomegalovirus (HCMV), also known as human betaherpesvirus 5 (HHV-5), is a member of the herpesvirus family (*Orthoherpesviridae*) and the *Betaherpesvirinae* subfamily (Davison et al., 2005; Stern-Ginossar et al., 2012; ICTV, 2024). Like other herpes viruses, such as herpes simplex virus (HSV), varicella zoster, and Epstein–Barr virus (EBV), HCMV is an enveloped virus, with a linear double-stranded DNA (230–250 kbp) encoding hundreds of proteins, for many of which the function and effect are unknown (Stern-Ginossar et al., 2012) (Fig. 1.1). HCMV causes lifelong latent infections after primary infection and periodically reactivates in immunocompetent individuals (more frequently in immunosuppressed patients), leading to episodes of intermittent virus excretion in body fluids, all of which are potential sources of virus transmission (Cannon et al., 2011; Griffiths and Reeves, 2021).

HCMV can be classified into five subgroups on the basis of genotypic variation in glycoprotein B (gB), which is found on the viral envelope and is encoded by the *gpUL55* (or *UL55*) gene. Essential for viral entry into hosts, cell-to-cell viral transmission, and the fusion of infected cells, eliciting a strong immune response, gB thus serves as a target for neutralizing and non-neutralizing antibodies, as well as for antiviral therapeutics (Nuévalos et al., 2023). The five primary gB genotypes are gB1, gB2, gB3, gB4, and gB5 (Huang et al., 2023). The HCMV genome encodes several other glycoproteins, including glycoproteins H (gH), L (gL), and O (gO). Conserved within the herpesvirus families (α, β, and γ), gB and the gH/gL heterodimer are essential for viral entry into the cell, which comprises two steps: receptor-binding and membrane fusion. The gH/gL-containing complexes determine cellular tropism and function as receptor-binding proteins: gH/gL/gO for entry into fibroblasts and gH/gL/UL128/UL130/UL131A (pentamer) for entry into epithelial or endothelial cells; gB acts as the fusogen, mediating membrane fusion (Burke and Heldwein, 2015; Chandramouli et al., 2015). After interaction with the receptor, the viral envelope fuses with the cell membrane, and the viral capsid is released into the intracellular environment (Fig. 1.2). The capsid is then transported to the nucleus, where it releases the viral genome via a nuclear pore (Knipe and Howley, 2020). HCMV has coevolved with humans for thousands of years. The HCMV genome exhibits several evolutionary phenomena, including antigenic variation, recombination, and gene loss (Suárez et al., 2019). HCMV has a broad

Fig. 1.1 Schematic representation of the HCMV virion structure



The human cytomegalovirus (HCMV) particle is composed of an outer lipid envelope containing viral glycoproteins (gB, gH, gL, gO and UL128/UL130/UL131A), a pleomorphic proteinaceous tegument layer, and an inner icosahedral nucleocapsid. The nucleocapsid encloses a large, linear, double-stranded DNA genome (230–250 kbp).

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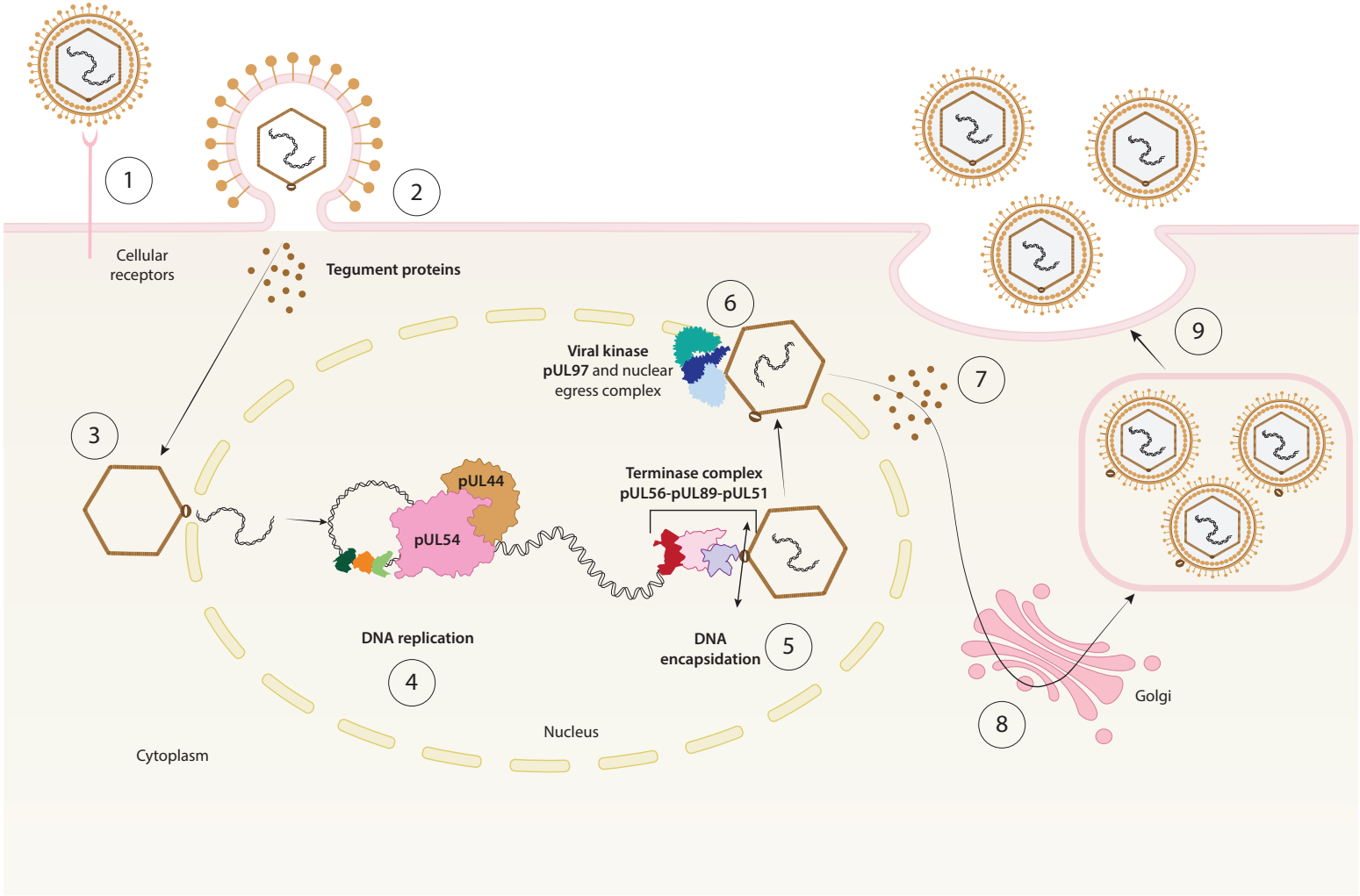
cell tropism and, through a variety of mechanisms, is capable of regulating and evading host cell responses to achieve effective viral replication (Cimato et al., 2024). Proteome studies have identified 59 proteins in the viral particle, some of which regulate processes involved in apoptosis, cell adhesion, immune response, metabolism, transport, and signalling (Varnum et al., 2004). [The Working Group noted that additional proteins are encoded by the viral genome (Viralzone, 2025).] During active (lytic) infection, phosphoprotein 65 (pp65) – a major target of $CD4^+$ and $CD8^+$ T-cell responses to HCMV – is synthesized abundantly (Malouli et al., 2014). HCMV establishes latency in myeloid cells of the bone marrow, causing a lifelong infection with sporadic reactivations (Jean Beltran and Cristea, 2014). Several studies have shown that, although at lower levels and not necessarily following the kinetics of the three transcriptional phases – immediate-early (IE), early (E), and late (L) – many HCMV proteins are expressed during latency (Dupont and Reeves, 2016; Cheng et al., 2017).

Fig. 1.2 The HCMV lifecycle



HCMV, human cytomegalovirus.

The stages of the life cycle include: 1. Virus attachment to the cell receptors by envelope glycoproteins. 2. Entry of virus capsid into cell cytoplasm. 3. Migration of capsid to cell nucleus and release of viral genome through nuclear pore. 4. Replication of viral DNA by the rolling circle method using the viral polymerase pUL54. 5. Encapsidation of the genome into capsids via the terminase complex. 6. Nuclear egress of capsid. 7. Formation of tegument by accumulation of tegument proteins around the nucleocapsid. 8. Passage of virions through the Golgi apparatus and acquisition of a primary envelope. 9. Budding and release of infectious virus particles.

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1.1.2 Discovery and historical perspective

The large, hence cytomegalic, cells with a central nuclear body surrounded by a clear halo (intranuclear inclusions) typical of HCMV infection were first observed by scientists in Germany in 1881, in sections of kidney, lungs, and liver of stillborn infants, and parotid glands of infants (Ribbert, 1904). About 50 years later, Wyatt et al. (1950) described 6 cases and reviewed 58 cases of a fatal congenital infection characterized by petechiae, hepatosplenomegaly, frequent inclusions in the respiratory tract, and intracerebral calcifications in 2 cases, and designating the condition as "generalized cytomegalic inclusion disease", although the viral infection as a cause of illness and death was not known for all cases (Wyatt et al., 1950).

In the 1950s, newly developed cell culture techniques allowed three scientists (Weller, Smith, and Rowe), working independently, to isolate viruses that produced similar cytopathic changes. The isolates were later exchanged and identified as cytomegalovirus. The virus was isolated from the salivary gland, kidney, and liver tissues of deceased infants (one of which was suspected to have congenital toxoplasmosis) and, serendipitously, from adenoid tissue of children (Weller et al., 1957). Cytopathic changes observed in inoculated adenoidal tissue cultures from three children were different from those observed in children with respiratory illness caused by adenovirus (Rowe et al., 1956). These isolates (AD169) originated the first so-called laboratory strain of HCMV, which in 1990 became the first complete betaherpesvirus genome to be sequenced (Chee et al., 1990).

In the 1960s, the use of the complement-fixation test to measure specific antibodies against HCMV suggested potential strain differences among isolates. For the next 20 years, complement-fixation tests were employed in studies on the epidemiology of HCMV, revealing the ubiquity of the infection in populations, despite being usually asymptomatic (Krech and Tobin, 1981; Brandt et al., 1984). HCMV was later recognized as a possible cause of a disease resembling infectious mononucleosis, which could also occur after blood transfusion, and as one of the most common opportunistic pathogens for patients undergoing bone marrow, stem cell, or solid organ transplantation (Pereyra and Rubin, 2004; Razonable and Humar, 2019; Cassaniti et al., 2021), and in patients with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) (Zhao et al., 2020).

1.1.3 Cell and tissue tropism

HCMV can infect a variety of cell types in the human host, including epithelial cells of glands and mucosal tissues; connective tissue cells (fibroblasts) in various organs; smooth muscle cells in the gastrointestinal tract; vascular endothelial cells; and various haematopoietic cell types ($CD34^+$ haematopoietic stem cells and cells of the myeloid lineage, including $CD14^+$ monocytes; Elder et al., 2019a). Ubiquitous in the body, fibroblasts and smooth muscle cells support viral replication and productive lytic infection, which is disseminated to other organ tissues via haematopoietic cells. Viral replication in almost every organ is observed in severe cases of intrauterine infection. Clinical manifestations in immunocompromised hosts are related to viral replication in the liver, gastrointestinal tract, lung, retina, or brain. Epithelial cells are assumed to play an important role in host-to-host transmission (Sinzger et al., 2008).

1.2 Methods of detection

The various diagnostic methods (direct and indirect) available for the detection of HCMV are described in this section (see also Table 1.1). Diagnosis essentially involves serology (measurement of IgG and IgM, most commonly with enzyme immunoassays) and nucleic acid

Table 1.1 Overview of methods of detection of HCMV

Method	Marker	Specimen	Strengths	Limitations
Serology	Antibodies, IgG and IgM	Serum	IgG marker of past infection	Poor specificity of IgM.
Antigenaemia	pp65	Blood	Sensitivity	Lack of automation and assay standardization, and interpretation was subjective.
Cell culture	Cytopathological effect	Blood, urine, saliva	Specificity	Low sensitivity, time-consuming.
PCR	DNA, less common: RNA	Blood, urine, saliva, tissue	High sensitivity, rapid	No distinction between free DNA and DNA in viral particle.
In situ hybridization	DNA	Tissue	Localization of viral DNA	Lower sensitivity than PCR, high cost, technical complexity.
Immunohistochemical staining	Viral proteins	Tissue	Localization of virion, specificity	Variable sensitivity depending on antibody/ antigen, pre-analytical step and fixation.

HCMV, human cytomegalovirus; Ig, immunoglobulin; PCR, polymerase chain reaction.

Modified from Razonable et al. (2020).

amplification tests (NAATs), primarily with quantitative polymerase chain reaction (qPCR).

1.2.1 Direct methods

(a) Detection of HCMV by culture

Culture of fetal specimens (amniotic fluid, chorionic villi, tissues) or body fluids (such as urine and saliva from neonates within the first 21 days of life) have been used for diagnosing congenital HCMV infection and were considered the gold standard before the introduction of molecular techniques. Human fibroblasts are typically used for cell culture and exhibit a cytopathic effect typical of HCMV. Definitive identification is performed via immunofluorescence. A virus-specific monoclonal antibody with fluorescent label is used to stain the infected cells, for example, against early proteins (IE1 or IE2) (Lee et al., 1999). Shell vial cultures are faster techniques in which the sample is centrifuged onto a cell monolayer, incubated from 12 to 72 hours, and then stained for protein detection. Culture is labour-intensive, takes up to 21 days to obtain results, and, despite high specificity, has a sensitivity that is lower than that with molecular techniques (Leber, 2024).

(b) Detection of HCMV by nucleic acid amplification test

NAATs, mainly real-time polymerase chain reaction (PCR), are used for the detection and diagnosis of HCMV in various populations. [The Working Group noted that these tests have become the gold standard for HCMV detection.] Commercially available assays for quantitative detection of HCMV in blood or plasma have been approved for viral load monitoring in immunocompromised adults or children, although wide variability in viral load reporting across assays precludes consensus on viral load thresholds for clinical guidance. Variability in viral load has been reported to result from variability in testing protocols, such as limits of detection and quantification, nucleic acid extraction method, gene target, and amplicon size (Razonable et al., 2020). Therefore, it is recommended that all HCMV quantitative nucleic acid testing (HCMV qNAT) be calibrated to the World Health Organization (WHO) standard (Fryer et al.,

2016) for the management of HCMV in solid organ transplantation (Kotton et al., 2025).

Two qualitative NAATs have been approved by the United States Food and Drug Administration (US FDA) for the diagnosis of congenital HCMV using saliva or urine in neonates (Tan et al., 2023). [However, most NAATs using dried blood spots are still laboratory-developed.] The sensitivity and specificity of NAATs are >90% for amniotic fluid, urine, or saliva, although positive results in saliva must be confirmed with urine testing because of potential contamination by HCMV-containing breast milk. The sensitivity of HCMV PCR using dried blood spots from neonates (aged >3 weeks) for HCMV screening ranged from 34.4% to 76.8%, depending on the extraction method used (Leber, 2024).

Laboratory methods for the detection of HCMV in tissues included PCR, nested PCR (nPCR), reverse transcriptase PCR, quantitative PCR (qPCR), and droplet digital PCR (ddPCR) (Peredo-Harvey et al., 2021), with the last two techniques being the most sensitive. Inconsistent results may be attributable to variations in the sensitivity of PCR tests (laboratory-developed or commercial) and the impact of tissue quality on genome extraction because of DNA fragmentation during formalin-fixed paraffin-embedded (FFPE) tissue preparation (Maleki et al., 2020).

Next-generation sequencing (NGS) can be used to detect different viral strains and analyse the whole genome (Martí-Carreras and Maes, 2019). Metagenomics can detect HCMV among all sequenced genomes of a sample. [The Working Group noted that NGS is not routinely used for the detection of HCMV infection.]

(c) Detection of HCMV proteins

In immunocompromised patients (such as recipients of solid organ transplants, patients with haematological malignancies, and patients with HIV infection), an assay for HCMV pp65 antigenaemia was commonly used to assess HCMV viraemia and monitor risk of HCMV disease (Hardie et al., 2013). In this assay, HCMV pp65 is detected and quantified by indirect immunofluorescence of isolated peripheral blood mononuclear cells (PBMCs). Post-transplant patients are monitored weekly in the first 100 days for evidence of HCMV reactivation, to allow for timely initiation of pre-emptive antiviral therapy. [The Working Group noted that detection of HCMV proteins has been replaced by NAAT methods in the clinical setting.] Limitations of the pp65 assay include that blood samples must be fresh (<24 hours), patients must have sufficient leukocyte counts, and reading of fluorescent slides is subjective.

HCMV pp65 detection in tissue has been the standard for many years, although it may have low sensitivity for detecting HCMV in the eye or the gastrointestinal tract. HCMV viral load is more sensitive for the detection of HCMV viraemia than is pp65 detection, although standardization of assays is still lacking (Ikewaki et al., 2003; Hardie et al., 2013; Kwon et al., 2015). Both HCMV pp65 and viral load measured by qPCR (see Section 1.2.1(b)) are markers of systemic reactivation. Despite the low predictive value for HCMV disease, a positive test result can aid in the diagnosis of end-organ disease without requiring biopsy (Nagata et al., 2011). Definitive diagnosis for most end-organ diseases requires obtaining tissue specimens for histopathology (to observe the cytopathic effect of the virus in infected cells) and immunohistochemistry (IHC) studies.

HCMV pp65 and other proteins, including immediate-early (IE1), early (pp52), and late (gB or UL55, pp150, pp28), have also been examined by IHC in tumour tissue samples from patients with glioblastoma (GBM) (e.g. Peredo-Harvey et al., 2021), breast cancer (Nakhaie et al., 2021a), and other types of tumour. Nevertheless, there are no established assays for studies on cancer tissues and there is disagreement on the validity of the experimental approaches, i.e. phase of the

replication cycle, status of the infection, if latent or lytic, tropism (see also Section 4).

1.2.2 Indirect methods

(a) Detection of anti-HCMV antibodies

Detection of immunoglobulin G (IgG) antibodies to HCMV in the absence of IgM antibodies indicates a previous, but not acute, infection. Detection of anti-HCMV IgM antibodies may indicate a recent infection but does not distinguish between primary versus non-primary HCMV infection (e.g. reactivation or superinfection) (Dollard et al., 2011). The sensitivity of IgM assays varies, and antibody cross-reactivity may occur (Miendje Deyi et al., 2000). Detection of anti-HCMV IgG antibodies in serum collected 2–4 weeks after a negative test for anti-HCMV IgG from a previous sample indicates primary infection. Measurement of the avidity of IgG (the overall strength of IgG binding to the HCMV antigen) can help to date the primary infection. The binding is weak in recent infections and increases with time. Therefore, detection of anti-HCMV IgG with low avidity can aid in the diagnosis of primary HCMV infection; the IgG response exhibits low avidity for up to 3–4 months and matures over time, reaching high avidity by 6 months post-primary infection (Leber, 2024). The threshold for determining low or high avidity differs from one test to another. However, for correct interpretation, accurate evaluation of IgG avidity is not possible for samples with very low IgG levels, and IgG avidity testing should be performed only on samples that are positive for both IgG and IgM (Razonable et al., 2020). In previous research, detection of new strain-specific IgG reactivity to polymorphic envelope proteins (gH and gB) has been used to identify reinfections in individuals with previously IgG-positive results. Serology is not used for diagnosing congenital HCMV infection in infants aged < 3 weeks, as NAATs on urine, saliva, or amniotic fluid are needed to establish HCMV infection in the fetus or neonate (Leber, 2024).

Enzyme-linked immunosorbent assay (ELISA) is currently the most common serological test for measuring antibodies to HCMV (CDC, 2024), but various serological assays exist, including bead-based immunoassays, complement fixation, radioimmunoassay, latex agglutination, anticomplement immunofluorescence, and indirect haemagglutination (Yeager, 1979; Mintz et al., 1980; Wilson et al., 1990; Tcherniaeva et al., 2018; Razonable et al., 2020). The sensitivity of commercial tests for anti-HCMV IgG ranges from 97% to 100%, and specificity ranges from 96% to 100%. Anti-HCMV IgM tests have variable sensitivity (54% to 100%) and specificity (62% to 100%) for the diagnosis of primary infection (Leber, 2024). IgM antibodies are typically produced within 1–2 weeks of primary infection and decline to undetectable levels within 4 months, although they may persist for up to 1 year. Non-primary infections (reinfection or reactivation) can also lead to IgM production (Razonable et al., 2020). IgG positivity is persistent over a lifetime except in rare cases of extremely immunocompromised patients. Measuring IgG is therefore a useful tool to detect past infection with HCMV. [The Working Group noted that in a low prevalence setting, it is recommended that both IgG and IgM be measured to diagnose primary infection. However, in high-prevalence populations, measurement of IgM is useless for the detection of non-primary infection, because tests for IgM are not always positive.]

(b) Detection of cell-mediated immunity against HCMV

There are several HCMV-specific assays for the assessment of cell-mediated immunity. [The Working Group noted that these methods do not measure HCMV itself, but the cell's immune system response towards it.] HCMV enzyme-linked immunosorbent spot assays measure

production of interferon gamma (IFNγ) by cells stimulated with HCMV-specific peptides. These tests have been used to assess the risk of HCMV reactivation in haematopoietic cell transplant recipients. Approved tests are commercially available in Europe, and these are mostly used for research in the United States of America (USA). These tests allow assessment of T-cell immune activity after ex vivo stimulation with HCMV antigens; IFNγ is released from activated cells and quantified by ELISA. Tests either require initial isolation of PBMCs or use whole blood, and the targeted cells can vary (e.g. $CD4^+$ and $CD8^+$ T cells; $CD8^+$ T cells only) (Nesher et al., 2016; Burton et al., 2018; Terlutter et al., 2018; Rogers et al., 2020). The HCMV antigens used include pp65 and IE1. Flow cytometry-based assays also measure IFNγ expression, using intracellular cytokine staining, after HCMV stimulation (Hall et al., 2022).

1.3 Epidemiology of HCMV infection

1.3.1 Transmission and diseases associated with HCMV infection

Contact with bodily fluids such as blood, saliva, urine, tears, seminal fluid, cervical secretions, and breast milk from infected individuals can lead to HCMV transmission.

Primary HCMV infection is often asymptomatic in healthy, immunocompetent individuals. A mild illness resembling infectious mononucleosis may occur. Prolonged febrile illness, accompanied by fatigue, night sweats, malaise, myalgias, headache, and laboratory abnormalities, such as leukocytosis and elevated transaminases, have been reported in case series. However, primary HCMV infection is infrequently considered in the differential diagnosis of prolonged fever, and estimates of disease incidence are lacking (Nolan et al., 2017). Although rare, severe organ involvement has also been reported in immunocompetent patients. In a review of 89 studies that included 290 immunocompetent adults, severe HCMV infection presented variously as colitis, meningitis, encephalitis, myelitis, nerve palsies, myeloradiculopathy, haemolytic anaemia, thrombocytopenia, uveitis, retinitis, hepatitis, pneumonitis, and thrombosis of the arterial and venous system (Rafailidis et al., 2008).

Mother-to-fetus transmission may occur as a consequence of maternal primary HCMV infection or non-primary infection (superinfection with a new HCMV strain or reactivation) in the periconceptional period or during pregnancy. The rate of vertical transmission after primary infection increases from approximately 30% during the first trimester to 70% in the third trimester of pregnancy (Enders et al., 2011), but limited data exist on the rate of vertical transmission after non-primary infections (Britt, 2015). Congenital HCMV infection is estimated to occur in approximately 5 per 1000 live births in high-income countries, and 14 per 1000 live births in low- and middle-income countries, with a pooled overall prevalence of congenital HCMV of 0.67%. The proportion of cases of symptomatic congenital HCMV among all cases of congenital HCMV at birth is 10.9% (Ssentongo et al., 2021; Khalil et al., 2025).

Infants born to HCMV-seropositive mothers may acquire HCMV through exposure to cervical secretions at birth or postnatally via breast milk. The immunological factors that lead to viral reactivation in HCMV-seropositive women late in pregnancy and in postpartum women are not well understood. The suppression of cellular immunity is thought to induce localized reactivation of HCMV in the mammary gland (Numazaki, 1997; Rabe et al., 2020). Perinatal or postnatal infection is usually asymptomatic in healthy neonates at term. However, infants with very low birth weight and premature infants are at risk of developing severe postnatal HCMV disease, characterized by hepatopathy, thrombocytopenia, neutropenia, petechiae, respiratory

distress syndrome, or sepsis-like syndrome (Lanzieri et al., 2013). Exposure to HCMV in breast milk has also been associated with a higher risk of necrotizing enterocolitis in infants with very low birth weight (Patel et al., 2020).

In immunocompromised patients, primary HCMV infection, superinfection, or reactivation can cause a myriad of clinical syndromes (Fowler et al., 2022). In patients with HIV/AIDS, HCMV retinitis is the most common opportunistic infection in the eye and the most common cause of vision loss (Steffen and Rice, 2020), even in patients receiving highly active antiretroviral therapy (HAART) (Sugar et al., 2012). HCMV is a common cause of pneumonitis in haematopoietic cell transplant recipients and is associated with rejection after solid organ transplantation and graft-versus-host disease in allogeneic haematopoietic cell transplant recipients (Nesher et al., 2016).

1.3.2 Prevalence

(a) Seroprevalence

Worldwide, the seroprevalence of anti-HCMV IgG is estimated at 83% for the general population, and 86% for women of reproductive age and for blood and organ donors. [These worldwide estimates are based on the results of a variety of studies conducted in different populations, with data lacking from some regions.] Anti-HCMV IgG seroprevalence tends to be highest (~90%) in countries in South America, the Eastern Mediterranean, Africa, and Asia, and lowest (50–70%) in western Europe and North America (Zuhair et al., 2019; see also Table 1.2 and Fig. 1.3). Anti-HCMV IgG seroprevalence is generally higher in females than in males and increases with age (Dana Flanders et al., 2024). [These trends have been observed in various countries, but the exact profiles are country-specific. For example, in high-prevalence settings, age-specific prevalence reaches high levels at younger ages.] Anti-HCMV IgG seroprevalence tends to be higher among people of lower socioeconomic status, and in developing countries compared with more developed countries (Zuhair et al., 2019). There could be a cohort effect related to development level and other factors. For example, in an analysis of data from the National Health and Nutrition Examination Survey (NHANES) in the USA it was reported that anti-HCMV IgG seroprevalence among children aged 1–5 years increased from 20.7% in 2011–2012 to 29.0% in 2017 to March 2020 (Lanzieri et al., 2022). Differences by race and Hispanic origin, poverty index, and number of children aged ≤5 years in the household were noted in the earlier years, but not in the more recent years. Breastfeeding remained the factor most strongly associated with HCMV seroprevalence, although data on childcare arrangements were not collected in this study (Lanzieri et al., 2022).

Geographical differences may be explained by several factors related to HCMV transmission: breastfeeding rates and duration, childcare arrangements, sexual activity, household crowding, and number of children (Cannon et al., 2010). Variability within regions and within countries is expected. In studies published during 2010–2020, anti-HCMV IgG seroprevalence for women of reproductive age ranged from 45.6% to 95.7% in Europe, 60.2% in Japan, 58.3% to 94.5% in Latin America, and 24.6% to 81.0% in North America (Fowler et al., 2022). Recent studies found that maternal anti-HCMV IgG seroprevalence was as high as 98.1% in Colombia (Rico et al., 2021), and 93.1% in Kenya (Otieno et al., 2019).

HCMV IgM seroprevalence was reported to be 1.0–4.6% in Europe, 0.8% in Japan, and 0–0.7% in Latin America (Fowler et al., 2022). In the USA, HCMV IgM seroprevalence among women is estimated at 2.3–4.5%. HCMV IgM positivity usually follows primary HCMV infection but also can appear during non-primary HCMV infection (Dollard et al., 2011). The proportion

Table 1.2 Estimated mean seroprevalence of HCMV in the WHO regions and globally per population group

WHO region	Mean seroprevalence % (95% CI)		
	General population	Women of reproductive age	Blood and organ donors
European Region	66% (56–74)	70% (63–76)	69% (61–77)
Region of the Americas	75% (64–84)	79% (69–87)	78% (67–87)
South-East Asia Region	86% (77–93)	89% (82–94)	88% (81–94)
African Region	88% (80–93)	90% (85–94)	90% (84–94)
Western Pacific Region	88% (81–93)	91% (86–94)	90% (85–94)
Eastern Mediterranean Region	90% (85–94)	92% (88–95)	92% (87–95)
Global	**83% (78–88)**	**86% (83–89)**	**86% (82–89)**

CI, confidence interval; WHO, World Health Organization.

Data from Zuhair et al. (2019).

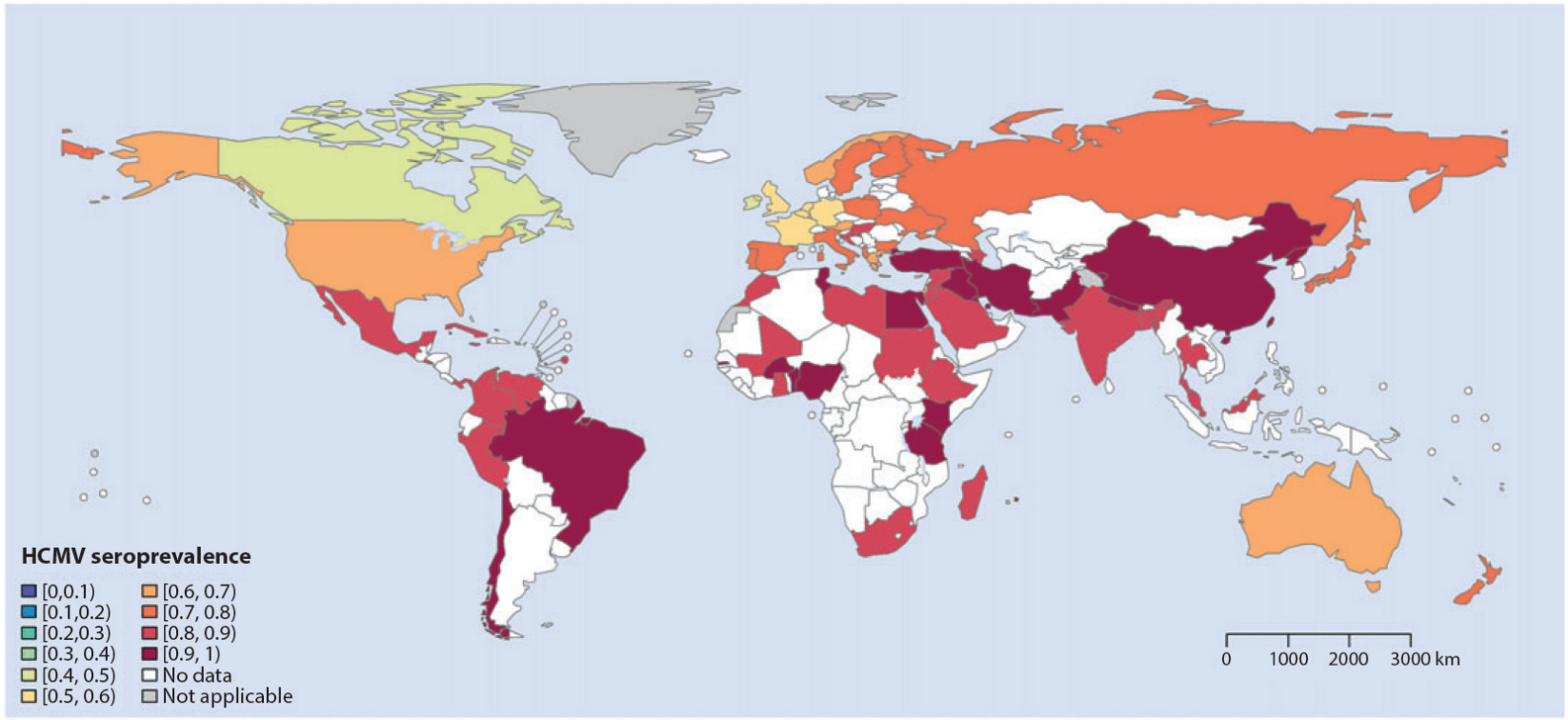
of HCMV IgM–positive associated with low anti-HCMV IgG avidity, suggesting primary HCMV infection, decreases with age: 33.0% in 12–19 years, 16.5% in 20–29 years, and 3.1% in 30–49 years (Dollard et al., 2011).

The annual incidence of primary infection ranged from 0.4% to 7% among pregnant women in the USA (Colugnati et al., 2007; Hughes et al., 2021) and was 1.8% in Canada (Balegamire et al., 2021). In Brazil, where maternal HCMV seroprevalence is ~98%, the incidence of primary HCMV infection among pregnant women who were IgG-seronegative during the first trimester of pregnancy was 20% (Mussi-Pinhata et al., 2018). But in pregnant women who were IgG-seropositive at the first prenatal visit, seroconversion to new HCMV strains was 35% among mothers who had infants with congenital HCMV infection versus 9% in the control group without congenital HCMV infection (Yamamoto et al., 2010). The annual incidence of primary HCMV infection was significantly higher among childcare workers (7.4%) as compared with health care workers (3.1%) (Balegamire et al., 2022). In a study conducted among adolescent girls (aged 10–17 years) in the USA, Finland, and Mexico, the annual incidence of primary HCMV infection and reinfection was 5% and 2–8%, respectively (Paris et al., 2023). [On very rare occasions, some HCMV-positive individuals might appear seronegative because the infection does not trigger a detectable antibody response. However, as this is very rare, the Working Group did not consider that this has a significant impact on the sensitivity of common test methods.]

(b) Prevalence of HCMV DNA

HCMV is commonly excreted in breast milk; infectious virus and HCMV DNA and RNA have been isolated from cell-associated and whey fractions in the breast milk of 40% to 97% of HCMV-seropositive lactating women (Lanzieri et al., 2013). Children with congenital HCMV infection exhibit the highest prevalence (80%) and longest duration of excretion of HCMV in urine and saliva, with a steep decline by age 5 years. Excretion of HCMV is more frequent in healthy children attending day care (23%) than in healthy children not attending day care (12%). Peak prevalences in children occurred at age 1–2 years, and HCMV was more prevalent in urine specimens than in oral secretions (Cannon et al., 2011). [The Working Group noted that the duration of HCMV excretion in different specimens can vary.]

Fig. 1.3 Estimated global HCMV seroprevalence in women of reproductive age



HCMV, human cytomegalovirus.

[The Working Group noted that reproductive age was not defined in this article. It is commonly defined as 15–49 years (WHO, 2025), but this may vary between studies.]

Reproduced from Zuhair M et al., Estimation of the worldwide seroprevalence of cytomegalovirus: a systematic review and meta-analysis, *Reviews in Medical Virology*, Volume 29, issue 3, John Wiley and Sons (Zuhair et al., 2019).

Later studies have found that the prevalence of HCMV excretion among children in the United Kingdom (UK) increases from 0% among neonates and infants aged < 2 weeks to 11.0% among those aged 2 weeks to 5 years and 5.2% among those aged 6–10 years (Fowler et al., 2022). In the USA, HCMV excretion in urine and saliva was reported to be 17.0% among children aged 0–47 months, with half of anti-HCMV IgG–seropositive children excreting HCMV in at least one fluid (Stowell et al., 2014). In day-care centres in France, among the 1770 enrolled children with samples suitable for analysis, the HCMV shedding prevalence was 40% and was highest in children aged between 12 and 18 months, in children with a history of HCMV infection in at least one parent, and in children from families with a mid-level income (Alain et al., 2020). In Colombia, the prevalence of congenital HCMV was 8.4 per 1000 live births. Among infants tested at age 3 and 6 months, 9.9% and 54.7%, respectively, were excreting HCMV in the urine, probably as a result of postnatal infection acquired via breast milk (Rico et al., 2021).

In a review of various studies, it was reported that adults with risk factors such as attendance at a clinic for sexually transmitted diseases had a higher prevalence of HCMV excretion (22%) than did adults without risk factors (7%); HCMV excretion was more common in the urine in the former group and in genital secretions in the latter (Cannon et al., 2011).

1.3.3 Treatment and vaccination

Most HCMV infections do not need therapeutic management; a mild infection in a healthy person will resolve without the need for treatment.

Treatment is considered in immunosuppressed patients (solid organ or haematopoietic stem cell transplant recipients and patients with AIDS) after primary infection and to prevent or control reactivation. Available molecules for treatment of HCMV infections target several essential steps of the viral replication cycle. Inhibitors of pUL54 viral polymerase, such as ganciclovir and its prodrug valganciclovir, are the most widely used. Ganciclovir activity requires activation via phosphorylation by the HCMV serine/threonine kinase UL97 followed by two phosphorylations by cellular kinases. In cases of haematological toxicity or emergence of resistance mutations, other inhibitors of viral polymerase such as foscarnet or cidofovir may be used as alternatives. More modern inhibitors have been developed that target the terminase complex (letermovir) or viral kinase UL97 (maribavir) and are indicated respectively for the prevention of reactivation in transplant patients and the management of infections that are refractory or resistant to polymerase inhibitors (Gourin et al., 2023). All these inhibitors are contraindicated in pregnant women.

Aciclovir, a viral polymerase inhibitor usually used to treat HSV infections, is offered to pregnant women with a primary HCMV infection in the first trimester, to prevent HCMV transmission (Chatzakis et al., 2024). Several studies showed promising results for hyperimmune globulins as a therapeutic agent for preventing antenatal HCMV transmission (Nigro et al., 2005), but two randomized controlled trials failed to show a significant effect of such therapy on transmission rates in pregnant women with a primary HCMV infection (Revello et al., 2014; Hughes et al., 2021). In cases of symptomatic congenital HCMV infection, neonates are treated with valganciclovir (Kimberlin et al., 2015).

No vaccine is available for HCMV, but several trials are ongoing (Akingbola et al., 2025).

1.4 Quality of exposure assessment in key epidemiological studies of cancer and mechanistic studies in humans

See Table S1.3 (Annex 1, Supplementary material for Section 1, Exposure characterization, available from: https://publications.iarc.who.int/667).

HCMV is widespread throughout the world, with varying seroprevalence (see Section 1.3). The Working Group reviewed the exposure assessment methods used in a selection of epidemiological studies on the association between cancer risk and pre-diagnostic HCMV infection. In additional studies reviewed in Section 2, HCMV infection was assessed in tissues after cancer diagnosis. Detection methods in blood and tissues are discussed in Section 1.2, and additional details on some methodological issues concerning the detection of HCMV in various tissues can be found in Section 4.

Two main aspects to be considered when judging the quality of exposure assessment in these studies were the methods and assays used to detect HCMV infection, and the sample storage conditions and preparation methods.

1.4.1 Detection methods

Different detection methods were used in these studies (for details, see Section 1.2). Indirect diagnostic methods for the measurement of anti-HCMV IgG or IgM were performed using commercial kits or in-house methods based on various technologies (ELISA, bead-based multiplex immunoassay, or not specified). Detection of IgG, which is an adequate method for the detection of previous HCMV infection, was commonly carried out using ELISA (Lehtinen et al., 2003; Cox et al., 2010; Amirian et al., 2013). Coghill et al. (2022) used an in-house bead-based multiplex immunoassay method that was not described in detail. [The Working Group could not judge the adequacy of the method because of the lack of information.] In the largest study on cancer risk associated with HCMV infection, Geris et al. (2022) assessed the serostatus of 247 318 recipients of solid organ transplants in the USA. [However, lack of information on the IgG antibody-detection method used prevented the Working Group from assessing the method quality.] There are several causes of variation in IgG titres among recipients of solid organ transplants, which may make interpretation of the results more difficult.

In two studies, IgM was measured in addition to IgG (Lehtinen et al., 2003; Amirian et al., 2013). [Amirian et al. (2013) reported a very high rate of detection of IgM positivity, which might indicate cross-reactivity or polyclonal stimulation. The Working Group noted that measurement of IgM is not informative because IgM is not a sensitive marker for HCMV replication.]

In one case–control study on non-Hodgkin lymphoma (NHL), it was not specified whether it was IgM or IgG that was measured by ELISA [the Working Group assumed that it was IgG] (Mueller et al., 1991). Antibody titres of anti-HCMV IgG were analysed in samples stored for an average of 63 months before diagnosis (Mueller et al., 1991). There is no agreement on the definition of high titres, and antibody levels may vary between two samples from the same patient taken at different times, depending on the clinical context and the state of the immune response at the time of collection.

Direct detection of HCMV proteins (pp65, CMV-IL0, and CMV-CXC-1) has also been performed with a bead-based multiplex immunoassay (Wiemels et al., 2022). Rates of detection of HCMV protein (~90%) in children were higher than those for congenital HCMV infection described in the literature, which were between 0.3% and 1% worldwide (Leruez-Ville and Ville, 2020). [The Working Group noted that detection of this protein in children could result from either infection of the child or transfer

from the mother. Considering that the detection frequencies were much higher than the usual rates for congenital HCMV, it was likely that some of the positive results were a consequence of maternal transfer; however, the contribution of both potential sources remained difficult to estimate. Therefore, the Working Group noted that the HCMV proteins measured in this study should not be interpreted as a measure of HCMV infection.] In addition, the performance of the in-house bead-based multiplex immunoassay was not described (Wiemels et al., 2022).

The NAATs used in these studies most often included qPCR (Gustafsson et al., 2006; Geris et al., 2023), although one used ddPCR and NGS (Francis et al., 2017). [The Working Group noted that NAAT-based methods are highly sensitive and are the preferred methods for the detection of ongoing viral replication (see Section 1.2.1(b)). Owing to their high sensitivity, there is a risk of false-positive signals resulting from contamination.]

In a cohort study on haematological malignancies and tumours of the central nervous system (CNS) in Sweden, Wiemels et al. (2019) analysed the association between specific International Classification of Diseases (ICD) codes for clinically apparent HCMV infection and malignancies in registries. The exposure assessment was based only on codes, without details on how infection was measured, and asymptomatic HCMV infections were not captured. In addition, the ICD code O35.3 is not specific for HCMV, as it includes other maternal infections. This made it difficult for the Working Group to judge the quality of the exposure assessment methods applied.

1.4.2 Samples analysed

Whatever the assay used, it is essential to consider the sample quality, which is influenced by storage conditions and duration. The collection of samples long before diagnosis in prospective studies is important to assess a temporal association between HCMV exposure and cancer outcomes, but this might result in long storage durations and therefore risk of sample degradation.

In some studies, there was prolonged sample storage with a risk of degradation. For example, in a nested case–control study, Cox et al. (2010) measured seroconversion in cases of breast cancer and controls in pre-diagnostic samples collected on average 8.5 years apart. Although the sample storage conditions described in this study were good, the long-term storage of samples (> 5 years) raised the question of serum stability, which also applied to other studies with long sample storage. Moreover, in some cases, after a positive serology result for the first sample, the second sample gave a negative result, which suggested a loss of quality in some samples.

In a case–control study nested in two large cohorts (the Norwegian Janus Serum Bank and United States Cancer Prevention Study II, CPS-II), Coghill et al. (2022) assessed the association between antibodies to six human herpes viruses (including HCMV) and glioma. An in-house bead-based multiplex immunoassay allowed the detection of antibodies to HCMV proteins pp150, pp52, and pp28. Samples analysed were collected at least 5 years, but some more than 20 years, before diagnosis. [The Working Group assumed that serology was performed once the case–control study population was assembled (i.e. after diagnosis) and not at the time of sample collection.] The long storage duration before analysis could be a limitation because of the use of an in-house assay that required the presence of high-quality HCMV proteins and that could thus lack sensitivity.

In several case–control studies, the role of HCMV was investigated in haematological malignancies, in particular, childhood ALL ($n = 5$), NHL, and chronic lymphocytic leukaemia (CLL). To assess the association between anti-HCMV IgG or IgM and ALL or other types

of childhood leukaemia, one study relied on maternal serum samples from Finland and Iceland that were stored under good conditions (Lehtinen et al., 2003).

In the other four studies on ALL, HCMV was measured in dried blood spots (or Guthrie cards) by PCR, NGS, or viral protein detection. Degradation of samples during long-term storage and the use of extraction methods that are not validated for HCMV can be an issue in the analysis of dried blood spots. In the earliest study, qPCR was used to analyse dried blood spots from 46 children who had developed ALL and from controls; no positive samples were found among the cases or controls (Gustafsson et al., 2006). Certain limitations can be highlighted, such as the storage conditions for dried blood spots and the extraction method used, which was not validated for HCMV detection. [The Working Group expected that these limitations would result in false-negative results.] In two additional studies, HCMV was detected in dried blood spots (Francis et al., 2017; Wiemels et al., 2022), indicating more adequate storage conditions or extraction methods. With an HCMV positivity rate (0.5%) similar to that already described for congenital HCMV and validated methods of detection, the large-scale study by Geris et al. (2023) (1189 cases of ALL and 4756 matched controls) seemed to be more robust than were other studies.

A case–control study on CLL was designed to analyse the role of polyomaviruses in CLL (Robles et al., 2015). For this purpose, the authors developed a multiplex serology assay that could also detect antibodies to HSV-1 and HCMV. Decreased HCMV seroreactivity was observed in cases of CLL, but sample collection was not described, and the results may reflect the global hypogammaglobulinemia described in patients with leukaemia.

All these studies used very different assays, and comparison was not always possible. Moreover, some results did not seem to correlate with the commonly reported prevalence values in the epidemiological literature on HCMV infection (e.g. Gustafsson et al., 2006, and Wiemels et al., 2022).

2. Cancer in Humans

Introduction

The evidence for cancer in humans for HCMV has not been previously evaluated by the *IARC Monographs* programme.

A systematic search was conducted in the PubMed database to identify relevant studies (see General Remarks in the present volume). The search terms and lists of retrieved studies are available from: https://hawcproject.iarc.who.int/assessment/713/.

Among human cancers, HCMV has been most frequently evaluated with respect to cancers of the CNS and the lymphohaematopoietic system, as well as with cancers of the digestive system, reproductive system, respiratory system, and breast. The studies included retrospective cohort or nested case–control studies in which HCMV status before cancer diagnosis was determined from stored specimens or medical records. In most studies, HCMV status was measured in cases after diagnosis; these included case–control studies making between-person comparisons, and case series making within-person comparisons of HCMV status in tumour and non-tumour tissue from the same individuals (hereafter referred to as "within-person comparison studies").

As obtaining brain tissue from healthy individuals without cancer or other brain conditions (controls) is complex, studies using convenience samples of controls (e.g. without matching) were described alongside more well-defined case–control studies. For other cancer outcomes, smaller case–control studies (fewer than 25 cases or controls) that also lacked a clear description of the control group recruitment or matching

procedures were considered to be uninformative (e.g. Yao et al., 1993; Fillet et al., 1995; Zhao et al., 1995; Harkins et al., 2002; Hermouet et al., 2003; Qian et al., 2005; Barzon et al., 2008; Harkins et al., 2010; Mohamed et al., 2014; Mehrabani-Khasraghi et al., 2016; Bakhtiyrizadeh et al., 2017; Loutfy et al., 2017; Sepahvand et al., 2019; Hunter-Schlichting et al., 2021; Youssry et al., 2022; Abdul-Razzaq et al., 2023; Khasawneh et al., 2024). Small studies ($n < 100$) of post-diagnostic samples reporting prevalence estimates of either 0% or 100% in both exposed cases and controls for all markers of HCMV tested, as true differences may be undetectable if close to 0% or 100% were also considered to be uninformative (e.g. Park et al., 2010; Zhang et al., 2011; Gupta et al., 2016; Geßner et al., 2021).

Case series without within-person comparisons were not routinely described or cited, apart from glioma, for which an assessment of the variability in detection within larger case series (including more than 50 cases) was deemed to be useful for interpreting comparative studies. Serology-based case series, ecological studies, individual case reports, and studies without cancer end-points or tumours were not considered informative for the present evaluation and are not described here.

The studies are presented by cancer types, with cohort or nested case–control studies described first, in chronological order, followed by case–control studies, within-person comparison studies, and meta-analysis. Case series are described in Annex 3 (Supplementary material for human cytomegalovirus, Section 2, Cancer in humans, online only, available from: https://publications.iarc.who.int/667).

2.1 Glioma and other cancers of the central nervous system

See Table 2.1 and Table S2.2 (Annex 3, Supplementary material for human cytomegalovirus, Section 2, Cancer in humans, online only, available from: https://publications.iarc.who.int/667).

There were three cohort studies (Wiemels et al., 2019; Coghill et al., 2022; Geris et al., 2022), eight case–control studies (Mitchell et al., 2008; Ranganathan et al., 2012; Amirian et al., 2013; Lin et al., 2016; Han et al., 2017; Ghaffari et al., 2021a; Fall et al., 2022; Peredo-Harvey et al., 2023), and one within-person comparison study (Scheurer et al., 2008) exploring the association between HCMV and glioma or other cancers of the brain (for details on the classification of tumours of the CNS, see WHO Classification of Tumours Editorial Board, 2021). A case–control study by Priel et al. (2015) was considered to be uninformative because of the small sample size and the lack of details on the exact tumour histologies included, both for the glioma cases and the non-glioma brain tumour controls, limiting the ability to fully assess the appropriateness of the methodology used. The case–control study by Wen et al. (2020) was considered to be uninformative, because the methods were not described in enough detail to allow the Working Group to fully evaluate the reliability of the study results.

Case series were considered relevant only if they were based on strong virological methods and included at least 50 cases of brain tumour (see the supplementary text in Annex 3). Table S2.2 also includes the results for prevalence of PCR, ISH, or IHC reported in case–control studies also examining tumour tissue, to compare the results across studies providing prevalence of HCMV content in tumour tissues. A case series reported by Wang et al. (2017a) was excluded because the results of HCMV testing on 64 cases of glioma were not reported, as this was not the primary focus of their analysis.

Table 2.1 Epidemiological studies on HCMV and glioma and other cancers of the central nervous system

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Wiemels et al. (2019) Record linkage, Sweden Children born in 1987–2014/ follow-up to 2015 Cohort	2 782 507 children born in 1987–2014, identified through the Medical Birth Registry, followed up until age 15 yr or 2015. Linkage with cancer registry, cause of death. *Exposure assessment method*: Clinically apparent HCMV infection was identified from medical records of patient registries for children ≥ 6 mo before diagnosis of malignancy and for their mothers during pregnancy.	CNS (childhood)	Prior (≥ 6 mo before diagnosis) maternal or childhood HCMV infection (prevalence, %): CNS-free cohort (n = 2 781 470) Cases (n = 933)	1037 1	0.037% 0.11%	None	*Exposure assessment critique*: Little detail in the exposure data. Adequacy of the test could not be determined because no information was available. ICD code O35.3 is not specific for HCMV but includes other maternal infections. As exposure was assessed through medical records, exposure was likely to be underestimated because HCMV testing is not systematic and is prompted by clinical signs. The frequency of active HCMV infections diagnosed (0.04%) was an order of magnitude lower than the expected frequency of congenital HCMV. *Design and analysis*: *Strengths*: Use of cohort design. *Limitations*: Only 1 exposed case was reported.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Geris et al. (2022) Transplant Cancer Match Study, USA 2000–2017 Cohort	247 318 patients within the Transplant Cancer Match Study with available anti-HCMV IgG serology status of donor (D) and recipients (R) at the time of transplant. Exclusion: history of cancer, HIV infections, multiple donors. Linkage to SEER cancer registry. *Exposure assessment method*: Measurement of anti-HCMV IgG, data obtained from Scientific Registry of Transplant Recipients. No information on tests used or timing.	CNS	HCMV donor (D) and recipient (R) HCMV serostatus (adjusted IRR): Low risk (R–D–) (reference) Intermediate risk (R+) High risk (R–D+)	 17 58 14	 1 0.89 (0.46–1.69) 0.60 (0.25–1.40)	Sex, age, race/ ethnicity, transplant organ type, EBV R/D status, SES	*Exposure assessment critique*: Lack of information on detection method precluded judgement of method quality. Used pre-transplant donor and recipient serostatus to assess risk of infection post-transplant rather than measured HCMV infection post-transplant, which could lead to misclassification of exposure. *Design and analysis*: *Strengths*: Use of an ideal high-risk population (immunosuppressed individuals are at a high risk of virus-associated cancer). *Limitations*: Small number of CNS tumours; no assessment of viral activity in tumour.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Coghill et al. (2022) Janus Serum Bank (Janus), Norway; CPS-II, USA Janus: NR; CPS-II: 1998–2001 to 2013 Nested case–control	*Source cohort*: Janus serum bank participants. CPS-II participants who provided samples in 1998–2001. *Cases*: 323 (Janus), 37 (CPS-II); glioma cases (lag was 3 yr in CPS-II and 5 yr in Janus) *Controls*: 323 (Janus), 74 (CPS-II); matched (1:1 in Janus, 2:1 in CPS-II) on birth date, sex, date blood drawn, and, in Janus only, county of residence. *Exposure assessment method*: Seropositivity for three different HCMV antigens (pp150, pp52, pp28) measured by GST capture assay combined with bead-based multiplex immunoassays in pre-diagnostic samples.	Brain (glioma)	HCMV pp150, 28, 52 positivity, Janus cohort (adjusted OR):			Matched variables	*Exposure assessment critique*: Limited validation for in-house assays precluded judgement of the adequacy of methods. Co-exposures other than viruses were not measured. *Other comments*: Some method details from Egan et al. (2021). *Design and analysis*: *Strengths*: Pre-diagnosis HCMV antibody measures; long follow-up time. *Limitations*: Based on circulating antibodies against HCMV and did not account for the presence or activity of the virus in tumour tissue.
			HCMV (pp150)	246	1.24 (0.85–1.79)		
			HCMV (pp52)	238	1.07 (0.74–1.54)		
			HCMV (pp28)	261	1.32 (0.88–1.98)		
		Brain (glioma)	Gliomas (adjusted OR):				
			CPS-II cohort: HCMV (pp150)	26	1.18 (0.53–2.66)		
			HCMV (pp52)	27	1.18 (0.53–2.66)		
			HCMV (pp28)	26	1.20 (0.52–2.79)		
		Brain (GBM)	GBM, Janus cohort (adjusted OR):				
			HCMV (pp150)	NR	1.00 (0.63–1.58)		
			HCMV (pp52)	NR	0.85 (0.54–1.34)		
			HCMV (pp28)	NR	1.04 (0.62–1.74)		
		Brain (GBM)	GBM, CPS-II cohort (adjusted OR):				
			HCMV (pp150)	NR	1.00 (0.40–2.53)		
			HCMV (pp52)	NR	1.00 (0.40–2.53)		
			HCMV (pp28)	NR	1.18 (0.44–3.20)		
		Brain (non-GBM glioma)	Non-GBM gliomas, Janus cohort (adjusted OR):				
			HCMV (pp150)	NR	1.86 (0.97–3.56)		
			HCMV (pp52)	NR	1.67 (0.88–3.16)		
			HCMV (pp28)	NR	1.92 (0.98–3.76)		
			HCMV (≥ 1 of the 3 antigens)	NR	2.08 (1.07–4.03)		
		Brain (non-GBM glioma)	Non-GBM gliomas, Janus cohort (5–12 yr from blood draws) (adjusted OR):				
			HCMV (pp150)	NR	4.0 (1.13–14.2)		
			HCMV (pp28)	NR	4.5 (0.97–20.8)		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Coghill et al. (2022) (cont.)		Brain (non-GBM glioma)	Non-GBM glioma, CPS-II cohort (adjusted OR):			Matched variables	
			HCMV (pp150)	NR	2.00 (0.36–11.2)		
			HCMV (pp52)	NR	2.00 (0.36–11.2)		
			HCMV (pp28)	NR	1.24 (0.25–6.06)		
			HCMV (≥ 1 of the 3 antigens)	NR	2.00 (0.36–11.2)		
Mitchell et al. (2008) Duke University, USA NR Case–control and case–case	*Cases*: 45 patients with GBM, with tumour and blood samples stored at the Duke tissue bank. *Controls*: 17 patients undergoing craniotomy for reasons other than GBM. Additionally, adjacent normal tissue from 33 cases. *Exposure assessment method*: HCMV DNA, using PCR in tissue samples; IHC for IE1-72 antibody; and ISH.	Brain (glioma)	Prevalence of HCMV DNA in peripheral blood (%):			None	*Strengths*: use of multiple detection methods including ISH and IHC. *Limitations*: Little detail on method and extent of age-matching process. *Other comments*: Additional results in Table S2.2[a].
			GBM cases (n = 20)	16	80%		
			Age-matched healthy controls (n = 17)	0	0%		
			Other brain tumours (n = 3)	0	0%		
			Metastatic tumours (n = 3)	0	0%		
		Brain (glioma)	Prevalence of HCMV DNA in fresh tissue, real-time PCR (%):				
			Control samples (template-negative, metastatic brain from breast or colon, meningiomas, acoustic neuroma, epidermoid tumours)	0	0%		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Ranganathan et al. (2012) University of Wisconsin, Madison, USA 1994–2009 Case–control	*Cases*: 75 GBM, 6 meningioma, 5 schwannoma, 5 oligodendroglioma; samples stored in the pathology archives of the Hospital and Clinics at the University of Wisconsin, Madison. *Controls*: 15 non-neoplastic brain samples from patients with epilepsy stored in the same biobank. *Exposure assessment method*: HCMV DNA, using PCR in tissue samples.	Brain (glioma)	Positivity of HCMV DNA in tissue (crude OR): Epilepsy (n = 8) GBM (n = 35)	 3 19	 [1] [1.98 (0.41–9.59)]	None	*Strengths*: Multiple viral gene products were targeted in the exposure assessment. *Limitations*: Only one detection method (PCR) was used, small sample size, lack of adjustment. The more frequent detection of viral genes in frozen samples than in paraffin-embedded archival tissue samples from the same patients indicated some level of misclassification when using archival specimens. *Other comments*: Additional results in Table S2.2[a]. Of 12 HCMV genomic loci tested, 9 were more prevalent in GBM samples than in other brain tumour types or in brain specimens from patients with epilepsy.
Amirian et al. (2013) Harris County, USA 2001–2006 Case–control	*Cases*: 362 histologically confirmed cases of glioma (ICD-O-3 codes 9380–9481). *Controls*: 462 cancer-free controls recruited through by random-digit dialling using standard methods, frequency-matched to cases on age (within 5 yr), race/ethnicity, and sex.	Brain (glioma)	Serum anti-HCMV IgG positivity (adjusted OR): Controls (n = 462) Cases (n = 362)	 270 207	 1 1.04 (0.78–1.39)	Age, sex, and race/ethnicity	*Exposure assessment critique*: IgM measurement was not useful in this setting. Very high detection rate of IgM positivity, which could be due to cross-reactivity or polyclonal stimulation.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Amirian et al. (2013) (cont.)	*Exposure assessment method*: Measurement of HCMV IgG and IgM levels in blood samples.	Brain (glioma)	Level of serum anti-HCMV IgG (reference was highest IgG levels), anti-HCMV IgG–positive individuals (OR):			Age, sex, and race/ ethnicity	*Design and analysis*: *Strengths*: Large number of glioma cases and frequency-matched random-digit dialling controls. *Limitations:* Examined only blood-based IgM and IgG anti-HCMV antibodies (prevalence and levels); however, these are standard markers of active and prior HCMV exposures, respectively, although IgM are not specific enough to be considered reliable.
			< 10 U/mL	NR	2.51 (1.42–4.43)		
			10–29 U/mL	NR	1.51 (0.89–2.58)		
			≥ 30 U/mL	NR	1		
			Trend-test *P* value, 0.0008				
		Brain (glioma)	Serum anti-HCMV IgM positivity (OR):				
			Controls	300	1		
			Cases	232	0.97 (0.72–1.31)		
		Brain (glioma)	Level of serum anti-HCMV IgM, anti-HCMV IgM–positive individuals (reference was highest IgM levels) (OR):				
			< 10 U/mL	NR	1.03 (0.56–1.88)		
			10–29 U/mL	NR	0.94 (0.53–1.66)		
			≥ 30 U/mL	NR	1		
Lin et al. (2016) George Washington University Hospital and the National Institutes of Health, USA NR Case–control	*Cases*: 63 unique adult brain tissue samples (including 45 GBM, 12 astrocytoma grade III, 2 astrocytoma grade II, 4 astrocytoma grade I). *Controls*: Brain samples from 49 patients with non-neurological disease (29 male patients). *Exposure assessment method*: ddPCR for HCMV DNA (UL55-gB).	Brain (astrocytoma)	Positivity frequency of HCMV DNA (UL55-gB) in brain specimens (%):			None	*Strengths*: Inclusion of a housekeeping gene (rpp30) in the ddPCR reactions and the validation of the multiplex PCR results in a single-plex reaction. *Limitations*: Demographic data were not reported for cases; controls had a wide age range; there was no matching or controlling for basic demographic variables.
			Cases (*n* = 63)	0	0%		
			Controls (*n* = 49)	1	[2%]		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Han et al. (2017) First Hospital of China Medical University, China 2012–2014 Case–control	*Cases*: 150 patients with glioma, including 33 astrocytoma (grade II), 22 anaplastic astrocytoma (grade III), 95 GBM, grade IV). *Controls*: 150; tumour free (with traumatic brain injury) controls matched on geography, ethnicity, age, gender. *Exposure assessment method*: PCR: HCMV gB (UL55) gene in blood samples and IHC for expression of the HCMV proteins IE1-72 and pp65 in tissue samples; ELISA.	Brain (glioma)	Prevalence of HCMV DNA (UL55-gB) in peripheral blood (%):			None	*Strengths*: Multiple detection methods and three viral targets, use of matched controls. *Limitations*: Only seropositivity was included for case–control comparisons. *Other comments*: See also Table S2.2[a].
			Glioma cases (n = 150)	48	32%		
			Controls (n = 150)	0	0%		
		Brain (glioma)	Presence of HCMV IgG (unconditional OR):			Geography, ethnicity, age, gender	
			Controls (n = 150)	108	1		
			Cases (n = 150)	112	[1.15 (0.69–1.91)]		
		Brain (glioma)	Presence of HCMV IgM (unconditional OR):				
			Controls (n = 150)	23	1		
			Cases (n = 150)	28	[1.27 (0.69–2.32)]		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Ghaffari et al. (2021a) Hazrate Rasoole Akram Hospital in Tehran, Islamic Republic of Iran 2014–2017 Case–control	*Cases*: 42 FFPE GBM samples from the hospital pathology department. Exclusion: glioma grades I–III or other brain tumour types or in patients who underwent antiviral therapy. *Controls*: 42 brain autopsies (cause of death different from brain disease) from the Iranian Legal Medicine Organization (January to September 2018). No matching was performed. *Exposure assessment method*: nPCR targeting the HCMV UL55 gene.	Brain (glioma)	Prevalence of HCMV (UL55 gB gene) (%): GBM (n = 42) Controls (n = 42) χ^2 test P value, 0.07	 3 0	 7.1% 0%	None	*Limitations*: No matching, no control for basic demographic variables. An important sex difference was present between cases and controls. Only one detection method and one viral target was used.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Fall et al. (2022) Neurosurgery Department of Hospital University (CHU) Hassan II of Fez, Morocco 2014–2019 Case–control	*Cases*: 102 adult patients with glioma, including 46 GBM, 36 astrocytomas (non-GBM), 3 oligodendrogliomas, and 17 ependymomas. *Controls*: 32 non-tumour patients (9 encysted haematoma, 13 meningeal haemorrhage, and 10 non-tumour primary cerebral epilepsy). No matching. *Exposure assessment method*: nPCR for HCMV IE gene promoter.	Brain (glioma)	Prevalence of HCMV by nPCR (%): Glioma (n = 102) Controls (n = 32) P value, 0.001	 31 0	 30.4% 0%	None	*Strengths*: Included all patients seen in a neurosurgery department. *Limitations*: Only one method for detection and one viral target. Small sample size, and the number of controls was only 30% that of cases.
Peredo-Harvey et al. (2023) Karolinska University Hospital, Sweden 2005–2009 Case–control	*Cases*: 59 patients with brain tumour, with plasma sample (25 GBM, 18 non-GBM astrocytomas, 4 secondary GBM, 1 ganglioglioma, 1 meningioma, and 10 metastatic tumours in the brain). *Controls*: 114 healthy individuals from the hospital blood centre (age-matched). *Exposure assessment method*: Serological assessment of HCMV IgG using ELISA.	Brain (GBM) Brain (astrocytoma)	Positivity to ant-HCMV IgG (unconditional OR): Age-matched healthy donors (n = 32) Primary GBM (n = 25) Positivity to HCMV IgG (unconditional OR): Age-matched healthy donors (n = 77) Astrocytoma II–III (n = 18)	 27 20 63 16	 1 [0.74 (0.19–2.91)] 1 [1.78 (0.37–8.63)]	Age	*Other results*: IgG levels were higher in patients with GBM (P = 0.008) or with astrocytoma grades II–III (P = 0.003) than in age-matched healthy controls. No cases or controls were positive for HCMV IgM. *Strengths*: Age-matching. *Limitations*: Small number of brain tumours. Reliance on a single detection method. Only seropositivity was assessed.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Scheurer et al. (2008) University of Texas MD Anderson Cancer Center, USA NR Within-person controls	*Cases*: 50 patients with glioma samples banked in a study on adult glioma at MD Anderson, including 21 GBMs, 12 anaplastic gliomas, and 17 low-grade gliomas. *Controls*: 27 samples with healthy tissue margin (7 GBM, 9 anaplastic, 11 low-grade glioma). *Exposure assessment method*: IHC for IE1, ISH (cocktail of 12 probes).	Brain (glioma)	HCMV positivity (IHC) in tumour cells versus vs normal tissue (%): GBM tumour cells Non-tumour cells of tumour tissue Trend-test *P* value, < 0.001 Non-tumour cells of tumour tissue	 NR NR NR	 79% 79% 4%	None NR	*Other comments*: Additional results: HCMV nucleic acids were detected by ISH in tumour cells and endothelial cells in the tumours, but not in control non-tumour brain cells (results in Table S2.2[a]). *Strengths*: Use of multiple detection methods, comparison by tumour histology types. ISH with multiple probe-cocktail. *Limitations*: IHC only targeted IE1. *Other comments*: Additional results: HCMV nucleic acids were detected by ISH in tumour cells and endothelial cells in the tumours, but not in control non-tumour brain cells (results in Table S2.2[a]).

CI, confidence interval; CNS, central nervous system; CPS-II, Cancer Prevention Study-II; D, donor; ddPCR, digital droplet PCR; EBV, Epstein–Barr virus; ELISA, enzyme-linked immunosorbent assay; FFPE, formalin-fixed paraffin-embedded; GBM, glioblastoma; HCMV, human cytomegalovirus; HIV, human immunodeficiency virus; ICD, International Classification of Diseases; IE1, immediate-early 1 protein; IgG, immunoglobulin; IHC, immunohistochemical; IRR, incidence rate ratio; ISH, in situ hybridization; mo, month(s); nPCR, nested polymerase chain reaction; NR, not reported; OR, odds ratio; PCR, polymerase chain reaction; PP, phosphoprotein; R, recipient; RT-PCR, reverse transcription-polymerase chain reaction; SEER, Surveillance, Epidemiology and End-Results; SES, socioeconomic status; Tx, transplant; USA, United States of America; vs, versus; yr, year(s).

[a] Table S2.2 is provided in Annex 3, Supplementary material for human cytomegalovirus, Section 2, Cancer in humans, online only, available from: https://publications.iarc.who.int/667).

There was also a meta-analysis (Farias et al., 2019) that reported pooled estimates of HCMV in glioma from 32 previously published studies, many of which were too small to be included in the present summary (e.g. Cobbs et al., 2002; Rahbar et al., 2015). Additionally, Peredo-Harvey et al. (2021) reported a pooled prevalence for HCMV in GBM samples across studies collected in a systematic literature review.

2.1.1 Cohort studies

Wiemels et al. (2019) conducted an analysis of a retrospective cohort study on the relationship between HCMV infections reported in the medical record and development of brain tumours, as part of a study primarily focused on childhood leukaemia and using data from several registries in Sweden (reviewed in Section 2.2). Of the 933 children diagnosed with a brain tumour, only one child had a prior diagnosis of HCMV disease (≥ 6 months) before the brain tumour diagnosis (0.11%) compared with 1037 children diagnosed with HCMV out of a population of 2 781 470 without CNS tumours (0.037%). [The Working Group noted that only one exposed case was detected, which reduced the informativeness of the study for the present evaluation, although the cohort design was considered to be a strength, and the lack of a large number of cohort studies meant that the Working Group considered the study to be of some informativeness. However, the Working Group also noted that not all infections would warrant medical attention or treatment, and exposure to the virus was likely to be underestimated using this method of exposure assessment.]

Geris et al. (2022) reported the analysis of a retrospective cohort study conducted through the Transplant Cancer Match Study that linked data from the Scientific Registry of Transplant Recipients (SRTR) (which includes all transplants carried out in the USA since 1987) with cancer data from 32 state and regional cancer registries. After exclusions, follow-up data were available for 247 318 solid organ transplant recipients through linkage to the cancer registries. Serological testing for anti-HCMV IgG antibodies in both the organ donor and the recipient before transplant was used to classify patients into three groups: negative for both donor and recipient (reference category), positive for the recipient regardless of donor serostatus, and positive for the donor and negative for the recipient (this latter was identified as the higher risk group). During follow-up, there were 89 tumours of the brain or other CNS tumours (0.75%). After adjusting for age, sex, socioeconomic status, EBV recipient and donor status, and the organ transplanted, there was no increase in risk of brain and other CNS tumours among the recipient-positive (incidence rate ratio, IRR, 0.89; 95% CI, 0.46–1.69) or the recipient-negative/donor-positive (IRR, 0.60; 95% CI, 0.25–1.40) groups, compared with the recipient-negative and donor-negative group. [The Working Group noted that a strength of this study was that transplant recipients who are immunosuppressed and are at higher risk of developing virus-associated cancers are an ideal high-risk group in which to evaluate this hypothesis. The Working Group also noted as a limitation that the small number of CNS tumours represented in the cohort limited the ability to examine risk for any subgroups of CNS cancers, specifically glioma. A further limitation was the lack of routine or longitudinal tracking of HCMV activity or disease in recipients. Also, circulating IgG antibodies against HCMV do not account for the presence or activity of the virus in the tumour tissue.]

Coghill et al. (2022) reported a nested case–control analysis from two large cohorts, the Janus Serum Bank in Norway and CPS-II in the USA. The Janus Serum Bank component included 323 cases of glioma diagnosed ≥ 5 years after blood collection and 323 cancer-free controls (one per case) individually matched to each case on birth date within 2 years, sex, date of blood draw

within 12 months, and county of residence. The CPS-II component included 37 cases of glioma diagnosed ≥ 3 years after blood collection and two cancer-free controls individually matched to each case on birth date within 1 year, sex, and date of blood draw within 6 months. A multiplex serological assay was used to measure IgG antibodies to six human herpes viruses, including HCMV pp150, pp52, and pp28 antigens. Control seropositivity ranged from 66% to 77% across the three antigens and the two cohorts. No associations were observed between HCMV antigens and GBM in either cohort. However, individuals with evidence of serological response to at least one of the three HCMV antigens were two times as likely to be diagnosed with a non-GBM glioma in the Janus (OR, 2.08; [95% CI, 1.07–4.03]; $P = 0.03$) and CPS-II (OR, 2.0; [95% CI, 0.36–11.2]) cohorts, although the estimated increases were very imprecise, particularly in the smaller CPS-II cohort (only 10 non-GBM tumours were included). These results were especially elevated for the pp150 and pp28 antigens (ORs, 1.86 [95% CI, 0.97–3.56] and 1.92 [95% CI, 0.98–3.76], respectively), within the Janus cohort. There was no reported evidence of a dose–response trend for any of the antigens (P for trend ranged from 0.23 to 0.41). Risk of non-GBM tumours was highest for the time period 5–12 years after blood draw (ORs, 4.0; [95% CI, 1.13–14.2; $P = 0.03$] for pp150 and 4.5 [95% CI, 0.97–20.8; $P = 0.05$]) for pp28 in the Janus cohort. [The Working Group noted that the pre-diagnosis measures of HCMV antibody and the long follow-up time afforded by these cohorts provided temporal support for a potential causal association. However, the association was based on circulating antibodies to HCMV and did not demonstrate the presence or activity of the virus in tumour tissue.]

2.1.2 Case–control studies

Mitchell et al. (2008) used three methods of detection (IHC, ISH, and PCR amplification of viral DNA) to detect HCMV in tumour and blood samples from 45 patients with GBM and in blood samples from 17 "normal" volunteers (patients with non-malignant conditions or health-care workers) recruited at Duke University, USA. They detected HCMV in > 90% of GBM tumours by IHC or ISH and in 61.7% by PCR. There was no immunoreactivity in surrounding "normal" brain. They also detected HCMV by PCR in the blood from 80% of GBM patients but none among the controls. [The Working Group noted as strengths of this study that the investigators used multiple detection methods, compared tumour areas with surrounding non-tumour brain tissue in the cases, and compared viral DNA in the blood in patients with GBM and in control participants. Given the hypervascularized nature of tumours, a high prevalence of virus in the blood, detected by PCR, could be expected. The controls were reportedly age-matched with the cases; however, the method and extent of the matching were not reported. Also, the controls ($n = 17$) were only included in an analysis of HCMV DNA in the blood compared with cases for whom blood samples were available ($n = 20$), which represents a small sample size. Additionally, the Working Group noted that in this study fewer tissue samples gave positive results by PCR (which should be more sensitive) than by IHC or ISH.]

Ranganathan et al. (2012) used PCR amplification of 12 viral gene products to detect HCMV in samples from 75 patients with GBM, 6 patients with meningioma, 5 patients with schwannoma, 5 patients with oligodendroglioma, and 15 control participants with non-neoplastic epilepsy recruited from the University of Wisconsin–Madison, USA. They reported that multiple HCMV viral genes (9 of the 12 tested) were more likely to be found in GBM samples (55%) than in other types of brain tumour (42%) or in brain

specimens from participants with epilepsy (34%). They also noted that the viral genome was more often detected in frozen samples than in paraffin-embedded archival tissue samples from the same patients. [The Working Group noted that an advantage of this study was that investigators targeted multiple viral gene products in their assessment. However, they used only one detection method (PCR), and the inclusion of smaller numbers of patients with non-GBM tumours and controls limited the interpretability of the results. Additionally, the more frequent detection of viral genes in frozen samples compared with paraffin-embedded archival tissue samples from the same patients indicated some level of misclassification when using archival specimens. The authors did not report on the age of the included case and control participants; however, given the description of the tumours included, they were assumed to be adults.]

Amirian et al. (2013) assessed levels of anti-HCMV IgM and IgG among 362 patients with newly diagnosed, histologically confirmed glioma (ICD-O-3 codes 9380–9481), identified between 2001 and 2006 by hospital physicians in and around Harris County, Texas, USA, and 462 cancer-free controls recruited through by random-digit dialling using standard methods, frequency-matched to cases on age (within 5 years), race/ethnicity, and sex. Anti-HCMV positivity was not associated with glioma risk (IgG OR, 1.04; 95% CI, 0.78–1.39; and IgM OR, 0.97; 95% CI, 0.72–1.31), after adjusting for age, sex, and race/ethnicity. However, among participants who were IgG-positive (57% of cases and 58% of controls), increasing anti-HCMV IgG levels were associated with decreasing glioma risk (*P* for trend = 0.0008), with those at the lowest level of anti-HCMV IgG (< 10 U/mL) having the highest risk of glioma (adjusted OR, aOR, 2.51; 95% CI, 1.42–4.43). [The Working Group noted as the primary strength of this study the inclusion of samples from a large number of adult cases of glioma and the frequency-matched controls identified by random-digit dialling (based on age, sex, and race/ethnicity). Although recent studies that use random-digit dialling may be biased because of lower participation, this study was conducted in an era when those types of controls were highly beneficial to case–control studies. This study examined only blood-based IgM and IgG anti-HCMV antibodies (prevalence and levels), without an assessment of tumour markers of HCMV. The finding that higher levels of IgG, among those who were seropositive, was associated with lower odds of glioma was in line with the results of studies on other markers of immune surveillance (i.e. prevalence of allergies and atopic disorders and IgG levels against those conditions) on the risk or odds of glioma.]

Lin et al. (2016) reported on the presence of multiple human herpes viruses, including HCMV, among 63 patients with astrocytoma of various grades (grade IV, 45; grade III, 12; grade II, 2; and grade I, 4) and 49 control patients with non-neurological disease recruited from the George Washington University Hospital and the National Institutes of Health, USA. All samples (except for tissue from one control) tested for HCMV (UL55 or gB) by ddPCR gave negative results. [The Working Group noted that the controls included in this study were deceased patients with a variety of non-neurological conditions for whom fresh or FFPE brain tissues collected at autopsy were available for analysis. The controls were both men and women ranging in age from 23 to 86 years (mean, 51.7 years); however, the mean age for the cases was reported as 35 years, and the sex ratio was not reported. Two advantages to the methods used in this study were the inclusion of a housekeeping gene (*rpp30*) in the ddPCR reactions and the validation of the multiplex PCR results via a single-plex reaction.]

Han et al. (2017) recruited 150 patients with glioma (grade II, 33; grade III, 22; and grade IV, 95) and 150 tumour-free control participants with traumatic brain injury, matched on

geography, ethnicity, age, and gender from the Neurosurgery Department of the First Hospital of China Medical University, China, between January 2012 and December 2014. To detect HCMV, they used nPCR to amplify the HCMV gB (UL55) gene in blood samples and IHC to assess expression of HCMV proteins IE1–72 and pp65 in tissue samples. Additionally, anti-HCMV IgG and IgM were assayed in serum samples collected from the patients. HCMV DNA was detected in the blood for 32% of patients with glioma, equivalently by tumour grade, but not in any control patients. Immunoreactivity to IE1–72, assessed in cases only, was observed in 78% of glioma specimens, including 73% of grade II, 68% of grade III, and 82% of grade IV tumours. Expression of pp65 was detected in 66% of glioma samples, including 64% of grade II, 59% of grade III, and 68% of grade IV samples. Anti-HCMV IgG was detected in the serum of 74.7% of patients with glioma (grade II, 79%; grade III, 77%; and grade IV, 73%) and in 72% of controls (OR, [1.15; 95% CI, 0.69–1.91]). Anti-HCMV IgM was detected in the serum of 18.7% of patients with glioma (15% of grade II, 18% of grade III, and 20% of grade IV) and in 15.3% of controls (OR, [1.27; 95% CI, 0.69–2.32]). [The Working Group noted important strengths for this study in that the investigators used multiple detection methods and three viral targets. Also, the cases and controls were matched on several relevant factors, most importantly age. One limitation was that the only comparison between cases and controls was by HCMV seropositivity; no tissue comparisons were made. Given the incomplete understanding of the mechanisms involved in the relationship between HCMV and glioma, having confirmation of the presence of the virus in tumour tissue would provide additional evidence regarding the potential activity, albeit not necessarily on the carcinogenicity, of the exposure.]

Ghaffari et al. (2021a) conducted a study on 42 FFPE tumour samples from patients with GBM and 42 samples of brain autopsies from participants without neurological disorders, collected between August 2014 and December 2017 from the Pathology Department of Hazrate Rasoole Akram Hospital in Tehran, Islamic Republic of Iran (subsequently referred to as "Iran"). Using an nPCR targeting the HCMV UL55 (gB) gene, HCMV DNA was detected in 3 out of 42 (7.1%) GBM samples and in none of the control group samples. [The Working Group noted several limitations for this study. First, no matching between cases and controls was performed. Although the controls were fairly similar to the cases in age (mean age, 40 and 47 years, respectively), 98% of the controls were male compared with only 69% of the cases, which meant that confounding by sex and residual confounding by age could not be ruled out. Second, the investigators used only one detection method against only one viral target. These limitations reduced the informativeness of the study for the present evaluation.]

Fall et al. (2022) conducted an analysis of 102 gliomas (GBM, 46; non-GBM astrocytoma, 36; oligodendroglioma, 3; and ependymoma, 17) collected between February 2014 and December 2019 at the Neurosurgery Department of the Hospital Centre of the University (CHU) Hassan II of Fez, Morocco. They compared these with brain tissues removed during neurosurgery or biopsy from 32 control patients without a tumour (encysted hematoma, 9; meningeal haemorrhage, 13; and primary cerebral epilepsy, 10). The status of isocitrate dehydrogenase genes *IDH1* and *IDH2* in fresh frozen tissue samples was determined using standard sequencing, and nPCR was used to detect HCMV by amplifying a region of the HCMV IE gene promoter. HCMV was detected in 30% of glioma samples and in none of the non-tumour control patients. Older patients (aged ≥ 50 years) had a higher prevalence (53%) of HCMV. [The Working Group noted several limitations of this study.

First, the investigators used only one method of detection against one viral target. Second, there were only 30% as many controls as cases included in the study, and the controls had other neurological conditions that may hinder comparability; however, the study included all consented patients seen at the hospital's neurosurgery service. There was also no description of the demographics of the control group, and no matching was performed.]

Peredo-Harvey et al. (2023) conducted a case–control analysis of anti-HCMV antibodies (IgG and IgM) from 59 patients with brain tumours (GBM, 25; non-GBM astrocytoma, 18; secondary GBM, 4; ganglioglioma, 1; meningioma, 1; and metastatic tumours in the brain, 10) compared with 114 age-matched healthy participants recruited from Karolinska University Hospital, Sweden, between 2005 and 2009. Anti-HCMV IgG antibodies were detected by ELISA in 80% of patients with GBM, 89% of patients with astrocytoma grades II–III, and 80% of healthy donors. IgG levels were significantly higher in patients with GBM and in patients with astrocytoma grades II–III than in age-matched healthy donors. All patients and healthy controls were negative for HCMV IgM. [The Working Group noted that a strength of this study was the age-matched control population for whom seroprevalence of IgG and IgM was compared with that for the cases. Two notable weaknesses, however, were the small numbers of each brain tumour type included in the study and the reliance on one viral detection method (serostatus) without investigating status in tumour tissue.]

2.1.3 Within-person comparison studies

Scheurer et al. (2008) reported on the detection of HCMV antigen and DNA in 50 brain samples containing glioma and non-tumour tissue from the same patients, collected from The University of Texas MD Anderson Cancer Center, USA (GBM, 21; anaplastic glioma, 12; and low-grade glioma, 17) using sensitive IHC (IE1) and ISH (cocktail of 12 probes) methods. By tumour grade, 100% of grade IV GBM samples were positive for HCMV compared with 75% of anaplastic glioma samples, and 82% of samples of low-grade tumours. By histology, 94% of astrocytic tumours were positive for HCMV compared with 85% of oligodendrocytic tumours and 33% of ependymal tumours. By IHC, the HCMV-positive cells were found to be primarily restricted to areas of tumour tissue (79%), and not the surrounding non-tumour tissue areas (4% positivity). By ISH, HCMV nucleic acids were detected in tumour cells and in endothelial cells within the tumours, but not in non-tumour brain cells. [The Working Group noted two strengths of this study, in that the investigators used two methods of detection (IHC and ISH) and that they made comparisons of HCMV prevalence by histological tumour type. The Working Group noted as limitations that the IHC method targeted only IE1, whereas the ISH method used a multiprobe cocktail, and that there was a low number of tumours of each histological type. The potential for reverse causation in within-person comparisons was also noted, potentially if the virus demonstrates tropism for immune cells, which could be enriched within a tumour for various reasons, including hypervascularization.]

2.1.4 Meta-analyses

Farias et al. (2019) conducted a meta-analysis that included 32 previously published studies. The studies included a total of 2190 cases: 1871 cases of glioma and 319 of non-glioma. The overall pooled estimate of HCMV positivity was 63% (95% CI, 56–70%), resulting in a meta-estimate (meta-OR) of the association between HCMV and glioma of 3.04 (95% CI, 1.7–5.3) after adjusting for comparative group type, viral marker, and laboratory diagnostic method. Of note, among the 31 studies pooled, there were eight studies (with number of cases ranging

from 17 to 58) in which no samples were identified as positive for HCMV. When limiting the pooled prevalence estimate to only those studies in which virus was detected in at least one sample, the prevalence increased to 70% (95% CI, 63–77%). No correlation between the association and histological subtype or tumour grade was reported. [The Working Group noted the importance of this meta-analysis in summarizing the prevalence of HCMV and the association with glioma across these studies, as the variation in detection across studies of various designs was considered useful for gauging the strength of comparative studies. One limitation was that the summary estimates spanned several types of glioma histology. Another limitation was the inclusion of studies that were not considered by the Working Group to be individually informative for the present evaluation.]

Peredo-Harvey et al. (2021) carried out a systematic review that included 81 previously published studies on the association between HCMV and GBM only. They reported on the prevalence of HCMV among 9444 samples from 81 research articles, including overall, by detection method, and by viral target when using IHC. The overall prevalence of HCMV was 38% in all samples tested and 36% among the tissue samples tested. The prevalence was higher when using IHC (48.2%) or immunofluorescence (54.8%) than with ISH (38.9%) or PCR (29.1%). [The Working Group noted that the strengths of this summary study were that it focused on one histological type (GBM), included a large number of patients or samples from a large number of published studies, and that it provided summary estimates by detection method and viral target used. These important aspects of the association between HCMV and GBM had not been previously assessed on this scale. A limitation was the inclusion of studies that the Working Group did not consider to be individually informative.]

2.2 Haematolymphoid malignancies

See Table 2.3.

This section describes studies with haematological and lymphoid cancer end-points in humans. The Working Group identified 12 studies that had pre-diagnostic samples, including six cohort studies (Mueller et al., 1991; Wolff and O'Donnell, 2001; Opelz et al., 2009; Wiemels et al., 2019; Geris et al., 2022; Namayandeh et al., 2022) and six studies nested within cohorts (Lehtinen et al., 2003; Katz et al., 2007; Wiemels et al., 2022) or within population-based sources (Gustafsson et al., 2006; Francis et al., 2017; Geris et al., 2023). There were also nine case–control studies that had post-diagnostic samples (Herne et al., 2003; Csire et al., 2007; Ballanger et al., 2009; Michos et al., 2009; Steininger et al., 2009; Omer et al., 2011; Robles et al., 2015; Kadry et al., 2016; Janković et al., 2022). A case–case comparison by Gallant et al. (2022), comparing prevalence by subtypes of ALL, was also considered to be informative.

Haematolymphoid malignancies in adults are described first, followed by those in children. Studies including both children and adults are described within the section on adults. Cohorts are described in chronological order, and case–control studies are organized by cancer type and then chronologically.

2.2.1 Haematolymphoid malignancies in adults

(a) Cohort or nested case–control studies

Mueller et al. (1991) conducted a study to examine pre-diagnostic serostatus of HCMV (and EBV), on average 63 months before diagnosis. They used a case–control design nested in a series of cohorts based in four existing serum biobanks of samples from healthy individuals (from Washington County, Maryland; Northern California; Norway; and the Hypertension Detection and Follow-up Repository serum bank,

Table 2.3 Epidemiological studies on HCMV and haematolymphoid malignancies

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Mueller et al. (1991) Four serum banks: Washington County, USA; Norwegian Cancer Society, Norway; the joint Northern California Kaiser-Permanente Medical Care Program, and Hypertension Detection and Follow-up Repository, USA Enrolment, 1974, 1973, 1964–1971, 1972–1973, respectively/ follow-up, NR Nested case–control	*Source cohort*: Four serum banks including > 240 000 individuals. *Cases*: 104 NHL cases identified through linkage with the cancer registry (Washington, Norway), medical data (Kaiser cohort), or unspecified methods (Hypertension Detection and Follow-up Repository). *Controls*: 259 individuals matched on sex, ethnicity, index date of serum collection, date of birth (2:1 except from the Washington County where 4 controls per case were selected). Individuals with a history of cancer were excluded. *Exposure assessment method*: HCMV antibodies detected by ELISA (dual reviewed) collected on average 63 mo before NHL diagnosis.	NHL (B-cell lymphoma)	Any elevation in titres of Ab to HCMV proteins (RR):			Sex, race/ethnicity, date serum collection, date of birth	*Exposure assessment critique:* Class of HCMV Ab not defined, but presumably IgG. No consensus on the definition of using high titres when multiple values were available. Very variable time between serum collection and diagnosis. Serum samples were stored under good conditions. *Comment*: Blood specimens were stored in the bank on average 63 mo (5.2 yr) from index date. *Design and analysis:* *Strengths:* Use of matched controls, adjustment for important confounders, use of pre-diagnostic sample. *Limitations:* Single, one-time measurement.
			None	NR	1		
			Any elevation	104	0.6 (0.3–1.2)		
		NHL (B-cell lymphoma)	Any elevation in titres of Ab to HCMV proteins (RR):			Sex, ethnicity, date of birth, date of serum draw, EBV antibodies	
			None	NR	1		
			Any elevation	104	0.4 (0.2–0.9)		

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Wolff and O'Donnell (2001) Georgetown University Medical Center, USA first era (pre-HAART): 1993–1995 second era: 1997–2000/ follow-up, NR Cohort	204 (first era), 114 (second era); all HIV-infected patients referred to the pulmonary and critical care service at Georgetown. The separation by era reflected stratification by use of HAART (common in second era). *Exposure assessment method*: Medical records indicating prior infection.	NHL	Prior history of HCMV infection, era 2 patients: (OR): No Prior HCMV disease χ^2 test *P* value, 0.013	 NR NR	 1 5.99 (1.27–28.33)	None	*Limitations:* Lack of control for HAART, which is a major driver of risk of NHL in this population. Small sample size.
Opelz et al. (2009) Collaborative Transplant Study, international (EU, North America, Australia) 1995–2007 Cohort	18 682 kidney Tx recipients within the Collaborative Transplant Study with known EBV status pre-Tx. *Exposure assessment method*: Medical records of prior infection.	NHL	Hospitalized for HCMV in year 1 post-Tx (adjusted OR): Not hospitalized Hospitalized for HCMV	 NR NR	 1 6.1 (2.0–18.4)	Age, gender, race, continent of residence, year of Tx, Tx number (first, second ...), deceased or living donor, immunosuppressive medication	*Strengths:* Strong methodology. Large sample size. *Limitations:* Confounding by EBV is an issue and might explain the results observed. *Other comments*: There was no significant difference in lymphoma rates according to HCMV+ or HCMV– serostatus among either EBV+ or EBV– recipients (log rank $P = 0.55$ and $P = 0.57$, respectively).

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Geris et al. (2022) Transplant Cancer Match Study, USA 2000–2017 Cohort	247 318 patients within the Transplant Cancer Match Study with available HCMV IgG serology at the time of Tx (solid Tx). Exclusion: history of cancer, HIV infections, multiple donors. Linkage to SEER cancer registry. *Exposure assessment method*: Measurement of anti-HCMV IgG, data obtained from Scientific Registry of Transplant Recipients.	NHL	HCMV status (R/D) (IRR):			Sex, age, race/ ethnicity, organ type, SES quintile, EBV R/D status	*Exposure assessment critique:* Lack of information on detection method precluded judgement of method quality. Used pre-Tx donor and recipient serostatus to assess risk of infection post-Tx rather than measuring HCMV infection post-Tx, which could lead to misclassification of exposure. *Design and analysis:* *Strengths:* Large sample size. *Limitations:* No assessment of viral activity in tumour.
			R (–); D (–)	48	1		
			R (+)	164	1.36 (0.66–2.80)		
			R (–); D (+)	58	1.56 (0.74–3.30)		
		NHL (DLBCL)	HCMV status (R/D) (IRR):				
			R (–); D (–)	267	1		
			R (+)	680	0.83 (0.69–1.00)		
			R (–); D (+)	246	0.74 (0.59–0.91)		
		HL	HCMV status (R/D) (IRR):				
			R (–); D (–)	19	1		
			R (+)	21	0.47 (0.19–1.18)		
			R (–); D (+)	21	0.75 (0.29–1.96)		
		ALL	HCMV status (R/D) (IRR):				
			R (–); D (–)	4	1		
			R (+)	11	0.95 (0.23–3.85)		
		AML	HCMV status (R/D) (IRR):				
			R (–); D (–)	13	1		
			R (+)	76	1.83 (0.84–3.95)		
			R (–); D (+)	23	1.58 (0.67–3.70)		
Namayandeh et al. (2022) Namazi hospital, Islamic Republic of Iran April 2018 to March 2019 Cohort	143 recipients of solid organ Tx at Namazi hospital followed up for PTLD (paediatric and adult combined). *Exposure assessment method*: Real-time PCR for HCMV and EBV DNA.	NHL (B-cell lymphoma, PTLD)	PTLD occurrence during follow-up (%):			None	*Limitations:* Small study; no covariate control; low positivity rates for both viruses; no serology so only assessed active viraemia; results not reported for HCMV mono-infected.
			HCMV-EBV coinfected patients (n = 18)	8	[44%]		
			Mono-infected patients (total n = 125)	1	[0.8%]		

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Omer et al. (2011) Children's Central Teaching Hospital and Medical City Teaching Hospital in Baghdad, Iraq 2006–2008 Case–control	*Cases*: 291; patients with leukaemia (acute and chronic) (age range, 1–72 yr; 66%, < 15 yr; median age, 26.4 yr). *Controls*: 350 healthy blood donors, patients attending primary clinic for vaccination, family members of patients with leukaemia. *Exposure assessment method*: Serological testing for anti-HCMV IgG using ELISA.	Leukaemia	HCMV seropositivity IgG (%):			None	*Strengths:* Availability of data by subtypes. *Limitations:* Comparison to controls not very informative due to the lack of matching or controlling for covariates. Use of post-diagnostic sample.
			All leukaemia cases	280	96.2%		
			ALL	193	97.5%		
			CLL	17	94.4%		
			AML	31	93.9%		
			CML	39	92.8%		
			Controls	320	91.4%		
			All leukaemia (children aged < 15 yr)	183	94.6%		
			All leukaemia (adults aged > 15 yr)	97	99.0%		
		Leukaemia	HCMV seropositivity IgM (%):				
			All leukaemia cases	35	12%		
			ALL	29	14.6%		
			CLL	1	5.6%		
			AML	2	6.1%		
			CML	3	7.1%		
			Controls	28	8%		
			All leukaemia (< 15 yr)	11	5.7%%		
			All leukaemia (> 15 yr)	24	24.5%		

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Csire et al. (2007) Hungary NR Case–control	*Cases*: 95 patients with monoclonal gammopathy (69 multiple myeloma, 16 MGUS, 10 WM). *Controls*: 44 gammopathy-negative control participants with NHL. *Exposure assessment method*: Serological testing for anti-HCMV IgG using ELISA.	Multiple myeloma and MGUS, WM	Seropositivity to anti-HCMV IgG (%):			None	*Other results:* Fisher exact test for comparison of each disease group with controls was $P > 0.05$. *Limitations:* No covariates considered. Potential confounding by age, sex, and HHV-8.
			Multiple myeloma ($n = 52$),	46	88%		
			MGUS ($n = 16$)	11	92%		
			WM ($n = 100$)	8	100%		
			Control (NHL) ($n = 27$)	18	67%		
		Multiple myeloma and MGUS, WM	HCMV by PCR (%):				
			Multiple myeloma ($n = 69$)	8	12%		
			MGUS ($n = 16$)	1	6%		
			WM ($n = 10$)	4	40%		
			Control (NHL) ($n = 44$)	4	9%		

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Steininger et al. (2009) Vienna, Austria 2004–2005 Case–control	*Cases*: 100 consecutive patients with CLL (Caucasian [White]), attending the referral centre at the University of Vienna. For additional comparison, an additional 100 CLL patients from University of California in San Diego were used in a separate analysis. *Controls*: 100 individuals matched (1:1) on age and gender, drawn from healthy Caucasian [White] patients attending the hospital virology clinic to determine virus-specific immunity after vaccination. *Exposure assessment method*: Serological testing for anti-HCMV IgG using ELISA.	NHL (CLL)	HCMV seropositivity (IgG), using CLL Vienna cases (OR): HCMV seropositivity	79	[2.84 (1.52–5.29)] Fisher exact test *P* value, 0.001	Age and gender	*Strengths:* Use of age- and sex-matched control group. Assessment of EBV. *Limitations:* Small sample size. Use of post-diagnostic sample. No adjustment for confounding by EBV, despite measuring EBV.
		NHL (CLL)	HCMV seropositivity (IgG), using CLL cases from USA (%): HCMV seropositivity	53	53%	None	

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Robles et al. (2015) Multicentre study, Spain 2010–2012 Case–control	*Cases*: 359 cases of CLL (incident and prevalent). *Controls*: 370 population-based, frequency-matched on age, sex, and region. *Exposure assessment method*: Multiplex serology with fluorescent-bead technology to measure Ab to viral antigens, pp150 for HCMV.	NHL (CLL)	HCMV seropositivity (IgG) (OR):			Sex, region, age	*Exposure assessment critique:* The performance of the in-house multiplex technique is not described in detail which precludes from judging the adequacy of methods. *Design and analysis:* *Strengths:* Controls matched on age, sex, and region. *Limitations:* Use of post-diagnostic sample. Potential confounding by immune status or infectious agents because of lack of adjustment. *Other comments*: Results for other viruses tested indicated a lower frequency of positivity in CLL cases than in controls.
			HCMV serostatus	261	0.51 (0.36–0.74)		
		NHL (CLL)	HCMV seroreactivity (IgG) (OR):				
			Tertile 1	129	1		
			Tertile 2	60	0.45 (0.30–0.68)		
			Tertile 3	72	0.52 (0.35–0.78)		

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Kadry et al. (2016) Egypt 2009–2011 Case–control	*Cases*: 100 newly diagnosed lymphomas (83 NHL, 14 HL) at Medical Oncology Clinics, National Cancer Institute, Cairo University. *Controls*: 100 healthy controls, matched on age and sex (recruitment not described). *Exposure assessment method*: Seropositivity for HCMV IgG and IgM using ELISA.	Lymphoma (type not specified) Lymphoma (type not specified) Lymphoma (type not specified)	HCMV seropositivity IgM and IgG (%): Cases (IgM+) Controls (IgM+) Cases (IgG+) Controls (IgG+) HCMV seropositivity IgG (OR): Negative Positive HCMV positivity by PCR (%): Cases Controls	 12 0 84 82 16 84 11 0	 12% 0% 84% 82% 1 [1.15 (0.55–2.41)] 11% 0%	Age and sex	*Limitations:* No risk calculations provided. Use of post-diagnostic samples. No separation of HL and NHL. Potential for confounding or reverse causation because of immune status.
Janković et al. (2022) Serbia NR Case–control	*Cases*: 83 B-cell leukaemia/lymphoma cases recruited at a single centre. *Controls*: 259 (77 after matching) population-based, without a history of cancer, matched on age and gender. *Exposure assessment method*: Seropositivity for IgG using ELISA.	Lymphatic and haematopoietic (B-cell leukaemia or lymphoma)	HCMV seropositivity (IgG) (OR): HCMV seropositivity	 75	 [0.12 (0.01–1.01)]	Adjusted for age and gender.	*Strengths:* Age and sex propensity score–matching. *Limitations:* Small sample size. Cases and controls analysed in two different laboratories with different methodologies. No further adjustments for immune-related factors.

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Herne et al. (2003) MD Anderson Cancer Center, USA 1992–2001 Case–control	*Cases*: 116 cases of mycosis fungoides or Sézary syndrome; biopsy stored at MD Anderson (1992–2001). *Controls*: 1322 healthy bone-marrow donors (1988–2001) evaluated at MD Anderson (at baseline, before donating). *Exposure assessment method*: Seropositivity for IgG using ELISA.	Cutaneous T-cell lymphoma (mycosis fungoides, Sézary syndrome)	HCMV IgG seropositivity, age < 55 yr (%): Cases (n = 116) Controls (n = 1322) P value of t-test comparison, 0.001	113 757	97.4% 57.3%	None	*Limitations:* Use of post-diagnostic sample. Lack of adjustment for confounding by patient characteristics (age, sex, SES, immune status).
Ballanger et al. (2009) Nantes, France NR Case–control	*Cases*: 27 patients with mycosis fungoides, 21 with Sézary syndrome, 24 with parapsoriasis. *Controls*: 124 healthy subjects with HCMV serology from the same laboratory in the same period. *Exposure assessment method*: Seropositivity for IgG using ELISA and HCMV DNA using PCR.	Cutaneous T-cell lymphoma	HCMV seropositivity IgG (%): Small plaque parapsoriasis (n = 12) Large plaque parapsoriasis (n = 13) Mycosis fungoides (n = 27) Sézary syndrome (n = 21) Healthy control participants (n = 124)	4 6 18 9 46	33.3% 46.1% 66.67% 42.86% 37.1%	None	*Limitations:* No matching or adjustment for age, sex, or immune status, and lack of information on the control group. *Other comments*: The only two HCMV DNA–positive biopsies were detected for 2 cases of Sézary syndrome at an advanced stage (after treatment); both were negative at initial sampling, just after diagnosis.

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Ballanger et al. (2009) (cont.)		Cutaneous T-cell lymphoma (parapsoriasis, mycosis fungoides, Sézary syndrome)	HCMV DNA PCR positivity in skin biopsy (%): HCMV DNA (*n* tested, 39)	2	[5%]	None	
Fine et al. (1985) UK NR Cohort	2570 mothers (age < 40 yr) who experienced infection in utero when pregnant, identified through previous study on antenatal infection or by direct contact with clinicians. Information on health of the offspring was obtained through contact with general practitioner. *Exposure assessment method*: Medical records of antenatal infection.	All cancers combined: childhood cancer	Maternal HCMV infection during pregnancy (*n*): Cases of childhood ALL	1	NC	None	The other cancer detected among HCMV in utero exposed is a single testicular cancer. *Limitations:* Small numbers of cases precluded adjustment for potential confounding. The methods are not well described.

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Lehtinen et al. (2003) Finnish Maternity Cohort (Finland), Rubella Screening Serum Bank (Iceland) Finland, 1983–1997; Iceland, 1975–1997 Nested case–control	*Source cohort*: Finnish Maternity Cohort (750 000 serum samples from 500 000 mothers) and Rubella Screening Serum Bank (75 000 serum samples collect from all pregnant Icelandic women, 1975–1997). *Cases*: childhood leukaemia cases (342 ALL, 61 non-ALL leukaemia) in the offspring (age < 15 yr), identified through cancer registries. *Controls*: 1261 mother–child (free of cancer) pairs matched (Finland, 3:1; Iceland, 4:1; incidence-density sampling) on index maternal age at serum sampling, date of specimen collection, offspring date of birth and gender. Samples collected at 12–14 wk of gestation. *Exposure assessment method*: Seropositivity for HCMV IgG and IgM was measured by ELISA in pre-diagnostic samples.	ALL (childhood) ALL (childhood) Childhood leukaemia (non-ALL) Childhood leukaemia (non-ALL)	Maternal pregnancy seropositivity to HCMV IgM (OR): Seropositive Maternal pregnancy seropositivity to HCMV IgG (OR): Seropositive Maternal pregnancy seropositivity to HCMV IgM (OR): Seropositive Maternal pregnancy seropositivity to HCMV IgG (OR): Seropositive	 10 75 5 78	 1.3 (0.9–1.9) 0.9 (0.7–1.2) 0.5 (0.1–1.6) 1.3 (0.6–2.8)	Matching variables	*Exposure assessment critique:* Good storage conditions for serum samples. Analysis of only one sample during the pregnancy. IgM is not a sensitive marker for HCMV replication. *Design and analysis:* *Strengths:* Large record-linkage study. Use of pre-diagnostic, maternal samples. *Limitations:* Lack of adjustment for EBV, which was associated with childhood ALL risk. No assessment of treatment-related effects.

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Katz et al. (2007) Children's Memorial Hospital, USA 1988–2002 Nested case–control	*Source cohort*: All patients undergoing heart transplantation at the hospital, receiving ongoing surveillance for post-Tx complications, including PTDL (n = 59) (age ≤ 19.5 yr at time of Tx). *Cases*: 9 patients who developed PTLD during follow-up. *Controls*: 18; matched (2:1) on age at Tx, time since Tx, under follow-up at the time of case diagnosis. *Exposure assessment method*: Seropositivity for IgG using ELISA in donors and recipients.	NHL (B-cell lymphoma), PTLD	Recipient HCMV seronegativity (OR): Recipient HCMV seronegativity	5	9.2 (1.5–55)	Sex, race, donor age, EBV, seronegativity prior Tx, EBV seroconversion, n rejection episodes, n times lympholytics were used Matching variables	*Strengths:* Controlled for critical covariates (sex, race, donor age, recipient diagnosis, donor HCMV seropositivity, recipient treatment for HCMV infection, EBV seropositivity at the time of PTLD diagnosis). *Limitations:* Small study with imprecise estimates.
		NHL (B-cell lymphoma), PTLD	Donor HCMV seropositivity (OR): Donor HCMV+	4	[1 (0.20–5.00)]		
			Trend-test P value, 0.16				
		NHL (B-cell lymphoma), PTLD	HCMV mismatch (D+, R–) (OR): Donor HCMV+, recipient HCMV–	2	[4.86 (0.38–62.63)]		
			Trend-test P value, 013				

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Gustafsson et al. (2006) Sweden 1980–2001 Case–control (with pre-diagnostic samples)	*Cases*: 48 children with ALL with available Guthrie card admitted for treatment at four different Swedish paediatric oncology centres from 1980 to 2001. *Controls*: 46 children without ALL, matched on age and birthplace, drawn from Swedish Medical Birth Register. *Exposure assessment method*: qPCR for HCMV DNA detection from Guthrie cards.	ALL (childhood)	HCMV DNA positivity (%): Cases Controls	 0 0	 0% 0%	Age and birthplace	*Exposure assessment critique:* The study used real-time PCR which is a validated, quantitative method for detection of HCMV DNA, though sensitivity may be lower than digital techniques, which may be important for stored Guthrie cards. The extraction method used was not validated for HCMV. *Design and analysis:* *Strengths:* Pre-diagnostic samples from Guthrie cards. Controls from population-based register. *Limitations:* There was no analysis of other exposures. Low sample size for detecting low prevalences.

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Francis et al. (2017) California Childhood Leukaemia Study, USA 1995 (end of enrolment for this analysis, NR); year of birth ranged from 1982 to 2006, with a median of 1993 Case–control (with pre-diagnostic samples)	*Cases*: 268 cases of ALL and AML from the California Childhood Leukaemia Study. *Controls*: 270 random sample from California Childhood Leukaemia Study control pool (cancer-free, randomly drawn from the California department of Public Health of Vital records, matched on date of birth, race, sex). Because of the use of random control samples, matching factors were used in adjustment set in logistic regression. *Exposure assessment method*: Next-generation sequencing and ddPCR methods were used to detect HCMV and other viruses in bone-marrow and neonate dried blood spot samples.	ALL (childhood)	HCMV PCR in neonatal bloodspots (OR):			Age, sex, race	*Exposure assessment critique:* ddPCR is very sensitive technique, which increases risk of contamination. Very high rates of HCMV positivity (~9%) correspond to levels of HCMV that are likely to be not clinically relevant, given that the rate of congenital HCMV infection is between 0.3% and 1% worldwide. *Design and analysis:* *Strengths:* Pre-diagnostic samples; large study; EBV as negative control exposure (OR for ALL, 1.0; 95% CI, 0.42–2.42); AML as negative control outcome; population-based. Use of blood spots and bone-marrow samples. *Limitations:* Single time point sample at birth. Lack of matching. Reverse causation possible for bone-marrow sample as at diagnosis.
			HCMV PCR positivity	26	3.71 (1.71–8.95)		
		ALL (childhood)	HCMV PCR in neonatal bloodspots, Hispanic (OR):			Age, sex	
			HCMV PCR positivity	15	5.90 (1.89–25.96)		
		ALL (childhood)	HCMV PCR in neonatal bloodspots, White, non-Hispanic (OR):				
			HCMV PCR positivity	71	2.10 (0.69–7.13)		
		ALL (childhood)	HCMV 454 DNA/RNA sequencing (viral particles) in pretreatment bone marrow, bone-marrow analysis (36 ALL, 10 AML patients) (OR):			None	
			HCMV positivity in AML	NR	1		
			HCMV positivity in ALL	NR	18 (2.04–159.09)		

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Francis et al. (2017) (cont.)							*Other comments*: In addition, total RNA sequencing screening of 91 ALL and 28 AML bone-marrow samples also demonstrated HCMV exclusively within ALL sequencing pools.
Wiemels et al. (2019) Record linkage, Sweden 1987–2014 to 2015 Cohort	2 782 507 children born 1987–2014, identified through the Medical Birth Registry, followed up until age 15 yr or 2015 through cancer registry. *Exposure assessment method*: Clinically apparent HCMV infection was identified from medical records of patient registries for children ≥ 6 mo before diagnosis of malignancy and for their mothers during pregnancy.	Lymphatic and haematopoietic (childhood) (ICD-9, 200–208)	Child HCMV infection (6 mo before diagnosis) or maternal HCMV infection records (HR): No Yes	 2256 9	 1 11.34 (5.89–21.83)	Attained age (timescale), congenital malformations, small or large for gestational age, congenital chromosome abnormalities	*Exposure assessment critique:* Little detail on the exposure data. Adequacy of test could not be determined because no information was available. ICD code O35.3 is not specific for HCMV but includes other maternal infections. As exposure is assessed through medical records, exposure is likely to be underestimated because HCMV testing is not systematic and is prompted by clinical signs. The frequency of active HCMV infections diagnosed (0.04%) was an order of magnitude lower than that than expected for the frequency of congenital HCMV.

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Wiemels et al. (2019) (cont.)							*Design and analysis:* *Strengths:* Large study. *Limitations:* Small number of cases with HCMV and malignancy; no stratification by cancer subtypes; EBV-related disease was not assessed; treatments for HCMV-related diseases or other viral illnesses were not evaluated.
Wiemels et al. (2022) Southern California, USA 1999–2009 Nested case–control	*Source cohort*: California biobank with available mid-pregnancy samples and matched neonatal blood spots. These were surplus specimens from the California Prenatal Screening programme, which offered voluntary screening for chromosomal and neural tube defects to pregnant women between 15 and 20 wk of gestation (participation rate, approximately 70%) (see Kharrazi et al., 2012).	ALL (childhood)	HCMV-IL10 protein levels at birth			Age at collection, year of birth, sex, race/ethnicity, birth weight, gestational age, plurality, birth order, mode of delivery, mother's age at delivery, mother's birthplace, other cytokines	*Exposure assessment critique:* Performance of the in-house bead-based multiplex immunoassay was not described. Positivity rates of HCMV protein (~90%) were much higher than expected based on the rate of congenital HCMV infection, which is between 0.3% and 1% worldwide. This could indicate that detection of the protein does not indicate clinically relevant infection, but protein could derive from maternal transfer.
			Continuous (per unit increase)	NR	1.27 (1.01–1.59)		
			Tertile 1	41	1		
			Tertile 2	40	0.96 (0.56–1.66)		
			Tertile 3	49	1.47 (0.85–2.54)		
		ALL (childhood)	HCMV-pp65 protein levels at birth			Age at collection, year of birth, sex, race/ethnicity, birth weight, gestational age, plurality, birth order, mode of delivery, mother's age at delivery, mother's birthplace	
			Continuous (per unit increase)	NR	1.05 (0.85–1.29)		
		ALL (childhood)	HCMV-vCXCL-1 protein levels at birth				
			Continuous (per unit increase)	NR	0.98 (0.79–1.21)		

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Wiemels et al. (2022) (cont.)	*Cases*: 130 children (aged 0–14 yr) with ALL, born 1999–2009, drawn from California Biobank, with available neonatal blood spot. *Controls*: 460, frequency-matched on year of birth, sex, and race/ethnicity, with available neonatal blood spot. *Exposure assessment method*: Bead-based multiplex immunoassays were used to measure cytokines and HCMV proteins in neonatal blood spots. The assays included calibration standards, quality control samples, and blanks.						*Design and analysis:* *Strengths:* Use of pre-diagnostic samples. *Limitations:* Multiple testing of different HCMV proteins; lack of analysis of diagnostic samples to assess HCMV persistence and presence in the disease. *Other comments*: Raw data were adjusted for batch, age of the blood spot, and level of protein extracted.
Geris et al. (2023) Michigan, USA 1987–2014 Case–control (with pre-diagnostic samples)	*Cases*: 1189 children (aged 0–14 yr, born in Michigan) with ALL, identified through the Michigan Cancer Surveillance Program. HCMV infection assessed by the Michigan BioTrust for Health.	ALL (childhood) ALL (childhood) (hyperdiploidy cases)	HCMV PCR in bloodspots: PCR positivity HCMV PCR in bloodspots, compared with all controls (n = 4756), using exact methods: PCR positivity	6 2	1.30 (0.52–3.24) 6.26 (1.44–27.19)	Maternal age at birth, maternal diabetes, birth weight, gestational age, congenital anomalies NR	*Exposure assessment critique*: Ambient storage temperature of dried blood spots could affect DNA quality but positivity rate was similar to that for congenital HCMV commonly detected, which indicated adequate detection.

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Geris et al. (2023) (cont.)	*Controls*: 4756, cancer-free, matched (4:1) on age, sex, mother's race/ethnicity, within a population-based study on congenital HCMV infection. *Exposure assessment method*: Quantitative PCR was used to detect HCMV DNA in dried blood spot samples (Guthrie cards) collected shortly after birth.						The percentage positivity for HCMV was an order of magnitude lower than that reported by Francis et al. (2017) because of the use of less sensitive methodology (positive prevalence in controls, 7.5 times higher in Francis et al. (2017) than in Geris et al.). *Design and analysis:* *Strengths:* Large number of cases and controls; "real-time" or "quantitative PCR". *Limitations:* Low proportion of cases and controls exposed, particularly for subtypes, led to imprecise estimates. *Other comments*: Only the "high hyperdiploidy" subtype demonstrated HCMV status at birth showing risk. For the hyperdiploidy analysis, unmatched controls were used, as the model with matched controls did not converge (0 exposed among controls).

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Michos et al. (2009) Greece 1996–2003 Case–control	*Cases*: 52 HL, 73 NHL; childhood cancer cases (aged 0–14 yr) identified through a network of six paediatric centres for haematolymphoid malignancies. *Controls*: 52 for HL, 73 for NHL; matched on age and gender, identified among children admitted at the same hospital as cases for minor non-infectious paediatric diseases. *Exposure assessment method*: Seropositivity for IgG using ELISA.	NHL (childhood) HL (childhood)	HCMV seronegativity (IgG) (OR): Seronegative HCMV seronegativity (IgG) (OR): Seronegative	39 NR	2.05 (0.83–5.03) 1.66 (0.55–5.03)	Age, gender, residence, rooms/ person, maternal education, maternal smoking during child life, breast fed, eye colour, sun protection, days spent annually at seaside resort, respiratory syncytial virus (RSV), influenza A and B, para-influenza type 1, adenovirus, Epstein–Barr virus (EBV), herpes human virus 6 (HHV6), and *Bartonella henselae* (B. henselae)	*Strengths:* Examination of and adjustment for multiple infections. *Limitations:* Use of post-diagnostic samples; no adjustment for confounding by immune status.

Table 2.3 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Gallant et al. (2022) California Childhood Leukaemia Study, USA NR Case–case	*Cases*: 743 ALL cases within the California Childhood Leukaemia Study, with bone-marrow samples (median age, 4.9 yr). *Controls*: 125; no controls were used, but the AML cases within the California Childhood Leukaemia Study were used as the comparator (median age, 4.6 yr). *Exposure assessment method*: HCMV DNA assessment of bone marrow.	ALL (childhood)	HCMV positivity by PCR in bone marrow (OR):			NR	*Strengths:* Evaluation of multiple ALL subtypes. *Limitations:* Pre-diagnostic samples or controls were not assessed.
			Negative	NR	1		
			Highest quintile	NR	2.5 (1–5.47)		
		ALL (childhood)	HCMV positivity by PCR in bone marrow (OR):			NR, additionally for ethnicity	
			Negative	NR	1		
			Highest quintile	NR	1.93 (0.44–1.29)		
		ALL (childhood) (hyperdiploid cases)	HCMV positivity by PCR in bone marrow, hyperdiploid compared with other ALL (OR):			Age, ethnicity	
			Negative	NR	1		
			Highest tertile	NR	2.71 (1.34–4.73)		

Ab, antibody(ies); ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; CI, confidence interval; CLL, chronic lymphocytic leukaemia; D, donor; ddPCR, digital droplet polymerase chain reaction; DLBCL, diffuse large B-cell lymphoma; EBV, Epstein–Barr virus; ELISA, enzyme-linked immunosorbent assay; EU, European Union; HAART, highly active antiretroviral therapy; HCMV, human cytomegalovirus; HHV-8, human herpesvirus 8; HIV, human immunodeficiency virus; HL, Hodgkin lymphoma; HR, hazard ratio; ICD, International Classification of Diseases; Ig, immunoglobulin; IL, interleukin; IRR, incidence rate ratio; MGUS, monoclonal gammopathy of undetermined significance; mo, month(s); NHL, non-Hodgkin lymphoma; NR, not reported; OR, odds ratio; PCR, polymerase chain reaction; pp, phosphoprotein; PTLD, post-transplant lymphoproliferative leukaemia; qPCR, quantitative polymerase chain reaction; R, recipient; RR, relative risk; SEER, Surveillance, Epidemiology and End Results; SES, socioeconomic status; Tx, transplant; UK, United Kingdom; US, United States; USA, United States of America; WM, Waldenstrom macroglobulinaemia; yr, year(s).

USA; totalling more than 240 000 participants). Participants who were later diagnosed with NHL ($n = 104$) were compared with matched control participants (matched on sex, ethnicity, birthdate, and date of serum collection; $n = 259$). The study found a reduced relative risk (RR) of NHL with anti-HCMV IgG seropositivity (RR, 0.6; 95% CI, 0.3–1.2), which was accentuated after adjustment for other EBV antibody measurements (RR, 0.4; 95% CI, 0.2–0.9) in patients with NHL compared with controls. [The Working Group noted that the aim of this study was mainly the investigation of EBV serological responses, which were elevated in cases versus controls. EBV is a well-recognized cause of NHL (being classified by IARC as *carcinogenic to humans*, Group 1; IARC, 2012), particularly in relation to adult-onset EBV infections. Upward bias for the estimates for HCMV because of confounding by EBV was considered likely, as the multivariable models that included serological responses to both viruses suggested a stronger inverse association between anti-HCMV IgG and NHL, and a stronger positive association between EBV IgM and NHL (ORs, 2.8 and 3.2 in univariable and multivariable analyses, respectively, controlling for HCMV). The Working Group considered the study to be of high quality because of the careful management of confounders by matching and pre-diagnostic measurement in this nested case–control study within a cohort; however, use of a single measurement and no longitudinal samples was a limitation.]

In a study by Wolff and O'Donnell (2001) in a severely immunocompromised population (i.e. patients with HIV in the 1990s), previous HCMV infection (defined by fulminant symptoms, i.e. documented prior HCMV pneumonitis, retinitis, colitis, or disseminated infection) was strongly associated with the development of NHL in patients treated between 1997 and 2001, a period in which HAART was more common than it is at present (OR, 5.99; 95% CI, 1.27–28.32). [The Working Group considered that strong confounding leading to upward bias may be present, as there was no adjustment for any other viral illnesses or for HAART therapy (which was strongly associated with NHL risk (OR, 15.11). The study was therefore considered to be of limited informativeness, particularly in the modern era of improved treatments for HIV.]

Opelz et al. (2009) conducted a large international study of solid organ (kidney) transplant recipients ($n = 18\,682$) in which hospitalization for HCMV disease in the year after kidney transplantation was associated with NHL (hazard ratio, HR, 6.1; 95% CI, 2.0–18.4; unadjusted for EBV status); however, this relation was not seen after controlling for EBV status before transplantation. Indeed, there was no significant difference in lymphoma rates according to pretransplantation HCMV serostatus (HCMV-positive or HCMV-negative) among either EBV-positive or EBV-negative kidney transplant recipients (log rank $P = 0.55$ and $P = 0.57$, respectively; HRs were not provided). [The Working Group considered this to be a high-quality study to explore the differential risk between EBV- and HCMV-naive participants placed into an immunosuppressive state. The results, including the strong HR between EBV status and NHL (HR, 3.6) among patients who had received a heart transplant, indicated that the association for HCMV was explainable by confounding by EBV.]

Geris et al. (2022) examined HCMV status before transplant in 247 318 pairs of donors and recipients in the Transplant Cancer Match Study (see Section 2.1). Compared with HCMV-seronegative donors with HCMV-seronegative recipients, HCMV-seronegative recipients with HCMV-seropositive donors had a lower risk of diffuse large B-cell lymphoma (DLBCL) in long-term follow-up (OR, 0.74; 95% CI, 0.59–0.91). The risk of DLBCL was also lower in the group of HCMV-seropositive recipients than in the group of HCMV-seronegative recipients with HCMV-seronegative donors (OR, 0.83; 95% CI, 0.69–1.00 for DLBCL). In contrast, for NHL

overall, imprecise increases in risk were noted in HCMV-seropositive recipients (OR, 1.36; 95% CI, 0.66–2.80) and in HCMV-seronegative recipients with seropositive donors (OR, 1.56; 95% CI, 0.74–3.30) compared with the group of HCMV-seronegative donors and recipients. [The Working Group noted the large sample size as a strength but considered the lack of routine or longitudinal tracking of HCMV activity or disease in recipients after transplantation as a drawback in this analysis. The Working Group also noted the lack of treatment data on anti-HCMV prophylaxis post-transplantation and considered that anti-HCMV prophylaxis (antiviral medication) may have off-target effects in reducing the incidence of EBV-driven lymphoma, which could potentially explain the inverse association between HCMV and DLBCL.]

In a study exploring HCMV and post-transplant lymphoproliferative disorder (PTLD) in Iran (Namayandeh et al., 2022), 143 solid organ transplant recipients were assessed for both HCMV and EBV by quantitative polymerase chain reaction (qPCR) methods using DNA isolated from patient sera. HCMV was found in 24 transplant recipients, EBV was found in 34, and both HCMV and EBV were found in 18. PTLD subsequently developed in eight of the 18 HCMV-EBV–coinfected patients, whereas only 1 of 125 non-coinfected patients contracted PLTD. [The Working Group noted the low positivity rates for both viruses and that seroprevalence was not assessed, suggesting that the assay did not capture history of infection – only current active viraemia. Additional limitations were the small sample size, the lack of covariate control, and lack of results for HCMV monoinfection.]

(b) Case–control studies with post-diagnostic samples

The case–control studies described below, grouped by type of outcome (B-cell lymphoma, T-cell lymphoma, adult leukaemia), are characterized by analysis based on samples collected at or after cancer diagnosis. [The Working Group noted that investigations on infection and cancer that begin with ascertainment of cancer cases can be affected by reverse causation because cancer weakens the immune system, or because of the higher risk of nosocomial or iatrogenic infection around the time of diagnosis.]

(i) Adult leukaemia

In a study in Iraq (Omer et al., 2011) that included 291 cases of leukaemia (adults, 33.7%; children, defined as aged < 15 years, 66.3%,) and 350 controls (healthy blood donors, vaccine recipients, and case family members), HCMV serological prevalence was similar in cases and controls (IgG, 96.2% and 91.4%; and IgM, 12% and 8%, for cases and controls, respectively). The study included patients with acute lymphoblastic leukaemia (ALL), acute myeloid leukaemia (AML), CLL, or chronic myeloid leukaemia (CML), with an average age of 26.4 years, including mostly cases aged < 15 years ($n = 193$), but with a considerable number ($n = 98$) aged ≥ 15 years. Patients with ALL ($n = 198$) had the highest frequency of anti-HCMV IgM (14.6%) when compared with patients with CLL (5.6%), AML (6.1%), or CML (7.1%). [The Working Group considered the comparison of leukaemia types to be a strength, but that the comparison with controls without leukaemia was less informative for this investigation because of the lack of matching, separation by age, or controlling for covariates.]

(ii) Adult lymphoma, B-cell lymphoma, and other B-cell malignancies

Csire et al. (2007) conducted a study of the involvement of four herpes viruses in cases of multiple myeloma ($n = 69$) and other monoclonal B-cell gammopathies ($n = 26$) compared with monoclonal gammopathy-negative controls with NHL ($n = 44$) by PCR (for DNA sequences) and serology. No differences were detected in HCMV DNA or in serological responses to HCMV or

other viruses between the study groups. [The Working Group noted that no covariates were considered, not even sex and age, which reduced study informativeness. Confounding by HHV-8 (Kaposi sarcoma virus) was possible, as HHV-8 DNA was more prevalent among cases of multiple myeloma and gammopathy than among controls, although no serological response to HHV-8 was observed.]

Steininger et al. (2009) enrolled 100 patients with CLL from University Hospital Vienna, Austria, and 100 controls selected from the same hospital (patients attending a virology clinic to determine virus-specific immunity after vaccination) and matched on age and gender; 79% of cases and 57% of controls showed seropositivity (IgG) to HCMV ($P = 0.001$; crude OR, [2.84; 95% CI, 1.52–5.29]). However, a second group of CLL cases ($n = 100$) from University of California in San Diego, USA, had a similar rate of HCMV seropositivity (53%) as the Vienna controls, but without comparison to a local matched control group. [The Working Group noted that, although the controls were age- and sex-matched to the 100 CLL cases in Vienna, the small sample size and lack of other information on potential confounders raised the possibility of confounding.]

In a study on adult CLL, 359 cases were compared with 370 population-based controls (frequency-matched on sex, region, and age) for serostatus (IgG antibodies) to HCMV (Robles et al., 2015). Although 261 cases were positive for anti-HCMV IgG, the frequency was lower than that in the age-, sex-, and region-matched controls (OR, 0.51; 95% CI, 0.36–0.74), as well as that for 10 other viruses tested in this study. [The Working Group noted that the matching was a strength but the lack of adjustment for other viruses was a limitation, as this may have accounted for general differences in viral exposure or immune responses and provided a more thorough assessment of the association for HCMV.]

In a study of seroreactivity and viral DNA detection to five viruses and lymphoma (Kadry et al., 2016), 100 patients with lymphoma (including 83 confirmed cases of NHL and 14 confirmed cases of Hodgkin lymphoma, HL) were compared with 100 age- and sex-matched controls in Egypt. Although the cases and controls were similarly seropositive for anti-HCMV IgG (84% and 82%, respectively), the cases were more often seropositive for anti-HCMV IgM (12% and 0%, respectively), an indication of HCMV primary infection, reinfection, or reactivation. [The Working Group noted that the study did not report whether the patients who were positive for anti-HCMV IgM were also those who were positive for HCMV DNA, but this would be expected. A patient with active lymphoma would be immunosuppressed, and the Working Group considered it unsurprising that cases may host an active superinfection, but without pre-diagnostic samples the Working Group could not assess whether the infection was etiologically important or nosocomial. A limitation was that the study did not present results separately for NHL or HL, limiting the informativeness for the present evaluation.]

In a study in Serbia of 83 cases of B-cell leukaemia or lymphoma and 77 controls (matched on age and gender), Janković et al. (2022) found HCMV seropositivity to be higher among controls (98.7%) than cases (90.4%; $P = 0.035$). [The Working Group calculated the unconditional OR in cases compared with controls as 0.12 (95% CI, 0.01–1.01). The Working Group noted as limitations that the small size of the study increased imprecision and prevented any further regression analysis or adjustment for covariates. A further notable limitation was that samples for cases and controls were analysed in separate laboratories with different methodology, limiting the informativeness of the analysis for the present evaluation.]

(iii) Cutaneous T-cell lymphoma

There were two case–control studies on HCMV and cutaneous T-cell lymphoma (CTCL) (Herne et al., 2003; Ballanger et al., 2009).

Herne et al. (2003) examined serostatus for HCMV and other viruses (human T-lymphotrophic viruses HTLV I and II, HIV-1, and EBV). The investigators compared 116 cases (mycosis fungoides or Sézary syndrome) with 1322 unmatched healthy bone marrow donors as controls. After restricting to individuals aged < 55 years, the cases had a significantly higher (97.4%) rate of HCMV seropositivity (IgG) than did the controls (57.3%). [The Working Group noted as limitations that there was no control for patient characteristics (age, sex, SES, education, race/ethnicity), which could lead to confounding, and the lack of information on the temporality of HCMV infection and whether the controls arose from the same population, reducing the informativeness of the study. Of note, the patients with CTCL had a higher rate of HCMV seropositivity than some published estimates in other known immunocompromised groups, such as adolescents with HIV (positivity rate, 79.7%) (Holland et al., 2000) or renal transplant patients (positivity range across racial/ethnicity groups, 80–56%) (Hirata et al., 1996).]

In a study in France, 48 participants diagnosed with CTCL were compared with 124 healthy control participants who were tested for HCMV (Ballanger et al., 2009). Seropositivity was assessed in the same laboratory for both cases and controls. Seropositivity for anti-HCMV IgG in the cases was lower than reported by Herne et al. (2003), at only 66.67% for mycosis fungoides and 42.86% for Sézary syndrome, but significantly higher than that in the controls, 37.1%, which may be related to the lower age of the controls or population exposure differences that were not addressed. HCMV sequences were detected in two patients' tumours but were absent from 37 other participants tested using real-time PCR. [The Working Group noted that no information was provided regarding control selection other than sex or age, and age and sex was not accounted for in the analysis, limiting the study informativeness.]

2.2.2 Childhood leukaemia and other childhood haematolymphoid malignancies

The section below contains a description of studies assessing childhood leukaemia or other childhood cancer as the outcome. Studies that included cancers in both adults and children (Omer et al., 2011) are described above in the section on adult haematolymphoid malignancies (Section 2.2.1).

(a) Cohort and nested case–control studies

In an early study that examined the incidence of cancer after antenatal viral infection (diagnosed in the mother before the birth of her child) (Fine et al., 1985), a cohort of 2570 pregnancies with antenatal infections of the mother were followed for cancer in the offspring. HCMV status was defined as clinical diagnosis in the mother before the child's birth. Among the 97 children exposed to HCMV in utero, two contracted cancer (one acute ALL and one testicular cancer). [The Working Group considered this study to be less informative for the present evaluation because of the small number of participants precluding multivariable regression analyses to control for confounders, and the lack of details provided on the source of the cohort.]

In a nested case–control study within a cohort of mother–child pairs in Finland and Iceland, Lehtinen et al. (2003) examined maternal HCMV IgM antibody responses for children who developed ALL and AML and for healthy matched controls. Maternal samples for a total of 352 children with ALL and 1216 matched controls (matched on the index mother's country and age at serum sampling, date of specimen collection;

and, for the offspring, date of birth and gender, 3 or 4 controls per case) were compared. HCMV IgM seropositivity was slightly higher in cases (10 cases had HCMV and ALL) than in controls (OR, 1.3; 95% CI, 0.9–1.9). [The Working Group noted that a strength of this study was the use of a large record-linkage study and the availability of maternal serology, which represented a pre-diagnostic sample; however, IgM in pregnancy is not considered a sensitive marker for recent HCMV infection or replication. As a limitation, the study did not present results for HCMV with adjustment for EBV seropositivity, which could have acted as a confounder as the study found EBV IgM seropositivity was associated with risk of ALL in the offspring (OR, 2.3). Similarly, the study did not provide any assessment of treatment-related effects such as the impact of antiviral medications.]

Within a cohort study of transplant recipients, Katz et al. (2007) assessed HCMV status in paediatric cardiac transplant patients who progressed to post-transplant lymphoproliferative disorder (PTLD) ($n = 9$) versus those who did not progress ($n = 86$). The 9 patients with PTLD were matched 1:2 on age to 18 controls at transplantation and time since transplant for further comparison. The patients who contracted PTLD were more likely to be seronegative for HCMV (OR, 9.2; 95% CI, 1.5–55) and EBV at transplant (for HCMV), but donor HCMV seropositivity or HCMV mismatch between donor and recipients (donor positive, recipient negative) was not associated with PTLD. [The Working Group noted that the authors carefully controlled for critical covariates (sex, race, donor age, recipient diagnosis, donor HCMV seropositivity, recipient treatment for HCMV infection, and EBV seropositivity at the time of PTLD diagnosis) but also that the small numbers of patients led to large imprecision in the study estimates.]

In an investigation in Sweden, Gustafsson et al. (2006) examined the presence of HCMV in neonatal blood spots (48 ALL cases and 46 controls) but did not detect HCMV infections in any participants, despite validating the assay's ability to detect HCMV in two children diagnosed with HCMV disease at birth. [The Working Group noted as strengths the use of pre-diagnostic neonatal blood spots with quantitative real-time PCR, which is a validated method for detection of HCMV DNA, although also noted the potentially limited sensitivity of this technique compared with digital PCR. Given that the positivity rate of congenital HCMV infection is very low, around 0.3–1%, the Working Group considered that the sample size may have been too small to adequately assess differences in HCMV prevalence, which reduced the study informativeness.]

Francis et al. (2017) examined HCMV status at the DNA level in Guthrie cards collected routinely shortly after birth, using a highly sensitive digital PCR technique (ddPCR) in the California Childhood Leukaemia Study (CCLS) (ALL and AML cases, $n = 268$; controls, $n = 270$). Controls represented a random sample of the CCLS cohort pool; hence matching factors (date of birth, sex, race) were adjusted for in the analysis. HCMV positivity by Guthrie card was associated with later development of ALL (OR, 3.7; 95% CI, 1.7–9.0). In analyses stratified by Hispanic identity, among whom the incidence of childhood ALL is elevated, the OR for Guthrie card HCMV positivity was higher (OR, 5.7) in Hispanic than in non-Hispanic White individuals (OR, 2.1). The presence of HCMV viral particles in pretreatment bone marrow at diagnosis was tested in 36 patients with ALL and compared with that in 10 patients with AML (454 sequencing). HCMV was the only virus that was more prevalent in ALL tumours than in AML (the control) (OR for viral particle detection, 18; 95% CI, 2.04–159.09). In addition, screening by total RNA sequencing of 91 ALL and 28 AML bone-marrow samples also demonstrated that HCMV was present exclusively within ALL sequencing pools. [The Working Group noted

as strengths the use of pre-diagnostic samples, represented by the availability of neonatal blood-spots, and the use of a highly sensitive digital PCR technique, used for neonatal screening. The lack of association between EBV at birth and ALL in the California study participants (the OR was 1.01 for the presence of EBV DNA sequences, comparing cases and controls) was considered to be an indication of a lack of confounding. The diagnostic analysis of the bone-marrow analysis strengthened confidence in the specificity of HCMV in comparison with other potential infectious agents, but did raise the possibility of reverse causation, as the bone-marrow sample was collected at diagnosis.]

Wiemels et al. (2019) (see Section 2.1) used record-linkage data for 2 782 507 children in Sweden. Exposure was examined based on a maternal HCMV-related clinical diagnosis (physician referral and clinical test, standard of care) during gestation and/or HCMV infection diagnosed during the perinatal period, as recorded in Swedish health registries. The rate of HCMV disease was reported to be 11 times as high during pregnancy or within neonates for children who later contracted haematolymphoid malignancies (defined as ICD-9, 200–208) (HR, 11.3; 95% CI, 5.9–21.8; 9 cases). [The Working Group noted that EBV-related disease and treatments for HCMV-related diseases or other viral illnesses were not assessed in this Swedish cohort, which was a limitation. The frequency of active HCMV infections diagnosed (0.04%) was an order of magnitude lower than the expected frequency of congenital HCMV – but this exposure misclassification was not expected to be differential between cases and controls. Limitations included the small number of cases with HCMV and malignancy, and a lack of stratification by subtypes of haematolymphoid malignancies (the outcome including both leukaemia and lymphoma).]

Wiemels et al. (2022) conducted a nested case–control study of childhood ALL ($n = 130$) within a California biobank cohort, assembled from available surplus specimens from the California Prenatal Screening programme, which offered voluntary screening for chromosomal or neonatal neural tube defects to all pregnant women between weeks 15 and 20 of gestation (participation rate, 70%). The controls were frequency-matched on year of birth, sex, and race/ethnicity. Neonatal blood spots were available and were used to test for HCMV proteins including pp65, CXCL1, and an HCMV-encoded homologue of human IL-10, using bead-based multiplex immunoassays. Higher levels of vIL-10 at birth were associated with subsequent risk of ALL (OR per unit increase, 1.27; 95% CI, 1.01–1.59; and OR per highest tertile versus lowest 1.47; 95% CI, 0.85–2.54), whereas other HCMV proteins were not. [The Working Group noted the use of pre-diagnostic samples as a strength. The prevalence of HCMV proteins of ~90% was much higher than expected and the source of these proteins was unclear without diagnostic samples for this sample set, but it is possible that they came from the mother, although the timing or presence of infection during pregnancy was unknown. As multiple HCMV proteins were assessed, inconsistencies for the three proteins clouded interpretation of the results, and concerns over multiple testing were noted.]

In a larger study in Minnesota, Geris et al. (2023) tested 1189 ALL cases and controls matched 4:1 per case on year of birth, sex, and mother's reported race and ethnicity. HCMV sequences were found in 6 cases of paediatric ALL and 21 controls, with an OR of 1.30 (95% CI, 0.52–3.24). Analyses by ALL subtype defined by cytogenetic classifiers revealed a high odds of HCMV DNA for one subtype of ALL (high hyperdiploidy), with an OR of 6.26 (95% CI, 1.4–27.2; 2 exposed cases). High hyperdiploidy is the most common cytogenetic sub-

type of ALL (about 30%). [The Working Group noted the use of real-time qPCR, which is a validated method for the detection of HCMV DNA, although also noting the reduced sensitivity of this method compared with digital techniques. This may have limited the detectability of the virus (< 1%; 0.4% in controls) in comparison with the study by Francis et al. (2017), in which a 7% positivity at birth (3% in controls) was reported. The Working Group also noted imprecision because of small numbers of cases, particularly for the cytogenetic ALL subtype.]

(b) Case–control or case–case studies with post-diagnostic samples

Michos et al. (2009) assessed the serological response to 10 infections, including HCMV, in a study on paediatric NHL ($n = 73$) and HL ($n = 52$) that included one age- and sex-matched control per case. Seronegativity for anti-HCMV IgG was positively associated with risk of NHL (OR, 2.05; 95% CI, 0.83–5.03) and HL (OR, 1.66; 95% CI, 0.55–5.03) in conditional logistic regression models including all 10 infections – most of which also had positive ORs for NHL (only significant for seronegativity for EBV and respiratory syncytial virus, RSV). [The Working Group considered the assessment and adjustment for multiple infections to be useful, and that the positive associations for other infections could indicate potential for confounding by immune status or function, which highlighted the importance of this adjustment.]

In a case–case investigation, Gallant et al. (2022) assessed PCR HCMV positivity in bone-marrow samples from children with ALL and AML, without comparing with healthy controls. Cases (743 ALL, 125 AML) were from the California Childhood Leukaemia Study (CCLS). There was partial overlap (47 cases) of the 91 pooled ALL samples used for RNA extraction in the study by Francis et al. (2017) with the 743 ALL samples used by Gallant et al. (2022). HCMV positivity was tested via PCR in bone-marrow aspirate samples obtained from participating hospitals. Compared with HCMV-negative cases, ALL cases were 2.5 times (95% CI, 1.00–5.47) as likely to have high HCMV DNA positivity (highest quintile) as were AML cases; however, the association weakened after further adjusting for ethnicity (OR, 1.93; 95% CI, 0.44–1.29). Among ALL subtypes, high-hyperdiploid cases were 2.71 times (95% CI, 1.34–4.73) as likely as other ALL subtypes (as a group) to be strongly HCMV-positive (highest tertile) than HCMV-negative, after adjusting for age and ethnicity. [The Working Group considered the evaluation of multiple ALL subtypes to be a strength, but noted the lack of pre-diagnostic samples or non-cancer controls as a limitation if these cancers have shared etiology.]

2.3 Breast cancer

See Table 2.4 and Table S2.5 (Annex 3, Supplementary material for human cytomegalovirus, Section 2, Cancer in humans, online only, available from: https://publications.iarc.who.int/667).

Two cohort studies (Cox et al., 2010; Geris et al., 2022) and two case–control studies (Richardson et al., 2004; Pandey et al., 2016) have investigated HCMV and breast cancer using serological methods. Four within-person comparison studies comparing HCMV DNA in breast cancer and non-cancerous breast tissue (Richardson et al., 2015; Mohammadizadeh and Mahmudi, 2017; Costa et al., 2019; Nakhaie et al., 2021b) were also included.

Nevertheless, within-person comparison studies with unclear case sources for the adjacent tissue or those making comparisons with tissues other than breast (e.g. lymph nodes), between breast cancer subtypes or histological characteristics, or with non-neoplastic tissues from other individuals were not considered to be informative (Harkins et al., 2010; Taher et al.,

Table 2.4 Epidemiological studies on HCMV and cancer of the breast

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Cox et al. (2010) Janus Serum Bank, Norway Recruitment, 1973–1991; diagnosis, 1982–2003 Nested case–control	*Source cohort*: Recruitment through routine health check-ups, screening tests for cardiovascular diseases, or from blood donors from the Red Cross Blood Donor Centre in Oslo (10% of the cohort). *Cases*: 399 cases of breast cancer (age ≥ 20 yr at diagnosis), identified through cancer registry linkage, with a blood sample collected ≥ 4 yr before diagnosis (index sample) and an additional blood sample collected 12 mo before the index sample ("early"). *Controls*: 399; alive and free of cancer (except skin) at cancer case diagnosis. A first pool of controls was selected by frequency matching on age among women with a blood sample within 2 mo of the index sample of the case.	Breast	CMV IgG positivity in sample 1 (early) (OR):			Matching variables, parity	*Exposure assessment critique:* Potential of degradation of serum samples during long-term storage. Unmeasured factors can influence IgG titres. *Design and analysis:* *Strengths:* Availability of two pre-diagnostic samples; ability to examine seroconversion between these two samples. *Limitations:* Lack of adjustment for potential confounders (BMI, HRT, alcohol use).
			Negative	NR	1		
			Positive	NR	0.84 (0.69–1.03)		
		Breast	CMV IgG positivity in sample 2 (index) (OR):				
			Negative	NR	1		
			Positive	NR	0.95 (0.78–1.15)		
		Breast	CMV IgG seroconversion between earlier and index sample (OR):			Matching variables, number of births	
			Did NOT serconvert	NR	1		
			Seroconverted	11	4.0 (1.1–14.4)		
		Breast	CMV IgG seroconversion between earlier and index sample (OR):			Number of births and age at birth first child	
			Parous women: Did not seroconvert	NR	1		
			Seroconverted	10	9.7 (1.2–77.3)		
		Breast	Unit change in HCMV OD between earlier and index sample (OR):			Matching variables, number of births	
			Unit change	NR	1.7 (1.1–2.5)		
		Breast	Unit change in HCMV OD, parous women (OR):			Number of births and age at birth of first child	
			Unit change	NR	2.0 (1.2–3.4)		

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Cox et al. (2010) (cont.)	Among this pool of controls, only controls with at least one earlier sample were selected and individually matched by duration of the sample storage of the "early" sample. *Exposure assessment method*: Seroconversion of HCMV was measured for IgG using ELISA in two pre-diagnostic samples, an average of 8.5 yr apart.						

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Geris et al. (2022) Transplant Cancer Match Study, USA 2000–2017 Cohort	247 318 (1 245 369 person-years); patients within the Transplant Cancer Match Study with available HCMV IgG serology at the time of Tx. Exclusion: history of cancer, HIV infections, multiple donors. Linkage to SEER cancer registry. *Exposure assessment method*: Measurement of HCMV IgG antibodies, data obtained from Scientific Registry of Transplant Recipients. No information on tests used or timing.	Breast	R and D HCMV status at the time of Tx (pre-Tx) (IRR): R (–), D (–) R (+), D (any status) R (–), D (+)	 103 464 96	 1 0.86 (0.65–1.14) 0.75 (0.52–1.07)	Sex, age, race/ethnicity, organ type, EBV recipient and donor status, SES	*Exposure assessment critique:* Lack of information on detection method precluded judgement of method quality. Used pre-Tx D and R serostatus to assess risk of infection post-Tx rather than measuring HCMV infection post-Tx, which could lead to misclassification of exposure. *Design and analysis:* *Strengths:* Use of an ideal high-risk population (immunosuppressed individuals are at a high risk of virus-associated cancer). *Limitations:* No assessment of viral activity in the tumour.

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Richardson et al. (2004) Australian Breast Cancer Family Study, Australia 1992–1995 Case–control	*Cases*: 466 (208 with plasma stored) cases of invasive breast cancer (aged < 40 yr), identified through Victorian or New South Wales Cancer registries. *Controls*: 408 (169 with plasma stored) women (aged < 40 yr) drawn from electoral roll list using proportional random sampling based on expected age distribution of cases. *Exposure assessment method*: Seropositivity for IgG using ELISA.	Breast	HCMV IgG seropositivity (OR):			Age, education, country of birth, state, marital status, BMI, height, age at menarche, number of live births, ever use of oral contraceptives, and history of breast cancer in first-degree relatives	*Strengths:* Confounding was unlikely. *Limitations:* IgG measurement post-diagnosis, reverse causation could not be excluded.
			HCMV negative	86	1		
			HCMV-positive	122	1.08 (0.68–1.71)		
		Breast	HCMV IgG value (OR):				
			Per a unit in OD increasing	NR	1.46 (1.06–2.03)		

–, negative; +, positive; BMI, body mass index; CI, confidence interval; D, donor; EBV, Epstein–Barr virus; ELISA, enzyme-linked immunosorbent assay; HCMV, human cytomegalovirus; HIV, human immunodeficiency virus; HRT, hormone replacement therapy; Ig, immunoglobulin; IRR, incidence rate ratio; mo, months(s); NR, not reported; OD, optical density; OR, odds ratio; R, recipient; SEER, Surveillance, Epidemiology and End Results; SES, socioeconomic status; Tx, transplant; USA, United States of America; yr, year(s).

2014; El-Shinawi et al., 2016; Rahbar et al., 2017; Cui et al., 2018; El Shazly et al., 2018).

Individual case series, without within-person comparisons, (e.g. Tsai et al., 2005) were not considered to be informative individually. However, a meta-analysis of multiple case series assessing HCMV prevalence using IHC, ISH, or PCR (see Section 2.3.4; Richardson et al., 2015) was considered to be informative for describing variations in the detection of HCMV.

2.3.1 Cohort studies

Cox et al. (2010) conducted a nested case–control study on breast cancer that was based in the Janus Serum Bank. The study comprised 399 cases of invasive breast cancer diagnosed in 1982–2003, identified through linkage to the Norwegian Cancer Registry, and randomly selected until they reached a sample size goal of 400, although one woman with ductal carcinoma in situ (DCIS) and her control were later excluded. Eligible cases were those aged ≥ 20 years, with a blood sample collected ≥ 4 years before diagnosis (index sample) and one 12 months before the index sample ("earlier sample"). Eligible controls were those who were alive and free of cancer (except basal cell or squamous cell carcinoma of the skin) at the time of the case diagnosis, with an available blood sample taken within 2 months of the case's index sample (or, if not available, at least 1 year earlier) and frequency-matched 1:1 on age (± 5 years). Anti-HCMV IgG was tested in the two samples, with a cut-off value of 0.2 optical density (OD). There was no association between individual time points (earlier or index samples) and breast cancer incidence; however, 11 cases and 3 controls seroconverted between the earlier and index sample; of these, 10 cases and 2 controls were parous women. The OR for breast cancer associated with seroconversion between the earlier and index sample was 4.0 (95% CI, 1.1–14.4) overall, adjusted for parity, and 9.7 (95% CI, 1.2–77.3) among parous women, adjusted for number of births and age at first birth. No association was observed among women who remained seropositive during the two time points (data not shown). [The Working Group noted as strengths the assessment of pre-diagnostic samples and seroconversion between two time points, as well as the adjustment for parity and matching factors. Although ORs were not adjusted for body mass index (BMI), BMI was not associated with breast cancer risk in this population. However, data on other potential confounders were unavailable; e.g. hormone replacement therapy or alcohol use. EBV seropositivity was also assessed, but no association was observed between either time point or seroconversion and breast cancer incidence, which can be considered to be a negative control.]

Geris et al. (2022) (see Section 2.1) examined HCMV status before transplantation in 247 318 pairs of donors and recipients in the Transplant Cancer Match Study, USA. A total of 663 breast cancers were identified, with adjusted RR estimates of 0.86 (95% CI, 0.65–1.14) for the recipient-positives and 0.75 (95% CI, 0.52–1.07) for the donor-positive, recipient-negatives, relative to donor- and recipient-negatives in an analysis. [The Working Group noted as a strength that the study was within transplant recipients, who may be at higher risk of viral-related cancers because they are undergoing immunosuppressive treatment. The Working Group also noted as a strength the adjustment for age, sex, socioeconomic status, EBV infection status, and the organ transplanted. A limitation of this registry-based study was the lack of breast cancer risk factors that might confound associations such as parity.]

2.3.2 Case–control studies

Richardson et al. (2004) investigated the relation between HCMV and breast cancer in the Australian Breast Cancer Family Study (ABCFS), a population-based case–control study

on breast cancers diagnosed at age < 40 years from 1992 to 1995. Controls were selected from electoral rolls, frequency-matched on age, and aged < 40 years at the time of invitation to the study. Of the 466 cases and 408 controls interviewed, plasma samples were available for 208 cases and 169 controls. Anti-HCMV IgG was measured, with positivity defined as OD > 0.2. ORs were adjusted for reference age (diagnosis age minus 1 year for the cases, and age at interview for the controls), history of breast cancer in first-degree relatives (verified), education, country of birth, state, marital status, BMI, height, age at menarche, number of live births, and use of oral contraceptives. No overall association was observed with seropositivity to HCMV (122 positive cases and 97 positive controls, OR, 1.08; 95% CI, 0.68–1.71). However, there was an increasing trend for the continuous IgG variable, with an OR of 1.46 (95% CI, 1.06–2.03) for each OD unit. No difference was noted by the authors in a sensitivity analysis removing 18 cases with a known germline mutation in *BRCA1* or *BRCA2*. [The Working Group noted as a strength that the adjustment set reduced confounding. However, reverse causation through reactivation of infection cannot be excluded because of the use of post-diagnostic samples. The Working Group did not consider IgG OD units to be a "dose" of the virus, in the way that DNA or RNA or viral protein levels could be considered, but higher titres could be related to infection with a new strain, especially during pregnancy when immunity wanes.]

Pandey et al. (2016) conducted a two-country multiethnic hospital-based case–control study that included consecutive patients with breast cancer recruited in Nagano, Japan, and São Paulo, Brazil. In Japan, controls were recruited from cancer-free participants seen for check-ups and matched on age (within 3 years) and residential area. In Brazil, controls were hospital patients who were free of cancer, matched on age (within 5 years) and ethnicity (405 matched pairs from Japan and 472 matched pairs from Brazil; described in Iwasaki et al., 2011). Levels of IgG antibody to gB were higher in controls (mean ± standard deviation, 767.2 ± 263.2) than cases (834.1 ± 327.9), particularly in Japan. The *P* value for the association between anti-HCMV gB IgG antibody levels and case status ($P < 0.001$, after adjustment for age and BMI in linear regression) was reported, but not the measure of the effect for that association. [The Working Group noted that it was not possible to derive a risk estimate from the data presented, which reduced the informativeness of the study for the present evaluation.]

2.3.3 Within-person comparison studies

Within-person comparison studies comparing HCMV DNA in breast cancer and non-cancerous breast tissue were considered to be less informative because of the potential for reverse causation and field effects for adjacent tissue; however, they were deemed useful for triangulation purposes, as the comparisons were not subject to confounding by traditional risk factors (e.g. age, BMI, parity).

In a study of patients from Christchurch Hospital, New Zealand, Richardson et al. (2015) examined 70 breast cancers and 70 paired samples of normal breast tissue (taken from areas with no fibrosis, at least 70–100 mm away from the tumour) from the Cancer Society Tissue Bank (to which all patients with cancer-related surgery are potential donors). DNA was extracted from frozen samples. None of the breast tumour tissue samples but two of the paired normal tissue samples were positive for HCMV using qPCR. [The Working Group noted the use of areas without fibrosis at least 70 mm away from breast tumour tissue to be a strength, by reducing field effects.]

In a study from Iran, 70 invasive breast cancer tumours and 26 samples of adjacent normal tissue were analysed with IHC for

HCMV IE antigen (of these, two with equivocal results were confirmed to be negative by PCR) (Mohammadizadeh and Mahmudi, 2017). The samples comprised FFPE breast tissue archived in the hospital pathology department in 2013–2016. All samples were negative for IE antigen expression. [The Working Group noted the small number of controls to be a limitation of the study.]

In a study in Oslo, Norway, Costa et al. (2019) assessed extensive HCMV IE and late antigen (LA) positivity (≥ 50% positive cells) via IHC in 75 patients with infiltrating breast cancer and 26 samples of adjacent, benign breast tissue samples. HCMV IE was identified in > 50% of cells within 73% ($n = 55$) of breast cancer tissue samples compared with only 8% ($n = 2$) of adjacent samples. HCMV-LA expression in > 50% of cells was present in 4% ($n = 3$) of breast cancer samples compared with 0% of the adjacent tissue samples. [The Working Group noted the small number of controls as a limitation.]

In another study from Iran, Nakhaie et al. (2021b) used nPCR to test 49 pairs of breast cancer tissue–normal adjacent tissue in fresh samples collected in various hospitals in the Ahvaz region (2020) and found that 16.3% of the breast cancers (8 of 49) and 2% of the adjacent normal tissue (1 of 49) were HCMV-positive. [The Working Group noted the small sample size as a limitation.]

2.3.4 Meta-analyses

In a meta-analysis, Richardson et al. (2015) evaluated case series of breast cancer assessed for HCMV using IHC (LA and IE antigen) (Harkins et al., 2010; Taher et al., 2013), ISH (Harkins et al., 2010) and PCR methods (PCR, nPCR, and real-time PCR) (Tsai et al., 2005; Harkins et al., 2010; Antonsson et al., 2012; Eghbali et al., 2012; El-Shinawi et al., 2013; Taher et al., 2013; Utrera-Barillas et al., 2013; Richardson et al., 2015). In nearly all studies (Tsai et al., 2005; Antonsson et al., 2012; Eghbali et al., 2012; Utrera-Barillas et al., 2013; Richardson et al., 2015), the prevalence of HCMV using PCR was close to zero; there were two exceptions (Tsai et al., 2005; El-Shinawi et al., 2013; Taher et al., 2013) for which prevalence rates ranged from 53% to 96%. In contrast, among studies using IHC and ISH, prevalences were consistently close to 100% (Harkins et al., 2010; Taher et al., 2013). [The Working Group considered that the meta-estimates were not interpretable, given the high heterogeneity in prevalence rates according to the type of assay used, and with reported prevalence ranging from 0% to 100% for real-time PCR studies. However, the meta-analyses did reveal high heterogeneity, which was itself useful information for the evaluation.]

2.4 Cancers of the digestive and respiratory systems, skin, and thyroid

See Table 2.6.

This section summarizes studies that have investigated the relation between infection with HCMV and cancers of the digestive system or other solid organ cancers.

There were two cohort studies on cancers of the digestive system or other solid organ cancers (Faivre et al., 2019; Geris et al., 2022). One was a small follow-up study of liver cancer in people with cirrhosis (Faivre et al., 2019), and the other was a large, retrospective linkage investigation of various cancer types in recipients of solid organ transplants (Geris et al., 2022). Case–control studies comparing HCMV infection status (according to antibody positivity, nucleic acid sequences, or viral proteins) in series of cancer cases and sets of controls were also included for liver cancer, including Lepiller et al. (2011) and a case–control component from Faivre et al. (2019), colorectal cancer (Chelbi et al., 2021), stomach cancer (Zhang et al., 2017a), and lung

Table 2.6 Epidemiological studies on HCMV and cancers of the digestive system and other organ sites

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Faivre et al. (2019) CIRCE study, France 2008–2012 Cohort	273 patients (aged > 35 yr) with cirrhosis, recruited within the CIRCE study. *Exposure assessment method*: Seropositivity for IgG using ELISA.	Liver (HCC)	HCMV antibody positivity and HCC risk during follow-up (HR): Positive	25	1.32 (0.58–2.98)	None specified	*Strengths*: Serology assessed before cancer diagnosis. *Limitations*: Small number of incident cases – imprecision in the estimates; lack of adjustment for confounding, including by age. Cirrhosis-based cohort.
Geris et al. (2022) Transplant Cancer Match Study, USA 2000–2017 Cohort	247 318 SOT recipients within the Transplant Cancer Match Study (linked to cancer registries). Analysis restricted after 2000, because before that data on HCMV status were unavailable for most participants. Individuals with cancer diagnosis before Tx were excluded. *Exposure assessment method*: Measurement of HCMV IgG antibodies, data obtained from Scientific Registry of Transplant Recipients. No information on tests used or timing.	Tongue	HCMV status of R or D (IRR): R (–), D (–) R (+) R (–), D (+)	 27 77 36	 1 1.03 (0.59–1.80) 0.90 (0.47–1.74)	Sex, age, race/ ethnicity, organ transplanted, EBV R/D status, SES	*Exposure assessment critique*: Lack of information on detection method precluded judgement of method quality. Used pre-Tx D and R serostatus to assess risk of infection post-Tx rather than measured HCMV infection post-Tx, which could lead to misclassification of exposure.
		Salivary glands	HCMV status of R or D (IRR): R (–), D (–) R (+) R (–), D (+)	 12 39 9	 1 0.87 (0.37–2.02) 0.74 (0.27–2.05)		
		Oesophagus	HCMV status of R or D (IRR): R (–), D (–) R (+) R (–), D (+)	 34 94 23	 1 0.78 (0.45–1.34) 0.78 (0.41–1.50)		
		Stomach cancer	HCMV status of R or D (IRR): R (–), D (–) R (+) R (–), D (+)	 21 167 35	 1 1.73 (0.96–3.12) 1.13 (0.56–2.30)		
		Small intestine	HCMV status of R or D (IRR): R (–), D (–) R (+) R (–), D (+)	 19 72 12	 1 0.65 (0.37–1.16) 0.23 (0.09–0.63)		

Table 2.6 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Geris et al. (2022) (cont.)		Colon and rectum	HCMV status of R or D (IRR):			Sex, age, race/ ethnicity, organ transplanted, EBV R/D status, SES	*Design and analysis*: *Strengths*: Use of an ideal high-risk population (immunosuppressed individuals are at a high risk of virus-associated cancer). *Limitations*: Small number of tumours for some cancer types. Circulating IgG antibodies against HCMV do not account for the presence or activity of the virus in the tumour tissue. For Kaposi sarcoma, no adjustment for HHV-8 was performed.
			R (–), D (–)	102	1		
			R (+)	434	1.17 (0.88–1.56)		
			R (–), D (+)	138	1.23 (0.89–1.70)		
		Anus	HCMV status of R or D (IRR):				
			R (–), D (–)	12	1		
			R (+)	86	1.24 (0.60–2.59)		
			R (–), D (+)	21	1.05 (0.44–2.50)		
		Liver	HCMV status of R or D (IRR):				
			R (–), D (–)	31	1		
			R (+)	217	1.37 (0.86–2.17)		
			R (–), D (+)	48	0.81 (0.46–1.45)		
		Intrahepatic bile ducts	HCMV status of R or D (IRR):				
			R (–), D (–)	9	1		
			R (+)	43	1.38 (0.52–3.67)		
			R (–), D (+)	23	2.13 (0.76–6.00)		
		Nose, middle ear, larynx	HCMV status of R or D (IRR):				
			R (–), D (–)	19	1		
			R (+)	110	1.62 (0.84–3.09)		
			R (–), D (+)	35	1.22 (0.57–2.61)		
		Lung	HCMV status of R or D (IRR):				
			R (–), D (–)	274	1		
			R (+)	1347	1.24 (1.05–1.46)		
			R (–), D (+)	311	0.94 (0.77–1.14)		
		Pancreas	HCMV status of R or D (IRR):				
			R (–), D (–)	47	1		
			R (+)	202	1.06 (0.70–1.62)		
			R (–), D (+)	51	0.98 (0.59–1.61)		

Table 2.6 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Geris et al. (2022) (cont.)		Soft tissue including heart	HCMV status of R or D (IRR):			Sex, age, race/ ethnicity, organ transplanted, EBV R/D status, SES	
			R (–), D (–)	13	1		
			R (+)	68	1.24 (0.63–2.44)		
			R (–), D (+)	28	1.50 (0.72–3.15)		
		Skin (non-melanoma)	HCMV status of R or D (IRR):				
			R (–), D (–)	51	1		
			R (+)	157	0.94 (0.61–1.43)		
			R (–), D (+)	46	0.89 (0.54–1.47)		
		Melanoma	HCMV status of R or D (IRR):				
			R (–), D (–)	125	1		
			R (+)	308	0.86 (0.67–1.11)		
			R (–), D (+)	124	0.83 (0.61–1.12)		
		Thyroid gland	HCMV status of R or D (IRR):				
			R (–), D (–)	69	1		
			R (+)	248	0.95 (0.66–1.37)		
			R (–), D (+)	70	1.07 (0.70–1.62)		
Lepiller et al. (2011) University hospital, France Incidence 2003–2009 Case–control	*Cases*: 50 adult patients with HCC and cirrhosis. *Controls*: 140; 41 with cirrhosis and 91 with neither cirrhosis nor HCC, matched by age and gender. *Exposure assessment method*: Seropositivity for IgG using ELISA.	Liver (HCC)	HCC association (OR):			Age (> 65 yr), gender, exposition to hepatotropic viruses	*Strengths*: Adjustment for age, sex and HBV/HCV status. *Limitations*: No adjustment for alcohol consumption.
			Seronegative	NR	1		
			HCMV IgG seropositive	NR	2.76 (1.30–5.86)		

Table 2.6 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Faivre et al. (2019) CIRCE study, France 2008–2012 Case–control	*Cases*: 159 HCC cases with available HCMV status. *Controls*: 273 cirrhosis controls without HCC among a broader cohort of 1178 patients included in the CIRCE study. *Exposure assessment method*: Seropositivity for IgG using ELISA.	Liver (HCC)	HCMV antibody positivity and HCC at baseline (HR): Positive	NR	0.67 (0.42–1.05)	Age, gender, HBV or HCV-related cirrhosis, known duration of cirrhosis, MELD score, diabetes	*Strengths*: The multicentre nature of the case and control selection. Adjustment for various factors. *Limitations*: Use of cirrhosis-based cohort. Cross-sectional analysis. *Other comments*: This was a case–control study using baseline data from Faivre et al. (2019), which then followed up the controls for HCC incidence (see above).
Chelbi et al. (2021) Tunisia Unspecified Case–control	*Cases*: 40 tumour samples, 100 blood samples; patients with colorectal cancer (80 with serum samples). *Controls*: 35 peri-tumoural tissue samples, 138 blood samples; healthy subjects (100 with serum samples). *Exposure assessment method*: Seropositivity for IgG and IgM according to ELISA and DNA markers in blood according to nPCR.	Colon and rectum	HCMV blood DNA (UL55) (crude OR): Controls (n = 138) Cases (n = 100)	 84 93	 [1] [8.54 (3.68–19.80)]	None	*Strengths*: Blood DNA and serology assays were used. *Limitations*: No tissue samples. Unspecified sampling details. Queries over higher than expected PCR positivity. *Other comments*: Among 93 PCR HCMV-positive cases, 75% of samples were positive for HCMV IgG ($P < 0.01$). However, no significant correlation between anti-HCMV IgM positivity and genome presence of HCMV was found.
		Colon and rectum	HCMV serum IgG (crude OR): Controls (n = 100) Cases (n = 80)	 56 78	 [1] [30.64 (7.13–131.69)]		
		Colon and rectum	HCMV serum IgM (crude OR): Controls (n = 100) Cases (n = 80)	 2 1	 [1] [0.62 (0.06–6.97)]		
		Colon and rectum	Tissue HCMV UL55 positivity (%): Controls (n = 35) Cases (n = 40)	 29 37	 82.86% 92.5%		

Table 2.6 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Zhang et al. (2017a) Wenzhou, China May 2014 to December 2014 Case–control	*Cases*: 102 (80 serum) patients with gastric adenocarcinoma who underwent resection. *Controls*: 80 (serum only) age- and sex-matched controls from donors described as healthy. *Exposure assessment method*: Chemiluminescence immunoassay of serology; nPCR in tissue.	Stomach (gastric adenocarcinoma)	HCMV IgG serology (%):			None	*Strengths*: Histological confirmation of tumour and normal specimens. Description and suitable distance of adjacent tissue from tumour margin. Age- and sex-matched controls. *Limitations*: Unclear description of control selection. Lack of further adjustment. UL55 only tested in 22 samples.
			Controls (n = 80)	78	97.5%		
			Cases (n = 80)	76	95%		
		Stomach (gastric adenocarcinoma)	HCMV IgM serology (%):				
			Controls (n = 80)	3	3.75%		
			Cases (n = 80)	5	6.25%		
		Stomach (gastric adenocarcinoma)	Tissue HCMV UL56 protein expression, within-person (%):				
			Controls (n = 102)	49	48.04%		
			Cases (n = 102)	71	69.61%		
			Trend-test P value, 0.003				
		Stomach (gastric adenocarcinoma)	Tissue HCMV UL77 protein expression, within-person (%):				
			Controls (n = 102)	19	18.62%		
			Cases (n = 102)	56	54.9%		
			Trend-test P value, 0.001				
		Stomach (gastric adenocarcinoma)	Tissue HCMV UL47 protein expression, within-person (%):				
			Controls (n = 102)	26	25.49%		
			Cases (n = 102)	56	54.9%		
			Trend-test P value, 0.002				

Table 2.6 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Sarshari et al. (2022) Tehran, Islamic Republic of Iran March 2019 to January 2021 Case–control and within-person comparison	*Cases*: 50 samples of gastric cancer tumours. *Controls*: 105 samples of chronic gastritis. *Exposure assessment method*: HCMV DNA using PCR in tissue samples.	Stomach	HCMV DNA in tissue (within- and between-person) (%): Stomach cancer (n = 50) Gastritis (n = 105) Adjacent to gastritis normal tissue (n = 85)	 7 13 1	 14% 12.3% 1.1%	None	*Strengths*: Within- and between-person comparisons. *Limitations*: Modest sample size. Gastritis controls. Within-person comparisons were prone to reverse causation.
Carpagnano et al. (2018) Foggia, Italy NR Case–control	*Cases*: 70 patients with lung cancer. *Controls*: 40 controls. *Exposure assessment method*: HCMV DNA using PCR in brushing samples and exhaled breath.	Lung Lung	HCMV DNA in brushing samples (%): Controls (n = 40) Cases (n = 70) HCMV DNA in exhaled breath condensate (%): Controls (n = 40) Cases (n = 70)	 6 7 5 7	 15% 10.77% 12.5% 10.77%	None	*Strengths*: Consecutive sampling from same ward. *Limitations*: No adjustment for age and smoking. Suspicion of lung cancer in controls before test.
Almeida et al. (2017) Brazil February 2011 to August 2014 Case–control	*Cases*: 100 samples of malignant thyroid nodule. *Controls*: 83 benign tissue samples (plus available tissue from contralateral lobe to tumour for 104 patients). *Exposure assessment method*: Seropositivity for IgG using ELISA.	Thyroid gland	HCMV seropositivity IgG (%): Malignant cases (n = 100) Benign (n = 83)	 88 76	 88% 91%	None	*Strengths*: Reasonable sample size for serological analysis. Complemented by within-person comparison. *Limitations*: Unclear description of tumour testing numbers used in HCMV analysis.

Table 2.6 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
van der Kuyl et al. (2005) Amsterdam, Kingdom of the Netherlands Post 1984 to unspecified end date Case–control	*Cases*: 94; selected from the Amsterdam Cohort Studies on HIV infection and AIDS. *Controls*: 87 patients with AIDS who did not develop Kaposi sarcoma. *Exposure assessment method*: HCMV DNA using PCR in peripheral blood mononuclear cells samples.	Kaposi sarcoma	HCMV DNA positivity, HHV-8–positive (OR):			None	*Strengths*: Allowed stratification by HHV-8. *Limitations*: Adjustment for other factors was not conducted. Unclear selection strategy. Timing of sampling may differ.
			Controls (n = 12)	7	1		
			Cases (n = 47)	15	0.33 (0.09–1.23)		
		Kaposi sarcoma	HCMV DNA positivity, HHV-8–negative (OR):				
			Controls (n = 75)	42	1		
			Cases (n = 47)	26	[0.97 (0.47–2.03)]		

–, negative; +, positive; AIDS, acquired immunodeficiency syndrome; CI, confidence interval; CIRCE, Cirrhose et risque de carcinome hépatocellulaire dans le grand-est; D, donor; EBV, Epstein–Barr virus; ELISA, enzyme-linked immunosorbent assay; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCMV, human cytomegalovirus; HCV, hepatitis C virus; HHV-8, human herpesvirus 8; HIV, human immunodeficiency virus; HR, hazard ratio; Ig, immunoglobulin; IRR, incidence rate ratio; KS, Kaposi sarcoma; MELD, model for end-stage liver disease; nPCR, nested polymerase chain reaction; NR, not reported; OR, odds ratio; PCR, polymerase chain reaction; R, recipient; SES, socioeconomic status; SOT, solid organ transplant; Tx, transplant; USA, United States of America; yr, year(s).

cancer (Carpagnano et al., 2018). Only one case–control study on Kaposi sarcoma (van der Kuyl et al., 2005) was considered to be informative, as other studies did not adequately control for confounding from HHV-8 (Kaposi sarcoma herpesvirus), which has been established as a very strong causative agent of Kaposi sarcoma. Two meta-analyses, one on colorectal cancer (Bai et al., 2016) and one on stomach cancer (Wang et al., 2020a), were also considered informative.

In addition to studies considered to be uninformative for reasons explained in the introduction to Section 2, two retrospective cohort studies by Nagel et al. (2023) and Rashid et al. (2023), who assessed history of HCMV diagnosis in medical records, were considered to be uninformative as there was a lack of clarity on the type of information extracted from the record on HCMV diagnosis, as well as other gaps in the description of the methodology and results. A case–control study by Lv et al. (2020), who also reported a meta-analysis, was also considered to be uninformative as stomach and colorectal cancers were combined as an outcome in both analyses. A case–control study on Kaposi sarcoma was considered to be uninformative because the exposure was HCMV and EBV combined (Amodio et al., 2011). A meta-analysis on lung cancer and various viral infections by Gupta et al. (2016) was considered to be uninformative as only one study on HCMV with no positive cases or controls was included. For stomach cancer, cross-sectional studies in which the reported results did not account for age, sex, and *Helicobacter pylori* status (Matveeva, 2013; Del Moral Hernández et al., 2019) were not considered to be informative.

A common limitation that the Working Group noted that applied to multiple studies was that serological assessment via antibody positivity is a crude and indirect marker of HCMV infection status.

2.4.1 Cohort studies

Faivre et al. (2019) measured IgG antibodies to HCMV among 273 patients with cirrhosis who did not have hepatocellular carcinoma (HCC) at baseline and were followed up for the occurrence of HCC. Analysis of the 25 cases of incident HCC found that the crude HR for HCMV antibody positivity was 1.32 (95% CI, 0.58–2.98). [The Working Group noted as limitations the small number of incident cases, leading to imprecision in the estimate; and the lack of adjustment for potential confounding by factors such as age. As cirrhosis can be an early phase in the development of HCC, the use of controls with cirrhosis would allow only estimation of a causal role after the development of cirrhosis.]

Geris et al. (2022) (see Section 2.1) examined HCMV status before transplant in 247 318 pairs of donors and recipients in the Transplant Cancer Match Study. There were 11 831 cases of incident solid cancers reported during follow-up, with the highest counts reported for cancers of the lung, prostate, and colorectum. The 95% confidence intervals of the adjusted RR of cancer in the HCMV-positive groups compared with the reference group included 1.0 for virtually all cancer types, with two exceptions. For lung cancer, the adjusted RR was 1.24 (95% CI, 1.05–1.46) in the recipient-positive group, and 0.94 (95% CI, 0.77–1.14) in the donor-positive, recipient-negative group. For cancer of the small intestine, the corresponding point estimates and their confidence intervals were 0.65 (95% CI, 0.37–1.16) and 0.23 (95% CI, 0.09–0.63), respectively. [The Working Group noted that transplant recipients all receive immunosuppressive treatment and are recognized to be at high risk of cancers with a known viral etiology, so are an ideal population in which to investigate possible relationships between viral infection and cancer. The Working Group also noted that, based on the large number of comparisons, the observed associations in this study are consistent with chance rather than

being true associations, and that IgM positivity is a crude and indirect marker of HCMV infection status post-transplantation. The result for Kaposi sarcoma was uninformative because of the lack of adjustment for HHV-8.]

2.4.2 Case–control and cross-sectional studies

(a) Hepatocellular carcinoma

Lepiller et al. (2011) reported on the seroprevalence of anti-HCMV IgG antibodies in hospital patients with HCC and cirrhosis, those with cirrhosis but without HCC, and those with neither. The prevalence was higher in patients with HCC (74%) than in the other two groups (54% and 57%, respectively). Multivariable models adjusting for age, sex, and infection with hepatitis B and C viruses (HBV and HCV) provided an OR for HCC associated with HCMV infection according to IgG of 2.76 (95% CI, 1.30–5.86). [The Working Group noted the adjustment for age, sex, and hepatitis as strengths, but also noted the lack of adjustment for alcohol as a limitation.]

In a cross-sectional case–control analysis of the baseline status of the cohort described above, Faivre et al. (2019) measured IgG antibodies to HCMV among 432 patients with cirrhosis of whom 159 had HCC. HCMV antibody positivity was similar between HCC cases and those without HCC, at 52.8% and 59.7%, respectively (adjusted OR, 0.67; 95% CI, 0.42–1.05). [The Working Group noted as strengths the multicentre nature of the case and control selection and the adjustment for various factors.]

(b) Colorectal cancer

Chelbi et al. (2021) tested for anti-HCMV IgG and IgM antibodies, and nucleic acid sequences via PCR, in blood samples from people with colorectal cancer and control participants without cancer recruited from three hospitals in Tunisia. There was a higher prevalence of IgG in available sera from 80 patients with cancer ($n = 78$, 97.5%) compared with 100 controls ($n = 56$, 56%), but very few participants were IgM-positive (cases, $n = 1$, 2.5%; and controls, $n = 2$, 2%). There was also a higher proportion of HCMV nucleic acid sequences detected in blood samples from 100 cases ($n = 93$, 93%) than in the 138 controls ($n = 84$, 60%), with a crude OR of 8.85 (95% CI, 3.82–20.50). [The Working Group queried the high prevalence of HCMV positivity by PCR in the blood specimens and noted that the paper lacked clarity in regard to the numbers involved in various aspects of the study. The mean age of the groups was very similar, although the proportion of female participants was higher among the controls. No other information was provided on characteristics of the cases and controls. HCMV antibodies are a crude and indirect marker of infection status.]

(c) Stomach cancer

Zhang et al. (2017a) conducted a case–control study on anti-HCMV IgG and IgM antibodies in 80 people who had stomach cancer and 80 control participants without cancer, matched on age and sex. They also conducted a tumour-adjacent normal tissue comparison as described in Section 2.4.3. For the case–control comparison, they found no difference in prevalence, at 95% (76 of 80) and 6.25% (5 of 80) respectively for the two antibody types in the cancer cases compared with 97.5% (78 of 80) and 3.75% (3 of 80) among the controls. [The Working Group noted that the tumour–normal comparison described later in this section was likely to be more informative than was the serology comparison. For the serology, matching on age and sex was noted as a strength, but no other information was provided on characteristics of the cases and controls, so it was not possible to determine the broader comparability of the two groups in regard to other risk factors for stomach cancer, such as smoking and *H. pylori* infection.]

Sarshari et al. (2022) conducted a case–control study on HCMV DNA in tissue from 50 people with stomach cancer and 105 control participants with gastritis. They also carried out a tumour-adjacent normal tissue comparison, as described in Section 2.4.3. HCMV DNA was found in 14% ($n = 7$) of stomach cancer tissues compared with 12.3% ($n = 13$) in gastritis tissues. [The Working Group noted the use of controls with gastritis as a limitation.]

(d) Lung cancer

Carpagnano et al. (2018) used PCR to test for HCMV DNA sequences using two different specimen collection methods (bronchial brushing and exhaled breath condensate) for each of 110 consecutive patients being investigated for lung cancer (70 cases of lung cancer, 40 control participants without lung cancer detected). HCMV DNA was detected in 10.77% of cases via either method compared with 15% of controls via brushing sampling and 12.5% of controls via exhaled breath sampling. [The Working Group noted the use of samples from consecutive patients from the same ward as a strength, but the suspicion of lung cancer as a potential bias towards the null and the lack of adjustment for age or smoking was a limitation.]

(e) Thyroid cancer

Almeida et al. (2017) found that anti-HCMV IgG antibody seropositivity was identified in 88% of 100 cases of malignant cancer of the thyroid gland compared with 91% among 83 control participants with benign thyroid nodules. There was no evidence of HCMV nucleic acid sequences in thyroid tumour specimens from individuals who were seropositive for anti-HCMV IgG. [The Working Group noted the sample size for serological analysis as a strength, but the unclear description of tumour testing numbers used in the HCMV analysis was deemed a limitation.]

(f) Kaposi sarcoma

van der Kuyl et al. (2005) reported on HCMV DNA prevalence within peripheral blood monocytic cell samples from within the Amsterdam Cohort Studies on HIV/AIDS, stratified by the presence of HHV-8 (classified by the *IARC Monographs* programme as *carcinogenic to humans*, Group 1, with *sufficient* human cancer evidence for Kaposi sarcoma; IARC, 2012). In HHV-8–negative samples, HCMV DNA was detected in a similar proportion of Kaposi sarcoma cases [26 of 47] and controls [42 of 75]. In samples that were positive for HHV-8, HCMV DNA was detected in fewer cases ([15 of 47]) than controls ([7 of 12]). [The Working Group noted the stratification by HHV-8 status as a strength but also noted the limited sample sizes within some strata.]

2.4.3 Within-person comparison studies

See Table S2.7 (Annex 3, Supplementary material for human cytomegalovirus, Section 2, Cancer in humans, online only, available from: https://publications.iarc.who.int/667).

Several reports have described studies in which the prevalence of HCMV nucleic acids or proteins was investigated in tumour tissue and compared with the prevalence in adjacent normal tissue. In a substantial proportion of such studies, markers of other infections, most often EBV and human papillomavirus (HPV), were also investigated.

(a) Colorectal cancer

Chen et al. (2012) used PCR to detect HCMV nucleic acid sequences in colorectal cancer specimens (69 of 163, 42%) compared with adjacent normal tissue at least 10 cm away from the tumour mass within fresh frozen tissue from surgical resections (14 of 163, 5.6%). In a further 54 archival samples, HCMV DNA was detected in 23 colorectal cancer samples and 2 adjacent-tissue samples. [The Working Group

noted as strengths the large number of cases, use of fresh frozen samples, and specification of the distance of adjacent tissue from the tumour.]

In a similar study, Dimberg et al. (2013) found HCMV sequences in 18 of 119 (15.1%) colorectal cancer specimens from Sweden and 33 of 83 (40%) from Viet Nam, as compared with 2 of 119 and 10 of 83, respectively, in the corresponding adjacent normal tissue (~5 cm from the tumour margin). [The Working Group considered that the specification of the distance from the tumour of the adjacent tissue, use of fresh frozen samples, and a moderate number of samples, were strengths, whereas the lack of paired analysis was noted as a limitation.]

Cai et al. (2016) found using IHC staining that HCMV protein US28 was expressed in 40 of 103 (39%) colorectal tumour specimens, as compared with 7 of 98 (7.1%) adjacent normal tissue specimens from the same people. [The Working Group considered that the large number of cases was a strength but noted that the distance between the adjacent tissue and the tumour was not stated, which did not allow the Working Group to assess the likelihood of field effects.]

Raouf et al. (2023) investigated the prevalence of HCMV nucleic acid and protein expression in 50 colorectal cancer specimen biopsies compared with adjacent normal tissue (> 5 cm from the tumour), and found higher levels in the tumours, at 32 of 50 (64.0%) versus 13 of 50 (26.0%) for HCMV nucleic acid, and 14 of 29 (48%, with 21 specimens unable to be assessed) versus 10 of 50 (20%) for HCMV IE and early proteins. [The Working Group noted that the distance of the adjacent tissue from the tumour was specified, which was noted as a strength, but that the number of samples was modest, particularly for the assessment for HCMV proteins by IHC.]

(b) Stomach cancer

Jin et al. (2014) assessed the expression of HCMV genes in 60 patients with stomach cancer undergoing surgical resection and found that mRNA related to UL133 was detected more frequently in cancer tissue (68.33%) than in adjacent tumour-negative resection tissue (45.00%) and other mRNAs were detected only in cancer tissue although at low rates. [The Working Group noted that the description of adjacent tissue was limited.]

Zhang et al. (2017a) investigated the prevalence of four different HCMV nucleic acid sequences in tumour tissue compared with adjacent normal tissue (at least 10 cm from the tumour-negative resection margin) in 102 people with stomach cancer. For all four target sequences, prevalence was higher in the tumour tissue, substantially so for three of them. [The Working Group noted as strengths the sample size, the description of the distance of the adjacent tissue from the negative resection margin and use of different sequences. IHC analysis was also carried out, but results were not reported quantitatively.]

Fattahi et al. (2018) similarly assessed HCMV nucleic acid detection in tumour tissue compared with adjacent tissue (> 5 cm from the tumour) in 63 people with stomach cancer. HCMV DNA was detected in tumour tissue from 16 of 63 [25.4%] people and in non-tumour tissue from five of 63 people [7.9%]; three of these people had HCMV DNA in both tissue types. [The Working Group noted that the specification of the distance of the adjacent tissue from the tumour was a strength.]

Sarshari et al. (2022) found that 7 of 50 (14%) stomach cancer tissues were HCMV DNA–positive compared with two of 42 (4.7%) adjacent normal tissues. In patients with gastritis, 13 of 105 (12.3%) were HCMV DNA–positive compared with 1 of 85 (1.1%) adjacent normal tissues.

Krishnamoorthy et al. (2024) investigated HCMV DNA in 42 patients with stomach cancer and found a prevalence of 6 of 42 [14.28%] in tumour tissues and 0 of 42 [0%] in adjacent normal tissues. [The Working Group noted the modest sample size and lack of description of the adjacent normal tissue distance as limitations.]

(c) Salivary gland cancer

Melnick et al. (2012) found that HCMV proteins (IE1 and pp65) were present in 38 of 39 mucoepidermoid tumour specimens, as compared with none detected in adjacent non-tumour salivary gland tissue.

Radunovic et al. (2016) used IHC staining and detected HCMV antigen in 65 of 92 salivary gland tumour specimens taken during surgery but not in adjacent tissue. [A comparison with 20 pathologically normal salivary gland specimens from autopsies of people without cancer in this organ was considered to be uninformative because of convenience sampling and comparability of autopsy samples. The reporting of the results for adjacent tissue was unclear.]

2.4.4 Meta-analyses

Bai et al. (2016) conducted a review and meta-analysis of studies on the prevalence of HCMV markers in series of colorectal cancers and, where available, control series. With various exclusions applied, the analysis was reduced to studies by Chen et al. (2012, 2015) and Dimberg et al. (2013), and yielded a pooled OR of 6.59 (95% CI, 4.48–9.69) for the presence of HCMV nucleic acid sequences. [The meta-analysis was considered to be of low informativeness, as the inclusion criteria did not align with those of the Working Group.]

Wang et al. (2020a) reviewed studies on associations between stomach cancer and viral infections and reported a pooled OR of 2.09 (95% CI, 1.14–3.84) based on five studies that used a variety of detection methodologies. [The Working Group noted that some of the studies included were not considered to be informative for the present evaluation, which reduced the informativeness of the meta-analysis.]

2.5 Cancers of the reproductive and urinary tract

See Table S2.8 (Annex 3, Supplementary material for human cytomegalovirus, Section 2, Cancer in humans, online only, available from: https://publications.iarc.who.int/667).

Four cohort studies and 26 retrospective case–control studies on HCMV and risk of cancers of the reproductive and urinary tract were identified. Case series were not routinely reported.

Fine et al. (1985) assessed cancer incidence in individuals exposed to viral infections in utero, as judged by medical records, compared with a control population; the study was considered to be uninformative, as only one malignant teratoma was observed and exposure misclassification could be substantial. A case–control study on bladder cancer by Hrbáček et al. (2023) was considered to be uninformative because of the use of prevalent cases, modest sample size (< 50 controls), and particularly the number of exposed cases ($n = 2$) or controls ($n = 0$). Additionally, the study was considered to be prone to confounding.

For cancers of the uterine cervix, vulva, vagina, and penis, a large number of studies (Kumar et al., 1980; Schmauz et al., 1989; Baldauf et al., 1992; Guijon et al., 1992; de Sanjosé et al., 1994; Dillner et al., 1994; Wang et al., 1994; Chan et al., 1999; Hsieh et al., 1999; Marinho-Dias et al., 2013) were considered to be uninformative because there was no adjustment for, or conditioning on, HPV, many types of which are classified by IARC as *carcinogenic to humans*, Group 1 (IARC, 2012), with *sufficient* evidence for cervical cancer in humans. For HPV 16, the

evidence is *sufficient* for cancers of the vulva, vagina, and penis in humans. The potential for residual confounding by HPV, which may induce upward bias, was noted as a common limitation of studies of cervical cancer. Two studies using pre-cancerous lesions for cervical cancer were also excluded (Brock et al., 1989; Broccolo et al., 2008). Zhang et al. (2023) conducted a systematic review with a meta-analysis on human herpesvirus infections and cervical carcinoma but were unable to account for HPV; this review was thus considered to be uninformative for the present evaluation.

2.5.1 Cohort studies

Akre et al. (1999) conducted a serology-based nested case–control study within a cohort of men who donated blood samples within the Janus project of Norway. Blood was collected until 1991. Cancer outcomes were assessed on the basis of linkage to the Cancer Registry of Norway. Among the blood donors, 81 cases who were diagnosed with invasive testicular cancer (after blood sample collection) until 1993 were each matched on birth date (within 1 year) to three blood donor controls who were alive and without a diagnosis of testicular cancer (total, 242 controls). ELISA was used to assess seropositivity and quantify IgG antibody titres to HCMV. In a conditional logistic regression analysis, an OR for seropositivity of 1.08 (95% CI, 0.60–1.94) was observed for testicular cancer cases compared with controls. An imprecise increased risk of HCMV was noted for seminoma cases (OR, 1.70; 95% CI, 0.80–3.59), whereas an imprecise decreased risk was noted for non-seminoma cases compared with controls. [The Working Group noted the nested case–control design with cancer registry linkage as a strength. The imprecision in estimates for individual testicular cancer types was noted as a limitation, together with the lack of adjustment for confounding by calendar time (as HCMV prevalence varied over time), and cryptorchidism.]

Bergh et al. (2007) conducted a nested case–control study on benign prostatic hyperplasia tissue from 201 individuals who later progressed to prostate cancer and 201 individuals who did not. No HCMV DNA was identified in either group, using nPCR. [The Working Group noted that the sample size was large enough to identify a modest prevalence of HCMV but may be underpowered for the detection of differences in prevalence at very low levels.]

Holl et al. (2008) conducted a nested case–control study within maternity cohorts from Finland, Sweden, and Iceland to assess whether congenital or neonatal infections were associated with testicular cancer risk in male offspring. In the maternity cohorts, serum samples were collected and stored 10–14 weeks (Finland and Iceland) or 7–18 weeks (Sweden) after pregnancy. Among offspring of mothers who had donated serum to the maternity cohorts, 66 histologically verified cases of testicular cancer were identified via linkage to the national cancer registries. Son–mother case pairs were matched to three or four son–mother control pairs in which the son was free of testicular cancer at the time the cases were diagnosed, based on the son's date of birth (within 1 month). A total of 258 control mothers were selected. IgG and IgM antibodies to HCMV were assessed via ELISA. The median times between maternal serum sample donation and testicular cancer diagnosis among the male offspring was 17 years (range, 0–26 years). Anti-HCMV IgG seropositivity was noted in 80% of index mothers and 81% of control mothers. In conditional logistic regression analyses, ORs of 0.95 (95% CI, 0.48–1.86) and 0.57 (95% CI, 0.07–4.64) were noted for anti-HCMV IgG and IgM, respectively, in mothers of cases compared with mothers of controls. [The Working Group noted the novelty of assessing son–mother pairs but noted imprecision in estimates and potential confounding because of a

lack of adjustment for calendar time and other immune- or pregnancy and birth-related factors, including cryptorchidism.]

Sutcliffe et al. (2012) conducted a nested case–control study within the USA-based Prostate Cancer Prevention trial of finasteride. Participants were offered annual study check-ups and biopsies if clinical suspicion arose during the follow-up period or at the end of the study. Cases diagnosed with prostate cancer after the second visit were frequency-matched to controls who were prostate cancer–free by age, treatment arm, and family history of prostate cancer. Anti-HCMV IgG antibodies were assayed via ELISA in serum collected during the second study visit, and demographic and lifestyle factors were assessed using a self-administered questionnaire. The analytical sample included 614 cases and 616 controls. The OR for anti-HCMV IgG seropositivity in prostate cancer cases compared with controls was 1.13 (95% CI, 0.89–1.45), estimated using unconditional logistic regression adjusted for matching factors and for race. [The Working Group noted the offer of regular check-ups to all participants, which was likely to reduce surveillance bias, as a strength.]

Geris et al. (2022) (see Section 2.1) examined HCMV status before transplantation in 247 318 pairs of donors and recipients in the Transplant Cancer Match Study. Adjusted IRRs for prostate cancer of 1.12 (95% CI, 0.93–1.34) and 0.86 (95% CI, 0.69–1.08) were observed in individuals deemed to be at intermediate and high risk of post-transplant HCMV infection, respectively. For bladder cancer, an increased IRR was noted in the groups at intermediate risk (adjusted IRR, 1.43; 95% CI, 0.92–2.22) and high risk (adjusted IRR, 1.58; 95% CI, 0.97–2.57) of post-transplant HCMV infections. For kidney cancer, no increase in incidence was observed in either the intermediate-risk (adjusted IRR, 1.01; 95% CI, 0.83–1.24) or high-risk groups (adjusted IRR, 0.89; 95% CI, 0.70–1.13). [The Working Group noted the large size of the study and adjustment for EBV status of donors and recipients as strengths for the assessment of prostate cancer. The other result provided for "genital cancer sites" as a broad group was not considered to be informative for the present evaluation, given the heterogeneity in terms of risk factors for the cancers included in this group.]

2.5.2 Case–control studies

(a) Prostate cancer

Martinez-Fierro et al. (2010) conducted a case–control study to assess the presence of viral genomes in prostate tissues from 130 individuals undergoing prostate biopsy because of clinical indication in 2006 or 2007. The case group included all 55 individuals with histologically confirmed prostate cancer and 75 control participants without histological evidence of prostate cancer. nPCR was used to assess the presence of DNA from various viruses, including HCMV. Viral genomes for HCMV were detected in six of the control tissue samples, but none of the prostate tumour tissue samples. [The Working Group considered the study to be of lower informativeness as the analysis did not account for age, which would be expected to lead to downward bias, because the mean age of cases was higher than that of controls and seroprevalence of HCMV is expected to increase with age. Additionally, controls were selected to receive biopsies based on clinical suspicion; the control group could have been enriched for HCMV compared with the general population.]

Hrbáček et al. (2011) conducted a single-centre case–control study among 434 men who had undergone open prostate surgery, including 329 individuals with prostate cancer and 105 control participants with benign prostatic hyperplasia. All individuals were from the same catchment area. The presence of antibodies to various infections in fasting blood samples taken on the morning of surgery was assessed using ELISA. Anti-HCMV seropositivity (IgG) was noted in

264 cases (80.2%) and 91 (86.7%) controls, with an unadjusted OR of 0.62 (95% CI, 0.32–1.14). Mean serum levels of HCMV antibodies were also lower in cases (3.839 optical density/cut off) than in controls (4.407 optical density/cut off; $P = 0.0004$). [The Working Group noted the younger mean age of the cases compared with the controls, which may lead to downward bias, and the potential bias towards the null from using controls with benign hyperplasia.]

Mikhaleva et al. (2023) conducted a case–control study involving 50 patients undergoing treatment for prostate cancer and 48 patients undergoing treatment for benign prostatic hyperplasia between 2019 and 2021. Real-time PCR on the surgical specimens was used to detect HCMV and other viruses. There was an imprecise increased prevalence of HCMV among patients with prostate cancer (16.0%) compared with patients with hyperplasia (4.2%; $P = 0.09$). [The Working Group noted as limitations the estimate imprecision and the potential bias towards the null from using controls with benign hyperplasia.]

(b) Testicular cancer

Mueller et al. (1988) conducted a serology-based case–control study that included male patients who had been treated or were being treated for testicular tumours at Karolinska Hospital, Sweden, between 1975 and 1984. The controls comprised 100 samples from healthy men selected at random from series of 500 sera from a blood bank. The sera of cases were assayed using ELISA for IgG antibodies to HCMV, and antibodies to a variety of other infections, at the National Bacteriological Laboratory, as part of routine practice. Serological samples from controls were analysed in 1983–1984 as part of a previous study of 500 individuals. Antibody positivity and arithmetic mean titre were estimated for all participants, with standard *t*-tests used to compare differences between cases and controls. An increased risk of HCMV antibody positivity was noted in cases of testicular cancer compared with controls (RR, 2.0; 95% CI, 1.1–3.6). An increased risk of testicular cancer was noted among individuals in the top quartile of HCMV antibody titre compared with individuals without detectable antibodies (unadjusted RR, 3.9; and RR, 4.9 after adjustment for other herpes viruses). [The Working Group noted the dose–response analysis as a strength but also noted potential confounding because of a lack of adjustment for pregnancy and birth-related factors, including cryptorchidism. However, the adjustment for other herpes viruses may indirectly control for immune function.]

Heinzer et al. (1993) assessed the seroprevalence of HCMV antibodies among 54 patients with testicular cancer and 54 age-matched control participants without cancer. Only two cases (4.2%) and no controls were classified as having an increased serum titre [threshold unclear] of HCMV antibodies. [The Working Group noted that a tissue analysis was also conducted, although this was in fewer than 25 cases ($n = 16$), so was not considered to be informative. The comparison of seroprevalence was hindered by the low number of exposed cases and controls, limiting the informativeness of this study.]

(c) Cervical cancer

Shen et al. (1993) conducted a case–control study that included 78 cases with cervical cancer and 55 controls with uterine leiomyoma (restricted to age 30–60 years to reduce age differences). Genital HCMV and HPV were assessed in cervical samples using PCR. The unadjusted OR for cervical cancer was 1.21 (95% CI, 0.38–3.94) in individuals with HCMV infection and without HPV infection. [The Working Group noted the analysis limited to HPV-negative women as a strength. The estimate imprecision was noted as a limitation.]

Koffa et al. (1995) conducted a hospital-based case–control study within two gynaecology departments in Greece. Tissue specimens were collected for 187 women with genital lesions, including 35 carcinomas of the cervix. The controls were selected from hospital records from the same departments on the basis of a Pap smear showing cervicitis. PCR was used to detect HCMV, HPV, EBV, and HSV DNA in FFPE tissue samples. HCMV DNA was detected in 12 of 35 cervical carcinoma cases (34.3%, $P = 0.017$) compared with 2 of 38 (5.3%) controls [HPV-adjusted OR, 4.64; 95% CI, 0.71–30.18]. Within HPV-negative samples, HCMV DNA prevalence was higher in cervical carcinoma cases (4 of 12, 33.3%) than in controls ([0 of 23, 0%]). In HPV-positive samples, the prevalence of HCMV DNA was 8 of 23 (34.8%) in cervical carcinoma cases [reflecting similar prevalence in cases regardless of HPV status], and 2 of 15 (13.33%) in cervicitis controls. [The Working Group noted the univariable analysis as a limitation but also noted that HPV-stratified estimates were provided, and that the association was present in HPV-negative samples. The Working Group did not consider the results for endometrial, ovarian, and vulval carcinoma to be informative because of the lack of directly comparable controls and corresponding tissue and the low number of cases of ovarian and vulval cancers.]

Ferrera et al. (1997) selected cases diagnosed with cervical cancer or carcinoma in situ between 1993 and 1995 in multiple cancer centres serving Tegucigalpa, Honduras. Cases were each matched to two age- and recruitment-time-matched controls who attended the same centre as the case, and for whom screening indicated a normal cervix. Blood samples collected for testing for sexually transmitted infections were assessed via ELISA for anti-HCMV IgG and antibodies to other infections. HPV DNA positivity in cervical scrapes was assessed using PCR. Data were available for analysis for 49 cases and 95 controls. The majority of the cases (89.8%) and controls (94.7%) were anti-HCMV IgG–seropositive. The OR after adjusting for the presence of HPV DNA, schooling, and previous cytological screening was 0.19 (95% CI, 0.00–6.8). [The Working Group noted the adjustment for HPV as a strength but considered the high imprecision in the estimates to be a notable limitation.]

(d) Ovarian, vulval, and endometrial cancers

Sherman et al. (1991) conducted a case–control study in which they identified cases with vulvar cancer from a population-based reporting system in western Washington, USA, and general population controls through random-digit dialling. Seropositivity to HCMV was assessed in blood samples, and other information such as sexual history was collected via interview. The adjusted RR of HCMV seropositivity was 1.4 (95% CI, 0.6–3.4) among invasive cases compared with controls. [The Working Group noted as strengths the population-based recruitment of cases and controls, and adjustment for sexual activity, but also noted the lack of adjustment for HPV directly as a limitation.]

Rådestad et al. (2018) conducted a regional hospital-based case–control study of 45 consecutive cases with presumed epithelial ovarian cancer and undergoing surgical resection, and 30 controls with benign ovarian cystadenoma. Surgical tissue samples were analysed by IHC for expression of HCMV proteins (IE protein and tegument protein pp65). HCMV IE proteins were detected in [75.5%] of ovarian cancers and 67% of benign cystadenomas. HCMV pp65 proteins were detected in [26%] of cases and 14% of benign cystadenomas. Additionally, plasma from fresh blood samples was assayed for anti-HCMV IgG and IgM using ELISA. Plasma was also assayed from 31 aged-matched women from the hospital biobank who were selected as controls. Anti-HCMV IgG seropositivity was identified in 77% of patients with ovarian cancer and 68% of healthy controls. HCMV IgM seropositivity was identified in 4 cases of ovarian cancer ([11.76%]) but

not in any of the healthy controls. [The Working Group noted as strengths the use of both tissue and plasma samples and use of consecutive cases from the only regional referral centre. However, the lack of analyses to adjust for confounding by age, which differed between cases and controls with benign disease, was noted as a limitation.]

Grabarek et al. (2023) conducted a case–control study that included 48 cases with ovarian cancer, 45 cases with endometrioid endometrial cancer, and 50 controls undergoing surgical removal of the uterus and adnexa for non-oncological reasons. Surgical tissue samples were analysed for HCMV and other viruses using real-time PCR. HCMV DNA positivity was more prevalent in the cases of endometrial cancer [28.9%] and ovarian cancer [18.75%] than in the controls (2%). Adjusted ORs for HCMV positivity were reported as 19.910 (95% CI, 2.481–159.690) for endometrial cancer cases compared with controls; 16.333 (95% CI, 1.670–159.760) for cases of surgery-only ovarian cancer; and 7.903 (95% CI, 0.881–70.8778) for cases of ovarian cancer later treated with chemotherapy, compared with controls. [The Working Group had concerns that the unclear adjustment strategy made it difficult to rule out a partial contribution of confounding and noted the large imprecision in estimates.]

2.6 Evidence synthesis for cancer in humans

The association between HCMV and cancer incidence was investigated in 20 cohort or nested case–control studies, or case–control studies with pre-diagnostic samples; 37 case–control studies; 15 within-person comparison studies; one case–case study; five meta-analyses; and various case series.

Cancers that were studied more extensively included glioma, childhood ALL, NHL, and cancers of the colorectum, breast, and prostate. There were fewer studies that were considered to be informative for other cancer types, including T-cell lymphoma, other leukaemia subtypes (AML, CML, adult ALL), and cancers of the testes, ovary, vulva, endometrium, uterine cervix, bladder, liver, stomach, salivary gland, and thyroid gland, and Kaposi sarcoma.

2.6.1 Studies evaluated

The studies reviewed presented evidence on several types of cancer. There was one retrospective cohort study, based on transplant registry data, that covered multiple cancer sites (Geris et al., 2022). The other studies on HCMV status before diagnosis focused on single types of cancer, and blood samples were tested for the presence of HCMV using serological methods (e.g. Cox et al., 2010; Coghill et al., 2022) or PCR methods (e.g. Francis et al., 2017; Geris et al., 2023), or assessed HCMV-related illness via clinical records (e.g. Opelz et al., 2009; Wiemels et al., 2019). In the few population-based case–control studies, serological testing for antibodies (e.g. Richardson et al., 2004; Amirian et al., 2013; Robles et al., 2015) was also used. The other case–control studies and within-person comparator studies were all based on hospital or clinic series and used various combinations of serological testing of blood samples (e.g. Mueller et al., 1988; Lepiller et al., 2011; Peredo-Harvey et al., 2023); detection of HCMV nucleic acid sequences (e.g. Martinez-Fierro et al., 2010; Han et al., 2017; Chelbi et al., 2021), including in both tumour and normal tissue from same individuals (e.g. Dimberg et al., 2013; Richardson et al., 2015; Nakhaie et al., 2021b); and detection of HCMV proteins (e.g. Zhang et al., 2017a; Rådestad et al., 2018), and similarly also in studies comparing tumour and normal tissue in the same individuals (e.g. Melnick et al., 2012). Case series were also considered to be potentially informative for glioma, because obtaining control tissue is particularly challenging, and these studies provided information on variability in detection

of the virus, but case series without a within-person comparison were not considered to be informative for other tumour sites. Few studies were able to collect comprehensive information on other risk factors for the cancer types under investigation.

The cohort studies and case–control studies were virtually all from high-income countries (Australia, Europe, USA), whereas some case–control and within-person comparison studies came from a much broader range of countries, including countries in Africa (e.g. Egypt, Tunisia), Asia (e.g. China, India, Iran), and South America (e.g. Brazil).

2.6.2 Exposure assessment quality considerations

HCMV infection can be acquired during pregnancy by vertical transmission or over the course of life. After primary infection, the virus is either in a latent phase, during which it does not replicate, or can reactivate intermittently (Griffiths and Reeves, 2021). The exposure assessment methods used in various epidemiological studies that have explored whether HCMV can cause cancer are reviewed in detail in Section 1.4. Various diagnostic methods have been used, including serological assays for IgG or IgM detection (ELISA, bead-based multiplex immunoassay), and/or direct detection of HCMV proteins and/or NAATs such as qPCR, ddPCR, and RNA transcript detection. Widely available methods for IgG measurement are sufficiently robust to detect whether a person has ever had an infection with HCMV. IgM measurement is a sensitive method that has been used as a marker for primary HCMV infection. However, it cannot distinguish primary infection from superinfection or reactivation (Dollard et al., 2011) and, because of cross-reaction and polyclonal stimulation, it is vulnerable to false-positive results, which limits its use for the detection of primary HCMV infections. Although antibody positivity as assessed via serology may indicate exposure of the individual to HCMV, it does not indicate exposure of specific cancer sites to HCMV, which may hinder causal interpretation.

Studies on tumour tissue may better reflect exposure of the specific site to HCMV. NAAT methods are very sensitive and preferred for the detection of ongoing HCMV replication (Razonable et al., 2020) (Section 1.2.1(b)). Regardless of the tests used, the time of first infection cannot be determined without sequential testing. Although NAAT methods are highly sensitive, they cannot localize viral particles within the tissue. Techniques such as ISH and IHC can detect the location of HCMV markers more specifically in relation to the tumour. However, IHC methods and in situ hybridization are less sensitive than NAAT techniques. The quality and duration of sample storage are critical factors in the reliability of results, so studies that used old samples that were stored for a long time may have limitations because of deterioration.

The performance of commercial assays has been more extensively documented, enabling their results to be evaluated with greater confidence than those of some in-house assays, for which publicly available information is limited or non-existent. For example, HCMV-protein positivity rates were extremely high (>90%) in one case–control study (Wiemels et al., 2022) compared with the rates of congenital HCMV infection of 0.3–1% reported by other sources. This could indicate that protein detection in this context primarily derived from maternal transfer, rather than infection in the infant. Further considerations can be found in Section 1.4.

2.6.3 Confounding, bias, and reverse causation

Several potential sources of confounding and bias were identified across the evaluated studies. Some of the cancer types that were investigated have well-established risk factors that were not measured and therefore not adjusted for in most reported studies. For childhood ALL and glioma, established risk factors that are likely to be related to HCMV exposure are not well understood, hindering the investigation of the role of confounding. Nevertheless, the Working Group considered potential confounding by other infections or immune system–related processes to be plausible for childhood ALL when proxies for these were not adjusted for. Similarly, the Working Group noted specific concerns over genetic variability in risk of ALL across diverse populations that may be prone to different levels of exposure to HCMV, leading to spurious associations. For other cancers, such as breast cancer, confounding by socioeconomic or modifiable risk factors (e.g. parity, BMI, and alcohol intake for breast cancer) was raised as a potential concern.

Several of the studies compared HCMV markers in tumour tissue and normal tissue, either from two distinct groups of people or paired within the same individuals. For such studies, the Working Group noted that interpersonal confounding was less likely but considered that the findings from such studies would be vulnerable to potential reverse causation. The observation of elevated prevalence of HCMV markers in tumour tissue compared with normal tissue may be a result of biological phenomena related to the presence of the tumour while having no implication for causation of the tumour by HCMV. For example, HCMV DNA may be more frequently present in tumour tissue than in normal tissue because of tropism for immune cells, which could be enriched within a tumour for various reasons, including hypervascularization. Studies using techniques such as ISH and IHC (e.g. Scheurer et al., 2008) were better able to identify the infection in the tumour cells themselves, rather than in tumour-related blood vessels, although studies using these methods were not available for all cancer outcomes.

Another issue noted by the Working Group was that the concordance of serological positivity and detection of HCMV nucleic acid sequences or proteins in tissue was rarely reported (e.g. as in the study by Chelbi et al., 2021), an analysis that could have provided additional confidence in the relevance of the serological results to organ-specific carcinogenicity. For some viral agents (e.g. HBV, HPV, Merkel cell polyomavirus) that have been determined to be causes of cancer (IARC, 2012; see the monograph on Merkel cell polyomavirus in the present volume), the integration of viral sequences into the genome of cancer cells, combined with evidence for the tumorigenic role of these sequences, has provided a clear means of interpreting the finding of increased levels of viral nucleic acid in tumour tissue, and the Working Group assessed this phenomenon for HCMV.

Another type of bias that may affect interpretation of the cross-sectional studies in which HCMV markers were investigated could arise from the possibility that the comparator–control sample is not representative of normal tissue. For example, in some studies the biopsies that provided the tissue samples in the comparator groups came from people who were under investigation for possible cancer (e.g. Martinez-Fierro et al., 2010) or had been diagnosed with a benign lesion (e.g. Hrbáček et al., 2011). Similarly, the adjacent tissue samples may also not be entirely representative of normal tissue. The difference in HCMV marker prevalence between the cancer and control groups might be reduced in these instances, making the detection of a true association less likely.

Selection issues are also a concern as some studies do not clearly specify the source of the control samples (e.g. Mohammadizadeh and Mahmudi, 2017; Chelbi et al., 2021; Ghaffari et al., 2021a), which raises the possibility of selection bias and lack of comparability between the case and control samples, which reduced the informativeness of these studies for the present evaluation.

Given these limitations, the most interpretable source of evidence for investigating the possible relation between HCMV infection and cancer is from studies that determined HCMV status before the diagnosis of cancer. This would include cohort studies, nested case–control studies, and case–control studies that could access pre-diagnostic specimens, although the lack of HCMV status in tissue samples before diagnosis was noted by the Working Group as a limitation in the evidence base.

2.6.4 Childhood acute lymphoblastic leukaemia

Six studies (including formal nested case–control studies) in which HCMV status was detected in biological samples obtained before cancer diagnosis (Lehtinen et al., 2003; Gustafsson et al., 2006; Francis et al., 2017; Wiemels et al., 2019; Wiemels et al., 2022; Geris et al., 2023) and one case–case study with post-diagnostic samples (Gallant et al., 2022) were considered to be informative for the evaluation of childhood ALL. An additional cohort study on haematolymphoid neoplasms combined, rather than specifically on ALL, was also considered for the evaluation of ALL (Wiemels et al., 2019).

The Working Group considered that three large studies with pre-diagnostic measures of HCMV by Lehtinen et al. (2003), Francis et al. (2017), and Geris et al. (2023) were the most informative for the evaluation of the association between HCMV and childhood ALL, although limitations were noted for each study. The nested case–control study in Finland and Iceland by Lehtinen et al. (2003) found a small increase in childhood ALL risk associated with maternal HCMV IgM (but not IgG) status at 12–14 weeks of gestation; however, upward bias due to confounding was possible as there was a stronger association between childhood ALL and EBV IgM and IgG status, and EBV was not adjusted for in the analysis for HCMV. In the two other most informative studies (Francis et al., 2017; Geris et al., 2023), stored neonatal blood spots (Guthrie cards) were tested for HCMV DNA. Francis et al. (2017), using ddPCR methodology with single HCMV copy sensitivity, found an increased risk of childhood ALL associated with HCMV positivity (OR, 3.71; 95% CI, 1.71–8.95) but no increase associated with EBV markers, which was considered to be an indication of a lack of confounding by EBV. Geris et al. (2023), using qPCR, found a small but imprecise increase in risk of ALL (OR, 1.30; 95% CI, 0.52–3.24) (with a larger but highly imprecise increase in risk of high hyperdiploidy subtypes based on 2 exposed cases).

Three further studies with pre-diagnostic measures of HCMV were considered to be of lesser informativeness. This included a nested case–control study by Wiemels et al. (2022), who found an association between a particular HCMV protein (HCMV-encoded vIL-10) in the stored neonatal blood spot (OR per unit increase, 1.27; 95% CI, 1.01–1.59); however, negative results were observed for two other HCMV-derived proteins studied – [pp65 OR, 1.05 (95% CI, 0.85–1.29); and vCXCL-1, OR, 0.98 (95% CI, 0.79–1.21)] – in association with ALL. Overall, the Working Group considered that the high positivity rate (90%) for HCMV proteins in neonatal blood may reflect past maternal infection at an unknown time period, which reduced the informativeness of this study. In a further small study, Gustafsson et al. (2006) assessed 48 ALL cases and 46 controls, using qPCR, the same PCR measurement method as used by

Geris et al. (2023), and detected no HCMV in stored Guthrie cards of either cases or controls, which minimized the informativeness of this study. Given that the positivity rate of congenital HCMV infection is around 0.3–1%, a much higher sample size would be needed to ensure enough exposure contrast to be informative. In a record-linkage study in Sweden, Wiemels et al. (2019) found a substantial increase in risk of all haematolymphoid malignancies combined (which were likely to include a substantial proportion of ALL) in children whose mothers had a history of HCMV during the pregnancy or who themselves had an infection that was diagnosed 6 months before cancer diagnosis (OR, 11.34; 95% CI, 5.89–21.83).

The limited knowledge of risk factors for ALL means that ruling out confounding with reasonable confidence is complex, and this includes potential confounding by infectious or immune system–related processes. In an additional study, Gallant et al. (2022) examined ALL and AML cases at diagnosis (AML cases were used as controls, in a case–case study design) and found that bone-marrow samples from ALL cases had a higher HCMV positivity rate than did those from AML cases, although with a weaker association after adjusting for ethnicity. In addition, in an analysis restricted to ALL cases, the bone-marrow samples from high hyperdiploid subtypes, when compared with those from all other ALL subtypes, were more strongly HCMV-positive, which is consistent with HCMV playing a selective role in this subtype, as suggested by Geris et al. (2023).

In summary, studies with cohort and nested case–control designs were broadly consistent in finding a modestly elevated risk of ALL in relation to various markers of exposure to HCMV in early infancy. In the three most informative studies of childhood ALL (Lehtinen et al., 2003; Francis et al., 2017; Geris et al., 2023), the results were broadly consistent in showing a modest increase in risk, although the results of other studies were varied (Gustafsson et al., 2006; Wiemels et al., 2019, 2022). The Working Group considered that the body of evidence suggested a credible positive association for HCMV infection but could not exclude the possibility that the association observed was a result of one or more unmeasured confounding factors. Also, confidence intervals around effect measures for some of the studies were wide, leading to the possibility that chance may be an explanation for the positive findings.

2.6.5 Glioma

One nested case–control study on glioma (Coghill et al., 2022), two cohort studies on grouped CNS cancers (Wiemels et al., 2019; Geris et al., 2022), eight case–control studies (Mitchell et al., 2008; Ranganathan et al., 2012; Amirian et al., 2013; Lin et al., 2016; Han et al., 2017; Ghaffari et al., 2021a; Fall et al., 2022; Peredo-Harvey et al., 2023), one within-person comparison study (Scheurer et al., 2008), and two meta-analyses (Farias et al., 2019; Peredo-Harvey et al., 2021) were considered to be informative. The Working Group could not dismiss the large number of case series with the aim of determining the prevalence of HCMV in brain tumour tissues, especially considering the difficulty in obtaining pre-diagnostic brain tissues for studies on the association. Therefore, 22 case series (Sabatier et al., 2005; Poltermann et al., 2006; Lucas et al., 2011; Fonseca et al., 2012; Rahbar et al., 2013; Baumgarten et al., 2014; Ding et al., 2014, 2019; Libard et al., 2014; Yamashita et al., 2014; Bhimrao et al., 2015; Bianchi et al., 2015; Huang et al., 2015; Sardi et al., 2015; Shamran et al., 2015; Malekpour Afshar et al., 2016; Stangherlin et al., 2016; Xing et al., 2016; Garcia-Martinez et al., 2017; Holdhoff et al., 2017; Yang et al., 2017; Limam et al., 2020) were considered to offer modest complementary information for the present evaluation.

Inconsistencies were noted in results from studies in which HCMV was measured before glioma diagnosis. In a nested case–control study in two cohorts from Norway (the Janus Serum Bank) and the USA (the CPS-II cohort study) with stored baseline blood specimens, Coghill et al. (2022) found a twofold risk of non-GBM glioma among people with IgG serology markers of HCMV infection. This association was observed in the two cohorts, although it was largely imprecise in CPS-II, and no associations were observed for the other five herpes viruses investigated (which served as negative control exposures), reducing concerns that the findings may be a result of bias or confounding by immunity-related factors. These studies had a long follow-up time and used pre-cancer diagnosis samples for the anti-HCMV IgG serological assessment, lending credibility to the positive findings. Geris et al. (2022), however, found no association between anti-HCMV IgG serological markers collected clinically from a cohort of organ transplant donors and recipients in the USA and a combined CNS tumour outcome. Although Geris et al. did not look at specific histologies, the majority (80%) would be likely to be glioma, but not non-GBM glioma (only about 30% of total brain tumours, Miller et al., 2021). Finally, Wiemels et al. (2019) identified one brain cancer in an individual with prior maternal or childhood HCMV infection, recorded in medical records, within a retrospective cohort record-linkage study in Sweden. Since this study used clinical reporting of HCMV disease in the medical record, it was likely that subclinical disease was missed. This reduces the number of exposed, and hence with only 1 exposed case identified, it was not possible to perform a statistical analysis, which reduced the informativeness of the study for the evaluation of glioma.

In one population-based case–control study (Amirian et al., 2013) and several other hospital-based case–control studies (for example, Han et al., 2017), the authors looked at serological evidence of HCMV infection (at diagnosis) and found no association with risk of glioma, GBM, or other nervous system tumours. However, given the immunosuppressive nature of glioma, immune markers in the cases could have been differentially suppressed, which could have biased the results in those studies in an undetermined direction. Several case–control studies and the within-person comparison study (Scheurer et al., 2008) found differences between the prevalence of HCMV markers (whether viral nucleic acids or proteins) in tumour and non-tumour tissue (for example, Mitchell et al., 2008; Han et al., 2017; Ghaffari et al., 2021a; Fall et al., 2022); however, others did not (Ranganathan et al., 2012; Lin et al., 2016).

Additionally, several case series reported high levels (70–100%) of these markers for gliomas overall (e.g. Ding et al., 2014; Libard et al., 2014), GBM (e.g. Rahbar et al., 2013; Bianchi et al., 2015; Huang et al., 2015; Shamran et al., 2015), and non-GBM (e.g. Scheurer et al., 2008; Libard et al., 2014; Shamran et al., 2015). However, there was considerable variability in the findings for HCMV detection across studies, with some studies reporting 0% or nearly 0% prevalence (Poltermann et al., 2006; Baumgarten et al., 2014; Yamashita et al., 2014; Lin et al., 2016; Garcia-Martinez et al., 2017; Holdhoff et al., 2017), as also shown in the published meta-analyses by Farias et al. (2019). This raised concerns for the Working Group over the reliability of studies indicating the presence of HCMV in brain tissue.

Few case–control studies examined glioma subtypes to assess whether risk varied by subtype. Moreover, subtype classifications differed across studies, and non-GBM tumours were often grouped together despite comprising a range of histological types. In addition, the variability in prevalence of HCMV in tumour tissue did not follow a clear pattern across glioma subtypes, with reported prevalence being higher in non-GBM gliomas in some studies but higher in GBM in others. Therefore, there were inconsistent

results among glioma subtypes across studies. As described in Section 2.6.3 above, interpretation of these observations, which were based on samples taken after diagnosis, needs to take account of the possibility that the increased level of detection of markers in tumour tissue is due to biological phenomena related to the presence of the tumour, rather than a causal role of HCMV. In this regard, one could expect to see higher levels of other viruses with immune cell tropisms in the tumour tissues; however, studies that used other viruses as negative-control exposures (e.g. EBV, HHV6, adenovirus) were lacking.

According to the methodological issues, although positive associations have been reported in some studies of this type, the results were inconsistent both across the current body of literature and with regard to glioma subtype. There were also inconsistencies both in the reported prevalence of HCMV in glioma tissues across studies and in the differences observed between tumour and non-tumour tissues across case–control studies and case series. Aside from these studies, there was only one (Coghill et al., 2022) involving a method of HCMV detection applied before cancer diagnosis in which a positive association was found with non-GBM glioma. Owing to these concerns, the Working Group could not draw a conclusion about the presence of a causal association between HCMV infection and glioma.

2.6.6 Non-Hodgkin lymphoma

There were four studies using pre-diagnostic samples – including a serum biobank study (Mueller et al., 1991), two studies of transplant recipients (Opelz et al., 2009; Geris et al., 2022), and one within people with HIV (Wolff and O'Donnell, 2001) – in which HCMV markers and risk of NHL or NHL subtypes were assessed. Eight studies using post-diagnostic samples assessed NHL overall (including PTLD) or NHL subtypes such as multiple myeloma or CLL (Csire et al., 2007; Katz et al., 2007; Michos et al., 2009; Steininger et al., 2009; Omer et al., 2011; Robles et al., 2015; Janković et al., 2022; Namayandeh et al., 2022).

Although positive associations between markers of HCMV and subsequent NHL risk were observed among transplant recipient cohorts (Opelz et al., 2009; Geris et al., 2022), these were deemed to be prone to upward bias due to confounding by EBV infection. Indeed, the association reported by Opelz et al. disappeared when stratifying on EBV status. Another study in people with HIV found an association that was likely to be confounded away from the null by profound immunosuppression induced by HIV and HAART therapy, as the study did not adjust for any other viral illnesses, or for HAART therapy (Wolff and O'Donnell, 2001). In a serum biobank study, Mueller et al. (1991) observed a reduced risk of NHL associated with anti-HCMV IgG seropositivity, which was accentuated when adjusted for other antibody measurements for EBV (OR, 0.4; 95% CI, 0.2–0.9). In contrast to the modest increased risk of NHL overall in Geris et al. (2022), they found that HCMV status was associated with reduced risk of DLBCL (OR, 0.74; 95% CI, 0.59–0.91), but this result was thought to be confounded in an unknown direction by EBV or antiviral treatments for active viral infections including EBV.

The other available studies used either post-diagnostic measurements or unmatched control sets, reducing their informativeness for NHL or NHL subtypes (e.g. Csire et al., 2007; Omer et al., 2011) and did not consistently find a positive association.

Given the lack of consistency of a positive association and the lack of studies that adequately considered the role of EBV and immunosuppression, the Working Group determined that no clear conclusion about a causal role of HCMV in NHL could be drawn from the findings.

2.6.7 Breast cancer

Two cohort or nested case–control studies (Cox et al., 2010; Geris et al., 2022), two case–control studies (Richardson et al., 2004; Pandey et al., 2016), four within-person comparison studies (Richardson et al., 2015; Mohammadizadeh and Mahmudi, 2017; Costa et al., 2019; Nakhaie et al., 2021b) and one meta-analysis (Richardson et al., 2015) were deemed informative.

A serum bank study in Norway (Janus Serum Bank) (Cox et al., 2010) found a strong association (OR, 4.0) between seroconversion to anti-HCMV IgG positivity (occurring between a sample taken 12 months before the index sample and the index sample) and the risk of incident breast cancer, particularly among parous women, but no association for baseline markers. A USA-based cohort study of transplant recipients (Geris et al., 2022) found no association between the anti-HCMV IgG serological status of donors and recipients and breast cancer risk in the recipients. A population-based case–control study from Australia of women with breast cancer aged < 40 years (Richardson et al., 2004) found no association with overall HCMV seropositivity but an increase in risk at increasing titres of IgG (per unit increase in OD). A small number of case–control and within-person comparison studies in which HCMV nucleic acid sequences or proteins in tumour tissue were compared with those in adjacent normal tissue had mixed results, with some reporting low levels of detection for both groups of specimens (Richardson et al., 2015), while others found very high levels of markers in the tumour tissue (Mohammadizadeh and Mahmudi, 2017; Costa et al., 2019). Generally, from several case series that were considered within the meta-analysis by Richardson et al. (2015), most findings on detection of nucleic acid sequence using real-time PCR found essentially zero prevalence (e.g. Antonsson et al., 2012 and primary data from Richardson et al., 2015), whereas studies detecting proteins using IHC and ISH found high prevalences, close to 100% (Harkins et al., 2010; Taher et al., 2013).

The one study that directly measured HCMV infection status before cancer diagnosis found an association of breast cancer risk with seroconversion but not baseline markers of exposure. Without supporting evidence from the other studies, the Working Group could not draw a conclusion about a credible causal role of HCMV in breast cancer development.

2.6.8 Colorectal cancer

One cohort (Geris et al., 2022), one case–control (Chelbi et al., 2021), and four within-person comparison studies (Chen et al., 2012; Dimberg et al., 2013; Cai et al., 2016; Raouf et al., 2023) were considered to be potentially informative.

The one retrospective cohort study, involving transplant recipients in the USA (Geris et al., 2022), found no strong association between IgG serostatus in recipients or donors and subsequent colorectal cancer risk in recipients. The case–control study and the within-person comparison studies (tumour versus normal tissue in the same individual) all found substantial differences between tumour and normal tissue in the prevalence of HCMV nucleic acid sequences and HCMV-related proteins. Interpretation of these observations needs to take account of the possibility that the increased level of detection of markers in tumour tissue was a result of biological phenomena related to the presence of the tumour, rather than a causal role of HCMV.

There was no evidence of an association between HCMV and colorectal cancer, apart from in studies relying on comparisons between tumour and normal tissue. Given the uncertainties related to the possibility of reverse causation with such studies, the Working Group could not draw any conclusion about a causal association

between HCMV infection and colorectal cancer risk.

2.6.9 Other cancer types

For prostate cancer, three cohort studies or nested case–control studies (Bergh et al., 2007; Sutcliffe et al., 2012; Geris et al., 2022) and three case–control studies (Martinez-Fierro et al., 2010; Hrbáček et al., 2011; Mikhaleva et al., 2023) were considered to be potentially informative. The study results did not show a consistent positive association, so the Working Group did not consider that there was evidence of a credible causal role of HCMV in prostate cancer development.

There were fewer than four studies on other haematolymphoid cancers, including adult myeloid leukaemia and adult ALL (Omer et al., 2011; Geris et al., 2022), HL (Michos et al., 2009; Geris et al., 2022), and T-cell lymphoma (Herne et al., 2003; Ballanger et al., 2009), or lymphoma overall without distinguishing subtypes (Kadry et al., 2016). Similarly, fewer than four studies were included on other solid tumours, including cancers of the testes (Mueller et al., 1988; Heinzer et al., 1993; Akre et al., 1999; Holl et al., 2008); ovary (Rådestad et al., 2018; Grabarek et al., 2023); liver (Lepiller et al., 2011; Faivre et al., 2019; Geris et al., 2022); salivary gland (Melnick et al., 2012; Radunovic et al., 2016; Geris et al., 2022); thyroid gland (Almeida et al., 2017; Geris et al., 2022); lung (Carpagnano et al., 2018); uterine cervix (Shen et al., 1993; Koffa et al., 1995; Ferrera et al., 1997) and endometrium (Grabarek et al., 2023); vulva (Sherman et al., 1991); and Kaposi sarcoma (van der Kuyl et al., 2005). For stomach cancer, one cohort (Geris et al., 2022 and two case–control studies (with additional within-person comparisons) (Zhang et al., 2017a; Sarshari et al., 2022), and three within-person comparisons were included (Jin et al., 2014; Fattahi et al., 2018; Krishnamoorthy et al., 2024). The study by Geris et al. (2022) covered a range of other cancers, including those of the kidney and bladder, larynx, pancreas, soft tissue, and skin (melanoma and non-melanoma). Findings did not indicate a consistent increase in risk or were of insufficient informativeness to permit conclusions to be drawn on the presence or absence of causal associations between exposure to HCMV infection and risk of cancer at any of these sites.

3. Cancer in Experimental Animals

3.1 Mouse

[The Working Group concluded that, because of the species specificity and large differences between HCMV and murine cytomegalovirus (MCMV), studies using MCMV would not provide meaningful information and were therefore not considered further. Studies involving other animal cytomegaloviruses were discounted for the same reason. Additionally, studies involving HCMV in humanized mice did not address the desired end-points and were therefore excluded.]

3.1.1 Topical application

In a well-conducted carcinogenicity study, groups of 62–75 female C57 mice (age not reported) were treated with 0.1 mL of UV-inactivated HCMV suspension that before inactivation contained HCMV at a median tissue culture infectious dose ($TCID_{50}$) of 10^5 $TCID_{50}$/mL, or an equivalent volume of control cell culture fluid, by vaginal insertion of saturated cotton tampons, three times per week for 90 weeks. [The Working Group noted that it was unclear why HCMV had been UV-inactivated, given that mice are not susceptible to HCMV infection.] The virus was propagated in monolayer cultures of human foreskin fibroblasts free of contaminating agents, including mycoplasma (Heggie et al., 1986). Cytological assessments

were performed on vaginal aspirate smears collected every 5 weeks. At the conclusion of the study, most of the mice were euthanized, and the complete reproductive tracts were subjected to histological analysis, with the evaluators blinded to the treatment groups. Mice that appeared ill and likely to die at unpredictable times were euthanized earlier to prevent loss of tissues from autolysis.

At the termination of the study, the incidence of invasive carcinoma of the cervix was significantly increased ($P < 0.01$, χ^2 test; [$P = 0.0117$, Fisher exact test]) in HCMV-exposed mice (6/59, 10%) compared with the control group (0/62, 0%). In addition, the incidence of invasive carcinoma of the cervix was significantly increased ($P < 0.01$, χ^2 test) at the interim time point of 60 weeks in exposed mice (7%) compared with the control group (0%). [The Working Group noted that the incidence at 60 weeks was given as a percentage, making it impossible to perform the Fisher exact test.]

Regarding pre-neoplastic lesions, the incidence of dysplasia was also significantly increased in exposed mice, starting from 15 weeks of treatment, compared with controls.

[The Working Group noted that this was a well-conducted study that used an adequate number of animals per group and an adequate duration of exposure and observation. However, only one sex was used, and one organ (cervix) was investigated. The Working Group considered that, given the use of UV-inactivated HCMV, this study was uninformative for evaluating the carcinogenicity of HCMV in experimental animals, and it was not tabulated.]

3.1.2 Initiation–promotion studies

Intracervical administration

In another study in which UV-inactivated HCMV strain AD-169 was used to elucidate the relationship between HCMV infection and cervical cancer, female Kunming mice (age, 2 months) were treated with gelfoam impregnated with 0.1 mL of UV-inactivated HCMV suspension that before inactivation contained 10^6 $TCID_{50}$/mL, or with an equivalent volume of control cell culture fluid, plugged with ophthalmic forceps into the cervix, four times per week, for 25 weeks. [The Working Group noted that it was unclear why HCMV had been UV-inactivated, given that mice are not susceptible to HCMV infection.] The virus was propagated in monolayer cultures of human embryonic lung cell line free of contaminating agents. There were 70 mice in the control group and 79 in the HCMV-treated group. A third group of 80 mice was used to study HCMV induction followed by croton oil promotion (HCMV plus croton oil) and was treated with HCMV as previously described, then with croton oil twice weekly for a duration of 6 weeks (Wang et al., 1997). All experimental groups were monitored for 220 days, after which the mice were euthanized. At the end of the study, the reproductive tracts were collected, fixed, sectioned, and stained with haematoxylin and eosin for histopathological examination.

The incidence of invasive carcinoma of the cervix was significantly increased in the HCMV-treated group – 12/79 (15%) ($P < 0.005$, χ^2 test; [$P = 0.0003$, Fisher exact test]) – as well as in the group treated with HCMV plus croton oil – 15/80 (18.8%) ($P < 0.005$, χ^2 test; [$P = 0.0001$, Fisher exact test]) – compared with the control group (0/70).

Regarding pre-neoplastic lesions, the incidence of pre-cancerous lesions [not otherwise specified] was significantly increased in both the groups treated with HCMV or HCMV plus croton oil.

[The Working Group noted that this was a well-conducted study that used an adequate number of animals per group and an adequate time of exposure and observation. However, only one organ (cervix) was investigated. The Working Group considered that, given the use

of UV-inactivated HCMV, this study was uninformative for evaluating the carcinogenicity of HCMV in experimental animals, and it was not tabulated.]

3.2 Evidence synthesis for cancer in experimental animals

The carcinogenicity of HCMV has been assessed in one study in female C57 mice by topical application (Heggie et al., 1986) and in one study in female Kunming mice by intracervical administration (Wang et al., 1997).

In both studies, there was a significant increase in the incidence of invasive carcinoma of the cervix (Heggie et al., 1986; Wang et al., 1997). [The Working Group noted that it was unclear why HCMV had been UV-inactivated, given that mice are not susceptible to HCMV infection. Given the use of UV-inactivated HCMV in a species that is not susceptible to infection by this virus, both studies were considered to be uninformative for the evaluation of the carcinogenicity of HCMV in experimental animals.]

4. Mechanistic Evidence

4.1 Virus infection, distribution, and cell interaction

HCMV causes a lifelong infection that is characterized by sporadic reactivations during which the virus is detectable in body fluids such as blood, urine, and saliva (Goodrum et al., 2021; Matthews et al., 2023). HCMV can infect many different cell types and organs. Infections are characterized by two main phases – lytic infection and latency (Goodrum et al., 2021; Matthews et al., 2023).

4.1.1 Viral replication

The infection of most cell types (including fibroblasts, endothelial cells, epithelial cells, and macrophages) typically leads to lytic virus replication, which is anticipated to result in virus production and to lead usually to host cell death, at the earliest stages of virus infection (Britt, 2008; Sinzger et al., 2008; Griffiths et al., 2015; Goodrum et al., 2021; Griffiths and Reeves, 2021; Matthews et al., 2023; Mocarski, 2024). Although HCMV can infect many different cell types, most replication data stemmed from studies using fibroblasts, which are killed by lytic replication (Sinzger et al., 2008; Goodrum et al., 2021).

HCMV host cell attachment is mediated by the binding of viral envelope glycoproteins to heparan sulfate glycosaminoglycans. This is followed by either viral gB-mediated plasma membrane fusion or gB-mediated endocytosis/macropinocytosis and subsequent endosomal membrane fusion. The capsid is then transported by microtubules to the nuclear membrane, where the HCMV genome is extruded through nuclear pores into the nucleus (Goodrum et al., 2021; Tyl et al., 2022).

As for all herpes viruses, the replicative lytic HCMV replication follows a cascade of events that can be categorized into three transcriptional phases: immediate-early (IE), early (E), and late (L) (Goodrum et al., 2021; Tyl et al., 2022; Matthews et al., 2023).

The IE phase is predominantly driven by the expression of the IE genes 72 (IE72, also known as immediate-early gene 1, IE1) and 86 (IE86, also known as immediate-early gene 2, IE2) from the major immediate-early promoter (MIEP). Depending on cell type and differentiation status, additional gene products can also play a role. IE gene expression is followed by the early phase and the onset of viral DNA replication. Finally, the HCMV late genes are expressed, and the late gene products contribute to virion assembly. HCMV replication is facilitated by

arresting the host cell cycle at the G1/S transition point (Goodrum et al., 2021; Tyl et al., 2022; Matthews et al., 2023).

The HCMV genome undergoes chromatin formation by associating with host cell histones. HCMV replication then breaks down into three phases: IE, E, and L. The IE phase begins in the absence of de novo synthesis of viral proteins. The HCMV IE proteins (IE1 and IE2) inhibit intrinsic and innate immune responses, such as interferon (IFN) expression and signalling, and premature cell death, and trigger phase E. In phase E, the circularized viral DNA is replicated by the viral polymerase pUL54. Viral DNA is produced as concatemers. During L phase, most of the genes encoding structural proteins are transcribed. The viral DNA encapsidation step is mediated by the terminase complex composed of proteins pUL56, pUL89 and pUL51. After this stage, the capsids containing the viral genome exit the nucleus in a process mediated by the nuclear egress complex (NEC). The NEC is composed of three proteins – pUL50, pUL53, and the viral kinase pUL97 (Ligat et al., 2018), which is responsible for phosphorylation not only of the core NEC proteins but also of lamin A/C (Tillmanns et al., 2025). Subsequently, the proteins produced during L phase aggregate around the capsid, forming the tegument. The viral particles then pass through the Golgi apparatus and become immature virions contained in a vesicle. Finally, they pass through the cell membrane by budding to acquire the definitive envelope and are released into the extracellular environment as mature virions (Knipe and Howley, 2020).

4.1.2 Latency

Lifelong virus persistence is a feature that HCMV shares with all other herpes viruses. Primary infections in the mother occur in only 1–4% of pregnancies; however, primary infections of the fetus during pregnancy carry a substantially higher risk of congenital transmission (up to ~50%) compared with non-primary infections (Davis et al., 2017). In immunocompetent individuals, subclinical HCMV reactivation is anticipated to be a frequent event. HCMV reactivations in immunocompromised individuals and during pregnancy contribute to HCMV pathogenesis. Phenomena such as "memory inflation", an expansion of HCMV-specific T-cell memory that can affect the immune response to other pathogens in elderly patients, suggest that the host immune response is a critical factor in maintaining HCMV latency in infected individuals. Moreover, impairments of the immune system, particularly T-cell function, are associated with an increased likelihood of detectable virus levels in the blood and with an increased risk of HCMV disease (Goodrum et al., 2021; Griffiths and Reeves, 2021).

During cellular latency, HCMV genomes are assumed to persist as episomes. HCMV latency is associated with MIEP repression and observed in undifferentiated cells, in particular in $CD34^+$ haematopoietic progenitor cells and $CD14^+$ monocytes (Elder and Sinclair, 2019; Poole and Sinclair, 2020; Matthews et al., 2023). Latency in $CD34^+$ haematopoietic progenitor cells is characterized by the absence of viral genome replication, resulting in a lack of lytic viral replication and no production of infectious virus particles (Goodrum et al., 2021; Matthews et al., 2023). The infection of $CD14^+$ monocytes results in so-called "quiescence". This quiescence differs from the long-term latency observed in $CD34^+$ haematopoietic progenitor cells. Although there is initially no lytic replication, $CD14^+$ monocytes are short-lived, and virus entry further promotes monocyte differentiation into macrophages that are permissive to HCMV lytic replication, eventually resulting in virus replication and cell death (Britt, 2008; Goodrum et al., 2021; Matthews et al., 2023). Notably, haematopoietic stem cell differentiation into cells of the myeloid lineage that support lytic HCMV replication such as dendritic cells and macrophages also results in

HCMV reactivation, lytic replication, and host cell death (Britt, 2008). Moreover, specific forms of stress (e.g. inflammation or hypoxia) can trigger HCMV reactivation (Goodrum et al., 2021; Matthews et al., 2023). Taken together, differentiation and stress can overcome both latency and quiescence in HCMV-infected cells and are anticipated to result in lytic replication and host cell death (Britt, 2008; Goodrum et al., 2021; Griffiths and Reeves, 2021; Matthews et al., 2023).

The molecular mechanisms contributing to HCMV latency are poorly understood. For example, it is not clear to what extent HCMV genomes are maintained in latent reservoirs via their tethering to chromosomes and/or regular subclinical reactivations. In $CD34^+$ haematopoietic progenitor cells, epidermal growth factor receptor (EGFR) signalling through the PI3K/AKT and MEK/ERK axes contributes to HCMV latency as well as a to switch in the levels of the anti-apoptotic proteins from Mcl-1 to Bcl-2 (Goodrum et al., 2021).

4.1.3 Abortive infection and replication cycles

Abortive infection means that an infected cell does not undergo a complete virus replication cycle that results in the production of infectious virus particles. There is evidence that HCMV infection can result in abortive replication cycles (de Gast et al., 1992; Gerna et al., 2000). [The Working Group noted that abortive infections may contribute to carcinogenesis, but such infections have not been well characterized.]

4.1.4 Tropism

HCMV has a broad cell and organ tropism. It can infect multiple organs and cell types (epithelial cells; myeloid cells including monocytes, macrophages, and dendritic cells; endothelial cells; and fibroblasts) (Sinzger et al., 2008; Goodrum et al., 2021). In immunocompromised patients, common sites of clinical manifestation and disease are the colon, the liver, the oesophagus, and the retina, the latter being particularly associated with HIV/AIDS (Goodrum et al., 2021; Griffiths and Reeves, 2021). In recipients of solid organ and haematopoietic cell transplantations, infections of the lungs (potentially leading to pneumonia) and of the CNS (encephalitis) are also known clinical manifestations (Goodrum et al., 2021; Griffiths and Reeves, 2021). Latent infection is established in a more limited set of cells, such as $CD34^+$ haematopoietic progenitor cells and $CD14^+$ monocytes (Goodrum et al., 2021).

4.1.5 Impact on cell death in the context of lytic and latent infection cycles

HCMV encodes a large number of proteins and other gene products that can interfere with host cell death and that are critical for its lytic and latent replication cycles (Michaelis et al., 2004; Goodrum et al., 2021; Mocarski, 2024). However, the lytic replication cycle typically results in host cell death (Britt, 2008; Sinzger et al., 2008; Griffiths et al., 2015; Goodrum et al., 2021; Griffiths and Reeves, 2021; Matthews et al., 2023; Mocarski, 2024). [The Working Group noted that it is not known whether processes involved in lytic virus replication might contribute to carcinogenesis by promoting the survival of infected cells. Many viruses that do not cause cancer (e.g. adenoviruses, influenza viruses, severe acute respiratory syndrome coronavirus) still encode proteins that can function as oncoproteins in isolation, because they are needed for virus replication. However, they are expressed in the context of a lytic replication cycle that eventually kills the host cell (Moore and Chang, 2010, 2024; Javier and Rice, 2011). The Working Group also noted that bystander effects of lytically infected cells in surrounding cells cannot be excluded, but not enough evidence has been found in this context.]

4.2 Evidence relevant to key characteristics of carcinogens

This section reviews the mechanistic data for the 10 key characteristics of carcinogens (Smith et al., 2016) encompassed by the agent HCMV. The evidence was available for all key characteristics of carcinogens, except for "is electrophilic or can be metabolized to an electrophile". The studies in exposed humans used to support the mechanistic evidence were evaluated for the quality of the study design, exposure assessment, and assay accuracy and precision and were found to reflect suitable methods for human environmental epidemiological studies. The evaluated human studies also accounted for important confounding and modifying variables, with studies not meeting this criteria being excluded. The Working Group's determination may also be buttressed by mechanistic evidence from human primary cells and tissues or from experimental systems.

4.2.1 Is genotoxic

(a) Exposed humans

In one study, the genotoxicity of HCMV was assessed in a physiological context in humans. The study was conducted in 685 HCMV-seronegative and 1307 HCMV-seropositive individuals (Giacconi et al., 2025). The quartile of HCMV-seropositive individuals with the highest HCMV-specific IgG levels displayed higher levels of DNA strand breaks and a lower DNA repair capacity (as determined by fluorimetric detection of alkaline DNA unwinding or FADU assay) in PBMCs than did the quartile with the lowest HCMV-specific IgG levels (Giacconi et al., 2025). [The Working Group noted that this study was of limited relevance, because there was no comparison between the level of DNA damage and DNA repair capacity in HCMV-seropositive versus HCMV-seronegative individuals.]

(b) Human cells

Since the 1980s, HCMV has been reported to induce chromosomal abnormalities, DNA damage, and DNA-damage responses (which can be caused by genetic damage to the host genome and/or the virus genome) in human primary cells and human cell lines (Lüleci et al., 1980; AbuBakar et al., 1988; Deng et al., 1992a, b; Gaspar and Shenk, 2006; Merchut-Maya et al., 2022), although conflicting reports exist (Nishiyama et al., 1982). In addition, specific DNA breaks have been detected in the 1q21 and 1q42 chromosome regions of the host cells (Fortunato et al., 2000; Nystad et al., 2008; Kuan et al., 2021, 2022). These findings were reported in different cell types, including fibroblasts, the retinal pigment epithelial cell line ARPE-19, and human peripheral blood lymphocytes (PBLs), which are all cell types that undergo lytic infection and for which neither latent nor abortive infections have been documented (Goodrum et al., 2021; Golconda et al., 2023). [The Working Group noted that lytic HCMV infection leads to apoptosis through a caspase-dependent pathway and cell death (Shen et al., 2004; Britt, 2008; Sinzger et al., 2008; Griffiths et al., 2015; Goodrum et al., 2021; Griffiths and Reeves, 2021; Matthews et al., 2023; Mocarski, 2024). Therefore, virus-induced DNA damage in cells undergoing lytic viral replication is not considered to be a relevant model in the context of carcinogenesis, because this DNA damage will not be maintained in surviving cells.]

Notably, some evidence shows that HCMV cell entry is sufficient to induce specific DNA breaks in the 1q21 and 1q42 chromosome regions in fibroblasts undergoing a lytic replication cycle (Fortunato et al., 2000; Nystad et al., 2008; Kuan et al., 2021). However, no data were available on whether similar DNA breaks are induced in cell types that are not killed by HCMV infection, i.e. in cells undergoing latent or abortive infection cycles. Little evidence is available showing that

HCMV can cause DNA damage in cancer cells that display an abortive replication cycle not resulting in the production of virus particles (Merchut-Maya et al., 2022).

Isolated HCMV proteins have been shown to induce or at least to contribute to DNA-damage formation (Shen et al., 1997; Siew et al., 2009; Zhang et al., 2015). [The Working Group noted that studying the effects of proteins outside the context of the HCMV replication cycle may not reflect the situation in HCMV-infected cells. Notably, many viruses that are not known to cause cancer (e.g. adenoviruses, influenza viruses, severe acute respiratory syndrome coronavirus) still encode proteins that can function as oncoproteins in isolation, because they are needed for virus replication. However, they are expressed in the context of a lytic replication cycle.]

Synopsis

[The Working Group noted that there was some evidence that HCMV can be genotoxic in infected cells. However, most of these findings derived from lytically infected cells that die as a consequence of HCMV replication, and in model systems that may not reflect fully physiologically relevant conditions. There was a lack of evidence as to whether the activities of HCMV in inducing oxidative stress or inflammation in infected cells could result in bystander genotoxicity to non-infected cells.]

4.2.2 Alters DNA repair or causes genomic instability

Humans

(i) Exposed humans

In one study in Denmark, biopsy samples were analysed from 20 primary GBMs in patients before any radiochemotherapy and from matching recurrent lesions in the same patients whose tumour relapsed after treatment; increased HCMV IE antigen protein levels were reported in recurrent GBM tissues after radiochemotherapy in 12 out of 20 patients (Merchut-Maya et al., 2022). [The Working Group noted that although this study investigated end-points associated with alterations in DNA repair mechanisms and genomic instability in human cells in vitro, the investigation of HCMV IE antigen levels in tissue biopsies of GBM after radiochemotherapy had some limitations. It seemed that HCMV antigens were detected in all 20 GBM samples, and the HCMV serostatus of these patients was not reported. Additionally, the processes underlying HCMV infection of cancer cells may differ from those in healthy cells, and increased HCMV activity may be influenced by the radiation treatment itself.]

(ii) Human cells

To test the hypothesis that HCMV infection modifies the sensitivity of cells to γ-radiation–induced genetic damage, human PBLs from patients with glioma and from healthy controls infected with HCMV strain AD169 were challenged with γ-radiation. The combination of irradiation and HCMV increased chromosomal instability in glioma cases compared with controls. HCMV alone did induce apoptosis in PBLs, and this was enhanced when HCMV was combined with γ-radiation (Rourke et al., 2010). [The Working Group noted that the study did not differentiate between different types of lymphocyte (T lymphocytes, B lymphocytes, natural killer (NK) cells), and there was no testing for virus production. Most types of lymphocyte are not considered to support lytic HCMV infection (Rice et al., 1984; Söderberg et al., 1993). Differences in the response to HCMV and radiation of PBLs derived from patients with glioma and from healthy controls may be a consequence of the disease rather than of HCMV-mediated effects on DNA repair. Additionally, HCMV serostatus was not documented in the study participants (Rourke et al., 2010).]

HCMV strain AD169, but not other HCMV strains, activated caspase-2, an apoptosis-related enzyme activated by DNA damage in infected fibroblasts. This effect is triggered by cell fusion, which is mediated by certain HCMV gB variants (Tang et al., 2019). [The Working Group noted that this finding may be an artefact of the HCMV laboratory strain AD169 that has lost many genes and provides a limited reflection of the behaviour of wildtype virus (Goodrum et al., 2021).]

Notably, most of the findings regarding HCMV-altered DNA damage response signalling were made in fully permissive cells undergoing a lytic replication cycle, i.e. in human primary fibroblasts, ARPE-19, and human umbilical vein endothelial cells (HUVECs) (Gaspar and Shenk, 2006; Luo et al., 2007; Kulkarni and Fortunato, 2011; E et al., 2011, 2014). Xiaofei and Kowalik (2014) showed increased replication stress and genomic instability upon HCMV infection in non-permissive U2OS cells. Ranneberg-Nilsen et al. (2006) showed altered DNA damage response signalling in HCMV-infected human primary fibroblast cells that were arrested in the G1/S phase of the cell cycle.

Lytic HCMV infection normally leads to cell death (Shen et al., 2004; Britt, 2008; Sinzger et al., 2008; Griffiths et al., 2015; Goodrum et al., 2021; Griffiths and Reeves, 2021; Matthews et al., 2023; Mocarski, 2024). [The Working Group noted that lytic HCMV infection may not be considered a relevant model in the context of carcinogenesis, because the alterations in DNA repair or genomic instability will not be maintained in surviving cells.] There were some indications that the HCMV infection of cancer cells can result in increased genomic instability (Michaelis et al., 2012; Bartek et al., 2017; Merchut-Maya et al., 2022). The long-term infection (200 passages) of UKF-NB-4 neuroblastoma cells with HCMV strain Hi91 resulted in substantial changes to the genome, as measured by comparison of global gene expression and genomic copy number variation with non-infected cells (Michaelis et al., 2012). [The Working Group noted that it was not clear whether this was caused by direct HCMV-mediated effects on the genome or was a consequence of the selection pressure exerted by the chronic infection on genetically unstable cancer cells. The Working Group also noted that UKF-NB-4 cells are permissive to HCMV infection and produce infectious virus. Hence, it is possible that chronic HCMV infection in the UKF-NB-4/HCMV Hi91 models is maintained by a balance of HCMV-producing cells undergoing lytic infection and non-infected dividing cells. In such a scenario, direct DNA damage would occur only in lytically infected cells that eventually die.]

In an investigation of HCMV infection of cancer cells, the DNA damage response, measured by immunofluorescence markers to assess the status of the DNA-damage response machinery, was shown to be different in HCMV-infected DAOY medulloblastoma cells compared with HCMV-infected fibroblasts (Bartek et al., 2017). In fibroblasts, 53BP1 (p53-binding protein 1) was localized in the cytosol, as it is typically found in HCMV-infected diploid, healthy, HCMV-permissive cells. In medulloblastoma cells, however, the physiological nuclear localization of 53BP1 was maintained (Bartek et al., 2017). [The Working Group noted that the authors did not investigate whether HCMV-infected DAOY cells underwent a productive, lytic replication cycle.] Notably, Fornara et al. (2016) reported a stem cell-like phenotype in human primary GBM cells infected with HCMV. In a study in D283 cells (another medulloblastoma cell line), it was shown that infection with HCMV was associated with enhanced endogenous DNA damage, as demonstrated by aberrantly slow replication fork progression. The D283 medulloblastoma cell line displayed an abortive HCMV replication cycle (Bartek et al., 2020). [The Working Group noted that it was not clear whether DNA

damage responses may reflect damaged host cell or genomic viral DNA.]

There was some additional evidence showing that isolated HCMV proteins such as UL76 and UL35 can induce alterations in DNA damage responses (Salsman et al., 2012; Costa et al., 2013). [The Working Group noted that studying the effects of proteins outside the context of the HCMV replication cycle may not reflect the situation in HCMV-infected cells.]

Synopsis

[Overall, the Working Group noted that in various experimental systems, HCMV infection induced genomic instability and altered DNA repair; however, most of the evidence was derived from studies performed in cells that undergo lytic replication. It remained unknown whether HCMV could exert such effects in cells that do not undergo lytic replication.]

4.2.3 Induces epigenetic alterations

(a) Exposed humans

Hsu et al. (2024) profiled DNA methylation in 79 female and 77 male individuals of different ethnicities before kidney (72 patients; median age, 52.3 years) and/or lung (84 patients; median age, 63.8 years) transplantation. The HCMV serostatus of the kidney and/or lung donors (D) and recipients (R) was 6 of 12 D−/R−, 18 of 13 D−/R+, 17 of 19 D+/R−, and 31 of 33 D+/R+. A minority of the patients developed HCMV viraemia after transplantation. The study found that the genome of HCMV-positive organ transplant recipients is hypermethylated at loci associated with neural development and polycomb group (PcG) protein binding and is hypomethylated at regions that are critical for the maturation of lymphocytes. [The Working Group noted that the HCMV-associated epigenetic profiles were predictive of recipient HCMV serostatus and, in lung transplant recipients, provided improved prediction of HCMV reactivation risk beyond serostatus alone, particularly in intermediate-risk cases. In addition, the Working Group noted that there was an association between HCMV and epigenetic alterations; however, patients receiving lung or kidney transplants had chronic diseases that might have altered DNA methylation.]

The aim of a study by Huang et al. (2015) was to identify the association between histone H3 acetylation levels and HCMV IE2-86kDa expression in various histological types of glioma. Tissue samples were obtained from 60 patients with glioma, including 25 patients with GBM, 16 patients with anaplastic glioma, and 19 patients with low-grade glioma, in addition to nine tissue samples obtained from the normal brain cortex, which were used as the controls. Expression of IE2-86kDa was associated with the degree of malignancy and global H3K9ac and H3K14ac levels in glioma tissue.

Epigenetic clocks have been developed to estimate "biological age" in an attempt to characterize an individual's physiological, physical, and mental capacities beyond chronological age. "Epigenetic age acceleration", i.e. a biological age that is higher than the chronological, has been associated with age-related conditions including cancer, cardiovascular disease, diabetes, and neurodegeneration (Perna et al., 2016; Humaira Amanullah et al., 2024). Viruses including HCMV have been reported to be able to cause epigenetic age acceleration (Poloni et al., 2022; reviewed in Humaira Amanullah et al., 2024). In the study by Poloni et al. (2022), ages ranged from 60 to 90 years, with 65 participants being HCMV-positive and 22 HCMV-negative. All participants were healthy and asymptomatic, and the two groups did not significantly differ in sex, age, BMI, or smoking status. Those who were HCMV-seropositive had a higher average "EpiAge" (65.34 years) than those who were HCMV-seronegative (59.53 years). The increased EpiAge correlated with decreased percentage ($P = 0.003$) and numbers ($P = 0.0199$) of $CD4^+$

T cells. [The Working Group noted that these findings provided some evidence regarding the ability of HCMV to induce epigenetic alterations. However, there is still no consistency between the methodologies used to analyse such epigenetic clocks.]

(b) Human cells

Epigenetic processes in HCMV-infected cells affect the chromatin of both the virus and the host cell (Matthews et al., 2023). O'Connor et al. (2014) conducted the first proteomic analysis of global histone post-translational modifications in HCMV-infected human primary fibroblasts. Several changes in histone H3 and H4 modification were observed over the course of a viral replication cycle, including a marked increase in H3K79me2 and H3K27me3K36me2, and a decrease in H4K16ac. These authors also observed upregulation of H3K79 methyltransferase (DOT1L) expression in HCMV-infected primary fibroblasts.

Epigenetically active HCMV proteins include IE2-86kDa (IE2), which interacts with several different histone-modifying enzymes, affecting histone acetylation and histone methylation (Bryant et al., 2000; Reeves et al., 2006; Park et al., 2007). Likewise, the HCMV IE1-72kDa (IE1) protein interacts with histone deacetylase 3 (HDAC3) to antagonize histone de-acetylation in human primary fibroblasts (Nevels et al., 2004). Structural investigations and findings derived from studies on HCMV-infected human fetal diploid (MRC-5) lung fibroblasts showed that the IE1 protein also binds to histones that form cellular nucleosomes (Mücke et al., 2014; Fang et al., 2016). Both HCMV proteins IE1 and pUL82 (pp71) bind to the histone chaperone Daxx and affect its function (Hwang and Kalejta, 2007; Reeves et al., 2010).

Further, HCMV strain AD169 downregulated the vitamin D receptor during lytic infection in primary human foreskin fibroblasts, probably by epigenetic mechanisms, as the effects were prevented by the HDAC inhibitor trichostatin A (Stecher et al., 2022). HCMV AD169 infection of primary human fibroblasts was also shown to increase cellular levels of PcG proteins and to cause PcG protein localization to viral replication compartments (Svrlanska et al., 2019). The catalytic activity of the PcG proteins was not required for virus replication.

HCMV infection of different cell types, including MRC-5 fibroblasts, human monocyte-derived macrophages, the human medulloblastoma cell line D324, and HUVECs, showed that HCMV infection also causes alterations to the host cell genome, in particular, global hypomethylation and a shift in the localization of DNA methyltransferase (DNMT1) from the nucleus into the cytoplasm (Esteki-Zadeh et al., 2012; Estekizadeh et al., 2018). Moreover, HCMV AD169 infection of HUVECs was found to suppress expression of regulator of G-protein signalling 5 (RGS5), probably by hypermethylation, as the effect could be reversed by the DNA methylation inhibitor decitabine (Zhang et al., 2020).

Moreover, there were numerous studies on epigenetic mechanisms underlying the malignancy of HCMV-infected cancer cells. For example, Estekizadeh et al. (2019) investigated the expression and localization of DNA methyltransferase 1 (DNMT-1) and the effects of DNA methylation inhibitor 5-azacytidine (5AZA) in the context of HCMV replication as well as the proliferation and invasion capacities of HCMV-infected GBM cells. Additionally, the expression of various HCMV proteins and DNMT-1 was assessed in GBM tissue specimens obtained from five patients. The authors observed changes in the intracellular localization of DMNT-1 in HCMV-infected cells compared with non-infected cells. Treating infected cells with 5AZA did not affect viral replication but significantly reduced cell invasion and proliferation ($P = 0.05$ and $P < 0.0001$, respectively). In contrast, 5AZA treatment of non-infected cells had no effect on

cell invasion ($P = 0.09$), although proliferation was significantly reduced ($P < 0.0001$).

El Baba et al. (2025) have reported that upregulation of enhancer of zeste homologue 2 (EZH2), the H3K27 methyltransferase in polycomb repressive complex 2, is associated with transformation of various primary cell types by HCMV.

[The Working Group noted that there were few studies on HCMV-induced epigenetic alterations in latently infected cells.] The AML cell line Kasumi-3 can be used as a model for latent HCMV infection (Forte et al., 2018). In this model, HCMV infection resulted in an overall downregulation of host cell gene expression associated with reduced H3K27 acetylation (Forte et al., 2021).

Synopsis

[The Working Group noted that, in the available studies, HCMV infection induced epigenetic alterations, including DNA methylation, histone acetylation, and histone methylation, across a range of relevant human primary cells and experimental systems. These epigenetic alterations are triggered by HCMV proteins that bind to histones and histone-modifying enzymes, such as candidate viral oncoproteins (e.g. IE1-72kDa), as well as by changes in the expression and intracellular localization of epigenetically active host cell proteins, such as PcG proteins (e.g. EZH2) and DNMTs. However, the Working Group noted that most findings were not confirmed in physiologically relevant cells that do not undergo lytic replication. In addition, the persistency of the epigenetic end-points in the context of carcinogenesis is a notable research gap.]

4.2.4 Induces oxidative stress

(a) Exposed humans

No data were available to the Working Group.

(b) Human cells

HCMV infection of a wide range of cell types, including human fibroblasts, coronary artery smooth muscle cells, clonal U937 sublines, U373 GBM cells, and $CD14^+$ monocytes, resulted in increased mitochondrial respiration and reactive oxygen species (ROS) generation (Suzuki et al., 1997; Speir et al., 1998a, b, 2000; Speir, 2000; Xiao et al., 2015; Karniely et al., 2016; Mao et al., 2016; Combs et al., 2020; Wang et al., 2020b; Perera and Sinclair, 2022; Amratia et al., 2024).

ROS contributed to virus replication and reactivation, and, consistent with this observation, antioxidants inhibited HCMV replication (Speir et al., 1998a, b, 2000; Speir, 2000; Xiao et al., 2015; Mao et al., 2016; Wang et al., 2020b; Amratia et al., 2024). Betsinger et al. (2021) reported that HCMV UL13 modulated mitochondrial cristae architecture and enhanced oxidative phosphorylation, but they did not provide evidence for ROS generation or immune cell death. In another study, Kim et al. (2018) reported that HCMV proteins (not further specified) contributed to the induction of ROS that may contribute to the death of immune cells (Kim et al., 2018).

However, HCMV-induced ROS generation seems to be, at least in part, counterbalanced by an antioxidative environment mediated via increased glutathione levels and Nrf2 signalling (Tilton et al., 2011; Lee et al., 2013a). Moreover, VHL/E strain infection of human aortic endothelial cells protected them from hydrogen peroxide (H_2O_2)-induced apoptosis during the first 3 days of infection, probably by mechanisms including extracellular signal-regulated kinase (ERK) signalling and subsequent Bcl-2 upregulation (Wang et al., 2006). [The Working Group noted that most of these findings were made during lytic infection.]

In one study only, HCMV β2.7 lncRNA was shown to reduce the levels of ROS in latently infected $CD14^+$ monocytes (Perera and Sinclair, 2022).

[The Working Group noted that, since lytic HCMV infection leads to cell death (Shen et al., 2004; Britt, 2008; Sinzger et al., 2008; Griffiths et al., 2015; Goodrum et al., 2021; Griffiths and Reeves, 2021; Matthews et al., 2023; Mocarski, 2024), virus-induced oxidative stress in cells undergoing lytic viral replication is not considered to be a relevant model, because oxidative stress will not be maintained in surviving cells.]

Synopsis

[The Working Group noted that in infected human cells, there was some evidence that HCMV induced oxidative stress. However, most of these findings were derived from studies in lytically infected cells that could die as a consequence of HCMV replication.]

4.2.5 Induces chronic inflammation

(a) Exposed humans

Generally, the longitudinal analysis of breast milk samples showed that HCMV reactivation can occur in healthy asymptomatic HCMV carriers and cause inflammation (Rabe et al., 2020). Breast milk samples were obtained longitudinally from four IgG-seropositive and three IgG-seronegative breastfeeding mothers of preterm infants. Cytokine profiling in the milk whey revealed elevated levels of MCP-2, CCL19, CCL20, IL-8, CXCL9, CXCL10, CXCL11, IL-17C, glycoprotein CD5, and TNFSF14 in HCMV-seropositive mothers compared with HCMV-seronegative mothers (Rabe et al., 2020).

Inflammatory bowel disease, which encompasses ulcerative colitis and Crohn disease, is linked to an increased colorectal cancer risk (Koyyala et al., 2025). A recent umbrella review ranked HCMV among the top 10 risk factors contributing to inflammatory bowel disease (Wu et al., 2025).

There were numerous studies on the presence of HCMV in inflamed tissues from patients with inflammatory bowel disease (e.g. Sipponen et al., 2011, in tissue biopsies from 47 patients with ulcerative colitis and 32 patients with Crohn disease plus 15 control individuals without inflammatory bowel disease; Wang et al., 2022, in tissue biopsies from 287 patients with inflammatory bowel disease and 50 control individuals); and Ciccocioppo et al., 2015, in tissue biopsies from 40 patients with inflammatory bowel disease and 40 control individuals). The intensity of histochemical staining for HCMV antigens, the viral load, and HCMV prevalence were reported to be correlated with disease severity (Sipponen et al., 2011; Wang et al., 2022). Moreover, high HCMV loads in diseased tissue were associated with the failure of standard corticosteroid-based therapies (Domènech et al., 2008; Ciccocioppo et al., 2015; Ciccocioppo et al., 2016). Patients with inflammatory bowel disease ($n = 14$) treated for HCMV colitis were found to be at an increased risk of re-hospitalization, therapy failure, and colectomy (Singh et al., 2024). Consistent with these findings, antiviral treatment was reported to be beneficial in corticosteroid-refractory inflammatory bowel disease in a meta-analysis including 333 patients from 15 observational studies (Shukla et al., 2015). Moreover, CD13-specific autoantibodies were detected in 16 out of 24 HCMV-seropositive patients with inflammatory bowel disease and in one out of four HCMV-seronegative patients with inflammatory bowel disease, but not in 15 healthy HCMV-seropositive controls or in five healthy HCMV-seronegative controls (Rahbar et al., 2006). [The Working Group noted that the exact clinical relevance of HCMV as a factor and prognostic marker in inflammatory bowel disease remains to be fully established. There is no clear differentiation between genuine HCMV colitis and disease flare-ups associated with HCMV reactivation, including its role as a causative factor in initiating inflammatory

bowel disease; thus, reverse causation could not be excluded (Maresca et al., 2023; Alhalabi and Alziadan, 2024; İnan-Hekimoğlu et al., 2024). Moreover, there is no final agreement on HCMV detection methods in patients with inflammatory bowel disease (Wang et al., 2025a).]

There was also evidence that HCMV contributes to chronic inflammatory processes in different pathological conditions, including cardiovascular disease (Lee et al., 2023). For example, high anti-HCMV IgG antibody titres and levels of inflammatory markers were associated with an increased risk of coronary artery disease (Kaftan et al., 1999). [The Working Group noted that many different pathophysiological conditions may underly the observed phenomena. In addition, reverse causation could not be excluded.]

Notably, HCMV was also reported to contribute to chronic inflammatory conditions beyond cardiovascular disease and inflammatory bowel disease. For example, inflammatory cytokine production was increased in periodontitis specimens from HCMV-positive patients (Botero et al., 2008), and elevated levels of HCMV-specific $CD8^+$ T cells were detected in patients with systemic sclerosis (Arcangeletti et al., 2018). Additionally, higher rates of active HCMV replication were reported in patients with psoriasis compared with healthy controls (Asadullah et al., 1999). A meta-analysis found no difference in HCMV seropositivity in patients with multiple sclerosis compared with controls (Zabalza et al., 2020). [The Working Group noted that although HCMV infection is associated with inflammatory processes, these conditions are not clearly defined as chronic inflammation. In addition, reverse causation could not be excluded.]

Several studies investigated end-points associated with chronic inflammation in patients with cancer. HCMV pp65 protein (52 of 79 patients, 65.8%) and HCMV DNA (43 of 79, 54.4%) were detected in glioma specimens, whereas neither was detected in eight control brain samples. Endocan (ESM1) expression was observed in 62 of 79 (78.5%) glioma specimens and correlated with higher tumour grade and pp65 positivity (Xing et al., 2016). [The Working Group noted that there were studies that failed to detect HCMV in a high fraction of glioma tissues (Sabatier et al., 2005; Baumgarten et al., 2014; Yamashita et al., 2014; Bhimrao et al., 2015; Malekpour Afshar et al., 2016; Garcia-Martinez et al., 2017; Holdhoff et al., 2017; Yang et al., 2017).] HCMV-infected tumour-associated macrophages have also been proposed to increase the malignancy of inflammatory breast cancer (Mohamed et al., 2022). Although one study reported HCMV infection in 100% of the examined samples of breast cancer tissue (Cui et al., 2018), another found HCMV DNA in 88.6% (39 of 44) of inflammatory breast cancer cases and in 73.6% (67 of 91) of tissues from non-inflammatory breast cancer cases (El-Shinawi et al., 2016). Moreover, HPV16, EBV, HSV-1, HSV-2, and HHV8 were detected at varying frequencies in this study, and the presence of DNA from multiple viruses was associated with elevated cancer aggressiveness (El-Shinawi et al., 2016). HCMV was also detected in 65 of 92 (70.6%) tissues from salivary gland cancer cases and associated with increased interleukin IL-6 levels (Radunovic et al., 2016). Furthermore, IE1 was detected in 44.6% (25 of 56) of colorectal cancer tissue samples, 41.7% (15 of 36) of colon adenoma samples, and 12.5% (7 of 56) of tissue samples from normal areas adjacent to the cancer tissues, and a correlation with increased toll-like receptor 2 expression was reported (Li et al., 2015). [The Working Group noted that these studies provided some evidence of HCMV-associated inflammatory processes associated with carcinogenesis, but it was not possible to conclude that inflammation observed in cancer tissues was related to HCMV only or to other conditions associated with cancer development. In addition, reverse causation could not be excluded.]

(b) Human cells

Initial studies in cell types such as endothelial cells, vascular smooth muscle cells, monocytes, macrophages, microglial cells, and retinal pigment epithelial cells suggested that HCMV infection results in inflammatory signalling and/or cytokine production (Almeida et al., 1994; Pulliam et al., 1995; Holberg-Petersen et al., 1997; Yurochko and Huang, 1999; Redman et al., 2002; Momma et al., 2003; Gravel and Servant, 2005). HCMV-infected primary human brain vascular pericytes were also shown to promote inflammation (Alcendor et al., 2012), and HCMV-infected primary fibroblasts may develop an immunological phenotype that resembles that of senescent cells (Wolf et al., 2012), which are known to contribute to chronic inflammation (Wang et al., 2025b). Moreover, there was evidence suggesting that certain HCMV-mediated proinflammatory processes are maintained during virus latency (van de Berg et al., 2010).

A substantial part of the available information focuses on the role of HCMV infection in inflammatory processes that are relevant to cardiovascular pathophysiology. HCMV infection of macrophages, endothelial cells, and fibroblasts was proposed to contribute to chronic inflammatory processes in the aorta and inflammatory abdominal aortic aneurysm (Yonemitsu et al., 1996). IL-6 produced by HCMV-infected cells was shown to promote angiogenesis and endothelial cell survival (Botto et al., 2011; Fiorentini et al., 2011). HCMV-associated proinflammatory immune responses appeared to include ROS produced by immune cells and immune response-associated tissue damage (Speir, 2000; Bolovan-Fritts et al., 2004, 2007; Caposio et al., 2007; Bolovan-Fritts and Spector, 2008; van de Berg et al., 2012; Gustafsson et al., 2018). Finally, HCMV-induced endothelial–mesenchymal transition may cause inflammation that increases the risk of cardiovascular disease (Chen et al., 2019). Increased IL-6 production was also reported in specimens of HCMV-positive inflammatory bowel disease tissue (Rahbar et al., 2003). Many studies also reported on the detection of HCMV in human cancer cells and its impact on inflammatory signalling and cancer malignancy (El Baba and Herbein, 2021; Mercado et al., 2024; Blanco and Muñoz, 2025). [The Working Group noted that although in vitro systems are valuable for identifying chronic inflammation–associated end-points targeted by HCMV, the data were not informative regarding the chronicity of these effects.]

Synopsis

[The Working Group noted that there was some evidence that HCMV infection is associated with chronic inflammatory processes in exposed humans. However, it remained unclear whether these findings could have been a consequence of reverse causation.]

4.2.6 Is immunosuppressive

(a) Exposed humans

HCMV is one of the clinically most relevant opportunistic pathogens in immunocompromised patients. However, HCMV may also have an impact on the immune system in immunocompetent individuals. HCMV reactivation that needs to be controlled by the immune system to avoid symptomatic HCMV disease is anticipated to be common (as reviewed by Goodrum et al., 2021; Griffiths and Reeves, 2021). [The Working Group considered that HCMV infection results in a continuous interplay between antiviral immune responses and a virus that has evolved to escape recognition by the adaptive and the innate immune system in multifaceted ways.]

There were several studies on the effects of HCMV infection on the immune system. For example, samples from anti-HCMV IgG–seropositive children were collected at age 6 months and 6 years and compared with samples from

HCMV-seronegative children. HCMV infection in early life was found to result in long-term, potentially lifelong alterations in $CD8^+$ and $CD4^+$ memory T-cell functions, but the impact of these changes was not investigated (Ekman et al., 2024).

There were also reports suggesting that HCMV may be associated with age-related decline in immune function, often referred to as immunosenescence, at older ages and, in particular, possibly as a consequence of the long-term interplay between HCMV and the immune system (Goodrum et al., 2021; Müller and Di Benedetto, 2024). Long-term infection was found in some studies to be associated with the expansion (or inflation) of T cells and NK cells and immune cell exhaustion, which has been speculated to affect the control of HCMV (and possibly other) infections. This may also result in HCMV reactivation at a higher frequency, which itself has been proposed to aggravate HCMV-mediated immunosenescence in a positive feedback loop (Hoffmann et al., 2015; Heath et al., 2016; Zieliński et al., 2017; Davies et al., 2022; Müller and Di Benedetto, 2024). [The Working Group noted that there were several studies on the effects of long-term HCMV infection on immune function, potentially causing immunosenescence in at least some patients, particularly at the end of life. However, there was no evidence suggesting that HCMV may induce immunosuppression at the level documented for other viruses such as HIV or any other known immunosuppressant, e.g. immunosuppressive drugs. Known immunosuppressants directly target immune functions. In contrast, HCMV infection may be associated with expansion or inflation of HCMV-specific immune cells that results in reduced numbers of immune cells directed against other pathogens and a reduced capacity to respond to them. Moreover, and given that HCMV is a major opportunistic pathogen, the significance of the available data may be affected by reverse causation.]

Prior HCMV infection can alter or accentuate the response to a directed immune challenge. For example, HCMV-infected young adults displayed elevated antibody levels and $CD8^+$ T-cell responses after influenza vaccination (Furman et al., 2015). [The Working Group noted that the ability of HCMV to change the course of common infections has not been studied. In addition, HCMV seems to have a multifactorial role in changing immune function.] Children with a greater frequency of physician-managed infections were hypothesized to be more likely to be HCMV-infected and to develop HCMV-positive ALL. HCMV infection during pregnancy has also been linked to alterations in neonatal cytokine profiles (Wiemels et al., 2022; Gallant et al., 2023) (see Section 2). A shift to a T-helper cell 17 (Th17) response was reported in HCMV-seropositive individuals after SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) infection (Frozza et al., 2023). $CD4^+$ and $CD8^+$ T cells from individuals with higher HCMV-specific T-cell responses displayed a Th17-dominant response upon stimulation by the SARS-CoV-2 spike and nucleocapsid proteins, which is typically considered to provide protection against bacteria and fungi but to be a dysfunctional response to SARS-CoV-2. However, the Th17-dominant response was not associated with differences in clinical outcomes in patients with COVID-19 (coronavirus disease 2019, caused by SARS-CoV-2) (Frozza et al., 2023). [The Working Group noted that, although the findings on vaccination response and frequency of infection supported the idea that HCMV affects the immune system, HCMV seems to have a multifactorial role in changing immune function and there was no consistent evidence of HCMV-mediated immunosuppression. In addition, the significance of the available data may be affected by reverse causation.]

Several end-points associated with immunosuppression have been studied in patients with cancer. In patients with breast cancer, HCMV

gB antibody levels were lower than in matched controls among Japanese patients and individuals of Japanese descent in Brazil, but not in other ethnicities, possibly indicating reduced HCMV control (Pandey et al., 2016). Further research suggested that these differences may be caused by different affinities of HCMV-encoded decoy Fcγ receptors (FcγR) for different genetic markers of IgG, i.e. immunoglobulin GM (γ marker) allotypes (Pandey et al., 2017). Also, adaptive NK cell responses may be relevant in the control of breast cancer, but the role of HCMV infection in this context remains elusive (Bordignon et al., 2023). Increased HCMV antibody titres were associated with increased $CD8^+$ T-cell levels and a lower $CD4^+/CD8^+$ cell ratio in patients with head and neck cancer (Nelson et al., 2022). [The Working Group noted that although these findings provided some evidence of an effect of HCMV on the immune system in patients with cancer, reverse causation may have contributed to the observed findings, i.e. the presence and effects of HCMV may be caused by pre-existing reduced immune function.]

(b) Human cells

T-cell function is critically involved in maintaining HCMV latency (Beninga et al., 1996; Manley et al., 2004; Lim et al., 2020; Hassanzadeh et al., 2023). However, HCMV inhibits the expression of major histocompatibility complex class I (MHC-I) molecules (Ahn et al., 1996). HCMV can also directly inhibit $CD4^+$ T-cell proliferation and function in models that include those in human primary cells (Fornara et al., 2013). HCMV infection can also induce regulatory T cells that reduce the proliferation and activation of $CD8^+$ T cells and $CD4^+$ T cells and induce apoptosis in these immune cell types. Moreover, HCMV-induced regulatory T cells enhanced the levels of CTLA-4, a dendritic cell ligand that inhibits T-cell activation, and promoted apoptosis in dendritic cells (Tovar-Salazar and Weinberg, 2020). Various primary human Th cell types including 1 and 22 have been described to contribute to the immune response to HCMV (Lim et al., 2020; Hassanzadeh et al., 2023).

HCMV also interferes with innate immune cells and the interplay between the innate and the adaptive immune response (Goodrum et al., 2021). For example, HCMV has been found to inhibit MHC-II expression and to modify MHC-II protein localization on antigen-presenting cells, particularly dendritic cells (Cebulla et al., 2002; Lee et al., 2006, 2011; Sandhu and Buchkovich, 2020). Moreover, HCMV can generally inhibit dendritic cell maturation and function (Moutaftsi et al., 2002). Consistent with this observation, dendritic cells isolated from immunocompetent individuals undergoing symptomatic HCMV infection displayed impaired immune phenotype and immune function (Frascaroli et al., 2006a).

HCMV infection can also inhibit monocyte differentiation into macrophages and dendritic cells (Gredmark and Söderberg-Nauclér, 2003; Gredmark et al., 2004a, b) and inhibit monocyte-mediated immune responses (Frascaroli et al., 2006b; Avdic et al., 2013, 2016; Planchon et al., 2024).

Furthermore, HCMV interacts with NK cells in different ways, and NK cells are important in the control of HCMV (Goodrum et al., 2021; Forrest et al., 2023). HCMV prevents NK cell-mediated lysis of infected cells by various mechanisms (Tomasec et al., 2005; Zou et al., 2005; Wilkinson et al., 2008). Moreover, adaptive NK cells are involved in HCMV infection control (Goodier et al., 2014; Luetke-Eversloh et al., 2014; Lee et al., 2015; Kared et al., 2018; Muccio et al., 2018; Peppa et al., 2018; Mujal et al., 2021; Rückert et al., 2022).

Finally, HCMV encodes a variety of immunoevasive genes. Among these are viral cytokines, such as vIL-10 (UL111A). These viral genes promote immunosuppression through multiple mechanisms, including induction of immunosuppressive host cytokines and interference with

innate immune responses, such as interferon signalling (Young et al., 2017; Elder et al., 2019b; Schwanke et al., 2023; Vlachava et al., 2023; Almeida et al., 2025; Pamela et al., 2025). [The Working Group noted that although the studies described above were informative in describing how HCMV may exert modulatory effects on end-points associated with the immune system, though it was not possible to conclude whether these alterations were surrogates for immunosuppression or were surrogates for the interplay between the antiviral immune response and individual infected cells (immune evasion).]

Synopsis

[The Working Group noted that there was some evidence that HCMV has an impact on the immune system in exposed humans, with supportive evidence from studies in human cells in vitro. The evidence from human cells was considered to be informative regarding the end-points targeted by HCMV. However, the evidence from mechanistic studies in exposed humans was not consistent and the Working Group could not determine whether the presence and effects of HCMV could be caused by pre-existing reduced immune function.]

4.2.7 Modulates receptor-mediated effects

(a) Exposed humans

In Norway, Rahbar et al. (2017) analysed paraffin-embedded biopsy specimens from patients with breast cancer, including breast cancer ($n = 62$), ductal carcinoma in situ (DCIS; $n = 19$), and adjacent normal breast tissue ($n = 42$), for the presence of HCMV DNA, RNA, IE, and LA. The study found a negative correlation between the expression of IE and that of estrogen receptor-α (ERα) ($n = 47$; $P = 0.02$) and progesterone receptor (PR) ($n = 44$; $P = 0.003$), with no significant association observed with human epidermal growth factor receptor 2 (HER2).

In contrast, Cui et al. (2018) in China examined samples from 146 patients with breast cancer and 40 control participants with normal breast tissue for the presence of HCMV DNA, IE, and LA. IE and LA proteins were highly expressed in all breast cancer tissue samples. A significant negative association was reported between IE expression and ERα (31 patients; $P < 0.05$), but no significant association with PR or HER2.

[The Working Group noted that receptor activation was not investigated in these studies. Moreover, reverse causation may have contributed to the observed effects.]

(b) Human cells

HCMV infection and replication are associated with complex signalling events, including receptor-mediated signalling (Mahmud et al., 2020; Goodrum et al., 2021). EGFR and PDGFRα (platelet-derived growth factor receptor alpha) have been suggested as entry receptors for HCMV (Wang et al., 2003; Soroceanu et al., 2008; Feldmann et al., 2021). EGFR transfection of EGFR-negative cells was reported to be sufficient to enable HCMV infection of cells that were previously not susceptible (Wang et al., 2003). Moreover, HCMV-mediated EGFR signalling was also reported to be involved in HCMV infection (Wang et al., 2003). However, other researchers were not able to reproduce this finding (Isaacson et al., 2007; Vanarsdall et al., 2012).

Similarly, there were conflicting data on the role of PDGFRα and PDGFRα signalling on HCMV entry. Although it was initially reported that HCMV entry depends on the presence of PDGFRα and on PDGFRα signalling (Soroceanu et al., 2008), the results of follow-up studies showed that PDGFRα was required only for host cell infection by mutant laboratory strains such as AD169 and not by wildtype viruses, and that inhibition of PDGFRα signalling did not interfere with HCMV replication (Michaelis et al., 2011; Vanarsdall et al., 2012; Wu et al., 2018). [The Working Group noted that the evidence

on the effects of HCMV on EGFR and PDGFRα signalling was inconclusive.]

In one study in human fibroblasts, it was shown that HCMV infection induces vitamin D resistance by downregulating the vitamin D receptor (VDR) through upregulation of the transcriptional repressor Snail1 (Rieder et al., 2017; Stecher et al., 2022).

Other studies reported on HCMV infection of cancer cells. Rahbar et al. (2020) investigated the impact of HCMV on prolactin (PRL) and its receptor (PRLR) in infected ovarian cancer cells (SKOV3). Their findings revealed that HCMV infection significantly increased levels of both PRL and PRLR protein and transcript. Additionally, tissue samples from nine patients with ovarian cancer in Sweden exhibited high expression of both PRLR and HCMV IE antigen. However, receptor activity was not determined.

Moon et al. (2008) demonstrated that androgen (methyltrienolone) induced activation of the HCMV MIEP in LNCaP cells via protein kinase A (PKA) activation.

Synopsis

[The Working Group noted that there was some evidence from the infection of human cells that HCMV modulates receptor-mediated effects, in particular, in cells that may be relevant in cancer development. Data from exposed humans focused on receptor abundance but not on receptor activation.]

4.2.8 Causes immortalization

Humans

(i) Exposed humans

There were numerous studies on the presence of HCMV DNA, RNA, and proteins in tumours in humans. Bartek et al. (2020) investigated the stemness properties of medulloblastoma cells in clinical specimens from a cohort of 25 paediatric patients with medulloblastoma. These patients were treated for newly diagnosed medulloblastoma, with 24 undergoing first-time surgical resections and one receiving secondary surgery because of tumour recurrence, the first surgery being performed 1 year previously. IHC analysis revealed subsets of cells expressing stem cell markers CD133 (22 out of 25 cases, 0.5–5% positive cells; 3 out of 25 cases, 7–20% positive cells), CD15 (10 out of 25 cases, < 5% positive cells; 15 out of 25 cases, 10–40% positive cells), and the candidate marker vascular endothelial growth factor receptor 2 (VEGFR2) (all cases, 0.5–20% positive cells; and 4 out of 25 cases, > 7% positive cells), compared with normal cerebellum tissue. Notably, no case specimen gave completely negative results for any of these three proteins. Tumours in this cohort exhibited high proliferative indices (Ki67 score), pronounced endogenous DNA damage (γH2AX marker), and consistent expression of both IE and late HCMV proteins in a modest fraction of tumour cells. [The Working Group noted that, despite its small size, this was a well-conducted study that suggested an association between HCMV and cell stemness.]

(ii) Human cells

Early evidence documented HCMV's ability to induce the transformation in vitro of human embryonic lung fibroblasts (Geder et al., 1976, 1977). Infection of human embryo lung cells with the Mj strain of HCMV led to long-term persistent infection, and occasional cell transformation. These cells induced nondifferentiated tumours when injected into weanling athymic nude mice. After the subcutaneous inoculation with 2×10^7 cells, progressively growing tumours appeared in six of nine mice after an average latent period of 19 days. The tumours were composed of small, polygonal cells with large nuclei and scanty cytoplasm, embedded in an abundant collagenous matrix. The cells were poorly differentiated but may have been of epithelial origin (Geder et al., 1977). [The Working Group noted that Geder

et al. (1977) did not perform functional assays to demonstrate anchorage-independent growth.]

Several studies have explored whether HCMV can confer stemness properties and transform human mammary cells. Kumar et al. (2018) studied the impact of HCMV strain DB (KT959235) on human primary mammary epithelial cells (HMECs). Upon infection, several molecular changes were observed, including inactivation of retinoblastoma (Rb) and p53 proteins, activation of telomerase activity, activation of proto-oncogenes such as Myc and Ras, and upregulation of cyclin D1 and Ki67 antigen. Prolonged culture of infected HMECs resulted in the formation of spheroid cell clusters referred to as HCMV-transformed HMECs, injection of which into immunodeficient NOD/SCID gamma mice led to the development of triple-negative breast tumours. [The Working Group noted that activation of telomerase activity has been observed in infected HMECs; however, no colony formation was observed using an anchorage-independent assay.]

Nehme et al. (2021) screened various clinical HCMV strains and demonstrated that, besides HCMV-DB, strains such as HCMV-PJ, HCMV-RM, and HCMV-BL induced colony formation, indicative of anchorage-independent growth. At about day 30–50 post-infection, dense cell aggregates appeared, followed by the emergence of morphologically distinct cells in cultures infected with HCMV-BL. Furthermore, HCMV infection led to the generation of polyploid giant cancer cells, which exhibited characteristics of embryonic stem cells and epithelial–mesenchymal plasticity (Herbein and El Baba, 2024). The acquisition of stemness markers was positively correlated with upregulation of EZH2 and Myc expression (Nehme et al., 2022). El Baba et al. (2022) confirmed that HCMV strains B544 and B693, isolated from triple-negative breast cancer biopsies with high EZH2 and Myc expression, conferred transforming and stemness properties on normal HMECs. [The Working Group noted the concept of "high risk" and "low risk" HCMV strains, which refers to distinct properties regarding transforming potential, and could potentially be linked to cancer types induced, and patient clinical outcomes.]

In addition to HMECs, "high-risk" HCMV strains, such as HCMV-DB and BL, can also induce the transformation and formation of polyploid giant cancer cells in long-term cultures of ovarian epithelial cells (El Baba et al., 2023) and prostate epithelial cells (Bouezzedine et al., 2023).

Several studies also investigated the mechanisms by which HCMV regulates stemness properties in GBM cells. Liu et al. (2017) found that primary GBM cells infected with HCMV in vitro exhibited characteristics of cancer stem cells, including an increased percentage of slow-growing cells, enhanced sphere-forming potential, and greater self-renewal capacity. [The Working Group noted that no markers of cancer stem cells were measured in this study.] Soroceanu et al. (2015) showed that IE proteins promoted stemness in GBM. More than 70% of IE-positive cells also expressed CD133. Co-localization of HCMV IE proteins with Nestin and Sox2, markers associated with glioma stem-like cells, was also observed. [The Working Group noted that HCMV IE1 expression increased the glioma stem-like phenotype. However, using a transgenic mouse xenograft it was not possible to show a direct relationship between HCMV infection and tumour initiation.] Wang et al. (2017b) demonstrated that the human glioma U251 cell line when infected with HCMV AD169 strain expressed stem cell markers (CD133, Nestin, Notch1) and had increased neurosphere-forming ability, although other stem cell markers (Sox2, Oct4, Myc, Klf4 and Bmi-1) were unaffected. Infection of human diploid fibroblasts (HDFs), including fetal lung-derived HL411 and MRC5 cells, and human malignant glioma cell lines (U178, U251, U343, U373, and U563) with HCMV or transfection with expression vectors encoding

HCMV IE-72 or IE-86 resulted in telomerase activation in both HDFs and malignant glioma cells, as shown by expression of hTERT (human telomerase reverse transcriptase) and telomerase activation assays (Stråat et al., 2009). [The Working Group noted that, because HCMV-infected fibroblasts die from virus-mediated lysis, the authors were unable to determine either the functional consequences of HCMV-mediated telomerase activation or whether hTERT expression or telomerase activation extended the lifespan of infected cells.] Conversely, both pharmaceutical telomerase inhibition and small interfering RNA (siRNA)-mediated knockdown of hTERT reduced HCMV gene expression, protein accumulation, DNA replication, and virion production (Cavanaugh et al., 2025). Fiallos et al. (2014) used U87 and T98G GBM cell lines and primary glioma stem-like cells (GSC) 387 and 3832 infected with HCMV strain AD169 to measure viral gene expression over several time points (up to 15 weeks) after infection. HCMV-infected GSCs outlived their uninfected counterparts, and this was associated with increased tumoursphere frequency and upregulation of stemness regulators such as SOX2, p-STAT3, and BMX. Treatment with IL-6 significantly upregulated HCMV gene expression in long-term infected glioma cultures. [The Working Group noted that HCMV-infected GSC showed stem cell markers and induced sphere formation; however, sphere formation over serial passages has not been demonstrated.]

Wolmer-Solberg et al. (2013) examined six neuroblastoma cell lines (SH-SY5Y, SK-N-BE(2), SK-N-AS, SK-N-DZ, Kelly, and IMR-32) and found that HCMV-positive cells also expressed CD133 or CD44, both of which are stem cell markers.

Additionally, HCMV infection activated the IL-6-JAK-STAT3 pathway in human primary hepatocytes and HepG2 cells, promoting cellular proliferation, hepatocyte transformation, and enhanced HepG2 tumoursphere formation (Lepiller et al., 2013). In colorectal cancer cells, HCMV infection activated epithelial–mesenchymal transition and WNT signalling pathways (Teo et al., 2017).

Of the many candidate HCMV oncoproteins, IE1 is unique in that it has been demonstrated to individually induce the transformation of human cells, including HMECs, astrocytes, and ovarian epithelial cells. Ovarian epithelial cells transformed by IE1 exhibit characteristics that are similar to those reported for ovarian epithelial cells that have been transformed by HCMV, including the formation of polyploid giant cancer cells. IE1-mediated transformation has been linked to downregulation of the Rb/p53 pathway and the upregulation of the Myc/EZH2 pathway (El Baba et al., 2025). [The Working Group noted that this study employed both gain- and loss-of-function assays to demonstrate the role of IE1 in cellular transformation and stemness. Although increased expression of stemness markers was demonstrated, the observation of sphere formation could not be considered robust.]

Synopsis

[The Working Group noted that there were few reported data on an association between HCMV infection and end-points relevant to the key characteristic "causes immortalization" (KC9) in human primary epithelial or glial cells. The molecular processes underlying these phenotypes appear to involve well-known cellular oncogene and tumour suppressor pathways, as well as the viral IE1 protein. However, the informativeness of the studies was reduced by some technical limitations.]

4.2.9 Alters cell proliferation, cell death, or nutrient supply

(a) Exposed humans

Several studies have linked HCMV infection to increased proliferation in human tissues. For instance, Wen et al. (2020) demonstrated a

correlation between HCMV IE1/2 expression and Ki67 levels in 78 high-grade gliomas. However, many of these studies were conducted outside the tumour context. For instance, Albano et al. (2017) describes a case–control study nested within two cohorts of neonates screened for HCMV: one cohort included 30 308 neonates screened for HCMV in saliva, and the other included 4712 neonates born to HCMV-seropositive mothers and screened for HCMV in cord blood. Of these, 59 neonates tested positive for HCMV and were matched 1:4 with HCMV-negative neonates on birth date and hospital. Markers of cord blood potency were examined in 19 HCMV-infected neonates and 76 controls. The results suggested that congenital HCMV infection had an effect towards higher proliferation on $CD45^+$ cells as well as $CD34^+$ and other haematopoietic progenitor cells.

[The Working Group considered that these studies were informative as they measured reliable markers of cell proliferation in tumour and normal tissue. However, reverse causation could not be ruled out.]

(b) Human cells

HCMV infections are associated with an intricate interplay between the virus and its constituents and host cell survival and proliferation (Goodrum et al., 2021). Early evidence indicated that the infection of human embryonal lung cells with viruses, including the HCMV laboratory strains AD169, C-87, and Davis, and clinical isolates induced upregulation and activation of the proto-oncogenes *MYC*, *JUN*, and *FOS* in a manner that was independent of replication and IE gene expression (Boldogh et al., 1990, 1991a, b, c). Follow-up research on the HCMV proteins IE1 and IE2 showed that they could also promote expression of MYC and FOS in JURKAT and THP-1 cells (Monick et al., 1992).

A range of HCMV proteins including IE1, IE2, pUS21, pUL36, UL37-encoded vMIA, and pUL38 were shown to inhibit apoptosis and other forms of cell death in various cell systems (Zhu et al., 1995; Tanaka et al., 1999; Yu and Alwine, 2002; Reboredo et al., 2004; McCormick et al., 2005; Pauleau et al., 2007; Zhang et al., 2013c; Hong et al., 2016; Luganini et al., 2018; Sun et al., 2018; Zhao et al., 2019; Fletcher-Etherington et al., 2020). Accordingly, HCMV has been described as interfering with host cell death via several mechanisms. HCMV infection has been shown to disrupt p53 function through several mechanisms, including the sequestration of p53 in the cytosol, as reported in one study (Kovacs et al., 1996; Tanaka et al., 1999; Hsu et al., 2004; Zhang et al., 2006; Kwon et al., 2012; Savaryn et al., 2013). Moreover, HCMV infection and HCMV proteins were shown to inhibit cell death via mechanisms that included upregulation of anti-apoptotic Bcl-2 family proteins, inhibition of pro-apoptotic Bcl-2 family proteins, and MEK/ERK signalling (Wang et al., 2006; Pauleau et al., 2007; Zhang et al., 2013c; Hong et al., 2016).

Notably, there are also conflicting results with regard to the impact of HCMV infection and proteins on cell viability. For example, IE1 and IE2 have been reported to protect cells from cell death, to have no effect on cell death, and to induce cell death (Zhu et al., 1995; Tanaka et al., 1999, Wang et al., 2000; Yu and Alwine, 2002; Zhao et al., 2019). Accordingly, infection of human foreskin fibroblasts with HCMV strain Towne induced mitochondrial apoptosis in one study (Lee et al., 2013b). Moreover, HCMV-miR-US4-5p induced apoptosis in human embryonic lung fibroblasts (Shao et al., 2017). The discrepancies between the findings of different studies were likely to be a consequence of variation in experimental set-ups, including the use of different HCMV strains. For example, the low-passage HCMV Merlin strain, but not laboratory strain AD169, induced pUL36-mediated degradation of mixed lineage kinase domain-like protein (MLKL), resulting in the inhibition of necroptosis in hTERT-immortalized primary human

fetal foreskin fibroblasts (Fletcher-Etherington et al., 2020).

However, lytic HCMV infection results in cell death (Shen et al., 2004; Britt, 2008; Sinzger et al., 2008; Griffiths et al., 2015; Goodrum et al., 2021; Griffiths and Reeves, 2021; Matthews et al., 2023; Mocarski, 2024), and HCMV-mediated inhibition of premature host cell death during the lytic infection of various HCMV-permissive cell types was found to be a prerequisite for efficient virus replication (Zhu et al., 1995; Kovacs et al., 1996; Reboredo et al., 2004; Wang et al., 2006; Terhune et al., 2007; Luganini et al., 2018; Sun et al., 2018; Fletcher-Etherington et al., 2020).

There was one report on productive HCMV TB40/E infection of terminally differentiated forebrain neurons derived from iPSCs that did not result in cell death. HCMV caused fusion in these cells, which expressed proliferation markers and lost their electrophysiological neural functions (Adelman et al., 2023).

Much less is known about the role of HCMV in the regulation of cell death during latent infection. Varying findings were reported on AKT activation in various HCMV-infected cell types (Cobbs et al., 2007; Zhang et al., 2022). However, PI3K/AKT signalling was reported to contribute to the maintenance of latent HCMV infection in $CD14^+$ monocytes and to extend their survival via upregulation of anti-apoptotic proteins, including MCL1, HSP27, and XIAP (Chan et al., 2010; Peppenelli et al., 2016, 2018). SIRT1 and SIRT2 were also described to interfere with cell death in human peripheral blood $CD14^+$ monocytes (Cheung et al., 2023; Miller et al., 2024) and SIRT1-induced mTORC1-mediated reprogramming of mRNA translation (Miller et al., 2024). In human $CD34^+$ haematopoietic progenitor cells, HCMV UL7, miR-US5–1, and miR-UL112–3p were found to inhibit apoptosis via inactivation of FOXO3a (Hancock et al., 2021). However, HCMV latency in $CD34^+$ haematopoietic progenitor cells and $CD14^+$ monocytes is anticipated to result normally in cell differentiation, leading to the reactivation of the lytic viral replication and host cell death (Collins-McMillen et al., 2018; Mikell et al., 2019; Diggins et al., 2021).

As well as having an effect on cell death signalling, HCMV also interferes with the host cell cycle during lytic infection. Early research revealed that HCMV can interfere with the cell cycle in human fibroblasts at various stages (Jault et al., 1995; Lu and Shenk, 1996). Consistent with this finding, HCMV pUL21A was demonstrated in MRC-5 cells and human foreskin fibroblasts to reduce cellular levels of cyclin A, which is involved in the transition through different cell cycle checkpoints (Caffarelli et al., 2013).

In addition, most studies reported that HCMV induced a G1/S cell cycle block (Petrik et al., 2006). Many HCMV proteins were shown to contribute to an HCMV-induced G1/S cell cycle block (including IE2, UL69, UL97, UL80, UL76, JIL, TRL5, UL101, UL12, UL3, and Us30) (Hayashi et al., 2000; Petrik et al., 2006; Paladino et al., 2014; Iwahori et al., 2015), and Rb inhibition and hyperphosphorylation were identified as a point of interference with the host cell cycle (Johnson et al., 2000; Iwahori et al., 2015). However, Rb function was also shown to be required for effective AD169 replication in primary human dermal fibroblasts (VanDeusen and Kalejta, 2015). Hence, Rb plays a multifaceted role in HCMV replication. Technically, HCMV does not appear to induce a canonical cell cycle block, but to mediate cell cycle transition into S phase, with a block before the duplication of the host cell genome, which is part of preparing the host cell metabolism for virus replication (Bresnahan et al., 1996; Salvant et al., 1998; Sinclair et al., 2000; Wiebusch and Hagemeier, 2001; Kalejta et al., 2003; Bogdanow et al., 2021; Goodrum et al., 2021; Betsinger et al., 2023).

There was also evidence that HCMV can induce replicative senescence in primary human fibroblasts via p16INK4a upregulation, inhibiting EGF-mediated human fibroblast proliferation by HCMV-miR-US5-2–mediated depletion

of GAB1 (GRB2-associated-binding protein 1), and that it can interfere with epithelial–mesenchymal transition in fibroblasts and ARPE19 cells (Zannetti et al., 2006; Oberstein and Shenk, 2017; Hancock et al., 2020).

There were also indications that HCMV infection can modify host cell metabolism during lytic infection (Rodríguez Sánchez et al., 2019).

[The Working Group noted that numerous studies have described effects of HCMV infection on endothelial cells and processes involved in angiogenesis.] HCMV infection of endothelial cells induced endothelial cell migration and tube formation by various mechanisms (Zhang et al., 2013a, b, 2017b, 2020). [The Working Group noted that these findings were derived from endothelial cells infected with HCMV strain AD169. Although endothelial cells are permissive to lytic infection with low-passage HCMV strains, they do not normally support the replication of laboratory HCMV strains that have been extensively passaged, typically in fibroblasts, such as AD169 or Towne (Slobbe-van Drunen et al., 1998; Gerna et al., 2003; Revello and Gerna, 2010). However, similar endothelial cell migration and tube formation have been observed using the clinical/clinical-like strain TB40/E-UL32 (Bentz and Yurochko, 2008).] AD169 infection of human vascular smooth muscle cells resulted in increased haematopoietically expressed homeobox (HHEX) levels, and HHEX promoted proliferation and inhibited apoptosis in human vascular smooth muscle cells (Li et al., 2016). [The Working Group noted that it was not clear whether AD169 in these experiments reflected the tropism of clinical virus isolates.]

Infection of various cell types with different HCMV strains resulted in the production of pro-angiogenic factors such as VEGF, and the virus-free supernatants of HCMV-infected cell cultures induced endothelial cell survival, endothelial cell migration, and endothelial cell tube formation (Reinhardt et al., 2005; Botto et al., 2011; Fiorentini et al., 2011; Soroceanu et al., 2011; MacManiman et al., 2014). Hence, there is a possibility that HCMV infection of cells in the microenvironment of pre-cancerous stages and early cancer stages may contribute to cancer formation and progression by promoting tumour vessel formation. [The Working Group noted that the described experiments did not cover the complete process of vessel formation.]

There was also a substantial amount of information on the effects of HCMV infection, HCMV proteins, and HCMV miRNAs on human cancer cells (Allart et al., 2002; Kim et al., 2003; Terrasson et al., 2005). In several studies, it was reported that HCMV and/or its constituents increased glioma cell proliferation, migration, and invasiveness, inhibited glioma cell death (Lee et al., 2005; Cobbs et al., 2007, 2014; Soroceanu et al., 2011; Matlaf et al., 2013; Wang et al., 2014; Costa et al., 2016; Tseliou et al., 2016; Hu et al., 2017; Liang et al., 2017, 2019; Heukers et al., 2018; van Senten et al., 2019; Zhao et al., 2019; Singh and Neumann, 2020; Wen et al., 2023), and promoted growth in glioma xenograft models (Hu et al., 2017; Heukers et al., 2018; van Senten et al., 2019; Wen et al., 2023). Moreover, HCMV infection of LN-18 GBM cells induced paracrine signalling that caused metabolic and epigenetic changes in uninfected fibroblasts in a transwell system (Harrison et al., 2022).

However, there were also conflicting results on the interaction of HCMV and GBM cells. In one study, HCMV strain Towne infection and IE1 expression were described as protecting HCMV-permissive U373MG and SAOS2 osteosarcoma cells (but not non–HCMV-permissive A549 lung cancer cells) from etoposide-induced apoptosis (Kim et al., 2003). This raised the question of whether the observed anti-apoptotic effects are part of a lytic replication cycle, as U373MG and SAOS2 are regularly used as models of lytic HCMV infection (Johnson et al., 2000; Murphy et al., 2000; Wiebusch and Hagemeier, 2001; Kwon et al., 2012; Iwahori et al., 2015). In this context, one study found that U373MG cells infected with

HCMV strain AD169 can undergo cell division (Al-Qahtani et al., 2020). Another study had previously shown that HCMV infection induced U373MG cell entry into early S phase, in which host cell DNA synthesis and cell division were blocked with the involvement of IE2 (Murphy et al., 2000).

HCMV TB40/E and AD169 infection of the GBM cell lines A172, TP365MG, and U251MG reduced cell viability in correlation with their permissiveness to lytic infection and did not protect from toxicity induced by temozolomide and carmustine (dos Santos et al., 2018). Whereas Cobbs et al. (2008) found that IE1 stimulated proliferation in U87 and U118 GBM cells but induced growth arrest in LN229 and U251 cells and immortalized human astrocytes, Singh and Neumann (2020) reported that persistent HCMV AD169 infection in LN229 cells increased their viability and proliferation and conferred marked resistance to the DNA-alkylating agent temozolomide.

There was also evidence that chronic HCMV infection could increase neuroblastoma cell malignancy in human cell lines (Cinatl et al., 1996, 1998; Scholz et al., 1999, 2000; Blaheta et al., 2004; Hoever et al., 2005). Moreover, the HCMV protein pUL37x1 protected SH-SY5Y cells from apoptosis at least in part via bax inhibition (Hong et al., 2016). Although the pro-malignant effects were initially reversible upon virus removal (Cinatl et al., 1998), long-term exposure to HCMV could also result in the establishment of a highly aggressive cell population in which the malignancy phenotype no longer depends on the presence of HCMV (Michaelis et al., 2012).

HCMV and its constituents can promote proliferation and invasiveness and/or inhibit cell death in human cell lines derived from colorectal cancer, multiple myeloma, cervical cancer, lung cancer, and osteosarcoma (Hsu et al., 2004; Wei et al., 2004; Lares et al., 2013; Savaryn et al., 2013; Cai et al., 2016; Teo et al., 2017; Härtel et al., 2021). Rahbar et al. (2021) showed a potential proliferative effect of HCMV in ovarian borderline tumour tissue.

TB40E and clinical HCMV isolates productively infected Caco-2 cells and inhibited bortezomib-induced apoptosis (Härtel et al., 2021). In the mesenchymal breast cancer cell lines MDA-MB-231 and SUM1315MO2, HCMV induced mesenchymal–epithelial transition in association with a delayed or perhaps abortive replication cycle, which may promote metastasis formation (Oberstein and Shenk, 2017). Various infection rates for HCMV strain Hi were detected in the prostate cancer cell lines DU145 (< 1% of cells), LNCaP (5%), and PC3 (30%). IE1/IE2-independent integrin beta1-mediated increased adhesion to endothelial cells, and collagen was detected in HCMV Hi–infected PC3 cells (Blaheta et al., 2006).

There were also reports of anti-cancer effects with HCMV. A clinical HCMV isolate induced apoptosis in MO7e acute megakaryoblastic leukaemia cells (Sindre et al., 2000). HCMV US28 induced caspase-dependent apoptosis in 293T (immortalized human embryonic kidney) cells, HeLa (cervical carcinoma) cells, and COS (immortalized monkey kidney) cells (Pleskoff et al., 2005), and AD169 caused apoptosis in HepG2 xenografts and significantly restricted HepG2 xenograft formation in nude mice (Kumar et al., 2016). Also, overexpression of HCMV UL138 caused apoptosis of stomach cancer cells, partially via interaction with heat shock protein 70 (Chen et al., 2016).

HCMV can also modify cancer cell behaviour via paracrine effects. HCMV IL-10 increased the proliferation of MCF-7 breast cancer cells (Bishop et al., 2015) and enhanced MDA-MB-231 breast cancer cell invasion into Matrigel (Valle Oseguera and Spencer, 2017). In contrast, overexpression of HCMV IL-10 inhibited proliferation of AGS, BGC-803, and SGC-7901 stomach cancer cells, and reduced colony formation, migration, and invasion in AGS and SGC-7901 cells (Liu et al., 2020).

Finally, HCMV infection of ARPE-19 and various breast cancer cell lines increased HSATII RNA levels, and high HSATII RNA levels were shown to contribute to breast cancer cell proliferation and migration (Nogalski and Shenk, 2020).

Synopsis

[The Working Group noted that there was some evidence that HCMV could alter cell proliferation and cell death and that HCMV could alter nutrient supply. It was also noted that HCMV-infected cells may promote angiogenesis. The Working Group noted some concerns with regard to the angiogenesis models used, which covered only certain steps of the process.]

5. Summary of Data Reported

5.1 Exposure characterization

Human cytomegalovirus (HCMV) is a member of the herpesvirus family *Orthoherpesviridae* that can infect people of all ages but most commonly during childhood. Worldwide anti-HCMV immunoglobulin G (IgG) prevalence is estimated at 83% for the general population, ranging from approximately 40% to 100% across various countries. In general, prevalence is higher in South America, Africa, and South Asia than in North America and Europe, and increases with age. After primary infection, HCMV remains in the body (latent phase) and can reactivate throughout the lifetime. HCMV infection is most often asymptomatic or causes only mild illness such as mononucleosis. HCMV is transmitted via body fluids such as saliva, blood, urine, semen, and breast milk, and can also be transmitted from mother to fetus during pregnancy. Although usually harmless in healthy people, HCMV can cause serious complications such as hepatitis, colitis, retinitis, pneumonia, and encephalitis in immunocompromised patients, including transplant recipients or people with uncontrolled human immunodeficiency virus (HIV) infection. Congenital HCMV infection is estimated to occur in 0.7% of live births and is the most common viral cause of sensorineural hearing loss and neurological disabilities in neonates. Diagnosis essentially involves serology (measurement of IgG and IgM, most commonly with enzyme immunoassays) and nucleic acid amplification tests (NAATs), primarily with quantitative polymerase chain reaction (qPCR). Treatment of HCMV infection is generally not necessary for immunocompetent people but includes antiviral drugs for immunocompromised patients, to prevent or treat infection.

5.2 Cancer in humans

The association between HCMV and various cancer types was investigated in multiple cohort (and nested case–control) studies and a large number of case–control studies. In addition, the evaluation included studies making within-person comparisons, case series, meta-analyses, and a case–case study. Cancer types that have been studied more extensively were glioma, childhood acute lymphoblastic leukaemia (ALL), non-Hodgkin lymphoma (NHL), and cancers of the colorectum, breast, and prostate gland. The only study that investigated multiple types of cancer used a retrospective cohort design to follow up transplant recipients for cancer occurrence. Cohort and nested case–control investigations determined exposure to HCMV either by testing blood samples for the presence of HCMV antibodies, nucleic acids, or proteins, or by reviewing medical records to retrieve information on HCMV-related illnesses. Although some case–control studies also tested for HCMV antibodies, most of them, and all of the within-person comparison studies, tested tissue specimens for the presence of HCMV nucleic acid sequences, HCMV proteins, or both. Few studies were able to collect comprehensive information on other risk factors for the cancer types under

investigation. The cohort studies and nested case–control studies were all from high-income countries, whereas the other studies reviewed came from a much broader range of countries, including those in Africa and Asia.

A major issue in evaluating the published evidence on the relation between HCMV and cancer is the interpretation of studies in which HCMV markers in tumour were compared with those in normal tissue, whether in separate case and control groups, or within the same individuals. Interpretation of findings from such studies is vulnerable to the possibility of reverse causation, whereby a difference in HCMV marker prevalence between tumour and normal tissue is driven by the biology of the tumour, rather than indicating a causal role for HCMV in tumour formation. This unresolved concern meant that the evidence arising from studies of this kind was not accorded substantial weight in the evaluations, and that the Working Group largely focused on the studies in which infection status (either laboratory markers or clinical records) was obtained before the diagnosis of cancer. A further issue in interpreting the studies in which HCMV infection markers were assessed in tumour tissue was the high degree of variation in findings across studies on the same cancer type, raising questions about the reproducibility of the tests used. Other methodological issues included lack of information, particularly for several of the tissue marker studies, on the source of case and comparator specimens, and on factors that might play a confounding role.

For childhood ALL, studies with cohort and nested case–control designs were broadly consistent in finding a modestly elevated risk in relation to various markers of exposure to HCMV in pregnancy and neonates. The three most informative studies of childhood ALL each found a modest increase in risk for at least one measure of HCMV exposure, although the results of other studies were varied. A further record-linkage study found an increased risk of all childhood haematological malignancies, which are likely to include a substantial proportion of ALL, for medically recorded HCMV diseases. The Working Group considered that the body of evidence suggested a credible positive association for HCMV infection but could not exclude the possibility that one or more unmeasured confounding factors contributed to the observed association. Also, confidence intervals around effect measures for some of the studies were wide, leading to the possibility that chance may be an explanation for the positive findings.

For glioma, there was one nested case–control study in which a modest positive association was found, in particular for non-glioblastoma glioma, whereas in other cohorts or case–control studies, generally, no positive association was found. There were inconsistencies both in the reported prevalence of HCMV in glioma tissues across studies and in the differences observed between tumour and non-tumour tissues across case series. In one cohort study on multiple cancer types in transplant patients, results were essentially negative by type of cancer in relation to HCMV donor and recipient serostatus. For NHL and prostate cancer, other cohort or nested case–control studies in which HCMV status was determined before cancer diagnosis did not find a credible association between HCMV and cancer. For breast cancer, there was one study in which a positive association was found, whereas in other studies the association was null. Collectively, for all other cancer types including these, findings did not indicate a consistent increase or were of insufficient informativeness to permit conclusions to be drawn on the presence or absence of causal associations between exposure to HCMV and risk of cancer at these other cancer sites.

5.3 Cancer in experimental animals

The two available studies were considered to be uninformative for the evaluation of the carcinogenicity of HCMV in experimental

animals, given the use of ultraviolet radiation–inactivated HCMV in a species that is not susceptible to infection by this virus.

5.4 Mechanistic evidence

HCMV causes a lifelong infection that is characterized by sporadic reactivations, during which virus is detectable in body fluids. HCMV can infect many different cell types and organs. Infections with the virus can be lytic or latent. There is also evidence of abortive infections, whereby cells do not undergo a complete virus replication cycle and the genome is not maintained; however, this has not been systematically investigated.

For the key characteristics of carcinogens, the Working Group overall considered that the evidence was suggestive in exposed humans, in human primary cells, and in experimental systems. A small minority of the Working Group considered the evidence to be consistent and coherent in human primary cells and experimental systems for two key characteristics of carcinogens.

There is suggestive evidence that HCMV is genotoxic. An increased number of DNA double-strand breaks in peripheral blood mononuclear cells was observed in one study in exposed humans. In human primary cells and in human cell lines, various strains of HCMV caused DNA strand breaks and chromosomal aberrations, which are induced by virus entry. In addition, chromosome aberrations and other markers of DNA damage were induced or enhanced by HCMV infection or the (co)expression of individual HCMV proteins, including immediate-early proteins IE1-72kDa, IE2-86kDa, and pUL76. However, several findings were derived from studies in lytically infected cells and cancer cell lines that die as a consequence of HCMV replication or that may not reflect physiologically relevant conditions.

There is suggestive evidence that HCMV alters DNA repair or causes genomic instability. There is a paucity of data available in exposed humans. There is evidence of alterations in DNA repair, as well as of genomic instability, in human primary cells and in human cell lines infected with HCMV or expressing individual viral proteins, including IE1-72kDa. However, it remains unknown whether HCMV can exert such effects in physiologically relevant cells that do not undergo lytic replication.

There is suggestive evidence that HCMV induces epigenetic alterations. A few studies in exposed humans showed changes in global DNA methylation patterns. Evidence has been provided for HCMV-induced epigenetic alterations in primary human cells and human cell lines. These alterations include global changes to DNA methylation patterns and post-translational histone modifications, such as the acetylation and methylation of histones H3 and H4. These epigenetic effects appear to be mediated by the up- or downregulation, or changes in intracellular localization, of epigenetic writers (e.g. DNA methyltransferase 1) and readers (e.g. PcG polycomb group proteins), as well as viral proteins that target histone-modifying enzymes, histone chaperones, and/or nucleosomes. Examples of these viral proteins include IE1-72kDa, IE2-86kDa, and pUL82 (pp71). However, it remains unknown whether HCMV can exert such effects in physiologically relevant cells that do not undergo lytic replication.

A small minority of the Working Group considered that the evidence of epigenetic alterations in human primary cells and human cell lines is consistent and coherent, because, in addition to epigenetic alterations observed in lytically infected cells, there were adequate findings in latently infected cells.

There is suggestive evidence that HCMV induces oxidative stress. No data were available in exposed humans. In several studies in human primary cells and in human cell lines,

the evidence suggests increases in mitochondrial respiration and in the generation of reactive oxygen species. HCMV pUL13 protein and other unspecified viral proteins appear to contribute to this phenotype. However, most of these findings are derived from studies in lytically infected cells that die as a consequence of HCMV replication and in systems that may not reflect physiologically relevant conditions.

There is suggestive evidence that HCMV induces chronic inflammation. HCMV infection is associated with several acute and chronic inflammatory processes in exposed humans. Several studies reported an association between inflammatory bowel disease and HCMV infection; however, it is unclear whether HCMV infection is a cause of the inflammatory bowel disease or a consequence, reflecting concerns about reverse causation. Findings in human primary cells and human cell lines were valuable in showing end-points associated with inflammation; however, they were not informative for demonstrating the chronicity of the effects.

There is suggestive evidence that HCMV is immunosuppressive. Several studies in exposed humans showed that HCMV infection affects the immune system and may be associated with an age-related decline in immune function. HCMV can interfere in different ways with the innate immune response, including via interferon expression and signalling. However, the evidence is inconsistent, and reverse causation could not be ruled out. Alterations in end-points associated with immunosuppression provided by human primary cells and in human cell lines were not informative without consistent evidence of immunosuppression in exposed humans, since such alterations could be a surrogate for the antiviral immune response.

There is suggestive evidence that HCMV modulates receptor-mediated pathways. There were a few studies available in exposed humans that indicated a negative correlation between HCMV IE gene expression and estrogen receptor-α (ERα) expression; however, evidence of HCMV-mediated receptor activation is lacking. The results of in vitro studies using primary human cells and human cancer cell lines suggest that HCMV may have the ability to regulate various receptors.

There is suggestive evidence that HCMV causes immortalization. There is a paucity of data in exposed humans. HCMV infection has been associated with increased stemness properties in human tumours, primary human cells, and human cell lines. The virus has also been shown to cause the transformation, and thus immortalization, of human primary epithelial and glial cells. Although these findings are suggestive, there are still concerns regarding the methodology used.

A small minority of the Working Group considered that the available studies provided consistent and coherent evidence that persistent HCMV infection can lead to the immortalization of human primary cells. These phenotypes appear to be caused by the activation of oncogenes (e.g. *MYC*) and the inhibition of tumour suppressor proteins (e.g. p53 and Rb) by the viral IE1-72kDa protein, as well as potentially other viral proteins.

There is suggestive evidence that HCMV alters cell proliferation, cell death, or nutrient supply. Data were available in exposed humans on cell proliferation end-points in tumours and in normal tissues. In addition, the results of numerous studies in human primary cells and in human cell lines suggest that HCMV alters cell proliferation, cell death, and nutrient supply end-points. However, findings were inconsistent across the studies.

6. Evaluation and Rationale

6.1 Cancer in humans

There is *limited* evidence in humans for the carcinogenicity of human cytomegalovirus. Positive associations have been observed between human cytomegalovirus and childhood acute lymphoblastic leukaemia.

6.2 Cancer in experimental animals

There is *inadequate* evidence in experimental animals regarding the carcinogenicity of human cytomegalovirus.

6.3 Mechanistic evidence

There is *limited* mechanistic evidence.

6.4 Overall evaluation

Human cytomegalovirus is *possibly carcinogenic to humans* (Group 2B).

6.5 Rationale

The Group 2B evaluation for human cytomegalovirus (HCMV) was based on *limited* evidence for cancer in humans. There was *limited* evidence that HCMV causes childhood acute lymphoblastic leukaemia (ALL) in humans. There were five available studies on HCMV exposure before diagnosis of ALL in children. A positive association was observed between HCMV exposure and childhood ALL in four of these studies, but chance, bias, and particularly confounding from other infection-related risk factors could not be ruled out as an explanation for the findings. For all other cancer types, the evidence was deemed *inadequate*, because the studies did not show consistent positive associations, were few in number, or were insufficiently informative to permit a conclusion to be drawn about the presence or absence of a causal association.

There was also *limited* mechanistic evidence, based on suggestive evidence for the key characteristics of carcinogens. While a small minority of the Working Group considered the mechanistic evidence to be *strong* on the basis of consistent and coherent evidence for the key characteristics of carcinogens "induces epigenetic alterations" in human primary cells and experimental systems, and "causes immortalization" in human primary cells, overall the Working Group judged these studies to provide only suggestive evidence. Evidence regarding cancer in experimental animals was *inadequate* because the two available studies were considered to be uninformative for the evaluation.

A minority of the Working Group supported a Group 2A classification for HCMV, based on a combination of *limited* evidence for cancer in humans and *strong* mechanistic evidence of the key characteristics of carcinogens, but these conclusions were not supported by the Working Group overall, thus leading to a Group 2B evaluation.

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MERKEL CELL POLYOMAVIRUS

1. Exposure Characterization

1.1 Identification

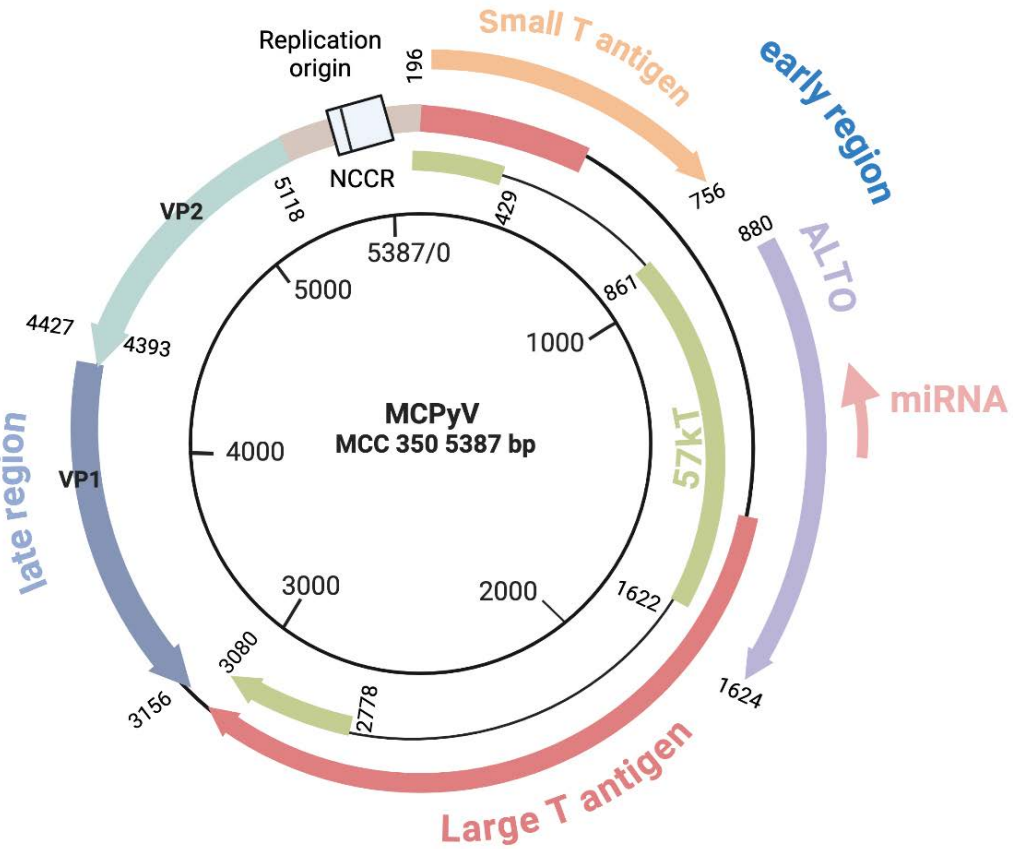
Structure, genome, and classification

In 2008, Patrick Moore, Yuan Chang, and colleagues identified a novel polyomavirus through digital transcriptome subtraction in Merkel cell carcinoma (MCC) primary tumours and metastases (Feng et al., 2008). Trabecular carcinoma of the skin, described by Toker (1972), shared features with MCC. Before 2008, given that MCC is more common in elderly and immunosuppressed individuals – a pattern suggestive of an infectious origin – some researchers searched for, but did not detect, oncogenic viruses such as Epstein–Barr virus (Shaw et al., 2006) and high-risk human papillomaviruses (HPVs) (Kanitakis et al., 2006).

The virus was subsequently named Merkel cell polyomavirus (initially abbreviated as MCV and ultimately as MCPyV) in reference to the shared dual epithelial/neuroendocrine phenotype of Merkel cells and MCC cells. The MCPyV genome was shown to be clonally integrated within the genome of MCC cells in approximately 80% of cases of MCC (Feng et al., 2008; Sastre-Garau et al., 2009; Schrama et al., 2019; Kervarrec et al., 2020; Starrett et al., 2020). MCPyV was classified by the International Committee on Taxonomy of Viruses (ICTV) in 2017 (Moens et al., 2017a) as *Alphapolyomavirus quintihominis* (realm: *Monodnaviria*; kingdom: *Shotokuvirae*; phylum: *Cossaviricota*; class: *Papovaviricetes*; order: *Sepolyvirales*; family: *Polyomaviridae*; genus: *Alphapolyomavirus*; species: *Alphapolyomavirus quintihominis*) (ICTV, 2025).

All polyomavirus species contain a single, circular, double-stranded DNA genome of approximately 5 kb encoding five to nine proteins (Houben et al., 2023), depending on the genus. The genome consists of two distinct transcriptional units located on opposite strands, i.e. the early region encoding the so-called T antigens and the late region encoding the capsid proteins (DeCaprio and Garcea, 2013) (Fig. 1.1). Between the early and late regions is located the non-coding control region (NCCR), which contains a bidirectional promoter and the origin of replication (Moens et al., 2017b). As such, the MCPyV genome (isolate MCC350, EU375803) is 5387 bp in length (Prado et al., 2018), and its early strand encodes two main early proteins, large T antigen (LT, 817 amino acids) and small T antigen (sT, 186 amino acids) which are characterized by a common N-terminus and a differentially spliced C-terminus (Fig. 1.1; see also Section 4.1). Two additional alternative splicing variants, the 57kDa T antigen (432 amino acids) (57kT) and a 28 kDa protein named alternative

Fig. 1.1 Circular genome map of MCPyV and its encoded gene products



The ~5.4 kb circular double-stranded DNA genome of Merkel cell polyomavirus (MCPyV) is organized into an early region, a late region, and a central non-coding control region (NCCR), which contains the origin of replication and bidirectional promoters that regulate transcription. The early region encodes several regulatory proteins: the small T antigen (sT), which modulates cellular signalling and promotes cell proliferation; the large T antigen (LT), essential for viral replication and cell cycle control; the 57kT isoform, produced by alternative splicing of the LT transcript and sharing the N-terminal region with LT; and ALTO (alternative T antigen), encoded in an overlapping reading frame within the LT locus. These early proteins play critical roles in viral replication, host manipulation, and, in oncogenic contexts, transformation. The late region encodes structural proteins: VP1 (major capsid protein, from 3156 to 4427 nucleotides) and VP2 (minor capsid protein, from 5118 to 4393 nucleotides), both required for virion assembly. The genome also encodes a viral microRNA driven by the late promoter, miR-M1, located antisense to the early region, which post-transcriptionally downregulates T-antigen expression.

The genome is shown in circular form, with transcriptional orientations, splicing events, overlapping reading frames (e.g. ALTO), and the position of the NCCR and replication origin indicated.

Adapted from Houben et al. (2023): Houben, R; Celikdemir, B; Kervarrec, T; Schrama, D. Merkel cell polyomavirus: infection, genome, transcripts and its role in development of Merkel cell carcinoma. *Cancers* 2023, 15, 444. https://doi.org/10.3390/cancers15020444. © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

LT open reading frame (ALTO) (248 or 250 amino acids), have been described (Carter et al., 2013; Korup-Schulz et al., 2017; Houben et al., 2023). The second strand of the genome encodes two late proteins, viral proteins VP1 and VP2, which are the major and minor capsid proteins, respectively. VP3, the third polyomavirus capsid protein, is not expressed because of the absence of an alternative start codon to initiate VP3 synthesis from VP2 messenger RNA (mRNA), as observed in other polyomaviruses (Schowalter and Buck, 2013). This late strand also encodes a viral microRNA (miRNA) that is involved in the

regulation of early-strand products (Seo et al., 2009; Lee et al., 2011).

Several MCPyV genotypes have been identified. Phylogenetic analyses demonstrate the existence of five major geographically related MCPyV genotypes (Europe/North America, [sub-Saharan] Africa, Oceania, South America, and Asia/Japan). None of these genotypes has been associated with a higher prevalence in virus-positive MCC (Freze Baez et al., 2014; Hashida et al., 2014; Martel-Jantin et al., 2014; Matsushita et al., 2014; Torres et al., 2018; Torres, 2020). The viral life cycle is described in Section 4.1.

1.2 Methods of detection

Identification of a current MCPyV infection is based on the detection of viral components by molecular biology techniques. The most commonly used method is detection of DNA via amplification by polymerase chain reaction (PCR). Current and past infections can be detected by the measurement of specific antibodies against viral capsid proteins, mainly VP1. Since the sequence diversity of known wildtype (WT) MCPyV isolates is very low, with >98.5% nucleotide identity across the viral genome (IARC, 2013), molecular methods based on viral DNA detection are preferred for cancer association studies (Iaria et al., 2015; Kervarrec et al., 2018). Because of the lack of commercially available assays, the detection of nucleic acids, viral proteins, and corresponding antibodies relies exclusively on in-house methodologies.

1.2.1 Detection of MCPyV DNA and RNA sequences

Viraemia is not part of the virus life cycle, which seems to be clearly skin-associated, and therefore detection of DNA in the blood is not useful for identifying MCPyV infection. Viral DNA can be extracted from formalin-fixed paraffin-embedded (FFPE) or fresh frozen tissue specimens, either from the tumour or not, or extracted from various other types of sample, such as skin swabs, blood, urine, and faeces. It is then detected by PCR, nested PCR, quantitative PCR (qPCR), digital PCR (including droplet digital PCR, ddPCR) (Arvia et al., 2017), rolling circle amplification (RCA) (Schowalter et al., 2010), fluorescence in situ hybridization (FISH) (Haugg et al., 2014), RNA in situ hybridization (RNA-ISH) (Wang et al., 2017) and techniques derived from CRISPR (clustered regularly interspaced short palindromic repeats) (Arora et al., 2020a). Of the different PCR amplification techniques, digital PCR was shown to have better sensitivity than did qPCR (Barchitta et al., 2022). PCR-based techniques use different primers specific to the genes encoding MCPyV LT, sT, and VP1 (for references, see Section 1.3.4 and Table 1.2). Methodological differences essentially relating to the primer pairs used across studies may explain some of the variation observed in the reported prevalence of MCPyV DNA (Eid et al., 2017). For PCR amplification from FFPE samples, as used in many studies, it has been reported that formalin fixation may fragment DNA and result in differences in amplification according to the size of the amplicons, and therefore lead to lower rate of detection than for fresh tissue samples (Kassem et al., 2009; Touzé et al., 2009). This is particularly important for samples containing very low copy numbers per cell (Pastrana et al., 2012). [The Working Group noted that comparison of MCPyV DNA prevalence and viral load between studies must be undertaken with caution, because no consensus primer sets have been established.]

The most effective approach to ascertain the virus-positive status of MCC is a multimodal one combining molecular detection of MCPyV genome by qPCR and immunohistochemistry (IHC) (Eid et al., 2017; Moshiri et al., 2017; Kervarrec et al., 2019a, b). Alternatively, the combination of LT and SRY-box transcription

factor 9 (SOX9) expression has been shown to distinguish MCPyV-positive from MCPyV-negative MCC (Torre-Castro et al., 2024). An indirect method of determining whether MCC is MCPyV-positive is by the dual detection of pRB and SOX2 expression (Thanguturi et al., 2022).

Detection of integrated papillomavirus sequences-PCR (DIPS-PCR) allows the detection of integrated MCPyV genomic sequences and the identification of integration sites in the human genome (virus–host junction) (Laude et al., 2010). Next-generation sequencing can be used to identify the presence of MCPyV and integration sites in the human genome (Harms et al., 2021).

1.2.2 Detection of antibodies against structural proteins

Most of the studies investigating anti-MCPyV antibodies use VP1, VP1 plus VP2 virus-like particles (VLPs), pseudovirions as antigens, or glutathione *S*-transferase (GST)–VP1 recombinant protein (capsomeres), which are produced in insect cells or human embryonic kidney-derived 293TT cells (Kean et al., 2009; Pastrana et al., 2009; Touzé et al., 2010; Chen et al., 2011; Viscidi et al., 2011; Li et al., 2015). Detection of antibodies (mainly IgG) against structural proteins is the recommended method for measuring current and past infection in the population. Quantification of the seroresponse is determined as an optical density value obtained with a single serum dilution or as a titre determined by serial dilutions. Most studies have been conducted with VP1 capsomeres or VLPs, because the major capsid epitopes are presented at the surface of the major protein (Fleury et al., 2015). Since no direct comparison of these two antigens in the same population has been performed, the difference in sensitivity is not known. In addition to VLPs and capsomere enzyme-linked immunosorbent assay (ELISA), neutralization assays using MCPyV pseudovirions produced in human embryonic kidney-derived 293TT cells, bead-based multiplex serological assays, and synthetic peptides have been used (Pastrana et al., 2009; Egan et al., 2021; Mazziotta et al., 2021a).

With regard to specificity, pre-incubation of serum samples with John Cunningham polyomavirus (JCPyV), BK polyomavirus (BKPyV), and lymphotropic polyomaviruses (LPyV or African green monkey polyomavirus) has shown that there is limited cross-reactivity between MCPyV and other human polyomaviruses (Kean et al., 2009; Tolstov et al., 2009; Touzé et al., 2010; Viscidi et al., 2011; Šroller et al., 2014). Pseudovirion neutralization assays also support the specificity of MCPyV reactivity (Pastrana et al., 2009). The pseudovirions containing luciferase reporter plasmid are composed of VP1 and VP2 proteins, hence the results are expressed in terms of anti-VP antibodies when used in neutralization and binding assays. Neutralizing titres are obtained by serial dilution of the serum sample.

1.2.3 Detection of antibodies to oncoproteins

Antibodies to MCPyV LT and sT are rarely detected in the general population, making them useless for detecting exposure to the virus. They are nonetheless relevant for assessing the presence of MCPyV in MCC and for monitoring tumour progression or potential disease recurrence in patients with MCC (Paulson et al., 2010, 2017; Samimi et al., 2016; Alexander et al., 2024). Such antibodies are detected and quantified by ELISA or bead-based multiplex techniques using GST–LT or GST–sT fusions. [The Working Group noted that ongoing research indicates that the two antigens have similar analytical and clinical performances.]

1.2.4 Detection of MCPyV proteins

Detection of MCPyV LT, using the monoclonal antibody CM2B4, is commonly used to determine the presence of MCPyV in MCC cells. This antibody was raised against a peptide located in the MCPyV LT unique region (MUR), in exon 2 (SRSRKPSSNASRGA). CM2B4 detects MCPyV LT and 57kT isoforms but not MCPyV sT (Busam et al., 2009). CM2B4 is recognized as the best antibody (sensitivity, 88%; specificity, 94%) for the detection of LT expression (e.g. see Shuda et al., 2011; Moshiri et al., 2017; Kervarrec et al., 2024). The AB3 antibody, raised against a recombinant LT protein (amino acids 1–260), was shown in the initial report to detect LT expression in 97% of MCC samples investigated (Rodig et al., 2012). Other authors have reported lower sensitivities of 80% for AB3 and 70% for CM2B4 (Yeni Erdem et al., 2025).

Detection of MCPyV sT can be achieved using an sT-specific antibody, CM5E1 (Shuda et al., 2011), or the 2T2 panT antibody, raised against the N-terminal region that is common to LT and sT (Wang et al., 2012).

Anti-VP1 monoclonal antibodies are available for research purposes (Pastrana et al., 2010; Fleury et al., 2015). Since the integration of MCPyV in MCC leads to the absence of expression of late products (Pastrana et al., 2009; Touzé et al., 2011), there is no rationale for the detection of VP1 in tumour tissue.

1.3 Epidemiology of MCPyV infection

1.3.1 Detection of MCPyV

An early investigation demonstrated that MCPyV DNA is detectable, in varying quantities, in a broad spectrum of tissue types (Loyo et al., 2010). The highest concentrations of MCPyV DNA are found in mucosal (especially oral) and skin swabs, fully assembled virions being released from healthy skin (Wieland et al., 2009; Foulongne et al., 2010; Loyo et al., 2010; Schowalter et al., 2010). Deep sequencing of DNA from skin swabs has revealed that MCPyV constitutes a major component of the skin virome (DNA viruses) (Foulongne et al., 2012), although it is found only infrequently in the blood virome (Ngoi et al., 2016; Mazzoni et al., 2017; Moustafa et al., 2017; Feng et al., 2023). The skin is now widely recognized as the primary site of active MCPyV replication (Hashida et al., 2016a; Bopp et al., 2021). MCPyV DNA is also frequently identified in human-occupied environments and in waste water (Bofill-Mas et al., 2010; Foulongne et al., 2011; Iaconelli et al., 2015; Di Bonito et al., 2017). Consequently, the potential for environmental contamination leading to false-positive detection must be taken seriously in PCR-based research on MCPyV (Cohen and Enserink, 2011).

1.3.2 Primary infection and transmission

MCPyV infection occurs early in childhood and is widespread to ubiquitous among adults (see Section 1.3.3) (Kean et al., 2009; Tolstov et al., 2009, 2011; Sadeghi et al., 2010; Touzé et al., 2010; Viscidi et al., 2011; Martel-Jantin et al., 2013; Nicol et al., 2013; Šroller et al., 2014; Vahabpour et al., 2016; Cason et al., 2018; Bopp et al., 2021). The virus is present on all skin surfaces and is part of the normal skin flora. The detection of the same DNA sequence on different skin surfaces from the same individual (Schowalter et al., 2010; Bopp et al., 2021) and the absence of variation in prevalence of DNA detection at different skin locations (Foulongne et al., 2010) suggest that MCPyV is often established as a persistent infection of the skin in clinically healthy people. The MCPyV viral load on the skin surface varies from as little as one 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). High viral loads have been observed in participants

aged >50 years in different anatomical locations and particularly on the forehead (Hashida et al., 2016a). [The Working Group noted that this could be expected as immunocompetency decreases with age.] In line with this, higher viral loads have been observed in men who are positive for human immunodeficiency virus (HIV) in whom the HIV infection is poorly controlled (Wieland et al., 2011). Furthermore, MCPyV antibody titres are highly correlated with DNA viral load on the skin, indicating that individuals with very high anti-MCPyV titres are those with persistent and active skin shedding of MCPyV virions (Faust et al., 2011; Pastrana et al., 2012).

The exact mode(s) of MCPyV transmission and the site(s) of initial infection have not yet been characterized (Loyo et al., 2010; Mertz et al., 2010; Schowalter et al., 2010; Chokwassanasakulkit and McMillan, 2024). Serological studies suggest that transmission occurs through close contact, as seropositivity in mothers and siblings has been significantly associated with seropositivity in other family members (Martel-Jantin et al., 2013). MCPyV 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 and during caring for the neonate (Martel-Jantin et al., 2013).

MCPyV DNA was detected in 0.6–1.3% of nasopharyngeal aspirates from children and in 2.1–8.5% from adults (Bialasiewicz et al., 2009; Goh et al., 2009; Kantola et al., 2009). MCPyV was also detected in 3.5–6% of samples of tonsil, [6.7%] of lung tissue, 2% of adenoid and 17.2% of bronchoalveolar and bronchoaspirates (Kantola et al., 2009; Babakir-Mina et al., 2010; Loyo et al., 2010; Cason et al., 2018). The presence of MCPyV 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.

In early studies, MCPyV was not found in cerebrospinal fluid, peripheral blood mononuclear cells, urine, plasma or blood, brain or prostate tissue, or frozen autopsy samples, regardless of whether the participants were immunocompetent or immunosuppressed (Kassem et al., 2008; Bluemn et al., 2009; Duncavage et al., 2009; 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 MCPyV DNA sequences in 15–25% of urine samples and in 0.1–12% of serum samples from studies of the general population or of groups of patients such as those who have undergone tonsillectomy (Kantola et al., 2009; Bofill-Mas et al., 2010; Husseiny et al., 2010). Using a much larger cohort ($n = 234$), a prevalence of 9.4% in cerebrospinal fluid of neurological patients has been reported (Delbue et al., 2019). In samples from healthy patients, the presence of MCPyV DNA in blood or plasma has been detected at variable prevalences ranging from 1.1% in Brazil to 31.2% in Pakistan (Mazzoni et al., 2017; Kamminga et al., 2018; Shahzad et al., 2019; Zucherato et al., 2023). The detection of MCPyV in serum raises the possibility of blood transmission. However, this route of transmission is not expected to play an important role as most adults have neutralizing antibodies against MCPyV (Pastrana et al., 2009; Fleury et al., 2015). The low genome copy number of MCPyV in the urine could be caused by contamination by skin when passing urine. MCPyV DNA has been detected in anal specimens in people living with HIV, with a prevalence of 32.6%, and MCPyV viral load was increased by fivefold in the context of high-risk HPV coinfection and by eightfold in people with HPV and HIV coinfections (Passerini et al., 2024). [The Working Group noted that increased detection rates in more recent studies are likely to be a result of the use of improved methods with higher sensitivity.]

MCPyV has been detected in urban sewage and river water, and is present in 85% of environmental surface samples, suggesting that viral DNA may be widespread on objects that come into contact with humans (Bofill-Mas et al., 2010; Foulongne et al., 2011; Di Bonito et al., 2015). The persistence of MCPyV DNA in environmental surface samples after deoxyribonuclease (DNase) treatment before nucleic acid extraction (Foulongne et al., 2011) suggests that the DNA is encapsidated, indicating the presence of potentially infectious viral particles. This observation was further supported by a study that showed that MCPyV DNA is likely to be shed from the skin in the form of assembled virions (Schowalter et al., 2010). [The Working Group noted that this reinforces the possibility that MCPyV could be transmitted to humans through environmental sources.]

1.3.3 Seroprevalence of MCPyV

MCPyV serology studies (see Table 1.1) show that a high proportion of adults have been exposed to MCPyV and that infection is acquired early in life (Carter et al., 2009; Kean et al., 2009; Pastrana et al., 2009; Tolstov et al., 2009; Sadeghi et al., 2010; Touzé et al., 2010, 2011; Viscidi et al., 2011; Martel-Jantin et al., 2013; Nicol et al., 2013; van der Meijden et al., 2013; Mazziotta et al., 2021b, 2023). Even though the methods used in most studies lead to quantitative results, most of the studies reported only seropositivity or seronegativity, according to an antigen-specific cut off value. The age-specific seroprevalence of MCPyV indicates strong exposure early in life, with a seroprevalence of 20% in children aged 1–5 years (Kean et al., 2009; up to 40% or 50% in some studies, see Table 1.1) and of 35–60% in children aged < 10–15 years (Tolstov et al., 2009; Chen et al., 2011, 2014; Viscidi et al., 2011; Martel-Jantin et al., 2013; Nicol et al., 2013) (see Table 1.2). Owing to transfer of maternal antibodies, data on seroprevalence under 2 years must be considered with caution (Martel-Jantin et al., 2013).

Prevalence of anti-VP1 antibodies ranging from 46% to 87.5% have been reported for adults in western Europe and the United States of America (USA) (Carter et al., 2009; Kean et al., 2009; Pastrana et al., 2009; Tolstov et al., 2009; Touzé et al., 2010, 2011; Nicol et al., 2013; Kamminga et al., 2018), and similar levels of seroprevalence have been observed in studies within northern Europe, eastern Europe, Western Asia, sub-Saharan Africa, and Eastern Asia (Sadeghi et al., 2012; Martel-Jantin et al., 2013; Šroller et al., 2014; Zhang et al., 2014; Vahabpour et al., 2016; Cason et al., 2018; Kamminga et al., 2018). [The Working Group noted that the differences in seroprevalence percentage among studies can be attributed to specificities in the population but also to the detection methods used and particularly the determination of the cut off value of the assay.] The level of circulating neutralizing antibodies against MCPyV is generally correlated with the overall MCPyV DNA load across wide areas of the skin (Pastrana et al., 2012). Among newly infected individuals, a transient increase in immunoglobulin (Ig) M antibodies is observed that declines over 1–2 years, and most individuals (65%) demonstrate a robust IgG seroconversion, with stable levels over time (Tolstov et al., 2011; Mazziotta et al., 2023). Follow-up studies demonstrated seroconversion events in children aged 1–4 years (Chen et al., 2011, 2014). In a cohort of very young Cameroonians, age of seroconversion has been identified at 17–18 months (Martel-Jantin et al., 2013).

MCPyV 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 MCPyV seroconversion in adulthood (Tolstov et al., 2011). Concerning HIV, anti-VP1 antibodies have been reported to be at higher titres in participants with HIV or

Table 1.1 Detection of MCPyV antibodies in healthy participants and in patients with cancer other than Merkel cell carcinoma or with skin diseases

Reference	Study location	MCPyV antigen used	Age (years)	No. of participants	Anti-MCPyV antibodies *n* (%)	Participant status
Children						
Jeles et al. (2022)	Hungary	6XHis-VP1	0.8–17.9	193	104 (53.8%)	Healthy (routine medical test)
Kean et al. (2009)	USA	GST-VP1	1–4	112	23 (20.5%)	Healthy
			5–17	517	182 (35.2%)	
Tolstov et al. (2009)	USA	VP1 + VP2 VLPs	2–15	81	38 (46.9%)	Patients with MCC or LCH, and healthy controls
Chen et al. (2011)	Finland	VP1 VLPs	1–4	298	19 (6.4%)	Healthy – children with otitis or acute lower respiratory tract infection
			5–13	26	9 (34.6%)	
			1–4	158	6 (4%)	
Viscidi et al. (2011)	Italy	VP1 VLPs	1–9	42	19 (45.2%)	Healthy (non-cancer) patients attending outpatient hospitals
			10–19	38	23 (60.5%)	
Vahabpour et al. (2016)	Iran	VP1 VLPs	2–10	53	13 (24.5%)	Healthy
			10–19	45	25 (55.5%)	
Cason et al. (2018)	Italy	GST-VP1	0–3	12	4 (34%)	Healthy (children who underwent adeno-tonsillar surgery)
			4–7	77	48 (62%)	
			8–17	9	4 (41%)	
Šroller et al. (2014)	Czechia	VP1 VLPs	6–19	326	172 (52.7%)	Healthy
Nicol et al. (2013)	Italy	VP1 VLPs	1–4	48	20 (41.7%)	Healthy
			5–19	250	196 (78.2%)	
Martel-Jantin et al. (2013)	Cameroon	VP1 VLPs	1–4	45	24 (53.3%)	Healthy
			5–14	201	138 (68.6%)	
			15–19	49	40 (81.6%)	
Adults						
Jeles et al. (2022)	Hungary	6XHis-VP1	18–85	359	249 (69.3%)	Healthy (routine medical test)
Carter et al. (2009)	USA	GST-VP1	42–86	76	40 (53%)	Patients with MCC and control participants
			24–77	451	268 (59.4%)	
Kean et al. (2009)	USA	GST-VP1	18–70s	1593	734 (46.1%)	Healthy
Pastrana et al. (2009)	USA	VP1 + VP2	47–75	48	42 (87.5%)	Patients with MCC and control participants
Tolstov et al. (2009)	USA	VP1 + VP2 VLPs	> 18	166	107 (64.5%)	Patients with MCC or LCH, and healthy controls
Sadeghi et al. (2010)	Finland	VP1 VLPs	18–45	462	212 (45.9%)	Healthy
Touzé et al. (2010)	Belgium	VP1 VLPs	18–25	101	78 (77%)	Healthy
Touzé et al. (2010)	Italy	VP1 VLPs	18–85	194	150 (77.5%)	Healthy
Faust et al. (2011)	Sweden, Austria	VP1 + VP2 VLPs	50–94	434	283 (65.2%)	Patients with skin diseases (not MCC)

Table 1.1 (continued)

Reference	Study location	MCPyV antigen used	Age (years)	No. of participants	Anti-MCPyV antibodies *n* (%)	Participant status
Tolstov et al. (2011)	USA	VP1 + VP2 VLPs	18–69	564	447 (79.3%)	Participants in the Multicenter AIDS Cohort Study
Touzé et al. (2011)	France, Italy	VP1 VLPs	22–90	82	70 (85.4%)	Patients with MCC and controls
Viscidi et al. (2011)	Italy	VP1 VLPs	20–70s	865	640 (74%)	Healthy (non-cancer) patients attending outpatient hospitals
Mazziotta et al. (2021b)	Italy	VP1 peptide	18–65	548	397 (72.4%)	Healthy
	Italy	VP2 peptide	18–65	548	372 (67.8%)	
Šroller et al. (2014)	Czechia	VP1 VLPs	20–64	665	456 (68.6%)	Healthy
Vahabpour et al. (2016)	Iran	VP1 VLPs	20–78	342	217 (63.5%)	Healthy
Zhang et al. (2014)	China	GST-VP1	25–65	5548	3382 (61.0%)	Healthy
Sadeghi et al. (2012)	Finland	VP1 VLPs	65–100	326	194 (59.6%)	Patients hospitalized for non-cancer, MCC, or skin disease
Nicol et al. (2013)	Italy	VP1 VLPs	20–100	530	462 (87.1%)	Healthy
Kamminga et al. (2018)	Kingdom of the Netherlands	GST-VP1	18–69	1044	855 (81.9%)	Healthy (blood donors)
Martel-Jantin et al. (2013)	Cameroon	VP1 VLPs	20–88	289	271 (93.7%)	Healthy

AIDS, acquired immunodeficiency syndrome; GST, glutathione *S*-transferase; LCH, Langerhans cell histiocytosis; MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus; USA, United States of America; VLP, virus-like particle; VP, viral protein; 6XHis-VP1, His-tagged viral protein 1.

acquired immunodeficiency syndrome (AIDS) (Vahabpour et al., 2017).

1.3.4 Prevalence of MCPyV DNA in tissue

Using PCR or nested PCR, MCPyV DNA has been detected in 0–28.1% of samples from a variety of skin locations (Feng et al., 2008; Dworkin et al., 2009; Mangana et al., 2010; Mertz et al., 2010; Mogha et al., 2010; Wieland et al., 2011; see Table 1.2). With more sensitive techniques such as qPCR or RCA, MCPyV sequences have been amplified in up to 100% of skin samples examined (Foulongne et al., 2010; Loyo et al., 2010; Schowalter et al., 2010; Faust et al., 2011; Wieland et al., 2011; see Table 1.2). MCPyV is ubiquitous and part of the skin virome of healthy individuals with no symptoms in the general population. Furthermore, detection of MCPyV DNA in 6 (30%) of 20 anal swabs and in 3 (50%) of six penile swabs has been observed (Wieland et al., 2009). In addition to the detection of MCPyV on the skin, high rates of MCPyV DNA detection have also been reported in the oral cavity, 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 MCPyV DNA has been reported to be lower in the skin than in the oral cavity mucosa (Loyo et al., 2010), but another group found the opposite result (Foulongne et al., 2010).

Low levels of MCPyV DNA can be amplified from many human tissues (Feng et al., 2008; Kantola et al., 2009; Bergallo et al., 2010; Loyo et al., 2010; Tagliapietra et al., 2020). This low level of viral DNA may indicate that MCPyV is systemically distributed, but not pathogenic, in most tissues, where it may undergo low-level replication, persist, or lie latent.

In summary, MCPyV is ubiquitous and part of the skin virome, with no symptoms in the general population. Elevated viral load has been found in the elderly (immunosenescence) and in immunosuppressed individuals such as transplant recipients, people living with HIV, and individuals with skin that has been damaged by ultraviolet radiation (UV) (UV-induced immunosuppression) (Houben et al., 2023).

1.3.5 Treatment and vaccination

[The Working Group noted that no vaccine is available for MCPyV and that no antiviral treatment against MCPyV infection has been developed.]

1.4 Critical review of exposure assessment in key epidemiological studies

The Working Group performed a detailed review of the exposure assessment methods used in five key epidemiological studies that assessed the association between MCPyV and MCC (Feng et al., 2008; Carter et al., 2009; Paulson et al., 2010; Viscidi et al., 2011; Faust et al., 2014) (see Table S1.3, Annex 1, Supplementary material for Section 1, Exposure characterization, online only, available from: https://publications.iarc.who.int/667). Three of these were retrospective case–control studies (Carter et al., 2009; Paulson et al., 2010; Viscidi et al., 2011) in which exposure measurements were done after ascertainment of outcome; one was a prospective nested case–control study (Faust et al., 2014); and another was a case series with comparator tissue from other individuals (Feng et al., 2008). Four of the studies (Feng et al., 2008; Carter et al., 2009; Paulson et al., 2010; Viscidi et al., 2011) were previously reviewed in *IARC Monographs* Volume 104 (IARC, 2013), and one new study was reviewed (Faust et al., 2014).

These studies had several strengths. Methods for exposure measurement were primarily robust and quantitative and included PCR or qPCR to identify and quantify MCPyV DNA (Feng et al., 2008; Carter et al., 2009; Viscidi et al., 2011), and

Table 1.2 Detection of MCPyV DNA in healthy skin or inflammatory skin lesions

Reference	Study location	Samples tested	Methods	Target primer/probe	Immunocompromised patients[a]	No. of participants	Detection of MCPyV DNA *n* (%)
PCR and nPCR							
Feng et al. (2008)	USA	Skin/inflammatory skin	PCR	LT/VP1	+/–	18	1 ([5.6%])
Dworkin et al. (2009)	USA	Normal skin adjacent (SCC patients)	PCR	LT/VP1	–	32	9 ([28.1%])
Wieland et al. (2009)	Germany	Perilesional healthy skin	nPCR	LT	+/–	34	8 ([23.5%])
Andres et al. (2010b)	Germany	Sun-exposed skin with seborrhoeic keratosis	PCR + SB	LT	NR	12	2 ([16.7%])
Andres et al. (2010a)	Germany	Inflammatory skin lesions	PCR + SB	LT	NR	23	2 (8.7%)
Mogha et al. (2010)	France	Skin	PCR	LT/VP1	–	20	2 (10.0%)
Mangana et al. (2010)	Switzerland	Normal skin	PCR	LT/VP1	NR	11	0 (0.0%)
Mertz et al. (2010)	Switzerland	Common wart	PCR	LT/VP1	–	10	1 (10%)
Wieland et al. (2011)	Germany	Forehead swabs (HIV-positive men)	nPCR	LT/VP1	+	210	124 (59.0%)
Wieland et al. (2011)	Germany	Forehead swabs (healthy controls)	nPCR	LT/VP1	–	239	118 (49.4%)
qPCR and RCA							
Garneski et al. (2009)	North America	Skin swabs	qPCR	LT	–	15	0 (0.0%)
Foulongne et al. (2010)	France	Skin swabs	qPCR	LT/VP1	–	70	56 (80.0%)
Schowalter et al. (2010)	USA	Skin swabs	RCA	NR	–	35	14 (40.0%)
Faust et al. (2011)	Sweden, Austria	Benign skin lesions	qPCR	LT	–	119	22 ([18.5%])
Bopp et al. (2021)	Germany	Forehead swabs	qPCR	VP1	–	109	74 (67.9%)
		Hand swab	qPCR	VP1	–	109	73 (67.0%)

HIV, human immunodeficiency virus; LT, large T antigen; MCPyV, Merkel cell polyomavirus; nPCR, nested polymerase chain reaction; NR, not reported; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; RCA, rolling circle amplification; SB, Southern blot hybridization; SCC, squamous cell carcinoma; USA, United States of America; VP1, capsid viral protein 1.

[a] –, no immunocompromised patients (only healthy immunocompetent patients); +, immunocompromised patients; +/–, both immunocompetent and immunocompromised patients.

Southern hybridization to confirm clonal integration of MCPyV in MCC (Feng et al., 2008). Other exposure characterization methods used were MCPyV neutralization (Faust et al., 2014), VLP ELISA (Viscidi et al., 2011), and detection of the presence of antibodies using antibody-binding assays for serum IgG antibodies to VP1 capsid, and small and large T antigen (sT and LT) oncoproteins (Paulson et al., 2010). VP1 antibodies and neutralizing antibodies were used to measure exposure to MCPyV, whereas antibodies to sT and LT are markers of MCC disease burden in about half of virus-positive patients with MCC (Paulson et al., 2010). The most recent case–control study was nested within a large population-based prospective biobank cohort and was able to assess whether the MCPyV neutralization and antibody presence were associated with subsequent MCC, and thus assess temporality of the association (Faust et al., 2014).

Achieving a sufficient sample size and assessing co-exposures was a challenge in these studies. In two of the case–control studies, it was not possible to assess important exposures such as immunosuppression (Paulson et al., 2010 and Faust et al., 2014), and one study included both immunocompetent and immunosuppressed controls (Feng et al., 2008). Age and other viral exposures (such as JCPyV and cutaneous HPVs) were assessed in most studies (Feng et al., 2008; Carter et al., 2009; Paulson et al., 2010; Viscidi et al., 2011; Faust et al., 2014).

Collectively, the retrospective case–control studies, the case series, and the prospective nested case–control study used robust quantitative techniques to measure exposure to MCPyV.

2. Cancer in Humans

Introduction

MCPyV was previously evaluated by the *IARC Monographs* in Volume 104 (IARC, 2013), in which it was classified as *probably carcinogenic to humans* (Group 2A), with a finding that there was *limited* evidence for a causal role of MCPyV with MCC in humans.

A systematic search was conducted in the PubMed database to identify relevant studies (see General Remarks in the present volume). The search terms and the lists of retrieved studies are available from: https://hawcproject.iarc.who.int/assessment/773/.

MCPyV has been mostly evaluated with respect to MCC (a rare but aggressive type of cancer that has been increasing in incidence) and less frequently with respect to other types of skin cancer (IARC, 2025). Studies on cancers of the respiratory and digestive system, haematological malignancies, and bladder cancer were also noted.

Case–control studies, serology-based cohort studies, and nested case–control studies, together with a large number of case series, were included. As with the previous evaluation of MCPyV (IARC, 2013), studies without a clear description of control selection that allowed the Working Group to assess comparability were considered for inclusion as case series, rather than as case–control studies. For case series, only those considered to offer complementary virological information on clonal integration were included, as this information was useful for causal interpretation of the evidence on cancer in humans. Ecological studies, individual case reports, and studies that did not report on cancer end-points or tumours were not considered to be informative for the evaluation and are not described here.

The studies included are described below by cancer type and then by study design (cohort studies first in chronological order, followed by case–control studies and then case series).

2.1 Merkel cell carcinoma

See Table 2.1.

2.1.1 Cohort studies on Merkel cell carcinoma

One nested case–control study, published since the previous evaluation, used serological methods to examine MCPyV in relation to subsequent MCC (Faust et al., 2014).

Faust et al. (2014) selected cases and controls nested within two large biobanks in Sweden and Norway. The Southern Sweden Microbiology Biobank contained serum samples from 1969 onward from individuals who underwent a blood draw mainly for viral testing or screening for blood donation or pregnancy. In Norway, the Janus Biobank included mainly participants in a population-based health survey (90%) as well as blood donors from 1972 onward. In Sweden, individuals in a biobank including approximately 524 000 individuals (1.5 million serum samples) were linked to the national cancer registry. Cases were defined as those diagnosed with MCC. Similarly, in Norway, individuals in the Janus Biobank, containing approximately 332 000 individuals (493 000 serum samples), were linked to the national cancer registry beginning in 1993. Before 1993, the pathology reports of those with International Classification of Diseases 7th revision (ICD-7) code of 191 were reviewed for MCC by a pathologist. All cases were required to have a serum sample collected ≥ 1 month before diagnosis. Four controls who were alive and free of skin cancer at the time of the case diagnosis were matched on age, sex, county (for Norway), subcohort, number of serial samples, length of follow-up, and date of the first sample. Two serological assays were performed. One was the pseudovirion-binding assay, with positivity defined as a mean fluorescence intensity (MFI) of > 250, and high IgG antibody levels defined as a parallel line of greater than one unit. The other was the neutralizing antibody test, with positivity defined as > 50% neutralization at a dilution of 1:10 000. Available tumours were tested for MCPyV DNA using real-time PCR. In total, 22 cases of MCC (providing 49 serum samples) and 76 controls (providing 85 serum samples) were selected. The odds ratio (OR) for MCPyV neutralization positivity was 5.3 (95% confidence interval, CI, 1.3–32.3) overall, compared with seronegativity. The OR for any level of MCPyV antibody was 2.6 (95% CI, 0.66–15.0) and for a high level of MCPyV antibody was 4.4 (95% CI, 1.3–17.4) overall, compared with seronegativity. [The Working Group noted that a correlation between viral load and antibodies levels had been demonstrated by two teams (Faust et al., 2011 and Pastrana et al., 2012).] No clear differences in ORs were observed by time since blood draw to diagnosis. Of the 12 tumours for which tissue was available, MCPyV DNA was detected in 58%, and all individuals with DNA-positive tumours were seropositive for MCPyV. However, three individuals with MCPyV DNA–negative tumours were seropositive.

[The Working Group noted as a key strength that in this the study serological assays were performed on sera collected before diagnosis, to reduce the likelihood of reverse causation. Other strengths included the use of high-quality cancer registry data (Barlow et al., 2009; Larsen et al., 2009) and the use of large biobanks, although as MCC is a rare malignancy the number of cases remained small and the biobank from Sweden involved individuals who had had a blood draw for other purposes. Although the potential for nondifferential misclassification of exposure when using serology was noted, the use of two correlated serological assays and an assessment of tumour DNA positivity from cases improved

Table 2.1 Epidemiological studies on infection with MCPyV and incidence of Merkel cell carcinoma

Reference, location, enrolment/follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Faust et al. (2014) Southern Sweden Microbiology Biobank, Sweden; and Janus Biobank, Norway Sweden: 1969 to NR; Norway: 1972 to NR Nested case–control	*Source cohort*: Southern Sweden Microbiology and Janus Biobank. *Cases*: 22 cases of MCC within linked nationwide cancer registry; with a blood sample collected and stored at the biobank for ≥ 1 mo before diagnosis. *Controls*: 76 controls matched (up to 4:1) on age, sex, and county. Alive and free of skin cancer at the time of case diagnosis. *Exposure assessment method*: Antibodies against MCPyV were detected by neutralization methods, defined as > 50% neutralizing activity at 1:10 000 dilution. Also, the presence of total IgG Ab was measured, defined as a mean fluorescence intensity of > 250 for all studied viruses. High Ab level was defined as parallel line > 1 unit. Real-time PCR to detect MCPyV DNA was used in available tumours. Other viruses, JCPyV and HPV, were used as controls.	Skin (MCC)	Baseline MCPyV-neutralizing Ab (OR):			Age, sex, county	*Exposure assessment critique*: Although the potential for some nondifferential misclassification of exposure when using serology is noted, the use of two correlated serological assays ($R^2 = 0.83$) and an assessment of tumour DNA positivity from cases improved confidence in the exposure assessment. The seropositivity in individuals with MCPyV DNA–positive tumours suggests a high sensitivity of serology for detecting tissue positivity. However, seropositivity in those without MCPyV DNA positivity raises the possibility that serology may not be specific for detecting MCPyV infection in the tumours themselves and could result in biased risk estimates towards the null. A strength was the use of other viruses as controls.
			Absence	12	1		
			Presence	10	5.3 (1.3–32.3)		
		Skin (MCC)	Baseline MCPyV Ab level (OR):				
			Any	19	2.6 (0.66–15)		
			High (> 1 unit)	10	4.4 (1.3–17.4)		
		Skin (MCC)	Baseline MCPyV-neutralizing Ab, women (OR):				
			Absence	NR	1		
			Presence	8	14.3 (1.7–677)		
		Skin (MCC)	Baseline MCPyV-neutralizing Ab, men (OR):				
			Absence	NR	1		
			Presence	2	1.3 (0.08–19.9)		
		Skin (MCC)	Baseline MCPyV-neutralizing Ab, < 10 yr of follow-up (OR):				
			Absence	NR	1		
			Presence	NR	4.2 (0.55–50.8)		
		Skin (MCC)	Baseline MCPyV-neutralizing Ab, > 10 yr of follow-up (OR):				
			Absence	NR	1		
			Presence	NR	7.33 (0.81–368)		

Table 2.1 (continued)

Reference, location, enrolment/follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histo-pathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Faust et al. (2014) (cont.)							*Design and analysis:* *Strengths*: Measurement of pre-diagnostic samples to assess temporality of the exposure. Controls were matched on multiple factors. The cohort was derived from large biobanks that allowed linkage to high-quality data from national cancer registries. Testing of tumour samples for MCPyV. *Limitations*: Limited sample size given the rarity of the malignancy. No results for Sweden or Norway separately. Not possible to adjust for other factors such as immunosuppression or UV radiation-related exposures and not fully accounted for by matching on county. Only 12 out of 22 tumours were available for PCR analysis. Could not compute the ORs associated with MCPyV DNA positivity.

Table 2.1 (continued)

Reference, location, enrolment/follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histo-pathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Carter et al. (2009) Fred Hutchinson Cancer Research Center, University of Washington, USA January to August 2008 Case–control	*Cases*: 41 patients with MCC who had plasma and serum samples stored in the Merkel Cell Carcinoma Repository of Patient Data and Specimens at the research centre. FFPE MCC tumour samples were also obtained. *Controls*: 76 (hospital-based controls); 451 (population-based controls). Hospital-based: age- and sex-matched controls selected from patients with available serum samples from previous case–control studies at Fred Hutchinson. Population-based: women previously selected for a study on the role of HPV in anogenital cancers, using random-digit dialling.	Skin (MCC)	Anti-MCPyV VP1 Ab, hospital-based controls:			Age, sex	*Exposure assessment critique*: MCPyV Ab were measured using adequate methods reducing misclassification. Associations with other polyomaviruses were tested. *Design and analysis:* *Strengths*: quantification of levels of anti-VP1; use of two control groups, including matched hospital-based controls. Use of a sensitive technique (real-time PCR) for the analysis of FFPE samples. Consistency between tumour DNA and Ab positivity checked and noted. *Limitations*: Imprecision in estimates. Population controls were not age- and sex-matched. *Other results*: A higher Ab positivity rate was noted for cases with MCPyV DNA detected (92%, 22/24), than for cases without MCPyV DNA detected in tumour tissue (29%, 2/7).
			Negative	5	1		
			Positive	36	6.6 (2.3–18.8)		
		Skin (MCC)	Anti-MCPyV VP1 Ab, population-based controls:			None	
			Negative	5	1		
			Positive	36	[4.92 (1.89–12.76)]		

Table 2.1 (continued)

Reference, location, enrolment/follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Paulson et al. (2010) Fred Hutchinson, USA and University Hospital of Wuerzburg, Germany, Fred Hutchinson: 2008–2010 Case–control	*Cases*: First set of 139 comprised consecutive patients with MCC with plasma and serum samples stored in the Merkel Cell Carcinoma Repository of Patient Data and Specimens, Fred Hutchinson Cancer Research Center The second set comprised 20 cases from the USA and 46 from Germany. MCC cases were not matched to controls, but had similar age and sex distribution as the first case set was added to the pool of cases. *Controls*: 530 population-based controls (Western Washington state), identified through random-digit dialling, frequency-matched on age and gender (4:1) to cases from Fred Hutchinson (set 1). *Exposure assessment method*: A case–control study using multiplex Ab-binding assays to measure serum IgG Ab to VP1 capsid, sT, and LT oncoproteins, to investigate an association between seropositivity and MCC, and between Ab titres and MCC tumour burden.	Skin (MCC)	Ab reactivity to the MCPyV capsid, using set 1 cases only (adjusted OR):			Age, gender	*Exposure assessment critique*: Cases and controls were taken from multiple sources at different times, raising the concern of differential misclassification. Nondifferential misclassification was unlikely, assay reproducibility was confirmed in a blinded manner. BKPyV was used for comparison. Assessing dose–response relation between Ab titres and tumour burden was considered a strength. *Design and analysis:* *Strengths*: A large sample size with a ratio of 4 controls for 1 case. The reproducibility of the serological assay was validated. Use of population-based controls. Ab against sT or LT MCPyV oncoproteins were detected. *Limitations:* The nature of the MCC samples (either FFPE or frozen) was not specified. *Other results*: Anti-MCPyV sT and anti-MCPyV LT were found in controls (0.9% and 2% respectively) as well as MCC cases (40.5% and 26% respectively).
			Ab reactivity to MCPyV capsid	127	5.5 (2.9–11.2)		
		Skin (MCC)	Ab reactivity to MCPyV T antigens, using set 1 and set 2 cases (adjusted OR):				
			Ab reactivity to LT oncoprotein	31	16.9 (7.8–36.7)		
			Ab reactivity to sT oncoprotein	51	63.2 (24.4–164.0)		

Table 2.1 (continued)

Reference, location, enrolment/follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Viscidi et al. (2011) Moffitt Cancer Center, Tampa, Florida, USA 2006–2008 Case–control	*Cases*: plasma samples from 33 patients with histologically confirmed MCC diagnosed and treated at the Moffitt Cancer Center. *Controls*: 37 patients undergoing skin cancer screening exams at the Moffitt Lifetime Cancer Screening facility who did not have any skin cancers. *Exposure assessment method*: Serological assessment of VP1 using ELISA and testing for DNA by real-time PCR.	Skin (MCC)	Prevalence of Ab to MCPyV VP1 (crude OR): Negative Positive	 3 30	 1 [4.4 (1.10–17.53)]	None	*Exposure assessment critique*: Differential misclassification was possible because of the different sources of cases and controls. Nondifferential misclassification was unlikely because the methods were adequate. Cross-reactivity with BKPyV and JCPyV was explored. *Design and analysis:* *Strengths*: The large sample size and the use of both DNA detection in tumours and quantification of anti-VP1 Ab. *Limitations*: Imprecise estimates. Adjusted ORs not provided. Ab identified in all six MCPyV-positive MCC samples but also in two of three MCPyV DNA–negative samples, indicating sensitivity of serology but not specificity in relation to MCPyV in the tumours. *Other results: P* value for prevalence of Ab to MCPyV VP1 after adjustment for age ($P = 0.32$). The mean level of MCPyV Ab was higher among cases than among controls after adjustment for age ($P = 0.04$).

Ab, antibody(ies); BKPyV, BK polyomavirus (human polyomavirus 1); CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; FFPE, formalin-fixed paraffin-embedded; HPV, human papillomavirus; Ig, immunoglobulin; JCPyV, John Cunningham polyomavirus (human polyomavirus 2); LT, large T antigen; MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus; mo, month(s); NR, not reported; OR, odds ratio; PCR, polymerase chain reaction; sT, small T antigen; USA, United States of America; UV, ultraviolet; VP1, viral protein 1; yr, year(s).

the exposure assessment. The seropositivity in individuals with MCPyV DNA–positive tumours reduced concerns over false-positive tumour results. However, seropositivity in individuals without MCPyV DNA positivity raised the possibility that seropositivity may over-represent MCPyV infection in the tumours themselves and could result in a bias of risk estimates towards the null. The Working Group noted that with the information provided it was not possible to compute ORs by tumour DNA positivity versus negativity. Results were not provided for the individual cohorts from Sweden and Norway, which precluded assessment of internal consistency of results. No adjustment was made for potentially confounding factors other than the matching factors, but given that the risk factors are uncertain, this was judged to be appropriate.]

2.1.2 *Case–control studies and case series of Merkel cell carcinoma*

Three case–control studies with a clear description of control selection were evaluated (Carter et al., 2009; Paulson et al., 2010; Viscidi et al., 2011). All three were included in the previous evaluation (IARC, 2013), and no further studies meeting the definition of case–control studies (see Introduction, Section 2) were identified. Inclusion of case series was based on an appraisal of virological strengths, including the assessment of the presence of integrated viral DNA, as integration allows the production of sT and LT antigens in large amounts; or the presence of mutated viral DNA, thus encoding truncated forms of LT antigens, which have enhanced proliferative properties. On this basis, the Working Group considered the following case series: Feng et al. (2008), Sastre-Garau et al. (2009), Laude et al. (2010), and Hashida et al. (2016b). Additional case series using immunohistochemical methods to assess expression of LT antigens (Shuda et al., 2009; Tolstov et al., 2009; Nakamura et al., 2010; Jung et al., 2011; Paik et al., 2011; Ota et al., 2012; Samimi et al., 2016; Santoro et al., 2022) were considered to be relevant but of lower informativeness and are thus described in Section A.1, Annex 4 (Supplementary material for Merkel cell polyomavirus, Section 2, Cancer in humans, online only, available from: https://publications.iarc.who.int/667). In a case series in which immunohistochemical methods were used, four presumed MCCs of the salivary gland were described (Choi et al., 2025).

Serological studies with quantitative assays were considered to offer complementary information for triangulation purposes, although these studies were of low informativeness individually so are also included in Section A.2, Annex 4 (Supplementary material for Merkel cell polyomavirus, Section 2, Cancer in humans, online only, available from: https://publications.iarc.who.int/667) (Pastrana et al., 2010; Touzé et al., 2011). More than 100 additional studies were reviewed by the Working Group, but those that were considered not to fit the case–control study definition and that did not meet the virological criteria mentioned above were deemed uninformative (e.g. studies of viral DNA in which mutation or integration were not assessed; studies on viral RNA only; or serological studies without quantification). Studies on viral RNA only, including those using in situ hybridization methods (e.g. Wang et al., 2017), were also considered to be uninformative, because viral RNA may also be present in clinically silent infection. On the basis of these inclusion and exclusion criteria, some studies that were previously described in Volume 104 (IARC, 2013) have not been included in the present volume (e.g. Touzé et al., 2009; Andres et al., 2010b).

There were three published meta-analyses on MCPyV and risk of MCC (Santos-Juanes et al., 2015; Wijaya et al., 2022; Volesky-Avellaneda et al., 2023). However, none were considered to be informative because they included studies that did not meet the criteria mentioned above

for individual studies and because the variability in study informativeness was too high.

(a) Case–control studies

Carter et al. (2009) performed a retrospective case–control study. Cases ($n = 41$) were consecutive patients with MCC from Fred Hutchinson Cancer Research Center, in Seattle, Washington, USA. Two sets of controls were used, one hospital-based and the other population-based. Blood samples from MCC patients were obtained from the Merkel Cell Carcinoma Repository of Patient Data and Specimens at the Fred Hutchinson centre. MCC tumour samples included excess surgical tissue or archival FFPE blocks that contained at least 50% tumour cells, as estimated by PCR. The hospital-based control group (group 1, $n = 76$) included age- and sex-matched controls selected from among patients with an available serum sample from previous case–control studies at the Fred Hutchinson centre. The population-based control group (group 2, $n = 451$) included women previously selected for a study on the role of HPV in anogenital cancers, using random-digit dialling, without matching on age or sex. A quantitative assay was used to detect antibodies against MCPyV viral protein 1 (VP1) and quantify titres in blood samples. The presence of MCPyV DNA was assessed in tumour samples via real-time PCR assays. Anti-MCPyV VP1 antibodies were detected in 88% (or 36 out of 41) of MCC cases and in 53% (40 of 76) of hospital-based matched controls (OR adjusted for age and sex, 6.6; 95% CI, 2.3–18.8) and 59.4% of controls from the general population [(OR, 4.92; 95% CI, 1.89–12.76)]. MCPyV DNA was detected in 77% (or 24 out of 31) MCC tumour tissue samples, with MCC tissue being unavailable for 10 cases.

[The Working Group noted that the strengths of this study included quantification of levels of anti-VP1 antibodies, use of two control groups (with similar antibody prevalence rates), and the analysis of MCPyV DNA in MCC tumours. A higher antibody positivity rate was noted in cases for which MCPyV DNA was detected in the tumour tissue than in cases for which MCPyV was not detected, which strengthened the exposure assessment. Limitations included imprecision in estimates and the lack of matching on age and sex for the population-based controls.]

Paulson et al. (2010) performed a retrospective case–control study on a set of 139 patients with MCC from the Merkel Cell Carcinoma Repository of Fred Hutchinson Cancer Research Center, Seattle, USA, for whom sera were collected between 1 January 2008 and 1 May 2010, and who were frequency-matched to 530 population-based controls (4 controls:1 case) selected through random-digit dialling from Western Washington state. An additional set of 66 patients with MCC with available sera (20 from the USA, collected between 2009 and 2010, and 46 from Germany, collected between 2001 and 2010) and comparable age and sex distribution (although this set of cases was not matched to the controls). FFPE samples were analysed for the presence of MCPyV DNA using a real-time qPCR assay and for expression of MCPyV LT by immunochemistry. Sera were analysed using an antibody-binding assay detecting antibodies specific for MCPyV sT, LT, and VP1 proteins. Anti-MCPyV VP1 antibodies were detected more frequently in MCC cases than controls (OR, 5.5; 95% CI, 2.9–11.2). Although anti-MCPyV sT and anti-MCPyV LT antibodies were rarely found in controls (0.9% and 2%, respectively), they were commonly detected in MCC cases (40.5% and 26%, respectively), with an OR for MCPyV sT antibodies of 63.2 (95% CI, 24.4–164.0) and an OR of 16.9 (95% CI, 7.8–36.7) for MCPyV LT antibodies. Anti-MCPyV sT antibodies were exclusively detected in cases with MCC samples containing MCPyV DNA versus those not containing MCPyV DNA.

[The Working Group considered that the strengths of the study were the relatively large study size, and the validated analysis of anti-

bodies against sT or LT MCPyV proteins together with MCPyV DNA in MCC tumours. The Working Group noted that the cases described by Paulson et al. (2010) overlapped with those in the study by Carter et al. (2009), but the extent to which they overlapped was unclear, and the use of different exposure metrics meant that both studies were informative.]

Viscidi et al. (2011) conducted a case–control study with 33 patients with MCC from the Cutaneous Oncology Program at Moffitt Cancer Center in the USA, between 2006 and 2008. A total of 37 cancer-free controls were selected from among patients attending the cancer screening programme at Moffitt's Lifetime Cancer Screening facility and/or the University of South Florida Family Medicine Clinic, Tampa. The controls were patients undergoing skin cancer screening examinations. Blood samples were analysed by ELISA. Antibodies against MCPyV VP1 were more frequently detected in MCC cases than in controls, with a crude OR of [4.4] (95% CI, [1.10–17.53]; $P = 0.02$). The OR adjusted for age was not provided, although the P value indicated that the association was not statistically significant ($P = 0.32$). Nevertheless, the mean level of MCPyV antibodies was higher for cases than for controls after adjustment for age ($P = 0.04$). Frozen MCC samples from 9 cases were assessed using quantitative PCR; of these, 6 were MCPyV DNA–positive.

[The Working Group noted that a strength of this study was the quantification of anti-VP1 antibodies and assessment of MCPyV DNA in tumour samples, but the limitations were imprecision in estimates and the lack of reporting of adjusted ORs. The comparison of tissue and serology for the detection of MCPyV DNA indicated that serology was highly sensitive but not specific in relation to tissue positivity, as two of the three MCPyV DNA–negative individuals were seropositive.]

(b) Case series of integration or mutation of MCPyV DNA

The prevalence of markers of MCPyV in tumour samples across all of the studies (case series, cohort, and case–control) is summarized in Table S2.2 (Annex 4, Supplementary material for Merkel cell polyomavirus, Section 2, Cancer in humans, online only, available from: https://publications.iarc.who.int/667).

Four case series assessed the integration or mutation of MCPyV DNA, which the Working Group considered to offer useful virological information in assessing a causal role; however, the studies shared limitations, including the modest number of MCC samples and lack of comparator or unclear comparability of the non-MCC samples.

Feng et al. (2008) assessed the presence and integration of MCPyV DNA in samples from 10 patients with MCC and in non-MCC skin specimens (six normal, 15 Kaposi sarcoma, one melanoma, one inflammatory) and an additional 59 samples from various body parts (including nine skin samples). The MCC tissues were frozen biopsy samples. The presence of MCPyV DNA was assessed using PCR with primer sets for the T antigen locus and VP1 gene, and the presence of integrated MCPyV DNA was studied by Southern blotting. MCPyV DNA was detected in 80% (8 of 10) of MCC biopsy samples, 16% (4 of 25) of samples of control skin tissue (one normal and three Kaposi sarcomas gave positive results) and in only 8% (5 of 59) control tissues from various sites. Integration of MCPyV DNA (with a clonal pattern) was demonstrated in six of the eight MCPyV DNA–positive MCC samples. [The Working Group considered that misclassification of exposure was unlikely owing to the use of multiple methods for exposure ascertainment and a large group of diverse controls.]

Sastre-Garau et al. (2009) analysed 10 MCC tumours and 1241 non-MCC epithelial or mesenchymal tumour samples (the non-MCC

skin cancers included other skin cancers, and cancers of the uterine cervix, ovary, colon, liver, breast, bone and soft tissue, and uveal tract) from the same hospital in France. Nine MCC samples were FFPE tissue biopsy specimens, and one was frozen in liquid nitrogen. Tumour cellularity of the samples was verified as being > 60%. Among the 10 cases, 12 samples were analysed from primary tumours (in 6 cases) and metastases (3 in skin and 3 in lymph nodes). Samples from three different lesions (one primary and two skin metastases) from one case were analysed. Samples were analysed for the presence of MCPyV DNA using three standard PCR and qPCR assays, and the presence of integrated MCPyV DNA was assessed either without identification of the integration site by FISH or with identification of the integration site inside the human genome by DIPS-PCR. MCPyV DNA was detected in 100% (10 of 10) of MCC cases and in none of the 1241 non-MCC tumour samples. Integrated MCPyV DNA was detected in 100% (10 of 10) of MCC cases, with a single integration per sample. In one sample analysed by FISH, a single fluorescent signal was observed in the nucleus of about 90% of the epithelial tumour cells, whereas no signal was found in adjacent non-tumour cells, with this observation corresponding to a clonal pattern of integration. No recurrent site of integration was identified, and a specific pattern was observed in each case. MCPyV DNA integration was observed on nine different chromosomes. In a single case for which a primary and two skin metastases were analysed, the MCPyV DNA integration sites were localized on the same chromosome in the three samples. Virus–host junctions were located inside the LT coding sequences in 6 cases, whereas integration occurred inside the VP1 coding sequence and regulatory region, respectively, in the other 2 cases. In all eight informative cases, the 3′ parts of integrated MCPyV LT sequences were either prematurely truncated (6 of 8 cases) or were fully conserved but harboured truncating mutations (deletion leading to a stop codon or punctual mutation generating a stop codon).

Laude et al. (2010) conducted a within-person comparison of frozen or FFPE samples of MCC and various other tissues, including squamous cell carcinoma (SCC), nasal swabs, urine, and blood samples, from 33 patients with MCC. The presence of MCPyV DNA was analysed by qPCR, the presence of mutated DNA was analysed by conventional DNA sequencing, and detection of integrated viral DNA was performed using the identification of integrated papillomavirus sequences by ligation-mediated PCR technique or junction PCR. MCPyV DNA was detected in 41 of 43 MCC samples from 31 out of 33 patients with MCC. Viral DNA was also detected in 1 out of 6 (16%) non-MCC cancer samples from five patients with MCC, in 27 out of 28 (96%) nasal swabs from all 21 patients with MCC, in 22 out of 38 (58%) of urine samples from 18 out of 28 (64%) patients with MCC, and in 20 out of 49 (41%) blood samples from 15 out of 30 (50%) patients with MCC. The presence of integrated DNA was observed in 6 cases of MCC, with the same integration site in the primary tumour and one metastasis of the same patient. The presence of mutated DNA harbouring truncating mutations was demonstrated in 9 out of 12 cases for which DNA was sequenced, with identical sequences in the primary tumour and metastasis from a single case. However, viral DNA harbouring LT antigen truncating mutations was not observed in non-MCC samples, except for one patient with metastatic disease for whom mutated DNA circulating in the blood was likely to correspond to circulating tumour DNA.

Hashida et al. (2016b) tested tumour and skin swab samples from non-cancerous skin (sun-exposed and unexposed) from six Japanese patients with MCC, using real-time qPCR. The presence of mutations was assessed by standard sequencing in 5 out of 6 cases of MCC, and integration status was assessed by DIPS-PCR in 1 case. MCPyV DNA was found in all samples and all skin swabs

from the 6 cases of MCC. MCPyV truncating mutations in MCC samples were found in 4 of 6 cases of MCC, whereas non-MCC skin samples displayed wildtype sequences without mutations or deletions. Integration was demonstrated in one sample of MCC. [The Working Group noted the availability of additional information from age-matched controls, although the description of how these were selected was unclear, and also noted that the comparison with controls did not include assessment of truncation or mutations, so was not further described.]

2.2 Other skin cancers

See Table 2.3.

2.2.1 Cohort and nested case–control studies on other skin cancers

One nested case–control study (Faust et al., 2016) and one cohort study (Amorrortu et al., 2021) assessed MCPyV serology before diagnosis and subsequent risk of cutaneous SCC and basal cell carcinoma (BCC).

In the nested case–control study on SCC and BCC of the skin, Faust et al. (2016) used similar methods to those used in the study on MCC by Faust et al. (2014). Individuals from the Southern Sweden Microbiology Biobank and Norwegian Janus Biobank were linked to the national registries for diagnoses of SCC of the skin in Norway and Sweden ($n = 633$), and BCC ($n = 1990$) in Norway. Cases were required to have a serum sample collected ≥ 1 month before diagnosis. One control, who was alive and free of SCC (and BCC, for Norway) at the time of the corresponding case diagnosis, was selected for each case on the basis of the criteria used in the earlier study (Faust et al., 2014). No association was observed between SCC or BCC in relation to baseline MCPyV seropositivity versus seronegativity (OR for SCC, 0.9; 95% CI, 0.7–1.2; and OR for BCC, 1.1; 95% CI, 1.0–1.3). Persistence (based on the first and last sample) was also evaluated in a subset of 531 cases of BCC and 256 cases of SCC and matched controls with two or more samples. Risk estimates were imprecise, but for those who were positive at baseline but negative at the last sampling point the ORs were slightly elevated for BCC (OR, 1.7; 95% CI, 0.8–3.7), but not for SCC (OR, 0.9; 95% CI, 0.3–2.5). [The Working Group noted that the lack of information on antibody levels limited the interpretation of this finding and that the results could be caused by diminishing immunity with age.] In contrast, for those who were seronegative at baseline but seropositive at the last sampling, the ORs were slightly raised for SCC (OR, 1.5; 95% CI, 0.5–4.6) but not for BCC (OR, 1.0; 95% CI, 0.5–1.9). Furthermore, the ORs were close to the null for those who were seropositive at both time points. [The Working Group noted that the national cancer registries in this study ascertained SCC and BCC, unlike many other national cancer registries, providing a unique opportunity to evaluate these malignancies in this study. Adjustment for other covariates was not mentioned and, given the immunosuppressive properties of UV, lack of adjustment for UV exposure could have biased risk estimates away from the null; however, it was unclear to what extent this was likely to occur. Nondifferential misclassification of exposure to the skin was possible because of the use of serology and the lack of assessment of DNA in skin samples.]

In the Virus in Skin Cancer (VIRUSCAN) study, Amorrortu et al. (2021) enrolled 1179 patients, aged ≥ 60 years, without a history of either SCC or BCC, from the University of Florida Dermatology Clinic, USA, in 2014–2017. Patients included those being seen for a dermatological condition or for whole-body screening for early detection of skin cancer, and the recruitment rate was 44.5%. Follow-up visits to identify new skin cancers were conducted every 6–12 months through September 2018, with 1008 patients completing at least one visit.

Table 2.3 Epidemiological studies on infection with MCPyV and incidence of other skin cancers

Reference, location, enrolment/follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Faust et al. (2016) Southern Sweden Microbiology Biobank, Sweden; and Janus Biobank, Norway Sweden: 1969 to NR; Norway: 1972 to NR Nested case–control	*Source cohort:* Southern Sweden Microbiology and Janus Biobank. *Cases*: 633 cases of SCC and 1990 cases of BCC from linked nationwide cancer registry, with blood sample collected before diagnosis. *Controls*: 633 controls for SCC, 1990 for BCC; matched (1:1) on age at serum sampling, sex, county; alive and free of skin cancer at the time of case diagnosis, with blood samples. *Exposure assessment method*: Seropositivity in assay for binding to MCPyV pseudovirions. The MFI cut-off value was 250 for seropositivity.	Skin (SCC)	MCPyV baseline seropositivity (OR):			None	*Strengths:* The prospective design allowed for the measurement of pre-diagnostic samples. Controls were matched on multiple factors. The cohort was derived from large biobanks that allowed linkage to national cancer registries that uniquely ascertained cases of incident SCC and BCC (in Norway). Persistent positivity was evaluated in a subset of 531 cases of BCC and 256 cases of SCC and their controls. *Limitations:* No information was available on MCPyV viral status of the tumours. Limited adjustment for potentially confounding, i.e. to UV-related factors.
			Negative	NR	1		
			Positive	460	0.9 (0.7–1.2)		
		Skin (SCC)	Repeated MCPyV serology, individuals with ≥ 2 samples (SCC, *n* = 256) (OR):			Matched on age at serum sampling, sex, county	
			First and last sample negative	56	1		
			First sample negative, last sample positive	10	1.5 (0.5–4.6)		
			First sample positive, last sample negative	7	0.9 (0.3–2.5)		
			First and last sample positive	183	0.9 (0.6–1.4)		
		Skin (BCC)	MCPyV baseline seropositivity (OR):			None	
			Negative	NR	1		
			Positive	1437	1.1 (1.0–1.3)		
		Skin (BCC)	Repeated MCPyV serology, individuals with ≥ 2 samples (BCC, *n* = 531) (OR):			Matched on age at serum sampling, sex, county	
			First and last sample negative	138	1		
			First sample negative, last sample positive	18	1.0 (0.5–1.9)		
			First sample positive, last sample negative	17	1.7 (0.8–3.7)		
			First and last sample positive	358	1.1 (0.8–1.5)		

Table 2.3 (continued)

Reference, location, enrolment/follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Amorrortu et al. (2021) VIRUSCAN, USA 2014–2017 Cohort	*Cohort*: 1008 patients without history of skin cancer, undergoing screening at least once over a 4-yr period at the University of South Florida (2014–2017). Visits occurred ever 6–12 mo until September 2018. *Exposure assessment method*: The study used multiplexed serological assays for Ab to the VP1 capsid protein of MCPyV and DNA-based multiplex methods to detect MCPyV in skin swabs, eyebrow and tumour samples. Seropositivity was defined in a separate case–control study, with a cut-point for MFI of > 5000.	Skin (SCC)	MCPyV baseline seropositivity (HR):			Age, sex, self-reported skin colour, hair colour and history of keratinocytic carcinoma	*Strengths:* The study involved active follow-up of the cohort with whole-body skin exams. Multiple substrates (eyebrow hair follicles and skin swabs) were used to assess MCPyV status. Risk estimates were adjusted for multiple potential confounders. Diagnoses of SCC and BCC were histologically confirmed by a single study dermatologist who graded the degree of solar elastosis in skin adjacent to the tumour. The lack of association between solar elastosis in the adjacent skin and overall HPyV prevalence in the tumour suggested that UV-related factors were unlikely to confound results. *Limitations:* Short duration of follow-up from a single centre, limiting precision of estimates. The manuscript describing the methods indicated that 1% of participants were organ transplant recipients. No adjusted results for combined forearm and eyebrow results were provided. There was low concordance between baseline positivity and subsequent tumour positivity.
			Negative	32	1.00		
			Positive	113	0.78 (0.53–1.17)		
		Skin (SCC)	MCPyV DNA in eyebrow hair (HR):				
			Negative	95	1.00		
			Positive	48	1.16 (0.81–1.65)		
		Skin (SCC)	MCPyV DNA in forearm skin swabs (HR):				
			Negative	29	1.00		
			Positive	107	1.39 (0.91–2.13)		
		Skin (SCC)	MCPyV DNA in either forearm skin swabs or eyebrow hair (HR):			Age and sex	
			Negative	NR	1.00		
			Positive	NR	1.66 (1.00–2.77)		
		Skin (SCC)	MCPyV DNA in SCC tumour tissue (%):			None	
			Positive	NR	14.5%		
		Skin (BCC)	MCPyV baseline seropositivity (HR):			Age, sex, self-reported skin colour, hair colour and history of keratinocytic carcinoma	
			Negative	25	1.00		
			Positive	101	0.94 (0.61–1.47)		
		Skin (BCC)	MCPyV DNA in eyebrow hair (HR):				
			Negative	91	1.00		
			Positive	38	0.94 (0.63–1.39)		
		Skin (BCC)	MCPyV DNA in forearm skin swabs (HR):				
			Negative	26	1.00		
			Positive	94	1.21 (0.77–1.90)		
		Skin (BCC)	MCPyV DNA in either forearm skin swabs or eyebrow hair (HR):			Age and sex	
			Negative	NR	1.00		
			Positive	NR	1.23 (0.72–2.12)		
		Skin (BCC)	MCPyV DNA in BCC tumour tissue (%):			None	
			Positive	NR	9.7%		
			Negative	3	1		
			Positive	30	[4.4 (1.10–17.53)]		

Table 2.3 (continued)

Reference, location, enrolment/follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Rollison et al. (2012) Moffit Dermatology, USA 2007–2009 Case–control	*Cases*: 173 confirmed cases of cutaneous SCC. *Controls*: 300 patients participating in skin cancer screening without a diagnosis of cancer or history of cancer. *Exposure assessment method*: Fluorescent bead-based multiplex serology, PCR.	Skin (SCC)	MCPyV seropositivity (adjusted OR):			Age and sex	*Strengths:* Quantification of anti-VP1 Ab. Analysis of MCPyV DNA in tumours. Stronger association between MCPyV seropositivity and cutaneous SCC when restricting to MCPyV DNA–positive cases. Adjustment for age and sex. *Limitations:* No matching or further adjustments for confounding by exposure to UV radiation.
			Negative	33	1		
			Positive	140	1.58 (0.96–2.60)		
		Skin (SCC)	MCPyV seroreactivity levels (adjusted OR):				
			Quartile 1	31	1		
			Quartile 2	39	1.44 (0.78–2.69)		
			Quartile 3	53	1.71 (0.94–3.11)		
			Quartile 4	50	1.53 (0.84–2.78)		
			Trend-test *P* value, 0.15				
		Skin (SCC)	MCPyV seropositivity, only including MCPyV DNA–positive SCC cases (adjusted OR):				
			Seronegative	7	1		
			Seropositive	48	2.49 (1.03–6.04)		
		Skin (SCC)	MCPyV seropositivity, only including MCPyV DNA–negative SCC cases (adjusted OR):				
			Seronegative	20	1		
			Seropositive	70	1.38 (0.76–2.48)		
Hampras et al. (2015) Moffitt Cancer Center, Florida, USA 2007–2009 Case–control	*Cases*: 171 cases of SCC identified through the screening programme. *Controls*: 300 individuals with negative skin cancer screening exam at the University of South Florida Family Medicine Clinic or Moffitt Cancer Centre (subset of the sample used by Rollison et al., 2012) with available MCPyV T-antigen serology results).	Skin (SCC)	Seropositivity to MCPyV antigen (adjusted OR):			Age and sex	The study used the same participants as used by Rollison et al. (2012), with a restriction to those with available MCPyV T-antigen serology results. *Additional results*: The ratio of N- to C-terminal copy numbers of MCPyV DNA for all SCC samples was close to 1.0, suggesting the absence of integrated MCPyV DNA.
			MCPyV sT	2	0.31 (0.06–1.62)		
			MCPyV LT exon 1	1	0.56 (0.05–5.93)		
			MCPyV LT exon 2	16	1.22 (0.57–2.62)		
			MCPyV LT	16	0.99 (0.48–2.08)		
		Skin (SCC)	Presence of MCPyV DNA in eyebrow hair vs absence (adjusted OR):				
			Presence	82	1.17 (0.76–1.78)		

Table 2.3 (continued)

Reference, location, enrolment/follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Hampras et al. (2015) (cont.)	*Exposure assessment method*: Multiplex serological assay and PCR analysis of eyebrow hair.	Skin (SCC)	Seropositivity to MCPyV, only including MCPyV DNA–negative SCC cases (adjusted OR):			Age and sex	*Strengths:* Large sample size. The analysis of MCPyV DNA integration and detection of Ab to sT and LT. The positive association between the presence of MCPyV DNA in eyebrow hair and MCPyV DNA in tumour tissue supported the utility of using MCPyV DNA in eyebrow hair as a proxy for tumour status. *Limitations:* Lack of adjustment beyond age and sex. The technique used to assess integration can underestimate integration.
			MCPyV sT	2	0.63 (0.12–3.32)		
			MCPyV LT exon 1	1	1.11 (0.11–11.61)		
			MCPyV LT exon 2	10	1.50 (0.63–3.53)		
			MCPyV LT	9	1.10 (0.47–2.6)		
			Presence of viral DNA in eyebrow hair vs absence	26	0.58 (0.34–1.01)		
		Skin (SCC)	Seropositivity to MCPyV, only including MCPyV DNA–positive SCC cases (adjusted OR):				
			MCPyV LT exon 2	5	1.06 (0.35–3.2)		
			MCPyV LT	6	1.06 (0.38–2.93)		
		Skin (SCC)	Presence of MCPyV DNA in eyebrow hair vs absence, only including MCPyV DNA–negative SCC cases (adjusted OR):				
			Presence	26	0.58 (0.34–1.01)		
		Skin (SCC)	Presence of MCPyV DNA in eyebrow hair vs absence, only including MCPyV DNA–positive SCC cases (adjusted OR):				
			Presence	42	4.05 (2.01–8.18)		

Ab, antibody(ies); BCC, basal cell carcinoma; CI, confidence interval; HPyV, human polyomavirus; HR, hazard ratio; Ig, immunoglobulin; LT, large T antigen; MCPyV, Merkel cell polyomavirus; MFI, mean fluorescence intensity; mo, month(s); NR, not reported; OR, odds ratio; PCR, polymerase chain reaction; SCC, squamous cell carcinoma; sT, small T antigen; USA, United States of America; UV, ultraviolet; VIRUSCAN, Viruses in Skin Cancer study; vs, versus; VP, viral protein; yr, year(s).

Biopsies were reviewed by a single dermatopathologist. Baseline serum samples underwent multiplex serology for antibodies to the MCPyV VP1 capsid protein. Eyebrow hair, forearm skin swabs, and tumour samples were analysed for viral DNA using multiplex assays. In the fully adjusted model that included age, sex, pigmentary characteristics, and history of a keratinocyte cancer, the hazard ratio (HR) for SCC was somewhat elevated for MCPyV DNA positivity in skin swabs (HR, 1.39; 95% CI, 0.91–2.13). For MCPyV DNA positivity in both eyebrow and skin swabs, the age- and sex-adjusted HR was 1.66 (95% CI, 1.00–2.77). [The impact of further adjustment for other factors was not provided, but the authors stated that the result was no longer statistically significant.] HRs for SCC and MCPyV seropositivity were below 1. HRs for MCPyV DNA in eyebrow samples, or for BCC and any of the measures, were close to the null.

[The Working Group noted the prospective active follow-up design with whole-body skin examinations as a strength of the study, together with the availability of covariates related to UV sensitivity and keratinocyte cancer history. The assessment of the presence of MCPyV DNA in eyebrow hair follicles and skin swabs collected at baseline, and additionally testing for MCPyV DNA in tumour tissue from the cases, was a notable strength. No association was observed between the level of solar elastosis in the adjacent skin and overall prevalence of human polyomaviruses, suggesting that UV-related factors were unlikely to confound results; however, solar elastosis results specific to MCPyV were not provided. The Working Group presumed that the study population was largely immunocompetent, as the original methods paper (Amorrortu et al., 2020) stated that only 1% of the study group were organ transplant recipients, although this was not mentioned in the current study. A limitation was that the duration of follow-up was relatively short (up to about 4 years), limiting the precision of estimates for SCC and BCC. Fewer than 5% of individuals with positive samples at baseline (serology, eyebrow hair, or skin swabs) went on to develop MCPyV-positive tumours, indicating low concordance.]

2.2.2 Case–control studies on other skin cancers

There were two case–control studies assessing MCPyV markers and SCC of the skin (Rollison et al., 2012; Hampras et al., 2015). Cases series were considered to be minimally informative for other skin cancers, although those in which the authors tested for MCPyV integration or mutation or performed IHC for detecting LT antigen are provided in Annex 4 (Supplementary material for Merkel cell polyomavirus, Section 2, Cancer in humans, online only, available from: https://publications.iarc.who.int/667).

Rollison et al. (2012) performed a case–control study including 173 cases of SCC and 300 controls recruited from the same university dermatology clinic (Moffit Cancer Centre, USA) between 2007 and 2009. Controls were individuals with no history of or current cancers and who were undergoing screening for skin cancer. Fresh frozen tumour tissue samples were analysed for the presence of MCPyV DNA by standard PCR using a single assay. The serum levels of antibodies against VP1 MCPyV were screened using a fluorescent bead–based assay. MCPyV DNA was detected in SCC tumour tissues in 38% (55 of 145) of cases. MCPyV seropositivity was associated with an increased odds of SCC overall (OR, 1.58; 95% CI, 0.96–2.60). The association between MCPyV seropositivity and MCPyV DNA–positive SCC was statistically significant (OR, 2.49; 95% CI, 1.03–6.04, adjusted for age and sex).

[The Working Group noted strengths of this study that were related to exposure assessment, including the quantification of anti-VP1 antibody levels and analysis of SCC MCPyV DNA in tumours. The stratified analysis, showing a

stronger association between MCPyV seropositivity and cutaneous SCC when restricting to MCPyV DNA–positive cases, supported the utility of serological assays. The lack of adjustment for potential confounding by UV radiation was noted as a limitation.]

Hampras et al. (2015) performed an additional analysis of the sample used by Rollison et al. (2012), restricting to individuals with available MCPyV T-antigen serology results (171 cases of SCC and 300 controls) or available eyebrow hair samples (169 cases of SCC and 292 controls). MCPyV viral load was determined in eyebrow hair samples and SCC fresh frozen samples using multiplex quantitative real-time PCR targeting the N-terminus and C-terminus of the MCPyV T-antigen sequence, and the ratio of N-terminal to C-terminal copy numbers of MCPyV DNA was determined to examine the presence of C-terminal deletions [which the Working Group considered to be an indirect hallmark of MCPyV DNA integration]. Antibodies against MCPyV sT and LT antigens were detected in the sera. No positive association was observed between seroreactivity to MCPyV sT or LT antigen and cutaneous SCC, either overall (OR for sT, 0.31; 95% CI, 0.06–1.62; and OR for LT, 0.99; 95% CI, 0.48–2.08) or when restricting the case set to MCPyV DNA–positive tumour cases (OR for LT antigen, 1.06; 95% CI, 0.38–2.93). [The Working Group noted that the OR for MCPyV sT antigen in MCPyV DNA–positive tumour cases could not be estimated.] The OR for cutaneous SCC status in individuals with MCPyV DNA in eyebrow hair was 1.17 (95% CI, 0.76–1.78). The ratio of C- to N-terminal copy numbers of MCPyV DNA for all SCC samples was close to 1.0, suggesting the absence of integrated MCPyV DNA.

[The Working Group noted that a strength of this study was the analysis of MCPyV DNA integration and detection of antibodies against sT and LT antigens. The positive association between the presence of MCPyV DNA in eyebrow hair and MCPyV DNA in tumour tissue supported the utility of using MCPyV DNA in eyebrow hair as a proxy for tumour status. A limitation of the study was the technique used to assess integration, which can lead to underestimation, because integration of MCPyV does not necessarily cause deletion of the LT C-terminus at the DNA level.]

2.3 Other cancers

See Table 2.4.

2.3.1 Cohort studies on other cancer types

Four nested case–control studies on non-skin cancer (one on non-Hodgkin lymphoma, NHL; two on lung cancer, and one on glioma) used serological approaches to detection of the VP1 capsid protein (Colombara et al., 2015, 2016; Teras et al., 2015; Egan et al., 2021). Two of the studies additionally tested for antibodies to sT oncoprotein (Colombara et al., 2015, 2016), although none of the studies examined MCPyV DNA in tumours.

Teras et al. (2015) conducted a nested case–control study on NHL within the American Cancer Society Cancer Prevention Study II (CPS-II), a nationwide cohort in the USA. [The Working Group noted that details of the methods used for selection of the original cohort were described by Calle et al. (2002).] The study included individuals who provided a blood sample between 1998 and 2001 and were followed for cancer by biennial questionnaires. A total of 279 individuals reported NHL that was medically verified. The median age at blood draw was 70 years, and the median age at NHL diagnosis was 74 years. A total of 557 controls were pair-matched 2:1 to the cases on sex, race, date of birth, and date of blood draw. Using a multiplexed serology approach shown to have high reproducibility, the authors tested for antibodies to the MCPyV VP1 capsid. No overall association was observed with seropositivity and NHL using an MFI cut off of ≥250. In an analysis of MCPyV seropositivity and NHL subtypes, an elevated OR was found

Table 2.4 Epidemiological studies on infection with MCPyV and incidence of cancers other than skin

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Teras et al. (2015) American Cancer Society CPS-II, USA Enrolment (blood drawn), 1998–2001/ follow-up dates, NR Nested case–control	*Source cohort*: Subgroup of CPS-II participants who provided a blood sample between 1998 and 2001. *Cases*: 279 cases of NHL self-reported through biennial questionnaire, verified through medical records or cancer registries. *Controls*: 577, incidence density–matched (2:1) on sex, race, birth, and blood draw dates. *Exposure assessment method*: Seropositivity was based on seroreactivity to the MCPyV VP1 capsid protein using multiplex serology.	NHL	MCPyV seropositivity (OR):			Sex, race, birth, and blood draw dates	*Strengths*: Prospective, matched nested case–control study design. Assessment of pre-diagnostic seropositivity and MFI as a continuous variable, with evaluation of nonlinearity. All participants were stated in the abstract as being immunocompetent (but details were not provided). *Limitations*: Relatively small sample sizes for NHL subtypes. No information on MCPyV status of the cancer cells. Limited adjustment for potentially confounding or modifying factors.
			Seronegative	82	1		
			Seropositive	197	1.09 (0.80–1.49)		
		NHL	MCPyV seropositivity (MFI values), among seropositive only (≥ 250 MFI) (OR):				
			Quartile 1	50	1		
			Quartile 2	50	1.01 (0.62–1.65)		
			Quartile 3	61	1.24 (0.77–2.01)		
			Quartile 4	61	0.72 (0.43–1.20)		
		NHL (DLBCL)	MCPyV seropositivity (OR):				
			Seronegative	19	1		
			Seropositive	49	1.17 (0.67–2.06)		
		NHL (follicular)	MCPyV seropositivity (OR):				
			Seronegative	14	1		
			Seropositive	32	1.05 (0.55–2.03)		
		NHL (SLL/CLL)	MCPyV seropositivity (OR):				
			Seronegative	24	1		
			Seropositive	42	0.80 (0.47–1.37)		
		Other NHL	MCPyV seropositivity (OR):				
			Seronegative	8	1		
			Seropositive	37	2.13 (0.97–4.70)		

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Colombara et al. (2015) CARET, 1985–2005 Nested case–control	*Source cohort*: CARET, trial to assess chemopreventive effect of supplementation on lung cancer in asbestos-exposed and smokers (asbestos-exposed were not included in this analysis). *Cases*: 200 cases of first primary lung cancer, identified through pathology review, cancer registry, and national death index. *Controls*: 200, frequency-matched on age and year of enrolment, intervention arm assignment, date of blood draw, and the number of serum freeze–thaw cycles. Alive and cancer-free at the time of case diagnosis. *Exposure assessment method*: Used a liquid bead-based microarray assay to test for Ab to both the MCPyV VP1 capsid protein and sT oncoprotein. MFI values of > 400 were considered to be seropositive.	Lung	Ab (MCPyV, VP1 protein) quartile level (mean lnMFI) (OR):			Age and year of enrolment, intervention arm assignment, date of blood draw, and the number of serum freeze–thaw cycles	*Strengths*: Matched, nested case–control study design. A high rate of pathological confirmation of cases (95%). Assessed the potential confounding or modifying effects of sex and family history of lung cancer, but no such effects were observed (data not shown). *Limitations*: Participants were all current or past heavy smokers. Limited statistical power to evaluate histological types of lung cancer. No available data on tumour MCPyV status.
			Lowest (4.24)	NR	1		
			Highest (10.6)	NR	0.79 (0.45–1.37)		
			Continuous (natural log transformed MFI)	NR	0.96 (0.88–1.04)		
			Trend-test *P* value, 0.28				
		Lung	Ab (MCPyV, sT protein) quartile level (mean lnMFI) (OR):				
			Lowest (0.04)	NR	1		
			Highest (5.73)	NR	0.72 (0.41–1.26)		
			Continuous (natural log transformed MFI)	NR	0.98 (0.89–1.06)		
			Trend-test *P* value, 0.58				

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Colombara et al. (2016) Yunnan tin miners cohort study, Yunnan, China Enrolment, 1992 and 1998/ follow-up until end of 2001 Nested case–control	*Source cohort*: 9295 Yunnan tin miners enrolled in a study on early lung cancer biomarkers, including annual screening exams. *Cases*: 183 cases of lung cancer in men, identified through annual examinations, hospital records, cancer registries, or death records. *Controls*: 217 men were frequency-matched on age and number of sample freeze–thaw cycles. *Exposure assessment method*: Used a liquid bead-based microarray assay to test for Ab to both the MCPyV VP1 capsid protein and sT oncoprotein. The MFI threshold for seropositivity was 200 for some viruses and 400 for others; which threshold was for MCPyV was not stated.	Lung	Ab quartile for MCPyV VP1 (mean lnMFI) (OR):			Age and the number of freeze–thaw cycles	*Strengths*: Prospective follow-up for lung cancers through active surveillance. Tested for effect modification by smoking history, radon exposure, and arsenic exposure using likelihood ratio tests. Assessed potential confounding by these factors and family history of lung cancer; no evidence of effect modification or confounding was found. Matched on age. *Limitations*: Study included only male miners. Underpowered to investigate histological type of lung cancer. No assessment of MCPyV DNA in tumour samples.
			Quartile 1 (5.03)	NR	1		
			Quartile 2 (8.50)	NR	0.67 (0.38–1.17)		
			Quartile 3 (9.74)	NR	0.80 (0.49–1.31)		
			Quartile 4 (12.57)	NR	0.90 (0.37–2.17)		
			Continuous (unit of lnMFI increase)	NR	1.00 (0.92–1.09)		
			Trend-test *P* value, 0.951				
		Lung	Ab quartile for MCPyV sT (mean lnMFI) (OR):				
			Quartile 1 (0.02)	NR	1		
			Quartile 2 (0.05)	NR	1.01 (0.58–1.76)		
			Quartile 3 (0.35)	NR	0.70 (0.40–1.22)		
			Quartile 4 (4.12)	NR	0.85 (0.48–1.48)		
			Continuous (unit of lnMFI increase)	NR	0.97 (0.88–1.08)		
			Trend-test *P* value, 0.623				

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Egan et al. (2021) CPS-II, USA; Janus Serum Bank, Norway CPS-II: Enrolment, 1998–2001/ follow-up until 2013; Janus: 1974–2007 Nested case–control	*Source cohort*: CPS-II and Janus Biobank. *Cases*: CPS-II: 37; Janus: 323; CPS-II: glioma cases diagnosed ≥ 3 yr after blood collection; Janus: glioma cases diagnosed ≥ 5 yr after blood collection. *Controls*: CPS-II: 74; Janus: 323; alive and no cancer history at the time of case diagnosis. CPS-II: matched on birth date, sex, date blood draw (2:1); Janus: matched (1:1) on birth date, sex, date blood draw, county. *Exposure assessment method*: Seropositivity was based on seroreactivity to the MCPyV VP1 capsid protein using multiplex serology. Seropositivity was defined as an Ab level ≥ 250 MFI.	Brain (glioma)	MCPyV, VP1 seropositivity, CPS-II cohort (OR):			Birth date, sex, date blood draw	*Strengths*: Used two prospective cohorts from the general population of two countries. Adjustment for seropositivity to other viruses did not influence the results. Evaluated effect modification by sex, type of glioma, latency, and calendar period. *Limitations*: Small sample sizes for the analyses of glioma subgroups and limited statistical power for analysis of Ab titres and to detect interactions. Limited assessment of potentially confounding factors such as immune competence. No information on MCPyV status of the tumours.
			Seronegative	12	1		
			Seropositive	25	1.29 (0.54–3.08)		
		Brain (glioma)	MCPyV, VP1 seropositivity, Janus serum bank (OR):				
			Seronegative	92	1		
			Seropositive	231	1.56 (1.10–2.19)		
		Brain (glioma)	MCPyV seropositivity (MFI values), Janus serum bank (OR):				
			Seronegative (< 250 MFI)	39	1		
			Seropositive, tertile 1	NR	1.73 (1.12–2.68)		
			Seropositive, tertile 2	NR	1.34 (0.86–2.07)		
			Seropositive, tertile 3	NR	1.63 (1.04–2.56)		
			Trend-test *P* value, 0.052				
		Brain (glioma)	MCPyV seropositivity (MFI values), Janus serum bank (OR):			Matching variables plus other polyomaviruses (BKPyV, JCPyV, HPyV6)	
			Seronegative	39	1		
			Seropositive, tertile 3	NR	1.61 (1.13–2.28)		

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Campello et al. (2011) Verona, Italy 2006–2008 Case–control	*Cases*: 64 cases of newly identified colon cancer, with fresh tissue and blood samples. *Controls*: 80 relatives of colon cancer cases, with biopsies, recruited through a screening programme, with fresh tissue and blood samples. *Exposure assessment method*: PCR.	Colon	DNA MCPyV (LT3, VP1 primer) (OR): Positive	4	4.47 (0.61–28.66)	Age	*Strengths*: Prospective recruitment of participants involved in screening; age adjustment; use of relatives attending screening as controls may control for socioeconomic and genetic factors. *Limitations*: Age adjustment may not account for non-overlap in ages. Bias expected from use of control samples from suspicious lesions. Small number of exposed cases.
Robles et al. (2012) Multicentre hospital-based (Epilymph), Spain 1998–2002 Case–control	*Cases*: 468 cases of incident lymphoma (from participating hospitals) with biological samples available for MCPyV testing. *Controls*: 552 patients were frequency-matched on age, sex, and centre, with a wide range of diagnoses on hospital admission, with biological samples available for MCPyV testing.	Lymphoma (type not specified) NHL (DLBCL) NHL (follicular) NHL (CLL)	MCPyV seropositivity (OR): Negative Positive MCPyV seropositivity (OR): Negative Positive MCPyV seropositivity (OR): Negative Positive MCPyV seropositivity (OR): Negative Positive	68 400 3 80 3 31 12 94	1 1.34 (0.95–1.88) 1 6.10 (1.88–19.75) 1 2.39 (0.71–8.03) 1 1.49 (0.80–2.75)	Age, sex, centre	*Strengths*: Large sample size; age-matched controls and adjustment by age; use of incident cases. *Limitations*: Ab levels not assessed.

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Robles et al. (2012) (cont.)		Multiple myeloma	MCPyV seropositivity (OR):				
			Negative	17	1		
			Positive	52	0.69 (0.38–1.26)		
		Hodgkin lymphoma	MCPyV seropositivity (OR):				
			Negative	7	1		
			Positive	38	1.65 (0.69–3.94)		
Gustafsson et al. (2012) Karolinska Institutet, Sweden 2000–2006 Case–control	*Cases*: 50; Guthrie cards of children who later developed ALL. *Controls*: 100 matched controls (birthplace, birth date). Controls were picked anonymously, two Guthrie card apart from cases to avoid contamination. *Exposure assessment method*: Real-time PCR.	Childhood leukaemia (ALL)	Prevalence MCPyV DNA–positive:			Birthdate and place of birth	*Strengths*: Good description of case and control selection, with matching on birthdate and place of birth. *Limitations*: No exposed cases or controls.
			Cases	0	0%		
			Controls	0	0%		
Polesel et al. (2012) Pordenone, Italy 2004–2007 Case–control	*Cases*: 114 cases of bladder cancer recruited from hospitals in Pordenone province. *Controls*: 140 hospital controls (wide spectrum of conditions, excluding diseases related to tobacco, alcohol, or diet). *Exposure assessment method*: Urine sample.	Urinary bladder	MCPyV DNA in urine sample (adjusted OR):			Sex, age, education, alcohol drinking, and tobacco smoking	*Strengths*: Adjustment for multiple potential confounders including tobacco smoking. High participation rate. *Limitations*: Use of hospital-based controls. Lack of analysis of MCPyV in tumours.
			MCPyV DNA–positive	3	0.51 (0.12–2.13)		
			MCPyV DNA–negative	8	1		

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Sitas et al. (2012) InterSCOPE collaboration: South Africa, Australia, central and eastern EU, Brazil, Iran, China 1995–2007 Case–control	*Cases*: 1561; oesophageal squamous cell carcinoma within established case–control studies with available serum. *Controls*: 2502 hospital controls (South Africa, EU, Brazil) and population controls (Iran, Australia, China) matched on age, sex (all), storage time (South Africa, EU, Brazil), neighbourhood or study centre (China, Iran, Brazil, EU).	Oesophagus (SCC)	Seropositivity to MCPyV VP1 (OR): Negative Positive	 300 1261	 1 0.88 (0.73–1.06)	Matching variables, education, rural/ urban residence, opium consumption (Iran only), mate consumption (Brazil only), snuff use (South Africa only)	*Strengths*: Large sample size covering several countries. Matching on age. *Limitations*: Ab levels were not determined.
Robles et al. (2013) Multicentre Case–control study, Spain 1998–2001 Case–control	*Cases*: 1135 cases of bladder cancer (age, 21–80 yr) with blood samples collected before treatment. *Control*: 982 hospital controls with diagnoses unrelated to known or suspected causes of bladder cancer (e.g. inguinal hernia, fractures) matched on sex, age, and region.	Urinary bladder Urinary bladder	MCPyV seroreactivity (OR): Negative Positive MCPyV seroreactivity level (OR): Tertile 1 (0.220–1.224) Tertile 2 (1.225–1.913) Tertile 3 (> 1.913) Trend-test *P* value, 0.001	 199 936 254 278 404	 1 1.05 (0.83–1.32) 1 1.13 (0.88–1.45) 1.48 (1.16–1.88)	Region, sex, age, ever smoked, batch	*Strengths*: A high response rate for cases (84%) and controls (88%). Adjustment for smoking, and matching on age. *Limitations*: No comparison with sera from cases of MCC as a positive control outcome. Hospital-based controls.

Table 2.4 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type (histopathology), incidence	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Robles et al. (2015) Multicentre study, Spain 2010–2012 Case–control	*Cases*: 359 cases of CLL (incident and prevalent). *Controls*: 370 population-based controls with no prior history of lymphoproliferative disorders, frequency-matched on age, sex, and region.	NHL (CLL)	Seropositivity to MCPyV (VP1) (OR):			Age, sex, region	*Strengths*: Large sample size; age-matched controls. Quantification of Ab levels. Analysis of LT Ab. Population-based controls. *Limitations*: Small number of exposed cases and controls for sT and LT antigens. Prevalent cases included.
			Negative	70	1		
			Positive	289	0.79 (0.54–1.16)		
		NHL (CLL)	Seroreactivity to MCPyV (VP1) (OR):				
			Tertile 1	123	1		
			Tertile 2	109	0.92 (0.63–1.34)		
			Tertile 3	57	0.46 (0.30–0.70)		
			Trend-test *P* value, < 0.001				

Ab, antibody(ies); ALL, acute lymphoblastic leukaemia; BKPyV, BK polyomavirus (human polyomavirus 2); CARET, Carotene and Retinol Efficiency Trial; CI, confidence interval; CLL, chronic lymphocytic leukaemia; CPS-II, Cancer Prevention Study II; DLBCL, diffuse large B-cell lymphoma; EU, European Union; HPyV6, human polyomavirus 6; JCPyV, John Cunningham polyoma virus (human polyomavirus 1); ln, natural log; LT, large T antigen; LT3, large T antigen protein 3; MCPyV, Merkel cell polyomavirus; MFI, mean fluorescence intensity; NHL, non-Hodgkin lymphoma; NR, not reported; OR, odds ratio; PCR, polymerase chain reaction; SCC, squamous cell carcinoma; SLL, small lymphocytic lymphoma; sT, small T antigen; USA, United States of America; VP1, major capsid protein/viral protein (VP1); yr, year(s).

in the category of "other NHL" (OR, 2.13; 95% CI, 0.97–4.37), and no association was detected for the other subtypes (chronic lymphocytic leukaemia/small lymphocytic lymphoma, CLL/SLL; diffuse large B-cell lymphoma; or follicular lymphoma).

[The Working Group noted the use of matching to reduce confounding as a strength but also noted a lack of adjustment for other potentially confounding factors such as immune status, although the population was referred to by the authors as "immunocompetent". The study had limited statistical power to examine effect modification or differences within the "other NHL" subtypes. No assessment of MCPyV status was performed in the cancer cells.]

Colombara et al. (2015) conducted a nested case–control study on lung cancer among participants in the Carotene and Retinol Efficacy Trial (CARET). CARET included 14 254 individuals who were heavy smokers, with ≥ 20 pack-years of smoking, who were not considered to be exposed to asbestos. Participants were recruited from study centres in Seattle, Portland, Irvine, San Francisco, Baltimore, and Groton, in the USA. Cases included 200 randomly selected individuals with incident lung cancer and a pre-diagnostic serum sample (366–1095 days before diagnosis, with two or fewer freeze–thaw cycles). Incidence-density sampling was used to select 200 controls who were alive and cancer free at the time the case was diagnosed, with a serum sample that had undergone two or fewer freeze–thaw cycles, and who were frequency-matched on age at enrolment, intervention arm, year of enrolment, date of blood draw, and number of freeze–thaw cycles. MCPyV serology for both the VP1 capsid protein and sT oncoprotein were performed using a bead-based multiplex assay. No associations were observed with MFI as quartiles or continuous.

[The Working Group noted that the study benefited from the use of incidence-density sampling for matching and assays for both VP1 and sT, and a high rate of pathological confirmation of lung cancers. However, limitations included that there were too few cases to robustly assess histological types of lung cancer. A further limitation was the potential for residual confounding, as participation was restricted to heavy smokers and the analyses were not adjusted for amount or duration of smoking.]

Colombara et al. (2016) conducted a nested case–control study of lung cancer selected from a cohort study on early biomarkers of lung cancer among 9295 current or retired employees of a tin-manufacturing company in China, who were enrolled between 1992 and 1998 and screened annually until the end of 2001. Participants were aged ≥ 40 years, had a history of ≥ 10 years of underground and/or smelting experience, and no previous malignancies (except non-melanoma skin cancer). Annual screening, along with hospitalization and death records, were used to identify lung cancer cases. Only 2 cases were women; therefore, the study was restricted to the 183 men with lung cancer. A total of 217 controls were identified who were frequency-matched on age and number of freeze–thaw cycles. Serum samples were tested for antibodies to both the MCPyV VP1 capsid protein and sT oncoprotein using a multiplex, liquid bead, microarray antibody assay. No associations were observed with MFI values for either antigen analysed either as quartiles or as a continuous variable (natural log transformed). In addition to matching factors, the authors evaluated the potential modifying or confounding effects of history of smoking, radon exposure, and arsenic exposure. No clear association was apparent within the boxplots for MCPyV prevalence by specific histological type of lung cancer (SCC, adenocarcinoma, small cell carcinoma, mixed/other, or not obtained), although no ORs were provided. [The Working Group noted that a strength of the study was the active follow-up of the cohort for new cases using lung cancer screening. An additional strength was the consideration of multiple potentially

confounding factors, although there was little evidence of confounding. A limitation was that the study had low statistical power to examine subtypes of lung cancer, and no ORs or confidence intervals were reported for these subtypes.]

Egan et al. (2021) conducted nested case–control studies that leveraged two prospective cohort studies: the United States CPS-II Nutrition Cohort of 32 609 cancer-free individuals enrolled between 1998 and 2001 and followed until 2013, and the Norwegian Janus Serum Bank (Langseth et al., 2017) of approximately 167 000 men and 158 000 women in which blood samples were collected between 1972 and 1991. From the CPS-II Nutrition Cohort, the authors identified 37 cases of glioma diagnosed ≥ 3 years after blood collection and selected 74 controls pair-matched (2:1) to the cases on year of birth, sex, and date of blood draw. From the Janus Serum Bank, they included cases and controls from a previous study of 323 cases of glioma diagnosed ≥ 5 years after blood collection and 323 controls matched on birth year, sex, date of blood draw, and county of residence, and who were alive and free of cancer (except BCC and SCC) at the time of case diagnosis. Multiplex serology was performed for the VP1 capsid protein of MCPyV, with an MFI cut off of > 250 for seropositivity. In both the CPS-II and Janus Serum Bank studies, elevated ORs for glioma were observed with MCPyV seropositivity (CPS-II OR, 1.29; 95% CI, 0.54–3.08 and Janus OR, 1.56; 95% CI, 1.10–2.19). In the Janus cohort, the ORs in the highest MFI tertile (OR, 1.63; 95% CI, 1.04–2.56) and lowest MFI tertile (OR, 1.73; 95% CI, 1.12–2.68) were increased compared to MFI < 250, with a *P* for trend (0.052) in increasing titre of MCPyV antibodies. Additional adjustment for seropositivity for the other polyomaviruses tested (BKPyV, JCPyV, and human polyomavirus 6, HPyV6) had little impact on the results (OR for MCPyV adjusted for BKPyV, JCPyV, and HPyV6, 1.61; 95% CI, 1.13–2.28). Results varied by type of glioma, with a more consistent association with glioblastoma than with non-glioblastoma.

[The Working Group noted that a strength of this study was that it included two cohorts from different regions of the world and found similar trends in both cohorts. Limitations were the small sample sizes for the analyses of glioma subgroups. Also, there was a lack of data on other tumour characteristics and other potentially confounding factors, i.e. those influencing immune competence. Confidence intervals were wide when assessed by time from blood collection to diagnosis, age, and calendar period of blood collection.]

2.3.2 Case–control studies on other cancer types

Seven case–control studies on other cancers were evaluated: there were single studies on colon cancer (Campello et al., 2011), lymphoma (Robles et al., 2012), acute lymphoblastic leukaemia (ALL) in children (Gustafsson et al., 2012), CLL in adults (Robles et al., 2015), and oesophageal cancer (Sitas et al., 2012), plus two studies on bladder cancer (Polesel et al., 2012; Robles et al., 2013). Case series in which the authors tested for MCPyV integration or mutation, or performed IHC for the LT antigen, are provided in Annex 4 (Supplementary material for Merkel cell polyomavirus, Section 2, Cancer in humans, online only, available from: https://publications.iarc.who.int/667), but were considered to be less informative for the present evaluation.

In addition, three meta-analyses of studies on non-skin tumours were identified (Geng et al., 2022; Goudarzi Far et al., 2022; Saadh et al., 2024), but these were considered to be uninformative because they included studies that did not meet the criteria above for individual studies, and the number of informative studies was low.

Campello et al. (2011) performed a monocentric case–control study in Italy, prospectively recruiting adults with colon cancer and their

first-degree relatives enrolled consecutively as part of a family screening programme. The authors analysed fresh tissue samples from 64 adults with colon cancer and fresh colonoscopy biopsies of suspicious lesions in the colonic mucosa from 80 relatives. MCPyV DNA was detected in 11 out of 144 (8.8%) colon specimens, with no statistical differences between cases and controls (6.3% positive), the OR being 4.47 (95% CI, 0.61–28.6). [The Working Group noted that strengths included the prospective recruitment of cases and the use of family relatives involved in screening as controls to reduce detection bias and to control for some socioeconomic and genetics-driven factors. Although the age adjustment would be expected to reduce confounding, the use of sons or daughters as controls reduced overlap in the age distributions, which may be problematic for adjusting for confounding from nonlinear age differences.]

Robles et al. (2012) conducted a case–control study on 468 cases of incident lymphoma and 552 controls matched on age, sex, and recruitment centre, testing for VP1 MCPyV antibodies by ELISA. MCPyV seroprevalence was high and similar in controls and in lymphoma cases (82% and 85%, respectively), with an OR of 1.34 (95% CI, 0.95–1.88) for all lymphomas. A notable difference in seropositivity compared with controls was observed only for diffuse large B-cell lymphoma cases, with OR of 6.10 (95% CI, 1.88–19.75). [The Working Group noted that this was a well-conducted study with a large sample size, although antibody levels were not assessed and confidence intervals were wide for lymphoma subtypes.]

Gustafsson et al. (2012) conducted a case–control study in 50 children with ALL and 100 children with no history of ALL as controls, chosen from the phenylketonuria laboratory, at Karolinska University Hospital, Huddinge, Sweden. In the laboratory, Guthrie cards are sorted by birth date and birthplace. Controls were selected by picking the Guthrie card placed at a distance of 2 cards from the case's card. This procedure of selecting controls allowed matching by birth date and birthplace while controlling for cross-card contamination. The cards were analysed for the presence of MCPyV DNA by standard and real-time PCR. MCPyV DNA was not detected in the blood spots of any of the ALL cases or matched controls. [The Working Group noted as a strength that the cases and controls were well described but noted that the sample size may have been too small to detect modest differences if exposure prevalence were low.]

Polesel et al. (2012) conducted a case–control study on bladder cancer in Pordenone Province, Italy, between August 2004 and July 2007. Cases comprised 124 patients aged ≥ 18 years with histologically or cytologically confirmed transitional cell carcinoma of the bladder and controls were 151 patients admitted to the same hospital as the cases who had acute conditions unrelated to smoking, alcohol consumption, or that required long-term dietary changes. Immunocompromised individuals (i.e. transplant recipients) were excluded from the study. Multiplex PCR was used to detect MCPyV DNA in first morning void samples, and included specific primers targeting the LT antigen gene. [The results for LT did not appear in the publication.] Detection of urinary MCPyV was 2.7% among 113 cases and 5.8% among 139 controls (OR, 0.51; 95% CI, 0.12–2.13, adjusted for adjusted for sex, age, education, tobacco smoking, and alcohol drinking). [The Working Group noted that the use of hospital-based controls was a limitation that could bias results towards the null. The lack of analysis of MCPyV in tumours was a further limitation. Strengths included the adjustment for multiple factors and low participation refusal rates (< 5%).]

Sitas et al. (2012) conducted a multicentre case–control study on oesophageal cancer (SCC) in South Africa, central and eastern Europe, Brazil, the Islamic Republic of Iran, and China. Participants had been recruited in six previous

different case–control studies, each of which included at least 100 cases and controls. MCPyV VP1 seropositivity was detected in 80.8% (1261 of 1561) of cases and 83.4% (2088 of 2502) of controls, with an adjusted OR of 0.88 (95% CI, 0.73–1.06). [The Working Group noted that a strength of the study was the large sample size covering several countries, although antibody levels were not assessed.]

Robles et al. (2013) conducted a case–control study on bladder cancer that included 1135 cases and 982 hospital controls recruited from five regions of Spain. Controls were matched to cases on sex, age at interview, ethnic origin, and region, and were individuals with diagnoses considered to be unrelated to bladder cancer, such as inguinal hernia or fractures. Anti-VP1 MCPyV was detected with a similar prevalence in controls (82.1%; n = 806) and cases (82.5%; n = 936) (OR, 1.05; 95% CI, 0.83–1.32). Among seropositive participants, the odds of bladder cancer were higher when comparing highest with lowest MCPyV seroreactivity tertiles (OR, 1.48; 95% CI, 1.16–1.88). [The Working Group noted the use of hospital-based controls as a limitation, but the high response rate in cases and controls as a strength.]

Robles et al. (2015) conducted a case–control study that included 359 cases of CLL and 370 population-based controls recruited between 2010 and July 2012 from seven centres in the Asturias, Barcelona, Cantabria, and Granada regions of Spain. Controls were matched on region, sex, and age. Levels of antibodies against sT antigen, LT antigen, and VP1 MCPyV was measured using bead-based technology. The prevalence of VP1 MCPyV antibodies was similarly high in controls and cases (83.8% and 80.5%, respectively; OR, 0.79; 95% CI, 0.54–1.16). Participants with CLL tended to have low levels of antibodies. None of the participants were seropositive for MCPyV LT antigen, and only two participants (one control and one with CLL) were seropositive for MCPyV sT antigen. [The Working Group noted that a strength of the study was the quantification of antibody levels and testing for antibodies to sT and LT antigens, but also noted the small number of exposed cases and controls and the use of prevalent cases as limitations.]

2.4 Evidence synthesis for cancer in humans

This section provides a synthesis of the evidence on the association between MCPyV and subsequent risk of cancer. Most of the available evidence was on MCC of the skin (Feng et al., 2008; Carter et al., 2009; Sastre-Garau et al., 2009; Shuda et al., 2009; Tolstov et al., 2009; Laude et al., 2010; Paulson et al., 2010; Jung et al., 2011; Paik et al., 2011; Touzé et al., 2011; Viscidi et al., 2011; Ota et al., 2012; Faust et al., 2014; Hashida et al., 2016b; Samimi et al., 2016; Santoro et al., 2022). Additionally, other cancer outcomes that were studied in association with MCPyV included other types of skin cancer (Rollison et al., 2012; Hampras et al., 2015; Faust et al., 2016; Amorrortu et al., 2021) and haematological malignancies, lung cancer, glioma, and bladder cancer (e.g. Gustafsson et al., 2012; Polesel et al., 2012; Robles et al., 2012; Sitas et al., 2012; Colombara et al., 2015, 2016; Teras et al., 2015; Egan et al., 2021). Case series of other malignancies were considered to be minimally informative.

2.4.1 Studies evaluated

The present evaluation of MCPyV included studies with prospective designs, which were not available in the previous evaluation. These, along with the case–control studies included in *IARC Monographs* Volume 104 (IARC, 2013) were considered to be informative. Case series were considered to be informative only if the presence of integrated MCPyV DNA was assessed, including mutations or truncations resulting

in truncated LT antigens, or expression of LT protein at levels detectable by immunochemistry.

In total, the evaluation included seven nested case–control studies, 12 case–control studies, and, for MCC, 25 additional case series. Epidemiological prospective studies included studies using data from two Nordic biobanks, various European countries, the USA, and China. Case–control studies included those from Europe, the USA, and one including data from multiple continents. The case series additionally included cases from Australia, Iran, Japan, and the Republic of Korea.

2.4.2 Exposure assessment

In the studies considered to be most informative for assessing the association between MCPyV infection and MCC, a range of robust, primarily quantitative, exposure assessment methods were used. Techniques were of high quality and included digital transcriptome subtraction, quantitative PCR (qPCR), Southern hybridization, MCPyV neutralization assays, and antibody-binding assays detecting serum IgG antibodies to VP1 and LT and sT antigens. In most prospective studies, it was not possible to test for the presence of MCPyV in all the tumours of the cases, limiting the ability to estimate adjusted risk estimates associated with seropositivity among MCPyV-positive versus MCPyV-negative tumours, which would have yielded more precise estimations of risk. Nonetheless, in some studies, a small subset of tumours were analysed (i.e. Faust et al., 2014, and Amorrortu et al., 2021). These indicated strong concordance between MCPyV seropositivity and MCPyV DNA positivity in MCC tumours (Faust et al., 2014), but lack of concordance between MCPyV seropositivity, MCPyV DNA in eyebrow hair or MCPyV DNA in skin swabs at baseline, and subsequent MCPyV DNA–positive keratinocyte carcinomas (SCC and BCC of the skin) (Amorrortu et al., 2021). The most recent study (Faust et al., 2014) was able to assess temporality by using pre-diagnostic serum samples from a population-based prospective cohort for a nested case–control analysis.

Southern hybridization, used by Feng et al. (2008), is a method that is able to confirm clonal integration of MCPyV DNA into the host genome. Direct sequencing of PCR products allows the identification of gene sequences encoding truncated forms of LT (tLT) antigen. This was coupled with the assessment of expression of oncogenic LT and sT antigens by IHC. The presence of antibodies to sT and LT in the serum was correlated with tumour burden.

Common limitations across studies included incomplete assessment of co-exposures, such as UV radiation and immunosuppression. Nevertheless, confounders such as age and sometimes other viral exposures (e.g. JCPyV, cutaneous HPVs) were routinely measured appropriately.

In conclusion, the studies collectively provided consistent, replicable evidence using multiple, validated exposure assessment methods, including measurement of viral integration.

2.4.3 Confounding and biases

Nested case–control studies within studies with prospective follow-up benefit from the comparability of the cases to controls, since they are both derived from the same study population, whereas retrospective case–control studies often rely on somewhat different populations for cases and controls, especially if controls are hospital-based.

The results of either prospective or retrospective studies can be influenced by confounding factors. Matching in case–control studies was one approach to minimizing confounding for age and sex, used by Carter et al. (2009) and Paulson et al. (2010). For MCC, aside from age and sex, risk factors are largely unknown, and thus confounding by known risk factors was

less of a concern. Similarly, although the *IARC Monographs* programme has found *limited* evidence that hydrochlorothiazide (a common antihypertensive medication) causes MCC (Volume 137; IARC, 2026), the association with MCPyV exposure is unclear, which reduced concerns about confounding. However, for skin cancers, including MCC, factors related to UV radiation could be confounders since UV radiation also has immunosuppressive properties. The extent to which this may be operating is unknown; however, in one study (Amorrortu et al., 2021) it was noted that the presence of solar elastosis (a biomarker of UV radiation damage) was unrelated to overall seroprevalence of human polyomaviruses. Additionally, the lack of a consistent association between MCPyV seropositivity and BCC or SCC argued against UV radiation–related factors biasing the results of MCC strongly away from the null.

The results of studies on lung cancer could have been influenced by incomplete adjustment for tobacco exposure. For example, Colombara et al. (2015) carried out a study in a cohort of heavy smokers but did not adjust for duration of cigarette smoking, whereas in a later study within a separate cohort (Colombara et al., 2016), they did adjust for history of smoking. Both studies reported similar, null results; thus, confounding by smoking duration was unlikely to explain the lack of association reported in the earlier study (Colombara et al., 2015).

Confounding related to immune competency was considered, although some studies indicated that the population was "immunocompetent" (e.g. Teras et al., 2015), which indicated that the extent of confounding may be minimal.

Nevertheless, selection bias related to immune competency and other characteristics was a concern. Selection bias was a particular concern for tissue-based studies in which samples from the cases of selected cancers were compared with control samples from individuals for whom the description or selection procedures were not provided in detail (e.g. convenience samples that have no description of matching or reporting of age to allow an assessment of comparability). Consequently, the prevalence of MCPyV among the controls may not reflect the population from which the cases arose. For instance, in some studies, other cancers, pre-cancers, or other medical conditions were used as a comparator, and if these conditions were also related to MCPyV infection or immune competency, it would have biased risks towards the null. Tissues other than skin may be less likely to harbour MCPyV and could lead to an overestimate of risk, but this was less likely. Therefore, case series with comparator tissue were considered to be informative when advanced virological approaches were applied, such as the assessment of integration or mutation, or viral titres. Concerns over such biases were reduced when adjacent "normal tissue" was evaluated for the same individual (e.g. Sastre-Garau et al., 2009). However, risk estimates may be attenuated if adjacent tissue were to contain virally infected pre-malignant cells from which the cancer arose (i.e. there were field effects).

2.4.4 Reverse causation

A key question is one of temporality, i.e. which came first, the virus or the cancer? Retrospective case–control studies in which blood samples are collected for serology after the cancer diagnosis, cannot address this question, and may be prone to reverse causation. Likewise, temporality cannot be established in studies in which the presence of MCPyV DNA was analysed in tumours and comparator tissues from other individuals, as it is unclear whether tumours are more susceptible to MCPyV infection than normal tissue, or whether the viral infection preceded the cancer. The availability of studies with long prospective follow-up was seen as a considerable strength, as the potential for reverse causation is minimal in such studies.

2.4.5 Merkel cell carcinoma

For MCC, one nested case–control study using MCPyV serology collected before diagnosis, to reduce the likelihood of reverse causation (Faust et al., 2014), was evaluated; this study was conducted after the previous evaluation (IARC, 2013). The Working Group evaluated three case–control studies on MCC in which MCPyV seropositivity and the presence of MCPyV DNA in MCC tumours were assessed, all of which were included in the previous IARC evaluation (Carter et al., 2009; Paulson et al., 2010; Viscidi et al., 2011). In one of these studies, Paulson et al. (2010) tested for antibodies specific for sT, LT, and VP1 MCPyV proteins. Additional case series assessed evidence of integration or truncating mutations to provide virological evidence (Feng et al., 2008; Sastre-Garau et al., 2009; Shuda et al., 2009; Tolstov et al., 2009; Jung et al., 2011; Paik et al., 2011; Ota et al., 2012; Samimi et al., 2016; Santoro et al., 2022), and some of these included within-person comparisons to assess specificity to the tumour site (Laude et al., 2010; Hashida et al., 2016b). An additional study on MCC in salivary glands was included (Choi et al., 2025).

Both the nested case–control study, measuring MCPyV markers before diagnosis, and the case–control studies, measuring MCPyV after diagnosis, reported a consistently positive association between MCPyV VP seropositivity and MCC. The study by Faust et al. (2014) provided prospective evidence of a positive association between MCPyV and MCC using two methods – MCPyV neutralization and pseudovirion binding assay to VP proteins. Higher antibody titres were associated with a higher OR for MCC than was antibody positivity overall. Similarly, in case–control studies by Carter et al. (2009), an elevated OR for MCC was observed in relation to VP seropositivity, with verification within the MCPyV-positive tumours. In an additional, partially overlapping case–control study, Paulson et al. (2010) tested for antibodies specific for sT, LT, and VP1 MCPyV proteins; each was associated with MCC, with stronger associations for the presence of antibodies to sT and LT. No MCPyV-negative tumours were seropositive for antibodies to either sT or LT antigen. Similarly, in the case–control study by Viscidi et al. (2011), an elevated OR for MCC was reported in relation to VP1 seropositivity versus seronegativity.

Additional studies consistently showed that MCC tumour cells contain integrated MCPyV or mutated DNA, indicative of a role during or before tumour initiation (Feng et al., 2008; Sastre-Garau et al., 2009; Laude et al., 2010; Hashida et al., 2016b). In these studies, truncated LT gene sequences integrated inside the human genome, or mutated LT gene sequences harbouring truncating mutations (i.e. full sequences with point mutations or frameshift mutations encoding truncating proteins), were found in MCC tumours but not in non-MCC tissue, either adjacent or not. Integration and mutation patterns were also distinct for each MCC case, but identical in primary tumours and metastases (Sastre-Garau et al., 2009; Laude et al., 2010) from the same individuals. The expression of LT proteins in MCC cells was reported in several cases series using immunohistochemical methods (Tolstov et al., 2009; Jung et al., 2011; Paik et al., 2011; Ota et al., 2012; Samimi et al., 2016; Santoro et al., 2022). A study by Choi et al. (2025) also found evidence of LT protein expression in 2 of 4 cases of MCC in the salivary gland.

A credible positive association between MCPyV and MCC was supported by consistent increases in risk seen across studies using varying designs with different potential for bias, and the detection of integrated MCPyV, truncating mutations, and LT antigen expression in MCC tumours. The availability of a prospective study showing an association between MCPyV seropositivity and MCC provided evidence of temporality between infection and cancer. The studies matched for known risk factors such as age and sex, and the lack of association with SCC

or BCC indicated a lack of major confounding by UV-related factors. Therefore, the Working Group could rule out bias, chance, and confounding with reasonable confidence.

2.4.6 Squamous cell carcinoma of the skin

The Working Group evaluated two studies measuring MCPyV before diagnoses (one nested case–control study and one cohort study) along with two case–control studies examining SCC of skin. Case series of MCPyV integration or truncation were considered to be less informative. In the nested case–control and cohort studies, MCPyV positivity, measured by serology (Faust et al., 2016) or MCPyV DNA in eyebrow hair or skin swabs (Amorrortu et al., 2021), was not consistently associated with risk of SCC of the skin. Slightly elevated risks were observed in certain subgroups or exposure measures, i.e. if they seroconverted between the two samples analysed or if MCPyV DNA was present in eyebrow hair. In a case–control study (Rollison et al., 2012), seropositivity was imprecisely associated with an increased risk of SCC, which was stronger in individuals with MCPyV-positive SCCs. A subsequent case–control study (Hampras et al., 2015) included analyses of MCPyV T-antigen serology and detection in eyebrow hair along with MCPyV viral load in eyebrow hair and SCC fresh frozen samples using real-time qPCR. A positive association was observed between MCPyV DNA in eyebrow hair and SCC risk, although no other positive associations were observed.

Overall, the Working Group did not consider that the body of evidence indicated a credible positive association between MCPyV and SCC of skin. MCPyV integration or truncating mutations in DNA from SCC tumours was not consistently identified in case series, and the presence of the same mutation in multiple tumour and non-tumour samples in a study raised the question of contamination.

2.4.7 Basal cell carcinoma of the skin

The association between MCPyV and BCC of skin was assessed in one nested case–control and one cohort study. In the nested case–control study, Faust et al. (2016) identified a small increased risk of BCC in individuals who were seropositive for MCPyV at baseline, and a weakly positive association was observed among those who were seropositive at baseline and seronegative later, with wide confidence intervals. The cohort study reported no association with BCC for either MCPyV serology, or presence of MCPyV DNA in eyebrow hair or skin swabs (Amorrortu et al., 2021).

In a case series, no MCPyV DNA mutations were identified in BCC tumours, and weak LT protein expression was detected via IHC in only a small percentage of tumours (2 of 88) (Mertz et al., 2013).

In summary, the Working Group did not consider that the body of evidence indicated a credible positive association between MCPyV and BCC of skin.

2.4.8 Glioma

Only one study was considered to be informative for the evaluation of glioma in relation to MCPyV infection. In a nested case–control study, Egan et al. (2021) examined risk of glioma using data from two cohorts, the CPS-II and Janus Serum Bank, finding consistency between results across cohorts, reporting elevated adjusted ORs of glioma with MCPyV VP1 seropositivity. Furthermore, adjustment for seropositivity to the other polyomaviruses tested (BKPyV, JCPyV, and HPyV6) did not appreciably alter the results. An increasing trend in risk with increasing MCPyV antibody titres was indicated (*P* for trend, 0.052), though this appeared to be non-monotonic. Additional findings in this study included a stronger consistent association with glioblastoma than with non-glioblastoma,

although no measures of viral integration were assessed in the tumours.

Considering the availability of only one study demonstrating a positive association between MCPyV and risk of glioma, without evidence of the detection of integrated MCPyV, truncating mutations, or LT antigen expression in glioma tumours, the Working Group did not consider that the body of evidence indicated a credible positive association.

2.4.9 Haemopoietic malignancies in adults

One nested case–control study (Teras et al., 2015) and two case–control studies (Robles et al., 2012, 2015) were considered to be informative. In the nested case–control study, Teras et al. (2015) found no overall association between MCPyV and NHL or specific NHL subtypes (CLL/SLL, diffuse large B-cell lymphoma, follicular lymphoma), but they did detect an imprecisely elevated OR for "other NHL" in seropositive individuals (OR, 2.13; 95% CI, 0.97–4.37). In one case–control study on lymphoma, Robles et al. (2012) found little evidence of an overall association, and an elevated OR for diffuse large B-cell lymphoma, with a wide but positive confidence interval. In another case–control study on CLL and antibodies to sT and LT antigens and VP1 MCPyV (Robles et al., 2015), there were no participants who were seropositive for MCPyV LT antigen and only a small fraction who were seropositive for MCPyV sT. No MCPyV truncating mutations were identified in one study on lymphoma (Toracchio et al., 2010), although the sample size was small. In one study on CLL, Pantulu et al. (2010) identified a MCPyV mutation and some evidence of integration by FISH (DNA), although the Working Group noted that some patients with CLL also have MCC, and contamination could not be ruled out.

In summary, the Working Group did not consider that the body of evidence indicated a credible positive association.

2.4.10 Lung cancer

For cancer of the lung, two nested case–control studies (Colombara et al., 2015, 2016) were considered to be informative. No association was reported in the two nested case–control studies on all types of lung cancer (Colombara et al., 2015, 2016) among participants in the CARET trial or in an occupational cohort study of tin miners that entailed active follow-up, including lung cancer screening. Laboratory analyses included both MCPyV serology for VP1 capsid protein and sT oncoprotein, and the analysis considered multiple confounding factors. Histology-specific analyses were performed by Colombara et al. (2016); no associations were observed by histological type, with limited statistical power. Analysis of lung tumours did show some signs of LT protein expression (by IHC) and integration (Xu et al., 2014; Behdarvand et al., 2017; Jin et al., 2024), although one study found no evidence of this (Trejo Bittar and Pantanowitz, 2016), and thus this finding was not considered to be consistent (see Annex 4, Supplementary material for Merkel cell polyomavirus, Section 2, Cancer in humans, online only, available from: https://publications.iarc.who.int/667).

In summary, the Working Group did not consider that the body of evidence indicated a credible positive association.

2.4.11 Other cancer types

Other cancer types investigated included oesophageal cancer in a case–control study in which the OR for MCPyV VP1 positivity was 0.88 (95% CI, 0.73–1.06) (Sitas et al., 2012). A case–control study on bladder cancer measuring anti-VP1 MCPyV by Robles et al. (2013) observed an elevated bladder cancer risk was observed (OR, 1.48; 95% CI, 1.16–1.88) in relation to highest versus lowest seroreactivity tertiles, but no evidence of an increased risk was observed in a case–control study using urinary measures

of MCPyV DNA in Italy (Polesel et al., 2012). In a case–control study on childhood ALL, no MCPyV DNA was found in neonate bloodspots from cases or controls (Gustafsson et al., 2012). Campello et al. (2011) identified an increased odds of colon cancer in MCPyV DNA–positive individuals (OR, 4.47; 95% CI, 0.61–28.66), although these were imprecise, and there were limitations regarding the recruitment of controls.

The sparsity of comparative epidemiological studies for these cancers meant that an evaluation of case series for these cancers was not considered to be warranted.

In summary, the Working Group did not consider that the body of evidence indicated a credible positive association between MCPyV and these other cancer types.

3. Cancer in experimental animals

See Table 3.1.

MCPyV was previously evaluated by the *IARC Monographs* programme in 2012, and the evaluation was published in Volume 104 (IARC, 2013). In its evaluation at that time, the Working Group concluded that there was *inadequate* evidence in experimental animals for the carcinogenicity of MCPyV. Since the previous evaluation of MCPyV, there have been new studies investigating the occurrence of cancer in experimental animals in relation to exposure to MCPyV.

3.1 Oncogenic activity of MCPyV T antigen assessed in transgenic mice and in mouse models of chemical carcinogenesis

The advent of transgenic mouse technology has greatly advanced the study of viral oncogenes by enabling in vivo analysis of viral oncoproteins in an animal model. MCPyV encodes two oncoproteins, sT and truncated LT antigens, which are consistently expressed in virus-positive MCC tumours. Building on models developed for other polyomaviruses, such as SV40, researchers have generated transgenic mice that express MCPyV T antigens in a tissue-specific manner. The use of these models has begun to elucidate the oncogenic potential of MCPyV sT and LT antigens, offering insights into viral mechanisms of cellular transformation and tumorigenesis in vivo. Several transgenic models have been developed to assess the transforming potential of MCPyV T antigens in epithelial tissues.

Shuda et al. (2015) developed a transgenic mice model (*ROSAsT*) with inducible expression of MCPyV sT, achieved by inserting a floxed-stop-floxed MCPyV sT sequence into the *ROSA26* locus, allowing for Cre-mediated, conditional expression of sT. Intraperitoneal administration of tamoxifen (0.02 mg/g) to *UbcCreERT2*; *ROSAsT*; *p53^flox/flox* mice simultaneously induced MCPyV sT expression and *p53* gene deletion. After tamoxifen treatment, 5 out of 26 (19%) mice survived beyond 60 days. Four (80%) of these five mice developed high-grade malignant tumours in both the spleen and liver. In contrast, no tumours were observed in control mice with *p53* gene deletion alone (*UbcCreERT2*; *p53^flox/flox* mice) up to 140 days after tamoxifen treatment. [These findings, although not statistically significant, indicated that expression of MCPyV sT and deletion of the tumour suppressor gene *p53* lead to the development of poorly differentiated, highly anaplastic tumours in the spleen and liver of mice. The Working Group noted that this study was strengthened by the inclusion of appropriate controls and an adequate observation period; however, it was limited by the small number of animals surviving to the end-point. Additionally, the sex of the mice was not reported, which limited the interpretation of potential sex-specific effects.]

Table 3.1 Studies of carcinogenicity with MCPyV in experimental animals

Study design Species, strain (sex) Age at start Duration Reference	Virus strain Dosing regimen No. of animals at start No. of surviving animals	Results	Significance	Comments
Full carcinogenicity Mouse (transgenic), C57BL/6 (NR) Embryos 140 days Shuda et al. (2015)	MCPyV sT *ROSAsT* with inducible expression of MCPyV sT and *p53* deletion *UbcCreERT2*; *p53^flox/flox* mice, *UbcCreERT2*; *ROSAsT*; *p53^flox/flox* mice *p53* gene deletion alone in *UbcCreERT2*; *p53^flox/flox* mice treated for ≤ 140 days with tamoxifen at 0.02 mg/g 10, 26 10, 5	*Spleen and liver*: All tumours 0/10, 4/26	[NS, Fisher exact test]	*Principal strengths*: Appropriate controls, adequate observation duration. *Principal limitations*: Limited number of animals surviving to the end-point, the sex of the mice was not reported. *Other comments*: These findings, although not statistically significant, indicated that expression of MCPyV sT and deletion of the tumour suppressor gene *p53* leads to the development of poorly differentiated, highly anaplastic tumours in the spleen and liver of mice.
Full carcinogenicity Mouse (genetically engineered), *K14Cre-MCPyV168* (M and F combined) Embryos 6 mo Spurgeon et al. (2015)	MCPyV trLT + sT *ROSA26-LSL-MCPyV168*, *K14Cre-MCPyV168* Transgenic 48, 50 48, 35	*Skin* Papilloma 0/48, 16/50*	[*$P < 0.0001$, Fisher exact test]	*Principal strengths:* Adequate number of animals used, randomly allocated in groups, both sexes used, adequate duration of observation, appropriate controls. *Principal limitations:* Males and females were combined. *Other comments:* The spontaneous development of tumours in the absence of exposure to external carcinogens supports the tumorigenic role of MCPyV T antigens in epithelial tissues. However, only benign lesions were observed in this model; no malignant progression was reported.
Initiation–promotion (tested as initiator) Mouse (transgenic), FVB/N genetic background (M and F combined) 4–6 wk 20 wk Spurgeon et al. (2021)	*K14Cre-MCPyV168* transgenic mice MCPyV trLT + sT *K14Cre-MCPyV168*, *K14Cre-MCPyV168* + TPA, *Rosa26-LSL-MCPyV168* + TPA 15 nmol TPA twice per week for 20 wk on a region of the dorsal skin 28, 12, 17 22, 12, 17	Squamous cell papilloma Tumour multiplicity: [0.45 ± 0.65], [0.45 ± 0.65], [0.01 ± 0.1]*	*$P = 0.05$, Wilcoxon rank-sum test, *K14Cre-MCPyV168* + TPA vs *Rosa26-LSL-MCPyV168* + TPA	*Principal strengths:* Appropriate negative and positive controls, an adequate number of animals per group, adequate observation duration, both sexes used. *Principal limitations:* Males and females were combined in the analysis. *Other comments:* The data on tumour multiplicity were assessed by the Working Group using the diagram in Fig. 2 of the original publication.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Virus strain Dosing regimen No. of animals at start No. of surviving animals	Results	Significance	Comments
Initiation–promotion (tested as promoter) Mouse (transgenic), FVB/N genetic background (M and F combined) 4–6 wk 20 wk Spurgeon et al. (2021)	*K14Cre-MCPyV168* transgenic mice MCPyV trLT + sT *K14Cre-MCPyV168*, *K14Cre-MCPyV168* + DMBA, *Rosa26-LSL-MCPyV168* + DMBA 0.3 µmol DMBA once, on a region of the dorsal skin 28, 14, 22 22, 14, 18	*Skin*: SCC Tumour multiplicity: [0.25 ± 0.85], [2.0 ± 1.5]**, [0.36 ± 1.1]*	**$P < 0.0005$, Wilcoxon rank-sum test, *K14Cre-MCPyV168* + DMBA vs *K14Cre-MCPyV168* no treatment; *$P < 0.001$, Wilcoxon rank-sum test, *K14Cre-MCPyV168* + DMBA vs *Rosa26-LSL-MCPyV168* + DMBA	*Principal strengths:* Appropriate negative and positive controls, an adequate number of animals per group, adequate observation duration, both sexes used. *Principal limitations:* Males and females were combined in the analysis. *Other comments:* The data on tumour multiplicity were assessed by the Working Group using the diagram in Fig. 2 of the original publication.
Initiation–promotion (tested as promoter) Mouse (transgenic), FVB/N genetic background (M and F combined) 4–6 wk 20 wk Spurgeon et al. (2021)	*K14Cre-MCPyV168* transgenic mice topical treatment MCPyV trLT + sT *K14Cre-MCPyV168*, *K14Cre-MCPyV168* + DMBA + TPA, *Rosa26-LSL-MCPyV168* + DMBA + TPA A region of the dorsal skin, once with 0.01 µmol DMBA and then twice per week with 15 nmol TPA for 20 wk 28, 17, 23 22, 16, 19	*Skin*: Squamous cell papilloma Tumour multiplicity: [0 ± 1.5], [11.8 ± 6.0]**, [6.1 ± 5.2]*	**$P < 0.0001$, Wilcoxon rank-sum test, *K14Cre-MCPyV168* + DMBA + TPA vs *K14Cre-MCPyV168* no treatment *$P < 0.04$, Wilcoxon rank-sum test, *K14Cre-MCPyV168* + DMBA + TPA vs *Rosa26-LSL-MCPyV168* + DMBA + TPA	*Principal strengths:* Appropriate negative and positive controls, an adequate number of animals per group, adequate observation duration, both sexes used. *Principal limitations:* Males and females were combined in the analysis. *Other comments:* The data on tumour multiplicity were assessed by the Working Group using the diagram in Fig. 2 of the original publication.
Co-carcinogenicity Mouse (transgenic), *K14Cre-MCPyV168* transgenic mice (M and F combined) 4–6 wk 20 wk Spurgeon et al. (2021)	MCPyV trLT + sT *K14E6/E7* + DMBA + TPA, *K14Cre-MCPyV168* + DMBA + TPA, *Rosa26-LSL-MCPyV168* + DMBA + TPA Topical application to the dorsal skin once with 0.01 µmol DMBA and then twice per week with 15 nmol TPA for 10 wk. Mice were held for an additional 5 wk to monitor malignant progression to SCC grade 1. 22, 17, 23 16, 16, 19	*Skin*: Tumour progression to grade 1 SCC No. of grade 1 foci/No. of foci scored: 4/20, 6/26, 8/24	NS	*Principal strengths:* Appropriate negative and positive controls, adequate number of animals, adequate observation duration, both sexes used. *Principal limitations:* Males and females were combined.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Virus strain Dosing regimen No. of animals at start No. of surviving animals	Results	Significance	Comments
Full carcinogenicity Mouse (transgenic), FVB/N and C57BL/6 genetic backgrounds (M and F combined) 3 wk 6 mo Spurgeon et al. (2022)	Transgenic MCPyV trLT, MCPyV-Rb$^{\Delta L}$, MCPyV-Rb^{WT} Transgenic 13, 19 13, 19	*Skin*: Squamous cell papilloma 0/13, 11/19*	*P = 0.0006, Fisher exact test	*Principal strengths:* Both sexes used, adequate duration of observation. *Principal limitations:* Males and females were combined, small number of animals per group.
Full carcinogenicity Mouse (transgenic), C57BL/6J background (M and F combined) Postnatal day 21 NR Verhaegen et al. (2015)	Transgenic MCPyV sT Control, K5-CreER;KLEst (iK5;KLEsT) Recombination leading to sT antigen expression was induced in postnatal day 21 mice by five daily oral gavage doses of tamoxifen at 75 mg/kg resuspended in corn oil. 3, 3 3, 3	*Skin*: SCC NR	NA	*Principal strengths:* Both sexes used. *Principal limitations:* Males and females were combined, duration of observation was not reported, small number of animals per group, neoplasm incidence was not reported. *Other comments:* Expression of the sT antigen alone was sufficient to induce SCC in situ in *iK5*;KLEsT models. Although the authors reported a significant increase in neoplasms, incidence was not reported, and statistics could not be performed by the Working Group.
Full carcinogenicity Mouse (transgenic), FVB/N genetic background (M and F combined) Embryos 22 wk Verhaegen et al. (2022)	Transgenic MCPyV sT + trLT, and Atoh1 *K5-CreERT2*; *R26-LSL-rtTA*; *tetO*-sT/trLT; *tetO-Atoh1*, *K5-CreERT2*; *R26-LSLrtTA*; *tetO*-sT/trLT; *tetO-Atoh1*; *Trp53*(WT/fl) Transgenic 15, 14 15, 8	*Skin*;MCC Tumour incidence: 0/14, 8/14*	[*P = 0.0007, Fisher exact test]	*Principal strengths:* Both sexes used, adequate duration of observation. *Principal limitations:* Males and females were combined. *Other comments:* All of the surviving SLAP mice (n = 8) developed MCC.

DMBA, 7,12-dimethylbenz[*a*]anthracene; F, female(s); M, male(s); MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus; mo, month(s); NA, not applicable; NR, not reported; NS, not significant; SCC, squamous cell carcinoma; SLAP, Src-like adaptor protein; sT, small T antigen; trLT, truncated large T antigen; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; vs, versus; wk, week(s).

In the model described by Spurgeon et al. (2015), *K14Cre-MCPyV168* transgenic mice were used to investigate the oncogenic mechanisms of MCPyV in MCC. The K14Cre-driven expression of sT and truncated LT antigens induced epithelial hyperplasia, increased proliferation, and caused spontaneous tumorigenesis in the skin. Of the *K14Cre-MCPyV168* male and female [(combined)] mice that survived weaning (35/50), nearly half (16/35, 45.7%) developed spontaneous cutaneous papillomas, with incidence being significantly higher than in their Cre-negative *ROSA26-LSL-MCPyV168* littermates [$P < 0.0001$, Fisher exact test]. Papillomas began to emerge in the *K14Cre-MCPyV168* mice between age 6 weeks and 3 months. Notably, 7 out of 16 (44%) of the affected mice developed multiple papillomas. The occurrence of these lesions appeared to be independent of sex and was observed in mice regardless of whether they were housed alone or in groups. The papillomas formed on the hairy regions of the skin, commonly appearing on the back, hind limbs, and around the base of the tail. [The Working Group noted that the spontaneous development of tumours in the absence of exposure to external carcinogens supports the tumorigenic role of MCPyV T antigens in epithelial tissues. However, only benign lesions were observed in this model; no malignant progression was reported.]

Regarding pre-neoplastic lesions, extensive epithelial hyperplasia was observed in the stratified epithelium of the whisker pads, footpads, and dorsal skin of both neonatal and adult *K14Cre-MCPyV168* mice. [The Working Group noted that this study was strengthened by the inclusion of an adequate number of animals, appropriate controls, an adequate duration of observation, and both sexes, although the results were not analysed separately by sex.]

To assess the role of MCPyV T antigens at various stages of skin cancer development, Spurgeon et al. (2021) employed a chemically induced (initiation, promotion, and progression) carcinogenesis model using 7,12-dimethylbenz[*a*]anthracene (DMBA) as an initiator and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as a promoter, or the combination of both. They tested the previously described *K14Cre-MCPyV168* transgenic mice, which express the MCPyV T antigens in K14-positive cells of the stratified epithelia, in parallel with two other mouse models used as controls: (i) *Rosa26-LSL-MCPyV168*, which lacks Cre recombinase and therefore does not express the MCPyV T antigens, thus serving as a negative control; and (ii) *K14E6/E7 HPV16* transgenic mice, which had been previously tested using the DMBA/TPA model and therefore served as a positive control. These mice were assigned to different treatment groups that were treated with TPA only, DMBA only, or a combination of DMBA and TPA (DMBA plus TPA). An additional group of untreated *K14Cre-MCPyV168* mice was also included to assess the baseline incidence of spontaneous tumours. At age 4–6 weeks, mice received a single topical application of 0.3 µmol DMBA (DMBA-only treatment group), 15 nmol TPA (TPA-only treatment group), or 0.01 µmol DMBA (DMBA plus TPA treatment group). One week later, mice in the DMBA plus TPA treatment group began receiving topical applications of 15 nmol TPA twice weekly for 20 weeks. All mice were examined every 2 weeks for tumour development.

The results demonstrated that *K14Cre-MCPyV168* mice developed more malignant tumours of the skin, characterized as SCC (grade 1), than did *Rosa26-LSL-MCPyV168* control mice in the DMBA-only treatment group, beginning at week 16 post-treatment and continuing to the 20-week end-point. The incidence of tumours in the TPA-only treatment group of *K14Cre-MCPyV168* mice was significantly higher than in *Rosa26-LSL-MCPyV168* mice. In the DMBA plus TPA group, tumour incidence in *K14Cre-MCPyV168* mice was significantly higher than in the *Rosa26-LSL-MCPyV168* mice

treated with DMBA plus TPA at all time points from week 10 onwards. In contrast, *Rosa26-LSL-MCPyV168* mice did not develop any tumours during the observation period, confirming the specificity of the response to T-antigen expression. Together, tumour incidence in all three treatment groups was significantly increased. [The Working Group noted that, although the authors described incidence and indicated significance, values were not reported. Tumour multiplicity was assessed from Fig. 2 of the original publication by Spurgeon et al. (2021).]

To assess tumour progression after the 20-week DMBA plus TPA treatment period, tumour-bearing *Rosa26-LSL-MCPyV168*, *K14E6/E7*, and *K14Cre-MCPyV168* mice were maintained without further treatment for an additional 5 weeks. Within 5 weeks, approximately 23% (6/26) of tumours in *K14Cre-MCPyV168* mice treated with DMBA plus TPA had progressed to grade 1 SCC. With extended observation to 8 weeks, one *K14Cre-MCPyV168* mouse developed multiple lesions, 60% of which were dysplastic and 40% were invasive SCCs (grades 1 and 3). [The Working Group noted that this study included appropriate negative and positive controls and used an adequate number of animals and observation duration. However, although both males and females were included in the study, the results for each were combined.]

The interaction between LT and the retinoblastoma tumour suppressor protein (pRb) was studied by Spurgeon et al. (2022). This follow-up study provided direct evidence for the pivotal role of the LT–pRb interaction in MCPyV-induced tumorigenesis. Using a K14-driven transgenic model expressing MCPyV T antigens, the researchers disrupted LT binding to pRb by introducing a homozygous mutation in the pRb-binding domain (MCPyV-Rb$^{\Delta L}$). Mice with intact LT–pRb binding (MCPyV-Rb^{WT}) exhibited pronounced epithelial hyperplasia and increased cell proliferation, and developed spontaneous papillomas on the dorsal skin and areas surrounding their hind legs. Tumours occurred in 11 out of 19 mice (58%), representing a significant increase ($P = 0.0006$, Fisher exact test) compared with mice with disrupted LT–pRb interaction (MCPyV-Rb$^{\Delta L}$) (0/13).

These findings supported a mechanistic role for the LT–pRb interaction in early stages of MCPyV-mediated skin tumorigenesis. [The Working Group noted that this study used both sexes and an adequate duration of observation; however, males and females were combined and the number of animals per group was small.]

A study by Verhaegen et al. (2015) corroborated these findings by showing that sT expression alone was sufficient to induce hyperplasia in basal keratinocytes and spontaneous tumours in the skin, using the Cre-inducible sT transgenic mouse model, designated *KLEsT,* expressing MCPyV sT under the control of the bovine K5 promoter, directing expression to basal keratinocytes in the skin. By crossing *KLEsT* mice with *K5-CreERT2* mice (Indra et al., 1999) carrying tamoxifen-inducible Cre, they generated *K5-CreERT2;KLEsT* (*iK5;KLEsT*) bi-transgenic mice. Treatment of these mice from postnatal day 21 with tamoxifen (at 75 mg/kg in corn oil as five daily doses by oral gavage) to activate sT expression led to significant epithelial hyperplasia and increased cellular proliferation. Over time, a subset of these mice developed lesions resembling SCC in situ (Bowen disease). [The Working Group noted that the incidence of these lesions was not reported.]

Regarding pre-neoplastic lesions, sT drove rapid epidermal hyperplasia, impaired differentiation, and increased proliferation. These findings supported a role for MCPyV sT in early skin tumorigenesis. [The Working Group noted that this study used both sexes; however, males and females were combined, the number of animals per groups was small, the duration of observation was not reported, and the incidence of neoplasms was not reported, making it impossible to perform statistical analysis.]

In a follow-up study, Verhaegen et al. (2017) employed multiple transgenic mouse models (4–14 animals per treated group [number of animals in control groups not specified]) to express MCPyV truncated LT and sT antigens, individually or in combination, under the control of the keratin 5 (K5) promoter, targeting expression to the epidermis or to epidermal cells reprogrammed into a Merkel cell lineage by Atoh1. Expression of sT antigen alone induced epidermal hyperplasia and dysplasia. Co-expression of MCPyV sT and Atoh1 led to the development of malignant small blue cell tumours that expressed typical MCC markers, including SOX2, K8, and K20. Notably, the addition of truncated LT did not modify the phenotypes observed in mice expressing sT alone or in combination with Atoh1. Furthermore, additional transgenic mouse models carrying mutations in the domains of sT that bind to protein phosphatase 2A (PP2A) or to FBXW7 were generated to assess their roles in tumour development.

The results showed that MCPyV sT is sufficient to initiate MCC-like tumours in late-stage mouse embryos. [The Working Group noted that no data on tumour incidence were provided.] The MCC-like phenotype did not require the sT domain that binds PP2A but was strictly dependent on the sT FBXW7-binding domain. Therefore, sT-driven tumour phenotypes depend on its interaction with FBXW7, but not with PP2A. [The Working Group noted that the sex of the mice was not reported, there was a small number of animals per group, the number of animals in the control group was not reported, and tumour incidence was not reported, making statistical analysis impossible to perform. Therefore, this study was considered to be uninformative for the evaluation of MCPyV carcinogenicity in experimental animals, and it was not tabulated.]

Verhaegen et al. (2022) extended previous findings by combining MCPyV T-antigen expression with Atoh1-induced reprogramming and *p53* deletion. A triple-transgenic model that included: (i) a hormone-inducible *Cre* allele; (ii) a *Cre*-inducible *rtTA* allele; and (iii) *tetO*-driven effector alleles was used. Crosses with *K5-CreERT2*, *R26-LSLrtTA*, and *tetO-Atoh1* mice were performed to generate *K5-CreERT2;R26-LSL-rtTA;tetO-sT/tLT;tetO-Atoh1* mice (which were designated as *SLA*), and corresponding mice lacking the *tetO-Atoh1* allele (designated as *SL*). Transgene expression with or without *Trp53* deletion was induced in *SLA* ($n = 15$; 4 males and 11 females) and *SLAP* ($n = 14$; 4 males and 10 females) mice starting at postnatal days 21–24 by continuous administration of tamoxifen chow at 400 mg/kg in the feed and doxycycline (200 µg/mL) in drinking-water containing 5% sucrose. Mice were monitored biweekly for skin phenotypes and tumour development. *SLA* mice were monitored for up to 12 months, during which no overt skin tumours were observed. *SLAP* mice were monitored until skin tumours developed or until euthanasia was required for humane reasons (6/14), which in two mice included growth of grossly evident internal tumours that were not MCCs.

Each of the remaining eight mice developed one or more grossly visible tumours with classic MCC histology and marker expression as observed in human tumours between 11 and 22 weeks after transgene induction in *SLAP* mice ($n = 8$). All tumours except for one (10/11) were scored as histologically consistent with human MCC by a board-certified dermatopathologist. Moreover, RNA-sequencing analysis of mouse tumours showed gene expression profiles that were closely similar to those of human MCCs. [The Working Group noted that the *SLAP* model represents the only model available in vivo in which MCC-like tumours developed that closely resembled human MCC. Although the number of *SLAP* mice studied was relatively small, the incidence of tumours was high, with all surviving mice developing tumours (8/8, 100%). The Working Group also noted that this study

used males and females, and an adequate duration of observation. However, males and females were combined.]

3.2 Evidence synthesis for cancer in experimental animals

The carcinogenic potential of MCPyV has been evaluated in multiple studies using male and female transgenic mice expressing MCPyV T antigens (Shuda et al., 2015; Spurgeon et al., 2015, 2021, 2022; Verhaegen et al., 2015, 2017, 2022).

Across these studies, a significant increase in neoplasm incidence was consistently observed. In particular, compared with controls, *K14Cre-MCPyV168* mice (Spurgeon et al., 2015) and *MCPyV-Rb*^{WT} mice (Spurgeon et al., 2022) exhibited a markedly higher incidence of spontaneous squamous cell papilloma. Expression of the sT antigen alone was sufficient to induce SCC in situ in *iK5;KLEsT* models (Verhaegen et al., 2015). [The Working Group noted that, although the authors reported a significant increase in neoplasms, incidence was not reported.] Moreover, co-expression of MCPyV T antigens with Atoh1-induced cellular reprogramming and *p53* deletion led to a marked increase in the incidence of tumours that closely resembled human MCC in both histology and gene expression (Verhaegen et al., 2022). In a separate study, conditional expression of sT antigen in combination with *p53* deletion in *UbcCreERT2; ROSAsT; p53^flox/flox* mice resulted in the development of high-grade tumours in both the spleen and liver after tamoxifen administration, although the increase was not statistically significant (Shuda et al., 2015). In a chemical carcinogenesis model for the assessment of tumour promotion and progression, *K14Cre-MCPyV168* mice treated with DMBA showed a significantly increased incidence of squamous papillomas, compared with control mice (Spurgeon et al., 2021).

[The Working Group noted that these experimental models were adequate for the present evaluation because of their appropriate design and use of well-characterized transgenic mouse models expressing MCPyV T antigens. They employed diverse and complementary strategies to investigate MCPyV-mediated carcinogenesis, including conditional and tissue-specific expression of viral oncogenes, co-deletion of tumour suppressor genes such as *Trp53*, chemical carcinogenesis protocols, and lineage reprogramming to simulate Merkel cell differentiation. Notably, both benign and malignant lesions, including tumours resembling human MCC, were observed across these models. Although some studies had limitations, such as the combined analysis of males and females or incomplete reporting of tumour incidence, the overall experimental designs were rigorous, incorporated appropriate control groups, and yielded consistent findings across independent studies and animal models.]

Verhaegen et al. (2017) carried out a study using transgenic mouse models to express MCPyV truncated LT and sT antigens, individually or in combination, under the control of the K5 promoter, targeting expression to the epidermis or to epidermal cells reprogrammed into a Merkel cell lineage by Atoh1. This study was considered to be uninformative for the evaluation of the carcinogenicity of MCPyV in experimental animals.

4. Mechanistic Evidence

4.1 Infection and cell tropism

In humans, although clonally integrated MCPyV has been identified in the majority of, but not all, MCCs, some studies have confirmed that MCPyV DNA can also be found in various non-MCC tissues, but with lower prevalence, viral load, and protein expression (Dimitraki and Sourvinos, 2022).

In cell culture, MCPyV-based viral particles can infect primary keratinocytes and a wide variety of transformed cell lines, but the most efficiently transduced cell lines do not support robust replication of native MCPyV virions (Schowalter et al., 2012).

MCPyV infection is, at least in cell culture experiments, stimulated by β-catenin and growth factor (e.g. epidermal growth factor, EGF; and fibroblast growth factor, FGF) signalling, which induces matrix metalloproteinase genes (Liu et al., 2016). When MCPyV viral particles are applied to human foreskin cell populations, viral entry has been observed in keratinocytes, mesenchymal cells, and fibroblasts, but completion of the full viral life cycle is restricted to dermal fibroblasts (Liu et al., 2016; Liu and You, 2020). It is still a matter of debate as to whether fibroblasts that are infected with MCPyV can give rise to MCC (Houben et al., 2023).

In order to replicate, MCPyV virions bind through VP1 to sulfated glycosaminoglycans on the cell surface, then interact with ganglioside GT1B, the cell entry receptor (Erickson et al., 2009; Neu et al., 2012; Bayer et al., 2020). Like most polyomaviruses, the internalization of MCPyV occurs through caveolar/lipid raft–dependent endocytosis, followed by transport to endosomes and the endoplasmic reticulum (ER) (Becker et al., 2019; Giannecchini, 2020). The ER facilitates capsid uncoating, and the viral genome is then transported via nuclear pore complexes into the nucleus with the help of capsid protein nuclear localization signals (Giannecchini, 2020; Wang et al., 2025). Thus, MCPyV is clonally integrated into the MCC genome, and viral integration precedes oncogenesis (Feng et al., 2008).

4.1.1 Genome organization

The MCPyV genome is a small, circular, double-stranded DNA molecule of approximately 5.4 kb in size, which is organized into three functional, regulatory regions (see Fig. 1.1) (Feng et al., 2008; Shuda et al., 2008; DeCaprio, 2021).

The non-coding control region (NCCR), also known as the regulatory region, is located between the early and late coding regions. The NCCR contains the origin of replication and promoters that regulate the transcription of early and late genes (Kwun et al., 2009; Harrison et al., 2011).

The early region is expressed immediately after infection and generates three early viral transcripts – LT antigen, sT antigen, and 57kT antigen – by alternative splicing of the early region (T-antigen locus), and the overprinted gene named ALTO (Feng et al., 2008; Carter et al., 2013). One viral miRNA, MCPyV-miR-M1, is expressed in the late phase and negatively regulates early protein expression (Seo et al., 2009). Finally, the early region generates circular RNA (circRNA), the most abundant being circ-MCPyV-T (Yang et al., 2021).

The late region is activated after viral DNA replication and encodes capsid proteins VP1 and VP2 (Schowalter and Buck, 2013). VP1, the major capsid protein, is essential for virion structure and host cell entry, and VP2, the minor capsid protein, is involved in virion assembly and DNA packaging (Schowalter and Buck, 2013; Moens et al., 2017b).

4.1.2 Viral replication cycle

MCPyV has an orderly gene-expression cascade during replication. By day 2, LT and 57kT proteins are expressed, followed by the sT and VP1/2 proteins. In the replication-defective viruses, VP1 and sT proteins cannot be detected, whereas expression of the spliced 57kT antigen is substantially lower, suggesting that early gene splicing and late gene transcription may be replication-dependent (Feng et al., 2011).

(a) Viral early-gene mutation and integration, and their roles in oncogenesis

In MCPyV-positive MCC, the MCPyV genome is integrated into the host DNA such that the functions of its early promoter and partial expression of the T antigens are preserved (Feng et al., 2008). MCPyV DNA is clonally integrated into the MCC genome. Integration typically occurs in a manner that removes the VP genes (at least partially) and preserves the native sT gene. The LT gene may be preserved in part or even in its full length, but in the latter case, it contains truncating mutations (stop codons, frameshifts, or deletions) that prevent expression of the full-length LT protein. These truncating mutations deprive LT of its replication-mediating function and are thought to arise before viral integration. When present, truncating mutations are likely to occur before integration (Schrama et al., 2019). Integration also allows high-level expression of sT and truncated LT proteins, which can be detected in MCC cells by IHC (Shuda et al., 2008), but not at the low levels present in non-MCC infected cells. The oncogenic potential of MCPyV is driven by enhanced expression of sT and truncated LT proteins after integration (Shuda et al., 2008). In an MCPyV transgenic mouse model in which MCC tumour-derived sT and truncated LT were expressed under the keratin 14 (K14) promoter in stratified squamous epithelial cells and Merkel cells of the skin epidermis, epithelial hyperplasia, increased proliferation, and spontaneous tumorigenesis were observed (see Section 3 and Section 4.2.8) (Spurgeon et al., 2015).

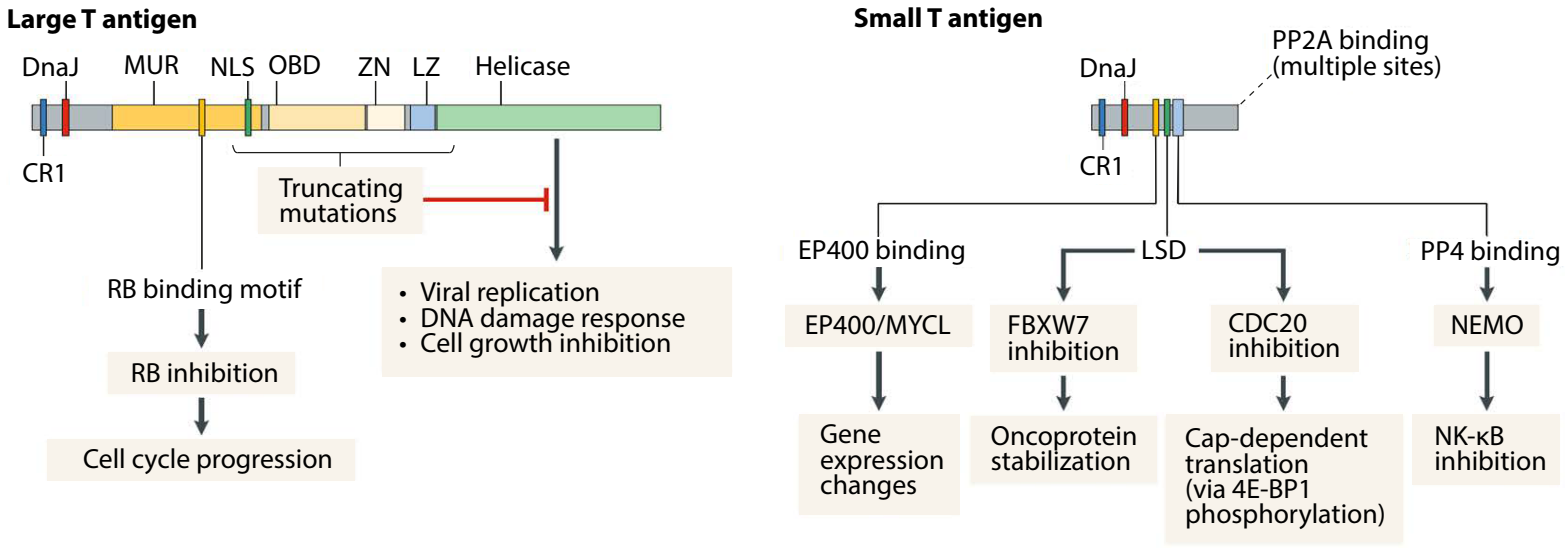
In MCPyV-positive MCC, the normal MCPyV life cycle is dramatically modified owing to random integration of the viral genome carrying a mutated LT antigen. The analysis of MCC cell lines and MCC primary tumours reveals distinct integration patterns, suggesting non-homologous end joining and microhomology-mediated break-induced replication as underlying mechanisms (see Section 4.2.1) (Czech-Sioli et al., 2020). These two events are now considered a hallmark of MCPyV-positive MCC. The presence of an MCPyV genome encoding a truncated LT is considered to be the main causal event for cancer evolution and persistence (Ahmed et al., 2021; DeCaprio, 2021; Yang and You, 2022). Events that are now considered a feature of MCPyV-positive MCC are: (i) a clonal pattern of integration of the viral genome within the tumour genome, indicating that viral integration occurs before tumour progression and is, therefore, essential for MCC development (Sastre-Garau et al., 2009; Schrama et al., 2019; Starrett et al., 2020); (ii) MCPyV LT is indispensable in maintaining the proliferation and survival of established MCC cell lines, as short hairpin RNA (shRNA) targeting both T antigens causes growth inhibition, which can be completely rescued by ectopic expression of LT alone (Angermeyer et al., 2013).

(b) Viral proteins and their functions

LT antigen initiates viral replication. Truncated forms of LT antigen retain the Rb-binding or LXCXE motif but lose the DNA-binding and helicase domains (Cheng et al., 2013). Truncated LT promotes cell proliferation more effectively than does full-length LT, highlighting its role in tumorigenesis (Cheng et al., 2013) (see Fig. 4.1). The interaction between LT and pRb is essential for tumour development in a mouse model, underscoring the importance of this interaction in MCC pathogenesis (Spurgeon et al., 2022).

sT antigen contributes significantly to the development and progression of MCPyV-positive MCC. sT antigen promotes viral lifecycle progression by activating mRNA expression and capsid protein production through interaction with transcriptional regulators and through stabilizing LT (Rapchak et al., 2022). sT has also been shown to carry iron/sulfur clusters that are needed for sT-mediated enhancement of LT-driven viral DNA replication, independent of its stability to stabilize LT (Tsang et al., 2016).

Fig. 4.1 Structure and cellular functions of the large and small T antigens of MCPyV



CDC20, cell division cycle protein 20; CR1, conserved region 1; DnaJ, mediates binding to heat-shock cognate 71 kDa protein; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; FBXW7, F-box/WD repeat-containing protein 7; LSD, large T-antigen stabilization domain; LZ, leucine zipper; MCPyV, Merkel cell polyomavirus; MUR, Merkel cell polyomavirus large T antigen unique region; NF-κB, nuclear factor kappa B; NEMO, NF-κB essential modulator; NLS, nuclear localization signal; OBD, origin binding domain; PP2A, protein phosphatase 2A; RB, retinoblastoma-associated protein; ZN, zinc finger domain.

Reproduced from Harms et al. (2018). Harms PW, et al. The biology and treatment of Merkel cell carcinoma: current understanding and research priorities. *Nature Reviews Clinical Oncology*, Volume 15, pp. 763–776. ©2018, Springer Nature Ltd.

Another study has shown that sT induces hyperphosphorylation of 4E-BP1 (Shuda et al., 2011; Kwun et al., 2015), a key regulator of cap-dependent translation, resulting in increased synthesis of proteins that promote cell growth and proliferation (see Section 4.2.8).

In another study, Cheng et al. (2017) identified the MYC homologue MYCL (L-MYC), its heterodimeric partner MAX, and the EP400 complex as sT-interacting proteins in a large-scale proteomic screen. They further showed that the sT–MYCL–EP400 complex binds to specific promoters (e.g. 4EBP1 and MYCL), increased expression of which contributes to cellular transformation and the generation of induced pluripotent stem cells. MCPyV sT also has an impact on cell motility and migration by affecting the actin cytoskeleton and promoting the formation of filopodia. This is caused via a mechanism involving the catalytic subunit of protein phosphatase 4 (PP4C). Furthermore, the authors showed that sT-induced cell motility is dependent upon the activities of Rho family GTPases Cdc42 and RhoA (Stakaitytė et al., 2018a) (see Section 4.2.8).

MCPyV sT is an essential contributor to MCPyV transforming activity (see Section 4.2.7) (Shuda et al., 2011; Verhaegen et al., 2017). Whereas LT is truncated in MCPyV-positive MCC, sT is typically wildtype and intact. Wildtype MCPyV sT contains 186 amino acid residues and shares the N-terminal 79 residues with LT. The remaining C-terminal region of sT is unique. A unique MCPyV sT domain, not present in the sTs of other human polyomaviruses, is the LT stabilization domain (LSD) at residues 91–95. This region is involved in inhibition of the proteasomal degradation of LT (Kwun et al., 2013).

In all mammalian polyomaviruses, sT binds to the serine-threonine PP2A complex. Mammalian PP2A consists of at least three subunits. Polyomavirus sT typically displaces the PP2A B subunit and forms a trimeric complex with the A and C subunits and inhibits the activity of the complex (Pallas et al., 1990). However, it is not clear whether the binding of MCPyV sT to PP2A contributes to its transforming potential (Kwun et al., 2015). MCPyV sT forms a complex with the MYC homologue MYCL (L-MYC) and its heterodimeric partner MAX (Cheng et al., 2017). In addition, sT and MYCL/MAX recruit the EP400 (p400) chromatin remodelling complex. The EP400 complex has both histone acetylation and chromatin remodelling activities that participate in transcription and DNA damage responses. One of the consequences of EP400 binding is reduced p53 activity in MCPyV-positive MCC tumours containing wildtype *TP53*. The sT–MYCL-EP400 complex increases levels of its target gene *MDM2*, an E3 ubiquitin ligase, which binds p53 and promotes its ubiquitination and subsequent degradation by the proteasome. In addition, the sT–MYCL-EP400 complex increases levels of MDM4 and CK1α (CSNK1A1), which cooperate to enhance the E3 ubiquitin ligase activity of MDM2, indicating that sT employs a dual mechanism to inactivate wildtype *TP53* in MCPyV-positive MCC (Cheng et al., 2017; Park et al., 2019). Overexpression of MCPyV sT in HEK293 cells led to increased levels of proteins involved in microtubule destabilization, particularly stathmin, resulting in a motile and migratory phenotype (Knight et al., 2015). [The Working Group noted that it is possible that deregulation of these target genes contributes to the highly metastatic potential of MCC.]

LT in MCC is always truncated, but its RB1 interaction domain is consistently preserved, indicating that this pattern is essential for MCC development (Shuda et al., 2008). In addition, (i) the growth of MCPyV-positive MCC cells generally depends on the expression of MCPyV oncoproteins (T antigens) (Houben et al., 2010); (ii) the transforming ability of MCPyV T antigens in vitro and in vivo has established them as oncogenes (Shuda et al., 2011; Spurgeon et al.,

2015; Verhaegen et al., 2017); and (iii) the lack of recurrent mutations in established human oncogenes in MCPyV-positive MCC (Harms et al., 2015; Goh et al., 2016; Starrett et al., 2017) further supports the critical role of viral oncoproteins in carcinogenesis. Although these arguments strongly support MCPyV T antigens as key drivers of MCC oncogenesis, testing this hypothesis is limited by the unknown cell of origin of MCC. However, accumulating evidence suggests a keratinocytic cell of origin (Sunshine et al., 2018; Gravemeyer et al., 2022; Kervarrec et al., 2022).

57kT protein is homologous to SV40 17kT protein (Comerford et al., 2012), which is known to promote host cell proliferation. 57kT does not support MCPyV replication (Kwun et al., 2009), and its role in infection or MCC development remains unclear.

Mutations in the sialic acid–binding pocket of MCPyV VP1 rendered the particle non-infectious but did not perturb binding to cells (Neu et al., 2012), providing evidence for a function of VP1 in cellular binding and entry. VP2 increases infectivity and plays an important role during infectious entry in some cell types but not in others (Schowalter and Buck, 2013). [The Working Group noted that it has not been suggested that VP1 and VP2 are involved in tumour initiation or progression. The Working Group further noted that when mutated or fragmented MCPyV genomes integrate into the host genome in such a way that VP1 and VP2 protein expression is abolished but sT and truncated LT expression is sustained, cellular functions are altered, thus driving tumour maintenance and progression.]

The ALTO protein supports persistent infection (Wang et al., 2024) and can modulate MCPyV infectious and tumorigenic potential through transcription regulation (Yang et al., 2021).

miR-M1 inhibits MCPyV replication and thus contributes to the maintenance of MCPyV genomes as episomes in the infected host cell by negatively regulating LT transcripts (Seo et al., 2009; Theiss et al., 2015).

4.1.3 *Experimental models of MCPyV infection*

In cell culture, MCPyV-based viral particles can enter primary keratinocytes and a wide variety of transformed cell lines, but the most efficiently transduced cell lines could not support robust replication of MCPyV virions (Schowalter et al., 2012). In cell culture experiments, matrix metalloproteinase genes have been shown to be stimulated by WNT/β-catenin and growth factor (e.g. EGF and FGF) signalling, stimulating MCPyV infection (Liu et al., 2016). When MCPyV viral particles were applied to a total cell population of the human foreskin, again viral entry was observed in keratinocytes and fibroblasts, but completion of the full viral cycle was restricted to dermal fibroblasts (Liu et al., 2016; Liu and You, 2020). [The Working Group noted that whether fibroblasts are cells that upon infection can give rise to MCC is a still a matter of debate.]

4.2 Evidence relevant to key characteristics of carcinogens

This section reviews the mechanistic data for the key characteristics of carcinogens (Smith et al., 2016) encompassed by the agent MCPyV. Evidence was available for the key characteristics "is genotoxic", "alters DNA repair and causes genomic instability", "induces epigenetic alterations", "induces chronic inflammation", "is immunosuppressive", "modulates receptor-mediated effects", "causes immortalization", and "alters cell proliferation, cell death, or nutrient supply". No data were available for the key characteristics "is electrophilic or can be metabolized to an electrophile" and "induces oxidative stress".

4.2.1 Is genotoxic

(a) Human

(i) Exposed humans

Integration of MCPyV into the host genome is a hallmark of MCPyV-positive MCC (Feng et al., 2008; Shuda et al., 2008). This viral integration is clonal, as demonstrated by consistent integration patterns within the same tumour and between primary tumours and their metastases in the same patient (Feng et al., 2008). [The Working Group noted that these observations indicated that viral infection and integration occur early in tumour development, before the clonal expansion of tumour cells.]

MCPyV preferentially integrates into regions of open chromatin, inserting into host DNA double-strand breaks through error-prone repair mechanisms. These include microhomology-mediated break-induced replication between the viral and host genomes (Czech-Sioli et al., 2020; Starrett et al., 2020) and non-homologous end joining (Czech-Sioli et al., 2020). Although integration can disrupt or deregulate nearby cancer-associated genes, the integration sites appear to be largely random. In some MCC tumours, viral integration occurs near oncogenes or tumour suppressor genes such as *PTPRG* (Feng et al., 2008), *TP53*, *TOP2A*, and *KDM6A* (Knepper et al., 2019), as well as *KMT2D* (Arora et al., 2020b). However, no recurrent integration events within these genes have been reported (Feng et al., 2008; Knepper et al., 2019; Schrama et al., 2019; Arora et al., 2020b; Czech-Sioli et al., 2020; Starrett et al., 2020).

(ii) Human cells in vitro

Tsang et al. (2014) demonstrated that MCPyV LT colocalizes with phosphorylated DNA damage response markers such as γH2AX, ATR, and pChk2 in nuclear foci of infected human U2OS osteosarcoma cells, indicating that MCPyV infection activates the DNA damage response to facilitate viral replication.

Li et al. (2013) further showed that MCPyV infection in U2OS cells triggers activation of the DNA damage response through both ATR and ATM signalling pathways, whereas MCPyV LT specifically activates the ATR pathway via its C-terminal helicase-containing region, leading to p53 phosphorylation and cell cycle arrest. Moreover, the C-terminal region of LT (amino acids 441–817) induces DNA damage in U2OS cells, as shown by comet assay and accumulation of single-stranded DNA-binding protein RPA70 in punctate nuclear foci. Additionally, Wu et al. (2019) reported that MCPyV sT induces hyperphosphorylation of ATM, 53BP1, CHK2, and γH2AX in HEK293 cells, indicating activation of the ATM pathway and suggesting an increase in double-strand DNA breaks. [The Working Group noted that this study did not assess the functional consequences of activation of the DNA damage response or double-strand DNA breaks, including potential mutagenesis or cell viability. Therefore, although these findings provided evidence of genotoxic stress induced by MCPyV sT in human cells, the downstream effects remained unclear. Together, these findings highlighted the involvement of both full-length LT and sT in activation of the DNA damage response via distinct signalling pathways. However, truncated LT lacking the C-terminal helicase domain does not induce DNA damage or activate the DNA damage response.]

(b) Experimental systems

In mice with K14-directed expression of tumour-derived MCPyV T antigens, Spurgeon et al. (2015) observed increased phosphorylation levels of γH2AX and ATM in papillomas, but not in the surrounding skin. Notably, MCPyV T antigens were expressed in the epithelium of both the papilloma and the adjacent skin, suggesting that expression of MCPyV T antigens alone is insufficient to activate the DNA damage response. In a related study, Verhaegen et al. (2015) showed that MCPyV sT induces

γH2AX and cleaved caspase 3 in the epithelium of K5 promoter–driven, sT-expressing mice, indicating both activation of the DNA damage response and increased apoptosis. Moreover, Kwun et al. (2017) demonstrated that MCPyV sT induces chromosome breakages in human fibroblast WI38 cell lines, as assessed by karyotype analysis. Consistent with its in vitro effect, MCPyV sT also promotes micronucleus formation in mice (Kwun et al., 2017).

Synopsis

[The Working Group noted that several studies supported the genotoxicity of MCPyV, primarily through viral integration and involvement in the DNA damage response, both of which contribute to genomic alterations and tumorigenesis. In exposed humans, integration of MCPyV with a truncating mutation in the LT gene into the tumour genome is a consistent and early event in MCPyV-positive MCC, with clonal integration patterns supporting a causal role in tumour initiation. Additionally, MCPyV full-length LT and sT can induce genotoxicity in human cells and mouse models. Full-length LT activates the ATR pathway through its C-terminal helicase domain, leading to p53 phosphorylation, cell cycle arrest, and DNA damage in human cells. In contrast, sT induces phosphorylation of ATM, 53BP1, CHK2, and γH2AX, and promotes chromosomal breakages in human cells. In vivo, sT expression also results in micronucleus formation in transgenic mice. However, truncated LT, which is the form found only in MCC tumours, does not appear to induce genotoxicity. Collectively, these findings suggested that sT is the primary driver of genotoxic stress in MCPyV-driven tumorigenesis.]

4.2.2 Alters DNA repair or causes genomic instability

(a) Humans

(i) Exposed humans

MCPyV-positive tumours exhibit fewer genomic alterations than do MCPyV-negative tumours, suggesting a degree of genomic stability in MCPyV-driven MCC (Paulson et al., 2009; Goh et al., 2016; Knepper et al., 2019; Horny et al., 2021; Torre-Castro et al., 2024). Array comparative genomic hybridization analyses have revealed fewer genomic deletions in MCPyV-positive tumours than in virus-negative tumours (Paulson et al., 2009), whereas genomic sequencing studies indicated limited copy number variation in MCPyV-positive MCC (Goh et al., 2016; Starrett et al., 2017; Horny et al., 2021). [The Working Group noted that MCPyV-positive and MCPyV-negative MCC tumours arise through distinct biological mechanisms. Virus-negative tumours are driven primarily by ultraviolet (UV)-induced DNA damage, whereas MCPyV-positive tumours are linked to viral oncogenesis. Therefore, direct comparisons between these two subtypes may not reflect virus-specific effects and could be confounded by differences unrelated to MCPyV.]

(ii) Human cells in vitro

Demetriou et al. (2012) demonstrated that MCPyV-positive MCC MKL-1 cells are hypersensitive to UV radiation and exhibit defective DNA repair when compared with MCPyV-negative UISO cells. After UV exposure, MKL-1 cells showed significantly reduced viability and lower DNA repair efficiency, indicated by increased levels of UV-induced DNA photoproducts and decreased expression of the DNA damage recognition protein XPC.

Consistent with the association between impaired DNA repair and MCPyV positivity of MCC cells, expression of truncated LT antigen in UISO cells inhibits DNA repair and cell cycle

arrest after UV radiation. In contrast, cells expressing full-length LT or sT exhibited repair efficiency comparable to that of vector controls (Demetriou et al., 2012). Beyond MCC cells, expression of truncated LT in normal human dermal fibroblasts also impaired the repair of UV-induced DNA damage and failed to trigger proper cell cycle arrest after UV exposure, supporting its role in defective DNA repair and cell cycle regulation, even in non-cancerous cells (Demetriou et al., 2012).

(b) Experimental systems

Additionally, research by Kwun et al. (2017) found that expression of MCPyV sT in NIH 3T3 cells induced genomic instability by interfering with the SCF^{Fbw7} E3 ubiquitin ligase complex. This interference resulted in the accumulation of c-Myc and additional cell cycle–regulatory substrates such as PLK1 and YAP, driving centrosome amplification, chromosomal breakage, and aneuploidy. Furthermore, MCPyV sT increased aneuploidy in vivo, as shown by the presence of micronuclei in mouse models (Kwun et al., 2017). These findings underscored the crucial role of MCPyV sT in driving genomic instability.

On the other hand, a recent study by Bachiri et al. (2025) highlighted a cooperative interaction between truncated LT and EHMT2 (a histone methyltransferase) in regulating DNA repair. EHMT2 is known to recruit DNA damage response proteins to sites of DNA damage for repair (Yang et al., 2017).

EHMT2 inhibition increased levels of p-CHK1, a marker of the single-strand break response, in U2OS (with or without LT), PeTa (with or without LT), MKL1 (with or without LT) and WaGa wildtype cells, but not in WaGa cells in which LT had been knocked down by shRNA. In MKL-1 and PeTa cells, sustained CHK1 phosphorylation after hydroxyurea recovery was observed in both wildtype and T-antigen knockdown cells when EHMT2 was inhibited, but T-antigen knockdown alone did not increase CHK1 activation. In WaGa cells, knockdown of T antigens increased the frequency of γH2AX foci (a marker of DNA double-strand breaks) upon etoposide treatment, suggesting impaired DNA repair. However, when EHMT2 was inhibited and T antigens were knocked down, γH2AX foci were reduced when compared with T antigen–knockdown alone, implying that T antigens may compensate for EHMT2 inhibition.

[The Working Group noted that these findings may indicate functional evidence that MCPyV T antigens are actively involved in the regulation of host DNA repair pathways. Through coordination with EHMT2, they help modulate the cellular response to DNA damage, particularly single-strand breaks and replication stress, thereby contributing to genomic maintenance and possibly MCC tumorigenesis.]

Synopsis

[The Working Group noted that MCPyV-positive MCCs exhibit fewer genomic alterations than do virus-negative tumours, suggesting a relatively stable genome. However, studies indicated that MCPyV T antigens can disrupt DNA repair processes and promote genomic instability. Truncated LT impairs DNA repair and disrupts cell cycle arrest mechanisms in human cancer cell lines and in primary fibroblasts. sT contributes to genomic instability by interfering with the SCF^{FBXW7} ubiquitin ligase complex, as demonstrated in rodent cell lines and transgenic mouse models. Additionally, truncated LT interacts with the histone methyltransferase EHMT2, a regulator of DNA repair. Disrupting EHMT2 or T-antigen function alters DNA damage response signalling, including sustained CHK1 phosphorylation and changes in γH2AX accumulation. Taken together, these findings indicated a role for truncated LT in modulating DNA repair and a role for sT in promoting genomic instability in experimental systems.]

4.2.3 Induces epigenetic alterations

(a) Humans

(i) Merkel cell carcinoma

One well-characterized mechanism involves EZH2, the catalytic component of polycomb repressive complex 2 (PRC2), which deposits the H3K27me3 histone mark to repress gene expression (Kim and Roberts, 2016). Durand et al. (2023) demonstrated that EZH2 expression and H3K27me3 levels are significantly elevated in MCPyV-positive MCCs compared with MCPyV-negative MCCs, suggesting a role for MCPyV in EZH2 upregulation and consequently increased H3K27me3 deposition.

In contrast to the study by Durand et al. (2023), Gujar et al. (2021) identified differentially methylated genes in MCPyV-positive and MCPyV-negative MCC tumours, particularly in pathways related to neuroendocrine differentiation. Additionally, the promoters of genes such as *KDM6B* and *EZHIP*, which are involved in histone modification and chromatin dynamics, were hypomethylated in MCPyV-positive MCCs. [The Working Group noted that MCPyV-positive and MCPyV-negative MCC tumours might arise through distinct biological mechanisms. Virus-negative tumours are driven primarily by UV-induced DNA damage, whereas MCPyV-positive tumours are linked to viral oncogenesis. Therefore, direct comparisons between these two subtypes may not reflect virus-specific effects and could be confounded by differences unrelated to MCPyV.]

(ii) Human cells in vitro

Mechanistic studies in human cells have shown that silencing MCPyV T antigens (both sT and LT) in MCPyV-positive MCC cell lines (WaGa and PeTa) leads to a reduction in EZH2 mRNA and protein levels (Durand et al., 2023; Khattri et al., 2023). Conversely, ectopic expression of MCPyV T antigens or truncated LT alone induced EZH2 expression in human primary dermal fibroblasts, whereas expression of sT alone or an Rb binding–defective LT mutant did not (Durand et al., 2023). This Rb binding–dependent activation of EZH2 was further confirmed in both BJ human foreskin fibroblasts and IMR90 lung fibroblasts (Khattri et al., 2023), underscoring the critical role of Rb inactivation in MCPyV-mediated EZH2 upregulation. However, the precise mechanism by which viral LT regulates EZH2 expression remains unclear. Furthermore, MCPyV-positive MCCs exhibit a dependency on EZH2 activity, as EZH2 knockdown strongly impairs tumour cell viability, whereas treatment with EZH2 inhibitors showed no effect under the conditions used (Khattri et al., 2023). In contrast to the above, Durand et al. (2023) reported that EZH2 inhibition reduced H3K27me3 levels and impaired the viability of MCPyV-positive cell lines. [The Working Group noted that these findings suggested that MCPyV-driven EZH2 activity is essential for the survival of MCPyV-positive MCC cell lines.]

Beyond EZH2, MCPyV also modulates histone demethylation through LSD1 (KDM1A), a histone demethylase involved in transcriptional regulation. Park et al. (2020) demonstrated that MCPyV sT antigen in complex with MYCL, MAX, and EP400 binds to the promoter of LSD1, leading to its upregulation. Notably, MCPyV-positive MCCs are highly sensitive to LSD1 inhibitors, whereas MCPyV-negative MCCs are resistant, as observed in cell viability experiments (Leiendecker et al., 2020; Park et al., 2020).

In MCPyV-positive MCCs, LSD1 inhibition disrupted the LSD1–CoREST complex, altering chromatin structure and transcriptional activities to suppress cell proliferation and promote neuronal differentiation, suggesting that MCPyV sustains an undifferentiated proliferative phenotype via LSD1 (Leiendecker et al., 2020). [The Working Group noted that it remains unclear whether the observed effect is dependent on LSD1 demethylase activity.]

In terms of DNA methylation, MCPyV-positive and MCPyV-negative MCCs displayed distinct methylation profiles (Gujar et al., 2021; Gravemeyer et al., 2022; Harms et al., 2022). Using DNA methylation arrays, Harms et al. (2022) reported reduced global DNA methylation in MCPyV-positive MCC cell lines compared with MCPyV-negative counterparts; however, the authors did not identify significantly enriched gene sets among the hypermethylated genes in MCPyV-positive MCC. Gravemeyer et al. (2022) found similar DNA methylation patterns in MCPyV-positive and MCPyV-negative MCC cell lines, but also identified hypomethylation at specific loci in MCPyV-positive cell lines, particularly in neuroendocrine lineage markers such as *SOX2* and *INSM1*, as well as in binding sites for the PRC2 components EZH2 and SUZ12 (Gravemeyer et al., 2022).

Whether MCPyV directly modulates DNA methylation to drive oncogenic transcriptional programmes remains unclear. Nonetheless, MCPyV-positive MCCs exhibit functional vulnerabilities linked to these DNA methylation changes. Treatment with the DNA demethylating agent decitabine selectively induced cell death in MCPyV-positive MCC cells while exerting minimal effects on MCPyV-negative MCC cells (Harms et al., 2022).

(b) Experimental systems

Studies using MCC xenograft models (MKL-1, WaGa, and PeTa) further supported the functional significance of MCPyV-driven epigenetic reprogramming in MCC. Pharmacological inhibition of EZH2 (by treatment with tazemetostat) and LSD1 (GSK-LSD1 and CPI-242) markedly reduced tumour growth in these in vivo models (Leiendecker et al., 2020; Park et al., 2020; Gartin et al., 2022). [The Working Group noted that only pharmacological approaches were used in the experimental mouse models. Although use of these inhibitors provided supportive evidence for the involvement of catalytic activities of these epigenetic regulators, off-target effects, particularly at higher concentrations, may confound the interpretation of the findings. Additional experimental strategies, including genetic knockdown or knockout plus rescue experiments using catalytically dead EZH2 mutants, and direct assessment of epigenetic changes in inhibitor-treated tumours would strengthen the conclusion that the observed effects were caused by the catalytic activities of these enzymes. Furthermore, the absence of MCPyV-negative cell lines in these studies limited the ability to determine whether the observed effects were specific to MCPyV-positive tumour cells. Similarly, the DNA demethylating agent decitabine significantly suppressed tumour growth in MCPyV-positive MKL1 xenografts but showed no effect in MCPyV-negative MCC40 xenografts (Harms et al., 2022).]

Synopsis

[The Working Group noted that several studies have reported upregulation of EZH2 and its associated histone mark H3K27me3 in MCPyV-positive MCCs. Functional studies in human cells demonstrated that MCPyV truncated LT upregulates EZH2 expression via its Rb-binding domain. In parallel, MCPyV sT promotes cell growth by recruiting MYCL, MAX, and EP400 to induce transcription of LSD1, another histone-modifying enzyme. Pharmacological inhibition of EZH2 or LSD1 suppresses tumour growth in MCPyV-positive MCC xenograft models, indicating functional dependence on these epigenetic regulators. Additionally, distinct DNA methylation profiles have been observed for MCPyV-positive and MCPyV-negative MCC tumours and cell lines; however, no significantly enriched gene sets have been identified specifically in MCPyV-positive MCC. Collectively, these findings provided some evidence that MCPyV induces epigenetic alterations in MCC cell lines, particularly through modulation of histone methylation via EZH2

and LSD1. The direct impact of MCPyV regulation on DNA methylation remains unclear.]

4.2.4 Induces chronic inflammation

(a) Human

(i) Exposed humans

Kumar et al. (2011) examined T-helper (Th) cell–mediated proliferation and cytokine responses to MCPyV VLPs in 30 randomly selected asymptomatic volunteers who were either seropositive or seronegative for MCPyV. The study found that MCPyV VLPs stimulated Th cell proliferation and cytokine responses to a greater extent in seropositive participants. The difference between seropositive and seronegative participants in their cytokine response to MCPyV antigen was particularly evident for interferon gamma (IFNγ) but was also significant for IL-10 and IL-13. [The Working Group noted that this study was performed in healthy participants without MCC.]

MCPyV virus-reactive $CD8^+$ and $CD4^+$ T-cell responses to MCPyV oncoproteins were detected (using intracellular cytokine cytometry, IFNγ-ELISPOT, and a novel HLA-A*2402-restricted MCPyV tetramer) in both MCPyV-positive MCC tumours and peripheral blood, in MCC patients (14 of 27) and control participants (5 of 13). Notably, MCPyV-specific CD8 T cells were more frequent in tumour tissue than in blood. In addition, $CD4^+$ T cells with the same MCPyV specificity were isolated from both an MCC primary tumour and a subsequent metastasis. Consistent with the tumour findings, peripheral blood mononuclear cell (PBMC) responses to T antigen were detected in 12 of 19 patients with MCPyV-positive MCC tumours, compared with only one of six patients with MCPyV-negative MCC tumours ($P = 0.07$). Finally, although tetramer-positive T cells were identified in PBMCs from two patients with MCPyV-positive MCC, these cells failed to produce IFNγ (Iyer et al., 2011).

In patients ($n = 26$) with advanced MCC receiving pembrolizumab, MCPyV-specific T cells decreased over time in patients who had a complete response to PD-1 therapy and increased in patients who had persistent disease. MCPyV-positive tumours had a more clonal intratumoral T-cell receptor repertoire than did virus-negative tumours ($P = 0.0001$). T-cell receptor clonality was significantly higher in MCPyV-positive MCC tumours than in MCPyV-negative MCC tumours, indicating a focused, antigen-driven T-cell response in MCPyV-positive tumours (Miller et al., 2018).

MCPyV-specific T-cell levels (CD8 T cells) were detected in blood from 7 of 11 patients with MCPyV-positive tumours. MCPyV-specific T-cell levels in the blood increased with MCC tumour burden and disease progression, and decreased after successful treatment (Afanasiev et al., 2013). [The Working Group noted that the authors did not specify the type of treatment received.]

MCPyV-positive MCC tumours showed a higher number of infiltrating $CD8^+$ T cells, $CD3^+$ T cells, $CD16^+$ natural killer cells, $CD163^+$ macrophages, and $FoxP3^+$ regulatory T cells compared with MCPyV-negative MCC tumours (Sihto et al., 2012; Harms et al., 2013; Lauttia et al., 2014; Wheat et al., 2014). [The Working Group noted that only the studies by Sihto et al. (2012) and Harms et al. (2013) compared MCPyV-positive and -negative tumours. In the study by Harms et al. (2013), MCPyV-positive tumours were associated with a significantly increased proportion of $CD8^+$ cells ($P = 0.01$), and a more moderately increased proportion of $CD3^+$ cells ($P = 0.10$). Sihto et al. (2012) showed that MCPyV-positive tumours had a higher count of $CD3^+$, $CD8^+$, $CD16^+$, $FoxP3^+$, and $CD68^+$ cells (all $P < 0.05$).]

(ii) Human cells in vitro

Infecting human primary dermal fibroblasts with MCPyV activated the innate immune response (Krump et al., 2021), inducing interferon-stimulated genes (ISGs) and inflammatory cytokines, and stimulating cGAS/STING/TBK1/IRF3 and nuclear factor kappa B (NF-κB) pathways. IFNγ-inducible protein 16 (IFI16), localized in MCPyV replication centres, did not significantly affect ISG induction, even when knocked out, whereas knocking out components of the cGAS/STING/TBK1/IRF3 and NF-κB pathways ablated ISG induction. [The Working Group noted that the innate immune response to MCPyV infection appeared to depend on the cGAS-STING-TBK1-IRF3 and NF-κB pathways rather than on IFI16.]

In human dermal fibroblast cells, derived from human foreskin tissues, Wang et al. (2023) showed that IFN-mediated induction of ISGs in response to MCPyV infection was not crucial to viral control. Type I IFN exerted a more direct effect on MCPyV infection post-entry, by repressing early viral transcription.

MCC cell lines and an immortalized human dermal fibroblast cell line were used to investigate the effects of MCPyV T antigens on IL-33 signalling (Rasheed et al., 2022). The authors showed that MCPyV T antigens induce IL-33 expression, activating the IL-33/ST2-IL1RAcP axis, which promotes pro-tumorigenic signalling in MCC.

[The Working Group noted that the specific cell of entry for MCPyV in initiating tumorigenesis has not yet been definitively established.]

The MCPyV-positive MKL2 cell line exhibited higher levels of expression of CCL17/TARC, a member of the CC-motif chemokine family, than did the MCPyV-negative MCC13 cell line (Rasheed et al., 2018).

Major histocompatibility complex class 1 (MHC-I) was found to be downregulated in five MCC cell lines (three MCPyV-negative and two MCPyV-positive) and, in patient samples, the expression of MHC-I was lower in MCPyV-positive MCCs than in MCPyV-negative MCCs. (Paulson et al., 2014). By inhibiting EZH2 (enhancer of zeste homologue 2), a histone methyltransferase responsible for repressive H3K27 trimethylation, H3K27me3 can restore human leukocyte antigen (HLA) class I expression in MCPyV-positive MCC cell lines. Reduction in H3K27me3 correlated with transcriptional activation and surface re-expression of HLA class I molecules (Durand et al., 2024).

EZH2 is expressed in most MCC tumours, with significantly higher levels in MCPyV-positive MCC tumours, resulting in increased H3K27me3 levels. Additionally, an LT mutant lacking the pRb-binding domain fails to induce EZH2 in MCPyV-positive MCC tumours (Durand et al., 2023).

A study using stable BJ-hTERT cell lines expressing different MCPyV T antigens (full-length LT, tumour-derived LT, tumour-derived LT plus sT, and tumour-derived LT lacking the pRb-interaction motif) showed that tumour-derived LT and tumour-derived LT plus sT increased cell proliferation (see Section 4.2.8) and upregulated inflammatory cytokine and chemokine genes, leading to elevated IL-8 secretion (Richards et al., 2015).

MCPyV LT expression in BJ-hTERT human fibroblasts caused a nucleolar stress response, followed by p21-dependent senescence and senescence-associated secretory phenotypes (SASPs), including modest upregulation of inflammatory cytokines IL-6 and IL-8. The LT-mediated senescence was independent of the DNA damage response and dependent on p21, and it shifted the gene expression profile towards a senescence-related gene profile. The senescence effect was reduced with senolytic navitoclax, a Bcl-2 anti-apoptosis family protein inhibitor (Pham et al., 2023).

Synopsis

[The Working Group noted that there was some evidence that MCPyV triggers a T-cell–mediated response in exposed healthy individuals and patients with MCC. A few studies in human primary cells indicated that MCPyV activates the innate immune response, including ISGs and inflammatory cytokine genes. In a few studies, it was reported that inflammatory cytokine genes were upregulated in MCC cell lines and immortalized human dermal fibroblasts.]

4.2.5 Is immunosuppressive

(a) Humans

(i) Exposed humans

MCC metastatic-site tumour samples were collected from patients before treatment with avelumab and were analysed by IHC and next-generation sequencing (D'Angelo et al., 2024). In MCPyV-positive tumours, high infiltration with $CD8^+$ T cells and immunosuppressive M2 macrophages was observed.

MCC tumours showed MHC-1 downregulation compared with surrounding tissues. Additionally, MHC-1 expression was significantly ($P < 0.01$) lower in MCPyV-positive than in MCPyV-negative tumours (Paulson et al., 2014).

MCC tumours are characterized by reduced expression of HLA class I, a frequent immune escape mechanism observed in MCC (Ritter et al., 2017). Reduced expression of HLA class I was associated with impaired expression of the antigen processing machinery, including LMP2, LMP7, TAP1, and TAP2, which can be epigenetically silenced by histone hypoacetylation.

(ii) Human cells in vitro

Using genome-wide transcriptome studies in human primary dermal fibroblasts, Ohnezeit et al. (2024) investigated the role of sT in MCPyV infections. They simulated infection and tumorigenesis conditions by ectopically expressing the early viral proteins (sT, LT, and truncated LT) individually or in combination at different time points. MCPyV sT suppressed IFN type I signalling by interfering with the IFN-stimulated gene factor 3 response (ISGF3). The authors showed that sT reduced the expression of IRF9 and STING1, two components of ISGF3. sT was also shown to downregulate genes involved in antigen processing and presentation of MHC-1, including HLA-A, HLA-B, HLA-E, TAP1, and B2M. In contrast, full-length and truncated LT induced the IFN type 1 pathway (Ohnezeit et al., 2024). [The Working Group noted that the authors speculated that sT counteracts the host antiviral response induced by LT or truncated LT, contributing to immune evasion of MCC tumour cells.]

MHC-1 downregulation was demonstrated in two out of two MCPyV-positive MCC cell lines (MKL-1 and WaGa) and one out of three of the MCPyV-negative cell lines (UISO, MCC13, and MCC26) (Paulson et al., 2014). IFNγ treatment restored MHC-I expression in MCPyV-positive cells, indicating reversibility (Paulson et al., 2014).

sT also was found to perturb the host antiviral immune response by inhibiting activation of the transcription factor NF-κB in 293 cells engineered to allow induced expression of MCPyV sT (Griffiths et al., 2013). The study showed that MCPyV sT binds to NEMO (NF-κB essential modulator) and disrupts NF-κB signalling. Interference with NF-κB signalling reduced pro-inflammatory cytokine production and weakened immune responses (Griffiths et al., 2013). [The Working Group noted that targeting of the NEMO protein impairs activation of the innate immune response, allowing for immune evasion and viral persistence.] Additionally, MCPyV sT was found to downregulate key genes involved in innate immunity, such as CCL20, CXCL9, IL-8, and TANK, a regulator of NF-κB signalling (Griffiths et al., 2013).

MCPyV LT and sT antigens reduced TLR9 mRNA and protein levels in cells expressing MCPyV early genes, preventing TLR9 from recognizing viral DNA and impairing the ability of the host to trigger an antiviral immune response in spontaneously immortalized human keratinocytes (NIKS) and RPMI 8226 human B lymphocytes. LT antigen expression inhibited TLR9 expression by downregulating the mRNA and protein levels of the transcription factor C/EBPβ (Shahzad et al., 2013) in epithelial and MCC-derived cells. sT also contributed to TLR9 downregulation, but it was not clear whether sT acted directly on the pathway or indirectly by stabilizing LT (Shahzad et al., 2013).

Ritter et al. (2017) reported that MCC tumours and MCC cell lines are characterized by reduced expression of HLA class I. In MCC cell lines, pharmacological inhibition of histone deacetylase by treatment with a combination of vorinostat plus mithramycin resulted in induction of global histone acetylation in MCC cell lines. Additionally, the expression of HLA class I was restored by vorinostat plus mithramycin.

The authors also reported that MHC class I chain–related proteins MICA and MICB are not expressed in MCPyV-positive MCC cell lines. Their promoters were shown to be silenced by histone hypoacetylation in MCPyV-positive WaGa cells. Expression of MICB and to a lower extent MICA was induced by histone deacetylase inhibitors. In this study, vorinostat alone or in combination with mithramycin A increased global acetylation and MICA and MICB promoter–specific histone H3 lysine 9 (H3K9) acetylation in MCC cell lines (Ritter et al., 2016).

[The Working Group noted that the above-mentioned evidence was derived from studies of MCC tumours and thus may not clearly reflect the tumorigenic effects of MCPyV.]

(b) Experimental systems

(i) Non-human mammalian systems in vivo

Dowlatshahi et al. (2013) analysed tumour-infiltrating lymphocytes (TILs) from primary and metastatic MCC tumours, using flow cytometry and functional assays to characterize the local immune-system microenvironment. Tumours contained both $CD4^+$ and $CD8^+$ T cells, including effector, memory, and regulatory subsets. These TILs exhibited markedly reduced activation, with low expression of CD69 and CD25 and frequent expression of PD-1, whereas the tumour microenvironment was enriched for $FOXP3^+$ regulatory T cells and PD-L1/PD-L2–expressing antigen-presenting cells, consistent with local immune suppression. In vitro stimulation with IL-2 and IL-15 restored T-cell activation, cytokine production, and cytotoxicity against MCC tumour cells, indicating the presence of tumour-reactive but functionally suppressed T cells. Consistently, MCC tumour fragments failed to grow in immunodeficient mice unless human T cells were depleted, demonstrating that endogenous T cells could restrain tumour growth when immune suppression was relieved. These findings established immune evasion and reversible T-cell dysfunction as key features of MCC, being observed in both virus-positive and virus-negative tumours (Dowlatshahi et al., 2013).

Ritter et al. (2017), as mentioned above, showed that MCC tumours and MCC cell lines are characterized by reduced expression of HLA class I, but also impaired antigen processing machinery that is silenced by hypoacetylation. In a mouse xenotransplantation model using MCPyV-positive WaGa cells in NOD.CB17/Prkdcscid mice, HDAC inhibition induced histone acetylation and stabilization of HLA class I. Treatment with vorinostat in combination with mithramycin increased the transcription of TAP2, LMP2, and LMP7 in xenotransplanted MCC tumours (Ritter et al., 2017). However,

MCPyV LT protein expression was not altered in MCC xenotransplants upon treatment with vorinostat plus mithramycin. [The Working Group noted that the MCC tumours in this study were not stratified, and results were not presented by MCPyV status.]

Synopsis

[The Working Group noted that low expression of MHC class I has been associated with MCPyV-positive tumours and cell lines. These findings suggest that MCPyV may promote immunosuppression. MCPyV sT can inhibit the IFN pathway through multiple factors, including downregulation of TLR9; disruption of NF-κB signalling; downregulation of genes involved in antigen processing and presentation, such as MHC I; and shaping a suppressive tumour microenvironment. However, the data accumulated to date were suggestive in experimental systems. There were few studies in human primary cells and in exposed humans.]

4.2.6 Modulates receptor-mediated effects

(a) Humans

(i) Exposed humans

[The Working Group noted that although several studies have reported that Wnt signalling is infrequently activated in MCC tumours, on the basis of limited nuclear β-catenin, which is a hallmark of canonical Wnt pathway activation (Liu et al., 2007; Panelos et al., 2009; Lill et al., 2013), more recent findings suggested that Wnt activation may play a role.]

Yang et al. (2025) identified significant enrichment of the Wnt signalling pathway among genes that were differentially expressed in MCC tumours and normal skin. Importantly, genes associated with the canonical Wnt pathway were upregulated in MCC tumours, whereas those linked to the non-canonical Wnt pathway were downregulated (Yang et al., 2025). [The Working Group noted that gene expression profiles of MCC tumours and normal skin samples were compared; it remained unclear whether activation of the Wnt signalling pathway was specifically associated with MCPyV-positive or MCPyV-negative status. Furthermore, the analysis was based on a single previously published gene-expression data set, limiting the strength of the conclusions.]

(ii) Human cells in vitro

Consistent with the observations in MCC tumours, Yang et al. (2025) showed that Wnt signalling is significantly enriched in fetal lung fibroblast (IMR90) and neonatal human dermal fibroblast cells (nHDFs) expressing MCPyV truncated LT and sT antigens. The viral T antigens upregulated several key regulators of canonical Wnt signalling, such as WNT3 and TCF3, whereas non-canonical Wnt ligands such as WNT5A/B were downregulated. Canonical Wnt signalling typically requires nuclear β-catenin to activate downstream target genes, whereas non-canonical Wnt signalling functions independently of β-catenin. Although nuclear β-catenin accumulation is rare in MCC, alternative mechanisms for canonical Wnt pathway activation, such as β-catenin–independent activation of T-cell factor (TCF) reported by Grumolato et al. (2013), cannot be ruled out in MCC.

Importantly, in MCPyV-positive MCC WaGa cells, silencing TCF expression or restoring WNT5B levels suppressed MCC marker expression and inhibited cell growth, indicating that Wnt signalling plays a crucial role in maintaining neuroendocrine differentiation and proliferation in MCC (Yang et al., 2025). [The Working Group noted that the functional assays and in vivo experiments were conducted exclusively in MCPyV-positive MCC cell lines, without inclusion of MCPyV-negative cell lines, thereby limiting the strength of the conclusions regarding the specificity of the observed effects to MCPyV status.]

Synopsis

[The Working Group noted that there were few studies in exposed humans and no data in human primary cells. However, there was some evidence that MCPyV may modulate the Wnt signalling pathway, contributing to the promotion of neuroendocrine differentiation and cell growth in MCC. Although Wnt signalling has been observed in MCC tumours, it remains unclear whether this activation is specifically associated with MCPyV-positive cases. Functional studies in human cells in vitro indicated that disruption of the Wnt pathway impairs MCC differentiation and survival; however, these effects were evaluated exclusively in MCPyV-positive MCC cell lines, limiting the ability to attribute them specifically to MCPyV status.]

4.2.7 Causes immortalization

(a) Humans

(i) Exposed humans

[The Working Group noted that no studies were available in exposed humans infected with MCPyV that showed cell immortalization.]

(ii) Human cells in vitro

The effects of MCPyV on cellular transformation have been primarily investigated in primary or immortalized rodent fibroblast cells. However, Shuda et al. (2011) demonstrated that expression of MCPyV sT sustained cell proliferation under serum-free conditions in immortalized human BJ-TERT fibroblast cells. However, sT alone was not sufficient to fully transform these cells, as demonstrated by the lack of colony formation in soft agar assays. Similarly, Cheng et al. (2017) showed that co-expression of MCPyV truncated LT and sT in immortalized human fibroblast cells (IMR90) induced anchorage-independent growth, as demonstrated by colony formation in soft agar. [The Working Group noted that evidence supporting the immortalization of normal human cells by MCPyV remains lacking. No data were currently available in human primary cells. Although Cheng et al. (2017) observed anchorage-independent growth in immortalized human fibroblasts expressing truncated MCPyV LT and sT, only a small number of colonies were formed. Furthermore, these effects were demonstrated under experimental conditions in which the cells also expressed dominant-negative p53 and hTERT, complicating the interpretation of MCPyV-specific transforming potential.]

(b) Experimental systems

Studies have demonstrated that MCPyV sT expression in rodent and human fibroblasts promotes anchorage-independent growth and serum-free proliferation (Kwun et al., 2013; Thevenin et al., 2024), in combination with truncated LT (Borchert et al., 2014); or evaluating p53DD/hTERT/MYC and LT in addition to sT (Berrios et al., 2016); or in low-serum conditions (Zhao et al., 2020). This transforming potential was at least partly attributed to its ability to induce 4E-BP1 hyperphosphorylation (Shuda et al., 2011), recruit MYCL to the EP400 complex (Cheng et al., 2017), and enhance aerobic glycolysis (Berrios et al., 2016).

Notably, MCPyV sT drives transformation through a mechanism that is independent of protein phosphatase 2A (PP2A) or heat-shock protein binding (Shuda et al., 2011). Shuda et al. (2011) demonstrated that an sT mutant defective in PP2A or heat-shock protein binding retained its transforming ability in Rat-1 cells, as assessed by focus formation and assays for colony growth on soft agar. Additionally, Kwun et al. (2013) showed that a mutation in the LSD domain abolished transformation of Rat-1 cells. The nuclear localization of sT is critical for anchorage-independent growth, increased glucose uptake, and reduced serum dependency in Rat-2 cells, underscoring its role in cellular transformation (Thevenin et al., 2024).

The role of LT in MCPyV-driven cellular transformation remains a subject of debate. Although truncated LT alone does not appear to be sufficient to drive full transformation, it has been shown to increase cellular proliferation and reduce doubling time under low-serum (1%) conditions in immortalized human BJ-hTERT fibroblasts and Rat-2 rodent fibroblasts (Cheng et al., 2013; Thevenin et al., 2024).

In contrast, Li et al. (2013) reported that truncated LT was capable of inducing anchorage-independent growth in NIH 3T3 mouse embryonic fibroblasts, whereas full-length LT could only promote this phenotype when co-expressed with dominant-negative p53.

Borchert et al. (2014) found that whereas truncated LT, in combination with sT, efficiently transformed baby rat primary kidney (pBRK) cells, full-length LT exhibited only weak transforming activity when co-expressed with sT. This suggested that MCC pathogenesis selects for LT truncation, eliminating the tumour-suppressive effects of its C-terminal domain while retaining the Rb-binding LxCxE motif for promoting oncogenesis.

[The Working Group noted that these findings suggested that although truncated LT may contribute to a pro-proliferative environment, additional genetic alterations or cooperating factors are likely to be required for complete cellular transformation.]

In vivo studies further supported MCPyV sT as the primary driver of transformation. Verhaegen et al. (2015) demonstrated that sT expression in transgenic mice induced epithelial transformation and dysplasia in an LSD domain–dependent, but PP2A-independent, manner. However, these models did not develop MCC-like tumours, suggesting that additional factors, such as truncated LT and other genetic alterations, may be required for full tumorigenesis. Indeed, Verhaegen et al. (2017, 2022) found that co-expression of MCPyV truncated LT and sT, together with ATOH1, initiated tumorigenesis in epidermal cells near the hair follicle stem cell niche. However, the development of MCC-like tumours required p53 loss, resembling the p53 inactivation observed in human MCPyV-positive MCCs (Verhaegen et al., 2022).

Synopsis

[The Working Group noted that there was evidence supporting the role of MCPyV in driving cellular transformation, particularly through the activity of MCPyV sT, which has been demonstrated to have clear transforming properties in both in vitro and in vivo models. However, direct evidence that the truncated LT alone is sufficient to induce immortalization remained limited.]

4.2.8 Alters cell proliferation, cell death, or nutrient supply

(a) Human cells and tissues

Stakaitytė et al. (2018b) used doxycycline-inducible i293 cells to induce expression of enhanced GFP (i293-EGFP) or EGFP-sT (i293-EGFP-sT). MCC13 cells (MCPyV-negative MCC cells) transiently transfected with EGFP or EGFP-sT were also used. Expression of sT induced cell motility in both cell lines, which was reduced by treatment with Cl^- channel modulators. Proteomic analysis identified upregulation of CLIC1 and CLIC4 upon induction of sT expression in i293 cells. When sT-expressing i293 or MCC13 cells were transfected with small interfering RNA (siRNA) specific for CLIC1 and/or CLIC4, sT-induced migration was reduced in both cell lines. In addition, cellular lysates from MCPyV-positive tumours showed a significant increase in CLIC1 and CLIC4 protein levels, when compared with MCPyV-negative non-tumour skin samples. Furthermore, gene expression profiles of tumours from 94 patients with MCPyV-positive or MCPyV-negative MCC identified a significant increase in CLIC1 ($P = 0.02$) and CLIC4 ($P = 0.002$) expression in MCPyV-positive

MCC tumours compared with MCPyV-negative MCC tumours. Lastly, CLIC1 expression was also significantly increased ($P = 0.04$) in metastatic MCPyV-positive MCC tumours compared with MCPyV-negative MCC tumours with a similar metastatic status. Together, these findings, implicated CLIC1 and CLIC4 function as being critical to MCPyV-induced metastatic processes (Stakaitytė et al., 2018b).

When expressed in fibroblasts (IMR90), sT promoted significant changes in gene expression, including induction of pro-glycolytic genes and elevation of aerobic glycolysis (Berrios et al., 2016).

Expression of MCPyV sT resulted in increased expression of the cellular microtubule-associated protein stathmin. Additionally, expression of EGFP-sT in MCC13 cells (a MCPyV-negative MCC cell line) changed the phosphorylation status of stathmin by its cellular phosphatase catalytic subunit PP4C when compared with cells transfected with EGFP only (Knight et al., 2015). The authors concluded that MCPyV sT promotes destabilization of the host cell microtubule network, leading to a motile and migratory cell phenotype (Knight et al., 2015).

MCPyV sT expression has also been shown to induce cell motility by remodelling the actin cytoskeleton in i293 and MCC13 cells expressing sT. Additionally, transient expression of sT in HEK293 cells promoted filopodia formation (Stakaitytė et al., 2018a).

The MCPyV-positive cell line MKL-1 showed hypersensitivity to UV radiation, impaired repair of DNA lesions, and impaired cell cycle arrest after UV radiation, compared with UISO cells (MCPyV-negative MCC cells) (Demetriou et al., 2012). Additionally, expression of mutant T antigen (truncated T antigen isolated from an MCC tumour, encoding both sT and truncated LT), but not wildtype T antigen (encoding sT, 57kT, and full-length LT) resulted in reduced DNA damage repair upon UV radiation and decreased expression of XPC. Both the expression of wildtype and the expression of mutated T antigen inhibited cell cycle arrest after UV radiation and resulted in decreased levels of p53 and p21. To further clarify the roles of LT and sT, wildtype LT and sT were separately expressed in non-infected UISO cells using expression vectors encoding the complementary DNA (cDNA) for each. In the cells expressing LT or sT, no impairment in the ability to repair cyclobutane pyrimidine dimers was observed. Cells expressing LT showed an increase in S-phase population after UV radiation, suggesting a G1 checkpoint defect, and cells expressing sT alone had a higher proportion of cells in S-phase relative to controls, but did not exhibit failure of cell cycle arrest after UV radiation.

In cultures of human foreskin BJ dermal fibroblasts cell lines and in MCPyV-positive MCC cancer cell lines MKL-1, MKL-2, MS-1, and WaGa, and MCPyV-negative MCC cancer cell lines MCC13, MCC26, and UISO, sT activates non-canonical NF-κB signalling by stabilizing NFκB2, leading to increased cell survival and proliferation (Zhao et al., 2020).

Shuda et al. (2014) have shown that sT contributes (with LT) to MCC carcinogenesis, stimulates progenitor Merkel cell proliferation in embryonic mice, and induces cancer cell transformation in a p53-null setting (Shuda et al., 2015).

In contrast to the study by Shuda et al. (2014), a study by Angermeyer et al. (2013) suggested that sT is not essential in MCPyV-induced MCC carcinogenesis and that LT is more critical in sustaining the growth and survival of MCPyV-positive MCC cells.

In immortalized human fibroblasts (BJ-TERT), the LXCXE motif of tumour-derived LT enhanced cellular proliferation and upregulated the transcription of cell cycle-related genes (Richards et al., 2015). Truncated LT antigen had growth-promoting activities that are more effective than those of full-length or 57kT versions of LT antigen in mouse and

human fibroblasts (BJ-TERT and MEF) (Cheng et al., 2013). Wildtype MCPyV LT is likely to mediate cellular senescence in immortalized human fibroblasts (BJ-TERT) by activating a nucleolar stress response, which results in the activation of the p53/p21 pathway and consequently cellular senescence (Pham et al., 2023). This senescence-inducing and proliferation-inhibiting function of wildtype LT is likely to be mediated by the LT C-terminus or the MUR domain (Cheng et al., 2013; Li et al., 2013; Nwogu et al., 2020; Pham et al., 2023).

[The Working Group noted that a single study was performed in human primary cells and primary tissues (however, from MCC tumours), and that the described effects came mostly from studies in human cell lines.]

In a xenograft mouse model, two MCC cell lines (MKL-1 and WaGa, both MCPyV-positive) were used to study vasculogenic mimicry (Lezcano et al., 2014). Experimentally induced xenograft tumours were histologically similar to seven tumours from six patients with MCC; of the seven, two were cutaneous primary lesions, two were recurrent cutaneous lesions, and three were lymph node metastases. Both xenograft tumours and patient tumours contained laminin networks associated with vascular endothelial-cadherin (CD144) and vascular endothelial growth factor receptor 1 (VEGFR-1). [The Working Group noted that only MCPyV-positive cell lines were used, and there was no comparison with MCPyV-negative cell lines.]

(b) Experimental systems

Non-human mammalian systems in vivo

In a mouse model, conditional co-expression of MCPyV T antigens (sT and LT) and ATOH1 in epidermal cells induced MCC-like lesions originating from hair follicles. These lesions were shown to accumulate p53. Additional deletion of *Trp53* permitted progression into gross tumours that expanded into the dermis without apparent epidermal involvement, mimicking human MCPyV-positive MCC tumours in terms of histology, marker expression, and transcriptomic profile. (Verhaegen et al., 2022).

In the model described by Spurgeon et al. (2015) (see Section 3), *K14Cre-MCPyV168* transgenic mice were used to investigate the oncogenic mechanisms of MCPyV in MCC. *K14Cre*-driven expression of sT and truncated LT antigens led to epithelial hyperplasia, increased proliferation, and spontaneous tumorigenesis in the skin. Of the *K14Cre-MCPyV168* mice that survived weaning (35/50), nearly half (16/35, 45.7%) developed spontaneous cutaneous papillomas, with a significant increase [$P = 0.0001$, Fisher exact test; see Section 3] in incidence when compared with that in the *Cre*-negative *ROSA26-LSL-MCPyV168* littermates. To evaluate the effect of MCPyV T-antigen expression on cell proliferation in vivo, the authors measured DNA replication using bromodeoxyuridine (BrdU) incorporation over a 1-hour period before the mice were euthanized. IHC was used to identify BrdU-positive cells in the epithelium, and the proportion of BrdU-positive cells was quantified. Remarkably, the *K14Cre-MCPyV168* mice showed an increase of fivefold in the total percentage of BrdU-positive cells in the ear epithelium, compared with controls (15.5%; $P = 0.036$). Additionally, MCPyV T-antigen expression altered the spatial distribution of proliferating cells from a strictly basal staining pattern to one in which BrdU-positive cells were observed throughout the parabasal and suprabasal layers of the stratified epithelium.

These results indicated that expression of MCPyV T antigens in the stratified epithelium enhanced cellular proliferation in a manner comparable to that observed with human papillomavirus HPV16 E6 and E7 proteins.

The same authors also used the *K14Cre-MCPyV168* mouse model (see Section 3) to investigate how sT and LT expression contribute to different phases of carcinogenesis (Spurgeon et al., 2021). The skin of *K14Cre-MCPyV168* mice

was treated with subcarcinogenic doses of the tumour initiator DMBA, or the tumour promoter TPA, or a combination of both. MCPyV LT and sT antigens synergized with the tumour initiator DMBA, but not the tumour promotor TPA, indicating a function in tumour promotion, but not initiation (Spurgeon et al., 2021).

Spurgeon et al. (2022) also studied the interaction between LT and the retinoblastoma tumour suppressor protein (Rb) in the same mouse model Spurgeon et al. (2022) (see Section 3). The authors employed the previously established *K14Cre-MCPyV168* mouse model and introduced an additional knock-in of an Rb mutant with severely impaired LT binding. They found that the previously observed phenotypes – hyperplasia, increased proliferation, and tumour development in the skin of sT- and LT-expressing transgenic mice – were dependent on an Rb that was capable of interacting with LT. This follow-up study therefore provided direct evidence for the critical role of the LT–pRb interaction in MCPyV-induced tumorigenesis.

LT-mediated pRb inactivation was critical in promoting tumour cell growth and proliferation in MCPyV-positive MCC (Hesbacher et al., 2016). Knockdown of LT induced growth arrest in MCPyV-positive MCC cells, which could be rescued by simultaneous knockdown of RB1.

Verhaegen et al. (2015) showed that sT expression alone was sufficient to induce hyperplasia and spontaneous tumours (see Section 3) in basal keratinocytes, using the Cre-inducible sT antigen transgenic mouse model, designated *KLEsT,* expressing MCPyV sT under the control of the bovine K5 promoter, targeting expression to basal keratinocytes in the skin. By crossing *KLEsT* mice with *K5-CreERT2* mice carrying tamoxifen-inducible Cre, the authors generated *K5-CreERT2;KLEsT* (*iK5;KLEsT*) bi-transgenic mice. Treatment of these mice at postnatal day 21 with tamoxifen to activate sT expression led to significant epithelial hyperplasia and increased cellular proliferation.

Synopsis

[The Working Group noted some evidence that in MCPyV-positive tumours there is induction of the expression of cell proliferation-related genes; however, the specific cell of entry for the virus has not been yet clarified in humans. In human primary cells there was a paucity of data. There was some evidence in human cells in vitro regarding the role of sT. Most of the evidence came from experimental systems in vivo. Epithelial hyperplasia and increased proliferation of the skin were observed in mice expressing MCPyV T antigens. In multiple human and rodent cell lines, sT activated NF-κB signalling, leading to increased cell survival, proliferation, and glucose uptake. Truncated LT and sT antigens contributed to growth-promoting activities in vitro and in vivo. The effect of MCPyV on alterations in nutrition supply has not been adequately addressed.]

4.2.9 Multiple key characteristics

[The Working Group noted that MCPyV can play a role in shaping the molecular landscape of MCC, with virus-positive and virus-negative tumours displaying distinct transcriptomic profiles and pathway alterations.] Transcriptomic analyses by Harms et al. (2013) revealed that MCPyV-positive tumours were enriched for immune response–related genes such as *CD3G*, *CD3D*, *ZAP70*, and *IGHM*, indicating a higher infiltration of TILs. This finding was corroborated by IHC, which demonstrated increased $CD8^+$ T-cell infiltration in MCPyV-positive tumours. Similar observations have been reported in multiple studies (Iyer et al., 2011; Paulson et al., 2011; Sihto et al., 2012).

In contrast, MCPyV-negative MCCs exhibited a more aggressive phenotype, driven by the upregulation of genes involved in notch (e.g. *DLL1*, *CTBP2*, *HES1*, *JAG1*, *JAG2*), receptor tyrosine kinase (e.g. *FGF14*, *FGF11*, *FGFR2*, *VEGFA*, *PDGFA*), and Wnt (e.g. *WNT4*, *WNT5A*)

signalling pathways, suggesting that they are activated specifically in virus-negative tumours (Harms et al., 2013). In a study that supported these molecular distinctions, Karpinski et al. (2023) identified 29 genes that were differentially expressed in virus-positive and virus-negative MCCs using the NanoString PanCancer panel, which includes 760 genes. Many of these genes – such as *TWIST1*, *CD44*, *COL6A3*, *COL11A1*, *CXCL8*, *INHBA*, *MMP1*, *NID2*, *SPP1*, *THBS1*, and *THY1* – are associated with epithelial–mesenchymal transition (EMT), a key process in cancer metastasis. Others are implicated in Wnt/β-catenin signalling (*CDH1*, *FZD9*, *WNT7B*, *WNT4*) and the immune response (*CXCL8*, *TNFAIP6*, *TREM2*, *FCGR2A*, *FCGR3A/B*). Using publicly available RNA-sequencing data, they confirmed EMT and Wnt pathway activation in MCPyV-negative tumours. Additionally, these tumours showed elevated expression of genes involved in cell cycle regulation, as well as components of the notch, TGFβ, and hedgehog signalling pathways, reinforcing the observations made by Harms et al. (2013) (Harms et al., 2015; Karpinski et al., 2023).

Richards et al. (2015) demonstrated that truncated LT and sT regulate overlapping but distinct gene sets in BJ-hTERT fibroblasts. Tumour-specific LT inactivated the RB tumour suppressor, promoting S-phase entry and upregulating E2F targets such as *CCNE*, *CDC25A*, *TYMS*, *RNASEH2A*, *CDT1*, and MCM complex components, which are key regulators of DNA replication and cell cycle progression. Notably, *CDKN2C*, a CDK4 inhibitor that enforces G1 arrest, was downregulated in BJ-hTERT fibroblasts expressing tumour-specific LT, further underscoring the role of tumour-specific LT in bypassing critical cell cycle checkpoints and facilitating uncontrolled proliferation. Beyond its impact on cell cycle control, tumour-specific LT also induced a broad set of chemokine, cytokine and interferon-stimulated genes, including *IL8*, *CXCL1*, *IL6*, *IL1β*, *CXCL6*, *CCL7*, *OAS3*, *OAS1*, and *ISG20*, implicating tumour-specific LT in the activation of immune signalling. Co-expression of sT further amplified the expression of these immune-associated genes, suggesting a synergistic role for MCPyV oncoproteins in modulating inflammatory and antiviral pathways. These findings supported the notion that MCPyV not only promotes cell proliferation through direct interference with tumour suppressor pathways but also reshapes the tumour microenvironment by activating immune-system signalling networks.

Yang et al. (2025) expanded on these findings by showing that expression of the MCPyV early region (sT and truncated LT) in IMR90 cells induced upregulation of canonical Wnt genes (*WNT3*, *TCF3*, *TCF7*) and downregulation of non-canonical Wnt genes (*WNT5A*, *WNT5B*, *WNT16*). Notably, non-canonical Wnt gene expression negatively correlated with neuroendocrine differentiation, suggesting that suppression of non-canonical Wnt signalling may be required for neuroendocrine features in MCC.

Macamo et al. (2024) showed that ectopic expression of MCPyV sT and LT in B-cell acute lymphocytic leukaemia REH and MCPyV-negative MCC13 cell lines modulated gene expression through DNA methylation. The sT antigen upregulated genes involved in ribosomal structure, DNA replication, extracellular matrix organization, and mitochondrial function while downregulating genes related to immune recognition, including those encoding MHC complex proteins, transmembrane receptors, and cell–cell adhesion molecules. Meanwhile, the LT antigen promoted the expression of genes associated with mRNA 3′-end processing, polyadenylation, receptor–ligand interactions, and post-synaptic membrane activity, while also suppressing genes involved in MHC complex function. [The Working Group noted that these findings highlighted the distinct yet complementary roles of sT and LT in reshaping the host epigenetic landscape to promote immune evasion.]

Park et al. (2019) showed that MCPyV oncoproteins can interact with key tumour suppressor pathways, notably the p53 axis. The authors demonstrated that ectopic expression of truncated LT in IMR90 lung fibroblast cells activated the p53 response by upregulating *ARF* and multiple p53 target genes such as *GDF15* and *CDKN1A* (*p21*). This activation was, in part, a downstream consequence of LT-mediated RB inactivation, which releases E2F transcription factors and leads to increased expression of ARF, a potent inhibitor of the E3 ubiquitin ligase MDM2 (Bates et al., 1998). By suppressing MDM2 activity, ARF stabilizes and activates p53, thereby promoting cell cycle arrest or apoptosis (Asker et al., 1999). Thus, LT binding to RB indirectly contributes to p53 activation via the E2F–ARF–MDM2 regulatory axis. However, co-expression of LT with sT dampened this p53 response. Mechanistically, sT assembles a transcriptional complex with MYCL and EP400 that directly upregulates MDM2 and CK1α (CSNK1A1); CK1α is a known activator of MDM4, a homologue of MDM2 that also suppresses p53 function (Shvarts et al., 1996). These coordinated actions enhanced the degradation of p53. Notably, MCPyV-positive MCC cell lines exhibit elevated levels of MDM4, and combined pharmacological inhibition of MDM2 and MDM4 synergistically reactivates p53, triggering robust apoptotic responses in both a virus-positive MCC cell line (MKL-1) and MKL-1 MCC xenograft models (Park et al., 2019). These findings highlighted the dual role of MCPyV oncoproteins in modulating the p53 pathway and underscored the therapeutic potential of targeting MDM2 and MDM4 in MCPyV-driven MCC.

In a study that further emphasized the oncogenic potential of sT, Schlemeyer et al. (2022) used RNA sequencing to show that silencing sT in WaGa cells altered the expression of genes involved in proliferation, migration, and extracellular matrix organization. Screening 371 surface proteins by flow cytometry and integrating transcriptomic data from WaGa cells with and without sT silencing, Schlemeyer et al. (2022) identified several surface markers that were differentially regulated by sT. Of these, CD47, a well-characterized "don't eat me" signal, was upregulated by sT, enabling cancer cells to inhibit macrophage-mediated phagocytosis, thereby contributing to evasion of immune destruction (Schlemeyer et al., 2022).

Synopsis

[The Working Group noted that truncated LT from MCPyV inactivated the RB tumour suppressor, thereby promoting S-phase entry and upregulating E2F target genes such as *CCNE* and *CDC25A*. Both truncated LT and sT further modulated the expression of genes involved in DNA replication, collectively enhancing the cell's proliferative capacity relevant to the key characteristic "alters cell proliferation, cell death, or nutrient supply" (KC10). In addition to driving proliferation, MCPyV oncoproteins influence receptor-mediated signalling pathways. For example, they modulate the expression of genes related to receptor–ligand interactions and regulate the Wnt signalling pathway, features that align with the key characteristic "modulates receptor-mediated effects" (KC8). The virus also exerts immunosuppressive effects. sT upregulates immunoinhibitory molecules such as CD47, a "don't eat me" signal that inhibits macrophage-mediated phagocytosis and downregulates components of the MHC class I pathway. Both LT and sT also suppress genes involved in immune surveillance. These immune evasion strategies are consistent with the key characteristic "is immunosuppressive" (KC7). Moreover, MCPyV oncoproteins activate pro-inflammatory and antiviral signalling pathways, consistent with the key characteristic "induces chronic inflammation" (KC9). LT induces the expression of cytokines and chemokines such as *IL6*, *IL8*, *CXCL1*, and *CCL7*, with these responses further amplified by co-expression

of sT. Although some of these responses may represent antiviral defence mechanisms, chronic activation may contribute to a tumour-promoting microenvironment. Another property of MCPyV-mediated oncogenesis is its capacity for epigenetic regulation. Both LT and sT have been shown to modulate gene networks through alterations in DNA methylation and chromatin remodelling ("induces epigenetic alterations", KC4), resulting in the repression of immune recognition genes and the activation of genes related to replication and cellular structure.]

4.3 Other relevant evidence

[The Working Group noted that microtubule destabilization is increasingly recognized as a hallmark of carcinogenesis because it causes disruption of key cellular functions such as mitotic fidelity, intracellular transport, and structural integrity. Microtubule destabilization promotes genomic instability, enhances cell motility and invasion, and accelerates tumour progression, making it a crucial mechanism exploited by many cancers to drive metastasis (Jordan and Wilson, 2004; Borys et al., 2020).]

The study by Knight et al. (2015) provided evidence that MCPyV sT antigen induces microtubule destabilization, thereby increasing cell motility, migration, and invasion. This effect was observed in HEK293 and MCPyV-negative MCC13 cells ectopically expressing sT. The underlying mechanism involved the interaction of sT with the cellular phosphatase PP4C, which dephosphorylates stathmin, a critical regulator of microtubule dynamics. In its unphosphorylated form, stathmin sequesters soluble tubulin, leading to microtubule depolymerization (Manna et al., 2009). Importantly, treatment with the microtubule-stabilizing drug paclitaxel reversed sT-induced microtubule destabilization and suppressed cell migration in MCC13 cells expressing sT, highlighting the importance of microtubule integrity in limiting tumour spread. In the MCPyV-positive MKL-1 cell line, silencing sT restored microtubule stability, as indicated by increased phosphorylated stathmin and acetylated tubulin levels. Additionally, stathmin depletion suppressed MCPyV sT-mediated cell motility, confirming its pivotal role in this process (Knight et al., 2015).

MCPyV sT expression has been demonstrated to induce aberrant mitosis and aneuploidy in human and mouse fibroblasts and transgenic mice (Kwun et al., 2017).

[The Working Group noted that, collectively, these findings indicated that MCPyV sT-driven microtubule destabilization may play a role in MCC progression, promoting a more aggressive tumour phenotype. In addition, the ability of paclitaxel to counteract these effects further confirmed the role of microtubule dynamics in MCPyV-positive MCC. The Working Group also noted that the described oncogenic strategy is not unique to MCPyV. Other polyomavirus T antigens, including mouse polyomavirus middle T and SV40 large T, also induce microtubule destabilization, leading to chromosomal instability and enhanced invasiveness (Pallas et al., 1990; Chang et al., 1997; Tei et al., 2009; Rathje et al., 2014).]

5. Summary of Data Reported

5.1 Exposure characterization

Merkel cell polyomavirus (MCPyV) was discovered in 2008 to be integrated in the genome of some Merkel cell carcinomas (MCCs) and is now classified as *Alphapolyomavirus quintihominis* within the polyomavirus family. It has a circular, double-stranded DNA genome. Molecular and seroepidemiological studies indicate that MCPyV is ubiquitous in human populations and is typically acquired in early childhood by close contact between humans. There is not much evidence on variations in prevalence across

specific populations. MCPyV persists in the skin as a major component of the normal skin virome without causing symptoms. Detection methods include polymerase chain reaction (PCR) for viral DNA, immunohistochemistry for large T antigen (LT) protein in tissues, and enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies (immunoglobulin IgG) to the major capsid protein VP1 (exposure marker) or the LT (MCC-associated marker) in serum. There is currently no vaccine or antiviral treatment for MCPyV infection.

5.2 Cancer in humans

As described in *IARC Monographs* Volume 104, research on the potential carcinogenic role of MCPyV initially focused on MCC; multiple case–control studies and case series from various countries reported associations and showed integration of the viral DNA, truncating mutations, and LT antigen expression in tumours. The evidence for MCC in humans was classified as *limited* in Volume 104. A notable gap in the evidence in the previous review was the lack of prospective studies. However, since that time, a prospective, nested case–control study has been conducted. Additionally, other types of skin cancer have also been investigated, including squamous and basal cell carcinomas of the skin, together with other types of cancer, e.g. bladder cancer, non-Hodgkin lymphoma (NHL), and lung cancer. Additional case series, including within-person comparisons, have been reported since the previous review by the *IARC Monographs* programme.

The studies considered to be most informative in assessing the association between MCPyV infection and MCC used a range of robust, primarily quantitative, exposure assessment methods. Techniques were of high quality and included digital transcriptome subtraction, PCR, Southern hybridization, immunohistochemistry, MCPyV neutralization assays detecting anti-VP IgG antibodies, and antibody-binding assays detecting serum IgG antibodies to VP capsid and LT and sT antigens.

The one prospective study on MCC was a nested case–control study with participants from two large biobanks in Sweden and Norway. In Norway, participants were those in a population-based health survey as well as blood donors. In Sweden, a large biobank was linked to the national cancer registry. All case participants were required to have a serum sample collected ≥ 1 month before diagnosis. Control participants were those who were alive and free of skin cancer at the time of the case diagnosis, matched on age, sex, county (for Norway), subcohort, number of serial samples, length of follow-up, and date of the first sample, in a ratio of 4:1. Two serological assays were performed: the pseudovirion-binding assay and the neutralizing antibody test. Elevated odds ratios for MCC were observed using both assays, with an odds ratio (95% confidence interval, CI) for MCPyV neutralization positivity of 5.3 (95% CI, 1.3–32.3) overall compared with seronegativity; 2.6 (95% CI, 0.66–15.0) for any level of MCPyV VP antibodies; and 4.4 (95% CI, 1.3–17.4) for high MCPyV antibody levels overall compared with seronegativity. Given the rarity of MCC tumours, even in this large cohort, the number of MCC cases was relatively small. However, the prospective design provided evidence of temporality (i.e., of infection before cancer occurrence). These results, combined with results from case–control studies, and the detection of integrated MCPyV DNA, truncating mutations, and LT antigen expression in MCC tumours, supported a credible positive association between MCPyV and MCC in humans, and meant that bias, chance, and confounding could be ruled out with reasonable confidence.

Two prospective studies (one nested case–control study and one cohort study), two case–control studies, and one within-person comparison of squamous cell carcinoma of the skin were considered to be informative, as were

one nested case–control study and one cohort study that assessed MCPyV and basal cell carcinoma. These studies from multiple countries (e.g. USA and in Scandinavia) did not provide consistent evidence of an association. Among studies that investigated other cancers, a prospective study examined risk of glioma, including data from two cohorts, and found an elevated risk of glioma, in particular non-glioblastoma, in relation to VP1 MCPyV seropositivity. A nested case–control study and a case–control study on NHL did not observe any overall associations but observed elevated odds ratios in a single subgroup with wide confidence intervals, although the subgroups differed between studies.

In a case–control study on chronic lymphocytic leukaemia and antibodies to sT antigen, LT antigen, and VP1 MCPyV, no seropositivity to MCPyV LT antigen was observed, and only a small fraction of individuals had seropositivity to MCPyV sT antigen. In the two nested case–control studies and two case–control studies on lung cancer that were considered to be informative – only one of which examined lung cancer histological types – there was no evidence of an association with MCPyV. The evaluated data on LT antigen expression in tumours were inconsistent. In a case–control study on oesophageal cancer, no association with MCPyV VP seropositivity was observed. In one case–control study on bladder cancer, an elevated odds ratio was observed in relation to the highest versus lowest tertiles of MCPyV VP1 seropositivity; whereas in another case–control study using urinary MCPyV DNA, with low detection levels, no evidence of an increased risk of bladder cancer was found. Although there were not extensive data on other types of tumour besides MCC, the available data on integration of MCPyV, mutations, or LT antigen expression in other tumours were inconsistent. Thus, although the nested case–control and case–control studies were considered to be well conducted, there were few studies carried out on each of the specific types of tumour, and the evidence regarding MCPyV alterations in tumours did not allow the Working Group to draw firm conclusions about the causal role of MCPyV in tumour types other than MCC.

5.3 Cancer in experimental animals

The tumorigenic potential of MCPyV in experimental animals has been investigated in seven studies using transgenic mouse models expressing MCPyV T antigens in a tissue-specific manner. Across these studies, a significant increase in neoplasm incidence was consistently observed. Specifically, *K14Cre-MCPyV168* mice and *MCPyV-RbWT* mice exhibited a significant increase in the incidence of spontaneous squamous cell papilloma of the skin, compared with controls. Expression of sT antigen alone was sufficient to induce squamous cell carcinoma of the skin in situ in *iK5;KLEsT* models. Moreover, co-expression of MCPyV T antigens with Atoh1 induced cellular reprogramming, and *p53* deletion led to a marked increase in incidence in a *K5-CreERT2;R26-lSL-rtTA;tetO-s-T/tLT;tetO-Atoh1* mouse model examining tumours that closely resembled human MCC in both histology and gene expression. In a separate study, conditional expression of sT antigen in combination with *p53* deletion in *UbcCreERT2;ROSAsT;p53^flox/flox* mice resulted in the development of high-grade malignant tumours in both the spleen and liver after the administration of tamoxifen. In a chemical carcinogenesis model of tumour promotion and progression, *K14Cre-MCPyV168* mice treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) only, 7,12-dimethylbenz[*a*]-anthracene (DMBA) only, or a combination of DMBA and TPA (DMBA plus TPA) showed a significantly increased incidence of squamous papilloma, compared with control mice.

5.4 Mechanistic evidence

After clonal integration of MCPyV in a cell, early viral transcripts, truncated LT antigen, and sT antigen are expressed. The truncated LT antigen retains the Rb-binding or LXCXE motif but not the helicase domain. sT antigen also contributes significantly to the development and progression of MCPyV-positive MCC.

There is consistent and coherent evidence that MCPyV exhibits key characteristics of carcinogens.

MCPyV is genotoxic. In exposed humans, integration of MCPyV DNA with a mutation in the LT gene into the tumour genome is a consistent and early event in MCPyV-positive MCC, and it has a clonal integration pattern. Full-length MCPyV LT and sT can increase γH2AX (a marker of DNA damage) and induce DNA double-strand breaks in human cell models. MCPyV sT induces micronucleus formation and chromosomal breakage in vivo. No data were available in human primary cells.

MCPyV alters DNA repair or causes genomic instability. In exposed humans, MCPyV-positive MCC tumours show fewer chromosomal alterations than do MCPyV-negative tumours, indicating relative genomic stability. No data were available in human primary cells. In human cancer cell lines and fibroblasts, truncated LT impairs DNA repair and disrupts cell cycle arrest mechanisms. In rodent cell lines and transgenic mouse models, sT induces centrosome amplification, chromosome breakage, and aneuploidy leading to genome instability.

MCPyV causes immortalization. No data were available in exposed humans, and there was a paucity of data in human primary cells. In immortalized human and rodent fibroblasts, MCPyV sT expression promotes anchorage-independent growth and serum-free proliferation. There were mixed results on the role of truncated LT in MCPyV-driven cellular transformation. However, in transgenic mice, sT expression induces epithelial transformation and dysplasia.

MCPyV alters cell proliferation, cell death, or nutrient supply. There was suggestive evidence in exposed humans, based on the expression of genes associated with cell proliferation pathways in MCPyV-positive tumours. There was a paucity of data in human primary cells. In engineered mouse models expressing MCPyV T antigens, epithelial hyperplasia and increased proliferation in the skin is observed. In multiple human and rodent cell lines, sT activates non-canonical NF-κB signalling, leading to increased cell survival, proliferation, and glucose uptake. Truncated LT and sT antigens contribute to growth-promoting activities in vitro and in vivo.

There is suggestive evidence that MCPyV induces epigenetic alterations in experimental systems. In exposed humans and human primary cells, there was a paucity of data. Both in vitro and in vivo, MCPyV induces epigenetic alterations in MCC cells, particularly through modulation of histone methylation via EZH2 and LSD1. However, the effect on DNA methylation is unclear.

There is suggestive evidence that MCPyV induces chronic inflammation. In exposed humans, the data suggested a T-cell mediated response against MCPyV in healthy individuals and patients with MCC. In human primary cells, a small number of studies suggested that MCPyV activates the innate immune response, including interferon-stimulated genes (ISGs) and inflammatory cytokine genes. In a few studies, it was reported that inflammatory cytokine genes are upregulated in MCC cell lines and immortalized human dermal fibroblasts.

There is suggestive evidence that MCPyV is immunosuppressive. The evidence in exposed humans and in human primary cells was sparse. Experimental studies have shown that MCPyV sT suppresses genes involved in MHC class I antigen processing and presentation, as well as interferon pathways, in human cells.

There is suggestive evidence that MCPyV modulates receptor-mediated effects. In exposed humans and human primary cells, there was a paucity of data. In one study, it was suggested that MCPyV modulates the Wnt signalling pathways. However, disruption of the Wnt pathway, which impairs MCC differentiation and survival, is observed exclusively in MCPyV-positive MCC cell lines, limiting the ability to attribute them specifically to MCPyV status.

No data were available for the other key characteristics of carcinogens.

6. Evaluation and Rationale

6.1 Cancer in humans

There is *sufficient* evidence in humans for the carcinogenicity of Merkel cell polyomavirus. Merkel cell polyomavirus causes Merkel cell carcinoma.

6.2 Cancer in experimental animals

There is *sufficient* evidence in experimental animals for the carcinogenicity of Merkel cell polyomavirus.

6.3 Mechanistic evidence

There is *strong* mechanistic evidence that Merkel cell polyomavirus exhibits key characteristics of carcinogens in exposed humans and in experimental systems.

6.4 Overall evaluation

Merkel cell polyomavirus is *carcinogenic to humans* (Group 1).

6.5 Rationale

The Group 1 evaluation for Merkel cell polyomavirus (MCPyV) is based on *sufficient* evidence for cancer in humans and also on the combination of *sufficient* evidence for cancer in experimental animals and *strong* mechanistic evidence of the key characteristics of carcinogens in exposed humans.

The human cancer evidence was *sufficient* for Merkel cell carcinoma (MCC) in relation to MCPyV exposure because of positive findings in several studies. In one nested case–control study within a cohort of patients in two Nordic countries, higher risk of MCC was observed in relation to several measures of exposure from prospectively collected samples. This was supported by positive findings from three non-nested case–control studies on MCPyV exposure in relation to MCC, as well as several case series that detected integrated MCPyV, truncating mutations, and LT antigen expression in MCC tumours.

The *sufficient* evidence for cancer in experimental animals was based on an increase in the incidence of benign (papilloma of the skin) and malignant (in situ squamous cell carcinoma of the skin; highly anaplastic tumours of the spleen and liver; and tumours that histologically and transcriptionally resemble human MCC) neoplasms in one species (mice, transgenic) and two sexes in multiple studies. The mechanistic evidence was *strong* in exposed humans because MCPyV is genotoxic. In addition, there was consistent and coherent evidence in experimental systems that MCPyV is genotoxic, alters DNA repair or causes genomic instability, causes immortalization, and alters cell proliferation.

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LIST OF ABBREVIATIONS

AAV	adeno-associated vector
AIDS	acquired immunodeficiency syndrome
ALL	acute lymphoblastic leukaemia
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AML	acute myeloid leukaemia
APRI	aspartate aminotransferase-to-platelet ratio index
ART	antiretroviral therapy
AST	aspartate aminotransferase
ATM	ataxia telangiectasia mutated
BCC	basal cell carcinoma
BKPyV	BK polyomavirus
BMI	body mass index
BrdU	bromodeoxyuridine
bw	body weight
CARET	Carotene and Retinol Efficacy Trial
CCLS	California Childhood Leukaemia Study
CD	cluster of differentiation
CHB	chronic hepatitis B
CHD	chronic hepatitis D
CI	confidence interval
CLL	chronic lymphocytic leukaemia
CML	chronic myeloid leukaemia
CNS	central nervous system
CRE	cAMP response element
CRISPR	clustered regularly interspaced short palindromic repeats
CstA	peptide transporter carbon starvation
CT	computed tomography
CTCL	cutaneous T-cell lymphoma
DAG	directed acyclic graph
DCIS	ductal carcinoma in situ
ddPCR	digital droplet polymerase chain reaction
DEG	differentially expressed gene
DLBCL	diffuse large B-cell lymphoma

EASL	European Association for the Study of the Liver
EBV	Epstein–Barr virus
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
EMT	epithelial–mesenchymal transition
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
EUROHEP	European Concerted Action on Viral Hepatitis
FFPE	formalin-fixed paraffin-embedded
FISH	fluorescence in situ hybridization
GGT	gamma-glutamyl transferase
GO	gene ontology
GSC	glioma stem-like cell
HAART	highly active antiretroviral therapy
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDAg	hepatitis D antigen
HDF	human diploid fibroblast
HDIN	Hepatitis Delta International Network
HDV	hepatitis D virus
HIV	human immunodeficiency virus
HL	Hodgkin lymphoma
HLA	human leukocyte antigen
HLC	hepatocyte-like cell
HMEC	human primary mammary epithelial cell
HPV	human papillomavirus
HR	hazard ratio
HSC	hepatic stellate cell
HSPG	heparan sulfate proteoglycan
HSV	herpes simplex virus
HUVEC	human umbilical vein endothelial cell
IARC	International Agency for Research on Cancer
ICD	International Classification of Diseases
ICD-O	International Classification of Diseases for Oncology
ICTV	International Committee on Taxonomy of Viruses
IDH	isocitrate dehydrogenase
IDU	injecting drug user
IE	immediate-early
IFN	interferon
Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin
IPA	ingenuity pathway analysis
IRR	incidence rate ratio

ISG	interferon-stimulated gene
ISH	in situ hybridization
JCPyV	John Cunningham polyomavirus
KC	key characteristic of carcinogens
LA	late antigen
LC	liver cirrhosis
LCM	laser capture microdissected
lncRNA	long noncoding RNA
LPyV	B-lymphotrophic polyomavirus or African green monkey polyomavirus
LSD	large T stabilization domain
MAVS	mitochondrial antiviral signalling
MCC	Merkel cell carcinoma
MCPyV	Merkel cell polyomavirus
MFI	mean fluorescence intensity
MH	malignant hepatocyte
MIEP	major immediate-early promoter
miRNA	microRNA
MLKL	mixed lineage kinase domain-like
mRNA	messenger RNA
MUR	Merkel cell polyomavirus large T unique region
NA	nucleoside analogue
NAAT	nucleic acid amplification test
NADHP	nicotinamide adenine dinucleotide phosphate, reduced
NCCR	non-coding control region
NEC	nuclear egress complex
NES	nuclear export signal
NGS	next-generation sequencing
NHANES	National Health and Nutrition Examination Survey
NHL	non-Hodgkin lymphoma
NK	natural killer
NLS	nuclear localization signal
NMH	non-malignant hepatocyte
NTCP	sodium taurocholate co-transporting polypeptide
OD	optical density
OR	odds ratio
ORF	open reading frame
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cell
PcG	polycomb group
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGFα	platelet-derived growth factor receptor alpha
PHH	primary human hepatocyte
PLI	proliferating cell nuclear antigen labelling index
PR	progesterone receptor
PTLD	post-transplant lymphoproliferative disorder
Q-MAC	quantitative microarray antibody capture
qPCR	quantitative polymerase chain reaction
RCA	rolling circle amplification
RIA	radioimmunoassay

RNP	ribonucleoprotein
ROS	reactive oxygen species
RR	relative risk
RT-PCR	reverse transcription-polymerase chain reaction
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
SASP	senescence-associated secretory phenotype
SCC	squamous cell carcinoma
SHCS	Swiss HIV Cohort Study
SIR	standardized incidence ratio
siRNA	small interfering RNA
SRE	serum response element
SRF	serum response factor
SRTR	Scientific Registry of Transplant Recipients
t-SNE	t-distributed stochastic neighbour embedding
TCF	T-cell factor
TCGA	The Cancer Genome Atlas
TCID50	tissue culture infectious dose
TIL	tumour-infiltrating lymphocyte
TOR	target of rapamycin
UK	United Kingdom
UL	unique long
ULN	upper limit normal
UPR	unfolded protein response
USA	United States of America
USB	uPA/SCID/beige
UV	ultraviolet
VEGF	vascular endothelial growth factor
VIRUSCAN	Virus in Skin Cancer
VLP	virus-like particle
WGCNA	weighted gene co-expression network analysis
WHO	World Health Organization

ANNEX 1. SUPPLEMENTARY MATERIAL FOR SECTION 1, EXPOSURE CHARACTERIZATION

These supplementary online-only tables are available from: https://publications.iarc.who.int/667.

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Hepatitis D virus

The following table was produced in draft form by the Working Group and was subsequently fact-checked, but not edited:

Table S1.3 Exposure assessment review and critique for studies on hepatitis D virus and hepatocellular carcinoma in humans

Human cytomegalovirus

The following table was produced in draft form by the Working Group and was subsequently fact-checked, but not edited:

Table S1.3 Exposure assessment review and critique for studies on human cytomegalovirus and cancer in humans

Merkel cell polyomavirus

The following table was produced in draft form by the Working Group and was subsequently fact-checked, but not edited:

Table S1.3 Exposure assessment review and critique for studies on Merkel cell polyomavirus and cancer in humans

ANNEX 2. SUPPLEMENTARY MATERIAL FOR HEPATITIS D VIRUS, SECTION 2, CANCER IN HUMANS

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Hepatitis D virus

Fig. S2.5	Meta-analysis for hepatitis D virus and hepatocellular carcinoma, including only cohort studies
Fig. S2.6	Meta-analysis for hepatitis D virus and hepatocellular carcinoma, excluding Raimondo et al. (1984)

ANNEX 3. SUPPLEMENTARY MATERIAL FOR HUMAN CYTOMEGALOVIRUS, SECTION 2, CANCER IN HUMANS

The supplementary online-only text and tables presented in Annex 3 (available from: https://publications.iarc.who.int/667, and listed below) were produced by the Working Group and were subsequently fact-checked but not edited.

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Text

Description of case series of glioma and central nervous system cancers that included at least 50 cases and used strong virological methods to assess human cytomegalovirus

Tables

Table S2.2	Prevalence of HCMV markers in glioma and tumours of the central nervous system
Table S2.5	Results from studies on HCMV and breast cancer: case–control studies without a reported risk measure and within-person control studies
Table S2.7	Within-person comparison studies on cancers of the digestive tract and HCMV
Table S2.8	Epidemiological studies on cancers of the reproductive and urinary tracts and HCMV

ANNEX 4. SUPPLEMENTARY MATERIAL FOR MERKEL CELL POLYOMAVIRUS, SECTION 2, CANCER IN HUMANS

The supplementary online-only text and tables presented in Annex 4 (available from: https://publications.iarc.who.int/667, and listed below) were produced by the Working Group and were subsequently fact-checked but not edited.

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Text

Description of immunohistochemistry-based case series; serological studies; and case series with assessment of large T antigen protein expression, DNA integration, or DNA mutation for non-Merkel cell carcinoma skin cancers or other non-skin cancers.

Table

Table S2.2 Prevalence of markers of Merkel cell polyomavirus in tumour samples

SUMMARY OF FINAL EVALUATIONS

Summary of final evaluations for Volume 139

Agent	Evidence stream			Overall evaluation
	Cancer in humans	Cancer in experimental animals	Mechanistic evidence	
Hepatitis D virus	*Sufficient*	*Inadequate*	*Strong*[a]	Group 1
Human cytomegalovirus	*Limited*	*Inadequate*	*Limited*	Group 2B
Merkel cell polyomavirus	*Sufficient*	*Sufficient*	*Strong*[b]	Group 1

[a] There was *strong* mechanistic evidence that the agent exhibits key characteristics of carcinogens in exposed humans and human primary cells.
[b] There was *strong* mechanistic evidence that the agent exhibits key characteristics of carcinogens in exposed humans and experimental systems.

IARC MONOGRAPHS

This volume of the *IARC Monographs* provides evaluations of the carcinogenicity of three viruses – hepatitis D virus (HDV), human cytomegalovirus (HCMV), and Merkel cell polyomavirus (MCPyV).

HDV is an RNA virus that requires hepatitis B surface antigen (HBsAg) as its envelope; thus, establishing an HDV infection necessitates prior or simultaneous infection with hepatitis B virus. Transmission can occur through contact with infected human blood or other infected body fluids, e.g. via sharing needles, transfusion of contaminated blood products, haemodialysis, or unprotected sex. The prevalence of HDV infection within HBsAg-positive populations ranges from 1% to 10% in most regions of the world, although it is much higher in some countries.

HCMV is a betaherpesvirus that is transmitted through body fluids such as saliva, blood, urine, semen, and breast milk, and from mother to fetus during pregnancy. Worldwide HCMV seroprevalence is estimated at 83% for the general population and is generally higher in South America, Africa, and South Asia. Infection can occur at all ages but is most common during childhood.

MCPyV is a polyomavirus that is ubiquitous in human populations. Infections are typically acquired in early childhood via close contact, and the virus persists as a major component of the normal skin virome.

An *IARC Monographs* Working Group reviewed evidence from cancer studies in humans, studies of cancer in experimental animals (for HCMV and MCPyV), and mechanistic studies to assess the carcinogenic hazard to humans of exposure to these agents and concluded that:

- Hepatitis D virus and Merkel cell polyomavirus are *carcinogenic to humans (Group 1)*;
- Human cytomegalovirus is *possibly carcinogenic to humans (Group 2B)*.

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