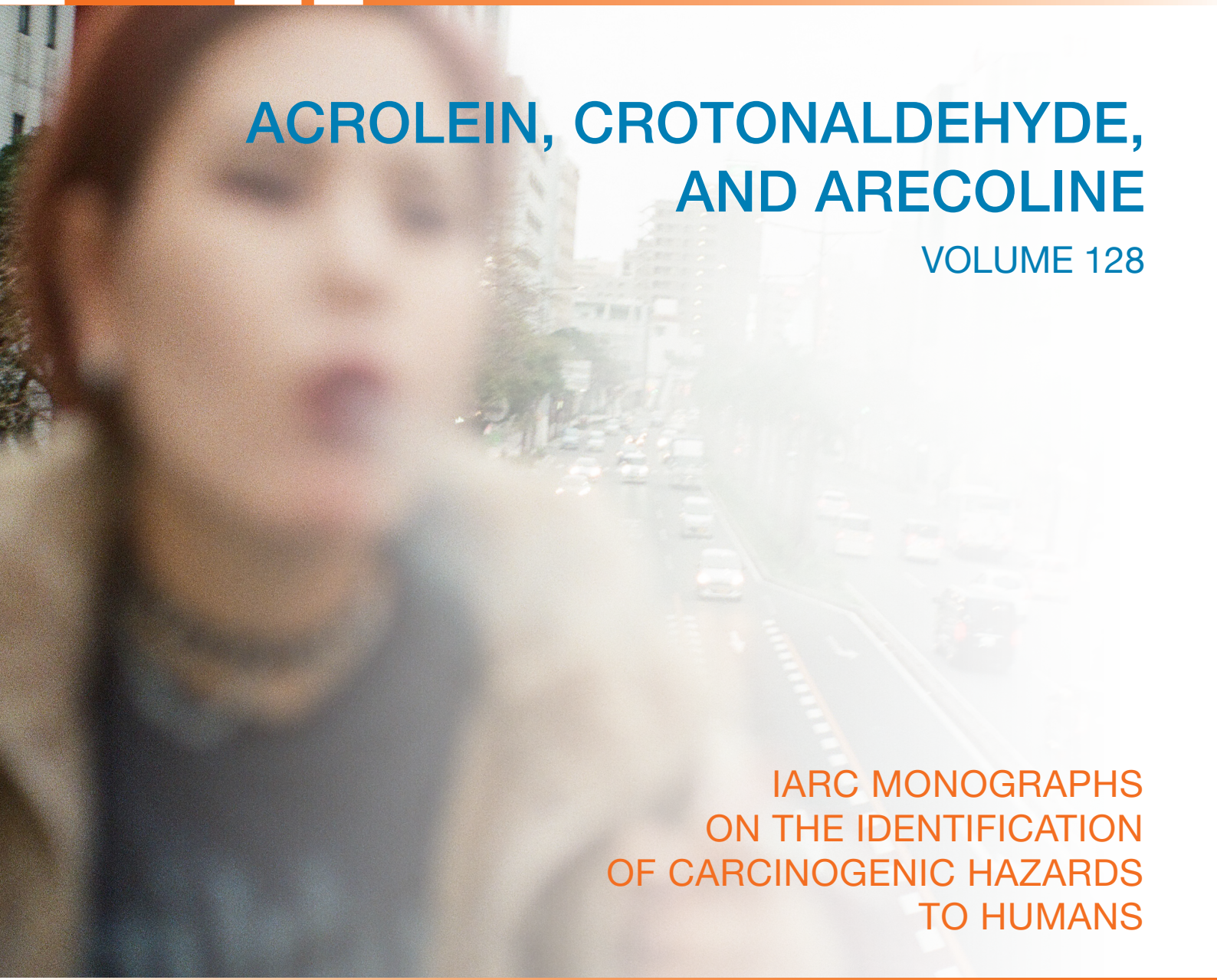
IARC MONOGRAPHS



ACROLEIN, CROTONALDEHYDE, AND ARECOLINE

VOLUME 128

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ON THE IDENTIFICATION
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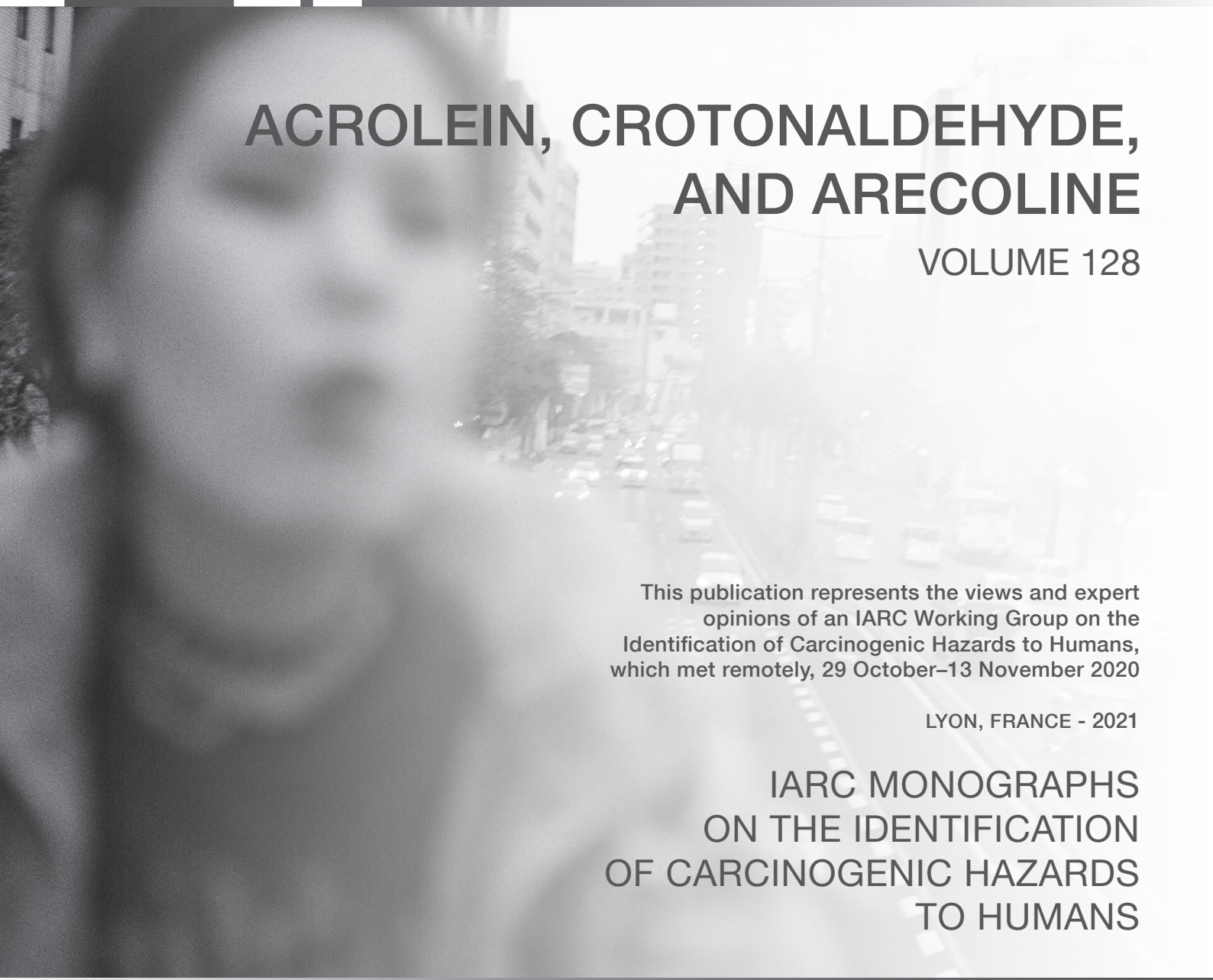

ACROLEIN, CROTONALDEHYDE, AND ARECOLINE

VOLUME 128

This publication represents the views and expert opinions of an IARC Working Group on the Identification of Carcinogenic Hazards to Humans, which met remotely, 29 October–13 November 2020

LYON, FRANCE - 2021

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.fr/.

This programme has been supported since 1982 by Cooperative Agreement U01 CA33193 with the United States National Cancer Institute, Department of Health and Human Services. Additional support has been provided since 1986 by the European Commission Directorate-General for Employment, Social Affairs, and Inclusion, initially by the Unit of Health, Safety and Hygiene at Work, and since 2014 by the European Union Programme for Employment and Social Innovation "EaSI" (2014–2020) (for further information please consult: https://ec.europa.eu/social/easi). Support has also been provided since 1992 by the United States National Institute of Environmental Health Sciences, Department of Health and Human Services. The contents of this volume are solely the responsibility of the Working Group and do not necessarily represent the official views of the United States National Cancer Institute, the United States National Institute of Environmental Health Sciences, the United States Department of Health and Human Services, or the European Commission.

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About the cover: A smoker on a bridge above a busy road, Japan. Sources of acrolein and crotonaldehyde include tobacco smoke and combustion of vehicle fuel.

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CONTENTS

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, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France, or via email at imo@iarc.fr, 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.fr/).

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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.fr/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.fr/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 & 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

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.

searching the literature and performing title and abstract screening, organizing conference calls to coordinate the development of pre-meeting 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.fr/).

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).

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

The Working Group meets at IARC for approximately eight days to discuss and finalize the scientific review and to develop summaries 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 end-point). 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 & 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 & 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 pre-neoplastic 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 & Bailer, 1989; Bieler & 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

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).

effects", are based on empirical observations of the chemical and biological properties associated with the human carcinogens identified by 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 & 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. s*ufficient 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-twenty-eighth volume of the *IARC Monographs* contains evaluations of the carcinogenic hazard to humans of acrolein, crotonaldehyde, and arecoline. Due to the coronavirus disease (COVID-19) pandemic, this meeting, which was scheduled to be held in Lyon, France, was held remotely.

Acrolein was considered previously by the *IARC Monographs* programme in 1978 (IARC, 1979), 1984 (IARC, 1985), 1987 (IARC, 1987), and most recently in 1995 (IARC, 1995), when it was evaluated as *not classifiable as to its carcinogenicity to humans (Group 3).* Crotonaldehyde was also evaluated by the *IARC Monographs* programme in 1995, when the Working Group concluded that it was *not classifiable as to its carcinogenicity to humans (Group 3)* (IARC, 1995).

Arecoline itself has not been previously evaluated by the *IARC Monographs* programme. However, arecoline is the most abundant alkaloid considered in the context of betel-quid and areca-nut chewing in *IARC Monographs* Volume 85 (IARC, 2004) and Volume 100E (IARC, 2012a). It is the primary active ingredient of the areca nut, which is classified as *carcinogenic to humans (Group 1)* (IARC, 2012a).

The Advisory Group to Recommend Priorities for the *IARC Monographs* that met in 2019 recommended that all three agents be evaluated with high priority (Marques et al., 2019). New data have become available, primarily bioassay and mechanistic evidence, and these data have been included and considered in the present volume. A summary of the findings of this volume appears in *The Lancet Oncology* (IARC Monographs Vol 128 Group, 2021).

Electrophilicity as a key characteristic of carcinogens

A characteristic feature of many carcinogens is that they are either direct-acting electrophiles or are metabolized to an electrophile. Electrophiles can bind to nucleophilic sites in DNA, forming adducts with DNA and potentially leading to DNA damage, including mutations, DNA strand breaks, and chromosomal aberrations. As described in a recent IARC Scientific Publication, multiple factors and mechanistic processes may play a role in determining whether electrophiles will result in carcinogenicity (IARC, 2019). A key factor is the extent of DNA binding of the electrophile, which can be affected by physiochemical properties (i.e. binding affinity for DNA or protein), time-dependent tissue concentrations, and the presence of alternative molecular targets (e.g. glutathione). In addition, the fate of the induced DNA lesion(s) may be influenced by various other molecular, cellular,

and physiological factors, including the physical properties of the lesion (e.g. persistence and mutagenic potential), the activities and effectiveness of relevant DNA repair processes, and the rates of cell division and cell death. Importantly, related mechanistic effects, including inhibition of topoisomerase II and inhibition of DNA repair, may also be triggered by some electrophiles, which can amplify the resulting DNA damage. Therefore, when considering whether a compound "is electrophilic" in a toxicologically meaningful way, its reactivity with biologically relevant nucleophiles, the nature of the resulting lesion(s), and the physiological context should be taken into account.

Evidence that an agent "is electrophilic" may come from a range of computational analyses, molecular and cellular experiments, and other types of studies. Relevant findings may predict inherent DNA reactivity of the parent compound or its metabolite(s), characterize the structure of the DNA adduct, and illustrate adduct formation during controlled experiments in experimental animals or after occupational or other exposures in humans. Studies on DNA-adduct formation may provide an essential part of this evidence, depending on the specificity of the adduct for the exposure and the outcome. As highlighted in the Preamble to the *IARC Monographs* and relevant for this key characteristic, data from studies investigating susceptibility to cancer in experimental animals or in humans can provide important support for mechanistic conclusions. For example, the Group 1 evaluations of trichloroethylene, *N*′-nitrosonornicotine, and nitrosomethylamino)-1-(3-pyridyl)-1-butanone (IARC, 2012a; 2014) were supported by studies showing that polymorphisms in metabolic enzymes affecting the formation or detoxification of electrophiles can influence cancer risk. The strength of these molecular epidemiology studies in supporting the conclusions of the Working Group relies on the evaluation of study quality, as elaborated in the Preamble, and is the focus of the collaborative review undertaken with Working Group members reviewing exposure characterization, studies of cancer in humans, and mechanistic data.

As further noted in the Preamble, evidence for a group of key characteristics of carcinogens described by Smith et al. (2016) can provide additional context and can strengthen mechanistic conclusions overall. For many Group 1 agents that are electrophilic (e.g. ethylene oxide; IARC, 2012b), or that can be metabolized to electrophiles (e.g. aflatoxins, vinyl chloride, aristolochic acid; IARC, 2012b, c), the mechanistic conclusions were strengthened by evidence that these agents are genotoxic. Studies of micronucleus induction in exposed workers or studies of molecular signatures in the DNA (e.g. the *TP53* mutation signature for aristolochic acid; IARC, 2012c) have been especially influential in this regard. For the agents evaluated in the present volume, all of which are electrophilic without requiring metabolic activation, there was evidence from a range of studies conducted in different systems and supporting multiple key characteristics of carcinogens. For two of the agents, acrolein and crotonaldehyde, there was supporting evidence for these mechanistic conclusions from studies on DNA adducts in humans; while providing important support, human studies on DNA adducts did not alone provide "strong" evidence from exposed humans of key characteristics of carcinogens. Acrolein is a strongly electrophilic α,β-unsaturated aldehyde (enal) that readily reacts with DNA bases and proteins. Acrolein is genotoxic; it alters DNA repair or causes genomic instability; it induces oxidative stress; it is immunosuppressive; it induces chronic inflammation; and it alters cell proliferation, cell death, or nutrient supply. Crotonaldehyde is an electrophilic bifunctional α,β-unsaturated aldehyde (enal) that can form cyclic adducts in DNA, DNA interstrand crosslinks, and DNA–protein crosslinks. Crotonaldehyde is genotoxic; it induces oxidative stress; and it induces chronic

inflammation. Arecoline is an electrophilic α,β-unsaturated ester that can undergo Michael addition with cellular nucleophiles. It is genotoxic; it alters DNA repair or causes genomic instability; and it induces oxidative stress.

Differentiating endogenous from exogenous exposures

For two of the agents considered in the present volume, acrolein and crotonaldehyde, human exposure may derive from both endogenous processes and exogenous sources. Findings were similar for some other aldehydes previously evaluated by the *IARC Monographs* programme, such as acetaldehyde and formaldehyde (IARC, 2012a, b).

As discussed in the monographs on acrolein and crotonaldehyde, many studies considered only tobacco smoking as an external exposure source to these chemicals and attributed findings in non-smokers (such as DNA adduct formation) to endogenous exposures. However, a diversity of exogenous exposures, including tobacco smoke as well as air pollution, diesel engine exhaust, inflammatory conditions, and a high fat diet, have been demonstrated in well-controlled experimental studies to increase the levels of aldehyde DNA adducts derived from acrolein and crotonaldehyde. Appropriate attribution of the effects of these diverse exogenous exposures is important in consideration of endogenous formation of acrolein and crotonaldehyde.

Endogenous formation of acrolein and crotonaldehyde is mechanistically plausible. For acrolein, endogenous formation may occur by several pathways, including the reaction of myeloperoxidase with hydroxyl-amino acids such as threonine; the oxidation of spermine and spermidine by amine oxidase; and peroxidation of polyunsaturated fatty acids (see the monograph on acrolein). Lipid peroxidation and metabolism, specifically from ω-3 polyunsaturated fatty acids, including docosahexaenoic acid, linoleic acid, and eicosapentaenoic acid, are also suggested endogenous sources of crotonaldehyde. As a result, aldehyde-derived DNA adducts are constantly formed in cellular DNA as endogenous background lesions.

The question remains as to how informative these endogenous processes are for cancer hazard assessment. Firstly, endogenous background levels of an agent pose a challenge in untangling external from internal exposures, especially if the external exposure can only be indirectly assessed from metabolites or biomarkers. The interpretation of data on such biomarkers is not always straightforward and it may be difficult to separate the contribution of endogenous formation of metabolites (e.g. originating from lipid peroxidation or inflammation) from exogenous sources. Secondly, decreased or increased adduct levels may result from alterations in endogenous processes, including during the toxic response to the agent or during the course of cancer development. For example, endogenous formation of formaldehyde may be important in the development of leukaemia in patients with Fanconi anaemia (IARC, 2012b). For these reasons, DNA adducts in human cancer cells cannot be considered as a marker of external exposure, since cells have undergone several cycles of molecular changes and selection that affect the internal concentration of adducts.

Data from high-throughput screening assays

The analysis of the in vitro bioactivity of acrolein and arecoline was informed by data from high-throughput screening assays generated by the Toxicity Testing in the 21st Century (Tox21) and Toxicity Forecaster (ToxCast) research programmes of the government of the

USA (Thomas et al., 2018). The results from these assays were uninformative regarding the carcinogenicity of these agents. Although neither programme includes assays for mutagenicity or DNA-adduct formation, they do include a few assays to detect end-points encompassed by the key characteristics of carcinogens (Smith et al., 2016), such as DNA repair, altered gene expression, oxidative stress, and modulated receptor-mediated effects. Nonetheless, a recent analysis of data from five such assays in Tox21 showed < 40% sensitivity for agents that are direct-acting genotoxicants in standard assays (i.e. Ames test, chromosomal aberrations in vitro, micronucleus formation in vivo) (Hsieh et al., 2019). These programmes are constantly being improved and new assays are included over time; however, at present, the general lack of metabolic capacity and the small number of genotoxicity assays limits the value of these high-throughput screening programmes for carcinogenicity assessments of genotoxic and other chemicals.

Scope of systematic review

Standardized searches of the PubMed database (National Library of Medicine, 2021) were conducted for the agent and for each outcome (cancer in humans, cancer in experimental animals, and mechanistic evidence, including the key characteristics of carcinogens). The literature trees for the agent, including the full set of search terms for the agent name and each outcome type, are available online.[1]

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[1] The literature trees for the present volume are available at: https://hawcproject.iarc.fr/assessment/436/ (acrolein), https://hawcproject.iarc.fr/assessment/646/ (crotonaldehyde), and https://hawcproject.iarc.fr/assessment/638/ (arecoline).

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ACROLEIN

1. Exposure Characterization

1.1 Identification of the agent

1.1.1 Nomenclature

Chem. Abstr. Serv. Reg. No.: 107-02-8

Deleted Chem. Abstr. Serv. Reg. No.: 25314-61-8

EC/List No.: 203-453-4

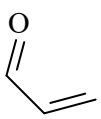
Chem. Abstr. Serv. name: 2-propenal

IUPAC systematic name: prop-2-enal

Synonyms: acraldehyde; acrylaldehyde; acrylic aldehyde; allyl aldehyde; ethylene aldehyde; propenal; 2-propenal; prop-2-en-l-al (IARC, 1995; O'Neil, 2013; ECHA, 2020).

1.1.2 Structural and molecular formulae, and relative molecular mass

Structural formula:



Molecular formula: C_3H_4O

Relative molecular mass: 56.06 (O'Neil, 2013).

1.1.3 Chemical and physical properties

Description: colourless to yellowish liquid with extremely acrid, pungent, and irritating odour, causing lachrymation (Verschueren, 1983; IARC, 1985; O'Neil, 2013)

Boiling point: 52.5–53.5 °C (Lide, 1993)

Melting-point: –86.9 °C (Lide, 1993)

Relative density: 0.8410 at 20 °C/4 °C (Lide, 1993)

Solubility: soluble in water (206 g/L at 20 °C), ethanol, diethyl ether, and acetone (IPCS, 1992; Lide, 1993)

Volatility: vapour pressure 29.3 kPa (220 mm Hg) at 20 °C (IPCS, 1992)

Flash-point: –26 °C (IPCS, 1992)

Stability: unstable in the absence of an inhibitor (IPCS, 1992); polymerizes, especially under light or in the presence of alkali or strong acid, to form disacryl, a plastic solid (O'Neil, 2013). Inhibited acrolein undergoes dimerization above 150 °C and highly exothermic polymerization also occurs in the presence of traces of acids or strong bases even when an inhibitor is present (IPCS, 1992)

Reactivity: reactions shown by acrolein include Diels–Alder condensation, dimerization and polymerization, additions to the carbon–carbon double bond, carbonyl

additions, oxidation, and reduction (IPCS, 1992; see also Section 4.2.1)

*Octanol/water partition coefficient (*P): log K_{ow}, –0.01 (O'Neil, 2013)

Odour perception threshold: 0.07 mg/m^3 (IPCS, 1992)

Conversion factor: 1 ppm = 2.29 mg/m^3 (IARC, 1995).

1.1.4 Technical products and impurities

Hydroquinone (IARC Group 3; IARC, 1999) at a concentration of 0.1–0.25% is typically used to stabilize commercially available preparations of acrolein (Etzkorn, 2009). Hydroquinone protects acrolein from polymerization, and also from hydrolysis in aqueous solutions (Kächele et al., 2014). Acrolein is available commercially with purities in the range of 90–98% and as solutions to be used as reference materials in water, methanol, and acetone (Chemical Abstracts Service, 2020). Impurities include water (up to 3.0% by weight; IPCS, 1992), acetaldehyde, and, depending on the production process, small amounts of propionaldehyde, acetone, propene oxide, and methanol, and traces of allyl alcohol and ethanol (Arntz et al., 2007).

1.2 Production and use

1.2.1 Production process

Acrolein was first prepared in 1843 by the dry distillation of fat (Redtenbacher, 1843). Commercial production of acrolein began in Germany in 1942, by a process based on the vapour-phase condensation of acetaldehyde and formaldehyde. This method was used until 1959, when a process was introduced for producing acrolein by vapour-phase oxidation of propene (Arntz et al., 2007). Several catalysts have been used in this process, including bismuth molybdate (Etzkorn, 2009). Propene oxidation is still the commercially dominant production process (Etzkorn, 2009), while research on more environmentally friendly methods of acrolein production from renewable feedstock such as glycerol, methanol, or ethanol is ongoing (Arntz et al., 2007; Etzkorn, 2009; Lilić et al., 2017). The oxidation of propene produces acrolein, acrylic acid, acetaldehyde, and carbon oxides.

1.2.2 Production volume

In 1975, global production of acrolein was approximately 59 000 tonnes (Hess et al., 1978). Worldwide production of acrolein in 1977 was estimated to have been 100–120 000 tonnes (IARC, 1979). The worldwide capacity for production of refined acrolein was estimated in the 1990s to be about 113 000 tonnes per year (Etzkorn et al., 1991). In 2007, the production capacity for acrolein in western Europe, USA, and Japan was estimated to total 425 000 tonnes per year (Arntz et al., 2007). In 2009, worldwide estimated acrolein production capacity was about 350 000 tonnes per year, which included acrolein made for captive use in methionine production (Etzkorn, 2009). Estimated global demand in 2018 was 620 000 tonnes (Zion Market Research, 2019).

Acrolein was listed by the Organisation for Economic Co-operation and Development (OECD) and the United States Environmental Protection Agency (US EPA) as a High Production Volume chemical for 2007 (IARC, 2019). About 100–1000 tonnes per year are manufactured and/or imported in the European Economic Area (ECHA, 2020).

The Chem Sources database lists 27 manufacturing companies worldwide, of which 12 are located in the USA and 5 in China (including Hong Kong Special Administrative Region) (Chem Sources, 2020).

1.2.3 Uses

Acrolein is an α,β-unsaturated aldehyde and a highly reactive, volatile organic chemical (see also Section 4.2.1). These properties contribute to the many reactions of acrolein and its commercial usefulness, either directly or (for the most part) as a chemical intermediate for the production of numerous chemical products. These include acrylic acid, which is used to make acrylates, and DL-methionine, an essential amino acid used as a feed supplement for livestock (Arntz et al., 2007; Faroon et al., 2008). Other important derivatives of acrolein are glutaraldehyde, pyridines, tetrahydrobenzaldehyde, allyl alcohol and glycerol, 1,4-butanedial and 1,4-butenediol, 1,3-propanediol, DL-glyceraldehyde, flavours and fragrances, and polyurethane and polyester resins (Sax & Lewis, 1987; Arntz et al., 2007).

The most important direct use of acrolein is as a biocide. It is used as an herbicide and to control algae, aquatic weeds, and molluscs in recirculating process water systems (at a concentration of 6–10 mg/L). It is also used to control the growth of microorganisms in liquid fuel, the growth of algae in oil fields, and the formation of slime in paper manufacture. Acrolein has been used in leather tanning and as a tissue fixative in histology (IPCS, 1992; IARC, 1995; Arntz et al., 2007; Etzkorn, 2009). Acrolein has also been used as a warning agent in methyl chloride refrigerants and other gases, in poison gas mixtures for military use, in the manufacture of colloidal forms of metals, and as a test gas for gas masks (IARC, 1979; Neumüller, 1979; O'Neil, 2013).

The market share for global acrolein production in 2017 was methionine use (61.2%), pesticide use (17.4%), glutaraldehyde use (7.3%), water treatment use (9.0%), and other applications (5.1%), with this distribution being stable (within 1%) for several consecutive years (Regal Intelligence, 2020).

1.3 Methods of detection and quantification

Methods for the analysis of acrolein in air, water, biological media including tissue, and food have been reviewed (IPCS, 1992; IARC, 1995; Shibamoto, 2008). Representative analytical methods for a variety of sampling matrices (air, water, cigarettes, foods and beverages, and biological specimens) are presented in Table S1.1 (Annex 1, Supplementary material for acrolein, Section 1, Exposure Characterization, web only; available from: https://publications.iarc.fr/602).

1.3.1 Air

Several reference procedures are available for the analysis of acrolein in air or gaseous emissions. These include ISO 19 701 (ISO, 2013) and ISO 19 702 (ISO, 2015) for the analysis of fire effluents, JIS K0089 (JIS, 1998) and VDI 3862 Part 5 (VDI, 2008) for the analysis of gaseous emissions, and MAK Air Monitoring Methods (Hahn, 1993). Official analytical methods for air analysis by the United States (US) National Institute for Occupational Safety and Health (NIOSH) (NIOSH 2501, NIOSH 2539) and Occupational Safety and Health Administration (OSHA) (OSHA 52) are available (NCBI, 2020). Methods for the analysis of mainstream cigarette smoke (see Section 1.4.2(b)), ISO 21 160 (ISO, 2018) and Health Canada Official machine smoking regime methods are also available. Protocols are required to standardize measurements of the emissions of toxic chemicals in mainstream cigarette smoke for regulatory purposes. Although ISO methods (from the International Organization for Standardization) have been widely used for decades, Health Canada and WHO have developed more intensive smoking conditions. The key differences between these protocols are that the ISO regime sets the machine to take 35 mL puffs every 60 seconds with ventilation holes left open, whereas the intensive regimes prescribe 50 mL

puffs every 30 seconds, and, importantly, all filter ventilation holes are blocked (WHO, 2012).

[The Working Group noted that the higher values provided by the Health Canada Official method correspond better to human exposure during smoking.]

High-performance liquid chromatography (HPLC) is the routine method to quantify acrolein derivatives obtained from sorbent matrix samplers, which may be used in conjunction with ultraviolet (UV), ion trap mass spectrometry (MS), and fluorescence detectors (Alberta Environment, 2011). Gas chromatography (GC) is the routine method to quantify acrolein pre-concentrated in pressurized sampling canisters and can be used with MS (GC-MS), flame ionization, and electron capture detectors (Alberta Environment, 2011).

1.3.2 Water

Similar chromatographic methods to those used for air analysis are applied to water. Several official analytical methods for water analysis are available from the US EPA (EPA-EAD 603: US EPA, 1984a; EPA-EAD 624: US EPA, 1984b; EPA-EAD 1624: US EPA, 1984c; EPA-RCA 5030C: US EPA, 2003; EPA-RCA 8015C: US EPA, 2007; EPA-RCA 8316: US EPA, 1994) and the United States Geological Survey (USGS) National Water Quality Laboratory (USGS-NWQL O-4127-96, Connor et al., 1996) (NCBI, 2020).

1.3.3 Soil

Standardized methods for analysing acrolein in soil were not identified. However, given the extent to which acrolein is expected to volatilize from soil based on its high vapour pressure and the irreversible binding of acrolein in soil, the lifetime of acrolein in soil may be too short for concern in the context of human exposure (ATSDR, 2007).

1.3.4 Food, beverages, and consumer products

Due to its high reactivity, direct analytical determination of acrolein is difficult, specifically in complex matrices such as foods and beverages (Kächele et al., 2014). Standardized methods for analysing acrolein in foods and beverages were not identified, but several methods with a focus on analysing alcoholic beverages and fat-based products are available (Table S1.1, Annex 1, Supplementary material for acrolein, Section 1, Exposure Characterization, web only; available from: https://publications.iarc.fr/602). Several different analytical approaches that mostly include derivatization have been suggested, typically based on HPLC or GC with various detectors including MS (Shibamoto, 2008). Several methods for acrolein analysis have applied solid-phase microextraction (SPME) for sample extraction and enrichment (Wardencki et al., 2003; Curylo & Wardencki, 2005; Saison et al., 2009; Osório & de Lourdes Cardeal, 2011; Lim & Shin, 2012; Kächele et al., 2014).

According to Kächele et al. (2014), acrolein standard solutions for calibrations should be stabilized by a suitable agent such as hydroquinone. The original hydroquinone content found in some commercial acrolein preparations as a stabilizer is not sufficient to prevent degradation if aqueous dilutions for trace analysis are prepared (Kächele et al., 2014).

1.3.5 Biological specimens

Several methods are available for the direct analysis of acrolein in saliva, urine and serum (Table S1.1, Annex 1, Supplementary material for acrolein, Section 1, Exposure Characterization, web only; available from: https://publications.iarc.fr/602) as well as the analysis of its metabolites or DNA and protein adducts (Table S1.2, Annex 1, Supplementary material for acrolein, Section 1, Exposure Characterization, web only;

available from: https://publications.iarc.fr/602). Of these, the urinary biomarkers *N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine(3-hydroxypropyl mercapturic acid, HPMA) and *N*-acetyl-*S*-(carboxyethyl)-L-cysteine (2-carboxyethylmercapturic acid, CEMA) appear to be most commonly determined, and can be detected using liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods (see Table S1.2, Annex 1, Supplementary material for acrolein, Section 1, Exposure Characterization, web only; available from: https://publications.iarc.fr/602).

Information regarding an internationally accepted validated biomarker for acrolein exposure was not available to the Working Group.

1.4 Occurrence and exposure

1.4.1 Environmental and natural occurrence

The incomplete combustion and heating of cooking oils produce acrolein, as does the photochemical degradation of 1,3-butadiene in the environment. Acrolein may also be formed endogenously (Faroon et al., 2008; see also Nath & Chung, 1994). Zhang et al. (2018) cited several sources of endogenous acrolein formation, the most important of which include the reactions of myeloperoxidase on hydroxyl-amino acids such as threonine, and the oxidation of spermine and spermidine by amine oxidase (Stevens & Maier, 2008), while other endogenous sources include peroxidation of polyunsaturated fatty acids (Uchida et al., 1998a) and oxidative ring opening of the anticancer drug cyclophosphamide and other oxazaphosphorine drugs such as ifosfamide (Brock et al., 1979). No quantitative data on endogenous production of acrolein were available to the Working Group.

Landfill leachate contained acrolein at a concentration of 0.07–2.1 ppm [0.07–2.1 mg/L] (Faroon et al., 2008). The US EPA lists acrolein as a pollutant in National Priority Superfund sites in at least 16 USA states; acrolein was detected at a concentration of 0.006–1.3 ppm [0.006–1.3 mg/L] in groundwater at half of these sites (Faroon et al., 2008). Because acrolein is highly reactive, it is not expected to bioaccumulate, but it can be formed in the environment as a breakdown product of other chemicals, in addition to occurring as a result of the direct emission of acrolein as a combustion product (Faroon et al., 2008).

1.4.2 Exposure in the general population

The most important sources of acrolein exposure in the general population include tobacco use and cooking with oil at high temperatures. Forest and residential fires, vehicle exhaust, and incinerators are other significant sources of acrolein exposure.

(a) Food, beverages, and cooking emissions

Acrolein concentrations measured in food, beverages, and cooking emissions are presented in Table 1.1.

Most food items are not considered to be major sources of acrolein in the general population. However, higher concentrations have been reported in certain food items, including frying fats and oils (mean acrolein concentration, 276 µg/L; maximum, 1389 µg/L; $n = 15$; see Table 1.1), and cooking food in hot oil has been shown to produce emissions containing acrolein, which can be a significant source of exposure.

An analysis by Umano & Shibamoto (1987) revealed that the two most important factors in the production of acrolein during cooking were cooking duration and cooking temperature, both of which were positively associated with acrolein production; the type of oil (i.e. sunflower, beef fat, soybean, corn, sesame, and olive, in increasing order of acrolein production) was less important. While little acrolein was formed under 240 °C, emissions increased 10-fold when the temperature was increased from 280 to 300 °C, and 3-fold from 300 to 320 °C. Temperatures in home

Table 1.1 Concentrations of acrolein in food, beverages, and cooking emissions

Item	Acrolein concentration (mean or range)	Country of study or purchase	Reference
Food			
French fries	1.97–4.85 mg/kg	Brazil	Osório & de Lourdes Cardeal (2011)
Domiati cheese	0.29–1.3 mg/kg	Egypt	Collin et al. (1993)
Doughnuts	0.1–0.9 mg/kg	USA	Lane & Smathers (1991)
Fried fish coating	0.1 mg/kg	USA	Lane & Smathers (1991)
Fruits	< 0.01–0.05 mg/kg	NR	Feron et al. (1991)
Vegetables	≤ 0.59 mg/kg	NR	Feron et al. (1991)
Frying fats and oils (15 tested)	Mean, 276; max., 1389 mg/kg	Germany	Kächele et al. (2014)
Beverages			
Lager beer, fresh (3 bottlings tested)	Mean, 1.6 µg/L	UK	Greenhoff & Wheeler (1981)
Lager beer, force aged (3 bottlings tested)	Mean, 5.05 µg/L	UK	Greenhoff & Wheeler (1981)
Lager beer (22 tested)	< 2.5–5.4 µg/L	Brazil	Hernandes et al. (2019)
Beer (9 tested)	All < 14 µg/L (LOD)	Germany	Kächele et al. (2014)
Wine (23 tested)	Mean, 0.7; max., 8.8 µg/L	Germany	Kächele et al. (2014)
Merlot wine	Mean, 15.9; max., 29.8 µg/L	Brazil	Ferreira et al. (2018)
Brandy/cognac (11 tested)	1.42–1.5 mg/L [1420–1500 µg/L]	Armenia	Panosyan et al. (2001)
Whiskey/bourbon (3 tested)	0.67–11.1 ppm [670–1110 µg/L]	USA	Miller & Danielson (1988)
Whiskey (15 tested)	Mean, 252; max., 915 µg/L	Germany	Kächele et al. (2014)
Vodka (4 tested)	All < 14 µg/L (LOD)	Germany	Kächele et al. (2014)
Absinthe (5 tested)	All < 14 µg/L (LOD)	Germany	Kächele et al. (2014)
Fruit spirits (28 tested)	Mean, 591; max., 2394 µg/L	Germany	Kächele et al. (2014)
Tequila (7 tested)	Mean, 404; max., 1205 µg/L	Germany	Kächele et al. (2014)
Asian spirits (16 tested)	Mean, 54; max., 477 µg/L	Germany	Kächele et al. (2014)
Grape marc (10 tested)	Mean, 487; max., 1808 µg/L	Germany	Kächele et al. (2014)
Mineral & table water (10 bottles)	All < 14 µg/L (LOD)	Germany	Kächele et al. (2014)
Water stored in cisterns	< 3–115 µg/L	Brazil	de Oliveira Moura et al. (2019)
Item (cooking oil)	**Acrolein concentration in air (µg/m^3)**	**Emission rate of acrolein (mg/kg food per hour)**	**Reference**
Emissions during cooking (for 5 minutes)			
Oil only (soybean)	57.9	26.67	Seaman et al. (2009)
French fries (soybean)	41.8	17.81	Seaman et al. (2009)
Chicken strips (soybean)	40	16.06	Seaman et al. (2009)
Battered fish (soybean)	64.5	27.04	Seaman et al. (2009)
Doughnuts (soybean)	32.4	12.9	Seaman et al. (2009)
Doughnuts (canola)	31.6	13.15	Seaman et al. (2009)
Doughnuts (corn)	26.4	10.68	Seaman et al. (2009)
Doughnuts (olive)	29.2	11.79	Seaman et al. (2009)
Doughnuts (no oil)	1.83	0.19	Seaman et al. (2009)

LOD, limit of detection; max., maximum; NR, not reported; ppm, parts per million.

cooking were reported to rarely exceed 200 °C. However, Hecht et al. reported that, among non-smoking Chinese women in Singapore who cook at much higher temperatures or cook more frequently than controls (women randomly selected from the Chinese Health Study), concentrations of urinary acrolein metabolites were about 50% higher than among women who cooked less frequently (see Table 1.2; Hecht et al., 2010, 2015).

Beer typically contains acrolein at a concentration of 1–5 µg/L, although higher concentrations (up to 25 µg/L) are found in the early stages of beer making, before processing to make the final product; the acrolein in other alcoholic drinks ranges from 0.02 to 11 µg/L, (Greenhoff & Wheeler, 1981; Ferreira et al., 2018; Hernandes et al., 2019). A study of 117 alcoholic beverages found that over half had detectable levels of acrolein (limit of detection, 14 µg/L), some at much higher concentrations (Kächele et al., 2014). None of 9 beers, 4 vodkas, and 5 absinthes tested had detectable concentrations, nor did only 21 out of 23 wines tested. However, over 85% of the 15 whiskey samples, 7 tequilas, 28 fruit spirits, and 10 grape marc samples tested were positive; the average acrolein concentration in all the samples was 276 µg/L, but some tequilas, fruit spirits, and grape marc were over 1000 µg/L (Kächele et al., 2014). Rainwater to be used as drinking-water and stored in polyethylene cisterns in Brazil was found to contain acrolein in 75% of the 36 cisterns tested, with concentrations up to 115 µg/L (de Oliveira Moura et al., 2019). No acrolein was detected in 10 bottles of mineral and table water in Germany (Kächele et al., 2014).

(b) Tobacco products and tobacco-related products

Acrolein is present in smoke from cigarettes, cigars, bidis, and hookahs, as well as in emissions from electronic cigarettes and "heatsticks" (Table 1.3). Average concentrations in mainstream smoke from bidis and small cigars are slightly higher than in cigarette smoke. The apparent variability in acrolein yield in mainstream smoke from cigarettes smoked according to the outdated ISO 3308 method is greatly reduced when using the Health Canada Intensive method recommended by WHO, with most products producing 100–200 µg of acrolein/rod. In general, sugars (which are natural components of tobacco and which may also be added during the manufacturing process) increase the emissions of acrolein in tobacco smoke by 20–70% (Talhout et al., 2006). Hookahs (waterpipes, narghile) produce approximately 900 µg of acrolein in mainstream smoke and 1100 µg of acrolein in sidestream smoke per session, which lasts for approximately 1 hour, meaning that secondhand acrolein exposure from waterpipes may exceed that from cigarettes, at 140 µg/rod (Al Rashidi et al., 2008; Daher et al., 2010). Although the fluid in electronic cigarettes ("e-liquid") does not contain acrolein, it is apparently formed during the heating of the fluid, at an amount that is dependent on the composition of the fluid and the temperature of the coil (Conklin et al., 2018); a single puff contains 3–15 ng of acrolein (Herrington & Myers, 2015). Increasing voltage from 3.8 V to 4.8 V increased the acrolein yield more than 4-fold (Kosmider et al., 2014), and the addition of humectants, sweeteners, and flavourings increased the production of acrolein from nondetectable to several hundred micrograms per gram of e-liquid (Khlystov & Samburova, 2016). [The Working Group noted that newer devices contain voltage/temperature controls that can increase the delivery of nicotine and also enhance acrolein production, indicating that acrolein exposures among current users may be much greater than reflected in the recent literature.] Heatsticks, which have been available in over 40 countries for the past 5 years, each discharge about 5 µg of acrolein in mainstream and 0.7 µg of acrolein in sidestream emissions (Cancelada et al., 2019). The acrolein exposure

Table 1.2 Levels of acrolein metabolite biomarkers measured in human urine

Study, country	Group (if applicable)	No. of samples HPMA/CEMA		HPMA Geometric mean (25th, 75th percentile) µg/g creatinine (unless otherwise stated)		CEMA Geometric mean (25th, 75th percentile) µg/g creatinine (unless otherwise stated)		Reference
		Unexposed[a]	Exposed	Unexposed	Exposed	Unexposed	Exposed	
Cigarette smoking								
NHANES 2005–2006, USA		2467/NR	601/NR	219 (140, 353)[b]	1089 (469, 2012)	78.8 (51.8, 121)	203 (111, 338)	Alwis et al. (2015)
PATH Study, USA	Cigarettes only	1571/1517	2284/2176	272.4	1143.5	98.14	271.5	Goniewicz et al. (2018)
European multicentre observational study, Germany, Switzerland, and UK	< 10 cigarettes/day		467/NR		1.12 mg/24 h			Lindner et al. (2011)
	10–19 cigarettes/day		557/NR		2.10 mg/24 h			
	≥ 20 cigarettes/day		135/NR		2.98 mg/24 h			
German university study		54/NR	40/NR	146[b]	884[b]			Eckert et al. (2011)
Multiethnic cohort study, USA	African American		362/NR		4123 (2341, 6808) [911 (517, 1505)][c,d]			Park et al. (2015)
	Native Hawaiian		329/NR		6007 (3947, 9606) [1328 (872, 2123)][c,d]			
	White		438/NR		6738 (3885, 1057) [1489 (859, 2422)][c,d]			
	Latino		449/NR		3480 (186, 5908) [769 (412, 1306)][c,d]			
	Japanese American		704/NR		5344 (3163, 8596) [11 851 (699, 1900)][c,d]			

Table 1.2 (continued)

Study, country	Group (if applicable)	No. of samples HPMA/CEMA		HPMA Geometric mean (25th, 75th percentile) µg/g creatinine (unless otherwise stated)		CEMA Geometric mean (25th, 75th percentile) µg/g creatinine (unless otherwise stated)		Reference
		Unexposed[a]	Exposed	Unexposed	Exposed	Unexposed	Exposed	
Betel-quid chewing								
Healthy subjects in a study of smoking, betel quid chewing and oral cancer, Taiwan, China	Cigarettes only		111/NR		5.8 [1282][e]			Tsou et al. (2019)
	Betel quid only		12/NR		3.6 [796][e]			
	Cigarettes + betel quid		107/NR		8.9 [1967][e]			
E-cigarettes								
PATH Study, USA	E-cigarettes only	1571/1517	212/198	272	315	98	108	Goniewicz et al. (2018)
	Cigarettes only				1144		272	
	E-cigarettes + cigarettes		767/739		1318		302	
Cooking								
Study of Chinese female regular home cookers, Singapore	Frequent home cooking vs random	50/NR	54/NR	1370 [303][d]	1959 [433][d]			Hecht et al. (2010)
	Cook > 7×/wk vs < 1×/wk	90/NR	95/NR	1901 [420][d]	2600 [575][d]			
Non-source–related								
Shanghai cohort Study, China	Control participants		392/NR		6712 (5845, 7707) [1483 (1292, 1703)][d]			Yuan et al. (2012)
National Children's Study, USA	Pregnant women	488/NR		240 µg/L[b]		71.8 µg/L[b]		Boyle et al. (2016)

Table 1.2 (continued)

Study, country	Group (if applicable)	No. of samples HPMA/CEMA		HPMA Geometric mean (25th, 75th percentile) µg/g creatinine (unless otherwise stated)		CEMA Geometric mean (25th, 75th percentile) µg/g creatinine (unless otherwise stated)		Reference
		Unexposed[a]	Exposed	Unexposed	Exposed	Unexposed	Exposed	
Pregnant women, Guatemala	Fasting	23/NR		268 (178, 399)[c]				Weinstein et al. (2017)
	After sauna	23/NR		572 (429, 1041)[c]				

CEMA, *N*-acetyl-S-(2-carboxyethyl)-L-cysteine (2-carboxyethylmercapturic acid); e-cigarette, electronic cigarette; HPMA, *N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine (3-hydroxypropyl-mercapturic acid); NR, not reported; vs, versus.

[a] Unexposed/exposed applies to exposures in subheadings (e.g. *Cigarette smoking*).

[b] Median.

[c] Median (interquartile range).

[d] pmol/mg creatinine [converted to µg/g creatinine].

[e] µmol/g creatinine [converted to µg/g creatinine].

Table 1.3 Concentrations of acrolein in smoke from tobacco products

Product and method details	Reported measurements				Reference
	Method: ISO 3308 (µg/product)		Method: Health Canada Intensive (µg/product)		
	Range	Median	Range	Median	
Cigarettes					
12 brands, mainstream smoke			51–223[a]	163[a]	Borgerding et al. (2000)
12 brands, sidestream smoke			342–523[b]	412[b]	Borgerding et al. (2000)
6 Thai & 2 US brands (90% market share)	79.9–181				Mitacek et al. (2002)
35 brands	30.8–82.6		139–213		Cecil et al. (2017)
3 brands + 1 reference cigarette	24.9–52.2	48.5	100–125	117	Eldridge et al. (2015)
Cigars					
Sheet-wrapped cigars (15 brands)	34.3–105		105–185		Cecil et al. (2017)
Bidis					
76 mm unfiltered bidi – one selected sample	67 µg				Hoffmann et al. (1974)
	Mean total yield	**Mean mainstream yield**	**Mean sidestream yield**	**Sidestream/mainstream yield ratio**	
Narghile/hookah (waterpipe)					
Narghile	145.5 µg/g tobacco				Al Rashidi et al. (2008)
Narghile, per session		892 µg			Al Rashidi et al. (2008)
Narghile, per session			1135 µg	0.7	Daher et al. (2010)
	Various metrics				
Electronic cigarettes					
Aerosol	0.003–0.015 µg/mL (≈20–230 g of acrolein per cigarette assuming 400–500 × 40 mL puffs)				Herrington & Myers (2015)
Aerosol from neat PG	< LOD (0.03×10^{-3} µg/puff)				Conklin et al. (2018)
Aerosol from neat VG	0.08 ± 0.002 µg/puff				Conklin et al. (2018)
Aerosol from 25–75% PG in VG	0.04 µg/puff				Conklin et al. (2018)
Aerosol – 'brand I' (unflavoured)	ND				Khlystov & Samburova (2016)
Aerosol – 'brand III' (unflavoured)	ND				Khlystov & Samburova (2016)
Aerosol – 'brand I' (flavoured)	172 ± 27 to 347 ± 37 µg/g of e-liquid				Khlystov & Samburova (2016)
Aerosol – 'brand II' (flavoured)	ND				Khlystov & Samburova (2016)
Aerosol – 'brand II' (flavoured)	ND to 237 ± 61 µg/g of e-liquid				Khlystov & Samburova (2016)

Table 1.3 (continued)

Product and method details	Reported measurements				Reference
	Mean mainstream emissions	Mean sidestream emissions	Mean environmental concentration	Range of % of conventional cigarette	
"Heatsticks"					
Heated tobacco device: "iQOS blue"[c]	5.4 ± 0.7 µg per heatstick	0.6 ± 0.3 µg per heatstick			Cancelada et al. (2019)
Heated tobacco device: "iQOS amber"	4.9 ± 0.6 µg per heatstick	0.8 ± 0.3 µg per heatstick			Cancelada et al. (2019)
Heated tobacco device: "iQOS yellow"	5.3 ± 0.7 µg per heatstick	0.7 ± 0.3 µg per heatstick			Cancelada et al. (2019)
Heated tobacco device: "iQOS"			4.6 ± 3.2 µg/m^3	1.8–2.3%	Ruprecht et al. (2017)

LOD, limit of detection; ND, not detected; PG, propylene glycol; VG, vegetable glycerin.

[a] Massachusetts machine smoking protocol.

[b] Sidestream smoke, Massachusetts machine smoking protocol. The median value was calculated by multiplying the median value for mainstream smoke by the median value for the sidestream/mainstream smoke ratios for the 12 commercial cigarette brands, which was 2.53.

[c] iQOS is a brand name.

from the heatsticks is reduced by a factor of about 10 compared with conventional cigarettes (Lachenmeier et al., 2018).

While acrolein metabolites (the mercapturic acids HPMA and CEMA) have been detected in the urine of 99% of Americans (Alwis et al., 2015), concentrations of these metabolites were three to five times higher in smokers than in non-smokers (Eckert et al., 2011; Lindner et al., 2011; Alwis et al., 2015; Goniewicz et al., 2018; Table 1.2), with concentrations increasing with the number of cigarettes smoked per day (Lindner et al., 2011) and with increasing urinary concentration of cotinine (a metabolite of nicotine) (Alwis et al., 2015). Acrolein metabolite concentrations were slightly higher in electronic-cigarette smokers than in non-smokers, but four times higher in dual users of cigarettes and electronic cigarettes (Goniewicz et al., 2018). Passive exposure to secondhand smoke led to comparable increases in urinary acrolein metabolites among hookah smokers and non-smokers alike after visiting a hookah lounge or attending a hookah social event at home (Kassem et al., 2018), probably due to the abovementioned high sidestream emission of acrolein from hookahs. Levels of urinary acrolein metabolites were significantly higher in children living with daily hookah smokers than in children from non-smoking homes (Kassem et al., 2014).

Park et al. (2015) reported significantly different concentrations of acrolein metabolites for smokers from different racial and ethnic groups. Similarly, the National Health and Nutrition Examination Survey (NHANES) found that the 25th percentile of the HPMA concentrations for tobacco smokers was greater than the 75th percentile for non-tobacco users, for all age groups, and that HPMA concentrations among non-tobacco users were similar for both sexes, and were lower for non-Hispanic White people and non-Hispanic Black people than for Mexican Americans or for people of other Hispanic origins or for other or multiple ethnicities. However, among Mexican Americans, metabolite concentrations for smokers were much lower (36%) than those of non-Hispanic White people (Alwis et al., 2015).

In Taiwan, China, healthy subjects who chewed betel quid had HPMA concentrations that were significantly elevated, but significantly lower than in cigarette smokers, and those who both smoked cigarettes and chewed betel quid had the highest urinary HPMA levels (3600, 5800, and 8900 pmol/mg creatinine [796, 1282, and 1967 µg/g creatinine], respectively, see Table 1.2 (see also Table 2.1); Tsou et al., 2019). In contrast, in patients with oral squamous cell carcinoma who both smoked cigarettes and chewed betel quid, urinary levels of HPMA were only 7% those of healthy people with matched smoking and betel-quid use history, despite the fact that their NNAL (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol)/creatinine levels were comparable.

(c) Indoor air

(i) In the home

Activities in the home, especially tobacco smoking and cooking with oils and fats heated to high temperatures, are the primary indoor source of acrolein (see Section 1.4.2(a)). Cooking can increase air concentrations of acrolein by 26 to 64 µg/m^3 (Seaman et al., 2009). Other indoor sources of acrolein include gas stoves, wood-burning fireplaces and stoves, burning candles, and incense. When indoor air in the home and outdoor air are measured simultaneously, the indoor concentration of acrolein is usually 2–10 times greater than the outdoor concentration. Azuma et al. (2016) reviewed surveys of Japanese homes and reported an average indoor concentration of acrolein of 0.267 µg/m^3, which was three times higher than the outdoor concentration, but much lower than that found in homes in other countries. A survey of 130 homes in Beijing, China, reported much higher

concentrations, with an average of 2.1 μg/m³, although neither smoking nor cooking occurred during the sampling period (Liu et al., 2014). A study of acrolein concentrations outdoors and inside occupied homes and unoccupied, newly constructed, model homes (expected to have high emissions from construction materials) reported morning indoor concentrations in occupied homes (2–8 μg/m³) that were generally more than 10 times higher than the outdoor concentrations (0.1–0.3 μg/m³) in Davis and surrounding towns in California, USA. Similarly, in new model homes, the indoor concentrations were also 10 times higher than the outdoor concentrations. The outdoor concentrations in occupied homes in Los Angeles averaged 5–10 times higher than those around Davis (0.8–1.7 μg/m³), but indoor concentrations were comparable (Seaman et al., 2007). The greatest increases in indoor acrolein concentrations in occupied homes in all three counties studied (Los Angeles, Placer, Yolo) were associated with cooking with fats and oils. Homes with frequent, regular cooking activity had the highest morning acrolein levels. In four unoccupied new houses, indoor acrolein concentrations were increased by 10-fold compared with those outdoors, although no cooking or smoking had taken place. However, the particle board and lumber used to construct these houses was found to emit acrolein (1–8 ng acrolein/g). The Relationship of Indoor, Outdoor and Personal Air (RIOPA) study of 398 homes in the USA found quite different average acrolein concentrations in the three cities studied. The average concentration in these cities ranged from 1.0 μg/m³ in Elizabeth, New Jersey, and 1.2 μg/m³ in Los Angeles, California, to 3.1 μg/m³ in Houston, Texas (Weisel et al., 2005).

In Prince Edward Island, Canada, acrolein concentrations were consistently two and a half times higher in homes with smokers than in homes without. Similarly, concentrations were higher in homes with new carpets than in those without new carpets. No significance was found for the presence of wood stoves, the type of heating, or painting (Gilbert et al., 2005). Subsequent studies of over 250 homes in Edmonton, Halifax, Regina, and Windsor, Canada, also found that homes with smokers had distinctly higher concentrations of acrolein than homes without, and also that indoor concentrations of acrolein were higher than outdoor concentrations; median indoor concentrations ranged from 1.3 to 8.1 μg/m³, while paired outdoor concentrations were more than 60% lower (ranging from 0.2 to 2.2 μg/m³) (Health Canada, 2020).

Other sources of acrolein in homes include burning incense and using kilns. Burning incense increases acrolein concentrations by 2.67–8.14 ppm/g [6000–19 000 μg/m³ per g] burned (Lin & Wang, 1994). Hirtle et al. (1998) measured acrolein concentrations greater than 20 ppb [46 μg/m³] in three homes with kilns.

Overall, the acrolein concentrations in homes ranged from less than 0.01 to 39 μg/m³, with median concentrations of 1 to 8 μg/m³.

(ii) Primary schools

In a study of 408 primary schools (attended by 6590 students) in France, 14% of the children were found to be exposed to acrolein at concentrations greater than 1.55 μg/m³ in their classrooms (Annesi-Maesano et al., 2012). [The Working Group noted that the aldehyde (acrolein, formaldehyde, and acetaldehyde) concentrations inside the classrooms in this study were greater than the outdoor concentrations in the same cities, which indicates that there might be indoor sources, but these were not identified. Possibilities include smoking by staff or emissions from building materials.] Similarly, a study of seven schoolrooms in Mira Loma, California, USA, reported that acrolein concentrations in the classroom were greater than outdoor concentrations. The authors attributed the higher indoor acrolein concentrations to building elements

such as carpet, drywall, and adhesives (Sawant et al., 2004).

(iii) Hospitality sites

Hospitality sites where smoking was permitted had higher indoor concentrations of acrolein. Measurements made in the 1970s and early 1980s in France found acrolein concentrations in cafés to be between 12 and 43 µg/m³. Acrolein concentrations in restaurants and taverns in the Netherlands were between 1 and 8 µg/m³, and concentrations in a car with three smokers increased from 13 µg/m³ with the windows open to ten times that level when the windows were closed (Triebig & Zober, 1984). Löfroth et al. (1989) reported acrolein concentrations on two evenings to be 21 and 24 µg/m³ in a tavern in the USA. [The Working Group noted that the advent of smoke-free regulations has presumably lowered these concentrations substantially.]

(d) Outdoor air pollution

The major sources of acrolein in the outdoor environment are forest fires and exhaust from motor vehicles and aircraft. Acrolein is released directly into the ambient air from vehicle exhaust and is also formed by photo-oxidation of 1,3-butadiene and other hydrocarbons (Faroon et al., 2008,). These reactions comprised an estimated 39% of total acrolein emissions in California, USA, in 2012 (OEHHA, 2018). Other sources of acrolein, which may be important in nearby local areas, include emissions from manufacturing processes such as pulp and paper, coal/gas/oil-fired power plants, waste-disposal emission, and the volatilization of biocides.

The seasonal effect for acrolein is opposite to that for many other pollutants in that concentrations decrease in winter. For example, the median summer concentration measured in several European cites was 2 µg/m³, while the median winter concentration was 0.6 µg/m³ (Campagnolo et al., 2017), which may be partially attributable to the decline in frequency of photochemical reactions with seasonal reduction in solar intensity. Outdoor concentrations of acrolein in the USA are typically 0.5–3.2 ppb [1–7 µg/m³] (Faroon et al., 2008), although acrolein concentrations measured outside 124 homes in Houston, Texas, averaged 17.9 µg/m³ (Weisel et al., 2005). Median concentrations in California were 0.041 µg/m³ in coastal areas, 0.068 µg/m³ in intermediate areas, 0.101 µg/m³ in the San Francisco Bay area, and 0.32 µg/m³ in the Los Angeles air basin (Cahill, 2014); concentrations outside 15 homes averaged 0.60 µg/m³ (Seaman et al., 2007). Based on measurements throughout the state, acrolein exposures in California increased between 2004 (0.51 ppb) [1.2 µg/m³] and 2014 (0.66 ppb) [1.5 µg/m³], although concentrations of volatile organic compounds other than aldehydes have declined, and acrolein emissions from gasoline-related sources decreased by two thirds between 1996 and 2012. The increase in acrolein emissions from non-gasoline related sources in 2012 was attributed primarily to a higher estimate of emissions from waste disposal (OEHHA, 2018).

Exhaust from gasoline- and diesel-powered vehicles is one of the most important, ubiquitous sources of acrolein in outdoor air. With the introduction of engine and fuel improvements due to stricter regulations to reduce exhaust emissions, this contribution has declined in North America and Europe. Schauer et al. (2002) reported that tailpipe emissions of acrolein from several gasoline-powered vehicles equipped with early catalytic converters (1981–1994) were greatly reduced compared with those from vehicles without these converters (1969–1970), from 3800 to 60 µg/km. The estimated acrolein emissions from on-road vehicles in the 48 contiguous states of the USA in 2007 were less than half the estimated emissions in 1996 (10 185 versus 21 266 metric tonnes/year). This decrease was almost entirely due to reductions from gasoline-powered vehicles and was attributed to changes in gasoline formulation

and implementation of stricter Tier 2 emission standards for light-duty vehicles (IARC, 2013).

(i) Local sources

Local sources may increase acrolein concentrations. The importance of nearby industry and traffic is illustrated by the results of 2 years of sampling in the Pittsburgh area, Pennsylvania, USA. Four locations were sampled every sixth day: one near downtown (near the city centre) with heavy traffic; one remote from both traffic and industry; and two in residential areas within 0.8 km of heavy industry. In the two residential areas near industry, acrolein concentrations were approximately double those in the rural area, while the downtown area had the highest average and 95th percentile concentrations (Logue et al., 2010). Other evidence of the importance of local sources included measurements made in the vicinity of a petrochemical plant: acrolein concentrations were 640 μg/m^3 at a distance of 1 km, and 2000 μg/m^3 at 100 m. Concentrations measured 50 m from a perfume factory ranged from 40 to 480 μg/m^3 (Izmerov, 1984).

Acrolein is used as a biocide in irrigation canals and volatilizes quickly after application. In the San Joaquin Valley of California, USA, a major agricultural area through which pass the 640 km California Aqueduct and numerous irrigation canals, an estimated 90 tonnes of acrolein were volatilized into the air in 2001 (CEPA, 2002). The estimate for 2012 was 33 tonnes (OEHHA, 2018). [The Working Group noted that no measurements of ambient acrolein concentrations were made while acrolein was in use, but these could affect local concentrations.]

(ii) Diesel and biodiesel

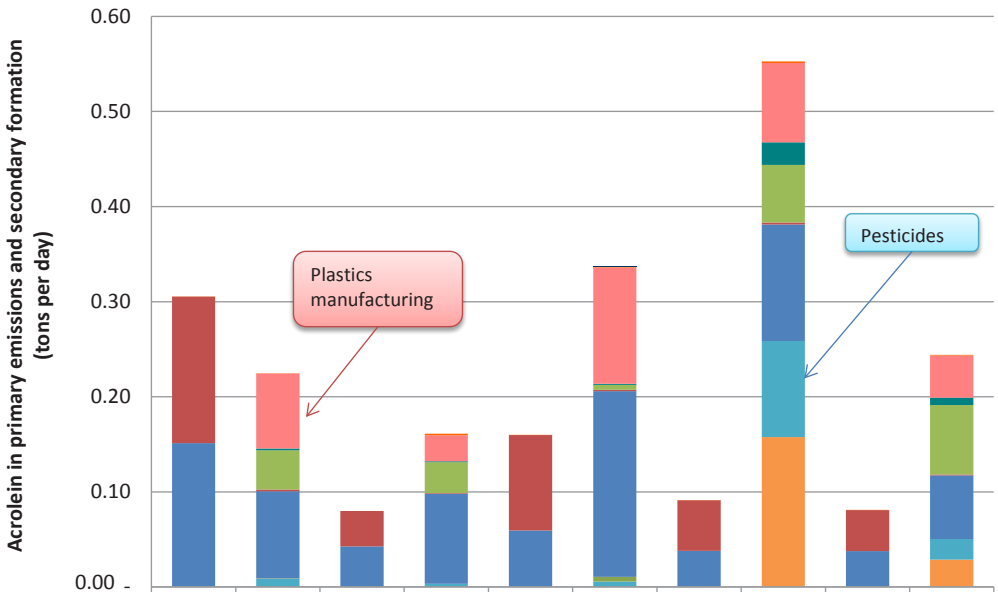
A study of emissions from the engines of two heavy-duty trucks found that both pollution-control technology and fuel were major determinants of acrolein emissions (Cahill & Okamoto, 2012). The truck engine built in 2008 was equipped with a diesel oxidation catalyst/diesel particulate filter, while the truck engine built in 2000 was not, although it complied with the environmental regulations of the time. Emissions from the truck engine without pollution controls (without the catalyst/filter) were 2–10 times greater than those from the engine with these controls, depending on the fuel type used; the difference was least for ultra-low sulfur diesel fuel (ULSD; a petroleum product) and greatest for soy biodiesel blend (50% soy biodiesel and 50% ULSD). More fuels were tested with the 2008 engine than with the 2000 engine. These included: ULSD, a soy biofuel, an animal biofuel, a "renewable" fuel (hydrotreated biofuel), and 50:50 blends of each of the biofuels and ULSD. Acrolein emissions from the renewable fuels (hydrotreated biofuel, and 50:50 hydrotreated biofuel and ULSD) were comparable to those from the petroleum-based fuel (ULSD); the animal biofuel and blend emitted 40% more acrolein than the ULSD fuel, and the soy biofuel emitted the most acrolein (two and a half to three times that of the ULSD).

(iii) Gasoline and other sources of acrolein

Gasoline- and diesel-powered road motor vehicles are the major quantified source of acrolein in outdoor air in Canada. Annual releases from these were estimated to be 209 000 to 2 730 000 kg. However, unquantified but possibly greater sources of acrolein are other vehicles that are not fitted with pollution-control devices, such as aircraft, railway and marine vehicles, as well as off-road motor vehicles, lawnmowers, and snowblowers. Other major anthropogenic sources include the oriented strand board industry (25 664 kg/year), pulp and paper mills (18 735 kg/year), waste incineration (2435 kg/year), and coal-based power plants (467–17 504 kg/year) (Environment Canada and Health Canada, 2000).

A recent evaluation of the sources of acrolein emissions in outdoor air in California, USA, reported that the contribution of gasoline, as both a primary and secondary pollutant,

Fig. 1.1 Primary and secondary sources of acrolein, 2012



	SC (G)	SC (NG)	SD (G)	SD (NG)	SF (G)	SF (NG)	SJV (G)	SJV (NG)	SV (G)	SV (NG)
CLEANING AND SURFACE COATINGS	-	-	-	-	-	< 0.01	-	-	-	-
FUEL COMBUSTION	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
INDUSTRIAL PROCESSES	-	0.08	-	0.03	-	0.12	-	0.08	-	0.04
MISCELLANEOUS PROCESSES	-	< 0.01	-	< 0.01	-	< 0.01	-	0.02	-	< 0.01
NATURAL SOURCES	-	0.04	-	0.03	-	< 0.01	-	0.06	-	0.07
ON-ROAD MOTOR VEHICLES	0.15	< 0.01	0.04	< 0.01	0.10	< 0.01	0.05	< 0.01	0.04	< 0.01
OTHER MOBILE SOURCES	0.15	0.09	0.04	0.09	0.06	0.20	0.04	0.12	0.04	0.07
PETROLEUM PRODUCTION AND DISTRIBUTION	-	< 0.01	-	-	-	< 0.01	-	< 0.01	-	< 0.01
SOLVENT EVAPORATION	-	< 0.01	-	< 0.01	-	< 0.01	-	0.10	-	0.02
WASTE DISPOSAL	-	-	-	-	-	-	-	0.16	-	0.03

G, gasoline sources; NG, non-gasoline sources; SC, South Coast (Los Angeles and surrounding counties); SD, San Diego County; SF, San Francisco Bay Area; SJV, San Joaquin Valley, southern part of Central Valley, a hot, dry agricultural region with major irrigation canals; SV, Sacramento Valley, northern part of Central Valley, also agricultural.
"Other mobile sources" of gasoline-attributed emissions include recreation boats, off-road equipment including garden and lawn, (each responsible for about 40%), off-road recreational vehicles such as motorcycles and all-terrain vehicles, and agricultural equipment and fuel storage.
Each region has two bars, the first for the gasoline sources and the second for all other sources. The first bar in each region represents the portion attributable to gasoline; especially notable here is that on-road motor vehicles, which have been tightly regulated, now contribute approximately half the gasoline-attributable acrolein, while the other half comes from "other mobile sources," such as recreational boats, off-road equipment for gardens and lawns (each contributes about 40% of this category), and off-road motorcycles and all-terrain vehicles. The second bar in each set illustrates the contribution of industrial processes, natural sources, such as wildfires, waste disposal, and "solvent evaporation," which refers to the volatilization of acrolein used as a biocide. This latter contribution appears significant only in the two agricultural regions of the Central Valley, the San Joaquin Valley, and the Sacramento Valley.
From OEHHA (2018), with permission.

declined significantly from 52% in 1996 to 28% in 2012. Despite this decline, the average exposure to ambient acrolein in California increased from 0.51 to 0.66 ppb [1.2 to 1.5 μg/m^3] (OEHHA, 2018). Fig. 1.1 presents the dominant sources of acrolein in three urban and two agricultural areas of California in 2012.

1.4.3 Occupational exposure

Workers may be exposed occupationally to acrolein during its manufacture and use as a chemical intermediate (see Section 1.2). However, as for the population at large, workplace exposures to acrolein occur primarily from

the formation of acrolein during the incomplete combustion of organic material such as tobacco, cooking oils, gasoline and diesel fuel, and forest and residential fires.

The National Occupational Exposure Survey estimated that approximately 1300 workers were potentially exposed to acrolein in the USA when the study was conducted in 1981–1983. Approximately one third of these workers were mechanics and repairers. Other occupations identified with potential exposure to acrolein included painters and spray painters, machinists, sheet metal workers, chemical technicians, janitors, and water and sewage treatment plant operators (NIOSH, 1990). [The Working Group noted that the survey did not include agricultural production, mining activity, or railroad transportation. During the subsequent 40 years, occupational exposures in manufacturing in the USA have evolved significantly and these numbers have probably changed substantially due to changes in product usage, export of chemical manufacturing, and automation, to name a few examples.] Between 1993 and 2009, 8 cases of acrolein-related illness from pesticide usage were identified in Washington State and California, USA (Rodriguez et al., 2013).

Occupational exposure to acrolein in firefighting, manufacturing, welding, food processing, and traffic-related occupations is presented in Table 1.4 and detailed below.

(a) Firefighting

Firefighters are exposed to high concentrations of acrolein produced during the incomplete combustion of burning materials. Structural fires and wildland fires are fought by distinctly different crews who have different exposure profiles. The exposures of wildland firefighters and urban firefighters are presented in Table 1.4.

The two distinct phases of fighting structural fires are: (i) knockdown, when the visible flames are extinguished; and (ii) overhaul, during which smouldering material is searched for embers and hidden flames. Jankovic et al. (1991) collected short-term personal samples from 22 fires, mostly residential, in the USA and reported that half the samples from during knockdown exceeded the short-term exposure limit (STEL) for acrolein at the time – 300 ppb [690 μg/m^3] – and that the maximum value was 3200 ppb [7330 μg/m^3]. Their data were similar to those reported by Burgess et al. in 1979 and plotted in the Jankovic publication. Together, these data provided a median of 500 ppb [1100 μg/m^3], with a 95th percentile of 5000 ppb [11 000 μg/m^3] and a maximum of 15 000 ppb [34 000 μg/m^3]. During knockdown, firefighters wear a self-contained breathing apparatus; some samples collected inside the breathing mask measured as high as 900 ppb [2000 μg/m^3]. During overhaul, when a self-contained breathing apparatus is not generally worn, measured acrolein concentrations were as high as 200 ppb [500 μg/m^3] in the Jankovic publication and 300 ppb [700 μg/m^3] in a study of 25 fires in the USA by Bolstad-Johnson et al. (2000). Of the 96 30-minute samples collected by Bolstad-Johnson et al. (2000), only 7 exceeded the limit of detection (11 ppb [25 μg/m^3]). The mean for these 7 samples was 123 ppb [282 μg/m^3].

Wildland firefighters do not wear respiratory protection. The three types of wildland firefighting are: (i) initial attack – the first day of a fire, during which all but 5% of fires are extinguished; (ii) project fires – the second and successive days of fighting those few fires that continue past the first day; and (iii) prescribed burns – intentionally set and controlled fires in an established area. In the USA, Reinhardt & Ottmar (2004) reported geometric mean (GM) acrolein concentrations of 1 ppb [2 μg/m^3] during 13–14 hour shifts for the initial attack day (45 samples) and also for the subsequent days (84 samples), while the GM during prescribed burns was 9 ppb [21 μg/m^3] (11.5-hour average shift, 200 samples), and the maximum concentrations were 11, 15, and 60 ppb [25, 34, and 140 μg/m^3], respectively. Similar results for prescribed burns in the USA

Table 1.4 Occupational exposure to acrolein

Job, task, or industry	Country	No. of sites	No. of samples	Acrolein air concentration	Reference
Firefighting					
Overhaul (structure fires)	USA	25	96	Mean, 0.123 ppm [282 µg/m³] Max., 0.3 ppm [687 µg/m³]	Bolstad-Johnson et al. (2000)
Initial attack – fireline (wildfires)	USA	NR	45	Geometric mean, 5 ppb [11.5 µg/m³] Max., 11 ppb [25 µg/m³]	Reinhardt & Ottmar (2004)
Project fires – fireline (wildfires)		NR	84	Geometric mean, 2 ppb [4.6 µg/m³] Max., 16 ppb [34 µg/m³]	
Prescribed burns – fireline (wildfires)		NR	200	Geometric mean, 15 ppb [34.4 µg/m³] Max., 98 ppb [225 µg/m³]	
Prescribed burns – pre- to post-shift time-weighted averages	USA	NR	65	Mean, 0.01 ppm [22.9 µg/m³] Max., 0.041 ppm [94 µg/m³]	Slaughter et al. (2004)
Manufacturing					
Phenol-formaldehyde resins (abrasive materials)	Poland	13	NR	Range, 0–0.003 mg/m³ [0–3 µg/m³]	Pośniak et al. (2001)
Phenol-formaldehyde resins (friction linings)		11	NR	Range, 0–0.01 mg/m³ [0–10 µg/m³]	
Plastics	USA	130[a]	23[b]	Mean[c], 39 ppb [89 µg/m³] Max., 240 ppb [550 µg/m³]	OSHA (2020)
Tyres and inner tubes		1	1 [b]	Max., 11 ppb [25 µg/m³]	
Copper foundries		17 [a]	6 [b]	Mean[c], 12 ppb [27 µg/m³] Max., 45 ppb [103 µg/m³]	
Photographic equipment		4 [a]	3 [b]	Mean[c], 1.2 ppb [2.7 µg/m³] Max., 1.8 ppb [4.1 µg/m³]	
Packing and crating		3[a]	3[b]	Mean[c], 6.7 ppb [15 µg/m³] Max., 8.5 ppb [19 µg/m³]	
Potters	Canada	10	50	Range, < 28–110 ppb [< 64–252 µg/m³]	Hirtle et al. (1998)
Welding and flame cutting					
Welding (unspecified)	USA	3[a]	1[b]	Max., 21 ppb [48 µg/m³]	OSHA (2020)
Food production					
Tortilla manufacturing	USA	8[a]	6[b]	Mean[c], 14 ppb [32 µg/m³] Max., 26 ppb [60 µg/m³]	OSHA (2020)
Food production including tortilla	USA	22[a]	13[b]	Mean[c], 29 ppb [66 µg/m³] Max., 74 ppb [169 µg/m³]	OSHA (2020)
Restaurants					
University catering kitchen	Iran	16	NR	Mean, 670 ppb [1534 µg/m³] Range, 210–910 ppb [481–2084 µg/m³]	Neghab et al. (2017)

Table 1.4 (continued)

Job, task, or industry	Country	No. of sites	No. of samples	Acrolein air concentration	Reference
Restaurants, hotels and burger chains	Norway	44	NR	Mean, 10 ppb [23 µg/m³] Max., 32 ppb [73 µg/m³]	Svendsen et al. (2002)
Gasoline and diesel exhaust-related exposures					
Bus drivers	Poland	10 drivers serving 5 bus lines	NR	Range, 0.01–0.035 mg/m³ [10–35 µg/m³]	Brzeźnicki & Gromiec (2002)
Toll station operators	USA	NR	6	Range, 0.031–0.14 µg/m³	Destaillats et al. (2002)
Toll station operators	Spain	15 attendants at 2 toll stations	17	Range, < 0.5–2.75 µg/m³]	Belloc-Santaliestra et al. (2015)
Highway construction	USA	12[a]	3[b]	Mean[c], 91 ppb [208 µg/m³] Max., 155 ppb [355 µg/m³]	OSHA (2020)
Transportation	USA	12[a]	2[b]	Mean[c], 9 ppb [21 µg/m³] Max., 20 ppb [46 µg/m³]	OSHA (2020)
Waste management and incineration					
Waste management	USA	3[a]	1[b]	Max., 13 ppb [29.3 µg/m³]	OSHA (2020)
Working near burn pit and incinerator operations at an airfield	Afghanistan	3 sites within close proximity	78	Site means, 9–19 ppb [21–44 µg/m³] Site maxima, 39–140 ppb [89–321 µg/m³]	Blasch et al. (2016)

Max., maximum; NR, not reported; ppb, parts per billion.

[a] Number of measurements.

[b] Number of measurements above the limit of detection.

[c] Mean value of measurements above the limit of detection was calculated by the Working Group.

were reported by Slaughter et al. (2004): a time-weighted average (TWA) mean of 10 ppb [23 µg/m³] and a maximum of 41 ppb [94 µg/m³] for 65 samples. Task-specific (~2 hours) concentrations ranged from < 1 ppb [< 2.3 µg/m³] at the engine and 5 ppb [11 µg/m³] while igniting the fire to 30 ppb [69 µg/m³] for the holding boss and 18 ppb [41 µg/m³] for others holding the fire within prescribed boundaries. A 30-minute exposure during direct attack to extinguish flames that had escaped these boundaries was 62 ppb [140 µg/m³] (Reinhardt & Ottmar, 2004).

(b) Manufacturing operations

The manufacture of acrolein can lead to very high exposures of 43–3526 ppb [98–4075 µg/m³] (Izmerov, 1984). Various plastic-manufacturing processes use or produce acrolein. Polyethylene extrusion operations and phenol–formaldehyde resins led to exposures under 13 ppb [< 30 µg/m³] (Tikuisis et al., 1995; Pośniak et al., 2001).

(c) Welding

In a study in Ukraine, Protsenko et al. (1973) found that, while metal untreated with primer emitted no measurable acrolein, some primers coated onto metals resulted in significant acrolein emissions during both gas cutting and automatic submerged arc welding, with acrolein concentrations reaching 447 ppb [1024 µg/m³]. While exposures during welding in new ship outfitting averaged 9 ppb [21 µg/m³], with maximum values reaching 28 ppb [64 µg/m³], exceeding the occupational exposure limit (OEL) for the European Union (EU), exposures during ship repair were even higher, reaching 64 ppb [150 µg/m³], and over half the shipbreaking samples exceeded the EU OEL, with one sample at 600 ppb [1400 µg/m³]. Although in most short-term (15-minute) samples collected in engine and garage repair shops acrolein was not detectable (i.e. < 65 ppb [< 150 µg/m³]), one sample contained acrolein at 260 ppb [595 µg/m³].

(d) Food processing, traffic-related, and other occupations

Exposures (summarized in Table 1.4) measured in restaurant kitchens are highly variable, probably reflecting emissions from cooking fuels. Similarly, those who work near gasoline exhaust, such as bus drivers, garage workers, and highway construction workers, and those who work at or near incineration facilities, have highly variable and significant exposures, from 10 ppb to > 100 ppb [~23 to > 230 µg/m³]. Klochkovskii et al. (1981) reported that 37% of 800 samples collected in quarry operations in an area of the former Soviet Union exceeded permissible limits, and that acrolein concentrations in exhaust gases and workplace air averaged 900–3100 ppb [2100–7100 µg/m³].

(e) Occupational exposure to acrolein from secondhand smoke

Workers, especially hospitality workers, may also be subject to significant exposures to acrolein in places where smoking is permitted. Acrolein concentrations in a tavern in North Carolina, USA, with moderately high levels of secondhand smoke (on average, particles, 430 µg/m³; and nicotine, 66 µg/m³) were measured at 21 µg/m³ and 24 µg/m³ on two sampling trips of 3–4 hours each (Löfroth et al., 1989). In open offices where smoking was allowed in Massachusetts, USA, the 90th percentile of weekly average concentrations of nicotine was 34 µg/m³ (Hammond et al., 1995), so office exposures may exceed 5 ppb [11 µg/m³] acrolein (Mitova et al., 2016). Ayer & Yeager (1982) reported that acrolein concentrations reached > 50 ppm [114 000 µg/m³] in the smoke plume of cigarettes. Thus, secondhand smoke can be an important source of both peak and TWA exposure to acrolein.

(f) Occupational Safety and Health Administration compliance data

OSHA maintains a publicly available database of industrial hygiene samples collected in the USA as part of its compliance monitoring programme, the Chemical Exposure Health Data (OSHA, 2020). The results for 1220 samples and blanks collected by OSHA inspectors and analysed for acrolein between 1984 and 2019 provide some information from inspections for those 35 years (OSHA, 2020). These values should be compared with the 8-hour TWA OSHA permissible exposure limit of 100 ppb [250 µg/m³], the EU OEL of 20 ppb [50 µg/m³] for 8-hour TWA and 50 ppb [114 µg/m³] STEL (for 15 minutes) as well as the American Conference of Governmental Industrial Hygienists (ACGIH) ceiling value of 100 ppb [250 µg/m³]. Only about 10% of the samples were above the limit of detection, and only 3 of the nearly 200 samples collected for less than 1 hour had detectable concentrations of acrolein, but of these 2 were of concern: the 15-minute sample was 115 ppb [263 µg/m³] acrolein and the 24-minute sample was 69 ppb [158 µg/m³], both in excess of the EU short-term limit of 50 ppb [120 µg/m³]; the limit of detection in air for these shorter-timed samples would have been higher than that for the 8 hour samples, but these values were not in the database. Because of the intense irritation caused by acrolein, the ACGIH recommends neither an 8 hour nor a 15-minute STEL, but, rather, a ceiling of 100 ppb [250 µg/m³] that should never be exceeded. [The Working Group noted that, while none of the OSHA samples contained detectable levels of acrolein after such short exposures, the higher concentrations clearly indicated that this recommendation was exceeded for many samples.] Of the samples with detectable levels of acrolein, 40% exceeded the EU OEL of 20 ppb [50 µg/m³], and half of these samples contained acrolein at more than twice that OEL (Table 1.4).

The highest acrolein concentration reported was from samples collected in late 2018 at a company that manufactured plastic pipes and pipe fittings. Four workers wore the sampling equipment for 90–180 minutes and their exposure concentrations were less than detectable, 17, 25, and 240 ppb [39, 57, and 550 µg/m³] (sampling times were 90, 140, 180, and 170 minutes, respectively). Only 17% of the 134 personal samples collected at approximately three dozen plastic-manufacturing establishments were above the limit of detection. Those samples that were detectable ranged from 3 to 240 ppb [7 to 550 µg/m³] acrolein, with an average of 39 ppb [89 µg/m³] and a median of 21 ppb [48 µg/m³]; one 24-minute sample averaged 69 ppb [158 µg/m³], above the EU STEL of 50 ppb [120 µg/m³] (Table 1.4; OSHA, 2020).

Over half of the personal samples collected from food production workers had detectable concentrations of acrolein, and both the mean and median values of those samples (29 and 25 ppb [66 and 57 µg/m³], respectively) exceeded the EU OEL of 20 ppb [50 µg/m³] (OSHA, 2020).

1.5 Regulations and guidelines

1.5.1 Exposure limits and guidelines

(a) Occupational exposure limits

Acrolein is a severe irritant to the eyes, mucous membranes, and the respiratory tract at concentrations lower than 1 ppm, and this is the basis for OELs. At higher concentrations, acrolein can cause pulmonary oedema and death (10 ppm; 23.3 mg/m³) (ATSDR, 2014; ACGIH, 2019).

In 1946, the ACGIH recommended that 8-hour TWA exposure to acrolein should not exceed 0.5 ppm [1100 µg/m³]. This value was lowered to 0.1 ppm [230 µg/m³] in 1963. In 1976, a STEL of 0.3 ppm [690 µg/m³] was added to this recommendation, and in 1998 both the TWA and the STEL were replaced by a ceiling value of 0.1 ppm [230 µg/m³] that should not be exceeded

for any duration. These ACGIH threshold limit values were intended as recommendations to industrial hygienists but have been adopted by many countries as OELs directly, by reference, or as the basis upon which national OELs were developed. Currently the EU has an 8-hour TWA OEL of 0.02 ppm or 0.05 mg/m^3 and a STEL of 0.05 ppm or 0.12 mg/m^3 (European Commission, 2017). Within the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) registration of acrolein, the derived no-effect level (DNEL) of long-term exposed workers was set at 0.2 mg/m^3 for both local and systemic effects, and the DNEL for long-term skin exposure at 0.08 mg/kg body weight (bw) per day (ECHA, 2020).

Table 1.5 presents the OELs for various countries. Many countries use the EU OEL of 0.02 ppm [0.05 mg/m^3], or the older ACGIH OEL (TWA, 0.1; STEL, 0.3) or the current ACGIH ceiling value of 0.1 ppm [0.23 mg/m^3].

(b) Environmental exposure limits

The US EPA reference concentration for inhalation exposures is 2×10^{-5} mg/m^3, and the reference dose for oral exposures is 0.5 µg/kg per day (US EPA, 2003). The US Agency for Toxic Substances and Disease Registry (ATSDR) set the minimal risk level for ingestion of acrolein at 4 µg/kg per day for 15–364 days on the basis of forestomach squamous epithelial hyperplasia in mice (ATSDR, 2007). The International Programme on Chemical Safety tolerable intake levels are 0.17 ppb [0.4 µg/m^3] for inhalation exposures and 1.5 µg/mL (corresponding to 7.5 µg/kg bw per day) for drinking-water exposures (IPCS, 1992).

For subchronic exposures, e.g. 8 hours, environmental guidelines were 0.03–4.8 ppb [0.07–11 µg/m^3], whereas OELs were 20–100 ppb [0.05–0.23 mg/m^3], although some guidelines suggested ceiling values of 100 ppb [230 µg/m^3] that should never be exceeded.

The occupational guidelines for acute exposures (50–100 ppb [120–250 µg/m^3]) are approximately 10–100 times the environmental guidelines for acute exposures. Acute exposure guideline levels (AEGLs) have been established for acrolein (National Research Council, 2010). The lethal level of exposure (AEGL-3) is reached after 10 minutes of exposure to acrolein at 6.2 ppm [14 000 µg/m^3], whereas exposure to acrolein for any duration from 10 minutes to 8 hours at 30 ppb [69 µg/m^3] leads to slight eye irritation and discomfort.

Table S1.3 (Annex 1, Supplementary material for acrolein, Section 1, Exposure Characterization, web only; available from: https://publications.iarc.fr/602) presents some guidelines for acrolein concentrations in the air.

1.5.2 Reference values for biological monitoring of exposure

A metabolite of acrolein (the mercapturic acid HPMA) has been measured as an indicator of exposure. The German Committee for the determination of occupational exposure limits (the "MAK-Commission") suggests a biological reference value for workplace substances (BAR) for HPMA of 600 µg/g creatinine in the urine in non-smokers (Jäger, 2019).

1.6 Quality of exposure assessment in key epidemiological studies

Table S1.4 and Table S1.5 (Annex 1, Supplementary material for acrolein, Section 1, Exposure Characterization, web only; available from: https://publications.iarc.fr/602) provide a detailed overview and critique of the methods used for exposure assessment in cancer epidemiology studies and mechanistic studies in humans that have been included in the evaluation of acrolein. Methods for the exposure assessment varied according to type of study. In the cancer studies in humans, two occupational

Table 1.5 Occupational exposure limits for acrolein in various countries

Country or agency	8-hour TWA		Short-term (15 minutes)		Ceiling		Reference
	ppm	mg/m³	ppm	mg/m³	ppm	mg/m³	
Argentina					0.1	0.23	ACGIH (2019), IOHA (2018)
Australia	0.1	0.23	0.3	0.69			IFA (2020)
Austria	0.02	0.05	0.05	0.12			IFA (2020)
Belgium	0.02	0.05	0.05	0.12			IFA (2020)
Brazil					0.1	0.23	ACGIH (2019)
Canada – Ontario					0.1		Government of Ontario (2020)
Canada – Québec	0.1	0.23	0.3	0.69			IFA (2020)
Chile					0.1	0.23	ACGIH (2019), IOHA (2018)
China						0.3	IFA (2020)
Columbia					0.1	0.23	ACGIH (2019), IOHA (2018)
Denmark	0.02	0.05	0.04	0.1			IFA (2020)
European Union	0.02	0.05	0.05	0.12			IFA (2020)
Finland	0.02	0.05	0.05	0.12			IFA (2020)
France	0.02	0.05	0.05	0.12			IFA (2020)
Germany – AGS	0.09	0.2	0.18	0.4			IFA (2020)
Hungary		0.23		0.23			IFA (2020)
India	0.1	0.25	0.3	0.8			Government of India (2015)
Ireland	0.02	0.05	0.05	0.12			IFA (2020)
Japan – JSOH	0.1	0.23					IFA (2020)
Latvia	0.02	0.05	0.05	0.12			IFA (2020)
Mexico					0.1	0.23	ACGIH (2019), IOHA (2018)
New Zealand	0.1	0.23					IFA (2020)
Poland		0.05		0.1			IFA (2020)
Republic of Korea	0.1	0.25	0.3	0.8			IFA (2020)
Romania	0.02	0.05	0.05	0.12			IFA (2020)
Singapore	0.1	0.23	0.3	0.69			IFA (2020)
South Africa	0.1	0.25	0.3	0.8			South Africa Department of Labour (1995)
Spain	0.02	0.05	0.05	0.12			IFA (2020)
Sweden	0.02	0.05	0.05	0.12			IFA (2020)
Switzerland	0.02	0.05	0.05	0.12			IFA (2020)
United Kingdom	0.02	0.05	0.05	0.12			IFA (2020)
USA – ACGIH					0.1	0.23	ACGIH (2019)
USA – Cal/OSHA					0.1	0.25	State of California (2020)

Table 1.5 (continued)

Country or agency	8-hour TWA		Short-term (15 minutes)		Ceiling		Reference
	ppm	mg/m³	ppm	mg/m³	ppm	mg/m³	
USA – NIOSH	0.1	0.25	0.3	0.8			IFA (2020)
USA – OSHA	0.1	0.25					IFA (2020)
Venezuela					0.1	0.23	ACGIH (2019), IOHA (2018)

ACGIH, American Conference of Governmental Industrial Hygienists; AGS, Ausschuss für Gefahrstoffe (German Committee on Hazardous Substances); Cal/OSHA, California Division of Occupational Safety and Health; JSOH, Japan Society for Occupational Health; NIOSH, National Institute for Occupational Safety and Health; OSHA, Occupational Safety and Health Administration; ppm, parts per million; TWA, time-weighted average.

cohort studies assigned exposure to acrolein, on the basis of expert evaluation of company records on the use of chemicals and also on job history information. No quantitative exposure assessment methods were applied. Other studies used internal markers of exposure to acrolein, based on urinary acrolein metabolites (HPMA), acrolein–DNA adducts from buccal cells, or analyses of acrolein–protein conjugates in serum samples (see Section 4.2.1 for further discussion of acrolein-derived DNA and protein adducts). In these studies, some information on possible external sources of exposure (e.g. smoking, betel-quid chewing, air pollution) was collected through questionnaires. The mechanistic studies in humans showed a partial overlap with the cancer studies in humans, applying internal markers of exposure showing similar limitations regarding assessment of external exposure.

1.6.1 Quality of exposure assessment in key cancer epidemiology studies

Two studies of occupational exposure identified workers exposed to acrolein using information from available records (Bittersohl, 1975; Ott et al., 1989a). No quantitative assessment of exposure was carried out in these studies.

Bittersohl (1975) investigated cancer frequency in an aldehyde factory in Germany and reported that the derivatives produced contained traces of acrolein; however, no evidence was provided that this resulted in any exposure of the workforce to acrolein. Employees were exposed to other chemicals at higher levels than acrolein.

Ott et al. (1989a) investigated risk of lymphohaematopoietic cancer in a complex chemical-manufacturing facility in the USA and assessed the potential for exposure of workers to 21 specific chemicals, including acrolein. Workers were assigned as having been exposed to acrolein if they worked in an area where acrolein was used for 1 day or more. This assessment was based on linking information on job histories with records that contained information on the historical use of chemicals in each department. Intensity of exposure was not assessed, but duration of exposure was estimated. There was no evidence provided of the airborne levels of acrolein in these production facilities. Among 200 production workers, 25 (12.5%) were judged to have been exposed to acrolein for at least 1 day and 3% were exposed to acrolein for 5 years or more (Ott et al., 1989b). Workers were likely to be exposed simultaneously to other chemical agents.

Four other studies assessed exposure to acrolein using internal markers. Yuan et al. (2012, 2014) estimated exposure to acrolein in two lung cancer case–control studies of smokers and non-smokers, respectively, nested within a cohort study of men in Shanghai, China. A single void urine sample was collected from each participant at baseline and analysed to determine the concentration of HPMA, and a range of other urinary biomarkers (including cotinine). Information on smoking was available. No assessment of external exposure to acrolein was carried out.

Tsou et al. (2019) investigated the role of acrolein in oral cancer and estimated exposure to acrolein through analyses of urinary HPMA and of acrolein–DNA adducts in buccal cells collected from cases and controls in Taiwan, China. Information was also collected on smoking history and betel-quid chewing. Buccal cells and urine samples were collected after diagnosis. There was no statistically significant difference in buccal acrolein–DNA adduct levels between healthy controls with different smoking and betel-quid chewing histories. The urinary HPMA concentration was statistically significantly correlated with smoking years and betel-quid chewing years. [The Working Group noted that it was not clear from the data to what extent the levels of buccal acrolein–DNA adducts and urinary HPMA levels are representative of historical exposure attributable to smoking and

betel-quid chewing. The Working Group was not certain whether acrolein–DNA adducts can be considered as a marker of exposure or effect, particularly since samples were collected and analyses carried out after diagnosis.]

Finally, Hong et al. (2020) investigated the role of endogenous exposure to acrolein in a case–control study of urothelial carcinoma patients with chronic kidney disease and healthy controls in Taiwan, China. Endogenous exposure to acrolein was estimated using acrolein–DNA adducts in DNA from tumour or normal urothelial cells, HPMA in urine, and acrolein–protein conjugates in serum samples. [The Working Group noted that information on smoking and air pollution was collected, but these exposures were considered only as confounders in the analyses. The Working Group was not certain whether acrolein–DNA adducts can be considered as a marker of exposure or effect. Markers of acrolein exposure were estimated in samples collected from cases and controls after diagnosis; hence it is not clear whether endogenous exposure to acrolein preceded tumour development or was a consequence of the urothelial carcinoma.]

1.6.2 Quality of exposure assessment in mechanistic studies in humans

Common elements of the human mechanistic studies were their cross-sectional nature, the small sample size (typically 10–20 participants) and the method-development design (e.g. to facilitate and optimize the measurement of certain acrolein adducts in various human tissues) (e.g. Nath & Chung, 1994; Chen & Lin, 2011; Alamil et al., 2020).

The majority of the studies investigated smokers (mainly relying on self-reports), assuming that smoking is the predominant source of exposure to acrolein in humans (Nath et al., 1998; Zhang et al., 2007; Bessette et al., 2009; Zhang et al., 2011; Weng et al., 2018; Yang et al., 2019b). Tsou et al. (2019) included other factors besides smoking, such as alcohol consumption or betel-quid chewing (also see Section 1.6.1 above for a detailed critique of Tsou et al. (2019) in the context of studies of cancer in humans). Wang et al. (2019) included fried food consumption in non-smokers, but insufficiently defined other external exposures.

Another large subset of studies investigated acrolein adducts in tumour tissues without considering any potential external exposure of the patients (Liu et al., 2005; Chen & Lin, 2011; Chung et al., 2012; Yin et al., 2013; Lee et al., 2014; Fu et al., 2018). Hence it is not clear whether external or internal exposure caused adduct formation, or adduct formation was a consequence of tumour development.

Several studies researched treatment with cyclophosphamide or other medicinal products of which acrolein is the principal metabolite (McDiarmid et al., 1991; Al-Rawithi et al., 1998; Takamoto et al., 2004). While external exposure attributable to the medicines is well characterized, all these studies failed to consider other external exposures except smoking.

Interestingly, endogenous exposure was not defined or assessed in most of the studies. Yang et al. (1999b) suggested that both endogenous and exogenous sources may contribute to the formation of acrolein–DNA adducts. [The Working Group noted that it was unclear whether the background exposure comes from endogenous formation or from a low external exposure such as air pollution, secondhand smoke, or consumption of fried food.] Noteworthy regarding endogenous exposure is the study of Ruenz et al. (2019), which placed non-smoking participants in defined living conditions, adhering to a defined diet, and which provided convincing evidence for substantial background exposure to acrolein that was independent of smoking, ingestion of heat-processed food, or other nearby environmental exposures such as exhaust gases or open fires.

2. Cancer in Humans

2.1 Descriptions of individual studies

See Table 2.1.

Six studies – one cohort study, two case–control studies, and three nested case–control studies in cohorts – have been published on the relationship between cancer and exposure to acrolein. Five other studies (mainly case reports) described bladder cancers or leukaemia occurring after use of the pharmaceutical cyclophosphamide (classified in IARC Group 1, *carcinogenic to humans*) or ifosfamide to treat cancer or autoimmune disease. These studies on pharmaceutical agents were determined by the Working Group to be uninformative because the role of acrolein in causing these cancers could not be distinguished from that of other metabolites. The quality of the exposure assessment in the six studies described below is detailed in Section 1.6.

Bittersohl (1975) reported on a small cohort of 220 workers exposed to multiple aldehydes or aldehyde derivatives including acrolein (in trace amounts) in a factory in the former German Democratic Republic, who were followed up from 1967 to 1972. There were 9 cases of cancer in men (5 squamous cell lung carcinomas, 2 squamous cell carcinomas of the oral cavity, 1 adenocarcinoma of the stomach, and 1 adenocarcinoma of the colon) and 2 cases in women (1 leukaemia and 1 cancer of ovary). There was no formal comparison group except a narrative comparison with incidence rates in the general population, source unspecified. [The Working Group noted that although cancer rates were reported to be higher in the cohort than in the population of the German Democratic Republic, the study did not quantify any excess, nor specify the population rate in the German Democratic Republic. Exposure was poorly defined, and no attempt was made to assess exposure (semi-) quantitatively by measurements of duration. No inference can be made regarding the association between acrolein exposure and cancer risk.]

In an occupational nested case–control study among male chemical workers in the USA, Ott et al. (1989a) reported on 129 workers who died from lymphohaematopoietic cancer and their controls (matched on hire decades), with the time scale being time since hire. Information on multiple chemical exposures was available (Ott et al., 1989b), with expert assessment of individual exposures based on jobs, including acrolein. Positive associations between acrolein exposure and non-Hodgkin lymphoma (NHL), multiple myeloma, and leukaemia were reported, based on small numbers of exposed cases ($n = 6$). Given the small sample size and multiple exposures, no inference was possible. [The Working Group noted that matching was based on hire decades. Implications for potential bias were not discussed in the paper. In addition, the exposure assessment was insufficient, limited to dichotomous (ever/never) classification, based on production records and not measured exposure, and exposure encompassed multiple chemicals in addition to acrolein.]

Yuan et al. (2012, 2014) published the results of two nested case–control studies within a cohort of 18 244 Chinese men enrolled in 1986–1989 in Shanghai, China. Besides in-person interviews, a spot urine sample was taken from each participant at baseline and stored until laboratory analysis. Incident cases of and deaths from lung cancer were identified through annual in-person interviews of all surviving participants, the local cancer registry, and the vital statistics office. The first study (Yuan et al., 2012) was a nested case–control study on lung cancer, limited to current smokers at enrolment, and based on follow-up through 2006. Urinary biomarkers related to smoking habits were measured at enrolment, including HPMA (an acrolein-derived, mercapturic acid metabolite), NNAL, cotinine and others. Overall, 343 cases and 392 controls were included in the analysis, after exclusion of

Table 2.1 Epidemiological studies of cancer in humans exposed to acrolein

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Bittersohl (1975) Former German Democratic Republic 1967–1972 Cohort	220 workers in the chemical industry for dimerization of aldehydes. Workers were exposed to acetaldehyde, crotonaldehyde, butyraldehyde, and/or acrolein (traces). Exposure assessment method: records; exposure was assumed based on employment within the aldehyde factory	Lung (squamous cell carcinoma), incidence	Men: NR	5	NR	None	*Exposure assessment critique*: Poorly defined exposure. No evidence of acrolein exposure provided. No separate exposure assessment for different chemical agents present in the factory. *Limitations*: no inference possible for lack of comparator.
		Oral cavity, incidence	Men: NR	2	NR	None	
		Stomach, incidence	Men: NR	1	NR	None	
		Colon, incidence	Men: NR	1	NR	None	
		Leukaemia, incidence	Women: NR	1	NR	None	
		Ovary, incidence	Women: NR	1	NR	None	

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Ott et al. (1989a) USA 1940–1978 Nested case–control	Cases: 52 cases of NHL, 20 cases of multiple myeloma, 39 cases of nonlymphocytic leukaemia, 18 cases of lymphocytic leukaemia; 129 deaths from lymphohaematopoietic cancers; in two chemical manufacturing plants; 29 139 men in the cohort Controls: 5 controls randomly selected per case (N not reported); incidence sampling design from the cohort. Exposure assessment method: expert judgement; 1020 substances, including acrolein, associated with different working areas; exposure was assumed based on whether a chemical substance was used at all in a production unit; no assessment of the intensity of exposure or estimation of cumulative exposure	NHL, mortality Multiple myeloma, mortality Nonlymphocytic leukaemia, mortality	Acrolein exposure (OR): Never Ever Acrolein exposure (OR): Never Ever Acrolein exposure (OR): Never Ever	 NR 2 NR 1 NR 3	 NR 2.6 NR 1.7 NR 2.6	Decade of hire (by matching in design)	*Exposure assessment critique*: No (semi-) quantitative exposure assessment carried out. Exposure was assumed based on assignment to production unit within factory. Exposure was not based on measurement of personal exposure. 21 chemicals were included, and workers are likely exposed to multiple agents (see Ott et al., 1989b). *Limitations*: cases had died, controls alive; small number of subjects exposed to acrolein.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Yuan et al. (2012) Shanghai, China enrolment, 1986–1989/ follow-up, 2006 Nested case–control	Cases: 343 cases of incident lung cancers and deaths, current smokers at enrolment, identified through annual in-person interviews and reviewed through Shanghai Cancer Registry and Shanghai Municipal Vital Statistics Office; lung cancer cases and matched controls from within a cohort of 18 244 Chinese men in Shanghai	Lung, incidence	Quartile of urinary HPMA (pmol/mg creatinine), current smokers at enrolment (OR): First quartile Second quartile Third quartile Fourth quartile Trend-test *P* value, 0.004	49 74 92 128	1 1.39 (0.86–2.23) 1.60 (1.00–2.58) 2.00 (1.25–3.20)	Age at baseline, neighbourhood of residence, duration of sample storage, number of cigarettes smoked per day, years of cigarette smoking at baseline	*Exposure assessment critique*: Internal exposure assessment only. All study subjects were smokers. Smoking history collected and included in the models. Urine samples were collected at baseline, so clearly preceded the health outcome; however, only one urine sample was collected. *Strengths*: study design; relatively large sample and long follow-up (20 yr); few losses to follow-up (4.6%); urinary biomarker was collected before disease occurrence; self-reported smoking status was verified by urinary cotinine. *Limitations*: intraindividual variation in exposure not captured; 35% of cases were not histologically confirmed.
	Controls: 392 participants in the Shanghai Cohort Study; one control was selected from the same cohort, current smoker at enrolment, alive and free of cancer and matched to the index case on age (± 2 yr), date of specimen collection (± 1 month) and neighbourhood of residence at enrolment. Exposure assessment method: exposure to acrolein was determined based on measurement of urinary metabolites of acrolein (HPMA); urine samples were collected at baseline survey of the cohort, in which the case–control study was nested; smoking information was also collected	Lung, incidence	Quartile of urinary HPMA (pmol/mg creatinine), current smokers at enrolment (OR): First quartile Second quartile Third quartile Fourth quartile Trend-test *P* value, 0.046	49 74 92 128	1 1.26 (0.77–2.05) 1.38 (0.84–2.26) 1.64 (1.01–2.66)	Age at baseline, neighbourhood of residence, duration of sample storage, number of cigarettes smoked per day, years of cigarette smoking at baseline, urinary total NNAL and PheT	

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Yuan et al. (2012) (cont.)		Lung, incidence	Quartile of urinary HPMA (pmol/mg creatinine), current smokers at enrolment (OR):			Age at baseline, neighbourhood of residence, duration of sample storage, number of cigarettes smoked per day, years of cigarette smoking at baseline, urinary total NNAL and PheT, total cotinine	
			First quartile	49	1		
			Second quartile	74	0.98 (0.59–1.65)		
			Third quartile	92	1.02 (0.61–1.72)		
			Fourth quartile	128	1.06 (0.62–1.8)		
			Trend-test *P* value, 0.772				
		Lung (squamous cell carcinoma), incidence	Quartile of urinary HPMA (pmol/mg creatinine), current smokers at enrolment (OR):			Age at baseline, neighbourhood of residence, duration of sample storage, number of cigarettes smoked per day, years of cigarette smoking at baseline	
			First tertile	NR	1		
			Second tertile	NR	NR		
			Third tertile	NR	2.56 (1.30–5.05)		
			Trend-test *P* value, < 0.05				
		Lung (squamous cell carcinoma), incidence	Quartile of urinary HPMA (pmol/mg creatinine), current smokers at enrolment (OR):			Age at baseline, neighbourhood of residence, duration of sample storage, number of cigarettes smoked per day, years of cigarette smoking at baseline, total cotinine	
			First tertile	NR	1		
			Second tertile	NR	NR		
			Third tertile	NR	NR		
			Trend-test *P* value: > 0.10				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Yuan et al. (2014) Shanghai, China enrolment, 1986–1989/ follow-up, 2008 Nested case–control	Cases: 82 cases of incident lung cancer in men, lifelong non-smokers aged 45–64 yr at enrolment; Shanghai Cohort Study consisted of 18 244 men (80% of eligible) who were aged between 45 and 64 yr at enrolment in 1986–1989 and resided in one of four small geographically defined communities in Shanghai, China. Controls: 83 members of the Shanghai Cohort Study without cancer, non-smokers and alive at the time of cancer diagnosis of the case; matched on age at enrolment (± 2 yr), year and month of urine sample collection (± 1 month) and neighbourhood of residence at recruitment. Exposure assessment method: exposure was determined based on measurement of urinary metabolites of acrolein (HPMA); urine samples were collected at baseline survey of the cohort, in which the case–control study was nested; there was no assessment of external exposure	Lung, incidence	Quartile of urinary HPMA, never-smokers (OR): First quartile Second quartile Third quartile Fourth quartile Trend-test *P* value, 0.79	21 19 19 21	1 0.97 (0.40–2.34) 0.98 (0.40–2.36) 1.13 (0.47–2.75)	Age at baseline, neighbourhood of residence at enrolment, years of sample storage and urinary cotinine level	*Exposure assessment critique*: Internal exposure assessment only. No evidence of external exposure. Smokers were excluded. Urine samples were collected at baseline, so clearly preceded the health outcome; however, only one urine sample at baseline was collected. *Strengths*: active follow-up with annual in-person interviews; after 22 yr loss to follow-up low, only 5%; urinary cotinine was also quantified to confirm non-smoking status. *Limitations*: relatively small sample size; 26% of cases not histologically confirmed; small number of cases of squamous cell cancer (n = 16); intraindividual variation in exposure not captured.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Tsou et al. (2019) Taiwan, China 2016–2018 Case–control	Cases: 97 cases of cancer of the oral cavity; hospital-based Controls: 230 healthy controls, not further described Exposure assessment method: questionnaire; information on smoking and betel-quid chewing history was collected during interviews with participants or relatives; urine samples were analysed for HPMA; acrolein–DNA adducts were measured in buccal cells or tumour tissues	Oral cavity, incidence	Acrolein–DNA adduct: Controls Ratio of cases vs controls	 222 80	 1 1.4 ($P < 0.001$)	None	*Exposure assessment critique*: Acrolein–DNA adducts and urinary HPMA were measured in samples from cases and controls; however, it is not clear to what extent acrolein–DNA adducts are a marker of effect, or of exposure. Both were measured at the time of cancer treatment (and similar period for controls). The authors indicated that urinary HPMA was significantly correlated with smoking history but no correlation coefficient was given and the correlation appeared weak in the graph. Acrolein–DNA adduct levels were higher in the tumour tissues than in the buccal swabs, but HPMA levels were lower. There was no indication whether and for how long the cases had stopped smoking or chewing before their samples were collected. *Strengths*: DNA adducts in buccal swabs for exposure assessment. *Limitations*: small sample size; controls not described.
		Oral cavity, incidence	Acrolein–DNA, cigarette smokers + betel-quid chewers: Controls Ratio of cases vs controls	 101 51	 1 1.3 ($P < 0.05$)	None	
		Oral cavity, incidence	Acrolein–DNA, cases: Buccal tissue Ratio of tumour tissue to buccal tissue	 NR NR	 1.8 ($P < 0.01$)	None	

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Tsou et al. (2019) (cont.)		Oral cavity, incidence	Mean urinary HPMA (μmol/g creatinine):			None	
			Controls, all	230	7.1		
			Controls, cigarette smokers only	111	5.8		
			Controls, betel-quid chewers only	12	3.6		
			Controls, cigarette smokers and betel-quid chewers	107	8.9		
			Cases	97	0.7 ($P < 0.001$, compared with all controls)		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Hong et al. (2020) Taiwan, China 2016–2019 Case–control	Cases: 62 cases of urothelial cancer; hospital-based, Taiwan, China; patients with CKD; no treatment with cyclofosfamide or ifosfamide Controls: 43 healthy controls; not described but did not have CKD or other diseases. Exposure assessment method: questionnaire; exposure of interest was endogenous exposure to acrolein due to chronic kidney failure, measured through Acr–PC, acrolein–DNA adducts, and by HPMA in urine; information on smoking and air pollution was collected. Smoking did not appear to contribute to higher levels of acrolein–DNA adducts or Acr–PC	Urinary bladder (urothelial cancer), incidence	Acrolein–DNA, cases:			None	*Exposure assessment critique*: Only considered endogenous exposure due to kidney failure. External exposure to smoking was considered but only as a confounder. Biomarkers were used for estimating exposure. Controls had higher levels of HPMA. Air pollution was not related to HPMA. No other external exposure considered. Biomarker measurements were appropriate for acrolein, but it is not clear from the results if endogenous acrolein levels are a result or a consequence of urothelial carcinomas. GSH levels were measured, but not GST activity. *Strengths*: measurement of specific DNA adducts by acrolein and *TP53* mutations. *Limitations*: controls were not described; small sample size; there is a serious flaw in the disproportion of non-smokers: all controls and 79% of cases.
			Normal urothelial cells	62	1		
			Ratio in tumour cells compared with normal cells	62	1.2 ($P < 0.001$)		
		Urinary bladder (urothelial cancer), incidence	Acrolein–DNA, non-smoking cases:			None	
			Normal urothelial cells	48	1		
			Ratio in tumour cells compared with normal cells	48	1.2 ($P < 0.001$)		
		Urinary bladder (urothelial cancer), incidence	Mean plasma Acr–PC (mM):			None	
			Controls	43	0.26		
			Cases	37	0.51 ($P < 0.001$)		
		Urinary bladder (urothelial cancer), incidence	Mean plasma Acr–PC (mM)			None	
			Controls	43	0.26		
			Cases with early-stage CKD	25	0.48 ($P < 0.001$)		
			Cases with late-stage CKD	12	0.56 ($P < 0.001$)		

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study design	Population size, description, exposure assessment method	Cancer type	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Hong et al. (2020) (cont.)		Urinary bladder (urothelial cancer), incidence	Mean plasma Acr–PC, non-smokers (mM):			None	
			Controls	43	0.26		
			Cases	26	0.52 ($P < 0.001$)		
		Urinary bladder (urothelial cancer)	Mean urinary HPMA (μmol/g creatinine):			None	
			Controls	43	1.16		
			Cases	33	0.83 ($P = 0.023$)		

Acr–PC, acrolein–protein conjugates; CI, confidence interval; CKD, chronic kidney disease; HPMA, *N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine (3-hydroxypropylmercapturic acid); NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NHL, non-Hodgkin lymphoma; NR, not reported; OR, odds ratio; PheT, r-1,t-2,3,c-4-tetrahydroxy1,2,3,4-tetrahydrophenanthrene; vs, versus.

cases and controls for whom urine samples were depleted or values for one or more mercapturic acid metabolites were missing. One control per case was selected from among cohort members who were current smokers at enrolment, free of cancer, and alive at the time of the cancer diagnosis of the index case, and further matched on age at enrolment, date of biological specimen collection, and neighbourhood of residence at recruitment. Comparing the highest with the lowest quartiles, risk of lung cancer associated with HPMA levels doubled in models adjusting for matching factors and number of cigarettes smoked per day and years of cigarette smoking at baseline. In models with further adjustment for metabolites of polycyclic aromatic hydrocarbons and tobacco-specific nitrosamines (NNAL) and/or cotinine, no association was found between HPMA and lung cancer. [The Working Group noted that there were multiple correlated exposures (biomarkers). Strengths of the study included: a relatively large sample and long follow-up (20 years); few losses to follow-up (4.6%); urinary biomarkers collected before disease occurrence; and self-reported smoking status verified by urinary cotinine. The 2-fold increase in risk of lung cancer was associated with the highest quartile of HPMA concentration, adjusted for only intensity and duration of smoking. However, this effect disappeared with further adjustment for other smoking biomarkers, indicating that acrolein represented a biomarker of smoking. The Working Group judged that this study was uninformative for an evaluation of the carcinogenicity of acrolein.]

The second study (Yuan et al., 2014) had a similar study design but extended follow-up through 2008 and included only never-smokers at baseline (82 cases of lung cancer and 83 controls; same design as in the Yuan et al., 2012). The same urinary biomarkers as in the previous paper were measured. There was no association between quartile of urinary HPMA concentration and lung cancer in never-smokers (fourth quartile versus first quartile: OR, 1.13; 95% CI, 0.47–2.75) in analysis adjusting for matching factors and urinary cotinine level. [The Working Group noted that only internal exposure was assessed, and since the participants were all non-smokers, the source of external exposure to acrolein was unclear. The Working Group also noted that urinary cotinine represents a short-term biomarker of passive smoking and therefore may not fully adjust for long-term secondhand smoke exposure.]

Tsou et al. (2019) measured acrolein–DNA adducts in buccal swabs from patients (n = 97) with cancer of the oral cavity. Acrolein–DNA adducts were also measured in buccal swabs from 230 healthy controls. Additionally, HPMA and NNAL were measured in the urine of the same 97 patients with cancer of the oral cavity and 230 healthy controls. For the patients with cancer, Tsou et al. (2019) also compared DNA–adduct levels in cancer biopsies with those in adjacent normal tissue collected from buccal swabs. Levels of acrolein–DNA adducts in buccal cells were 1.4 times higher in cases than in controls ($P < 0.001$). The ratio was 1.3 among smokers and betel-quid chewers only ($P < 0.05$). Levels of acrolein–DNA adducts were 1.8 times higher in cancer biopsy specimens than in buccal swabs from adjacent normal tissue ($P < 0.01$). However, there was no significant difference in levels of acrolein–DNA adducts among healthy controls with different cigarette smoking or betel-quid chewing histories. Smoking and betel-quid chewing were associated with significantly higher levels of HPMA. Levels of urinary HPMA were lower among cases (0.7 µmol/g creatinine) than among controls (7.1 µmol/g creatinine) ($P < 0.001$), with a similar difference observed when only smokers and chewers were considered. There was no adjustment for covariates. [The Working Group noted that, overall, the paper suggests that HPMA (but not acrolein–DNA adducts) is associated with smoking and betel-quid chewing, and acrolein–DNA adducts are

associated with oral cancer (cross-sectionally). There were lower levels of HPMA in the urine of cases than in controls (irrespective of smoking/chewing status). The cross-sectional nature of the study and the fact that specimens were collected after cancer diagnosis in cases make causal inference difficult.]

Hong et al. (2020) in a case–control study in Taiwan, China, included 62 patients with urothelial carcinoma and 43 healthy controls. All cases and none of the controls had chronic kidney disease (CKD), the rationale being that CKD patients have a high risk of bladder cancer and altered metabolism that increases susceptibility to chemical exposures. Urinary HPMA, plasma acrolein–protein conjugates, DNA adducts formed by acrolein, and *TP53* mutations in frozen tissue samples were measured. Tumour biopsies showed levels of acrolein–DNA adducts that were 1.2 times higher than those in adjacent normal tissue in urothelial carcinoma patients overall ($P < 0.005$). The same ratio and P value were also found in cases and controls who were non-smokers. Levels of acrolein–DNA adducts were correlated with CKD severity. Also, levels of plasma acrolein–protein conjugates were twice as high in cases as in controls ($P < 0.001$). Similar results were observed for acrolein–protein conjugates in plasma in study participants with different degrees of severity of CKD and in non-smokers. Urinary HPMA levels were lower in cases (0.83 μmol/g creatinine) than in controls (1.16 μmol/g creatinine) ($P = 0.023$), this observation being attributed to binding of HPMA to glutathione (GSH) as a cellular defence mechanism. [The Working Group noted that controls were not described, and cases were all affected by CKD. The only endogenous exposure considered was due to kidney failure, while external exposure to smoking was considered only as a confounder. The study also had a small sample size, considerable age difference between cases and controls, and short follow-up period. There appeared to be a disproportionate number of non-smokers included: all controls and 79% of cases.]

2.2 Evidence synthesis for cancer in humans

The epidemiological evidence available on acrolein in relation to cancer in humans included one occupational cohort study (Bittersohl, 1975), three nested case–control studies in occupational or population-based cohorts (Ott et al., 1989a; Yuan et al., 2012, 2014), and two hospital-based case–control studies (Tsou et al., 2019; Hong et al., 2020). There was little consistency in the cancer sites evaluated across these studies, with studies variously examining cancers of the lung (Bittersohl, 1975; Yuan et al., 2012, 2014), oral cavity (Bittersohl, 1975; Tsou et al., 2019), bladder (Hong et al., 2020), or lymphohaematopoietic cancers (Ott et al., 1989a).

2.2.1 Exposure assessment

The quality of the exposure assessment carried out within the available studies was of concern, as detailed in Section 1.6. For the studies that considered occupational exposure to acrolein (Bittersohl, 1975; Ott et al., 1989a, b), no quantitative exposure assessment was carried out, and therefore no exposure–response analyses could be performed. In addition, study participants were simultaneously exposed to multiple, undifferentiated chemical agents, reducing the informativeness of a comparison of cancer risk between exposed and unexposed groups.

The remaining studies investigated acrolein mainly from a mechanistic point of view and looked at urinary metabolites (mercapturic acids) (Yuan et al., 2012, 2014; Tsou et al., 2019; Hong et al., 2020), acrolein–DNA adducts (Tsou et al., 2019; Hong et al., 2020), and/or acrolein–protein conjugates measured in serum (Hong et al., 2020). These studies did not consider external exposure to acrolein explicitly. Although information on

smoking was available in some studies and may have been an important source of acrolein exposure, these studies adjusted for smoking through restriction or statistical adjustment (Yuan et al., 2012, 2014; Hong et al., 2020).

2.2.2 Cancers of the lung, oral cavity, and other sites

Two case–control studies (Yuan et al., 2012, 2014) nested in a population-based cohort studied several biomarkers in relation to lung cancer (one among current smokers, one among non-smokers). There was matching by smoking habits and adjustment for markers of smoking (NNAL, cotinine, and urinary HPMA) but the study did not investigate the etiological involvement of acrolein per se.

One case–control investigation (Tsou et al., 2019) studied acrolein–DNA adducts in buccal swabs of patients with cancer of the oral cavity compared with healthy controls and found higher levels in cancer cases. However, adducts were not associated with tobacco smoking or betel-quid chewing, and thus were unlikely to be markers of those exposures. Urinary HPMA (a metabolite of acrolein) was associated with smoking and betel chewing. There were lower levels of HPMA in the urine of cases than in controls (irrespective of smoking/chewing status), attributed by the authors to HPMA binding to GSH as a cellular defence mechanism.

The three other studies were considered uninformative – one occupational cohort (Bittersohl, 1975), one nested case–control study on lymphohaematopoietic cancer in an occupational cohort (Ott et al., 1989a), and a case–control study on urothelial carcinoma in patients with CKD (Hong et al., 2020) – due to small numbers, poor external exposure assessment, and flaws in design.

Taken together, these studies provide little evidence of a positive association between acrolein exposure and cancer in humans. Some of the available studies were of a mechanistic nature, i.e. they investigated the role of a urinary mercapturic acid metabolite of acrolein in smokers with null results after controlling for other smoking-related biomarkers. In other studies, the design, including external exposure assessment, was poor.

3. Cancer in Experimental Animals

In previous evaluations, the *IARC Monographs* programme concluded that there was *inadequate evidence* in experimental animals for the carcinogenicity of acrolein (e.g. IARC, 1995).

Studies of carcinogenicity with acrolein in experimental animals are summarized in Table 3.1.

3.1 Mouse

3.1.1 Inhalation

In a study that complied with Good Laboratory Practice (GLP), groups of 50 male and 50 female B6D2F$_1$/Crlj mice (age, 6 weeks) were treated with acrolein (purity, > 98.3%; 1.42% acetaldehyde identified by GC-MS) by inhalation with whole-body exposure for 6 hours per day, 5 days per week, for up to 99 weeks. (JBRC, 2016a, b, c). The concentration in the exposure chambers was set to 0 (clean air, control), 0.1, 0.4, or 1.6 ppm (v/v) for males and females. The mean air concentrations, based on monitoring every 15 minutes, were the target values and the coefficients of variation were within 0.6%. The survival rates for all groups (including both male and female control groups) were decreased due to the development of renal lesions and/or amyloid deposition but were not affected by exposure to acrolein. When the survival rates for the male and female control groups were lower than 25%, the study was terminated by

Table 3.1 Studies of carcinogenicity with acrolein in experimental animals

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Mouse, B6D2F$_1$/Crlj (M) 6 wk 93 wk JBRC (2016a, b)	Inhalation (whole-body) > 98.3% Clean air 0, 0.1, 0.4, 1.6 ppm 6 h/day, 5 days/wk 50, 50, 50, 50 11, 15, 14, 15	*Nasal cavity*: adenoma Incidence: 0/50, 0/50, 0/50, 1/50 (2%)	NS	Principal strengths: multiple dose study; use of males and females; study complied with GLP. Historical control data in B6D2F$_1$/Crlj male mice for nasal cavity adenoma: 1/499 (0.2%; range, 0–2%); the incidence of hyperplasia of the respiratory tract (nasal cavity) was significantly increased in treated animals compared with controls; the Working Group considered hyperplasia of the respiratory tract to be a pre-neoplastic lesion.
		Lymph node: malignant lymphoma Incidence: 1/50, 3/50, 2/50, 4/50	NS	
Full carcinogenicity Mouse, B6D2F$_1$/Crlj (F) 6 wk 99 wk JBRC (2016a, b)	Inhalation (whole-body) > 98.3% Clean air 0, 0.1, 0.4, 1.6 ppm 6 h/day, 5 days/wk 50, 50, 50, 50 11, 18, 14, 19	*Nasal cavity*: adenoma Incidence: 0/50, 0/50, 0/50, 16/50 (32%)*	*P < 0.0001, Fisher exact test; P < 0.0001, Peto trend test (prevalence method) and Cochran–Armitage trend test	Principal strengths: multiple dose study; use of males and females; study complied with GLP. Historical control data in B6D2F$_1$/Crlj female mice for malignant lymphoma: 169/500 (33.8%; range, 28–46%); uterus histiocytic sarcoma: 114/500 (22.8%; range, 18–34%); nasal cavity adenoma: 0/500; the incidence of hyperplasia of the respiratory tract (nasal cavity) was significantly increased in treated animals compared with controls; the Working Group considered hyperplasia of the respiratory tract to be a pre-neoplastic lesion.
		Lymph node: malignant lymphoma Incidence: 12/50 (24%), 8/50 (16%), 6/50 (12%), 17/50 (34%)	P = 0.0347, Cochran–Armitage trend test	
		Uterus Histiocytic sarcoma Incidence: 6/50 (12%), 13/50 (26%), 14/50 (28%)*, 6/50 (12%)	*P = 0.0392, Fisher exact test	
		Endometrial stromal polyp Incidence: 1/50, 1/50, 2/50, 3/50	NS	
		Liver: histiocytic sarcoma Incidence: 0/50, 2/50, 0/50, 3/50	NS	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Mouse, CD-1 (M) 8 wk 18 mo Parent et al. (1991a)	Oral administration (gavage) 94.9–98.5% (hydroquinone, 0.25–0.31%) Deionized water 0, 0.5, 2.0, 4.5 mg/kg bw per day 1×/day 70, 70, 70, 75 NR	*All sites*: no significant increase in the incidence of tumours		Principal strengths: use of males and females; use of multiple doses; large number of animals per group. Principal limitations: all major tissues and gross lesions from the control and high-dose groups were examined microscopically; only the lungs, liver, kidneys, and gross lesions from the groups at the low and intermediate dose were examined microscopically; histopathological data from mice found dead or killed because moribund were to have been collected according to the protocol, but data were not reported; dosing volume not reported; trend towards reduced survival, and decreased survival in the group at the highest dose.
Full carcinogenicity Mouse, CD-1 (F) 8 wk 18 mo Parent et al. (1991a)	Oral administration (gavage) 94.9–98.5% (hydroquinone, 0.25–0.31%) Deionized water 0, 0.5, 2.0, 4.5 mg/kg bw per day 1×/day 70, 70, 70, 75 NR	*All sites*: no significant increase in the incidence of tumours		Principal strengths: use of males and females; use of multiple doses; large number of animals per group. Principal limitations: microscopic examination was reported for all major tissues and gross lesions from the control and high-dose groups, but only for the lungs, liver, kidneys, and gross lesions from the groups at the low and intermediate dose; histopathological data from mice found dead or killed because moribund were to have been collected according to the protocol, but data were not reported; dosing volume not reported.
Full carcinogenicity Mouse, B6C3F$_1$ (M) Neonatal (8 days) 12 mo Von Tungeln et al. (2002)	Intraperitoneal injection NR DMSO 0, 150 nmol Injections with one-third and two-thirds of the total dose in 30 µL DMSO at age 8 and 15 days, respectively 24, 23 24, 23	*Liver* Adenoma Incidence: 0/24, 1/23 Carcinoma Incidence: 0/24, 0/23 Adenoma or carcinoma (combined) Incidence: 0/24, 1/23 Multiplicity: 0, 2.0	 NS NA NS NR	Principal strength: use of males and females. Principal limitations: use of single dose; lack of body-weight data; rationale for dose not given, only data regarding liver tumours were reported.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (F) Neonatal (8 days) 12 mo Von Tungeln et al. (2002)	Intraperitoneal injection NR DMSO 0, 150 nmol Injections with one-third and two-thirds of the total dose in 30 µL DMSO at age 8 and 15 days, respectively 23, 24 23, 23	*Liver* Adenoma Incidence: 0/23, 0/23 Carcinoma Incidence: 0/23, 0/23	 NA NA	Principal strength: use of males and females. Principal limitations: use of single dose; lack of body-weight data; rationale for dose not given; only data regarding liver tumours were reported.
Full carcinogenicity Mouse, B6C3F$_1$ (M) Neonatal (8 days) 15 mo Von Tungeln et al. (2002)	Intraperitoneal injection NR DMSO 0, 75 nmol Injections with one-third and two-thirds of the total dose in 30 µL DMSO at age 8 and 15 days, respectively 24, 24 24, 24	*Liver* Adenoma Incidence: 4/24, 5/24 Carcinoma Tumour incidence: 0/24, 0/24 Adenoma or carcinoma (combined) Incidence: 4/24, 5/24 Multiplicity: 1.3, 1.0	 NS NA NS NR	Principal strength: use of males and females. Principal limitations: use of single dose; lack of body-weight data; rationale for dose not given; only data regarding liver tumours were reported.
Full carcinogenicity Mouse, B6C3F$_1$ (F) Neonatal (8 days) 15 mo Von Tungeln et al. (2002)	Intraperitoneal injection NR DMSO 0, 75 nmol Injections with one-third and two-thirds of the total dose in 30 µL DMSO at age 8 and 15 days, respectively 24, 24 24, 24	*Liver* Adenoma Incidence: 0/24, 0/24 Carcinoma Incidence: 0/24, 0/24	 NA NA	Principal strength: use of males and females. Principal limitations: use of single dose; lack of body-weight data; rationale for dose not given; only data regarding liver tumours were reported.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Mouse, NR, "partly inbred albinos" (F) ~3 mo ≤ 21–24 mo Steiner et al. (1943)	Subcutaneous injection NR Sesame oil 0.2 mg 1×/wk in 0.1 mL sesame oil for 24 wk 15 0 (11, 6, 3 and 1, at 12, 15, 18 and 21 mo, respectively)	*Subcutaneous tissue*: sarcoma Incidence: 0/15	NA	Principal limitations: use of females only; small number of mice; use of single dose; lack of body-weight data; limited information on sesame oil control group (see below); histopathological reporting limited to the induction of sarcomas; poor survival; justification of the dose was not provided. The authors stated: "at 12 months, the number of mice [sex distribution unspecified] living that had been injected with unheated sesame oil, used as vehicle for other substances in these experiments, was 61. None developed tumours at the site of injection".
Initiation–promotion (tested as initiator) Mouse, S NR NR 21–22 wk Salaman & Roe (1956)	Skin application NR Acetone 0, 12.6 mg (total dose) Untreated (control) or 1×/wk application for 10 wk of 0.5% acrolein (in [presumably] 0.3 mL acetone); 25 days after 1st application, 1×/wk application of 0.17% croton oil for 18 wk (dose reduced to 0.085% for the 2nd and 3rd application) 20, 15 19, 15	*Skin*: papilloma Incidence: 4/19, 2/15 No.: 4, 3	[NS] NR	Principal limitations: small number of mice per group; use of a single dose; limited reporting; justification of the dose was not provided.
Full carcinogenicity Rat, F344/DuCrlCrlj (M) 6 wk 104 wk JBRC (2016d, e)	Inhalation (whole-body) > 98.3% Clean air 0, 0.1, 0.5, 2 ppm 6 h/day, 5 days/wk 50, 50, 50, 50 41, 40, 37, 42	*Nasal cavity*: squamous cell carcinoma Incidence: 0/50, 0/50, 0/50, 1/50 (2%)	NS	Principal strengths: multiple-dose study; used males and females; study complied with GLP. Historical control data in F344/DuCrlCrlj male rats for nasal cavity squamous cell carcinoma, 0/599; the incidence of hyperplasia of the respiratory tract (nasal cavity) was significantly increased in treated rats compared with controls; the Working Group considered hyperplasia of the respiratory tract to be a pre-neoplastic lesion.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Rat, F344/DuCrlCrlj (F) 6 wk 104 wk JBRC (2016d, e)	Inhalation (whole-body) > 98.3% Clean air 0, 0.1, 0.5, 2 ppm 6 h/day, 5 days/wk 50, 50, 50, 50 43, 42, 41, 34	*Nasal cavity* Squamous cell carcinoma or rhabdomyoma (combined) Incidence: 0/50, 0/50, 0/50, 6/50 (12%)*	*P = 0.0133, Fisher exact test; P < 0.0001, Peto trend test (prevalence method and combined analysis) and Cochran–Armitage trend test	Principal strengths: multiple -dose study; used males and females; study complied with GLP. Historical control data in F344/DuCrlCrlj female rats for nasal cavity squamous cell carcinoma, 0/600; nasal cavity rhabdomyoma, 0/600; pituitary gland adenoma, 165/599 (27.5%; range, 22–42%); the incidence of hyperplasia of the respiratory tract (nasal cavity) was significantly increased in treated rats compared with controls; the Working Group considered hyperplasia of the respiratory tract to be a pre-neoplastic lesion.
		Rhabdomyoma Incidence: 0/50, 0/50, 0/50, 4/50 (8%)	$P \leq 0.0007$, Peto trend test (prevalence method) and Cochran–Armitage trend test	
		Squamous cell carcinoma Incidence: 0/50, 0/50, 0/50, 2/50 (4%)	NS	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Rat, F344/DuCrlCrlj (F) 6 wk 104 wk JBRC (2016d, e) (cont.)		*Pituitary gland* Adenoma or adenocarcinoma (combined) Incidence: 14/50, 17/50, 21/50, 17/50 Adenoma Incidence: 14/50 (28%), 15/50 (30%), 20/50 (40%), 17/50 (34%) Adenocarcinoma Incidence: 0/50, 2/50, 1/50, 0/50	 $P = 0.0215$, Peto trend test (standard method) $P = 0.0115$, Peto trend test (standard method) NS	
Full carcinogenicity Rat, F344 (M) 7–8 wk ≤ 124–132 wk Lijinsky & Reuber (1987)	Oral administration (drinking-water) NR, stabilized with hydroquinone (concentration, NR) Tap water 0 (control), 100 (for 124 wk), 250 (for 124 wk), 625 (for 104 wk) mg/L 5×/wk for 104–124 wk 20, 20, 20, 20 NR	*Liver*: tumours Incidence: 2/20, 8/20*, 0/20, 3/20	*[$P < 0.0324$, one-tailed Fisher exact test]	Principal strengths: long-term study (> 2 yr); use of males and females; use of multiple doses. Principal limitations: small number of rats per group; variable duration of treatments between groups; body-weight and survival data not reported. Median week of death: 115 (range, 92–124) (control), 119 (83–130), 116 (53–130), and 129 (95–132) wk, respectively; total acrolein consumption: 0, 1.2, 3.1, and 6.5 g, respectively; liver tumours were mainly neoplastic nodules, with a few hepatocellular carcinomas.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Rat, F344 (F) 7–8 wk ≤ 124–132 wk Lijinsky & Reuber (1987)	Oral administration (drinking-water) NR, stabilized with hydroquinone (concentration, NR) Tap water 0 (control), 625 (for 104 wk) mg/L 5×/wk for 104 wk 20, 20 NR	*Liver*: tumours Incidence: 2/20, 4/20 *Adrenal gland*: tumours Incidence: 1/20, 5/20	NS NS	Principal strengths: long-term study (> 2 yr); use of males and females. Principal limitations: small number of rats per group; variable duration of treatments between groups; body-weight data and survival not reported; use of a single dose. Median week of death: 118 (range, 82–124) (control), and 117 (58–132) wk, respectively; total acrolein consumption: 0 and 6.5 g, respectively; liver tumours were mainly neoplastic nodules, with a few hepatocellular carcinomas.
Full carcinogenicity Rat, Sprague-Dawley (M) ~6 wk 102 wk Parent et al. (1992)	Oral administration (gavage) 94.9–98.5% (hydroquinone, 0.25–0.31%) Deionized water 0.0, 0.05, 0.5, 2.5 mg/kg bw 1×/day 70, 70, 70, 70 NR	*Adrenal gland* Cortical adenoma Incidence: 0/60, 4/60, 3/60, 0/60 Cortical carcinoma Incidence: 0/60, 0/60, 1/60, 1/60	 NS NS	Principal strengths: use of males and females; multiple dose study; long-term study. Principal limitations: all major tissues and gross lesions from the control and high-dose groups were examined microscopically; only the lungs, liver, kidneys, and gross lesions from the groups at the low and intermediate dose were examined microscopically; reporting of adrenal gland tumours only. 10 rats per dose group were killed after 1 year.
Full carcinogenicity Rat, Sprague-Dawley (F) ~6 wk 102 wk Parent et al. (1992)	Oral administration (gavage) 94.9–98.5% (hydroquinone, 0.25–0.31%) Deionized water 0.0, 0.05, 0.5, 2.5 mg/kg bw 1×/day 70, 70, 70, 70 NR	*Adrenal gland*: cortical adenoma Incidence: 2/60, 3/60, 0/60, 0/60	NS	Principal strengths: use of males and females; multiple dose study; long-term study. Principal limitations: trend towards reduced survival, and decreased survival in the high-dose group; all major tissues and gross lesions from the control and high-dose groups were examined microscopically; only the lungs, liver, kidneys and gross lesions from the low- and mid-dose groups were examined microscopically; reporting of adrenal gland tumours only. 10 rats per dose group were killed after 1 year.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Initiation–promotion (tested as initiator) Rat, F344 (M) 5 wk 32 wk Cohen et al. (1992)	Intraperitoneal injection 97% (containing 3% water and 200 ppm hydroquinone) Distilled water Acrolein at 2 mg/kg bw followed by control diet (negative control), 0 mg/kg bw followed by uracil (sham control), 2 mg/kg bw followed by uracil, 0.2% FANFT followed by uracil (positive control) Intraperitoneal injection of 2 mg/kg bw acrolein or of distilled water, 2×/wk for 6 wk; followed by 3% uracil in the diet for 20 wk then 6 wk of control diet, or by control diet for 26 wk 30, 30, 30, 30 NR	*Urinary bladder* Papilloma Incidence: 0/30, 8/30, 18/30*, 9/30 Carcinoma Incidence: 0/30, 1/30, 1/30, 21/30	*[$P < 0.02$, Fisher exact test; acrolein + uracil group vs sham control (uracil only)] [NS]	Principal strength: sufficient duration. Principal limitations: use of single dose; data from stomach, lungs, oesophagus, liver, and kidney were not reported.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Initiation–promotion (tested as promoter) Rat, F344 (M) 5 wk 53 wk Cohen et al. (1992)	Intraperitoneal injection 97% (containing 3% water and 200 ppm hydroquinone) Distilled water Untreated (negative control), FANFT followed by distilled water (sham control), 2 mg/kg bw acrolein, followed by acrolein (see comments for regimen), FANFT followed by acrolein (see comments for regimen) Intraperitoneal injection of 2 mg/kg bw acrolein or of distilled water, 2×/wk for 6 wk, or 0.2% FANFT in the diet for 6 wk; followed by intraperitoneal injection of acrolein (see comments for regimen) or of distilled water until experimental wk 53, or by control diet until experimental wk 53 30, 30, 30, 30 NR	*Urinary bladder* Papilloma Incidence: 0/30, 0/30, 0/30, 0/30 Carcinoma Incidence: 0/30, 1/30, 0/30, 0/30 Simple or papillary/nodular (combined) hyperplasia Incidence: 0/30, 14/30, 16/30*, 22/30 Simple hyperplasia Incidence: 0/30, 14/30, 14/30*, 22/30 Papillary/nodular hyperplasia Incidence: 0/30, 0/30, 2/30, 0/30	NA [NS] *$P < 0.001$, Fisher exact test; acrolein + acrolein vs negative (untreated) control *$P < 0.001$, Fisher exact test; acrolein + acrolein vs negative (untreated) control NS	Principal limitations: use of single dose; data from stomach, lungs, oesophagus, liver, and kidney were not reported. The protocol (originally for a 100 wk-study) had to be revised for the two acrolein-treated groups, because of severe toxicity, and the acrolein treatment regimen was revised as follows: intraperitoneal injections of acrolein at 2 mg/kg bw, 2×/wk, during experimental wk 1–9; 1.5 mg/kg bw, 1× at experimental wk 10; 1.5 mg/kg bw, 2×/wk, during experimental wk 11–17; and 1.0 mg/kg bw, 1× at experimental wk 18, 2× at experimental wk 19, and 1× at experimental wk 20 and 21.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Hamster, Syrian golden (M) 6 wk 81 wk Feron & Kruysse (1977)	Inhalation (whole-body) NR Filtered air 0 (unexposed control), 9.2 mg/m^3 7 h/day, 5 days/wk for 52 wk 30, 30 7 (at 80 wk), 7 (at 80 wk)	*Respiratory tract*: all tumours (nasal cavity, larynx, trachea, bronchi or lung, combined) Incidence: 0/30, 0/30	NA	Principal strengths: use of males and females. Principal limitations: small number of animals per group; short duration of exposure; use of single dose; histopathological data were reported only for respiratory tract tumours; justification of the dose was not provided; lower survival. 15 hamsters per group also received intratracheal instillations of 0.2 mL 0.9% saline 1×/wk for 52 wk; the entire respiratory tract, grossly visible tumours, and gross lesions suspected of being tumours were examined microscopically; in acrolein-treated animals, inflammation and epithelial metaplasia of the nasal cavity were observed.
Full carcinogenicity Hamster, Syrian golden (F) 6 wk 81 wk Feron & Kruysse (1977)	Inhalation (whole-body) NR Filtered air 0 (unexposed control), 9.2 mg/m^3 7 h/day, 5 days/wk for 52 wk 30, 30 16 (at 80 wk), 13 (at 80 wk)	*Respiratory tract*: all tumours (nasal cavity, larynx, trachea, bronchi or lung, combined) Incidence: 0/30, 1/30	NS	Principal strengths: use of males and females. Principal limitations: small number of animals per group; short duration of exposure; use of single dose; histopathological data were reported only for respiratory tract tumours; justification of the dose was not provided. 15 hamsters per group also received intratracheal instillations of 0.2 mL 0.9% saline 1×/wk for 52 wk; the entire respiratory tract, grossly visible tumours, and gross lesions suspected of being tumours were examined microscopically; in acrolein-treated animals, inflammation and epithelial metaplasia of the nasal cavity were observed.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Co-carcinogenicity Hamster, Syrian golden (M) 6 wk 81 wk Feron & Kruysse (1977)	Inhalation (whole-body) NR Filtered air 0 mg/m³ acrolein + 0.175% B[*a*]P, 9.2 mg/m³ acrolein + 0.175% B[*a*]P, 0 mg/m³ acrolein + 0.35% B[*a*]P, 9.2 mg/m³ acrolein + 0.35% B[*a*]P, 0 mg/m³ acrolein + NDEA, 9.2 mg/m³ acrolein + NDEA Exposure to acrolein was 7 h/day, 5 days/wk for 52 wk; together with either weekly intratracheal instillations of a suspension of 0.175 or 0.35% B[*a*]P (in 0.2 mL 0.9% saline), or 1×/3 wk subcutaneous injections of 0.0625% NDEA in 0.2 mL saline 30, 30, 30, 30, 30, 30 8 (at 80 wk), 7 (at 80 wk), 11 (at 80 wk), 9 (at 80 wk), 1 (at 80 wk), 7 (at 80 wk)	*Respiratory tract*: all tumours (nasal cavity, larynx, trachea, bronchi or lung, combined) Incidence: 4/29, 7/30, 19/30, 19/29, 12/29, 10/30 No.: 5, 7, 27, 29, 15, 11	NS NS	Principal strengths: use of males and females. Principal limitations: small number of animals per group; short duration of exposure; use of single dose; histopathological data were reported only for respiratory tract tumours; justification of the dose was not provided; lower survival. 15 hamsters per control group also received intratracheal instillations of 0.2 mL 0.9% saline 1×/wk for 52 wk; the entire respiratory tract, grossly visible tumours, and gross lesions suspected of being tumours were examined histologically; in acrolein-treated animals, inflammation and epithelial metaplasia of the nasal cavity were observed.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Co-carcinogenicity Hamster, Syrian golden (F) 6 wk 81 wk Feron & Kruysse (1977)	Inhalation (whole-body) NR Filtered air 0 mg/m³ acrolein + 0.175% B[*a*]P, 9.2 mg/m³ acrolein + 0.175% B[*a*]P, 0 mg/m³ acrolein + 0.35% B[*a*]P, 9.2 mg/m³ acrolein + 0.35% B[*a*]P, 0 mg/m³ acrolein + NDEA, 9.2 mg/m³ acrolein + NDEA Exposure to acrolein was 7 h/day, 5 days/wk for 52 wk; together with either weekly intratracheal instillations of a suspension of 0.175 or 0.35% B[*a*]P (in 0.2 mL 0.9% saline), or 1×/3 wk subcutaneous injections of 0.0625% NDEA in 0.2 mL saline 30, 30, 30, 30, 30, 30 21 (at 80 wk), 17 (at 80 wk), 18 (at 80 wk), 17 (at 80 wk), 11 (at 80 wk), 20 (at 80 wk)	*Respiratory tract*: all tumours (nasal cavity, larynx, trachea, bronchi or lung, combined) Incidence: 3/27, 8/29, 7/24, 15/30, 11/27, 11/28 No.: 3, 8, 9, 22, 13, 15	NS NS	Principal strengths: use of males and females. Principal limitations: small number of animals per group; short duration of exposure; use of single dose; histopathological data were reported only for respiratory tract tumours; justification of the dose was not provided. 15 hamsters per control group also received intratracheal instillations of 0.2 mL 0.9% saline 1×/wk for 52 wk; the entire respiratory tract, grossly visible tumours, and gross lesions suspected of being tumours were examined histologically; in acrolein-treated animals, inflammation and epithelial metaplasia of the nasal cavity were observed.

B[*a*]P; benzo[*a*]pyrene; bw, body weight; DMSO, dimethyl sulfoxide; F, female; FANFT, *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide; GLP, Good Laboratory Practice; h, hour; M, male; NA, not applicable; NDEA, *N*-nitrosodiethylamine; NR, not reported; NS, not significant; mo, month; ppm, parts per million; vs, versus; wk, week.

necropsy; this was done at week 93 for males and week 99 for females. Survival in the groups at 0, 0.1, 0.4, and 1.6 ppm was: for males, 11/50, 15/50, 14/50, and 15/50, respectively, at week 93; and for females, 11/50, 18/50, 14/50, and 19/50, respectively, at week 99. Body weights of male mice at 1.6 ppm were significantly decreased from the first week of exposure and throughout the exposure period compared with the control value. The relative final body weight in males at 0.1, 0.4, and 1.6 ppm were 89%, 95%, and 83% of the control value, respectively. Body weights of female mice at 1.6 ppm were significantly decreased from the first week of exposure until week 82, compared with the control value. The relative final body weight in females at 0.4 ppm was slightly but significantly increased. The relative final body weight for females at 0.1, 0.4, and 1.6 ppm was 104%, 111%, and 101% of the control value, respectively. All mice underwent complete necropsy, and all organs and tissues were examined microscopically.

In treated male mice, there was no significant increase in the incidence of any tumour. One (1/50, 2%) adenoma of the nasal cavity was observed in a male at 1.6 ppm; this incidence was at the upper bound of the historical control range (incidence, 1/499 (0.2%); range, 0–2%).

In female mice, the incidence of malignant lymphoma was significantly increased with a positive trend (P = 0.0347, Cochran–Armitage test). The incidence of histiocytic sarcoma of the uterus was 6/50 (12%, control), 13/50 (26%, 0.1 ppm), 14/50 (28%, 0.4 ppm), and 6/50 (12%, 1.6 ppm). The incidence in the group at 0.4 ppm (28%) was significantly increased (P = 0.0392, Fisher test) compared with the control value. [The Working Group noted that this increase did not indicate a clear dose–response relationship. The Working Group considered that this increase may have been related to treatment.] The incidence of adenoma of the nasal cavity was 0/50 (control), 0/50 (0.1 ppm), 0/50 (0.4 ppm), and 16/50 (32%, 1.6 ppm) and showed a significant positive trend ($P < 0.0001$, Peto test prevalence method and Cochran–Armitage test). The incidence in the group at 1.6 ppm was significantly increased ($P < 0.0001$, Fisher test) compared with the value for the control group and was clearly in excess of the value for historical controls (0/500).

Regarding non-neoplastic lesions in the respiratory tract (see also Section 4 of this monograph), for males at 1.6 ppm there was a significant increase in the incidence and/or severity of: eosinophilic change, inflammation, squamous cell metaplasia, regeneration, and hyperplasia in the respiratory epithelium; respiratory metaplasia and atrophy in the olfactory epithelium; respiratory metaplasia in the nasal glands; hyperplasia in the transitional epithelium; atrophy and adhesion in the turbinate; and exudate in the nasal cavity was observed. For females at 1.6 ppm, there was a significant increase in the incidence and/or severity of: inflammation, squamous cell metaplasia, regeneration, and hyperplasia in the respiratory epithelium; respiratory metaplasia and atrophy in the olfactory epithelium; respiratory metaplasia in the nasal glands; and exudate in the nasal cavity was observed. The incidence of inflammation and hyperplasia in the respiratory epithelium was also increased in female mice at 0.4 ppm. [The Working Group considered the hyperplasias of the respiratory tract observed in both males and females to be pre-neoplastic lesions.]

[The Working Group noted this was a GLP study conducted with multiple doses, and with both males and females.]

3.1.2 Oral administration (gavage)

In a study performed by Parent et al. (1991a), groups of 70–75 male and 70–75 female CD-1 mice (age, 8 weeks) were given acrolein (purity, 94.9–98.5%; containing 0.25–0.31% hydroquinone as a stabilizer) at a dose of 0 (control, deionized water only), 0.5, 2.0, or 4.5 mg/kg body weight (bw) per day by daily gavage [dosing

volume not reported] for 18 months. In treated males, there was a significant negative trend in survival, and a significant decrease in survival in the group of males at the highest dose. Excess mortality was reported in all groups and attributed to trauma during gavage dosing, mis-dosing, or reasons unknown. [The number of surviving animals was not provided.] In males treated with the highest dose, a significant reduction in body-weight gain was observed. Histopathological examination was reported for all major tissues and gross lesions from mice in the control group and at the highest dose, but only for the lungs, liver, kidneys, and gross lesions from mice in the groups receiving the lowest and intermediate dose. In addition, tumour incidence was reported only for about half of the experimental animals (271/570) killed at 18 months. For males, data were reported for 30, 29, 30, and 27 animals for controls and each dose group respectively. For females, data were reported for 42, 30, 40, and 43 animals for controls and each dose group, respectively. [According to the protocol, histopathological data from mice found dead or killed in a moribund state were to be collected, but data were not shown.]

No significant increase in the incidence of tumours was observed.

[The Working Group noted the principal strengths of the study: the use of males and females, the large number of mice per group at start, and the use of multiple doses. The principal limitations of the study were that data were obtained from a limited number of mice assessed for histopathology after killing; that full histopathological examination was performed only for mice in the control group and at the highest dose; and that survival was lower in treated males.]

3.1.3 Intraperitoneal injection

In the first experiment in a carcinogenicity study by Von Tungeln et al. (2002), which focused on the induction of liver and lung tumours in newborn mice, groups of 23 male and 24 female B6C3F$_1$ mice (age, 8 days) were given two intraperitoneal injections of acrolein [purity not reported] at a total dose of 150 nmol. One third [50 nmol] and two thirds [100 nmol] of the total dose were given in dimethyl sulfoxide (DMSO) at age 8 and 15 days, respectively. Control groups of 24 males and 23 females were given intraperitoneal injections of DMSO only. There was no significant effect on survival. The mice were killed at age 12 months and underwent a complete necropsy; livers, lungs, and gross lesions were examined microscopically. Only one male mouse, in the treated group, developed liver adenomas (controls, 0/24; treated, 1/23). No liver tumours were observed in treated females and control females.

In a second experiment in the study by Von Tungeln et al. (2002), groups of 24 male and 24 female B6C3F$_1$ mice (age, 8 days) were given two intraperitoneal injections of acrolein at a total dose of 75 nmol. One third [25 nmol] and two thirds [50 nmol] of the total dose were given in DMSO at age 8 and 15 days, respectively. Control groups of 24 males and 24 females were given intraperitoneal injections of DMSO only. There was no significant effect on survival. Mice were killed at age 15 months. Control and treated males developed liver adenomas (incidence: controls, 4/24; treated, 5/24). No liver tumours were observed in control or treated females. [The Working Group noted the principal strength of the study: the use of males and females. The principal limitations were that a single dose was used, justification for the dose used was not given, only data regarding liver tumours were reported, and body-weight data were not provided.]

3.1.4 Subcutaneous injection

A group of 15 female mice [strain not reported, referred to as "partly inbred albinos", of unspecified age ("around 3 months")] were given weekly subcutaneous injections of 0.2 mg of acrolein [purity not reported] in sesame oil for 24 weeks (total dose, 4.8 mg) to assess the induction of sarcoma. Survival was poor, with 11, 6, 3, and 1 mice alive at 12, 15, 18, and 21 months, respectively. After 21–24 months, no sarcomas were observed (Steiner et al., 1943). [The Working Group noted the principal limitations of the study: the small number of animals, poor survival, use of females only, use of a single dose, limited reporting on a sesame oil control group, lack of body-weight data, absence of justification for the dose used, and unspecified histopathological assessment for tumours other than sarcoma. The study was considered inadequate for the evaluation of the carcinogenicity of acrolein.]

3.1.5 Initiation–promotion

A group of 15 strain S mice [sex and age not reported] was given 0.5% acrolein [purity not reported] in acetone by skin application, once per week, for 10 weeks (total dose, 12.6 mg). Twenty-five days after the first application, the mice were given 0.17% croton oil [purity not reported] in acetone by skin application, once per week, for 18 weeks (on the second and third week of treatment the dose was reduced to 0.085%). A group of 20 control animals was given croton oil only by skin application following the same schedule: 0.17% croton oil in acetone was applied once per week, for 18 weeks (on the second and third week of treatment, the dose was also reduced to 0.085%). One control mouse died before the end of the study. After experimental weeks 21–22, no increased incidence of papilloma of the skin was observed in mice initiated with acrolein compared with controls (Salaman & Roe, 1956). [The Working Group noted the principal limitations of the study: the limited reporting of the study, absence of justification for the dose used, and the use of a small number of animals and a single dose. The study was considered inadequate for the evaluation of the carcinogenicity of acrolein.]

3.2 Rat

3.2.1 Inhalation

In a study that complied with GLP, groups of 50 male and 50 female F344/DuCrlCrlj rats (age, 6 weeks) were treated by inhalation with acrolein (purity, > 98.3%; 1.42% acetaldehyde identified by GC-MS) by whole-body exposure for 6 hours per day, 5 days per week, for 104 weeks (JBRC, 2016d, e, f). The concentration in the exposure chambers was set to 0 (clean air, control), 0.1, 0.5, or 2 ppm for males and females and was monitored every 15 minutes. The mean air concentrations (± standard deviation) for these groups were 0.10 ± 0.00, 0.50 ± 0.00, and 2.01 ± 0.02 ppm, respectively. At 104 weeks, survival of females at 2 ppm was significantly decreased, compared with controls. Survival in the groups at 0, 0.1, 0.5, and 2 ppm was: for males, 41/50, 40/50, 37/50, and 42/50, respectively; and for females, 43/50, 42/50, 41/50, and 34/50, respectively. Male rats at 2 ppm showed a decrease in body-weight gain from the first week of exposure and throughout the exposure period, compared with controls. The relative final body weight in males at 0.1, 0.5, and 2 ppm was 96%, 99%, and 88% of the control value, respectively. Body weights of female rats at 2 ppm were slightly but significantly decreased (maximum, 10%) from the first week of exposure and throughout the exposure period, compared with controls. The relative final body weight in females at 0.1, 0.5, and 2 ppm was 101%, 98%, and 95% of the control value, respectively. All rats underwent complete necropsy, and all organs and tissues were examined microscopically.

In treated male rats, there was no significant increase in the incidence of any tumours. One (1/50, 2%) squamous cell carcinoma of the nasal cavity was observed in the group of males at 2 ppm, which was in excess of the value for historical controls (0/599). [The Working Group considered that this rare squamous cell carcinoma of the nasal cavity may have been related to exposure.]

Of the females, two rats (2/50, 4%) developed squamous cell carcinoma of the nasal cavity in the group at 2 ppm. Although not significantly increased compared with controls, the incidence of this rare tumour exceeded the historical control rate (0/600). The incidence of rhabdomyoma of the nasal cavity was 0/50 (control), 0/50 (0.1 ppm), 0/50 (0.5 ppm), and 4/50 (8%, 2 ppm), and showed a significant positive trend ($P \leq 0.0007$, Peto test (prevalence method) and Cochran–Armitage test), and the incidence in the group at 2 ppm exceeded the historical control rate (0/600). The incidence of squamous cell carcinoma or rhabdomyoma (combined) of the nasal cavity was 0/50 (control), 0/50 (0.1 ppm), 0/50 (0.5 ppm), and 6/50 (12%, 2 ppm), and showed a significant positive trend ($P < 0.0001$, Peto test (prevalence method and combined analysis) and Cochran–Armitage test). The incidence in the group at 2 ppm (12%) was significantly increased ($P = 0.0133$, Fisher test) compared with the control value. [The Working Group considered that rare squamous cell carcinomas and rhabdomyomas of the nasal cavity observed in female rats were related to exposure. The Working Group also noted that these two tumours have different histotypes.] The incidence of adenoma in the pituitary gland was 14/50 (28%, control), 15/50 (30%, 0.1 ppm), 20/50 (40%, 0.5 ppm), and 17/50 (34%, 2 ppm), and showed a significant positive trend ($P = 0.0115$; Peto test, standard method). The incidence of adenocarcinoma of the pituitary gland was 0/50 (control), 2/50 (4%, 0.1 ppm), 1/50 (2%, 0.5 ppm), and 0/50 (0%, 2 ppm). The incidence of adenoma or adenocarcinoma (combined) of the pituitary gland, was 14/50 (28%, control), 17/50 (34%, 0.1 ppm), 21/50 (42%, 0.5 ppm), and 17/50 (34%, 2 ppm), and showed a significant positive trend ($P = 0.0215$; Peto test, standard method); however, the incidence of adenocarcinoma of the pituitary gland was not significantly increased. [The Working Group considered that the occurrence of adenoma and/or adenocarcinoma of the pituitary gland may not be related to exposure, because of the high background incidence of adenoma of the pituitary gland in ageing rats, because the increased incidence was seen in females only, and because the incidence of adenocarcinoma of the pituitary gland was not significantly increased.]

Regarding non-neoplastic lesions in the respiratory tract (see also Section 4 of the present monograph), for males at 2 ppm there was a significant increase in the incidence and/or severity of: inflammation and squamous cell metaplasia in the respiratory epithelium; eosinophilic change, respiratory metaplasia, and atrophy in the olfactory epithelium; respiratory metaplasia in the nasal glands; hyperplasia in the transitional epithelium; adhesion in the turbinate; goblet cell hyperplasia; inflammation with foreign body; oedema in the lamina propria; and proliferation of striated muscle was observed in the nasal cavity. For females at 2 ppm, there was a significant increase in the incidence and/or severity of: inflammation and squamous cell metaplasia in the respiratory epithelium; respiratory metaplasia and atrophy in the olfactory epithelium; respiratory metaplasia in the nasal glands; hyperplasia in the transitional epithelium; goblet cell hyperplasia; inflammation with foreign body; and oedema in the lamina propria was observed in the nasal cavity. [The Working Group considered that hyperplasias of the respiratory tract observed in both males and females were pre-neoplastic lesions.]

[The Working Group noted this was a GLP study conducted with multiple doses and using males and females.]

3.2.2 Oral administration (drinking-water)

Groups of 20 male and 20 female Fischer 344 rats (age, 7–8 weeks), were given drinking-water containing acrolein at a concentration of 0 mg/L (control), 100 mg/L (males only), 250 mg/L (males only), or 625 mg/L [purity not reported] stabilized with hydroquinone [concentration not reported], for 5 days per week (the other 2 days per week, the rats were given tap water) for 124 weeks (except for 104 weeks for the highest dose) (Lijinsky & Reuber, 1987; Lijinsky, 1988). The rats were killed at age 124–132 weeks There was little or no difference in survival [data were not reported]. [Body-weight and water-consumption data were not reported.] Histopathological examination was performed on all lesions, major tissues, and organs. There was a significant increase in the incidence of liver tumours (mainly neoplastic nodules, and a few hepatocellular carcinomas) in the group of males at the lowest dose compared with controls. [The Working Group noted the principal strengths of the study: this was a long-term study (> 2 years) that used multiple doses in males, and both males and females. The principal limitations were the small number of animals per group, the variable duration of treatments between groups, and the use of a single dose in females.]

3.2.3 Oral administration (gavage)

In a study by Parent et al. (1992), groups of 70 male and 70 female Sprague-Dawley rats (age, about 6 weeks), received acrolein (purity, 94.9–98.5%; stabilized with 0.25–0.31% hydroquinone; in deionized water) at a dose of 0 (control), 0.05, 0.5, or 2.5 mg/kg bw by daily gavage for up to 102 weeks. The dosing volume was 10 mL/kg bw. Excess mortality was reported in all groups; this was attributed to trauma during gavage dosing, mis-dosing, or reasons unknown. Ten rats of each sex per dose group were killed after 1 year, and surviving rats were killed [the number of surviving rats was not reported] after 102 weeks. There was a negative trend in survival and a decrease in survival at the highest dose that was significant among males and females during the first year, but only significant in females throughout the entire treatment period. There was no significant effect on body weight. All major tissues and gross lesions from rats in the control group and at the highest dose were examined microscopically; only the lungs, liver, kidneys, and gross lesions from the groups at the lowest and intermediate dose were examined microscopically. In treated males, the incidence of cortical cell adenoma of the adrenal gland was increased, but the effect was not statistically significant. Cortical cell carcinoma of the adrenal gland was seen in two male rats, one in each group at the intermediate and highest dose. In females, the incidence of cortical cell adenoma of the adrenal gland in treated rats was not significantly increased, and no adrenal gland carcinomas were observed in any group. [The Working Group noted the principal strengths of the study: this was a long-term study (> 2 years) that used multiple doses, and both males and females. The principal limitations were the reduced survival among treated females; that histopathological data were reported only for adrenal gland tumours; and that full histopathological examination was performed only for rats in the control group and at the highest dose, while only the lungs, liver, kidneys, and gross lesions from rats in the groups at the lowest and intermediate dose were examined microscopically.]

3.2.4 Initiation–promotion

To evaluate the initiating activity of acrolein, two groups of 30 male Fischer 344 rats (age, 5 weeks) were given intraperitoneal injections of acrolein (purity, 97%; containing approximately 3% water and 200 ppm hydroquinone; in distilled water), at a dose of 0 (sham control, distilled water) or 2 mg/kg bw, twice per week,

for 6 weeks, followed by feed containing 3% uracil for 20 weeks, and then control feed for 6 weeks. Another group (negative control group) of 30 male rats was given intraperitoneal injections of acrolein at a dose of 2 mg/kg bw, twice per week, for 6 weeks, followed by control feed for 26 weeks. A positive control group of 30 male rats was given feed containing 0.2% *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) for 6 weeks, followed by feed containing 3% uracil for 20 weeks, and then control feed for 6 weeks. The rats were killed at experimental week 32. The stomach, lungs, oesophagus, liver, kidneys, and bladder were processed for histopathological examination. A significant increase [$P < 0.02$, Fisher exact test] in the incidence of urinary bladder papilloma was observed in rats initiated with acrolein and then exposed to the promotor uracil, compared with sham controls. The incidence of urinary bladder carcinoma was not significantly increased (Cohen et al., 1992). [The Working Group noted that the principal strength of the study was the sufficient duration. The principal limitations were the use of a single dose, and that data from stomach, lungs, oesophagus, liver, and kidneys were not reported.]

To evaluate the promoting activity of acrolein, two groups of 30 male Fischer 344 rats (age, 5 weeks) were given feed containing 0.2% FANFT for 6 weeks during the first phase, followed by intraperitoneal injections of acrolein (purity, 97%; containing 3% water and 200 ppm hydroquinone; in distilled water) at 0 (sham control, distilled water) or various concentrations (described below) during the second phase. Another group (acrolein-only group) received intraperitoneal injections of acrolein for the first and second phases. The intraperitoneal injections of acrolein were given as follows: 0 or 2 mg/kg bw, twice per week, during experimental week 1–9; 0 or 1.5 mg/kg bw, once at experimental week 10; 0 or 1.5 mg/kg bw, twice per week, during experimental week 11–17; and 0 or 1.0 mg/kg bw, once at experimental week 18, twice at experimental week 19, and once at experimental weeks 20 and 21. A negative control group was given the control feed only. The rats were killed at experimental week 53. The stomach, lungs, oesophagus, liver, kidneys, and bladder were processed for histopathological examination. No papilloma or carcinoma of the urinary bladder developed in any of the four groups of rats, apart from one rat bearing a carcinoma in the FANFT-only group (sham control). Regarding pre-neoplastic lesions, there was a significant increase ($P < 0.001$) in the incidence of simple or papillary/nodular (combined) hyperplasia of the urinary bladder in the acrolein-only group (16/30) compared with the negative (untreated) control group (0/30) (Cohen et al., 1992). [The Working Group noted that the principal limitations of the study were the use of a single dose, and that data from stomach, lungs, oesophagus, liver, and kidneys were not reported.]

3.3 Hamster

3.3.1 Inhalation

In a study by Feron & Kruysse (1977), groups of 30 male and 30 female Syrian golden hamsters (age, 6 weeks), were treated with acrolein at 0 mg/m^3 (filtered air, control), or 9.2 mg/m^3 [purity not reported] by inhalation with whole-body exposure for 7 hours per day, 5 days per week, for 52 weeks, and the hamsters were then killed at 81 weeks. Half of the hamsters also received intratracheal instillations of 0.2 mL of 0.9% sodium chloride, once per week, for 52 weeks [but mortality and tumour results were reported and combined for all 30 animals of each sex per group]. Survival in males was low, but acrolein exposure did not affect survival rate. Seven male controls, 7 treated males, 16 control females, and 13 treated females were alive at 80 weeks. All hamsters were subject to full necropsy, but only the entire respiratory tract, grossly visible tumours, and gross lesions suspected of being tumours

were examined microscopically. No respiratory tract tumours were observed in any group, apart from a single papilloma of the trachea that was found in a treated female. Exposure to acrolein vapour caused inflammation and a slight to moderate degree of epithelial metaplasia in the nasal cavity. [The Working Group noted that the principal strength of the study was the use of males and females. The principal limitations were the small number of animals per group; the short duration of the exposure; absence of justification for the dose used; the lower survival in males; reporting of pathological data only for respiratory tract tumours; and the use of a single dose.]

3.3.2 Administration with known carcinogens

In a study by Feron & Kruysse (1977), groups of 30 male and 30 female Syrian golden hamsters (age, 6 weeks), were treated with acrolein [purity not reported] at a concentration of 0 mg/m^3 (filtered air, control groups), or 9.2 mg/m^3 by inhalation with whole-body exposure for 7 hours per day, 5 days per week, for 52 weeks, together with either weekly intratracheal instillations of a suspension of 0.175% or 0.35% benzo[*a*]pyrene (B[*a*]P, purity > 99%) in 0.9% sodium chloride (B[*a*]P total dose, 18.2 or 36.4 mg/animal) or subcutaneous injections of 0.0625% *N*-nitrosodiethylamine (NDEA) in 0.2 mL of 0.9% sodium chloride once per 3 weeks (NDEA total dose, 2.1 µL/animal), and the hamsters were then killed at 81 weeks. Half of the control animals received also intratracheal instillations of 0.2 mL of 0.9% sodium chloride, once per week, for 52 weeks [but mortality and tumour results were reported and combined for all 30 control animals per sex]. Survival in males was low, but acrolein exposure did not affect survival rate. All hamsters were subject to full necropsy, but only the entire respiratory tract, grossly visible tumours, and gross lesions suspected of being tumours were examined microscopically.

There were no significant differences in the number or incidence of total respiratory tract tumours, or in the incidence of tumours of the nasal cavity, larynx, trachea, bronchi, or lungs. Tumours appeared slightly earlier in the groups of males and females exposed to acrolein plus NDEA compared with their respective NDEA-only controls. Exposure to acrolein vapour caused inflammation and a slight to moderate degree of epithelial metaplasia in the nasal cavity. [The Working Group noted the principal strength of the study: the use of males and females. The principal limitations were: the small number of animals per group; the short duration of the exposure; that justification for the dose was not provided; the lower survival in males; that pathological data were reported only for respiratory tract tumours; and the use of a single dose.]

3.4 Evidence synthesis for cancer in experimental animals

The carcinogenicity of acrolein has been assessed in one study that complied with GLP in male and female mice and rats treated by inhalation with whole-body exposure. The carcinogenicity of acrolein in mice and rats was also evaluated by other routes of exposure in studies that did not comply with GLP. Specifically, in mice, there was one study in males and females treated by oral administration (gavage), and there were two studies in newborn males and females treated by intraperitoneal injection. In addition, one study in females treated by subcutaneous injection and one initiation–promotion study (sex not reported) were available, but these studies were judged to be inadequate for the evaluation of the carcinogenicity of acrolein in experimental animals. In rats, there were two studies in males and females treated by oral administration (one drinking-water study and one gavage study), and two initiation–promotion studies in males. The carcinogenicity of acrolein has been assessed

in hamsters in one study in males and females treated by inhalation with whole-body exposure, both in the presence and absence of two known carcinogens.

In the inhalation study that complied with GLP in B6D2F1/Crlj mice, acrolein significantly increased the incidence of histiocytic sarcoma of the uterus in treated females, but without a clear dose–response relationship, and caused a significant positive trend in the incidence of malignant lymphoma in treated females. In treated females, there was also a significant positive trend and significant increase in the incidence of nasal cavity adenoma, which is a very rare tumour in the mouse strain used in the study (JBRC, 2016a, b, c). In the inhalation study that complied with GLP in F344/DuCrlCrlj rats, there was a significant positive trend in the incidence of rhabdomyoma of the nasal cavity and of squamous cell carcinoma or rhabdomyoma (combined) of the nasal cavity in treated females. The incidence of squamous cell carcinoma or rhabdomyoma (combined) of the nasal cavity was also significantly increased in treated females. Both tumour types are very rare in the rat strain used in the study (JBRC, 2016d, e, f).

In studies of oral administration, acrolein administered by gavage to male and female CD-1 mice or Sprague-Dawley rats did not cause an increased tumour incidence (Parent et al., 1991a). When administered in drinking-water in a study in male Fischer 344 rats, acrolein increased the incidence of liver tumours (mainly benign) (Lijinsky & Reuber, 1987; Lijinsky, 1988).

When tested by intraperitoneal injection, acrolein did not cause an increased tumour incidence in newborn B6C3F$_1$ mice (Von Tungeln et al., 2002). Acrolein increased the incidence of urinary bladder papilloma in male Fischer 344 rats when administered as a tumour initiator with uracil as a tumour promoter (Cohen et al., 1992). The incidence of urinary bladder tumours was not increased when acrolein was tested as a promoter in male Fischer 344 rats, with FANFT as the initiator (Cohen et al., 1992).

Tumours did not occur in mice treated with acrolein by subcutaneous injection (Steiner et al., 1943), and acrolein did not increase the incidence of skin tumours in an initiation–promotion study in strain S mice, with croton oil as the promoter (Salaman & Roe, 1956). However, these studies were judged to be inadequate for the evaluation of the carcinogenicity of acrolein in experimental animals.

In Syrian golden hamsters treated by inhalation with whole-body exposure, acrolein did not increase the incidence of tumours, either in the presence or absence of known carcinogens (B[*a*]P or NDEA) (Feron & Kruysse, 1977).

4. Mechanistic Evidence

4.1 Absorption, distribution, metabolism, and excretion

4.1.1 Humans

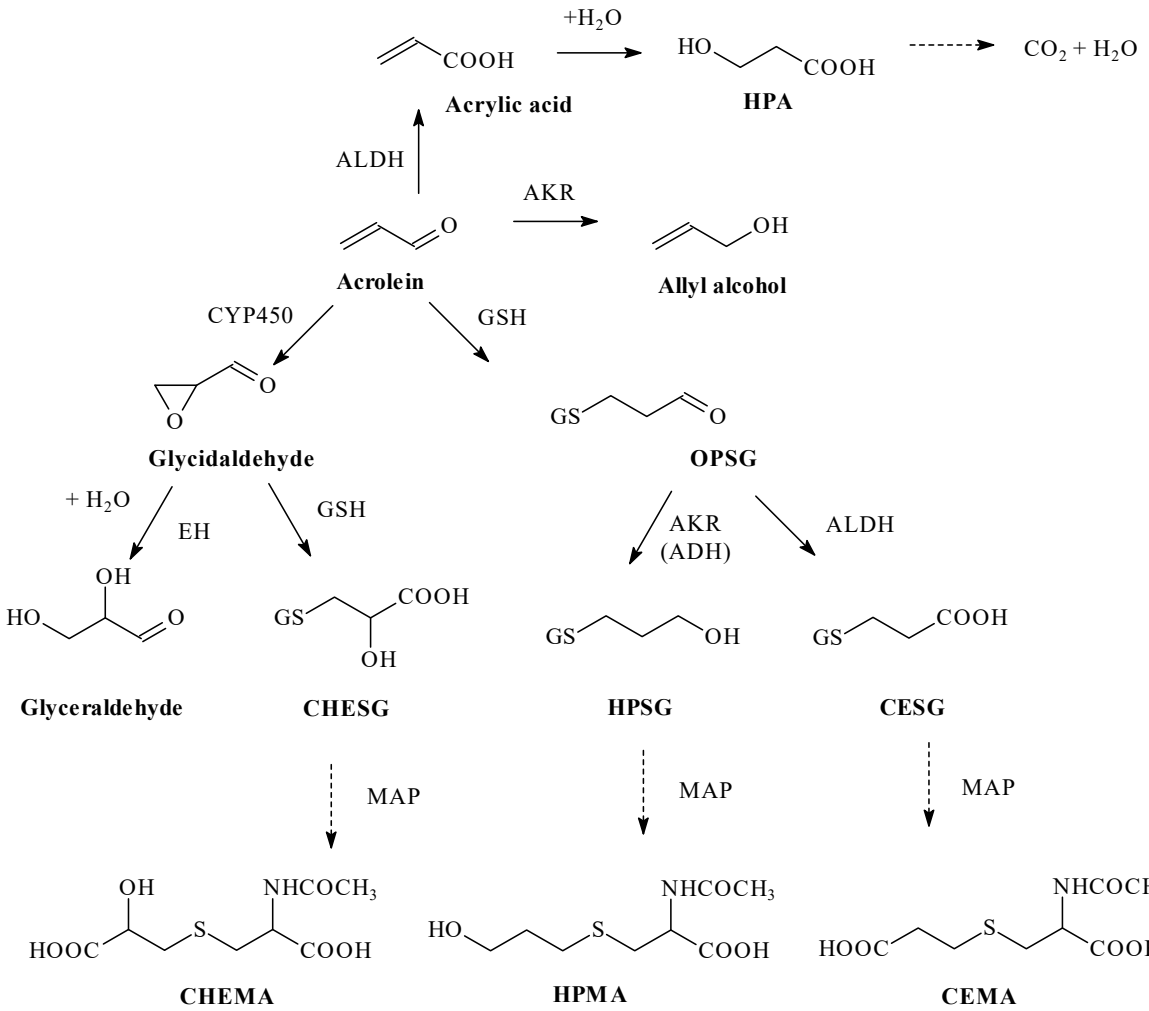
(a) Exposed humans

No data on the absorption or distribution of acrolein by inhalation were available to the Working Group.

The main metabolic pathways of acrolein are depicted in Fig. 4.1.

Two acrolein-derived mercapturic acids, HPMA and CEMA, were found in the urine of both smokers and non-smokers, HPMA being consistently the more common. Tobacco smokers showed significantly higher levels of both HPMA and CEMA (Alwis et al., 2012, 2015). A significant increase in acrolein-derived urinary mercapturic acids was also reported shortly after being served heat-processed food containing acrolein (Wang et al., 2019; Watzek et al., 2012). These mercapturic acids were also found in a limited exploratory toxicokinetic study on a single subject within

Fig. 4.1 The main pathways of acrolein metabolism



ADH, alcohol dehydrogenase; AKR, aldo-keto reductase; ALDH, aldehyde dehydrogenase; CEMA, *N*-acetyl-*S*-(carboxyethyl)-L-cysteine (2-carboxyethylmercapturic acid); CESG, *S*-(2-carboxyethyl)glutathione; CHEMA, *N*-acetyl-*S*-(2-carboxy-2-hydroxyethyl)-L-cysteine (2-carboxy-2-hydroxyethylmercapturic acid); CHESG, *S*-(2-carboxy-2-hydroxyethyl)glutathione; CYP450, cytochrome P450; EH, epoxide hydrolase; GSH, glutathione; HPA, 3-hydroxypropanoic acid; HPMA, *N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine (3-hydroxypropylmercapturic acid); HPSG, *S*-(3-hydroxypropyl)glutathione; MAP, mercapturic acid pathway; OPSG, *S*-(3-oxopropyl)glutathione.
Adapted from Patel et al. (1980), Parent et al. (1998), and Kurahashi et al. (2014).

24 hours after oral uptake of acrolein at a dose of 7.5 µg/kg bw in drinking-water. For HPMA and CEMA, respectively, elimination half-times were 8.9 hours and 11.8 hours, and maximum urinary concentrations reached 2 hours after ingestion were 1.61 and 1.05 µmol/g creatinine (Watzek et al., 2012). A similar elimination half-time for acrolein (9 hours) based on urinary metabolite HPMA profile was reported in a study on subjects who were served fried food containing acrolein (Wang et al., 2019).

Acrolein can be produced endogenously, including as a result of lipid peroxidation (Nath & Chung, 1994; Stevens & Maier, 2008).

Acrolein can be excreted unchanged in exhaled air (Andreoli et al., 2003; Ligor et al., 2008; Ruenz et al., 2019).

Free acrolein was also found in the urine (Al-Rawithi et al., 1998; Takamoto et al., 2004)

and saliva (Korneva et al., 1991) of patients treated with cyclophosphamide; acrolein is a metabolite of cyclophosphamide. Once formed, acrolein appeared to be rapidly excreted in urine because its urinary concentration peaked shortly (1–12 hours) after treatment with cyclophosphamide (Takamoto et al., 2004).

A role of glutathione *S*-transferases (GSTs) in detoxification of acrolein was demonstrated by a randomized clinical trial in which a significant increase in the excretion of HPMA was observed in individuals who received 2-phenethyl isocyanate, an inducer of GST mu 1 (GSTM1) and GST theta 1 (GSTT1), compared with controls (Yuan et al., 2016). The role of GSH and GSTs is discussed further below (see Section 4.1.2).

(b) In vitro

A low absorption rate of 0.480 ± 0.417 μg/cm^2 in 30 minutes was observed in experiments with human skin in vitro at 153 ppm (351 mg/m^3) of acrolein in air (Thredgold et al., 2020).

The reaction of acrolein with GSH in vitro is efficiently catalysed by human GST ε, μ, and π, the last one isolated from human placenta being the most catalytically active (Berhane & Mannervik, 1990). Significant differences were found in catalytic efficiency (k_{cat}/K_m) between four allelic variants of the π isoenzyme (hGSTP1-1) (Pal et al., 2000). However, in a search for genetic variants related to acrolein metabolism to mercapturic acids (GST polymorphism) by a genome-wide association study, no association with HPMA levels in smokers after adjusting for total nicotine equivalents was found (Park et al., 2015). [The Working Group noted that these results, together with the known high electrophilic reactivity of acrolein, suggest that its conjugation with GSH leading eventually to the excretion of HPMA is mainly a spontaneous non-catalysed process.]

Acrolein can be reduced by human aldo-keto reductases with catalytic efficiencies that vary greatly among the superfamily members. Thus, aldose reductase (EC 1.1.1.21) catalysed acrolein reduction with k_{cat}/K_m = 1.09 μM^{-1} min^{-1} and was significantly induced (7–20-fold) towards a variety of aldehydes by acrolein (Kolb et al., 1994). Human aldo-keto reductase AKR1A showed a much lower catalytic activity (k_{cat}/K_m = 0.29 × 10^{-3} μM^{-1} min^{-1} (Kurahashi et al., 2014), whereas AKR1B1 and ABR1B10 showed k_{cat}/K_m values of 0.12 and 1.07 μM^{-1} min^{-1}, respectively (Shen et al., 2011). AKR1B1, which is ubiquitously expressed in humans, also efficiently reduced the acrolein–GSH conjugate (*S*-(3-oxopropyl)glutathione, OPSG) with k_{cat}/K_m = 0.355 μM^{-1} min^{-1}, whereas AKR1B10 expressed mainly in the gastrointestinal tract showed a much lower catalytic efficiency, k_{cat}/K_m = 0.004 μM^{-1} min^{-1} (Shen et al., 2011). Downregulation of the *AKR1B10* gene increased the susceptibility of a colorectal cancer cell line to cytotoxicity caused by acrolein (Yan et al., 2007).

4.1.2 Experimental systems

(a) In vivo

Due to high electrophilicity and solubility in water, a significant portion of inhaled acrolein is taken up in the upper respiratory tract. Experiments on Fischer 344 rats with surgically isolated upper respiratory tract in vivo showed that the nasal uptake efficiency decreased with increasing acrolein concentration, time of exposure, and inspired air flow rate (Morris, 1996; Struve et al., 2008). At the inspired air flow rate of 100 mL/minute, the uptake efficiency averaged over an 80-minute exposure period was 98%, 68%, and 50% at 0.6, 1.8, and 3.6 ppm, respectively. At 300 mL/minute these values fell to 85%, 48%, and 38%, respectively (Struve et al., 2008). Somewhat lower time-averaged values were obtained earlier by Morris (1996), namely, 62%, 38%, and 28% at the exposure concentrations 0.87, 4.4, and 8.7 ppm, respectively (inspiratory flow rate, 200 mL/minute).

GSH concentrations in nasal epithelium were markedly lowered in a concentration-dependent

manner in rats exposed for 80 minutes. However, when the rats were pre-exposed to acrolein at 3.6 ppm during 14 days (6 hours per day, 5 days per week), the depletion was nearly compensated by an adaptive response (Struve et al., 2008). A marked depletion in rat nasal GSH was also reported earlier by Lam et al. (1985). [The Working Group noted that these results indicate a marked influence of tissue reactivity on uptake in the upper respiratory tract.]

Mercapturic acids, namely, HPMA and CEMA, were identified in the urine of rats dosed subcutaneously (Kaye, 1973; HPMA only) or orally with acrolein (Draminski et al., 1983; CEMA only), as well as in mice after inhalation and intraperitoneal injection (Linhart et al., 1996). Due to its high electrophilic reactivity, acrolein forms protein adducts in vivo (Gan & Ansari, 1989; Kautiainen et al., 1989; see also Section 4.2.1). A gradual accumulation of protein-adducted acrolein was reported in mice exposed to acrolein by inhalation at 1.5 ppm for 30 minutes twice per day for 3 weeks. At the same time, a gradual increase in urinary HPMA excretion was observed (Tully et al., 2014).

The metabolism and disposition of [2,3-^{14}C]acrolein was studied in male and female Sprague-Dawley rats treated by oral and intravenous administration (Parent et al., 1996a, 1998). The rats were divided into five groups of 5 males and 5 females and were given a single dose of [2,3-^{14}C]acrolein intravenously at 2.5 mg/kg bw, or orally by gavage at 2.5 or 15 mg/kg bw. One group of rats was pre-exposed to unlabelled acrolein for 14 days at 2.5 mg/kg-day before oral administration of [2,3-^{14}C]acrolein at 2.5 mg/kg bw. Urine, faeces, and expired air were collected for 7 days. In all exposure groups, about 26–31% of the radiolabel was exhaled as carbon dioxide while < 1.2% was tissue-bound. Rats given a single intravenous injection of [2,3-^{14}C]acrolein at 2.5 mg/kg bw excreted 66–69% of the radiolabel in urine and < 2% in faeces. The main urinary metabolites were identified by HPLC/MS analysis using authentic standards as 3-hydroxypropanoic acid, HPMA, CEMA, and *N*-acetyl-*S*-(2-carboxy-2-hydroxyethyl)-L-cysteine (2-carboxy-2-hydroxyethylmercapturic acid, CHEMA) and traces of malonic acid. After oral doses, less radiolabel was excreted in the urine (lower dose, 52%; higher dose, 36.5%) and more in the faeces (lower dose, 13%; higher dose, 31%). Two additional urinary metabolites, oxalic and malonic acid, were identified (Parent et al., 1998). No significant effect on the excretion pattern was observed after pre-treatment with acrolein. The main portion of radiolabel was excreted within 48 hours after dosing, but excretion was delayed in the group receiving the higher oral dose. The analysis of faeces did not reveal any distinct peaks in the excretion of radiolabel over time. [The Working Group noted that faeces probably contained polymers of acrolein or polysaccharide, or protein adducts resulting from the reaction of acrolein with food components.]

A computational fluid dynamics model was developed to predict nasal dosimetry of acrolein in rats and humans using parameters adjusted to fit experimental uptake efficiency data from Struve et al. (2008) and Morris (1996). In humans, calculated nasal uptake efficiencies for inhaled acrolein were 16% and 28% at exposure concentrations of 3.6 ppm and 0.6 ppm, respectively, and were consistently lower than those in rats. These predictions capture the overall trend of increased uptake when exposure concentrations decrease (Schroeter et al., 2008). [The Working Group noted that because of oral breathing, delivery of acrolein to the lower respiratory tract could be higher in humans than in rats, which are obligate nasal breathers.]

(b) In vitro

Acrolein reacts spontaneously with GSH to form OPSG (Esterbauer et al., 1975; Mitchell & Petersen, 1989; Horiyama et al., 2016), which is subsequently oxidized by rat liver aldehyde

dehydrogenase (ALDH) to *S*-(2-carboxyethyl)glutathione (CESG) and, in a lesser extent, reduced by rat liver alcohol dehydrogenase (ADH) to *S*-(3-hydroxypropyl)glutathione (HPSG) as the affinity of ADH (K_m=877 μM) was low compared with the high-affinity cytosolic (K_m = 310 μM) and mitochondrial (K_m = 198 μM) ALDH forms (Mitchell & Petersen, 1989). Rat AKR7A1 catalysed reduction of both acrolein and its GSH conjugate. Chinese hamster V79 cells expressing rat AKR7A1 were efficiently protected against acrolein-induced mutations (Gardner et al., 2004) (see Section 4.2.2b).

The carbonyl group of acrolein can be oxidized by ALDH and reduced by aldo-keto reductases (AKR). Recombinant mouse ALDH1a1 and ALDH 3a1 efficiently oxidized acrolein to acrylic acid, ALDH1a1 showing comparable catalytic efficiency (V_{max}/K_m >> 23) but a higher affinity (K_m = 23.2 μM) than ALDH3a1 (K_m = 464 μM) (Makia et al., 2011). Significant catalytic ALDH activities were found in the microsomes, cytosol, and mitochondria of rat liver (Rikans, 1987). In mitochondria, two different ALDH activities were found: a high-affinity one with K_m = 0.017 mM, V_{max} = 42.2 nmol min^{-1} mg^{-1}, and a low-affinity one with K_m = 0.430 mM, V_{max} = 29.2 nmol min^{-1} mg^{-1}. Similarly, in the cytosolic fraction, there was a high-affinity ALDH form with K_m = 0.026 mM, V_{max} = 14.9 nmol min^{-1} mg^{-1}, and a low-affinity form with K_m = 0.725 mM, V_{max} = 7.1 nmol min^{-1} mg^{-1}. In the microsomes, a single low-affinity ALDH activity with K_m = 1.5 mM and V_{max} = 30.5 nmol min^{-1} mg^{-1} was reported. Hence, the low K_m ALDH in mitochondria was found to have the highest metabolic activity (Rikans, 1987). [The Working Group noted that both oxidation by ALDHs and reduction by AKRs are important detoxication pathways in the metabolism of acrolein.]

Metabolic activation of acrolein to glycidaldehyde and its detoxification to acrylic acid were described in rat liver and lung preparations by Patel et al. (1980). Notably, oxidation to acrylic acid was not observed in the lung preparations. Glycidaldehyde is a substrate for epoxide hydrolases as well as for cytosolic GSTs in rat lung and liver (Patel et al., 1980). However, metabolic activation was not necessary for conjugation with GSH. A weak increase in GSH conjugation as measured by GSH depletion was observed only when cytochrome P450 (CYP) in rat liver microsomes was induced by pre-treatment of rats with phenobarbital (Garle & Fry, 1989). Experiments with [^{14}C]-labelled acrolein proved its covalent association with rat microsomal CYP and further metabolism to an epoxide (Marinello et al., 1984). [The Working Group noted that conjugation of glycidaldehyde with GSH should lead to urinary CHEMA, a confirmed metabolite of acrolein.]

4.2 Evidence relevant to key characteristics of carcinogens

This section summarizes the evidence for the key characteristics of carcinogens (Smith et al., 2016), including whether acrolein is electrophilic or can be metabolically activated to an electrophile; is genotoxic; alters DNA repair or causes genomic instability; induces oxidative stress; is immunosuppressive; induces chronic inflammation; alters cell proliferation, cell death, or nutrient supply; induces epigenetic alterations; modulates receptor-mediated effects; and causes immortalization.

4.2.1 Is electrophilic or can be metabolically activated to an electrophile

(a) DNA binding

(i) Studies in humans

Acrolein is a chemically reactive aldehyde that directly interacts with DNA as a result of its α,β-unsaturated carbonyl function. As further described in Section 4.2.1(b), it forms four isomeric α- and γ-hydroxy-1,N^2-

propano-2′-deoxyguanosine adducts (α-OH-PdG and γ-OH-PdG, two of each), and their ring-opened precursors (Chung et al., 1999). Acrolein-induced DNA adducts have been found in various tissues in studies in humans, including lung, buccal cells, leukocytes, peripheral blood, liver tissues, sputum, brain tissues, bladder, and urothelial mucosa (Weng et al., 2018; Zhang et al., 2007; Yang et al., 2019a; Chung et al., 2012; Nath et al., 1998; Bessette et al., 2009; Wang et al., 2019; Tsou et al., 2019; Zhang et al., 2011; Yin et al., 2013; McDiarmid et al., 1991; Alamil et al., 2020; Nath & Chung, 1994; Fu et al., 2018; Chen & Lin, 2011; Liu et al., 2005; Lee et al., 2014; Hong et al., 2020; see Table 4.1 and Table 2.1). Furthermore, significantly higher levels of acrolein–DNA adducts were found in bladder tumour tissues, hepatocellular carcinoma tissues, and brain tissues from patients with Alzheimer disease (Liu et al., 2005; Lee et al., 2014; Fu et al., 2018; see Table 4.1 and Table 2.1).

Acrolein–DNA adduct formation has been detected in the leukocytes of 40% of a group of patients treated with cyclophosphamide compared with none of the controls (McDiarmid et al., 1991). Several studies reported higher levels of acrolein–dG adducts in the buccal cells and lung tissues of tobacco smokers (Nath et al., 1998; Zhang et al., 2007; Weng et al., 2018). Similar results have been reported using immunochemical, ^{32}P-postlabelling 2D thin-layer chromatography/high-performance liquid chromatography (TLC/HPLC) and LC-MS/MS methods for measuring acrolein–dG adducts in buccal cells (Nath et al., 1998; Weng et al., 2018; Wang et al., 2019). [The Working Group noted that different methods were used in these studies, which may account for differences in levels detected.] Using the immunochemical method and ^{32}P-postlabelling 2D TLC/HPLC, Weng et al. (2018) reported that γ-OH-PdG accumulated significantly more in smokers than in non-smokers (Weng et al., 2018). Using the LC-MS/MS method, Chung et al. (2012) also confirmed that γ-OH-PdG is the major acrolein–DNA adduct formed in the human lung tissues. Alamil et al. (2020) reported higher levels of acrolein–DNA adducts in peripheral blood in a smoker than in a non-smoker. On the other hand, it has been reported that two isomers, α- and γ-OH-PdG, formed almost equally in the lung tissues of smokers and non-smokers; and that the level of acrolein–DNA adducts in smokers is not significantly different from that in non-smokers (Ma et al., 2019; Yang et al., 2019a). The levels of acrolein–dG adducts detected were about 10–100 times lower than those reported by other laboratories, and the levels of acrolein–DNA adducts in leukocytes and lungs were similar (Chung et al., 2012; Ma et al., 2019; Zhang et al., 2011; Weng et al., 2018; Yang et al., 2019a; Alamil et al., 2020). [Since tobacco smoke contains substantial amounts of acrolein (see Section 1.4.2(b)), the Working Group noted that the lack of differences in acrolein–DNA adduct formation in both lung tissues and leukocytes of smokers and non-smokers may be explained by other exposure sources.]

(ii) Human cells in vitro

There is ample evidence demonstrating that acrolein can adduct DNA in various primary human cells and in cultured human cell lines in vitro (Wilson et al., 1991; Feng et al., 2006; Pan et al., 2009, 2012, 2016; Greenspan et al., 2012; Wang et al., 2012; see Table 4.2). Feng et al. (2006) reported that acrolein treatment in normal human bronchial epithelial cells and normal human lung fibroblasts induces acrolein–DNA adducts that were preferentially formed at lung cancer *TP53* mutational hotspots, and that acrolein preferentially adducts guanines at cytosine methylation CpG sites. Wang et al. (2009a, 2012), using shuttle vectors containing the *supF* gene, showed that cytosine methylation at CpG sites enhanced acrolein–DNA adduct formation and mutations at these sites; and that in human lung cells, acrolein induced γ-OH-PdG (95%)

Table 4.1 Acrolein-derived DNA adducts in exposed humans

Biosample	Location, setting	Exposure level and no. of exposed and controls	Adduct frequency (analytical method) Response (significance)	Comments	Reference
Lung	Normal lung tissue of tobacco smokers (obtained from marginal tissues during tumour resection) and non-smokers (obtained from the Lung Tissue Research Consortium of the National Heart Lung and Blood Institute)	Smokers (n = 41) Non-smokers (n = 13)	Adducts/10^5 dG (^{32}P postlabelling TLC/HPLC) γ-OH-PdG: 1–24 in smokers vs 1–8 in non-smokers (statistically significant; P value, NR)	Lung, buccal cells, and sputum samples are from different individuals; smoking histories were from < 20 to > 50 packs/year.	Weng et al. (2018)
Lung	Normal tissue obtained at surgery from The Cancer Center Tissue Procurement Facility, University of Minnesota	Smokers (n = 5) Ex-smokers (n = 23)	Adducts/10^9 dG (LC-MS/MS) γ-OH-PdG: 49 in smokers vs 25 in non-smokers	Average calculated by the Working Group; ex-smokers quit smoking 1 mo to 26 yr; samples were from self-reported smokers; moderately well-defined exposure; other sources of acrolein exposure except smoking not considered.	Zhang et al. (2007)
Lung	Tissues obtained during surgery for lung cancer through the Tissue Procurement Facility, University of Minnesota	Smokers (n = 24) Non-smokers (n = 13)	Adducts/10^9 dG (LC-MS/MS) γ-OH-PdG: 20 in smokers vs 15 in non-smokers (NS)	Moderately well-defined exposure; other sources of acrolein exposure except smoking not considered.	Yang et al. (2019a)
Lung	Tissues obtained after surgery from the Histopathology & Tissue Shared Resource of the Lombardi Comprehensive Cancer Center, Georgetown University	n = 5	Adducts/10^9 dG (LC-MS/MS) γ-OH-PdG: 4–10	Poorly defined exposure; unclear whether the adducts were from endogenous exposure or from any unknown external exposure.	Chung et al. (2012)
Buccal cells	Buccal mucosa from subjects free of lung cancer at the time of the initial screening	Smokers (n = 33) Non-smokers (n = 17)	Adducts/10^7 dG (immunochemical method) γ-OH-PdG: 10–250 in smokers vs 5–15 in non-smokers (P < 0.0001)	Lung, buccal cells, and sputum samples are from different individuals; smoking histories were from < 20 to > 50 packs/year.	Weng et al. (2018)
Buccal cells (gingival tissue)	Samples from surgery at a periodontal clinic of New York University Dental Center, New York	11 smokers (4M, 7F); 12 non-smokers (8M, 4F)	Adducts/10^6 dG (^{32}P postlabelling and HPLC) Adduct levels significantly higher in smokers 1.36 ± 0.90 than in non-smokers 0.46 ± 0.26 (P = 0.003)	Small study; self-reported exposure.	Nath et al. (1998)

Table 4.1 (continued)

Biosample	Location, setting	Exposure level and no. of exposed and controls	Adduct frequency (analytical method) Response (significance)	Comments	Reference
Buccal cells	Smokers	$n = 5$	Adducts/10^7 dG (loss-triple stage with linear quadruple ion trap MS) > 5 per 10^7 unmodified DNA bases in buccal cell DNA	Tobacco smokers, smoking > 20 cigarettes per day, and on a non-controlled diet.	Bessette et al. (2009)
Buccal cells	Healthy subjects after consumption of fried food from three commercial restaurants	$n = 19$	Acr–dG (immunochemical method) Fried food causes a 50% increase in Acr–dG levels, 2–24 hours after meal ($P < 0.005$)	Urinary HPMA, 30% increase; poorly defined exposure.	Wang et al. (2019)
Buccal cells	Healthy subjects Patients with oral squamous cell carcinoma	$n = 222$ $n = 80$	Acr–dG (immunochemical method) 25% increase ($P = 0.001$)	Moderately well-defined exposure. Sources of acrolein exposure other than smoking, alcohol drinking, and betel-quid chewing were not considered.	Tsou et al. (2019)
Leukocytes	Samples from smokers and non-smokers obtained at the University of Minnesota Tobacco Use Research Center	Smokers ($n = 25$) Non-smokers ($n = 25$)	γ-OH-PdG/10^9 nucl (LC-MS/MS) Adduct levels: smokers, 7.4 ± 3.4 adducts/10^9 nucl; non-smokers, 9.8 ± 5.5 adducts/10^9 nucl; (NS)	No difference between smokers and non-smokers; poorly defined exposure; unclear whether the adduct levels are from endogenous exposure or from any unknown external exposure.	Zhang et al. (2011)
Leukocytes	Provided by five subjects	$n = 5$	γ-OH-PdG/10^8 nucl (LC-MS/MS) 7.5–11 adducts/10^8 nucl (mean, ~9.0 ± 1.3 adducts/10^8 nucl)	Acr–dA, Acr–dC and etheno–DNA also detected. Poorly defined exposure; unclear if the adduct levels are from endogenous exposure or from any unknown external exposure.	Yin et al. (2013)
Leukocytes	Patients treated with cyclophosphamide Untreated matched patients	$n = 12$ $n = 15$	Acr–dG (immunochemical methods) 6 positive results in cyclophosphamide treated patients vs 0 in matched controls ($P = 0.003$)	Moderately well-defined exposure; sources of acrolein exposure other than smoking were not considered.	McDiarmid et al. (1991)

Table 4.1 (continued)

Biosample	Location, setting	Exposure level and no. of exposed and controls	Adduct frequency (analytical method) Response (significance)	Comments	Reference
Peripheral blood	Smoker vs non-smoker	$n = 1$ $n = 1$	Acr–dG/10^7 nucl (LC-MS/MS) 4.1 in smokers, NR in non-smokers	Reduced FA–dG and MDA–dG detected; compared single samples, one a heavy smoker, for first-level validation of methods; no measurements supporting smoking extent provided (e.g. cotinine); smoker was said to have regularly smoked 30 cigarettes/day; acrolein adducts not seen in non-smoker.	Alamil et al. (2020)
Liver	Autopsy samples from Colombia University, New York	$n = 5$	γ-OH-PdG/10^6 nucl (^{32}P postlabelling and HPLC) 0.03–0.74 adducts/10^6 dG	The health status of these individuals was unknown; demonstration study.	Nath & Chung (1994)
Liver	Tissues obtained after surgery from the Histopathology & Tissue Shared Resource of the Lombardi comprehensive Cancer Center, Georgetown University	$n = 5$	γ-OH-PdG/10^9 nucl (LC-MS/MS) 1.11 adducts/10^9 dG	Poorly defined exposure; unclear if the adduct levels are from endogenous exposure or from any unknown external exposure.	Chung et al. (2012)
Liver	Liver biopsies or HCC specimens from patients who had liver biopsies or curative resection of HCC as part of standard medical care; Georgetown University Medical Center		γ-OH-PdG (immunostaining)	Biomarker for predicting the risk of human HCC recurrence.	Fu et al. (2018)
	HCC patients	$n = 90$	High γ-OH-PdG levels in the HCC specimens were strongly correlated ($P < 0.0001$) with poorer survival in these patients.		
	HCC recurrence 500 days after surgery	$n = 45$	Patients with tumours with low γ-OH-PdG experienced a significantly longer HCC recurrence-free survival than patients with tumours with high γ-OH-PdG ($P < 0.007$)		

Table 4.1 (continued)

Biosample	Location, setting	Exposure level and no. of exposed and controls	Adduct frequency (analytical method) Response (significance)	Comments	Reference
Sputum	From subjects free of lung cancer at the time of the initial screening	Smokers (n = 22) Non-smokers (n = 8)	Adducts/10^7 dG (immunochemical method) γ-OH-PdG: 5–240 in smokers vs 5–130 in non-smokers ($P < 0.05$)	Lung, buccal cells, and sputum samples were from different individuals; smoking histories were from < 20 to > 50 packs/year.	Weng et al. (2018)
Saliva	Healthy individuals	n = 27	γ-OH-AdG/10^8 nucl (LC-MS/MS) 13–218 adducts/10^8 dG	Etheno–dG detected (68–752 adducts/10^8 nucl); no information on external exposure.	Chen & Lin (2011)
Brain	Brain specimens removed at autopsy from subjects with Alzheimer disease and age-matched control subjects	Alzheimer disease, n = 8 (4M, 4F) Controls, n = 5 (3M, 2F)	γ-OH-PdG/10^6 nucl (LC-MS/MS) γ-OH-PdG: 5.1 in specimens from patients with Alzheimer disease vs 2.8 in healthy participants ($P < 0.025$)	Poorly defined exposure; unclear whether the adduct levels are from endogenous exposure or from any unknown external exposure.	Liu et al. (2005)
Bladder mucosa	Bladder tumours Normal urothelial mucosa	n = 10 n = 19	63 ± 25/10^7 dG in bladder tumours vs 25 ± 10 in normal urothelial mucosa ($P < 0.001$)	External exposure not defined.	Lee et al. (2014)
	CKD early CKD late	n = 40 n = 22	30% increase ($P < 0.01$)		
Urothelial tissue (non-smokers)	Normal tissue Tumour tissue	n = 48 n = 48	30% increase ($P < 0.005$)		

Acr, acrolein; CKD, chronic kidney disease; dG, deoxyguanosine; F, female; FA, formaldehyde; HCC, hepatocellular carcinoma; LC-MS/MS, liquid chromatography-tandem mass spectrometry; M, male; MDA, malondialdehyde; mo, month; MS, mass spectrometry; NR, not reported; NS, not significant; nucl, nucleotide; γ-OH-PdG, γ-hydroxy-1,N^2-propano-2′-deoxyguanosine; TLC/HPLC, thin-layer chromatography/high-performance liquid chromatography; vs, versus; yr, year.

Table 4.2 Acrolein-derived DNA adducts in human cells in vitro

End-point	Tissue, cell line	Result[a]	Concentration (LEC or HIC)	Comments	Reference
DNA adducts (^{32}P-postlabelling)	Xeroderma pigmentosum fibroblasts, GM 5509	+	1 μM		Wilson et al. (1991)
DNA adducts (^{32}P-postlabelling)	Primary normal bronchial fibroblasts, human skin fibroblasts from a cystic fibrosis patient, GM 4539	+	100 μM	Only one concentration tested.	Wilson et al. (1991)
DNA adducts (^{32}P-postlabelling)	Lung epithelial cells and fibroblasts	+	5–20 μM	Acrolein induced DNA damage at *TP53* mutational hotspots and inhibited DNA repair.	Feng et al. (2006)
DNA adducts (^{32}P-postlabelling)	Colon HT-29 cells	+	20 μM		Pan et al. (2009)
DNA adducts (^{32}P-postlabelling)	Colon HT-29 cells	+	200 μM		Pan et al. (2012)
DNA adducts (LC-MS/MS-MRM)	BEAS-2B (bronchial epithelial cells)	+	20 μM		Greenspan et al. (2012)
DNA adducts (TLC/HPLC)	Normal bronchial epithelial cells, normal lung fibroblasts, cultured lung adenocarcinoma cells (A549)	+	25–100 μM		Wang et al. (2012)
	Human colon cancer HCT116 + ch3 cells	+	200 μM		Pan et al. (2016)

HIC, highest ineffective concentration; LC-MS, liquid chromatography-mass spectrometry; LEC, lowest effective concentration; MS-MRM, mass spectrometry multiple reaction monitoring; NT, not tested; TLC/HPLC, thin-layer chromatography/high-performance liquid chromatography.

[a] + All tests were conducted without metabolic activation.

and α-OH-PdG (5%). These studies also investigated subsequent mutagenesis as well as effects on DNA repair (see Sections 4.2.2 and 4.2.3).

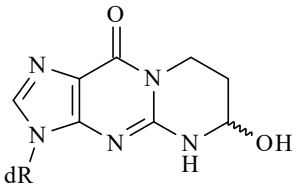
(iii) Experimental systems: reactions with deoxyribonucleosides

Acrolein is a strong electrophile and readily undergoes reactions with deoxyribonucleosides forming covalent adducts via Michael addition. The reactions of acrolein with deoxyguanosine, deoxyadenosine, and deoxycytidine have been well studied. The electron-rich purine bases are more reactive towards acrolein than are pyrimidine bases. These reactions involve an initial nucleophilic attack of a nitrogen in the bases to the terminal (β) olefinic carbon of acrolein, followed by the addition of a second nitrogen to the aldehydic carbon, leading to the formation of a new ring structure. The end products consist of a class of structurally unique cyclic adducts (Chung et al., 1986). Specifically, upon reaction with deoxyguanosine, acrolein yields cyclic 1,N^2-propano-2′-deoxyguanosine (PdG) adducts as a pair of regioisomers, designated as α and γ-OH-PdG (formerly as Acr–dG 1/2 and 3, respectively), depending on which deoxyguanosine nitrogen is involved in the Michael addition (Chung et al., 1984; Fig. 4.2). The α-isomers are a pair of diastereomers that exist in equilibrium due to interconversion via ring opening. The reaction of acrolein with deoxyadenosine yields cyclic 1,N^6-propano-2′-deoxyadenosine derivatives (1,N^6-PdA) with the possible formation of either 9- or 7-OH substituted regioisomers (Sodum & Shapiro, 1988, Smith et al., 1990a; Pawłowicz et al., 2006a); however, studies were mostly focused on the 9-OH-isomer (Fig. 4.2). The 9-OH-1,N^6-PdA adduct can further react with another acrolein molecule, forming a 2:1 adduct (Pawłowicz et al., 2006a); [the Working Group noted that such adducts are unlikely to be formed under physiological conditions in vivo]. In addition to the above-mentioned adducts, the exocyclic amino group in deoxyadenosine can be involved in two Michael additions with two acrolein molecules, followed by intramolecular aldol condensation, which gives rise to a 6-(3-formyl-1,2,5,6-tetrahydropyridyl) substituted adduct (Pawłowicz et al., 2006b). The reaction of acrolein with deoxycytidine forms a 3,N^4-substituted cyclic adduct (7-hydroxy-3,N^4-propano-2′-deoxycytidine) as a pair of diastereomers (Chenna & Iden, 1993). However, only one of the two possible regioisomers, the one resulting from Michael addition of the endocyclic N3 to the acrolein β-carbon, has been described (Fig. 4.2). Alkylated adducts, sometimes 2:1 adducts with deoxyadenosine and thymidine, which result from Michael addition to acrolein without subsequent ring closure, have also been described; these appear to be minor products (Lutz et al., 1982; Chenna et al., 1992; Pawłowicz et al., 2006a; Pawłowicz & Kronberg, 2008). Interestingly, under strenuous conditions (DMSO at 100 °C for 5 days) γ-OH-PdG, one of the cyclic adducts of acrolein with deoxyguanosine, can further react with another molecule of deoxyguanosine forming a cyclic bis-nucleoside, γ-OH-PdG–dG (Kozekov et al., 2001). [The Working Group noted that, despite the somewhat harsh conditions, the identification of the bis-nucleoside adduct suggests the possibility that interstrand dG–acrolein–dG crosslinks can be formed in duplex DNA.] Table 4.3 summarizes the reported reaction conditions between acrolein and deoxyribonucleosides/deoxyribonucleotides and the identity of the resulting adducts.

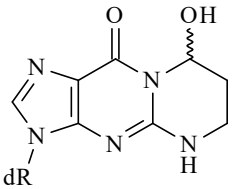
(iv) Experimental systems: reactions with DNA in vitro

Using synthetic adducts from the reactions with deoxyribonucleosides as reference standards, several studies, mostly with calf thymus DNA, have shown that acrolein can also modify DNA, forming some of the same adducts as with deoxyribonucleosides. Adducts formed in the acrolein-modified DNA have been detected and quantified, mainly after hydrolysis, by a variety

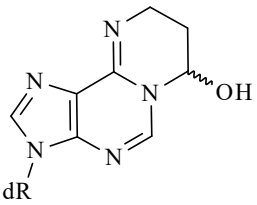
Fig. 4.2 Structures of the major acrolein–deoxyribonucleoside adducts



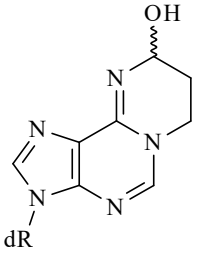
α-hydroxy-1,N^2-propano-2′-deoxyguanosine
(α-OH-PdG)



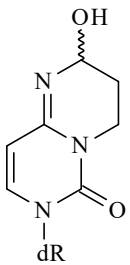
γ-hydroxy-1,N^2-propano-2′-deoxyguanosine
(γ-OH-PdG)



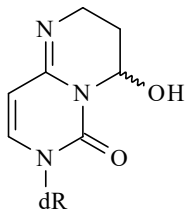
7-hydroxy-1,N^6-propano-2′-deoxyadenosine



9-hydroxy-1,N^6-propano-2′-deoxyadenosine



7-hydroxy-3,N^4-propano-2′-deoxycytidine



9-hydroxy-3,N^4-propano-2′-deoxycytidine

dR, 2′-deoxyribosyl.
Compiled by the Working Group.

of methods, including HPLC with fluorescence detection, ^{32}P-postlabelling, immune-based assays, or LC-MS/MS (Chung et al., 1984; Liu et al., 2005; Pawłowicz et al., 2006a; Pawłowicz & Kronberg, 2008; Pan et al., 2012; Chen et al., 2019a). The levels of adduct modification in these reactions are considerably lower than those with the monomers; however, the levels of modification may be significantly increased using denatured or single-stranded DNA, or oligomers. As the most nucleophilic base in DNA, guanine reacts to the greatest extent, in what constitutes a major pathway of DNA modification by acrolein.

Unlike its reactions with the monomers, the formation of cyclic adducts by acrolein with deoxyguanosine and deoxyadenosine in DNA appears to be regioselective. For example, γ-OH-PdG predominates over the α-isomer in DNA (Chung et al., 1984). Similarly, 9-OH-1,N^6-PdA was reported to be the product in acrolein-modified DNA, not the 7-OH-isomer (Smith et al., 1990a). Studies were carried out to

Table 4.3 Detection of acrolein-derived adducts with deoxynucleosides or deoxynucleotides in acellular systems

Nucleoside or deoxynucleotide	Conditions	Adduct	Reference
dG	PBS at 37 °C	γ-OH-PdG & α-OH-PdG	Chung et al. (1984)
dA		N^6-alkylated dA (Michael addition)	Lutz et al. (1982)
dA 5′-mp	PBS at 37 °C	9-OH-1,N^6-PdA-5′p	Smith et al. (1990a)
dA 3′,5′-bp	PBS at 37 °C	9-OH-1,N^6-PdA-3′,5′-bp	Smith et al. (1990a)
T	PBS at 37 °C	N3-alkylated	Chenna et al. (1992)
dC	PBS at 37 °C	7-OH-1,N^6-PdC	Chenna & Iden (1993)
dU	PBS at 37 °C	N3-alkylated	Chenna & Iden (1993)
dG/ γ-OH-PdG	DMSO at 100 °C	γ-OH-PdG–dG	Kozekov et al. (2001)
dG 5′-mp	ω-3 and ω-6 olyunsaturated fatty acids with ferrous sulfate/tris buffer at 37 °C	γ-OH-PdG	Pan & Chung (2002)
dA	PBS at 37 °C	1:1 and 2:1 (acrolein:dA) 1,N^6-PdA 1:1 and 2:1 (acrolein:dA) N^6-alkylated	Pawłowicz et al. (2006a, b)
T	PBS at 37 °C	1:1 N3-alkylated and four 2:1 (acrolein:T) N3-alkylated	Pawłowicz & Kronberg (2008)

1,N^6-PdA, 1,N^6-propano-2′-deoxyadenosine; 7-OH-1,N^6-PdC, 7-hydroxy-1,N^6-propano-2′-deoxycytosine; 9-OH-1,N^6-PdA, 9-hydroxy-1,N^6-PdA; α-, γ-OH-PdG, α-, γ-hydroxy-1,N^2-propano-2′-deoxyguanosine; dA, deoxyadenosine; dA 5′-mp, dA 5′-monophosphate; dA 3′,5′-bp, dA 3′,5′-bisphosphate; dC, deoxycytosine; dG, deoxyguanosine; dG 5′-mp, dG 5′-monophosphate; DMSO, dimethyl sulfoxide; dU, deoxyuridine; PBS, phosphate-buffered saline; T, thymidine.

shed light onto the molecular basis for the regioselectivity. Possible explanations involve the tertiary structure of DNA and/or an intermediacy of the Schiff's base between acrolein and amines (Chung et al., 2012). The 2:1 adduct of acrolein with deoxyadenosine, but not thymidine or deoxycytidine, was also observed in the reactions with DNA in vitro (Pawłowicz et al., 2006b; Pawłowicz & Kronberg, 2008). The formation of cyclic adducts of acrolein involves covalent binding with the nitrogens that participate in hydrogen bonding in the double helical structure of DNA.

Interestingly, the cyclic bis-nucleoside adduct of γ-OH-PdG (γ-OH-PdG–dG) described above was also found in a DNA duplex containing γ-OH-PdG in a 5′-CpG sequence context with the exocyclic amino group of deoxyguanosine in the opposite strand, resulting from interstrand crosslinking in oligonucleotide or DNA (Kozekov et al., 2001, 2010; Minko et al., 2009). Although the crosslinking product can undergo reversible reaction, it was sufficiently stable to be isolated for structural characterization. Table 4.4 summarizes the reactions of acrolein with oligomers and DNA.

(v) Experimental systems: DNA adduct formation in tissues and cells

See Table 4.5 and Table 4.6.

Most in vivo studies of the acrolein-derived DNA adducts in cells and tissues have focused on γ-OH-PdG. The only acrolein-derived DNA adduct other than γ-OH-PdG reported to be formed in vivo is 9-OH-1,N^6-PdA (Kawai et al., 2003). It has been shown that γ-OH-PdG can be formed in DNA in vivo from acrolein derived from two major sources: environmental exposure, such as tobacco smoke; and endogenous production, such as lipid peroxidation and polyamine oxidation. Although diet may also be a possible source, its importance has been

Table 4.4 Detection of acrolein-derived adducts with oligonucleotides and DNA

Oligomers or DNA	Conditions	Adduct	Detection method	Reference
ct-DNA	PBS pH 7 at 37 °C	γ-OH-PdG	HPLC-fluorescence	Chung et al. (1984)
ct-DNA	Tris pH 8.5 at 37 °C	γ-OH-PdG	CapLC-nanoESI-MS/MS	Liu et al. (2005)
ct-DNA	PBS pH 7.4 at 37 °C	1:1 9-OH-1,N^6-PdA; 2:1 1,N^6-PdA; 1:1 N^6-alkylated dA; 2:1 N^6-alkylated dA	LC-ESI-MS/MS	Pawłowicz et al. (2006b)
ct-DNA	PBS pH 7.4 at 37 °C	1:1 N3-alkylated T	LC-ESI-MS/MS	Pawłowicz & Kronberg (2008)
ct-DNA	PBS pH 7.0 at 37 °C	γ-OH-PdG-dG (crosslinking)	LC-ESI-MS/MS	Kozekov et al. (2010)
ct-DNA/plasmid pSP189 ct-DNA	PBS pH 7.0 at 37 °C LPO	γ-OH-PdG α-, γ-OH-PdG, and other LPO-derived cyclic adducts	ELISA/slot blot UHPLC/ESI-IT-MS	Pan et al. (2012) Chen et al. (2019a)

ct, calf thymus; dA, deoxyadenosine; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; LPO, lipid peroxidation; α-, γ-OH-PdG, α-, gamma-hydroxy-1,N^2-propano-2′-deoxyguanosine; 1,N^6-PdA, 1, N^6-propano-2′-deoxyadenosine; 9-OH-1,N^6-PdA, 9-hydroxy-1, N^6-PdA; CapLC-nanoESI-MS/MS, capillary liquid chromatography-nano electrospray ionization-tandem mass spectrometry; PBS, phosphate-buffered saline; UHPLC/ESI-IT-MS, ultra high-HPLC ESI-ion trap multistage mass spectrometry.

Table 4.5 Detection of acrolein-derived DNA adducts in experimental animals in vivo

Adduct	Species	Tissue	Exposure	Method of detection	Reference
γ-OH-PdG	Dog	Lymphocytes	Cyclophosphamide (6.6 mg/kg)	^{32}P-Postlabelling	Wilson et al. (1991)
γ-OH-PdG	Mouse Rat	Liver Liver	None None	^{32}P-Postlabelling	Nath & Chung (1994)
γ-OH-PdG	Mouse Rat	Skin Brain Lung Kidney Colon Prostate Mammary gland Leukocytes	None	^{32}P-Postlabelling	Nath et al. (1996)
γ-OH-PdG	Rat	Liver	None	LC-MS/MS	Fu et al. (2018)
γ-OH-PdG	Rat	Liver	None	UHPLC/ESI-IT-MS	Chen et al. (2019a)
γ-OH-PdG	Cockerel	Aorta	Acrolein inhalation (0, 1, and 10 ppm)	^{32}P-Postlabelling	Penn et al. (2001)
γ-OH-PdG	Mouse	Lung/bladder	Sidestream smoke	Immunoassay/^{32}P-postlabelling	Lee et al. (2015)
γ-OH-PdG	Mouse	Lung/bladder	Tobacco smoke	Immunoassay/^{32}P-postlabelling	Weng et al. (2018)
Not identified	Rat	Lung	Diesel-exhaust inhalation	HPLC-MS/MS	Douki et al. (2018)
γ-OH-PdG	Mouse	Liver	High-fat diet	IHC and LC-MS/MS	Coia et al. (2018)
1,N^6-PdA	Rat	Kidney	Ferric nitrilotriacetate	IHC	Kawai et al. (2003)

1,N^6-PdA, 1,N^6-propoanodeoxyadenosine; γ-OH-PdG, γ-hydroxy-1,N^2-propano-2′-deoxyguanosine; IHC, immunohistochemistry; HPLC-MS/MS; high-performance liquid chromatography-tandem mass spectrometry. UHPLC/ESI-IT-MS, ultrahigh-HPLC electrospray ionization-ion trap multistage mass spectrometry.

Table 4.6 Detection of acrolein-derived DNA adducts in experimental systems in vitro

Adduct	Cells	Acrolein concentration	Method of detection	Reference
γ-OH-PdG	*Salmonella typhimurium* TA100 and TA104	1, 4, 7, 10, 13 mM	ELISA	Foiles et al. (1989)
γ-OH-PdG	Chinese hamster ovary	1 mM	ELISA	Foiles et al. (1990)
1,N^6-PdA	Rat liver epithelial cells	5, 10, 25, 50 μM	Immunoassay	Kawai et al. (2003)
γ-OH-PdG	*Sphingobium* spp. strain KK22	10 mM	LC-ESI-MS/MS	Kanaly et al. (2015)

ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting, flow cytometry; LC-ESI-MS/MS, liquid chromatography-electrospray ionization-tandem mass spectrometry; 1,N^6-PdA, 1,N^6-propano-2′-deoxyadenosine; γ-OH-PdG, gamma-hydroxy-1,N^2-propano-2′-deoxyguanosine.

questioned by a study in which integrated quantitative structure–activity relationship–physiologically based kinetic/dynamic (QSAR-PBK/D) modelling was used to predict formation of γ-OH-PdG (Kiwamoto et al., 2015). As acrolein is an oxidation product of lipid peroxidation from ω-3 and -6 polyunsaturated fatty acids, the acrolein-derived adducts can be formed upon incubation of these fatty acids in the presence of deoxyguanosine under oxidative conditions (Pan & Chung, 2002; Kawai et al., 2003). As lipid peroxidation occurs continuously in vivo as part of normal physiological processes, acrolein-derived DNA adducts are constantly formed in cellular DNA as endogenous background lesions. Several methods have been developed to detect acrolein-derived DNA adducts in vivo, including ^{32}P-postlabelling, LC-MS/MS, and immunohistochemistry. The availability of monoclonal antibodies against acrolein-derived deoxyadenosine and deoxyguanosine adducts has facilitated the development of immune-based methods, such as immunohistochemistry, immunocytochemistry, and dot blot, for detecting these adducts in cells and tissues (Kawai et al., 2003 and Pan et al., 2012). However, it is generally agreed that LC-MS/MS is by far the most specific and most sensitive method for adduct detection and identification in vivo.

Acrolein-derived DNA adducts, including γ-OH-PdG, have been detected in various experimental animals in vivo (see Table 4.5). γ-OH-PdG was detected by a ^{32}P-postlabelling method in DNA of peripheral blood lymphocytes obtained from a dog given a therapeutic oral dose of cyclophosphamide at 6.6 mg/kg (Wilson et al., 1991). Studies later showed γ-OH-PdG is an endogenous background DNA lesion in livers of rodents and humans without known treatment and exposure (Nath & Chung, 1994; Nath et al., 1996). γ-OH-PdG was also detected in rats and mice given control feed (Fu et al., 2018; Chen et al., 2019a). Exposure of cockerels to acrolein (1 and 10 ppm) for 6 hours via inhalation gave rise to γ-OH-PdG in the aortic DNA (Penn et al., 2001). Exposure to tobacco smoke (mainstream, ~75 mg/m^3, 6 hours per day, 5 days per week, for 12 weeks; or sidestream, 500 μg/m^3, 6 hours per day, 5 days per week, for 8 or 16 weeks) and automobile exhaust was shown to induce γ-OH-PdG formation in the rodent lung (Lee et al., 2015; Weng et al., 2018; Douki et al., 2018). A small, but significant, increase in levels of acrolein-derived DNA adducts was found in the lung DNA of rats exposed to diesel exhaust; however, the data on the specific identity of the adduct were not reported (Douki et al., 2018). The notion that DNA adducts of acrolein can be derived from endogenous sources, such as lipid peroxidation, has been reinforced by recent studies showing that the levels of γ-OH-PdG are significantly increased in liver DNA of mice fed a high-fat diet (Coia et al., 2018). This study further demonstrated that the elevated hepatic

formation of γ-OH-PdG in mice fed a high-fat diet parallels the increased risk of developing hepatocellular carcinoma in these mice. The only other acrolein-derived DNA adduct in vivo so far reported is 9-OH-1,N^6-PdA. This adduct was found in rat kidney, using an iron-induced kidney carcinogenesis model under oxidative stress conditions in which rats were exposed to ferric nitrilotriacetate (Kawai et al., 2003). However, the structural identity of the adduct was not unequivocally established in this study because the adduct was detected by a monoclonal antibody raised against acrolein-modified DNA, not specifically 9-OH-1,N^6-PdA.

Acrolein-derived DNA adducts, including γ-OH-PdG, have also been assessed in various experimental cell types in vitro (see Table 4.6). Using a monoclonal antibody developed against crotonaldehyde-derived cyclic deoxyguanosine adducts structurally analogous to γ-OH-PdG (Foiles et al., 1987), an early study demonstrated the detection of γ-OH-PdG in *Salmonella typhimurium* strains TA100 and TA104 exposed to acrolein at the concentration range in which mutations were induced (Foiles et al., 1989). The first study detecting γ-OH-PdG in mammalian cells was reported using enzyme-linked immunosorbent assay (ELISA) in Chinese hamster ovary cells exposed to acrolein at a high concentration (1 mM) (Foiles et al., 1990). This concentration, however, was too toxic for scoring mutations. Later, monoclonal antibodies were raised against 1,N^6-PdA, using acrolein-modified DNA (Kawai et al., 2003), and against γ-OH-PdG, using specifically γ-OH-PdG-conjugated bovine serum albumin (Pan et al., 2012; see Table 4.4). More recently, a DNA adductomics approach was applied to the study of γ-OH-PdG in the soil bacterium *Sphingobium* spp. strain KK22 (Kanaly et al., 2015). This study demonstrated the potential of LC-MS/MS in DNA adductomics as a promising tool to study γ-OH-PdG and other related adducts in cells.

(b) Interactions with cellular proteins

(i) Reactions with amino acids and proteins in vitro

See Table 4.7.

Acrolein shows a strong propensity to react with amino acids or proteins via Michael addition, considerably more so than with DNA bases. Cysteines and the thiols of amino acids and proteins are the major sites for covalent binding with acrolein. Because the thiols are known to play important roles in enzyme activities and redox homeostasis, their facile interactions with acrolein can profoundly alter cellular functions. On the other hand, compounds with the mercapto (-SH) group, like GSH and cysteine, are widely used as effective scavengers of acrolein, with aim of reducing its adverse effects in cells or animals (Rees & Tarlow, 1967; Gurtoo et al., 1981; Wildenauer & Oehlmann, 1982).

The N-alkylation of proteins by acrolein may also occur. These reactions, through the side-chain amino group of lysine or a ring nitrogen of histidine, are kinetically less favourable than conjugation with -SH groups. Unlike reactions with cysteines, N-alkylation is irreversible, and the end products are usually quite stable (Cai et al., 2009). Reactions of acrolein with lysine have been investigated extensively with 3-formyl-3,4-dehydropiperidine (FDP), a 2:1 adduct, as a notable product that may serve as a potential biomarker of acrolein exposure detectable by a monoclonal antibody (Uchida et al., 1998a, b). The formation of FDP lysine adducts in histone has been associated with the inhibition of chromatin assembly mediated by acrolein (Fang et al., 2016). Furthermore, acrolein can form a Schiff base with the amine of lysine, followed by Michael addition yielding *N*-(3-methylpyridium)lysine via 2:1 addition (Furuhata et al., 2003; Kaminskas et al., 2005) and intra- and inter-protein crosslinks (Burcham & Pyke, 2006; Ishii et al., 2007; Minko et al., 2008). In addition to lysine, acrolein can also react with histidine by nucleophilic attack

Table 4.7 Reactions of acrolein with amino acids and proteins

Source	Amino acid or protein	Adduct	Detection method	Reference
Cyclophosphamide and acrolein	Rat hepatic microsomal CYP450	Not identified	Radioactivity with gel electrophoresis	Marinello et al. (1984)
Acrolein	[^{3}H-lysine]albumin	Not identified	Radioactivity	Thakore et al. (1994)
Acrolein	Synthetic peptide	Not identified	HPLC-MS	Carbone et al. (1997)
Acrolein	Lysine and low-density lipoprotein	(3-Formyl-3,4-dehydropiperidino)lysine	HPLC/MS/amino acid analysis IHC (mAb5F6)	Uchida et al. (1998a, b)
Acrolein	Histidine	3-Formylethylhistidine	LC-MS and NMR	Pocker & Janjić (1988), Uchida et al. (1998a)
Acrolein	BSA	Michael adduct	Spectrophotometric method for detection of DNPH derivative	Burcham et al. (2000)
Acrolein	BSA	*N*-(3-Formyl-3,4-dehydropiperidino)lysine	HPLC-MS/amino acid analysis	Furuhata et al. (2002)
Acrolein	Peptide (B chain of insulin)	*N*-(3-Methylpyridinium)lysine	ESI-LC/MS mAb5F6	Furuhata et al. (2003)
Acrolein	BSA	Lysine mono-Michael adduct versus Schiff base and FDP cyclic adduct	ESI-MS	Kaminskas et al. (2005)
Acrolein	Bovine pancrease Ribonuclease A	Crosslinking dimerized proteins	Gel electrophoresis	Burcham & Pyke (2006)
Acrolein	Actin	Cys374	LC-ESI-MS/MS	Dalle-Donne et al. (2007)
Acrolein	Peptide (B chain of insulin)	Crosslinking adducts	LC-ESI-MS/MS	Ishii et al. (2007)
Acrolein–dG or –dA adduct	KWKK peptide	Crosslinking adducts	Gel electrophoresis	Minko et al. (2008)
Acrolein	Insulin peptides	Cys, lysine, histidine, intra-molecular Schiff base	ESI-MS, ESI-MS/MS	Cai et al. (2009)
Acrolein/lipid oxidation	BSA/LDL	*N*-(3-Propanal)histidine	ESI/LC/MS/MS	Maeshima et al. (2012)
Acrolein	Recombinant histone/ H2a and H4	Lysine FDP adduct	LC-MS/MS	Fang et al. (2016)
Acrolein	Human serum albumin	Michael addition adducts	Biotin affinity tag LC-MS/MS	Coffey & Gronert (2016)
Acrolein	Lysozyme and human serum albumin	Histidine/cysteine/lysine adducts	LC-MS/MS	Afonso et al. (2018)

BSA, bovine serum albumin; CYP450, cytochrome P450; DNPH, dinitrophenyl hydrazine; ESI-MS, electrospray ionization-tandem mass spectrometry; FDP, *N*(ε)-3-formyl-3,4-dehydropiperidine; HPLC-MS, HPLC, high-performance liquid chromatography-mass spectrometry; LC, liquid chromatography; LDL, low-density lipoprotein; mAb, monoclonal antibody; NMR, nuclear magnetic resonance spectroscopy.

on the imidazole ring nitrogen (Pocker & Janjić, 1988; Maeshima et al., 2012). A recent study demonstrated that acrolein may be one of the aldehydes in tobacco smoke responsible for the inhibition of the enzymes involved in DNA repair by targeting these proteins via direct binding (Weng et al., 2018). Acrolein is a major metabolic product of certain anticancer drugs, such as cyclophosphamide, and early studies showed that the bladder toxicity of cyclophosphamide can be effectively attenuated by GSH or other SH-containing small compounds, whereas its therapeutic efficacy was not affected by GSH (Gurtoo et al., 1981). The reaction products were studied and compared between acrolein versus 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) and a synthetic peptide 128 (Carbone et al., 1997). Ample evidence shows that dithiothreitol and hydralazine also inhibit acrolein-induced cellular toxicity through their interactions with acrolein (Rees & Tarlow, 1967; Cox et al., 1988; Burcham et al., 2000; Burcham & Pyke, 2006; Chen et al., 2016). The chemical basis for the inhibition is the -SH conjugation with the former and formation of a hydrazone derivative with the latter; both reactions can effectively block acrolein's ability to bind cellular target proteins.

The identification of the binding sites of acrolein to protein is important because this knowledge may help understand the molecular basis underlying the toxicity caused by acrolein. To this end, LC-MS/MS-based proteomic methods have been developed in the past decade (Spiess et al., 2011; Coffey & Gronert, 2016; Afonso et al., 2018; Chen, Liu et al., 2019b). The application of proteomics in the determination of protein binding sites has been demonstrated with the use of model proteins as well as in cells treated with acrolein (Table 4.7).

(ii) Protein binding in human cells in vitro

Enhanced protein binding of acrolein has been demonstrated in exposed human bronchial epithelial cells (Caito et al., 2010), in human serum albumin (Colombo et al., 2010) and with the lactate dehydrogenase isozymes in human saliva (Avezov et al., 2014). Further investigations have revealed effects on protein function. For example, acrolein formed Michael adducts with sirtuin 1 (SIRT1) and reduced its activity (Caito et al., 2010). The evidence for protein dysfunction is ample; for example, Biswal et al. (2003) showed that acrolein modified c-JUN, preventing its dimerization and consequently preventing AP-1-promoter binding, and that acrolein modified B[*a*]P-induced TP53 and reduced its transcription transactivation activity. In human T cells, acrolein caused modification at Cys-61 and Arg-307 sites in p50 and IkB phosphorylation, consequently preventing DNA binding of NF-kB and reducing the expression of interleukins IL2 and IL10, interferon gamma (INFγ), tumour necrosis factor α (TNFα), and granulocyte-macrophage colony-stimulating factor. In human lung cells, acrolein at noncytotoxic levels can cause acrolein–Cys binding and consequently Hsp90 crosslinks (Burcham et al., 2007).

(iii) Protein binding in experimental animal cells and tissues

See Table 4.8.

To identify target proteins and binding sites in cells and tissues of rodents exposed to acrolein the methods currently used include immunohistochemistry, immunocytochemistry, Western blot, and LC-MS/MS. Because direct exposure to acrolein can cause overt toxicity, studies in vivo are often carried out with cancer chemotherapeutics, cigarette smoke, ethanol, and diet as indirect sources of acrolein (Gurtoo et al., 1981; Wildenauer & Oehlmann, 1982; Günther et al., 2008; Conklin et al., 2009; Chen et al., 2016). The availability of a monoclonal antibody to acrolein-modified keyhole limpet haemocyanin, with the lysine binding as an epitope, has greatly facilitated studies of acrolein-bound proteins in cells and tissues (Uchida et al., 1998a, b). The antibody was specifically developed

Table 4.8 Detection of acrolein-derived adducts in proteins in experimental animal cells and tissues

Source	Cell or animal	Protein target	Detection method	Reference
Cyclophosphamide	Mouse/rat	Hepatic proteins	Radioactivity	Gurtoo et al. (1981)
Cyclophosphamide	Rabbit liver microsomes Human erythrocytes	Membrane and cytoplasm	Radioactivity with SDS polyacrylamide Gel electrophoresis	Wildenauer & Oehlmann (1982)
Cyclophosphamide	SCID mouse	Implanted CT26 tumour cells	IHC	Günther et al. (2008)
Tobacco smoke or Acrolein	Mouse lung, plasma, aorta		Western blot	Conklin et al. (2009)
Acrolein	Human lung epithelial cells	Proteome	LC-MS/MS	Spiess et al. (2011)
Acrolein	F344 rat	Cardiac mitochondria	Aldehyde-specific chemical labelling and LC-MS/MS	Chavez et al. (2011)
Endogenous	F344 rat	Cardiac mitochondria	Proteomics NanoLC MALDI-MS/MS	Han et al. (2012)
Alcohol or acetaldehyde	Rat hepatoma H4IIEC cells	FDP-lysine adduct	ICC	Chen et al. (2016)
Diet with 5% ethanol	Male C57BL/6J mouse	Hepatic proteins	IHC	Chen et al. (2016)
Acrolein	Proteomes of human lung cancer H1299 cells	> 2300 proteins > 500 cysteines	Aldehyde-directed aniline-based probe by LC-MS/MS	Chen et al. (2019b)

GST, glutathione-*S*-transferase, ICC; immunocytochemistry; IHC, immunohistochemistry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; SDS, sodium dodecyl sulfate.

for lysine-adducted proteins. In recent years, LC-MS/MS-based proteomics has also been used to identify hundreds, if not thousands, of protein targets in cells and tissues (Spiess et al., 2011; Chavez et al., 2011; Han et al., 2012; Chen et al., 2019b; Table 4.8).

4.2.2 Is genotoxic

(a) Humans

(i) Exposed humans

No data were available to the Working Group.

(ii) Human cells in vitro

See Table 4.9.

Using the alkaline elution assay, acrolein-induced DNA strand breaks were observed in primary human bronchial epithelial cells (Grafström et al., 1986, 1988), human normal skin fibroblasts (CRL 1508) as well as xeroderma pigmentosum fibroblasts (Dypbukt et al., 1993), human myeloid leukaemia cells (K562) (Crook et al., 1986), and human lymphoblastoid cells (Namalwa) (Eisenbrand et al., 1995). [The Working Group noted that some of these experiments (Grafström et al., 1986; Dypbukt et al., 1993) were carried out at concentrations of acrolein that induced excessive cytotoxicity.] The frequency of phosphorylated H2AX proteins (γH2AX), an indicator of DNA double-strand breaks, was found to be significantly increased in acrolein-treated lung epithelial adenocarcinoma cells (A549) (Zhang et al., 2017) and human bronchial epithelial cells (BEAS-2B) (Zhang et al., 2020), and positive results were obtained in the comet assay for DNA damage in human normal lung fibroblasts (IMR-90) (Luo et al., 2013), A549 cells (Zhang et al., 2018), BEAS-2B cells (Zhang et al., 2020), human Burkitt lymphoma B lymphocytes (Raji) (Yang et al., 1999a), liver hepatoma cells (HepG2) (Li et al., 2008a), and retinal epithelial cells (ARPE-19) (Li et al., 2008b). [The Working Group noted that one experiment (Yang et al., 1999a) was carried out with acrolein at concentrations up to 500 μM with no measure of cytotoxicity.]

Acrolein-induced DNA–protein crosslinks were reported in bronchial epithelial cells (Grafström et al., 1986, 1988), in HepG2 cells (Li et al., 2008b), and in Burkitt lymphoma cells (EBV-BL) (Costa et al., 1997), but a negative result was reported in human promyelocytic leukaemia cells at a concentration (i.e. 100 μM) that resulted in a study-specific cell viability of 58% (Schoenfeld & Witz, 2000). [The Working Group noted that some of these experiments (Grafström et al., 1986; Schoenfeld & Witz, 2000) were carried out at concentrations of acrolein that induced excessive cytotoxicity.] Additionally, a negative result was reported in BEAS-2B cells exposed to acrolein at 7.5 μM; however, at this same concentration acrolein significantly enhanced the level of DNA–protein crosslinks observed when co-administered with formaldehyde (Zhang et al., 2020).

Acrolein induced a dose-dependent increase in *HPRT* mutant frequency in human DNA-repair-deficient xeroderma pigmentosum fibroblasts (Curren et al., 1988) and normal human bronchial epithelial cells (BEAS-2B) (Zhang et al., 2020), but failed to elicit a positive response in normal human fibroblasts when tested up to 2 μM (Curren et al., 1988). A positive result was obtained for micronucleus formation in lung A549 cells (Zhang et al., 2018) and BEAS-2B cells (Zhang et al., 2020), and for sister-chromatid exchanges in human primary lymphocytes (Wilmer et al., 1986). All studies in human cells were carried out in the absence of exogenous metabolic activation.

In eight experiments, plasmids containing the *supF* gene were reacted with acrolein and were then transfected into various human cell types to allow for repair and replication; the *supF* mutant frequency was subsequently assessed in *Escherichia coli*. Six experiments reported positive results (Feng et al., 2006; Kawanishi et al., 1998; Wang et al., 2009a, 2013a; Lee et al., 2014)

Table 4.9 Genetic and related effects of acrolein in human cells in vitro

End-point	Tissue, cell line	Results[a]	Concentration (LEC or HIC)	Comments	Reference
DNA strand breaks (alkaline elution)	Primary human bronchial epithelial cells	(+)	0.1 mM [5.6 µg/mL]	Concentration tested induced excessive cytotoxicity; single concentration tested.	Grafström et al. (1986)
DNA strand breaks (alkaline elution)	Primary human bronchial epithelial cells	+	30 µM [1.7 µg/mL]	Single concentration tested.	Grafström et al. (1988)
DNA strand breaks (alkaline elution)	Human xeroderma pigmentosum fibroblasts, CRL1223	(+)	100 µM [5.6 µg/mL]	Vehicle not reported; concentrations tested induced excessive cytotoxicity.	Dypbukt et al. (1993)
DNA strand breaks (alkaline elution)	Human normal skin fibroblasts, CRL1508	(+)	300 µM [17 µg/mL]	Vehicle not reported; concentrations tested induced excessive cytotoxicity.	Dypbukt et al. (1993)
DNA strand breaks (alkaline elution)	Human myeloid leukaemia, K562 cells	+	5.4 µM [0.3 µg/mL]		Crook et al. (1986)
DNA strand breaks (alkaline elution)	Human lymphoblastoid, Namalwa cells	+	50 µM [2.8 µg/mL]		Eisenbrand et al. (1995)
DNA double strand breaks (γH2AX)	Human lung epithelial carcinoma, A549	+	80 µM [4.5 µg/mL]		Zhang et al. (2017)
DNA double strand breaks (γH2AX)	Human bronchial epithelial cells, BEAS-2B	+	7.5 µM [0.42 µg/mL]		Zhang et al. (2020)
DNA damage (comet assay)	Human normal lung fibroblasts, IMR-90	+	4 µM [0.22 µg/mL]	Minimal information in description of comet method; single concentration tested.	Luo et al. (2013)
DNA damage (comet assay)	Human, retinal epithelial cells, ARPE-19	+	75 µM [4.2 µg/mL]	Minimal information in description of methods (i.e. pH of lysis etc.); no quantification of the level of DNA damage (binary approach used: nuclei with tails vs those without).	Li et al. (2008b)
DNA damage (comet assay)	Human Burkitt lymphoma B lymphocytes, Raji	(+)	500 µM [28 µg/mL]	No cytotoxicity assessment; minimal information in description of methods (i.e. pH of lysis etc.); comets classified into three size classes; vehicle not reported.	Yang et al. (1999a)
DNA damage (alkaline comet assay)	Human liver hepatoma, HepG2	+	12.5 µM [0.7 µg/mL]		Li et al. (2008a)
DNA damage (alkaline comet assay)	Human lung epithelial carcinoma, A549	+	55 µM [3 µg/mL]	This was the lowest concentration tested.	Zhang et al. (2018)
DNA damage (alkaline comet assay)	Human bronchial epithelial cells, BEAS-2B	+	1 µM [0.056 µg/mL]		Zhang et al. (2020)
DNA–protein crosslinks (method not specified)	Primary human bronchial epithelial cells	(+)	0.1 mM [5.6 µg/mL]	Single concentration tested, which induced excessive toxicity; minimal information in description of methods.	Grafström et al. (1986)

Table 4.9 (continued)

End-point	Tissue, cell line	Results[a]	Concentration (LEC or HIC)	Comments	Reference
DNA–protein crosslinks (modified alkaline elution assay)	Primary human bronchial epithelial cells	+	30 µM [1.7 µg/mL]	Single concentration tested.	Grafström et al. (1988)
DNA–protein crosslinks (alkaline comet assay, ProtK modified)	Human liver hepatoma, HepG2	+	50 µM [2.8 µg/mL]		Li et al. (2008a)
DNA–protein crosslinks (SDS/KCl precipitation assays)	Human bronchial epithelial cells, BEAS-2B	–	7.5 µM [0.42 µg/mL]	This was the only concentration tested; significantly enhanced DNA–protein crosslinks when co-exposed with formaldehyde.	Zhang et al. (2020)
DNA–protein crosslinks (SDS/KCl precipitation assay)	Human promyelocytic leukaemia cells, HL60	(–)	100 µM [5.6 µg/mL]	Cell viability at this dose was 58%; single concentration tested.	Schoenfeld & Witz (2000)
DNA–protein crosslinks (SDS/KCl precipitation assay)	Human Burkitt lymphoma cells, EBV-BL	+	150 µM [8.4 µg/mL]	Concentrations at which DNA–protein crosslinks were found were highly cytotoxic when assessed 4 days later by trypan blue exclusion.	Costa et al. (1997)
Gene mutation (*HPRT*)	Human xeroderma pigmentosum fibroblasts	+	0.2 µM [0.01 µg/mL]		Curren et al. (1988)
Gene mutation (*HPRT*)	Human normal fibroblasts	–	2 µM [0.1 µg/mL]		Curren et al. (1988)
Gene mutation (*HPRT*)	Human bronchial epithelial cells, BEAS-2B	+	7.5 µM [0.42 µg/mL]	Single concentration tested.	Zhang et al. (2020)
Micronucleus formation (CBMN)	Human bronchial epithelial cells, BEAS-2B	+	4 µM [0.22 µg/mL]		Zhang et al. (2020)
Micronucleus formation (CBMN)	Human lung epithelial carcinoma, A549	+	55 µM [3 µg/mL]	This was the lowest concentration tested.	Zhang et al. (2018)
Micronucleus formation (non-CBMN)	Human bronchial epithelial cells, BEAS-2B	+	7.5 µM [0.42 µg/mL]	Single concentration tested.	Zhang et al. (2020)
Sister-chromatid exchanges	Human primary lymphocytes	+	5 µM [0.28 µg/mL]		Wilmer et al. (1986)
Forward mutation (*supF*)	Plasmid pSP189 (exposed acellularly); transfected into normal human lung fibroblasts	+	100 µM [5.6 µg/mL]	Plasmids were reacted with acrolein at 37 °C and then transfected into human cells for replication and repair.	Feng et al. (2006)

Table 4.9 (continued)

End-point	Tissue, cell line	Results[a]	Concentration (LEC or HIC)	Comments	Reference
Forward mutation (*supF*)	Plasmid pMY189 (exposed acellularly); transfected into a normal human fibroblast cell line (W138-VA13)	+	26 mM [1456 µg/mL]	Plasmids were reacted with acrolein at 37 °C and then transfected into human cells for replication and repair.	Kawanishi et al. (1998)
Forward mutation (*supF*)	Plasmid pSP189 (exposed acellularly); transfected into human repair-proficient fibroblasts (GM637) and human repair-deficient (XPA) fibroblasts (GM4427)	–	1 mM [56 µg/mL]	Plasmids were reacted with acrolein and transfected into human cells for replication and repair.	Kim et al. (2007)
Forward mutation (*supF*)	Plasmid pSP189 (exposed acellularly); transfected into immortalized normal human lung fibroblasts (CCL-202)	+	0.5 mM [28 µg/mL]	Plasmids were reacted with acrolein at 37 °C and then transfected into human cells for replication and repair.	Wang et al. (2009a)
Forward mutation (*supF*)	Plasmid pSP189 (exposed acellularly); transfected into normal human lung fibroblasts	+	0.5 mM [28 µg/mL]	Plasmids were reacted with acrolein at 37 °C and then transfected into human cells for replication and repair.	Wang et al. (2013a)
Forward mutation (*supF*)	Plasmid pSP189 (exposed acellularly); transfected into immortalized normal human bladder cells (UROtsa) or normal human lung fibroblasts (CCL-202)	+	0.5 mM [28 µg/mL]	Plasmids were reacted with acrolein at 37 °C and then transfected into human cells for replication and repair.	Lee et al. (2014)

CBMN, cytokinesis-blocked micronucleus; γH2AX, phosphorylated gamma-histone 2AX; HIC, highest ineffective concentration; HPRT, hypoxanthine-guanine phosphoribosyltransferase; KCl, potassium chloride; LEC, lowest effective concentration; NA, not applicable; NT, not tested; PrtK, protein kinase; SDS, sodium dodecyl sulfate; vs, versus.

[a] +, positive; –, negative; +/–, equivocal (variable response in several experiments within an adequate study); (+) or (–), positive/negative in a study of limited quality.

All studies in human cells in vitro were carried out in the absence of exogenous metabolic activation.

and two experiments reported negative results (Kim et al., 2007). In one of these studies, Feng et al. (2006) sequenced mutations in the recovered plasmid from normal human lung fibroblasts and found that > 50% of the acrolein-induced base substitutions in the *supF* gene were G→T transversions. In the *supF* gene of acrolein-reacted plasmids recovered from human lung fibroblasts (CCL-202), primarily G→T transversions (53%) were observed, followed by G→A transitions (30%), and G→C transversions (12%); moreover, they found that mutational hotspots occurred in sequences with runs of Gs, and that the mutations across the *supF* gene mapped to the same sequence locations as those where the acrolein-derived adducts formed (Wang et al., 2009a). In another *supF* shuttle-vector study, of the acrolein-exposed plasmids recovered from a transformed normal human fibroblast cell line (W138VA13), 76% of mutations were base substitutions (46% single substitutions, 30% tandem or multiple substitutions), 21% were deletions, and 2% were insertions. Of the base substitutions, it was found that G→T predominated (44%), followed by G→A (24%), and G→C (12%) (Kawanishi et al., 1998).

A study in human xeroderma pigmentosum group V (XPV) cells transfected with a plasmid containing the α-OH-PdG adduct found that there was inaccurate translesion synthesis by both polymerases η and κ (Yang et al., 2003). Only marginal miscoding (< 1%) was observed for translesion synthesis across the γ-OH-PdG adduct in normal human fibroblasts, HeLa cells, xeroderma pigmentosum group A (XPA), and group V (XPV) cells (Yang et al., 2002a; Yoon et al., 2018). Another study in XPA cells transfected with plasmids containing either the α- or the γ-OH-PdG adduct found that the α-OH-PdG adduct strongly blocked DNA synthesis and induced base-pair substitutions (predominantly G→T) with an overall miscoding frequency of 10.4–12.5%, whereas the γ-OH-PdG adduct had neither effect (Yang et al., 2002b).

In one acellular study, human DNA polymerase ι was found to replicate past γ-OH-PdG in an error-free manner (Washington et al., 2004a), whereas in another acellular study, γ-OH-PdG was found to cause a significant replication block to human polymerase η (i.e. 100 times lower efficiency than dGTP), and caused misincorporation frequencies of approximately 10^{-2} to 10^{-1} (Minko et al., 2003).

(b) Experimental systems

(i) Non-human mammals in vivo

See Table 4.10.

A negative result was obtained for formation of DNA–protein crosslinks in the nasal respiratory mucosa of male Fischer 344 rats exposed to acrolein by inhalation for 6 hours; however, acrolein enhanced the level of DNA–protein crosslinks when rats were co-exposed to both acrolein and formaldehyde (Lam et al., 1985). No significant increase in the frequency of dominant lethal mutations was observed in male ICR/Ha Swiss mice exposed to acrolein as a single intraperitoneal injection (Epstein et al., 1972). In the micronucleus assay, a significant increase of 1.4-fold in the frequency of micronucleated polychromatic erythrocytes was observed in the bone marrow of male Sprague-Dawley rats treated with acrolein at 5 mg/kg bw per day by gavage, six times per week, for 30 days (Aydın et al., 2018). There was no dose-dependent increase in the frequency of micronucleated normochromatic erythrocytes in male and female B6C3F_1 mice exposed to acrolein at 10 mg/kg bw per day by gavage for 14 weeks. However, a significant increase of 2-fold in the frequency of micronucleus formation was observed in the female mice at 5 mg/kg bw per day (Irwin, 2006).

(ii) Non-human mammalian cells in vitro

See Table 4.11.

An increase in the frequency of DNA strand breaks was observed via the alkaline elution assay in Chinese hamster ovary (K1) cells (Deaton et

Table 4.10 Genetic and related effects of acrolein in non-human mammals in vivo

End-point	Species, strain (sex)	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
DNA–protein crosslinks	Rat, F344 (M)	Nasal respiratory mucosa	–	1.0 mg/m^3 [2 ppm]	Inhalation, 6 h	Acrolein enhanced the level of DNA–protein crosslinks when rats were co-exposed to both acrolein and formaldehyde.	Lam et al. (1985)
Dominant lethal mutation	Mouse, ICR/Ha Swiss (M)	Early fetal death/implants	–	2.2 mg/kg bw	Intraperitoneal 1×		Epstein et al. (1972)
Micronucleus formation	Rat, Sprague-Dawley (M)	Bone marrow (polychromatic erythrocytes)	+	5 mg/kg bw per day	Gavage, 6×/wk for 30 days; killed at day 30.	Significant increase but only 1.4-fold control value; single dose tested.	Aydın et al. (2018)
Micronucleus formation	Mouse, B6C3F$_1$ (M)	Blood (normochromatic erythrocytes)	–	10 mg/kg bw	Gavage daily, 5×/wk for 14 wk		Irwin (2006)
Micronucleus formation	Mouse, B6C3F$_1$ (F)	Blood (normochromatic erythrocytes)	–	10 mg/kg bw	Gavage daily 5×/wk for 14 wk	Positive at a single dose (2-fold, 5 mg/kg bw per day) but no dose trend; no analysis of target tissue exposure was reported.	Irwin (2006)

bw, body weight; d, day; F, female; h, hour; HID, highest ineffective dose; lowest effective dose; M, male; NT, not tested; ppm, parts per million; wk, week.

[a] +, positive; –, negative; +/–, equivocal (variable response in several experiments within an adequate study); (+) or (–), positive/negative in a study of limited quality.

Table 4.11 Genetic and related effects of acrolein in non-human mammalian cells in vitro

End-point	Species, tissue, cell line	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
DNA strand breaks (alkaline elution)	Chinese hamster ovary K1	+	NT	0.022 mM [1.2 µg/mL]		Deaton et al. (1993)
DNA strand breaks (alkaline elution)	Mouse, leukaemia L1210	(+)	NT	NR	Concentration at which a positive response was observed caused substantial cytotoxicity.	Eder et al. (1993)
DNA damage (alkaline comet assay)	Mouse, Leydig cells TM3	+	NT	7.4 µM [0.4 µg/mL]	LEC was the lowest concentration tested; LEC reported was for tail% DNA. LEC for OTM and tail length was 13.6 µM.	Yildizbayrak et al. (2020)
DNA damage (alkaline comet assay)	Rat, primary hepatocytes	(–)	NT	44.1 mM [2500 µg/mL]	Concentrations tested induced excessive cytotoxicity.	Kuchenmeister et al. (1998)
DNA and/or protein crosslinks (alkaline comet assay)	Rat, primary hepatocytes	(+)	NT	11 mM [616 µg/mL]	94% of cells had condensed spot in middle of cell characteristic of DNA and/or protein crosslinks; concentrations tested induced excessive cytotoxicity.	Kuchenmeister et al. (1998)
DNA–protein crosslinks	African green monkey kidney cell, CV-1	(+)	NT	0.5 mM [28 µg/mL]	No cytotoxicity assessment.	Permana & Snapka (1994)
DNA–protein crosslinks	Rat, nasal mucosal cells	(+)	NT	3 mM [168 µg/mL]	No cytotoxicity assessment.	Lam et al. (1985)
Gene mutation (*Hprt*)	Chinese hamster lung fibroblasts V79	+	NT	1 µM [0.056 µg/mL]		Smith et al. (1990b)
Gene mutation (*Hprt*)	Chinese hamster lung fibroblasts V79	+	NT	20 µM [1 µg/mL]	Only concentration tested.	Gardner et al. (2004)
Gene mutation (*Hprt*)	Chinese hamster lung fibroblasts V79 expressing rat AKR7A1	–	NT	20 µM [1 µg/mL]	Only concentration tested.	Gardner et al. (2004)
Gene mutation (*Hprt*)	Chinese hamster ovary (CHO)	+	NT	30 µM [1.7 µg/mL]	Control value not explicitly stated however response appears to be robust.	Cai et al. (1999)
Gene mutation (*Hprt*)	Chinese hamster ovary (CHO)	–	(+)	89 µM [5 µg/mL]	Elevated mutant frequencies observed at some concentrations but no clear concentration–response relationship.	Parent et al. (1991b)
Gene mutation (*Hprt*)	Chinese hamster ovary (CHO)	–	NT	100 µM [5.6 µg/mL]	No methods reported; HIC was cytotoxic.	Foiles et al. (1990)

Table 4.11 (continued)

End-point	Species, tissue, cell line	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Gene mutation (*Tk*$^{+/-}$)	Mouse, lymphoma L5178Y/*Tk*$^{+/-}$	+	NT	10 µM [0.56 µg/mL]		Demir et al. (2011)
Gene mutation (*cII*)	Mouse, embryonic fibroblasts from BigBlue TGR mouse	–	NT	100 µM [5.6 µg/mL]		Kim et al. (2007)
Chromosomal aberrations	Chinese hamster ovary (CHO)	–	NT	10 µM [0.56 µg/mL]	Only concentration tested that was non-toxic.	Au et al. (1980)
Chromosomal aberrations	Chinese hamster ovary (CHO)	–	–	17.9 µM [1 µg/mL]		Galloway et al. (1987)
Sister-chromatid exchange	Chinese hamster ovary (CHO)	+	–	10 µM [0.56 µg/mL]		Au et al. (1980)
Sister-chromatid exchange	Chinese hamster ovary (CHO)	+	–	17.9 µM [1 µg/mL]	Reported as a weak positive.	Galloway et al. (1987)

AKR, aldo-keto reductase; Hprt, hypoxanthine-guanine phosphoribosyltransferase; HIC, highest ineffective concentration; LEC, lowest effective concentration; NT, not tested; OTM, olive tail moment; Tk, thymidine kinase.

[a] +, positive; –, negative; +/–, equivocal (variable response in several experiments within an adequate study); (+) or (–), positive/negative in a study of limited quality.

al., 1993) and mouse leukaemia (L1210) cells, although the latter study noted that the tested dose caused substantial toxicity (Eder et al., 1993). An alkaline comet assay in mouse Leydig cells gave a positive result for DNA damage (i.e. comet tail intensity) at the lowest concentration tested (i.e. 7.4 μM) (Yildizbayrak et al., 2020). At a dose that was higher by nearly 6000-fold (i.e. 44.1 mM), an alkaline comet assay in rat primary hepatocytes gave a negative response when cells were analysed for comet tail length/intensity. However, 94% of cells had a condensed nucleus characteristic of compounds that cause DNA and/or protein crosslinks (Kuchenmeister et al., 1998). Acrolein-induced DNA–protein crosslinks were also observed in African green monkey kidney cells (CV-1) (Permana & Snapka, 1994) and in rat nasal mucosal cells (Lam et al., 1985). [The Working Group noted that these experiments were carried out with acrolein at high concentrations that either induced excessive cytotoxicity (Kuchenmeister et al., 1998; Lam et al., 1985), or at which cytotoxicity was not assessed (Permana & Snapka, 1994).]

Acrolein was found to be mutagenic, with a positive result for *Hprt* mutations in two assays in Chinese hamster lung fibroblasts (V79) (Smith et al., 1990b; Gardner et al., 2004). However, a negative result was obtained in V79 cells that express the rat aldo-keto reductase enzyme AKR7A1 (Gardner et al., 2004). [The Working Group noted that AKR7A1 catalyses the reduction of acrolein to alcohols, indicating that rat AKR7A1 protects against acrolein-induced mutagenicity (see Section 4.1.2b).] The frequency of acrolein-induced *Hprt* mutants was also analysed in Chinese hamster ovary cells, with one study reporting a positive response at 30 μM (Cai et al., 1999). Another study reported elevated mutant frequencies at some doses, but with no clear dose–response relationship when acrolein was tested at up to 89 μM with and without metabolic activation (rat liver S9) (Parent et al., 1991b). An additional study reported negative results for *Hprt* mutations in Chinese hamster ovary cells (Foiles et al., 1990). A significant increase in the frequency of $Tk^{+/-}$ mutations was reported in mouse lymphoma (L5178Y) cells (Demir et al., 2011), but a negative response was reported for the induction of *cII* mutations in mouse embryonic fibroblasts from the Big Blue mouse (Kim et al., 2007). Chromosomal aberrations and sister-chromatid exchanges were both assessed in two different studies in Chinese hamster ovary cells, with both reporting a negative response for chromosomal aberrations, and a positive result for sister-chromatid exchanges (Au et al., 1980; Galloway et al., 1987).

Using shuttle vectors containing either adduct isomer, the α- and γ-OH-PdG adducts were found to be mutagenic in African green monkey kidney (COS-7) cells, with a similar percentage mutagenicity observed for both isomers (i.e. 8.3% and 7.4%, respectively) (Sanchez et al., 2003). The γ-OH-PdG adduct was found to be significantly mutagenic in plasmid-transfected COS-7 cells; primarily transversions were observed, but also transition mutations (Kanuri et al., 2002).

(iii) Non-mammalian experimental systems

See Table 4.12.

In *Drosophila melanogaster*, largely positive results were obtained in SMART eye and wing spot mutation studies after exposure to acrolein in feed (Sierra et al., 1991; Demir et al., 2013; Vogel & Nivard, 1993), or via inhalation (Vogel & Nivard, 1993). Acrolein was also tested in *Drosophila* for the ability to induce sex-linked recessive lethal mutations, with negative results for all four feeding assays, but when acrolein was administered by injection, two out of three assays gave positive results (Zimmering et al., 1985, 1989; Sierra et al., 1991; Barros, et al., 1994a, b). Acrolein did not induce sex chromosome loss in *Drosophila* when administered either by injection or via feed (Sierra et al., 1991).

In *Saccharomyces cerevisiae,* acrolein did not induce DNA strand breaks and interstrand

Table 4.12 Genetic and related effects of acrolein in non-mammalian experimental systems

Test system (species, strain)	End-point	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Drosophila melanogaster	SMART wing spot mutation	+	NA	10 mM [560 µg/mL] (feed)		Sierra et al. (1991)
Drosophila melanogaster	SMART wing spot mutation	+	NA	10 mM [560 µg/mL] (feed)		Demir et al. (2013)
Drosophila melanogaster	SMART eye spot mutation	+	NA	5 mM [280 µg/mL] (feed)		Sierra et al. (1991)
Drosophila melanogaster	SMART eye spot mutation	+	NA	8.9 mM, 500 ppm [500 µg/mL] (inhalation)	Vehicle was ethanol.	Vogel &Nivard (1993)
Drosophila melanogaster	SMART eye spot mutation	–	NA	80 mM [4480 µg/mL] (feed)	Vehicle was ethanol.	Vogel &Nivard (1993)
Drosophila melanogaster	Sex-linked recessive lethal mutation	–	NA	5 mM [280 µg/mL] (feed)		Sierra et al. (1991)
Drosophila melanogaster	Sex-linked recessive lethal mutation	–	NA	10 mM [560 µg/mL] (feed)		Barros et al. (1994a, b)
Drosophila melanogaster	Sex-linked recessive lethal mutation	–	NA	14.3 mM [800 µg/mL] (feed)		Zimmering et al. (1989)
Drosophila melanogaster	Sex-linked recessive lethal mutation	–	NA	53.6 mM [3000 µg/mL] (feed)		Zimmering et al. (1985)
Drosophila melanogaster	Sex-linked recessive lethal mutation	–	NA	3.6 mM [200 µg/mL] (injection)		Zimmering et al. (1985)
Drosophila melanogaster	Sex-linked recessive lethal mutation	+	NA	3 mM [168 µg/mL] (injection)		Sierra et al. (1991)
Drosophila melanogaster	Sex-linked recessive lethal mutation	+	NA	3 mM [168 µg/mL] (injection)		Barros et al. (1994a, b)
Drosophila melanogaster	Sex chromosome loss	–	NA	5 mM [280 µg/mL] (feed)		Sierra et al. (1991)
Drosophila melanogaster	Sex chromosome loss	–	NA	5 mM [280 µg/mL] (injection)		Sierra et al. (1991)
Saccharomyces cerevisiae	DNA strand breaks and interstrand crosslinks	–	NT	0.1 mM [5.6 µg/mL]		Fleer & Brendel (1982)
Saccharomyces cerevisiae S211 and S138	Reverse mutation	–	NT	100 µg/mL		Izard (1973)

Table 4.12 (continued)

Test system (species, strain)	End-point	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Salmonella typhimurium TA1535 pSK 1002	DNA damage SOS (*umu*) induction assay	–	NT	5.6 µg/mL [0.1 mM]		Benamira & Marnett (1992)
Salmonella typhimurium TA1535	Reverse mutation	–	(+)	0.005 µg/mL		Hales (1982)
Salmonella typhimurium TA1535	Reverse mutation	–	–	13 µg/mL		Haworth et al. (1983)
Salmonella typhimurium TA1535	Reverse mutation	–	–	17 µg/plate		Florin et al. (1980)
Salmonella typhimurium TA1535	Reverse mutation	–	–	17 µg/plate		Florin et al. (1980)
Salmonella typhimurium TA1535,	Reverse mutation	–	–	28 µg/plate		Loquet et al. (1981)
Salmonella typhimurium TA1535	Reverse mutation	–	–	43 µg/plate		Lijinsky & Andrews (1980)
Salmonella typhimurium TA1535 (vapour protocol)	Reverse mutation	–	–	0.5 mL/chamber		Irwin (2006)
Salmonella typhimurium TA1535 (pre-incubation)	Reverse mutation	–	–	16 µg/plate		Irwin (2006)
Salmonella typhimurium TA100	Reverse mutation	+	+	10 µg/plate	Inconsistent dose–response relationship.	Parent et al. (1996b)
Salmonella typhimurium TA100	Reverse mutation	–	–	17 µg/plate		Florin et al. (1980)
Salmonella typhimurium TA100	Reverse mutation	–	–	28 µg/plate		Loquet et al. (1981)

Table 4.12 (continued)

Test system (species, strain)	End-point	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Salmonella typhimurium TA100	Reverse mutation	–	(+)	38 µg/mL		Haworth et al. (1983)
Salmonella typhimurium TA100	Reverse mutation	–	–	43 µg/plate		Lijinsky & Andrews (1980)
Salmonella typhimurium TA100	Reverse mutation	+	NT	224 µg/mL		Foiles et al. (1989)
Salmonella typhimurium TA100	Reverse mutation	–	–	NR		Basu & Marnett (1984)
Salmonella typhimurium TA100	Reverse mutation	+	Toxic	NR		Eder et al. (1993)
Salmonella typhimurium TA100	Reverse mutation	+	NT	NR	Solvent NR.	Khudoley et al. (1987)
Salmonella typhimurium TA100	Reverse mutation	+	NT	NR	Solvent NR.	Eder et al. (1990)
Salmonella typhimurium TA100 (vapour protocol)	Reverse mutation	–	–	1 mL/chamber		Irwin (2006)
Salmonella typhimurium TA100 (pre-incubation)	Reverse mutation	–	–	6 µg/plate without activation, 16 µg/plate with activation	Slight toxicity at highest dose without activation.	Irwin (2006)
Salmonella typhimurium TA100 (liquid suspension)	Reverse mutation	+	–	2.1 µg/mL –S9; HIC, 4.2 µg/mL +S9		Lutz et al. (1982)
Salmonella typhimurium TA104	Reverse mutation	+	NT	224 µg/mL		Foiles et al. (1989)
Salmonella typhimurium TA104	Reverse mutation	+	NT	14 µg/plate		Marnett et al. (1985)
Salmonella typhimurium TA1537	Reverse mutation	–	–	13 µg/mL		Haworth et al. (1983)
Salmonella typhimurium TA1537	Reverse mutation	–	–	17 µg/plate		Florin et al. (1980)

Table 4.12 (continued)

Test system (species, strain)	End-point	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Salmonella typhimurium TA1537	Reverse mutation	–	–	43 µg/plate		Lijinsky & Andrews (1980)
Salmonella typhimurium TA1538	Reverse mutation	–	–	43 µg/plate		Lijinsky & Andrews (1980)
Salmonella typhimurium TA1538 (pre-incubation)	Reverse mutation	NT	–	16 µg/plate		Irwin (2006)
Salmonella typhimurium TA97 (vapour protocol)	Reverse mutation	–	–	0.5 mL/chamber		Irwin (2006)
Salmonella typhimurium TA98	Reverse mutation	+	–	8.4 µg/plate		Lijinsky & Andrews (1980)
Salmonella typhimurium TA98	Reverse mutation	(+)	+	10 µg/plate	Weak positive (2-fold). Inconsistent dose–response relationship.	Parent et al. (1996b)
Salmonella typhimurium TA98	Reverse mutation	–	–	13 µg/mL		Haworth et al. (1983)
Salmonella typhimurium TA98	Reverse mutation	–	–	17 µg/plate		Florin et al. (1980)
Salmonella typhimurium TA98	Reverse mutation	–	–	28 µg/plate		Loquet et al. (1981)
Salmonella typhimurium TA98	Reverse mutation	–	–	NR		Basu & Marnett (1984)
Salmonella typhimurium TA98	Reverse mutation	+	+	NR		Claxton (1985)
Salmonella typhimurium TA98	Reverse mutation	+	NT	NR	Solvent NR.	Khudoley et al. (1987)
Salmonella typhimurium TA98 (vapour protocol)	Reverse mutation	–	–	1 mL/chamber		Irwin (2006)

Table 4.12 (continued)

Test system (species, strain)	End-point	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Salmonella typhimurium TA98 (pre-incubation)	Reverse mutation	–	–	16 µg/plate		Irwin (2006)
Salmonella typhimurium TA102	Reverse mutation	–	NT	NR		Marnett et al. (1985)
*Salmonella typhimurium hisD3052/nop*KM101	Reverse mutation	–	–	NR		Basu & Marnett (1984)
Escherichia coli HB101pUC13	DNA–histone crosslinks	+	NT	8.4 µg/mL		Kuykendall & Bogdanffy (1992)
Escherichia coli PQ37, SOS chromotest	DNA damage	+	NT	NR	Solvent NR.	Eder et al. (1990)
Escherichia coli PQ37, SOS chromotest	DNA damage	+	NT	NR		Eder et al. (1993)
Escherichia coli PQ37, SOS chromotest	DNA damage	–	NT	NR		Eder & Deininger (2002)
Escherichia coli PQ37, SOS chromotest	DNA damage	+	NT	NR	Ethanol used as solvent.	Eder & Deininger (2002)
Escherichia coli AB1157	Reverse mutation	+	NT	56 µg/mL [1 mM]		Nunoshiba &Yamamoto (1999)
Escherichia coli JTG10	Reverse mutation	+	NT	56 µg/mL [1 mM]	Strain lacks glutathione; mutation frequency higher than in AB1157 strain.	Nunoshiba &Yamamoto (1999)
Escherichia coli WP2 (*uvr*A)	Reverse mutation	–	(+)	50 µg/plate	Weak positive (2-fold); inconsistent dose–response relationship.	Parent et al. (1996b)
Escherichia coli WP2 (*uvr*A)	Reverse mutation	(+)	NT	NR	Reported as weak mutagenicity.	Hemminki et al. (1980)

Table 4.12 (continued)

Test system (species, strain)	End-point	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Plasmid pIF101 (acellular)	Reverse mutation A→C (*lacZ*)	+	NT	0.010 mM [0.56 µg/mL]	Plasmids were reacted with acrolein at 37 °C then transfected into AlkB proficient and deficient *E. coli* for mutation scoring.	Dylewska et al. (2017)
Plasmid pIF105 (acellular)	Reverse mutation A→T (*lacZ*)	+	NT	0.005 mM [0.28 µg/mL]	Plasmids were reacted with acrolein at 37 °C then transfected into AlkB proficient and deficient *E. coli* for mutation scoring.	Dylewska et al. (2017)
Plasmid pIF106 (acellular)	Forward mutation A→G (*lacZ*)	+	NT	0.005 mM [0.28 µg/mL]	Plasmid were reacted with acrolein at 37 °C then transfected into AlkB proficient and deficient *E. coli* for mutation scoring.	Dylewska et al. (2017)
Calf thymus DNA (acellular)	DNA damage (fluorescent screen for changes in DNA melting and annealing behaviour)	+	NA	100 mM [5600 µg/mL]		Kailasam & Rogers (2007)

AlkB, alpha-ketoglutarate B-dependent dioxygenase; A, adenine; C, cytosine; G, guanine; T, thymine; HIC, highest ineffective concentration; LEC, lowest effective concentration; NA, not applicable; NR, not reported; NT, not tested; ppm, parts per million; S9, 9000 × *g* supernatant.

[a] +, positive; –, negative; +/–, equivocal (variable response in several experiments within an adequate study); (+), positive in a study of limited quality.

crosslinks in one study (Fleer & Brendel, 1982), or reverse mutations in another study (Izard, 1973).

Acrolein has been evaluated in multiple assays in several *Salmonella* tester strains sensitive to base-pair substitutions (i.e. TA1535, TA100, TA104) and frameshift mutations (i.e. TA1537, TA1538, TA97, and TA98). However, only one assay was carried out in TA102, a strain that is used specifically for the detection of crosslinking agents. In the TA1535 base-pair substitution strain, a negative response was observed in the SOS induction assay (Benamira & Marnett, 1992) and the results were negative for reverse mutation (Hales, 1982; Haworth et al., 1983; Florin et al., 1980; Loquet et al., 1981; Lijinsky & Andrews, 1980; Irwin, 2006). In the TA100 strain, the results were mixed positive (Parent et al., 1996b; Haworth et al., 1983; Foiles et al., 1989; Eder et al., 1993; Khudoley et al., 1987; Eder et al., 1990; Lutz et al., 1982) or negative (Florin et al., 1980; Loquet et al., 1981; Lijinsky & Andrews, 1980; Basu & Marnett, 1984; Irwin, 2006), with the positive responses mainly occurring without metabolic activation (rat liver S9). Notably, only one pre-incubation assay was carried out with TA100 and a negative result was reported (Irwin, 2006). However, a positive result was obtained in TA100 when acrolein was tested in a liquid suspension assay (Lutz et al., 1982). Of the two assays reported in TA104, both gave positive results without metabolic activation (S9) (Foiles et al., 1989; Marnett et al., 1985). In the frameshift strains, all three TA1537 assays gave negative results (Haworth et al., 1983; Florin et al., 1980; Lijinsky & Andrews, 1980), both results in TA1538 were negative (Lijinsky & Andrews, 1980; Irwin, 2006), the one TA97 experiment gave negative results (using the vapour protocol) (Irwin, 2006), and four positive results (Lijinsky & Andrews, 1980; Parent et al.,1996b; Claxton, 1985; Khudoley et al., 1987) and six negative results were reported in TA98 (Haworth et al., 1983; Florin et al., 1980; Loquet et al., 1981; Basu & Marnett,1984; Irwin, 2006). A negative result was obtained in the crosslink strain TA102, but the highest tested dose was not reported (Marnett et al., 1985). The more sensitive pre-incubation version of the Ames assay was not carried out with any frameshift strains without metabolic activation.

In *E. coli*, a positive result for DNA-histone crosslinks was reported (Kuykendall & Bogdanffy, 1992). Several studies reported positive results for acrolein in the SOS chromotest (Eder et al., 1990, 1993; Eder & Deininger, 2002), whereas a negative response was observed in the SOS chromotest when DMSO was used as the solvent. Additional studies in *E. coli* reported positive results for reverse mutations (Nunoshiba & Yamamoto, 1999; Hemminki et al., 1980), as well as one experiment with a negative result without metabolic activation and a weak positive result with metabolic activation (Parent et al., 1996b). In one study, three different plasmids containing different mutational targets in the *lacZ* gene were reacted with acrolein and then transfected into *E. coli* for mutant frequency assessment; positive results were observed for all three mutation types (i.e. A→C, A→T, and A→G) (Dylewska et al., 2017). An increase in DNA damage, assessed via a fluorescence-based screen quantifying changes in DNA melting/annealing behaviour, was observed in calf thymus DNA reacted with acrolein in an acellular system (Kailasam & Rogers, 2007).

In the study of Kanuri et al. (2002), described above, the γ-OH-PdG adduct was found to be significantly less mutagenic in *E. coli* than in COS-7 cells transfected with the same plasmid (i.e. 0.96% mutations in *E. coli* versus 6.3–7.4% in COS-7). In *E. coli*, a study by Yang et al. (2001) found that DNA polymerase III catalysed translesion synthesis across the γ-OH-PdG adduct in an error-free manner, but that DNA polymerase I did so in an error-prone manner, with incorporation frequencies opposite the γ-OH-PdG adduct of 93% for deoxyadenosine triphosphate (dATP), 88% for deoxyguanosine triphosphate

(dGTP), 7% for deoxycytidine triphosphate (dCTP), and 5% for deoxythymidine triphosphate (dTTP). Additionally, γ-OH-PdG was found to inhibit DNA synthesis in *E. coli* (Yang et al., 2001). In another study with *E. coli* transformed with bacteriophage vectors containing an 8-hydroxy-1,N^2-propano-2′-deoxyguanosine (OH-PdG) adduct (no stereochemistry specified), the correct base was inserted under all conditions (VanderVeen et al., 2001).

In an acellular study, γ-OH-PdG was found to cause a significant replication block to yeast polymerase η (i.e. 190 times lower efficiency than deoxyguanosine), although incorporation opposite the adduct was relatively accurate (Minko et al., 2003). In an acellular study, both α- and γ-OH-PdG caused a significant replication block to yeast DNA pol η, with α-OH-PdG being a significantly stronger blocking lesion as pairing with dCTP was strongly inhibited (Sanchez et al., 2003). When assayed for nucleotide incorporation frequency, dCTP was primarily incorporated across from both lesions, but extension with other deoxynucleoside triphosphates (dNTPs) was also observed at almost identical ratios for both stereoisomers (Sanchez et al., 2003). In other acellular studies, yeast Rev1 was demonstrated to replicate past γ-OH-PdG in an error-free manner (Washington et al., 2004b; Nair et al., 2008).

In an acellular study with bacteriophage DNA polymerase T7$^-$ and HIV-1 reverse transcriptase, OH-PdG adducts (stereochemistry not specified) were found to be miscoding, with dATP being preferentially incorporated instead of dCTP (Zang et al., 2005). In another acellular study, *Sulfolobus solfataricus* Dpo4, the prototypic Y-family DNA polymerase, was capable of bypassing γ-OH-PdG adducts in a primarily error-free manner (Shanmugam et al., 2013).

4.2.3 Alters DNA repair

(a) Humans

No studies on exposed humans were available to the Working Group.

Acrolein was found to inhibit the DNA repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT) in human bronchial fibroblasts in two studies (Krokan et al., 1985; Grafström et al., 1986). [The Working Group noted that, as aldehydes are highly reactive towards thiols, this inhibition is probably due to acrolein reacting with and inhibiting the methyl-acceptor cysteine residue in MGMT (Grafström et al., 1986).] In a study using human normal skin fibroblasts and DNA-repair deficient XPA fibroblasts, it was concluded that acrolein inhibited nucleotide excision repair since there was an accumulation of DNA single-strand breaks in acrolein-treated normal skin fibroblasts, which only increased after a recovery period in fresh medium (Dypbukt et al., 1993). Indeed, several studies found that acrolein treatment causes concentration-dependent inhibition of nucleotide excision repair in primary normal human lung fibroblasts (NHLFs) (Feng et al., 2006; Wang et al., 2012), primary normal human bronchial epithelial cells (NHBEs), human lung adenocarcinoma cells (A549s) (Wang et al., 2012), and in immortalized human urothelial (UROtsa) cells (Lee et al., 2014). Acrolein also causes concentration-dependent inhibition of base excision repair in NHBEs, NHLFs, A549s (Wang et al., 2012), and UROtsa cells (Lee et al., 2014), and of mismatch repair in HeLa (epithelial adenocarcinoma) cells (Wang et al., 2012). A subgenotoxic concentration of acrolein (i.e. 50 μM) has also been demonstrated to inhibit the repair of gamma-irradiation–induced DNA damage in human B-lymphoid cells, and the repair inhibition increased with acrolein dose (Yang et al., 1999b).

Acrolein treatment reduced the expression level of certain DNA repair genes in A549 cells (Sarkar, 2019). Other studies did not find an effect on gene expression but showed that acrolein reacts rapidly with and directly inhibits DNA repair proteins (Wang et al., 2012; Lee et al., 2014). More specifically, in NHBE, NHLF, A549, and UROtsa cells, acrolein treatment caused a dose-dependent reduction in the expression of repair proteins (i.e. XPA, XPC, human 8-oxoguanine DNA glycosylase (hOGG1), PMS2, and MLH1) that are crucial for nucleotide excision repair, base excision repair, and mismatch repair (Wang et al., 2012; Lee et al., 2014). Pre-treatment of cells with proteasome inhibitors reduced the level of protein degradation, and pre-treatment with an autophagy inhibitor caused partial reduction in the degradation of DNA repair proteins; however, repair capacity was not rescued (Wang et al., 2012; Lee et al., 2014). [The Working Group noted that these results indicate that acrolein protein modification alone is capable of causing DNA-repair protein dysfunction, and that this modification results in DNA-repair protein degradation both by proteasomes and by autophagy; see also Section 4.2.1(a).]

Wang et al. (2012) found that both α-OH-PdG and γ-OH-PdG adducts were not efficiently repaired in acrolein-exposed NHBEs and NHLFs. A study using HeLa whole-cell extracts found that α-OH-PdG and γ-OH-PdG adducts were not efficiently removed by base excision repair (Yang et al., 2002b). In a study of nuclear extracts from unexposed human normal skin fibroblasts and DNA-repair deficient human XPA cells transfected with acrolein-treated plasmids, it was found that acrolein–dG adducts (i.e. a mixture of α-OH-PdG and γ-OH-PdG) are substrates for nucleotide excision repair proteins, but are repaired at a much slower rate than other similar adducts, and that this is probably because of poor recognition and/or excision of the lesions in DNA (Choudhury et al., 2013).

(b) Experimental systems

No data were available to the Working Group.

4.2.4 Induces oxidative stress

(a) Humans

No in vivo data were available to the Working Group.

In vitro studies using human retinal pigmented epithelial and lung fibroblast cell lines have demonstrated that acrolein induces a variety of biochemical changes, including decreased nuclear protein levels of nuclear factor erythroid 2-related factor 2 (NRF2; [NFE2L2, nuclear factor, erythroid 2-like 2]) (retinal pigmented epithelial cells only), decreased superoxide dismutase and glutathione peroxidase activities, lowered cellular GSH levels, and increased generation of reactive oxygen species (ROS) and protein carbonyls (Jia et al., 2007, 2009; Li et al., 2008a). Haem oxygenase-1 (*HO-1* [*HMOX1*]) gene expression is induced in human bronchial epithelial cells (HBE1 cells) after acrolein exposure, and acrolein-induced HO-1 protein levels are attenuated by pan-protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) inhibitors (Zhang & Forman, 2008). Exposure of human cultured liver (HepG2) or retinal pigment epithelial cells to acrolein results in endoplasmic reticulum stress, mitochondrial disruption, and oxidative stress (Li et al., 2008a, b; Mohammad et al., 2012). Human primary bronchial epithelial cells exposed to acrolein vapour (0.1 and 0.2 ppm) for 30 minutes had increased IL17 expression (Johanson et al., 2020).

(b) Experimental systems

See Table 4.13.

Multiple in vivo studies in rodents have shown that acrolein administration via multiple routes of exposure, including oral administration, inhalation, and intraperitoneal injection, results in decreased tissue GSH concentrations

Table 4.13 Effects of acrolein on markers of oxidative stress in non-human mammals in vivo

End-point	Species, strain (sex)	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
GSH SOD, GPx	Rat, Wistar (M)	Liver	↓	2.5 mg/kg bw per day	Gavage, 45 days		Arumugam et al. (1997)
PCO, TBARS GSH	Rat, Sprague-Dawley (M)	Spleen, thymus, PMN Spleen, thymus, PMN	↑ ↓	5 mg/kg bw per day	Oral, 6 days/wk for 30 days		Aydın et al. (2018)
GPx NPSH GST	Rat, Wistar (M)	Nasal cavity	– ↑ ↓	0.25 ppm 0.67 ppm 1.4 ppm	Inhalation (nose-only), 6 h/day for 3 days		Cassee et al. (1996)
GSH	Rat, F344 (M)	Nasal cavity Tracheobronchial mucosa	↓	0.2 ppm 0.2 ppm	Inhalation (nose-only), 6 h		Cichocki et al. (2014)
GSH	Rat, F344 (M)	Liver	↓	89 µmol/kg (0.1 mL/100 g bw) [31.5 mg/kg]	Intraperitoneal, 1×		Cooper et al. (1992)
GSH	Mouse (F)	Liver	↓	4.5 mg/kg	Intraperitoneal, 1×	Qualitative statistics only.	Gurtoo et al. (1981)
GSH 8-OHdG	Mouse, C57BL/6 (M)	Lung	↓ ↑	10 ppm 10 ppm	Inhalation (whole-body), 12 h		Kim et al. (2018)
Lipid peroxides, TBARS	Mouse, *ApoE*$^{-/-}$ (M)	Serum, peritoneal macrophages	↑	3 mg/kg per day	Oral (drinking-water), 1 mo	Genetic background not provided.	Rom et al. (2017)
ROS	Mouse, C57BL/6 (F)	Lung	↑	5 µmol/kg bw [0.06 mg/kg bw]	Intranasal, 1×	Elevated at day 7 post-exposure only (not day 28).	Sun et al. (2014)
8-Isoprostane Total antioxidant capacity	Mouse, gp$^{91phox(-/-)}$ (M)	Liver	↑ ↓	0.5 µg/kg per day	Intraperitoneal, 7 days		Yousefipour et al. (2013)
8-isoprostane Total antioxidant capacity	Mouse, gp$^{91phox(+/+)}$ (M)	Liver	↑ ↓	0.5 µg/kg per day	Intraperitoneal, 7 days		Yousefipour et al. (2013)

Table 4.13 (continued)

End-point	Species, strain (sex)	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
8-isoprostane Total antioxidant capacity	Mouse, $gp^{91phox(-/-)}$ (M)	Liver	↑ ↓	0.5 mg/kg per day	Intraperitoneal, 7 days		Yousefipour et al. (2017)
8-isoprostane Total antioxidant capacity	Mouse, $gp^{91phox(+/+)}$ (M)	Liver	↑ ↓	0.5 mg/kg per day	Intraperitoneal, 7 days		Yousefipour et al. (2017)

bw, body weight; F, female; h, hour; GPx, glutathione peroxidase; GSH, reduced glutathione; GST, glutathione-*S*-transferase; HID, highest ineffective dose; LED, lowest effective dose; M, male; mo, month; NPSH, nonprotein sulfydryl groups; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; PCO, protein carbonyls; PMN, polymorphonuclear leukocytes; ppm, parts per million; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; wk, week.

[a]↑, increase; ↓, decrease; –, no effect.

(Arumugam et al., 1997; Aydın et al., 2018; Cassee et al., 1996; Cichocki et al., 2014; Cooper et al., 1992; Gurtoo et al., 1981; Kim et al., 2018). Oral and parenteral rodent studies have shown evidence of lipid peroxidation or protein carbonyl production after short-term (up to 1 month) exposure (Aydın et al., 2018; Rom et al., 2017; Sun et al., 2014; Yousefipour et al., 2013, 2017). A significant increase in levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) has been reported in the mouse lung after inhalation of acrolein (Kim et al., 2018). Intraperitoneal exposure of wildtype and *gp91*^{phox} knockout mice with acrolein at 0.5 µg/kg provided evidence that increased oxygen radical generation occurs via NAD(P)H oxidase activation (Yousefipour et al., 2013). In vitro studies with bovine pulmonary artery endothelial cells have likewise shown that acrolein causes increased generation of oxygen radicals by NAD(P)H oxidase activation (Jaimes et al., 2004).

4.2.5 Is immunosuppressive

(a) Humans

No data in exposed humans were available to the Working Group.

In vitro studies show that acrolein exposure is associated with apoptosis and necrosis in human alveolar macrophages and with inhibition of IL1β, TNF, and IL12 release from cells (Li et al., 1997). Human T cells treated with acrolein also demonstrated suppressed cytokine production and T-cell responses (Lambert et al., 2005). Human alveolar macrophages infected with *Mycobacterium tuberculosis* and exposed to acrolein have a reduced ability to clear these bacteria (Shang et al., 2011).

(b) Experimental systems

See Table 4.14.

Multiple studies in rodents have assessed whether acrolein inhalation alters bacterial-induced mortality, bactericidal activity, or innate immune function (Aranyi et al., 1986; Astry & Jakab, 1983; Jakab, 1993; Danyal et al., 2016; Hristova et al., 2012; Leach et al., 1987). Most of these studies have used short-term exposures (e.g. < 10 days).

Splenic cells isolated from naïve female C57/BL6 mice that were subsequently exposed to acrolein exhibited decreased T- and B-cell proliferation (Poirier et al., 2002). Immunosuppression by acrolein has been attributed to GSH depletion and interactions with redox-sensitive signalling pathways such as NF-κB or JNK (Lambert et al., 2005; Valacchi et al., 2005; Kasahara et al., 2008).

4.2.6 Induces chronic inflammation

(a) Humans

No data were available to the Working Group.

(b) Experimental systems

See Table 4.15.

Chronic inhalation (6 hours per day, 5 days per week, for 104 weeks) of acrolein was associated with mild inflammation in the nasal respiratory epithelium in rats and mice (JBRC, 2016d, e, f; see also Section 3). A 1-year study in hamsters treated with acrolein by inhalation (7 hours per day, 5 days per week, for 52 weeks) was also associated with mild inflammation in the nasal respiratory epithelium (Feron & Kruysse, 1977; see Table 3.1). [The Working Group noted that changes in cell proliferation in response to acrolein exposure have not been evaluated in experimental systems.] Multiple studies in rodents with short-term or subchronic exposures to acrolein via inhalation have shown that acrolein produces airway inflammation (Johanson et al., 2020; Kasahara et al., 2008; Wang et al., 2009b; Liu et al., 2009a, b; Sithu et al., 2010). Accumulation of monocytes, macrophages, and lymphocytes in the lung interstitium and mucous cell metaplasia are common features seen in many rodent inhalation studies with acrolein (Kutzman et al., 1985; Borchers et

Table 4.14 Immunosuppression after acrolein exposure in non-human mammals in vivo

End-point	Species, strain (sex)	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Streptococcus zooepidemicus induced mortality	Mouse, CD_1 (F)	Lung	–	0.1 ppm	Inhalation (whole-body); 3 h	Single exposure concentration.	Aranyi et al. (1986)
Streptococcus zooepidemicus induced mortality	Mouse, CD_1 (F)	Lung	↑	0.1 ppm	Inhalation (whole-body); 3 h/day for 5 days	Single exposure concentration.	Aranyi et al. (1986)
35S-*Klebsiella pneumoniae* clearance	Mouse, CD_1 (F)	Lung	–	0.1 ppm	Inhalation (whole-body); 3 h	Single exposure concentration.	Aranyi et al. (1986)
35S-*Klebsiella pneumoniae* clearance	Mouse, CD_1 (F)	Lung	↓	0.1 ppm	Inhalation (whole-body); 3 h/day for 5 days	Single exposure concentration.	Aranyi et al. (1986)
Staphylococcus aureus clearance	Mouse, Swiss (F)	Lung	↓	3 ppm	Inhalation (whole-body); 8 h		Astry & Jakab (1983)
Staphylococcus aureus or *Proteus mirabilis* clearance	Mouse, Swiss (F)	Lung	–	2.5 ppm	Inhalation (nose-only); 4 h/day for 4 days	Single exposure concentration.	Jakab (1993)
Antigen-induced inflammation	Mouse, C57BL/6J (NR)	Lung	↓	5 ppm	Inhalation; 1 or 4 h	Single exposure concentration.	Danyal et al. (2016)
Innate macrophage function	Mouse, C57BL/6J (M)	Lung	↓	5 ppm	Inhalation (whole-body); 4 h	Single exposure concentration.	Hristova et al. (2012)
Listeria monocytogenes-induced mortality	Rat, Sprague-Dawley (M)	Lung	–	3 ppm	Inhalation (whole-body); 6 h/day, 5 days/wk for 3 wk	Exposure associated with nasal pathology.	Leach et al. (1987)
Antibody plaque-forming cells			–				

F, female; h, hour; HID, highest ineffective dose; LED, lowest effective dose; M, male; NR, not reported; ppm, parts per million; wk, week.

[a] ↑, increase; ↓, decrease; –, no effect.

Table 4.15 Inflammatory responses after acrolein exposure in non-human mammals in vivo

End-point	Species, strain (sex)	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Respiratory epithelial inflammation	Mouse, Crj:BDF1 (M,F)	Nasal cavity	↑	1.6 ppm	Inhalation (whole-body), 6 h/day, 5 days/wk, 104 wk		JBRC (2016a)
Respiratory epithelial inflammation	Rat, F344 (M, F)	Nasal cavity	↑	2.0 ppm	Inhalation (whole-body), 6 h/day, 5 days/wk, 104 wk		JBRC (2016d)
CxCl2, IL6, IL17β, and TNF expression	Mouse, 129S1/SvlmJ (F) Mouse, A/J (F) Mouse, BALB/cByJ (F) Mouse, C3H/HeJ (F) Mouse, C57BL/6J (F) Mouse, DBA/2J (F) Mouse, FVB/NJ (F)	Lung	↑ CxCl2: (C57BL/6J and FVB/NJ only) ↑ IL6: (129S1/SvlmJ; BALB/cByJ; C57BL/6J and A/J only) ↑ IL17β: (129S1/SvlmJ; BALB/cByJ; and C57BL/6J only)	1 ppm	Inhalation (whole-body), 6 h/day, 4–5 days/wk, 11 wk	Single exposure concentration; TNF data incompletely reported.	Johanson et al. (2020)
Cell count and cytokine level	Mouse C57BL/6J (M)	BALF	–	5 ppm	Inhalation (whole-body); 6 h/day for 3 days	Single exposure concentration.	Kasahara et al. (2008)
Total cells, macrophages, neutrophils; TNFα, CINC-1	Rat, Sprague–Dawley (M)	BALF Lung	↑ ↑	2.5 ppm 2.5 ppm	Inhalation (whole-body); 6 h/day, 7 day/wk for 2 or 4 wk	Single exposure concentration.	Wang et al. (2009b)
Macrophage, neutrophil, leukocytes, TNFα, KC (IL8 homologue)	Mouse, Kunning (M)	BALF	↑	4 ppm	Inhalation (whole-body), 6 h/day, 7 days/wk, for 21 days	Single exposure concentration.	Liu et al. (2009a)
Mucin, macrophage, neutrophil, TNFα, IL8, IL1β	Rat, Sprague-Dawley (M)	BALF	↑	3 ppm	Inhalation (whole-body), 6 h/day, 7 days/wk, 2 wk	Single exposure concentration.	Liu et al. (2009b)
TNFα, IL6, IL1β	Mouse, C57BL/6 (M)	Lung	–	5 ppm	Inhalation (whole-body), 6 h	Single exposure concentration.	Sithu et al. (2010)
TNFα, IL6, IL1β	Mouse, C57BL/6 (M)	Lung	–	1 ppm	Inhalation (whole-body), 6 h/day for 4 days	Single exposure concentration.	Sithu et al. (2010)
Wet weight, oedema	Rat, F344 (M, F)	Lung	↑	1.4 ppm	Inhalation (whole-body), 6 h/day, 5 days/wk for 13 wk		Kutzman et al. (1985)

Table 4.15 (continued)

End-point	Species, strain (sex)	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Macrophages, neutrophils	Mouse, FVB/N, (M)	BALF	↑	3 ppm	Inhalation (whole-body), 6 h/day, 5 days/wk, 3 wk	Single exposure concentration.	Borchers et al. (1999)
Macrophages	Mouse, C57BL/6J, (NR) Mouse, MME (+/+), (NR) Mouse, MME (–/–), (NR)	BALF	↑ ↑ ↑	3 ppm	Inhalation (whole-body), 6 h/day, 5 days/wk, 3 wk	Single exposure concentration.	Borchers et al. (1999)
CD8+ lymphocytes, macrophage	Mouse, C57BL/6J (NR)	Lung	↑	2 ppm	Inhalation (whole-body), 6 h/day, 5 days/wk for up to 12 wk	Single exposure concentration.	Borchers et al. (2007)
CD8+ lymphocytes, macrophage	Mouse, $Cd8^{-/-}$ (NR)	Lung	↑	2 ppm	Inhalation (whole-body), 6 h/day, 5 days/wk for up to 12 wk	Single exposure concentration.	Borchers et al. (2007)
Mucous cell metaplasia Macrophage accumulation	Mouse, C57BL/6J (F)	Lung	↑ ↑	2 ppm	Inhalation (whole-body), 6 h/day, 5 days/wk for up to 4 wk	Single exposure concentration.	Borchers et al. (2008)
Mucous cell metaplasia Macrophage accumulation	Mouse, γδ T-cell deficient (F)	Lung	↑ ↑	2 ppm	Inhalation (whole-body), 6 h/day, 5 days/wk for up to 4 wk	Single exposure concentration.	Borchers et al. (2008)
Mucous cell metaplasia Macrophage accumulation	Mouse, αβ T-cell deficient (F)	Lung	↑ –	2 ppm	Inhalation (whole-body), 6 h/day, 5 days/wk for up to 4 wk	Single exposure concentration.	Borchers et al. (2008)
Interstitial inflammation, neutrophil infiltration, congestion, and oedema	Mouse, C57BL/6 (M)	Lung	↑	10 ppm	Inhalation (whole-body), 12 h	Single exposure concentration.	Kim et al. (2018)
Nasal epithelial inflammation and metaplasia	Rat, Wistar (M, F) Hamster, Golden Syrian (M, F) Rabbit, Dutch (M, F)	Nasal cavity	↑ ↑ ↑	0.4 ppm 1.4 ppm 4.9 ppm	Inhalation (whole-body), 6 h/day, 5 days/wk for 13 wk		Feron et al. (1978)
Olfactory epithelial inflammation	Rat, F344 (M)	Nasal cavity	↑	1.8 ppm	Inhalation (whole-body), 6 h/day 5 days/wk for 13 wk		Dorman et al. (2008)

Table 4.15 (continued)

End-point	Species, strain (sex)	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Respiratory epithelial cell inflammation	Rat, F344 (M)	Nasal cavity	↑	1.8 ppm	Inhalation (whole-body), 6 h/day 5 days/wk for 13 wk		Dorman et al. (2008)
Mucus hypersecretion	Rat, Sprague-Dawley (M)	BALF	↑	3 ppm	Inhalation (whole-body), 6 h/day for 12 days	Single exposure concentration.	Chen et al. (2013a)
Inflammatory cells	Mice, C57BL/6 (M)	BALF	–	5 ppm	Inhalation (whole-body), 4 h/day, 4 days/wk for 2 wk	Single exposure concentration.	O'Brien et al. (2016)
Total protein	Mouse, 129S1/SvlmJ (F) Mouse, A/J (F) Mouse, BALB/cByJ (F) Mouse, C3H/HeJ (F) Mouse, C57BL/6J (F) Mouse, DBA/2J (F) Mouse, FVB/NJ (F)	BALF	All strains (↓)	1 ppm	Inhalation (whole-body), 6 h/day, 4–5 days/wk for 11 wk	Single exposure concentration.	Johanson et al. (2020)
Total protein Total cells Total protein	Rat, Wistar, (M)	BALF BALF NALF	↑ ↑ ↑	4 ppm 4 ppm 4 ppm	Inhalation (nose-only), 4 h/day for 2 days		Snow et al. (2017)
Total protein Total cells Total protein	Rat, Goto Kakizaki (M)	BALF BALF NALF	↑ ↑ ↑	2 ppm 4 ppm 4 ppm	Inhalation (nose-only), 4 h/day for 2 days		Snow et al. (2017)
Total cells; neutrophils; TNFα, IL1α; IL1β; KC	Mouse. BALB/c (M)	BALF	↑	1 mg/kg	Oropharyngeal aspiration	Relevance of route of exposure (anaesthetized).	Ong et al. (2012)
Bladder wet weight (oedema)	Mouse, Swiss (M)	Urinary bladder	↑	75 µg/bladder	Intravesical, 1×	Relevance of exposure route.	Batista et al. (2006)
Bladder wet weight (oedema)	Mouse, Swiss (M)	Urinary bladder	↑	75 µg/bladder	Intravesical, 1×	Relevance of exposure route.	Batista et al. (2007)
Bladder wet weight (oedema)	Mouse, C57 (F)	Urinary bladder	↑	6 µg/bladder	Intravesical, 1×	Relevance of exposure route.	Bjorling et al. (2007)
Bladder wet weight (oedema)	Mouse, C3H/HeJ (F)	Urinary bladder	↑	6 µg/bladder	Intravesical, 1×	Relevance of exposure route.	Bjorling et al. (2007)

Table 4.15 (continued)

End-point	Species, strain (sex)	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Bladder wet weight (oedema)	Mouse, C3H/OuJ (F)	Urinary bladder	↑	6 µg/bladder	Intravesical, 1×	Relevance of exposure route.	Bjorling et al. (2007)
Bladder wet weight (oedema)	Rat, Wistar (M)	Urinary bladder	↑	75 µg/bladder	Intravesical, 1×	Relevance of exposure route.	Macedo et al. (2008)
Bladder wet weight (oedema)	Rat, Wistar (F)	Urinary bladder	↑	1 mM (400 µL)/ bladder	Intravesical, 1×	Relevance of exposure route.	Merriam et al. (2011)
Bladder wet weight (oedema)	Rat, Wistar (F)	Urinary bladder	↑	75 µg/bladder	Intravesical, 1×	Relevance of exposure route.	Wang et al. (2013b)

BALF, bronchoalveolar lavage fluid; CINC, cytokine-induced neutrophil chemoattractant; F, female; h, hour; HID, highest ineffective dose; IL, interleukin; KC, mouse homologue for human IL8; LED, lowest effective dose; M, male; NALF, nasal lavage fluid; NR, not reported; TNF, tumour necrosis factor; ppm, parts per million; wk, week.

[a] ↑, increase; ↓, decrease; (↓), decrease in a study of limited quality; –, no effect.

al., 1999, 2007, 2008). Interstitial inflammation, neutrophil infiltration, congestion, and oedema were reported in mouse lung (Kim et al., 2018). Increased inflammation has also been reported in rat respiratory epithelial cells and in the rat, hamster, and rabbit olfactory epithelium after acrolein inhalation (Feron et al., 1978; Dorman et al., 2008). Mucus hypersecretion has been observed in rats after acrolein inhalation (Chen et al., 2013a), and effects on bronchoalveolar lavage fluid have variously been observed in studies in mice and rats (O'Brien et al., 2016; Johanson et al., 2020; Snow et al., 2017). Oropharyngeal administration of acrolein in mice results in pulmonary inflammation as shown by the associated increase in elevated macrophage and neutrophil counts in the bronchoalveolar lavage fluid, and increased expression of production of cytokines, including interleukins IL1α, IL1β, IL6, IL17, and TNF, IFNγ, and monocyte chemotactic protein 1 (MCP-1) (Ong et al., 2012).

Some in vivo studies in rodents have investigated the role of acrolein in cyclophosphamide-induced inflammation and haemorrhagic cystitis. These studies rely on an injection of acrolein directly into the urinary bladder (Batista et al., 2006, 2007; Bjorling et al., 2007; Macedo et al., 2008; Merriam et al., 2008; Wang et al., 2013b). [The Working Group noted that these studies use a route of exposure that is unlikely to occur in humans and they involve acute exposures.]

4.2.7 Alters cell proliferation, cell death, or nutrient supply

(a) Humans

No data in exposed humans were available to the Working Group.

In human cell lines, several studies suggested that acrolein is capable of inhibiting tumour suppressor genes and activating proto-oncogenes either by directly binding and modulating the protein or by disrupting signalling cascades that promote cell proliferation. Acrolein inhibited both DNA-binding activity and luciferase-reporter activity of the tumour suppressor TP53, in a B[*a*]P induction study using human lung adenocarcinoma cells (A549) (Biswal et al., 2003). The inhibitory effect of acrolein occurred in the absence of altered TP53 protein levels under basal or induced conditions (i.e. a 48-hour pre-treatment with B[*a*]P), which is probably the result of direct binding of the TP53 protein by acrolein, as well as the alteration of the intracellular redox status.

Acrolein was demonstrated to both covalently modify and inhibit phosphatase and tensin homologue (PTEN) in MCF-7 breast ductal carcinoma cancer cells (Covey et al., 2010). Perturbation of Wnt/β-catenin signalling in human embryonic kidney cells (HEK-293), which favour the retention of active protein kinase AKT, was demonstrated in both time- and dose-dependent manners. Acrolein inactivation of PTEN lead to an increase in activity of the proto-oncogene AKT, which can increase cellular proliferation and survival (Covey et al., 2010). AKT activity was further explored in a human colon carcinoma cell line (HCT 116), and in MCF-7 cells: acrolein was generated endogenously as a by-product of myeloperoxidase catalysis (quantified spectrophotometrically) and resulted in the accumulation of higher amounts of phospho-Ser^{473}AKT; when a PI3K inhibitor (wortmannin) or a myeloperoxidase inhibitor (resorcinol) were added, phospho-Ser^{473}AKT formation was suppressed (Al-Salihi et al., 2015).

Acrolein caused differential inhibition and modification (covalent adducts) of pyruvate kinase, the enzyme involved in the last step of anaerobic glycolysis, in MCF-7 cells and in a cell-free system. This change in activity has been reported during the transformation of cells to a proliferative or tumorigenic phenotype (Sousa et al., 2019).

(b) Experimental systems

A significant increase in the incidence and/or severity of respiratory tract metaplasia and hyperplasia was observed in B6D2F$_1$/Crlj mice and F344/DuCrlCrlj rats exposed to acrolein by inhalation for 2 years (JBRC, 2016a, b, c, d, e, f) and is discussed in Sections 3.1 and 3.2 of the present monograph.

Feron & Kruysse (1977) reported an increase in epithelial metaplasia of the nasal cavity epithelium in Syrian golden hamsters repeatedly inhaling acrolein vapour (4 ppm) for 7 hours per day, 5 days per week, for 52 weeks. In a subacute toxicity study (6 hours per day, 5 days per week, for 13 weeks) in hamsters, rats, and rabbits, squamous metaplasia was only observed in rats treated with the intermediate dose of 1.4 ppm (Feron et al., 1978). [The Working Group noted high mortality in the group at the higher dose.]

Fischer 344 rats treated with acrolein (0.6 or 1.8 ppm) by inhalation for up to 65 days presented with respiratory epithelial hyperplasia and squamous metaplasia (Dorman et al., 2008). In the most sensitive location, the lateral wall, respiratory epithelial cell proliferation occurred in the two highest dose groups as detected by proliferating cell nuclear antigen (PCNA) immunohistochemistry (Dorman et al., 2008). In a separate inhalational study, acrolein (3 ppm) induced goblet cell hyperplasia in the bronchial epithelium in lungs of male Sprague-Dawley rats exposed for 6 hours per day, 7 days per week, for 2 weeks (Liu et al., 2009b). Acrolein (≥ 0.1 µM) elicited a similar increase in goblet cell number in a differentiated lung epithelium model, which mirrored, to some extent, the goblet cell hyperplasia observed in animal inhalation models and after human chronic exposure (Haswell et al., 2010).

In Sprague-Dawley rats, acrolein (3 ppm) inhalation for 3 weeks led to metaplastic, dysplastic, and hyperplastic changes in the mucous, respiratory, and olfactory epithelium of the nasal cavity (Leach et al., 1987). These changes were prominent on the septum and in the anterior and ventral areas.

In Sprague-Dawley rats given a single exposure or a sustained 3-day exposure to acrolein at 0.2 or 0.6 ppm via inhalation, a concentration-dependent increase in the proportion of 5-bromo-2′-deoxyuridine (BrdU)-labelled nasal epithelial, tracheal epithelial, or free lung cells was observed (Roemer et al., 1993). Although significantly increased compared with control at both time-points, the single exposure elicited a stronger proliferative response (Roemer et al., 1993). Similar treatment-related proliferative increases were measured by both BrdU and PCNA labelling in nasal epithelium of albino Wistar rats that were treated (≤ 0.67 ppm) for 6 hours per day, for 3 days (Cassee et al., 1996). These rats also presented with slight disarrangement, necrosis, thickening, and desquamation of respiratory/transitional epithelium (Cassee et al., 1996). [The Working Group noted necrosis associated with the highest dose.]

A single inhalational exposure (5 ppm for 10 minutes) of BALB/c mice to acrolein led to a sustained increase in levels of vascular endothelial growth factor protein that persisted for 8 weeks (Kim et al., 2019). A single inhalation exposure of Sprague-Dawley rats to acrolein (3 ppm for a 12-day period of 5 days of treatment, 2 days of rest, and another 5 days of treatment) significantly activated the Ras/ERK pathway in bronchial epithelial cells, which functions downstream of epidermal growth factor. This finding was accompanied by an increase in goblet cell hyperplasia and metaplasia, which were significantly inhibited by simvastatin, a Ras inhibitor (Chen et al., 2010).

In oral gavage studies in B6C3F$_1$ mice and F334/N rats, acrolein treatment led to lesions associated with uncontrolled cell growth. Squamous epithelial hyperplasia in the forestomach and hyperplasia of bone marrow cells were observed in rats treated with acrolein at

≤ 10 mg/kg bw 5 days a week for 2 weeks; mice in the dose groups treated with ≤ 10 mg/kg bw developed squamous epithelial hyperplasia of the forestomach (Irwin, 2006). [The Working Group noted the high mortality in the groups of rats and mice at the highest dose.]

Forestomach epithelial hyperplasia was observed in male and female F344/N rats and $B6C3F_1$ mice given acrolein (≤ 10 mg/kg bw) by gavage once per day, 5 days per week, for 14 weeks (Auerbach et al., 2008). The pro-toxicants, allyl acetate and allyl alcohol, which are metabolized to acrolein, were also investigated. Periportal hepatocyte hypertrophy was observed in rats treated with allyl acetate and allyl alcohol, but not acrolein. Both species treated with the highest dose of allyl acetate exhibited forestomach epithelial necrosis. [The Working Group noted the 100% mortality of this dose group for all species and sexes; the Working Group also noted low (93.3%) purity of allyl acetate.]

In a mouse model of intestinal cancer, *Apc*$^{min/+}$ mice were either treated with water or dextran sodium sulfate to induce a model of colitis. Colonocytes isolated from mice treated with dextran sodium sulfate were found to have covalent acrolein–protein adducts on the PTEN tumour suppressor from endogenously generated acrolein (myeloperoxidase catalysis), which corresponded with the activation of the *Akt* protooncogene in these samples (Al-Salihi et al., 2015).

In studies in hypertension-resistant and salt-induced rats treated with acrolein (≤ 1.4 ppm) via inhalation for 6 hours per day, 5 days per week, for 62 days, bronchiolar epithelial hyperplasia was reported that was sometimes accompanied by squamous metaplasia and fibrosis (Kutzman et al., 1984). [The Working Group noted the toxicity associated with the highest dose, and the model of hypertension that was used for this study.]

4.2.8 Other key characteristics of carcinogens

(a) Induces epigenetic alterations

Several studies in experimental systems investigated the effect of acrolein on histone modification. Acrolein inhibited acetylation of the N-terminal tails of cytosolic histones H3 and H4 in vitro, compromising chromatin assembly in immortalized human bronchial epithelial and lung adenocarcinoma cell lines (Chen et al., 2013b). Interestingly, the effect of acrolein was specific to unmodified and newly synthesized histones; post-translational modifications seemed to protect the histone from being targeted. The mechanism behind these phenomena was further investigated by the same research group. Fang et al. (2016) determined that acrolein reacts and forms covalent adducts with lysine residues in an immortalized human bronchial epithelial cell line (BEAS-2B), including those residues important for chromatin assembly, therefore preventing these sites from undergoing physiological modifications (see Section 4.2.1). Promoter histone modifications of the *FasL* gene were enhanced by acrolein in the human liver hepatocarcinoma HepG2 cell line and in primary rat hepatocytes both alone and when co-treated with the HIV antiretroviral zidovudine (Ghare et al., 2016). When the acrolein scavenger hydralazine was added to the experiment, promoter-associated epigenetic changes were inhibited. Global DNA methylation and accumulation of DNA damage because of silencing of DNA repair genes was observed in acrolein-treated C57BL/6 mouse bladder tissue and in cultured mouse bladder muscle cells (Haldar et al., 2015, 2016). Cox et al. (1988) showed that DNA methylase isolated from the liver and urothelium of rats (strain not reported), treated with acrolein, was inhibited by 30–50% but the mechanism behind the inhibition was unclear.

(b) Modulates receptor-mediated effects

Several receptors appear to be activated or modulated by acrolein, although the studies are limited in both number and specificity. Thyroid hormone co-treatment with acrolein, administered both as a single compound and as a component of cigarette smoke, acts as a partial agonist for the thyroid receptor through recruitment of the nuclear coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SCR1) (Hayashi et al., 2018). Again, independently or as a component of cigarette smoke extract, acrolein was able to recruit GRIP1 or SCR1, but this time to peroxisome proliferator-activated receptor-α (PPARα) to induce transcriptional changes (Matsushita et al., 2019).

In male Fischer 344 rats given acrolein intraperitoneally with phenobarbital, α-, 2β-, 6β-, 16α-, and 16β-hydroxylation of testosterone and androstenedione was decreased (Cooper et al., 1992). This was the result of acrolein impairing the induction of CYP by 45%.

(c) Causes immortalization

Acrolein significantly increased soft agar anchorage-independent-growth colony formation, a characteristic of tumorigenic cell transformation, in immortalized human bronchial epithelial cells (BEAS-2B) and bladder urothelial cells (UROtsa) (Lee et al., 2015).

(d) Multiple characteristics

Gene expression changes in response to acrolein exposure were investigated in several studies. Data suggested a coordination of several of the key characteristics, namely induces oxidative stress, induces chronic inflammation and, to some degree, alters DNA repair in epithelial tissue or cells.

In normal human bronchial epithelial cells treated with acrolein for up to 24 hours, a combination of high-content screening and genome-wide transcriptomics revealed induction of genes associated with cellular stress followed by proliferation, and to a lesser extent, senescence networks (Gonzalez-Suarez et al., 2014). Interestingly, NRF2 was consistently activated despite the lack of observed increases in ROS. Furthermore, an increase in phosphorylation of histone 3 (pH3) levels was not accompanied by changes in cell number, suggesting the presence of cell cycle arrest at G_2/M. Rats exposed for 6 hours to acrolein by inhalation (nose-only) exhibited similar patterns of protein and gene expression (Gonzalez-Suarez et al., 2014). In addition to the nuclear accumulation of Nrf2 protein, antioxidant genes (i.e. NAD(P)H quinone dehydrogenase 1, *Nqo1*; catalytic subunit of glutamyl cysteine ligase, *Gclc*; and haem oxygenase 1, *Hmox1*) were upregulated at much lower acrolein concentrations than those required to induce the expression of proinflammatory genes (i.e. chemokine-induced neutrophil chemoattractant-1, *Cinc1*; and interleukin 6, *Il6*) (Cichocki et al., 2014).

Three studies investigated the transcriptional response to acrolein in human adenocarcinoma lung epithelial (A549) cells at various time-points. Over the course of 4 hours, a strong initial downregulation of genes was observed, possibly in response to DNA damage, followed by an increase in gene upregulation in which pro-inflammatory and pro-apoptotic pathways were dominant (Thompson & Burcham, 2008). Overall, these results indicate a dysregulation in several key characteristics of carcinogens including apoptosis, cell cycle control, and cell signalling. In a 2-hour exposure study in the same cells (A549), acrolein given alone or as a mixture with other short-chain aldehydes resulted in only one upregulated gene, *HMOX1*, a key gene in oxidative stress response (Cheah et al., 2013). A 24-hour treatment of A549 cells with acrolein at half maximal inhibitory concentration (IC_{50}) induced a robust expression of DNA repair genes, but this failed to rescue cells from

apoptosis, even after acrolein washout and a recovery period (Sarkar, 2019)

4.3 Data relevant to comparisons across agents and end-points

Acrolein is one of approximately 1000 chemicals tested across the full assay battery of the Toxicity Testing in the 21st Century (Tox21) and Toxicity Forecaster (ToxCast) programmes supported by the US EPA (2020). In vitro assay descriptions to map Tox21 and ToxCast screening data in the context of the 10 key characteristics were previously summarized by Chiu et al. (2018). Results in this data set only include one active hit out of 235 assays. Acrolein was active in the antioxidant response element assay designed to target transcription factor activity, specifically mapping to the *NRF2* gene using a positive control of naphthoflavone. *NRF2* encodes the transcription factor NRF2, which regulates genes containing antioxidant response elements (ARE) in their promoters; this is probably the result of acrolein inducing oxidative stress. [The Working Group noted one flag for < 50% efficiency with this assay.]

5. Summary of Data Reported

5.1 Exposure characterization

Acrolein is a High Production Volume chemical that is used as a reactive intermediate and in the manufacture of numerous chemical products, including acrylic acid and methionine. It is directly used as a biocide, specifically, as an herbicide in recirculating water systems. Acrolein is formed during combustion of fuels, wood, and plastics, and is present in cigarette smoke and vapours from electronic cigarettes. In kitchens, high-temperature roasting and deep-fat frying produce measurable amounts of acrolein in the air. Acrolein is also formed during fermentation and is found in various alcoholic beverages. Acrolein is routinely measured in studies monitoring outdoor air pollution, and it has been identified in various combustion emissions in numerous reports. Firefighters are also exposed to acrolein. Occupational and environmental exposure guidelines exist for acrolein.

The urinary metabolite *N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine (3-hydroxypropylmercapturic acid, HPMA) has been applied to estimate exposure, and a reference value for workplace substances is available. However, it is often challenging to differentiate endogenous from exogenous exposure due to the various external sources including air pollution, exposure to secondhand smoke, and consumption of fried and fermented foods.

5.2 Cancer in humans

One occupational cohort study, two hospital-based case–control studies, and three nested case–control studies in occupational or population-based cohorts were available, with little consistency in the cancer sites evaluated. The study in an occupational cohort, one case–control study on urothelial cancer in patients with chronic kidney disease, and one nested case–control study on lymphohaematopoietic cancer in an occupational cohort were uninformative due to small numbers, poor external exposure assessment, and flaws in design. The other case–control study detected higher levels of acrolein–DNA adducts in buccal swabs of patients with oral cancer compared with healthy controls, but the study did not find an association between adduct levels and external exposures, including tobacco smoking or betel chewing. Finally, two nested case–control studies in a population-based cohort studied several biomarkers (including metabolites of acrolein) in relation to lung cancer among current smokers and non-smokers respectively, without demonstrating a direct

etiological involvement of acrolein. In summary, all studies were judged to be uninformative and did not provide evidence on a causal relationship between acrolein exposure and cancer in humans. The studies were either of poor quality regarding design or exposure assessment, or they were of a mechanistic nature.

5.3 Cancer in experimental animals

Exposure to acrolein caused an increase in the incidence of either malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in two species.

In an inhalation study in B6D2F_1/Crlj mice, female mice exposed to acrolein showed a significant positive trend in the incidence of malignant lymphoma.

In an inhalation study in F344/DuCrlCrlj rats, there was a significant positive trend in the incidence of rhabdomyoma of the nasal cavity and of squamous cell carcinoma or rhabdomyoma (combined) of the nasal cavity in females exposed to acrolein. There was also a significant increase in the incidence of squamous cell carcinoma or rhabdomyoma (combined) of the nasal cavity. Rhabdomyoma of the nasal cavity and squamous cell carcinoma of the nasal cavity are very rare tumours in the strain of rats used in the study.

5.4 Mechanistic evidence

The available data on absorption and distribution of acrolein in humans are scarce. Acrolein is absorbed after inhalation or oral exposure. A slow absorption rate from air was observed in experiments with human skin in vitro. In humans, the delivery of acrolein to the lower respiratory tract can be higher than in rats; in rats, which are obligate nasal breathers, a significant portion of acrolein (up to 98%) is absorbed in the upper respiratory tract. Acrolein is a reactive electrophile that reacts spontaneously with cellular glutathione as well as with nucleophilic sites in proteins and DNA. It is efficiently metabolized by three detoxification pathways: (i) conjugation with glutathione leading eventually to HPMA and *N*-acetyl-*S*-(carboxyethyl)-L-cysteine (2-carboxyethylmercapturic acid, CEMA), which are excreted in urine; (ii) reduction by aldo-keto reductases to allyl alcohol; and (iii) oxidation by aldehyde dehydrogenases to acrylic acid, which is further converted to 3-hydropropionic acid and thereby enters physiological catabolism. Metabolic activation by cytochrome P450s (CYPs) to glycidaldehyde is a minor metabolic pathway leading to 2-carboxy-2-hydroxyethylmercapturic acid through glutathione conjugation. Acrolein is excreted in urine, exhaled air, and faeces. Excretion half-time in humans is approximately 9 hours as measured by urinary HPMA levels. Small amounts of acrolein from both endogenous and exogenous sources have been detected in exhaled air. In rats, 26–31% of both intravenous and oral doses were exhaled as carbon dioxide.

There is consistent and coherent evidence that acrolein exhibits key characteristics of carcinogens. Acrolein is a strongly electrophilic α,β-unsaturated aldehyde (enal) that readily reacts with DNA bases and proteins forming DNA and protein adducts in vivo and in vitro. Among these adducts, the most widely studied are the cyclic deoxyguanosine adducts, which are formed as a pair of α and γ regioisomers, α- and γ-hydroxy-1,N^2-propano-2′-deoxyguanosine (α- and γ-OH-PdG). γ-OH-PdG has been consistently detected in humans in various samples (including from lung, liver, brain, urothelial mucosa, and saliva), as well as in experimental animals, with detected levels dependent on species, tissue types, exposure, and physiological conditions. Elevated levels of acrolein-derived adducts are found in tobacco smokers, or under chronic inflammatory conditions, such as non-alcoholic fatty liver

disease. This indicates their formation by acrolein from tobacco smoke; their presence in tissues of non-smokers is indicative of acrolein formation by endogenous processes, including lipid peroxidation. In acrolein-treated human lung cells, acrolein–DNA adducts were preferentially formed at lung cancer *TP53* mutational hotspots, and acrolein preferentially adducted guanines at cytosine methylation CpG sites. Acrolein-derived DNA adducts have been detected in the liver of untreated rodents as well as in various tissues of rodents exposed to cigarette smoke, automobile exhaust, or a high-fat diet. Acrolein-derived DNA adducts have also been detected in dogs exposed to cyclophosphamide, and in cockerels exposed to acrolein.

Acrolein is genotoxic. No data in humans in vivo were available. In several studies in human primary cells, acrolein consistently induced DNA strand breaks and DNA–protein crosslinks. In cultured human cell lines, acrolein consistently induced DNA strand breaks, mutations, and micronucleus formation, and was suggestive of inducing DNA–protein crosslinks. A limited number of in vivo studies of genotoxic end-points were available and were largely negative; however, across many in vitro experimental systems acrolein was found to consistently induce DNA strand breaks, DNA–protein crosslinks, mutations, and sister-chromatid exchanges. In *Salmonella* strains tested without metabolic activation, acrolein induced both base-pair substitution and frameshift mutations. The mutagenicity of acrolein has also been demonstrated in experiments with plasmid DNA.

Acrolein alters DNA repair or causes genomic instability. No data in humans in vivo were available. Multiple studies in human cells have demonstrated that acrolein directly inhibits proteins in three major DNA-repair pathways. Acrolein induced concentration-dependent inhibition of nucleotide excision repair, base excision repair and mismatch repair in primary human lung fibroblasts and bronchial epithelial cells, as well as in cultured human lung and urothelial cells. Acrolein inhibited the DNA repair enzyme O^6-methylguanine–DNA methyltransferase in human bronchial fibroblasts. It also inhibited excision repair due to the accumulation of DNA single-strand breaks in normal skin fibroblasts.

Acrolein induces oxidative stress. No in vivo human data were available. In vitro studies using multiple human- and rodent-derived cells showed that acrolein induces biochemical changes consistent with depletion of glutathione and increased generation of ROS and protein carbonyls, indicative of oxidative stress. Multiple studies in rodents have likewise shown that acrolein administration via multiple routes of exposure including inhalation, oral, and intraperitoneal injection resulted in decreased tissue glutathione concentrations, and increased lipid peroxidation and protein carbonyl production. A statistically significant increase in levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) has been reported in rodent lung DNA after acrolein inhalation.

Acrolein is immunosuppressive. No data in humans in vivo were available. In studies in vitro with human immune cells, acrolein exposure can impair cytokine release and result in cytotoxicity. Human alveolar macrophages exposed to acrolein have reduced ability to clear *Mycobacterium tuberculosis* infection. Multiple studies in rodents have demonstrated that acrolein inhalation alters bacterial-induced mortality, bactericidal activity, or innate immune function. Mouse splenic cells exposed to acrolein exhibited decreased T- and B-cell proliferation.

Acrolein induces chronic inflammation. No data were available in humans, but acrolein exposure can produce chronic inflammation in rodents. Shorter (i.e. acute to subchronic) rodent studies showed that acrolein administration via multiple routes, including oral and inhalation, produces inflammation at the site of entry.

Additionally, acrolein alters cell proliferation, cell death, or nutrient supply. No in vivo data

were available in humans. In vitro studies using multiple human and rodent cell types showed that acrolein inhibited tumour suppressor genes and activated proto-oncogenes either by directly binding and modulating the protein or by disrupting signalling cascades that promote cell proliferation. Hyperplasia, metaplasia, and dysplasia were seen in the respiratory system of rodents exposed chronically or acutely by inhalation. After chronic exposure, rodents treated with acrolein by oral gavage developed forestomach epithelial hyperplasia.

There is suggestive evidence that acrolein induces epigenetic alterations via DNA methylation and histone modification. One study using mouse tissues and cells treated with acrolein reported alteration of global DNA methylation and accumulation of DNA damage because of silencing of DNA repair genes. This result was consistent with findings in mouse tissues and cells. DNA methylase was inhibited in two different rat strains. In vitro studies using human- and rodent-derived cells suggest that acrolein compromises chromatin assembly through inhibition of acetylation of the N-terminal tails of cytosolic histones.

Acrolein was essentially without effects in the assay battery of the Toxicity Testing in the 21st Century (Tox21) and Toxicity Forecaster (ToxCast) research programmes.

6. Evaluation and Rationale

6.1 Cancer in humans

There is *inadequate evidence* in humans regarding the carcinogenicity of acrolein.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of acrolein.

6.3 Mechanistic evidence

There is *strong evidence* that acrolein exhibits multiple key characteristics of carcinogens, primarily from studies with human primary cells and studies in experimental systems, supported by studies in humans for DNA adducts.

6.4 Overall evaluation

Acrolein is *probably carcinogenic to humans (Group 2A)*.

6.5 Rationale

The *Group 2A* evaluation for acrolein is based on *sufficient evidence* of cancer in experimental animals and *strong* mechanistic evidence. The *sufficient evidence* of carcinogenicity in experimental animals is based on an increased incidence of either malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in two species. There is *strong* evidence that acrolein exhibits multiple key characteristics of carcinogens; acrolein is electrophilic; it is genotoxic; it alters DNA repair or causes genomic instability; it induces oxidative stress; it is immunosuppressive; it induces chronic inflammation; and it alters cell proliferation, cell death, or nutrient supply. The supporting data that acrolein exhibits these key characteristics comes primarily from studies with human primary cells and studies in experimental systems, and is supported by studies in humans for DNA adducts.

The evidence regarding cancer in humans is *inadequate*. The few available studies related to acrolein exposure and human cancer were inconsistent in the cancer sites evaluated, and most studies were small. All had poor assessment of external exposure to acrolein or could not distinguish the effects of acrolein exposure from other constituents of cigarette smoking.

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CROTONALDEHYDE

1. Exposure Characterization

1.1 Identification of the agent

1.1.1 Nomenclature

Chem. Abstr. Serv. Reg. No.: 4170-30-3 (*E*/*Z*); 15798-64-8 (*Z*); 123-73-9 (*E*)

Chem. Abstr. Serv. name: 2-butenal (*E*/*Z*); (*Z*)-2-butenal; (*E*)-2-butenal

EC/List No.: 224-030-0; 204-647-1 (*E*)

IUPAC name: but-2-enal (*E*/*Z*); (*Z*)-but-2-enal; (*E*)-but-2-enal

ICSC No.: 0241 (ILO, 2018)

RTECS No.: GP9499000 (NIOSH, 2019)

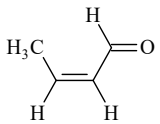
DSSTox substance ID: DTXSID8024864 (US EPA, 2020a)

Common name: crotonaldehyde

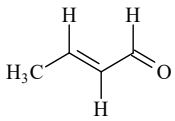
Synonyms: 2-butenaldehyde; crotonal; crotonic aldehyde; crotylaldehyde; 1-formylpropene; propylene aldehyde; methylpropenal; 3-methylacrolein; β-methylacrolein; BDQ; Topanel; butenal; topanelca; 2-butenal; bu-2-tenal; NCI-C56279; Topanel CA (ChemicalBook, 2019; NCBI, 2020).

1.1.2 Structural and molecular formulae, and relative molecular mass

Structural formula:



Z- (cis-) isomer



E- (trans-) isomer

Molecular formula: C_4H_6O

Relative molecular mass: 70.09

1.1.3 Chemical and physical properties

Description: pure crotonaldehyde is a colourless liquid with a suffocating odour, which degrades when exposed to light and air and turns pale yellow as it oxidizes to a peroxide and then to crotonic acid. It can polymerize in the presence of small amounts of mineral acids. If heated with alkali chemicals, it will also polymerize, condense, or resinify (Celanese Corporation, 2011; NCBI, 2020). The information below pertains to mixtures of the *trans-* (*E-*) and *cis-* (*Z-*) isomers of crotonaldehyde, unless stated otherwise.

Boiling point: 104–105 °C (Lide, 1993)

Melting point: –74 °C (Lide, 1993)

Relative density: 0.8495 at 20 °C/4 °C (Lide, 1993)

Solubility: soluble in water (150 g/L at 20 °C), acetone, benzene, diethyl ether, and ethanol; miscible with gasoline, kerosene, solvent naphtha, and toluene (Eastman Chemical Co., 1991; Lide, 1993; Larrañaga et al., 2016)

Vapour pressure: 32 mm Hg [4.3 kPa] at 20 °C; relative vapour density, 2.4 (air = 1) (Budavari, 1989; Eastman Chemical Co., 1994)

Flash point: 13 °C (closed cup) (ILO, 2018)

Stability: readily dimerizes when pure; slowly oxidizes to crotonic acid (Budavari, 1989); polymerizes to become inflammable and explosive (Eastman Chemical Co., 1994)

Reactivity: lower explosive limit, 2.15% at 24 °C; reacts violently with bases, strong oxidizing agents, and polymerization initiators (Eastman Chemical Co., 1994)

*Octanol/water partition coefficient (*P*)*: log K_{ow}, 0.63 (United States National Library of Medicine, 1994)

Odour perception threshold: 0.035–0.2 ppm [0.10–0.57 mg/m^3] (European Commission, 2013)

Conversion factor: 1 ppm = 2.87 mg/m^3 (IARC, 1995).

1.1.4 Technical products and impurities

Commercial crotonaldehyde is stabilized with 0.1–0.2% BHT (butylated hydroxytoluene, 2,6-di-*tert*-butyl-4-methylphenol) and is available at purities of 90–99%. One common commercial product consists of > 95% *trans*- (*E*-) and < 5% *cis*- (*Z*-) isomer and contains 0.1–0.2% BHT and 1% water as stabilizers (Sigma-Aldrich, 2020a); another commercial product that is > 99% *trans*- (*E*-) isomer is stabilized with 0.1–0.2% BHT and 1% water (Sigma-Aldrich, 2020b). A typical specification for crotonaldehyde is as follows: minimal purity, 90%; acidity (as crotonic acid), 0.15% maximum; water content, 8.5% maximum; aldol, 0.1% maximum; butyraldehyde, 0.02% maximum; low boiling-point compounds (including acetaldehyde, see IARC, 1987; and butyraldehyde), 0.20% maximum; butyl alcohol, 0.15% maximum; and high-boiling-point compounds, 1.0% maximum (Blau et al., 1987; Eastman Chemical Co., 1993; Spectrum Chemical MFG Corp., 1994).

1.2 Production and use

1.2.1 Production process

Crotonaldehyde is usually produced by the aldolization reaction of acetaldehyde, catalysed by one of various basic catalysts, e.g. alkali metal or alkaline earth metal catalysts, ammonium salts, zeolites, molecular sieves and claylike materials, followed by dehydration of the acetaldol and distillation (Blumenstein et al., 2015).

1.2.2 Production volume

Crotonaldehyde is a High Production Volume chemical according to the Organization for Economic Co-operation and Development (OECD) (OECD, 2020) and the United States Environmental Protection Agency (US EPA) (US EPA, 2020a). Less than 500 tonnes were used in the USA in 1977 (Baxter, 1979). In 1986, 1990, 1994, and 1998, between 10 and 50 million pounds [4500–22 700 tonnes] were produced annually in the USA. However, production fell to between 1 and 10 million pounds [450–4500 tonnes] in 2002 (NCBI, 2020). In 2012, 2013, and 2014 only two companies reported the use of crotonaldehyde to the US EPA, and each reported producing less than a million pounds [less than 450 tonnes] in each of those years (US EPA, 2020a).

In 2020, there were two major producers of crotonaldehyde in the USA, one in Germany, and another in western Europe (Market Watch, 2020; NCBI, 2020; US EPA, 2020a). The major producer and user of crotonaldehyde is currently China

(ResearchMoz, 2020), although production and use is growing in India. The global crotonaldehyde market was valued at US $244 million in 2019.

Crotonaldehyde was on the Pollutant Release and Transfer Register (PRTR) of Canada with a threshold use of 10 000 kg/year (no facilities reported), until it was removed in 2018 (Government of Canada, 2019). It remains on the National Pollutant Release Inventory (NPRI) of Japan with a threshold use of 1000 kg/year (three facilities), and the USA Toxics Release Inventory (TRI) with a threshold manufacturing or processing use of 11 340 kg/year or other use of 4536 kg/year (seven facilities) at the time of reporting (OECD, 2014).

1.2.3 Uses

Crotonaldehyde is a reactive chemical, with an aldehyde functional group that is conjugated to the olefinic double bond, and is a reducing agent. These characteristics make crotonaldehyde particularly versatile and useful for synthesizing other chemicals for diverse industries. The main use of crotonaldehyde in the past was in the manufacture of *n*-butanol (Baxter, 1979). In 1964, 88% of crotonaldehyde was used for the synthesis of *n*-butanol, 10% for *n*-butyraldehyde, and 2% for crotonic acid and sorbic acid (NCBI, 2020). Its predominant use today is as an intermediate in organic chemical synthesis and in the production of sorbic acid and intermediates such as crotonic acid (Blau et al., 1987), crotyl alcohol, *n*-butanal, as well as *n*-butanol (Celanese Corporation, 2011; Blumenstein et al., 2015). The primary industries that use crotonaldehyde as an intermediate are pharmaceuticals, rubber, chemicals, leather, food, and agriculture (Coherent Market Insights, 2020).

Crotonaldehyde is used in the synthesis of sorbic acid, a food preservative, and vitamin E (Blumenstein et al., 2015). Crotonaldehyde reacts with urea to form crotonylidene ureas, which are slow-release fertilizers, and is also used to make pesticides (Celanese Corporation, 2011).

Crotonaldehyde is an intermediate in the synthesis of chemicals including quinaldines, thiophenes, pyridines, and 3-methoxybutanol, which is a speciality solvent used in lacquers and varnishes to control viscosity, drying behaviour, and gloss. Crotonaldehyde can also be used to control polymerization. Other products include pharmaceuticals, resins, paints and coatings, dyestuffs, rubbers, adhesives, and chemicals used to tan leather (Blumenstein et al., 2015).

The *E*-isomer of crotonaldehyde is listed by the US EPA among the chemicals associated with hydraulic fracturing (US EPA, 2020b). Owing to its pungent odour and strong lacrimating properties, crotonaldehyde is also used as a warning agent in fuel gases and for locating breaks and leaks in pipes (Budavari, 1989) as well as in the purification of lubricating oils (NCBI, 2020). It can be used as a solvent for vegetable and mineral oils, fats, waxes, resins, and polyvinyl chloride (Celanese Corporation, 2011; NCBI, 2020).

1.3 Methods of detection and quantification

Methods for the detection and quantification of crotonaldehyde have evolved steadily since the agent was last evaluated by the *IARC Monographs* programme in Volume 63 (IARC, 1995). Techniques are now available to measure crotonaldehyde in air, water, foodstuffs, and biological specimens. Other methodologies estimate human exposure via metabolites, and both protein and DNA adducts. Table 1.1 summarizes these methods by sample matrix and indicates sample requirements and sensitivity parameters. These techniques are sufficiently sensitive to measure concentrations reliably in ambient air, water, food, and in human biological specimens, and can distinguish background levels from higher exposures (e.g. to combustion

Table 1.1 Representative methods for the detection and quantification of crotonaldehyde, its metabolites, and its DNA adducts

Sample matrix	Sample collection/preparation	Assay procedure	Limit of detection (unless otherwise stated)	Reference
Crotonaldehyde				
Air	DNPH-impregnated XAD and glass fibre filters; sampling rate, 30 mL/min; exposure time, 8 h	GC-FID and GC-ECD after sampling with immediate derivatization	FID, 2–20 µg/m³ ECD, 0.2–4 µg/m³	Otson et al. (1993)
Urban outdoor and indoor air	DNPH-silica sorbent tubes and US EPA Method TO-5 DNPH solution in midget impinger; sampling rate, ~0.5 L/min; sampling time, 2–3 h; maximum sample volume, 80 L	HPLC-UV after derivatization with DNPH	1.59 µg/m³ (US EPA Method TO-5) 1.02 µg/m³ (cartridge)	Williams et al. (1996)
Air	DNPH-impregnated silica gel, sampling rate 300 mL/min, sampling time, 1 h	Electrochromatography	0.26 mg/L [0.26 g/m³]	Fung & Long (2001)
Air	Passive button sampler; silica gel impregnated with DNPH, sampling time, 7 days	HPLC-UV – RP C18 method after DNPH derivatization	Calibration curve range, 0.05–10 ppm [0.14–29 mg/m³]	Liu et al. (2001)
Air in cigarette smoking chamber	DNPH-coated silica gel, flow rate, 200 mL/min; thermal desorption tubes, flow rate, 60 mL/min; sampling time, 4 h	HPLC-DAD-UV	LOQ, 0.62 µg/m³	Liu et al. (2017)
Air	DNPH-coated C_{18} cartridges; flow rate, 0.55–0.77 L/min; sampling times, 4, 5, 8, and 11 h; sampling volumes, 0.14–0.37 m³	HPLC-UV-visible detector	26 ng/sample; LOQ in ambient air, 0.06 ppb [0.17 µg/m³]	Grosjean et al. (1996)
Air	Tedlar bags and carbox tubes (thermally desorbed); flow rate, 100 mL/min; sampling time, 2 min	GC/MS	0.079 ng/sample; 0.02 ppb [0.080 µg/m³]	Ahn et al. (2014)
Air vapours and particulate	DNPH-coated diffusion cell and DNPH-coated filter in line; flow rate, 1.0 L/min; sampling time, 60 min	GC-TSD GC-MS GC-ECD	1.03 ng/mL [1.03 g/m³] 0.53 ng/mL [0.53 g/m³] 0.006 ng/mL [0.006 g/m³]	Dugheri et al. (2019)
Automobile exhaust	Two impingers connected in series; flow rate, 0.5 L/min	GC-ECD	LOQ, 0.15 µg in 2 mL of absorption solution	Nishikawa et al. (1987)
Water	PFBOA derivatization/hexane extraction	GC-ECD GC-MS-SIM	1.2 µg/L 11.2 µg/L	Glaze et al. (1989)
Food products	Oil sample, 5 g; headspace PFPH derivatization/solvent extraction DNPH derivatization/solvent extraction	Isotopic dilution/GC-MS Isotopic dilution/GC-MS Isotopic dilution/HPLC-MS/MS	3 µg/kg; LOQ, 9 µg/kg 2 µg/kg; LOQ, 6 µg/kg 1.5 µg/kg; LOQ, 4.5 µg/kg	Granvogl (2014)
Food products: fried clams	Lipid/oil sample, 1 g; solvent extraction of food product DNPH derivatization	HPLC-MS/MS	75 nM; LOQ, 300 nM	Liu et al. (2020)
Food products: oils	Oil sample: 10 mg; DNPH derivatization/SPE	HPLC-MS/MS	2.5 ng/mL; LOQ, 8 ng/mL	Suh et al. (2017)

Table 1.1 (continued)

Sample matrix	Sample collection/preparation	Assay procedure	Limit of detection (unless otherwise stated)	Reference
Food products: liquors	Beverage sample: 4.0 mL; DNPH derivatization	HPLC-UV-visible PDA	10–50 µg/L	Nascimento et al. (1997)
Human serum	Serum separation: 3000 rpm for 10 min; sample volume, 250 µL; acidification with 330 µL of 0.1 M HCl/SPME/ headspace	GC-MS	0.147 µg/L; LOQ, 0.147 µg/L	Silva et al. (2018)
Human serum	Sample volume, 100 µL; fluorescent derivatization	HPLC-PO-CL	~4–6 nmol/injection	Ali et al. (2014)
Metabolites				
Human urine: metabolite HMPMA	Sample volume in assay 50 µL urine, diluted 1:10 with buffer (50 µL undiluted urine + 25 µL working mixed internal standard + 425 µL 15 mM ammonium acetate, pH 6.8)	UPLC-ESI-MS/MS	2.00 ng/mL	Alwis et al. (2012)
Human urine: metabolite HMPMA	Sample volume, 400 µL; 96-well plate/SPE	HPLC-MS/MS	0.82 ng/mL	Carmella et al. (2013)
DNA adducts				
Human oral tissue: DNA adduct CdG	Gingival tissue or buccal mucosa samples, 50–300 mg each; DNA extraction; ^{32}P-postlabelling	HPLC	LLR, 0.026 µmol/mol CdG	Nath et al. (1998)
Human saliva: DNA adduct CdG	Sample volume, 3 mL; DNA sample, 25 µg; SPE	HPLC-NSI-MS/MS	LOQ, 0.5 pg	Chen & Lin (2011)

CdG, crotonaldehyde-derived 1,N^2-propanodeoxyguanosine; DAD, diode array detector; DNPH, 2,4-dinitrophenylhydrazine; ECD, electron-capture detector; ESI, electrospray ionization; FID, flame-ionization detector; GC, gas chromatography; h, hour; HCl, hydrochloric acid; HPLC, high-performance liquid chromatography; HMPMA, *N*-acetyl-*S*-(3-hydroxy-1-methylpropyl)-L-cysteine; ICP, inductively coupled plasma; LLR, lowest level recorded; LOQ, limit of quantification; MS, mass spectrometry; min, minute; NSI, nanospray ionization; PDA, photodiode array detector; PFBOA, O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine; PFPH, pentafluorophenylhydrazine; PO-CL, peroxyoxalate chemiluminescence; ppb, parts per billion; ppm, parts per million; RP, reversed phase; SIM, selected ion monitoring; SPE, solid-phase extraction; SPME, solid-phase microextraction; TSD, thermionic specific detector; UPLC, ultra high-performance liquid chromatography; UV, ultraviolet.

products, crotonadehyde-containing foodstuffs, or in occupational settings). However, sensitivity varies across methods, and not all methods – including National Institute for Occupational Safety and Health (NIOSH) method 3516 (NIOSH, 1994) – have sufficient sensitivity to measure air levels in occupational settings, i.e. at the current American Conference of Governmental Industrial Hygienists (ACGIH) recommended a threshold limit value (TLV) of 0.3 ppm [0.86 mg/m^3] (ACGIH, 2020).

1.3.1 Air

Air sampling for the measurement of crotonaldehyde concentrations was most often done by drawing air through impingers or midget bubblers (NIOSH, 1994; Zervas et al., 2002; Zhang et al., 2019a). A significant advance has been the quantitative chemical trapping of crotonaldehyde for the analysis of the corresponding hydrazone by high-performance liquid chromatography (HPLC) (Zhang & Smith, 1999) or gas chromatography (GC) (Otson et al., 1993). 2,4-Dinitrophenylhydrazine (DNPH) can be used in the impinger collection fluid (Zervas et al., 2002) or dried upon glass-fibre filters (OSHA, 1990), passive samplers (Otson et al., 1993), silica gel (Zhang & Smith, 1999; Fung & Long, 2001; Mitova et al., 2016), or octadecane sampling cartridges (Grosjean et al., 1996). Prepared DNPH tubes are available commercially (Williams et al., 1996; Liu et al., 2017). Ahn et al. (2014) used both polyester aluminium film sampling bags and sorbent tubes packed with carbon black to collect the air above fried fish to measure crotonaldehyde levels. Air from the bag was directly analysed by gas chromatography-mass spectrometry (GC-MS) while the crotonaldehyde on sorbent tubes was thermally desorbed before injection. Various forms of GC-MS and liquid chromatography-mass spectrometry (LC-MS) have been employed to detect crotonaldehyde from air samples collected as described above.

1.3.2 Water

Methods for the analysis of crotonaldehyde in water have involved derivatization of samples with pentafluorobenzyl hydroxylamine before hexane extraction and injection into GC equipped with a ^{63}Ni electron-capture detector (ECD) or GC-MS (Glaze et al., 1989). Wang et al. (2009) used DNPH derivatization before HPLC but did not detect crotonaldehyde in any rainwater samples tested (other aldehydes were detected). Baños & Silva (2009) evaluated six solid-phase extraction systems for the analysis of aldehydes in water. They described a continuous DNPH derivatization and pre-concentration step before analysis with LC-MS/MS. However, they found no crotonaldehyde in several samples of swimming pool water in which other aldehydes were detected.

1.3.3 Soil

No data were available to the Working Group.

1.3.4 Food, beverages, and consumer products

Methods for the analysis of crotonaldehyde in food are similar to those used for air, with the exception that crotonaldehyde must either first be extracted from the food matrix, or the headspace above the matrix must be sampled. Granvogl (2014) reported on three different methods involving isotopic dilution that gave good agreement and similar limits of detection and quantification in heat-processed fats and oils, and fried food. In the first method, headspace was sampled directly into the GC-MS. The second method involved derivatization with pentafluorophenylhydrazine, followed by extraction and injection into the GC-MS. The third method involved derivatization with

DNPH, followed by extraction and injection into a HLPC-MS system. Because of ease of application, the first method was used for the analysis of food products. The latter two methods were more sensitive than the first method but involved pre-analytical steps.

1.3.5 Biological specimens

Several methods are available for the direct analysis of crotonaldehyde in saliva, urine, and serum, as well as for the analysis of crotonaldehyde metabolites or DNA and protein adducts (Table 1.1). The urinary biomarker *N*-acetyl-*S*-(3-hydroxy-1-methylpropyl)-L-cysteine (HMPMA; 3-hydroxy-1-methylpropylmercapturic acid) was commonly analysed. *N*-Acetyl-*S*-(3-carboxy-1-methylpropyl)-L-cysteine (CMEMA; 2-carboxy-1-methylethylmercapturic acid) has also been analysed. Both can be detected using LC-MS/MS methods.

No data on a validated biomarker for crotonaldehyde were available to the Working Group.

1.4 Occurrence and exposure

1.4.1 Environmental and natural occurrence

Crotonaldehyde occurs naturally in a ubiquitous fashion. It is produced endogenously by plants and animals (including humans) as part of lipid peroxidation and metabolism (WHO; IPCS; IOMC, 2008). Crotonaldehyde has been measured in gases emitted by volcanoes (Graedel et al., 1986), but has also been detected as a biogenic emission from pine trees (0.19 μg/m^3) and deciduous forests (0.49 μg/m^3) (Ciccioli et al., 1993). Scotter et al. (2005) measured levels of crotonaldehyde in the headspace of fungal cultures. Detectable crotonaldehyde levels (mean, 0.106 μg/m^3; standard deviation, SD, 0.005 μg/m^3) were found in the air of a room when people were exercising, but not when they were resting (< 0.0636 μg/m^3) (Mitova et al., 2020).

Crotonaldehyde is found in burned and unburned tobacco (Bagchi et al., 2018), as a combustion product of burning wood and plastic, cooking fires (3.8–91.6 mg/kg fuel), and automobile exhaust (0.07–1.35 ppm [0.20–3.87 mg/m^3], depending on engine size and operating conditions) and diesel exhaust (15–27 mg/kWh energy produced, depending on fuel type and operating conditions) (Nishikawa et al., 1987; Zhang & Smith, 1999; Song et al., 2010).

An overview of exposure measurements of crotonaldehyde in outdoor air, indoor air and dust, and water is provided in Table 1.2.

1.4.2 Exposure in the general population

Important sources of exposure to crotonaldehyde in the general population include tobacco and tobacco-related products, indoor and outdoor air, food, and beverages. Table 1.3 presents concentrations of crotonaldehyde in cigarettes, engine emissions, and other sources. Table 1.4 presents data on levels of crotonaldehyde biomarkers in humans. including from studies of known exposures (e.g. to tobacco products) and from studies in which the exposure source was not characterized (e.g. in children). Studies on DNA adducts in humans (e.g. Chen & Lin, 2009) are further addressed in Section 4.2.1. Table 1.5 presents crotonaldehyde concentrations measured in food and beverages.

(a) Tobacco products and tobacco-related products

See Table 1.3 and Table 1.4.

Cigarette smoke is a major source of exposure to crotonaldehyde (Counts et al., 2004; Carmella et al., 2009). The amount of crotonaldehyde measured per cigarette varies widely depending on the source of the tobacco and the sampling protocol (Hammond & O'Connor, 2008; see Table 1.3). The mean concentration of

Table 1.2 Concentrations of crotonaldehyde in outdoor air, indoor air and dust, and water

Sample	Concentration (μg/m³) mean ± SD unless specified otherwise	Country or region	Reference
Volatile emissions from Chinese arborvitae *Thuja orientalis*	Identified but not quantified	USSR	Isidorov et al. (1985)
Air of forest areas of northern and southern Europe and a remote site in the Himalaya region	0.19 0.49 3.32 1.41 2.19 0.24	Storkow (Germany) Castel Porziano (Italy) K2-A (Nepal) K2-B (Nepal) K2-C1 (Nepal) K2-C2 (Nepal)	Ciccioli et al. (1993)
Ambient air	Annual average, 13.1 (range, 0.8–151.2)	Eastern Himalaya, India	Sarkar et al. (2017)
Ambient air	Range, 0.009–0.112	Northern California, USA	Seaman et al. (2006)
Ambient air	0.30 ± 0.10 ppb [0.86 ± 0.29]	Los Angeles, USA	Grosjean et al. (1996)
Ambient air, downtown	0.05 ppb [0.14]	Porto Alegre, Brazil	Grosjean et al. (1999)
Air outside urban residences: Spring Summer Fall Winter	 0.20 ± 0.07 0.51 ± 0.17 0.32 ± 0.19 0.44 ± 0.36	New Jersey, USA	Liu et al. (2006)
Air adjacent to a six-lane level roadway	2.17–3.71% of total aldehydes excluding acetone	Raleigh, NC, USA	Zweidinger et al. (1988)
Ambient air at the Oakland–San Francisco Bay Bridge toll plaza (occupational exposure)	Morning: 0.147 ± 0.004 Afternoon: 0.093 ± 0.002	San Francisco, USA	Destaillats et al. (2002)
Polluted air	442 ± 22.2 ppb [1.29 ± 0.064]	Osaka, Japan	Kuwata et al. (1979)
Urban roadside sites	 3.5 ± 2.6 1.4 ± 0.4 1.6 ± 0.8 ND	London, UK Ealing, residential Ealing, commercial Wood Green, residential Wood Green, commercial	Williams et al. (1996)
Air samples in rooms with: no people 3 persons (morning) 3 persons, no prior air purge (afternoon) 3 persons, prior air purge (afternoon)	 < 0.0636 < 0.0636 < 0.0636 < 0.0636	Neuchatel, Switzerland	Mitova et al. (2020)
Indoor air samples from 234 homes Personal exposure concentrations	0.7 1.3	Elizabeth, New Jersey, Houston, Texas, and Los Angeles County, California, USA	Liu et al. (2007)

Table 1.2 (continued)

Sample	Concentration (μg/m³) mean ± SD unless specified otherwise	Country or region	Reference
Indoor dust from 389 children's bedrooms	Quantified in 80% 0.9 μg/g (range, 0.01–10 μg/kg)	Värmland, Sweden	Nilsson et al. (2005)
Hospital indoor and outdoor air	0.16 (range, ND–0.37)	Guangzhou, China	Lü et al. (2010)
Indoor air risk-assessment demonstration for analytical laboratories	0.00835	Kanpur, India	Dhada et al. (2016)
Indoor air subjected to heated tobacco products Indoor air subjected to cigarettes	Median, < 0.182 Median, 2.04	Neuchatel, Switzerland	Mitova et al. (2016)
Train carriage air	Range, 2.6–3.6	Hangzhou, China	Lu & Zhu (2007)
Air in a closed room (27 m³) during burning of 5 kg of polypropylene	1.1 ppm [3200]	Borehamwood, Herts, England	Woolley (1982)
Volatile emissions from burning wood (cedar, red oak, and green ash) in a fireplace	[0.043 g/kg] (range, ND–0.116 g/kg)	Warren, Michigan, USA	Lipari et al. (1984)
Colours and chemicals production plant (occupational exposure)	General area: ND–3200 Personal samples: 1900–2100	East Hanover, NJ, USA	NIOSH (1982)
Diesel-fuelled automobile exhaust	0.01 ppm [290]	Warren, Michigan, USA	Lipari & Swarin (1982)
Automobile exhaust gas at different engine speeds	0.51 ppm [1475; range, 200–3870]	Gifu, Japan	Nishikawa et al. (1987)
Vapours emitted from polyurethane foam	Crotonaldehyde identified	Ottawa, Canada	Krzymien (1989)
Exhaust from a compressed natural gas heavy-duty engine Exhaust from a diesel engine	0.12 mg/kWh 0.42 mg/kWh	Naples, Italy	Gambino et al. (1993)
Emissions from polyethylene resin samples in a 30 000 m³ applications area during extrusion operations	Area: < 0.02 to < 0.05 ppm [< 60 to < 140] Personal: < 0.03 to < 0.05 ppm [< 90 to < 140]	Calgary, Canada	Tikuisis et al. (1995)
Emissions from a two-stroke chain saw engine using ethanol and ethanol-blended gasoline	0.012–0.063 g/kWh	Umeå, Sweden	Magnusson et al. (2002)
Industrial emission sources from 77 companies	8.66 ± 27.7 ppb [24.9 ± 79.5]	An-San and Si-Hung city, Republic of Korea	Kim et al. (2008)
Polyester-manufacturing plant, wastewater	5.64 mg/L, estimated	Brazil	Caffaro-Filho et al. (2010)
Ship diesel engine emissions: standard diesel fuel heavy fuel oil	 18 ± 4 μg/MJ 43 ± 13 μg/MJ	Rostock, Germany	Reda et al. (2014)
Air in the process chimney of a waste-treatment plant	8 ± 3 (range, 3–14)	Barcelona, Spain	Gallego et al. (2016)

ND, not detected; ppb, parts per billion; ppm, parts per million; SD, standard deviation.

Table 1.3 Concentrations of crotonaldehyde in cigarettes, engine emissions, and other sources

Source	No. of samples	Concentration		Country or region	Reference
		Average concentration (unless otherwise stated)	Range (unless otherwise stated)		
Cigarettes and related exposures					
Breathing-zone concentrations of cigarette smoke in garages (occupational exposure):				USA	Zhang et al. (2003)
smokers in garages	22	0.96± 0.94 mg/m^3	NR		
non-smokers in garages	31	0.53± 0.79 mg/m^3			
smokers control	11	0.29± 0.48 mg/m^3			
non-smokers control	22	0.25± 0.34 mg/m^3			
Mainstream cigarette smoke (ISO machine-smoking regimen)	5 studies 9 samples	13.9 µg/cig 9.8 µg/cig	95% CI, 11.1–16.6 95% CI, 6.1–13.5	Richmond, USA	Counts et al. (2004)
Mainstream smoke of "light" cigarettes (modified ISO machine-smoking regimen)	7	33% reduction	26–47% reduction	Canada	Gendreau & Vitaro (2005)
Smokeless tobacco:				Indianapolis, Dallas, Austin, Minneapolis, USA, and Sweden	Stepanov et al. (2008)
traditional products	5	2.98 µg/g dry weight	0.98–6.35 µg/g dry weight		
new products	12	9.12 µg/g dry weight	0.55–19.4 µg/g dry weight		
Mainstream cigarette smoke:	NR	Total, 12.5 ± 1.8 µg/cig	NR	Kentucky, USA	Eschner et al. (2011)
ISO machine-smoking regimen		Vapour phase, 8 ± 1 µg/cig			
"Canadian Intense" machine-smoking protocol		Vapour phase, 49 ± 7 µg/cig			
Mainstream cigarette smoke ("Canadian Intense" machine-smoking protocol)	95	55.4 µg/cig (n = 61)	35.4–75.1 µg/cig (n = 95)	USA	Bodnar et al. (2012)
Mainstream cigarette smoke:				Bayreuth, Germany (cigarette brands sold worldwide)	Eldridge et al. (2015)
ISO machine-smoking regimen	39	4.8–12.1 µg/cig[a]	SD, 0.7–1.5		
"Canadian Intense" machine-smoking protocol	40	37.9–47.1 µg/cig[a]	SD, 3.5–4.3		
Mainstream smoke of cigarettes (ISO machine-smoking regimen)	148	1.9–20.5 µg/cig [a]	NR	19 different laboratories	ISO (2018)
Mainstream smoke of:				Greece and USA	Farsalinos et al. (2018)
regular heated tobacco product	5	1.4–3.0 µg/stick[b]	SD, 0.4–0.7		
menthol heated tobacco product	5	1.9–3.3 µg/stick[b]	SD, 0.2–0.9		
e-cigarettes	5	ND µg/12 puffs	ND		
regular cigarettes	5	40.5–65.7 µg/cig[b]	SD, 8.6–14.6		

Table 1.3 (continued)

Source	No. of samples	Concentration		Country or region	Reference
		Average concentration (unless otherwise stated)	Range (unless otherwise stated)		
Mainstream smoke of:				Various locations	Cai et al. (2019)
experimental cigarettes	48	19.79 µg/cig	NR		
cigarettes from Chinese market	163	42–67 µg/cig	NR		
Heated tobacco products:				France	Cancelada et al. (2019)
mainstream smoke	9	4.9–5.2 µg/heatstick[c]	SD, 0.5–0.6		
sidestream smoke	9	0.3–0.4 µg/heatstick[c]	SD, 0.2		
Tobacco heatstick	32	< 3.0 µg/stick	NR	Sigmaringen, Germany	Mallock et al. (2018)
E-cigarette refill solutions	45	[0.19 µg/mL]	ND–0.75 µg/mL[c]	Republic of Korea	Lee et al. (2020)
	30	[0.26 µg/mL]	ND–1.35 µg/mL[c]	USA	
	15	ND	ND	Japan	
Engine exhaust					
Exhaust from a one-cylinder diesel research engine	3	0.04 [0.11] ± 0.088 [± 0.25] ppm [mg/m^3]	NR	Waukesha, Wisconsin	Creech et al. (1982)
Jet engine exhaust	7	0.009 [0.03] ppm [mg/m^3]	0–0.051 [0–0.15] ppm [mg/m^3]	Tokyo, Japan	Miyamoto (1986)
Other sources					
Barbecue charcoal combustion	4	42.5 [122] ppb [µg/m^3]	11.5–121 [33.0–347] ppb [µg/m^3]	Republic of Korea, Indonesia, China, Malaysia	Kabir et al. (2010)
Steel protective paints (polyvinyl butyral)	1	6 mg/m^3		Turku, Finland	Henriks-Eckerman et al. (1990)
Aspergillus flavus	5	Very low, < 75 cps		Christchurch, New Zealand	Scotter et al. (2005)
Aspergillus fumigatus	5	Very low, < 75 cps			
Candida albicans	5	NR	Low, 0 to < 300 cps		
Cryptococcus neoformans	3	NR	Low, 0 to < 1000 cps		
Fusarium solani	3	NR	Low, 0 to < 1000 cps		
Mucor racemosus	3	NR	Low/moderate, < 75 to < 300 cps		

Table 1.3 (continued)

Source	No. of samples	Concentration		Country or region	Reference
		Average concentration (unless otherwise stated)	Range (unless otherwise stated)		
Scented candles:		Burning (before lighting):		Republic of Korea, USA, and China	Ahn et al. (2015)
clean cotton	2	0.15 (0.15) [0.43 (0.43)] ppb[μg/m^3]			
floral	2	0.15 (4.72) [0.43 (13.5)] ppb[μg/m^3]			
kiwi melon	2	0.15 (0.15) [0.43 (0.43)] ppb[μg/m^3]			
strawberry	2	109 (1.2) [313 (3.44)]			
vanilla	2	0.15 (0.15) [0.43 (0.43)] ppb[μg/m^3]			
plain	2	8.54 (2.2) [24.5 (6.31)] ppb[μg/m^3]			

cig, cigarette; cps, counts per second; e-cigarette, electronic cigarette; ND, not detected; NR, not reported; ppb, parts per billion; ppm, parts per million; SD, standard deviation.

[a] Range of means.

[b] Range of means of three different puffing regimens.

[c] Range of means of different products.

Table 1.4 Levels of crotonaldehyde biomarkers in humans

Sample and source	Biomarker	No. of samples	Average concentration (unless otherwise stated)	Range (unless otherwise stated)	Country or region	Reference
Urine after smoking: cellulose acetate filter-tipped charcoal filter-tipped cigarettes	HMPMA	 20 19	 6220 µg/24 h 5152µg/24 h	 SD, 3063 SD, 2517	Munich, Germany	Scherer et al. (2006)
Urine of: e-cigarette smokers conventional cigarette smokers non-smokers (stopped)	HMPMA	 60 20 20	 750 µg/24 h 2320 µg/24 h 299 µg/24 h	 SD, 466 SD, 1405 SD, 166	Richmond, USA	Frost-Pineda et al. (2008)
Urine of smokers, 3–56 days after stopping smoking	HMPMA	17	242–331 nmol/24 h	SD, 83–153	Minneapolis, USA	Carmella et al. (2009)
Urine of Chinese non-smoking women who regularly cook at home	HMPMA	54	1158 pmol/mg creatinine	NR	Singapore	Hecht et al. (2010)
Urine of smokers who: developed lung cancer did not develop lung cancer	HMPMA	 343 392	 GM, 7915 pmol/mg creatinine GM, 5749 pmol/mg creatinine	 95% CI, 6906–9071 95% CI, 5022–6581	Shanghai, China	Yuan et al. (2012)
Urine from cigarette smokers	HMPMA	2613	3302 pmol/mL	SD, 3341	Minnesota, south California and Hawaii, USA	Carmella et al. (2013)
Urine of non-smokers, who: developed lung cancer did not develop lung cancer	HMPMA	 80 82	 GM, 1750 pmol/mg creatinine GM, 1714 pmol/mg creatinine	 95% CI, 1425–2150 95% CI, 1384–2123	Shanghai, China	Yuan et al. (2014)
Urine of never-smoking Chinese women who regularly cook at home: ≤ ×1/week ≥ ×7/week	HMPMA	 90 95	 GM, 894 pmol/mg creatinine GM, 1167 pmol/mg creatinine	 IQR, 749–1067 IQR, 1022–1332	Singapore	Hecht et al. (2015)
Urine of adults aged ≥ 20 yr: male non-smokers male smokers female non-smokers female smokers	HMPMA	 1244 (all men) 1084 (all women)	 GM, 485 ng/mL GM, 848 ng/mL GM, 488 ng/mL GM, 1162 ng/mL	 95% CI, 436–540 95% CI, 706–1017 95% CI, 433–549 95% CI, 993–1360	USA	Jain (2015b)

Table 1.4 (continued)

Sample and source	Biomarker	No. of samples	Average concentration (unless otherwise stated)	Range (unless otherwise stated)	Country or region	Reference
Urine of cigarette smokers:	HMPMA				Minnesota, southern California and Hawaii, USA	Park et al. (2015)
African American		361	Median, 2948 pmol/mL	IQR, 1418–5194		
Native Hawaiian		329	Median, 2766 pmol/mL	IQR, 1473–4493		
White		440	Median, 2535 pmol/mL	IQR, 1423–4492		
Latino		452	Median, 1986 pmol/mL	IQR, 1079–3602		
Japanese American		702	Median, 2134 pmol/mL	IQR, 1037–3507		
Urine of pregnant women with no smoke exposure or some smoke exposure	HMPMA	362 + 93	Median, 342 ng/mL	NR–17 700 ng/mL	New York, North Carolina, Utah, California, Pennsylvania, Wisconsin	Boyle et al. (2016)
Urine of cigarette smokers who switched to e-cigarettes:	HMPMA	20			Silesia, Poland	Goniewicz et al. (2017)
after 1 week			632 µg/g creatinine	IQR, 312–856		
after 2 weeks			616 µg/g creatinine	IQR, 331–706		
Urine of:	HMPMA				USA	Bagchi et al. (2018)
users of combusted tobacco		867	Median, 1.63 mg/g creatinine	IQR, 0.68–3.29		
non-users		3825	Median, 0.313 mg/g creatinine	IQR, 0.23–0.45		
Urine of adolescents:	HMPMA				San Francisco, USA	Rubinstein et al. (2018)
e-cigarette smokers		67	Median, 149 ng/mg creatinine	0–793		
e-cigarette & tobacco smokers		16	Median, 185 ng/mg creatinine	110–438		
non-smokers		20	Median, 100 ng/mg creatinine	0–522		
Urine after consumption of broccoli-sprout beverages	HMPMA	48	Median, 0.481–0.486 nmol/mg creatinine	IQR, 0.319–0.721 (SFR) IQR, 0.312–0.904 (GRR)	Qidong, China	Kensler et al. (2012)
Urine after consumption of:	HMPMA				Qidong, China	Egner et al. (2014)
broccoli-sprout beverages		137	GM, 1312 pmol/mg creatinine	IQR, 829–1790		
placebo		130	GM, 1510 pmol/mg creatinine	IQR, 880–1959		
Salivary DNA	CdG	27	7.5 adducts/10^8 nucleotides	SD, 12	Ming-Hsiung, Taiwan, China	Chen & Lin (2011)
Urine of children aged 6–11 yr:	HMPMA				USA	Jain (2015a)
males		203	GM, 338 ng/mL	95% CI, 298–382		
females		214	GM, 311 ng/mL	95% CI, 276–351		

CI, confidence interval; e-cigarette, electronic cigarette; GRR, glucoraphanin-rich; h, hour; HMPMA, *N*-acetyl-*S*-(3-hydroxy-1-methylpropyl)-L-cysteine; IQR, interquartile range; GM, geometric mean; NR, not reported; SD, standard deviation; SFR, sulforaphane-rich.

Table 1.5 Concentrations of crotonaldehyde in food and beverages

Item	No. of samples	Concentration		Country of study or purchase	Reference
		Average concentration	Range		
Rice seeds	12	~10–20 ng/g	~2–5 ng/g	Nanchang, China	Shenzao et al. (2018)
Carrot roots	> 8	0.04–0.1 mg/kg	NR	Piikkiö, Finland	Linko et al. (1978)
Apples, guavas, grapes, strawberries, tomatoes	NR	< 0.01 ppm [mg/kg]	NR	NR	Feron et al. (1991)
Cabbage, carrots, celery leaves, cauliflower, Brussels sprouts		0.02–0.1 ppm [mg/kg]	NR		
Bread, cheese, milk, meat, fish, beer		0–0.04 ppm [mg/kg]	NR		
Wine		0–0.7 [mg/L]	NR		
Heavily salted cod	14	1.02 µM/kg	NR	Canada	Yurkowski & Bordeleau (1965)
Whole-grain soft wheat	1	Detected but not quantified		USA	McWilliams & Mackey (1969)
Heated beef fat	1	Detected but not quantified		Tokyo, Japan	Yamato et al. (1970)
Bottled beer	NR	17 µg/L	NR	Takasaki, Japan	Hashimoto & Eshima (1977)
Beer	3	1.33 ppb [µg/L]	0.77–1.82 ppb [µg/L]	London, UK	Greenhoff & Wheeler (1981)
Scotch whisky (brand x)	4	0.03 ± 0.01 ppm [mg/L]	NR	Oxford, OH, USA	Miller & Danielson (1988)
Scotch whisky (brand y)	4	0.21 ± 0.02 ppm [mg/L]	NR		
Kentucky bourbon	3	0.04± 0.002 ppm [mg/L]	NR		
Vodka	3	< 0.02 (SD, NR)	NR		
Alcoholic beverages	NR	Detected but not quantified		Baltimore, USA	Theruvathu et al. (2005)
Mothers' milk	12	Identified in 1 sample		Bridgeville, PA; Bayonne, NJ; Jersey City, NJ; and Baton Rouge, LA, USA	Pellizzari et al. (1982)
Soymilk, ultra-high pressure homogenized	2	Detected but not quantified		Barcelona, Spain	Poliseli-Scopel et al. (2013)
Fish oil	10	1.0–21.7 (range of averages) µg/g	SD, 0.1–1.0	Lake Alfred, FL, USA	Suh et al. (2017)
Olive oil, extra virgin	3	0.067 ± 0.006 mg/kg	NR	Cordoba, Spain	Garrido-Delgado et al. (2011)
Olive oils, extra virgin	251	Detected but not quantified		Italy	Melucci et al. (2016)

Table 1.5 (continued)

Item	No. of samples	Concentration		Country of study or purchase	Reference
		Average concentration	Range		
Volatile components of raw and stir-fried fruits	7	Detected but not quantified		Chengu, China	Zhong et al. (2015)
Canola oil (180 °C)	2	1.0–1.7 mg/h per L oil	0.1–0.2[a]	Alicante, Spain	Fullana et al. (2004)
Canola oil (240 °C)	2	1.2–2.5 mg/h per L oil	0.1–0.6[a]		
Extra virgin olive oil (180 °C)	2	0.9–2.8 mg/h per L oil	0.1–0.4[a]		
Extra virgin olive oil (240 °C)	2	2.9–5.1 mg/h per L oil	0.1–0.3[a]		
Olive oil (180 °C)	2	0.9–1.9 mg/h per L oil	0.1–0.2[a]		
Olive oil (240 °C)	2	0.8 mg/h per L oil	0.1[a]		
Frying process, clam	18	1.44–2.20 µg/g	SD, 0.02–0.11	Dalian, Liaoning, China	Liu et al. (2020)
Cooking oil fumes of soybean oil, sunflower oil, rapeseed oil, and palm oil when cooking potatoes and pork loin	8	Quantified together with other aldehydes		Taiwan, China	Peng et al. (2017)
Coffea arabica flowers	3	Detected but not quantified		Bucaramanga, Colombia	Stashenko et al. (2013)

NR, not reported; ppb, parts per billion; ppm, parts per million; SD, standard deviation.

[a] Standard error of the mean.

crotonaldehyde in cigarettes from the Chinese market was 42–67 µg/cigarette (Cai et al., 2019). Zhang et al. (2019a) analysed the gas phase of mainstream smoke from 16 different brands of Chinese flue-cured cigarettes and reported an average crotonaldehyde concentration of 13.4 µg/cigarette under the International Organization for Standardization ISO 3308 machine-smoking regimen (35 mL puff volume, 2 second puff duration, 60 second puff interval). Similar concentrations were reported by Ding et al. (2016) and Sampson et al. (2014) when using the ISO regimen, whereas using the "Canadian Intense" protocol (55 mL puff volume, 2 second puff duration, 30 second puff interval) gave values that were 2–5 times higher; however, levels as high as 228 µg/cigarette have been reported previously. Brands originating in the USA appear to contain higher levels of crotonaldehyde, with Ding et al. (2016) reporting average levels of 25–72 [mean, 48] µg/cigarette in 10 USA brands under the "Canadian Intense" protocol. [The Working Group noted that the "Canadian Intense" method may provide higher values that correspond better to human exposure during smoking.] Several research groups have reported lower levels of crotonaldehyde in mainstream smoke when electronic cigarettes were machine-smoked (Farsalinos et al., 2018; Mallock et al., 2018).

Among smokers in the National Health and Nutrition Examination Survey (NHANES) study conducted by the United States Centers for Disease Control and Prevention (CDC), there was an increase in HMPMA concentration with increasing number of cigarettes smoked. Approximately 20% of participants were smokers (Bagchi et al., 2018). The urinary HMPMA concentrations in smokers were about 5 times higher than in non-smokers (1.63 versus 0.313 mg/g creatinine). HMPMA was detected in 99.9% of all urine samples (Bagchi et al., 2018). Median concentrations were 419 and 369 µg/L for the 2011–12 and 2013–14 sampling periods, respectively, while the 95th percentiles were 3700 and 3040 µg/L, respectively (NHANES, 2019). [The Working Group noted that the latter values are likely to include smokers and/or persons with significant occupational exposure.] The lowest concentrations were reported for non-Hispanic Black people (median, 253 µg/g creatinine for non-users and 1070 µg/g creatinine for users of exclusively smoked tobacco products; interquartile range, 195–356 and 489–1870 µg/g creatinine, respectively). These data indicate widespread crotonaldehyde exposure within the population and confirm that tobacco smoke is a major source of exposure (Bagchi et al., 2018; see Table 1.4).

Alwis et al. (2012) analysed urinary HMPMA concentrations in 1203 non-smokers and 347 smokers. They found the average (± SD) concentrations to be 429 µg/L (± 478 µg/L) in non-smokers and 1992 µg/L (± 2009 µg/L) in smokers, a highly significant difference. Carmella et al. (2009) studied HMPMA concentrations in 17 people who quit smoking. They found that concentrations were reduced by 80% when re-sampling occurred on the next return visit after 3 days (allowing an estimate of the maximum possible half-life of 36 hours for HMPMA) and then remained at approximately this level for the next 56 days of follow-up. Scherer et al. (2006) conducted a study of HMPMA comparing regular-filter cigarettes to those with a charcoal filter. HMPMA concentrations in week 1 were lower in smokers using cigarettes with charcoal filters than in smokers using cigarettes with regular filters. However, the difference disappeared when the groups crossed over after 1 week, although the glutathione-depleting activity of smoke passed through the charcoal filters was significantly less than of smoke passed through regular filters.

Park et al. (2015) studied HMPMA in more than 2200 smokers of five ethnicities. They found a significant difference between the ethnic groups, with native Hawaiians having the highest geometric mean concentrations of HMPMA and Latinos the lowest at 2759 and 2210 pmol/mL

urine, respectively. These data strongly suggest an ethnic influence on exposure effect.

Pluym et al. (2015) measured both HMPMA and CMEMA concentrations in three groups: non-smokers, light smokers (≤ 10 cigarettes/day) and heavier smokers (> 10 cigarettes/day). They reported a robust concentration–response relationship for HMPMA but not for CMEMA. Median concentrations in non-smokers were 18.9 (range, 9.7–64.4) µg/g creatinine for HMPMA, and 201 (range, 104–756) µg/g creatinine for CMEMA. These values were 95.9 (range, 55–268) µg/g creatinine and 226 (range, 125–408) µg/g creatinine in light smokers, and 121.7 (range, 57–220) µg/g creatinine and 226 (range, 121–299) µg/g creatinine in heavier smokers. In addition, there was only a weak correlation between HMPMA and CMEMA concentrations, and no correlation between CMEMA and cotinine concentrations.

(b) Indoor air

Indoor cooking can be a source of airborne exposure to crotonaldehyde. Zhang & Smith (1999) studied the emissions from 22 different methods of cooking in China and found that crotonaldehyde production ranged from not detected to 92 mg/kg fuel for wood used in a brick stove with a flue. Relatively large amounts (up to 88 mg/kg fuel; mean, 60 mg/kg fuel) were produced when liquefied petroleum gas was used as fuel while coal and coal briquette fuels produced the lowest levels. Consistent with these data, Weinstein et al. (2020) reported a non-significant 4% reduction in urinary HMPMA concentrations in women in Guatemala when wood-burning stoves were replaced by liquefied petroleum gas-powered stoves (from 193 µg/g creatinine with wood-burning stoves to 186 µg/g creatinine with liquefied petroleum gas). Mitova et al. (2020) found a mean concentration of 2.06 µg/m³ (SD, ± 0.01 µg/m³) in Switzerland where people warmed a cheese dish on an electric hotplate. Ahn et al. (2014) reported that crotonaldehyde concentrations ranged from 4.96 to 51.7 ppb [14.2 to 148 µg/m³] when mackerel were pan-fried using butane as a fuel in the Republic of Korea. Hecht et al. (2015) compared HMPMA concentrations in non-smoking women in Singapore who cooked once per week or less frequently with a wok (including boiling, stir frying, and deep frying) with those who cooked between 2 and 6 times per week and with those who cooked 7 times per week or more frequently. They reported a highly significant trend with increasing wok use, with the groups at either extreme (< 1 meal/week versus > 7 meals/week) having a geometric mean of 894 (95% confidence interval, CI, 749–1067) pmol/mg creatinine versus 1167 (95% CI, 1022–1332) pmol/mg creatinine). There was also an effect of the oil used to cook, with rapeseed oil (829 pmol/mg creatinine) and sunflower oil (1329 pmol/mg creatinine) being the extremes.

Ochs et al. (2016) reported that varnishing a door during apartment renovation was the source of an increase in crotonaldehyde concentrations that peaked at 80 µg/m³ but dissipated rapidly thereafter.

Lu & Zhu (2007) measured crotonaldehyde concentrations aboard six carriages in different trains during the 2004 Spring Festival in China when tens of millions of people used the train system; they reported concentrations of between 2.6 and 3.6 µg/m³.

(c) Outdoor air pollution

Grosjean et al. (1996) reported that concentrations of crotonaldehyde in outdoor air in Los Angeles, California, USA, peaked at about 0.5 ppb [1.4 µg/m³] with an average concentration of 0.3 ppb [0.86 µg/m³]. Concentrations seemed to increase with traffic, consistent with reports of crotonaldehyde in the exhaust of gasoline and diesel engines (Nishikawa et al., 1987; Zervas et al., 2002; Song et al., 2010). Similarly, Dugheri et al. (2019) reported that crotonaldehyde concentrations in four roads with heavy traffic in Florence,

Italy, were 0.8–1.3 μg/m³ (mean, 1.0 μg/m³), while in a low-traffic area, the mean concentration was 0.2 μg/m³. The bulk of the crotonaldehyde was found in the vapour phase.

(d) Food and beverages

See Table 1.4 and Table 1.5.

Crotonaldehyde is present in many foodstuffs, including vegetables (Brussels sprouts, cabbages, carrots, cauliflower, celery leaves; at concentrations of 0.02–0.1 ppm [mg/kg]), fruits (apples, grapes, guavas, tomatoes and strawberries; at > 0.01 ppm [mg/kg]), dairy products and meats (milk, bread, cheese, meat, clams and fish), beer, and wine (at 0–0.07 ppm [mg/kg, mg/L]) (Feron et al., 1991; Liu et al., 2020). Whisky and vodka contain from < 0.02 to 0.21 ppm [mg/L] (Miller & Danielson, 1988). Fruit intake was significantly associated with increased urinary HMPMA levels in the NHANES survey (Bagchi et al., 2018).

Recent data indicated that heated cooking oil is a significant source of exposure to crotonaldehyde in food. In a study conducted in Germany, Granvogl (2014) reported that while cooking oils differ intrinsically due to composition, the amount of crotonaldehyde in each oil increases significantly with temperature (100–180 or 220 °C) and heating time. Concentrations of crotonaldehyde in the oils ranged from below 9 μg/kg in unheated oils to 34 mg/kg [34 000 μg/kg] for linseed oil heated to 180 °C for 24 hours. Foods cooked in these oils also contained crotonaldehyde, albeit at lower concentrations. Both potato chips and doughnuts cooked in rapeseed oil contained twice as much crotonaldehyde as those cooked in olive oil (24.8 and 18.2 μg/kg, and 12.6 and < 9 μg/kg, respectively). Liu et al. (2020) measured crotonaldehyde concentrations in clams before and during deep frying in China. They found that the concentration of crotonaldehyde increased with both oil temperature and cooking time, from 0.04 μg/g for fresh clams to 1.46 μg/g for clams fried at 180 °C for 15 minutes. Crotonaldehyde concentrations in pre-marinated control clams also increased over 15 minutes when they were fried at 160 °C.

(e) Exposures in infants and children

See Table 1.4.

Regarding infants, El-Metwally et al. (2018) compared urinary concentrations of HMPMA in newborns in cribs versus those born pre-term and placed in incubators (median ages, 16 and 11 days, respectively). Median concentrations did not differ (394 μg/L versus 376 μg/L, respectively), suggesting that there were relatively high crotonaldehyde exposures in neonatal intensive care units (compared with children aged 6–11 years, see above). Boyle et al. (2016) studied 488 pregnant women and reported a 50th percentile HMPMA value of 342 μg/L which was virtually identical to the value of 352 μg/L reported by NHANES for girls and women aged 6–11 years, 12–19 years, and ≥ 20 years (NHANES, 2019). Boyle et al. reported that the highest value measured was 17 700 μg/L (5% of their sample were tobacco smokers).

1.4.3 Occupational exposure

One of the largest current commercial uses of crotonaldehyde is in the production of sorbic acid as a food preservative (E200), and crotonic acid (European Commission, 2013). However, no data could be found on workers' exposure during this process. A survey conducted by NIOSH (1983) suggested that fewer than 400 workers (metal-plating machine operators in the transportation equipment industry, and separating, filtering, and clarifying machine operators in the chemicals and allied products industry) had potential exposure to crotonaldehyde in the USA, but no measurements were made. Since that time, it has become appreciated that far more workers are exposed to crotonaldehyde via exposure to pyrolysis products. Therefore, studies have been

performed in cooks, coke-oven workers, and traffic officers, at toll booths, and particularly on firefighters, but have focused on biological monitoring rather than air concentrations.

The *IARC Monographs* programme in its previous evaluation of crotonaldehyde (Volume 63; IARC, 1995) noted that a variety of measurements for the compound were made in 24 Finnish businesses and that all measurements were below the Finnish standard at the time of 6 mg/m³. Linnainmaa et al. (1990) found concentrations of 0.23 mg/m³ near a doughnut-frying station in a Finnish bakery. In a chemical plant in the USA, area samples ranged from not detected to 3.2 mg/m³, with two personal samples of 1.9 and 2.1 mg/m³ (NIOSH, 1982). Crotonaldehyde was detected at concentrations of 1–7 mg/m³ in a plant producing aldehydes in Germany. More recently, Zhang et al. (2003) measured exposure to crotonaldehyde via inhalation in parking-garage workers (n = 53) and controls (n = 33) and reported that smoking parking-garage workers had a mean crotonaldehyde air concentration of 0.96 µg/m³ (SD, ± 0.94 µg/m³) and non-smoking parking-garage workers' mean concentrations were 0.53 µg/m³ (± 0.79 µg/m³). Smoking controls were exposed to crotonaldehyde at 0.29 µg/m³ (± 0.48 µg/m³), and non-smoking controls at 0.25 µg/m³ (± 0.32 µg/m³). Destaillats et al. (2002) measured concentrations at toll booths in San Francisco, USA, and reported concentrations (mean ± SD) of 0.061 ± 0.012 µg/m³ and 0.093 ± 0.002 µg/m³ in the afternoon and 0.147 ± 0.004 µg/m³ in the morning.

For firefighters, Dills et al. (2008) reported crotonaldehyde concentrations as high as 4.3 mg/m³ in the overhaul smoke of a demonstration fire (wood with polyvinyl chloride), when water was used to knock down the fire. In another demonstration-fire study (household materials), Jones et al. (2016) reported concentrations as high as 0.07 ppm [0.2 mg/m³] during the overhaul phase (smouldering) of the exercise. In a third demonstration study, Kirk & Logan (2015) measured concentrations between 1 and 11 µg/m³ off-gassing from a structural firefighting ensemble for 24 hours after four hostile attack evolutions (resin-bonded wood panels).

Frigerio et al. (2020) measured urinary CMEMA and HMPMA concentrations in coke-oven workers, but there was no statistical difference between concentrations in workers and controls, the latter being slightly higher.

1.5 Regulations and guidelines

1.5.1 Exposure limits and guidelines

(a) Occupational exposure limits

Occupational exposure regulations and guidelines for various countries and states are given in Table 1.6. Crotonaldehyde is a potent irritant of the skin, eyes, and mucous membranes throughout the respiratory tract. The ACGIH TLV of 0.86 mg/m³ for crotonaldehyde is based on analogy with formaldehyde as an irritant. The TLV is a ceiling level, i.e. a level that should never be exceeded. Crotonaldehyde is also given a "skin" notation by ACGIH indicating that there are data suggesting that the liquid is well-absorbed through the skin (ACGIH, 2020). Although the TLVs are established to provide professional guidance for practicing industrial hygienists, they have been adopted by many governmental regulatory agencies. The TLV for crotonaldehyde was last updated by the ACGIH in 1998 with the ceiling value being adopted. As can be seen from Table 1.6, values established before 2000 are significantly higher than those promulgated after 2000, the sole exception being United States Occupational Safety and Health Administration (OSHA) and NIOSH. Furthermore, the pre-2000 limits are time-weighted averages as opposed to the ceilings that should never be exceeded for many values set after 2000.

Table 1.6 Occupational exposure limits for crotonaldehyde[a] in various countries

Country or agency	Year	Concentration (mg/m³)	Interpretation	Notation, category
Australia	2018	5.7	TWA	
Austria	2011	1	MAK	
		4	STEL	
Argentina[b]	2019	0.86	Ceiling	Skin A3 Carcinogen
Belgium	2009	0.87	STEL	
Bulgaria[b]	2019	0.86	Ceiling	Skin A3 Carcinogen
Canada – Alberta	2001	5.8	TWA	
Canada – Ontario	2020	0.86	Ceiling	Skin
Canada – Quebec	2020	5.7	TWA	Skin A3 Carcinogen
China	2019	12	MAC	
Columbia[b]	2019	0.86	Ceiling	
Denmark	1999	6	TWA	Skin
European Union – SCOEL	2013			Skin
Finland	2000	0.29	TWA	Skin
		0.87	STEL	
France	2016	6	VLEP	
Germany – MAK	2006			Skin 3B Carcinogen
Ireland	2007	6	TWA	
		18	STEL	
Jordan[b]	2019	0.86	Ceiling	Skin A3 Carcinogen
New Zealand[b]	2019	0.86	Ceiling	Skin A3 Carcinogen
Norway	2013	6	TWA	Skin
Philippines	1993	6	TWA	
Poland	2018	1	TWA	Skin
	2018	2	STEL	
Portugal	2004	0.86	Ceiling	
Republic of Korea[b]	2019	0.86	Ceiling	Skin A3 Carcinogen
Romania	2018	25	STEL	
Singapore[b]	2014	5.7	PEL (long-term)	Skin A3 Carcinogen
Spain	2019	0.87	STEL	Skin
Switzerland	2005	1	MAK-W	Skin
United Kingdom	1993	6	LTEL	
		18	STEL	
USA –ACGIH TLV[c]	2019	0.86	Ceiling	
USA – OSHA PEL	2019	6	TWA	Skin A3 Carcinogen
USA – NIOSH REL	2019	6	TWA	
USA – Connecticut	2011	0.12	Ambient air	
USA – Nevada	2011	0.143	Ambient air	
USA – North Dakota	2011	0.18	Ambient air	

Table 1.6 (continued)

Country or agency	Year	Concentration (mg/m³)	Interpretation	Notation, category
USA – Virginia	2011	0.10	Ambient air	
Viet Nam[b]	2019	0.86	Ceiling	Skin A3 Carcinogen

ACGIH, American Conference of Governmental Industrial Hygienists; LTEL, long-term exposure limit (8 hours); MAC, maximum allowable concentration (ceiling value); MAK, MAK-W, Maximale Arbeitsplatz-Konzentration (maximum workplace concentration), in the workplace air which generally does not have known adverse effects on the health of the employee nor cause unreasonable annoyance even when the person is repeatedly exposed for 8 hours daily assuming on average a 40-hour working week; PEL, permissible exposure limit; PEL (long-term), permissible exposure level over an 8-hour working day and a 40-hour working week; REL, recommended exposure limit; SCOEL, Scientific Committee on Occupational Exposure Limits; STEL, short-term exposure limit, based on a 15 minute average; TLV, threshold limit value, the level to which a worker may be repeatedly exposed, day after day, over a working lifetime without adverse health effects; TWA, 8-hour time-weighted average; VLEP, *Valeur limite d'exposition professionnelle* (8-hour occupational exposure limit value).

[a] Includes *trans*- (*E*-), *cis*- (*Z*-), and a mixture of both.

[b] Use ACGIH TLVs as local regulations.

[c] Based on analogy with formaldehyde.

From ACGIH (2020); Pohanish (2012); European Commission (2013); Finland Ministry of Social Affairs and Health (2018); Ontario Ministry of Labour, Training and Skills Development (2020).

(b) Environmental exposure limits

Crotonaldehyde has not been widely regulated in the environment. As with acrolein and other reactive aldehydes, occupational guidelines for acute exposures (100–300 ppb) [0.29–0.86 mg/m³] are approximately 10 to 100 times the environmental guidelines for acute exposures (1–5 ppb [2.9–14 µg/m³] or for subacute exposures).

In 2008 the National Advisory Committee for Acute Exposure Guideline Levels (AEGLs) for Hazardous Substances of the United States National Academy of Sciences evaluated crotonaldehyde exposure concentrations and times that could be classified as nondisabling (AEGL-1), disabling (AEGL-2), and lethal (AEGL-3) (National Research Council, 2007). These are presented in Table 1.7. Note that AEGL-3, which is lethal, is reached after 10 minutes exposure to crotonaldehyde at 44 000 ppb (44 ppm) [130 mg/m³], whereas exposure for any duration of time from 10 minutes to 8 hours to 190 ppb [0.55 mg/m³] leads to slight eye irritation and discomfort.

1.5.2 Reference values for biological monitoring of exposure

There are currently no regulations or guidelines for measuring levels of crotonaldehyde metabolites or other biomarkers in biological samples. While there have been important studies involving metabolites in smokers, there are very few data related to metabolite concentrations, air concentrations, or effect markers (e.g. DNA adducts and metabolites), which are the parameters needed to provide guidance relevant for occupational exposure. In addition, there remain other data gaps that prevent development of such a biological exposure index. This includes data on metabolite elimination half-life, which is needed to recommend the timing of sample collection (ACGIH, 2020). One alternative for guidance is using "population" reference values based on the 95th percentile levels in the general population (ACGIH, 2020). [The Working Group noted that there appeared to be ample data to establish a population value for crotonaldehyde.]

Table 1.7 Summary of acute exposure guideline levels for crotonaldehyde

Classification	10 minutes	30 minutes	1 hour	4 hours	8 hours	End-point	Reference
Classification AEGL-1a (nondisabling)	0.19 ppm (0.55 mg/m³)	0.19 ppm (0.55 mg/m³)	0.19 ppm (0.55 mg/m³)	0.19 ppm (0.55 mg/m³)	0.19 ppm (0.55 mg/m³)	Mild eye irritation in humans	NIOSH (1982)
AEGL-2 (disabling)	27 ppm (77 mg/m³)	8.9 ppm (26 mg/m³)	4.4 ppm (13 mg/m³)	1.1 ppm (3.2 mg/m³)	0.56 ppm (1.6 mg/m³)	Impaired pulmonary function, NOAEL for bronchiole lesions	Rinehart (1967)
AEGL-3 (lethal)	44 ppm (130 mg/m³)	27 ppm (77 mg/m³)	14 ppm (40 mg/m³)	2.6 ppm (7.4 mg/m³)	1.5 ppm (4.3 mg/m³)	Lethality NOEL	Rinehart (1967)

AEGL, acute exposure guideline levels; NOAEL, no-observed-adverse-effect level; NOEL, no-observed-effect level; ppm, parts per million.
From National Research Council (2007).

1.6 Quality of exposure assessment in key epidemiological studies

Table S1.6 and Table S1.7 (Annex 2, Supplementary material for crotonaldehyde, Section 1, Exposure Characterization, web only; available from: https://publications.iarc.fr/602) provide a detailed overview and critique of the methods used for exposure assessment in cancer epidemiology studies and mechanistic studies in humans that have been included in the evaluation of crotonaldehyde. Only four studies of human cancer were identified: one occupational cohort and three nested case–control studies, two of lung cancer and one of colorectal cancer. The occupational cohort study assigned exposure based on expert evaluation of company records on the use of chemicals and on employment. The two case–control studies on lung cancer were nested within the same general population cohort and assessed exposure by measuring urinary metabolites (HMPMA). The case–control study on colorectal cancer applied an untargeted adductomics approach. The majority of the mechanistic studies in humans can be considered demonstration studies, as noted below.

1.6.1 Quality of exposure assessment in key cancer epidemiology studies

Bittersohl (1975) investigated cancer frequency in an aldehyde factory and assigned exposure to crotonaldehyde based on employment records. Quantitative crotonaldehyde measurements, available for some departments, were not used to quantify exposure intensity or cumulative exposure. Workers were likely to be exposed simultaneously to other chemical agents (e.g. acrolein, see the first monograph in the present volume). The two nested case–control studies of lung cancer (Yuan et al., 2012, 2014) assessed exposure by measuring a urinary metabolite of crotonaldehyde, HMPMA. A single void urine sample was collected from each participant at baseline, and these urine samples were analysed for cases and controls to determine the concentration of HMPMA. Information on smoking was available from a questionnaire, and smokers were studied separately from non-smokers.

The nested case–control study on colorectal cancer applied an untargeted approach to measure Cys34 adducts of albumin to crotonaldehyde in human serum. Serum samples were collected at time of recruitment to the cohort. Information on body mass index and lifestyle factors such as smoking, alcohol drinking, and

meat consumption was collected by questionnaire. The formation of crotonaldehyde adducts was not related to external exposures, such as smoking, and was instead attributed to endogenous production after oxidation of membrane lipids by reactive oxygen species.

1.6.2 Quality of exposure assessment in mechanistic studies in humans

As noted above, the majority of the mechanistic studies can be considered demonstration studies, simply reporting that it is possible to use the technique described to detect the particular biomarker in human samples (Nath & Chung, 1994; Nath et al., 1996, 1998; Zhang et al., 2006; Chen & Lin, 2009, 2011; Garcia et al., 2013; Alamil et al., 2020). Most studies were early validations and are not used to assess carefully the relationship between external exposure and mechanistic end-points. For compounds like crotonaldehyde that have widespread environmental sources, are produced endogenously, and are also present in basic foods and beverages, careful documentation of food, tobacco, and alcohol consumption, and significant exposure to automotive exhaust is required to determine contributions from different exposure sources. This was lacking in several studies. If samples are collected from cases (Grigoryan et al., 2019), the potential exists that the disease itself could cause differences in DNA adduct levels and/or that exposure for the cases may have changed between the time the case was identified and the time that the sample collected. In all these studies, only a single exposure marker was reported at a single point in time, making it difficult or impossible to assess exposure sources and duration, since the marker is then used as an outcome.

2. Cancer in Humans

2.1 Descriptions of individual studies

See Table 2.1.

One cohort study and three nested case–control studies in cohorts have been published on the relationship between cancer and exposure to crotonaldehyde.

Cohort studies

Bittersohl (1975) recorded cancer cases in a small cohort of 220 workers in an aldehyde factory in the former German Democratic Republic who were diagnosed between 1967 and 1972. Workers who left the factory for whatever reason were not included. Measurements in some factory departments showed values of crotonaldehyde of 1–7 mg/m^3. Four different cancer types were observed in nine men (5 cases of squamous cell lung carcinoma, 2 cases of squamous cell carcinoma of the oral cavity, 1 case of adenocarcinoma of the stomach, and 1 case of adenocarcinoma of the colon). Two cases in women (one leukaemia and one cancer of the ovary) were excluded from analysis due to short duration of exposure to aldehydes. There was no formal comparison group; a comparison was made with incidence rates in the general population of the former German Democratic Republic (source not reported). [The Working Group noted that the study design was weak. Not all those ever employed in the factory were included, only those currently employed (possible selection bias), and a small number of cases (9 cases) at four different sites were recorded. Exposure was based on measurements in some unspecified departments and there were multiple undifferentiated exposures experienced by workers. The exposure–disease association was not quantified because comparison rates for the general population were not provided.]

Table 2.1 Epidemiological studies of cancer in humans exposed to crotonaldehyde

Reference, location, enrolment/ follow-up period, study-design	Population size, description, exposure assessment method	Cancer type	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Bittersohl (1975) Former German Democratic Republic 1967–1972 Cohort	220 workers employed in an aldehyde factory operating since 1936; included men presently in the factory Exposure assessment method: quantitative measurements; workers exposed to multiple aldehyde derivatives containing traces of crotonaldehyde; exposure was assumed based on employment within the aldehyde factory, with measurable airborne levels of crotonaldehyde (1–7 mg/m^3)	Lung (squamous cell carcinoma)	Men, NR	5	NR	None	*Exposure assessment critique*: Poorly defined exposure. No attempt to assess exposure (semi-) quantitatively by measurements of duration. No separate exposure assessment for different chemical agents present in the factory, hence it is not possible to separate the effect of different chemical agents. *Strengths*: cancers among workers in the factory were recorded. *Limitations*: small sample size; selection bias, as only presently employed workers were included; relationship with the exposure could not be established; calculation of RR was not possible.
		Oral cavity, incidence	Men, NR	2	NR	None	
		Stomach, incidence	Men, NR	1	NR	None	
		Colon, incidence	Men, NR	1	NR	None	
		Leukaemia, incidence	Women, NR	1	NR	None	
		Ovary, incidence	Women, NR	1	NR	None	

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study-design	Population size, description, exposure assessment method	Cancer type	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Yuan et al. (2012) Shanghai, China Enrolment, 1986–1989; follow-up through 2006 Nested case–control	Cases: 343 cases of incident lung cancer and deaths; current smokers at enrolment, identified through annual in-person interviews and reviewed through Shanghai Cancer Registry and Shanghai Municipal Vital Statistics Office; cohort of 18 244 men aged 45–64 yr at baseline Controls: 392 participants in the Shanghai Cohort Study; one control was selected from the same cohort, current smoker at enrolment, alive and free of cancer and matched to the index case by age (± 2 yr), date of specimen collection (± 1 mo) and neighbourhood of residence at enrolment Exposure assessment method: exposure to crotonaldehyde was determined based on measurement of its urinary metabolite HMPMA; urine samples were collected at baseline survey of the cohort in which the case–control study was nested; smoking information was also collected.	Lung, incidence	Quartile of urinary HMPMA (pmol/mg creatinine) (OR):			Age at baseline, neighbourhood of residence, duration of sample storage, number of cigarettes smoked per day, years of cigarette smoking at baseline	*Exposure assessment critique*: No external exposure assessment. All study subjects were smokers. Smoking history was included as confounder in the analyses. Urine samples were collected at baseline, so clearly preceded the health outcome; however, only one urine sample was collected. Cancer risk was evaluated at increasing metabolite levels. *Other comments*: urinary levels of HMPMA were statistically significantly associated with increased risk of lung cancer; however, after adjustment for cotinine, a biomarker of nicotine, there was no longer an association. *Strengths*: active follow-up with annual in-person interviews; relatively large sample and long follow-up (20 yr); few losses to follow-up (4.6%); urinary biomarker was collected before disease occurrence; self-reported smoking status verified by urinary cotinine. *Limitations*: intraindividual variation in exposure not captured; 35% of cases were not histologically confirmed.
			First quartile	47	1		
			Second quartile	74	1.34 (0.83–2.17)		
			Third quartile	94	1.58 (0.98–2.56)		
			Fourth quartile	128	1.95 (1.22–3.12)		
			Trend-test *P* value, 0.004				
		Lung, incidence	Quartile of urinary HMPMA (pmol/mg creatinine) (OR)			Age at diagnosis and place of residence, smoking intensity and duration, duration of urine samples storage before laboratory analysis, urinary total NNAL and PheT	
			First quartile	47	1		
			Second quartile	74	1.19 (0.73–1.95)		
			Third quartile	94	1.33 (0.81–2.18)		
			Fourth quartile	128	1.58 (0.96–2.57)		
			Trend-test *P* value, 0.058				
		Lung, incidence	Quartile of urinary HMPMA (pmol/mg creatinine) (OR):			Age at diagnosis and place of residence, smoking duration and intensity, duration of urine samples storage before laboratory analysis, total urine cotinine	
			First quartile	47	1		
			Second quartile	74	0.90 (0.53–1.52)		
			Third quartile	94	0.95 (0.56–1.62)		
			Fourth quartile	128	0.97 (0.56–1.66)		
			Trend-test *P* value, 0.956				

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study-design	Population size, description, exposure assessment method	Cancer type	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Yuan et al. (2014) China, Shanghai enrolment, 1986–1989/ follow-up through 2008 Nested case–control	Cases: 82 cases of incident lung cancer in men, lifelong non-smokers aged 45–64 yr at enrolment; Shanghai Cohort Study consisted of 18 244 men (80% of eligible), aged 45–64 yr at enrolment and resided in one of four small geographically defined communities in Shanghai, China Controls: 83 participants in the Shanghai Cohort study without cancer, non-smokers and alive at the time of cancer diagnosis of the case; matched by age at enrolment (± 2 yr), year and month of urine sample collection (± 1 mo), and neighbourhood of residence at recruitment. Exposure assessment method: in-person questionnaire (for smoking status); exposure to crotonaldehyde was determined based on measurement of its urinary metabolite HMPMA; urine samples were collected at baseline survey of the cohort in which the case–control study was nested.	Lung, incidence	Quartile of HMPMA (OR): First quartile Second quartile Third quartile Fourth quartile Trend-test *P* value, 0.99	24 17 19 20	1 0.75 (0.31–1.83) 0.8 (0.33–1.97) 1 (0.41–2.41)	Age at baseline, neighbourhood of residence at enrolment, years of sample storage and urinary cotinine level	*Exposure assessment critique*: Internal exposure assessment only. No information on external exposure. Only never-smokers were included. Urine samples were collected at baseline, so clearly preceded the health outcome; however, only one urine sample at baseline was collected (intraindividual variations). *Strengths*: active follow-up with annual in-person interviews; long follow-up (22 yr); losses to follow-up low (5.4%); self-reported smoking status was confirmed by urinary cotinine levels. *Limitations*: no external exposure assessment; relatively small sample size; 26% of cases not histologically confirmed.

Table 2.1 (continued)

Reference, location, enrolment/ follow-up period, study-design	Population size, description, exposure assessment method	Cancer type	Exposure category or level	Exposed cases or deaths	Risk estimate (95% CI)	Covariates controlled	Comments
Grigoryan et al. (2019) Turin, Italy, EPIC study Enrolment, 1993 through 1997/follow-up, ≤ 14 yr Nested case–control	Cases: 57 men and women aged 36–65 yr at enrolment Controls: 72 men and women aged 36–64 yr at enrolment; included 47 case–control pairs matched by age, sex, and enrolment year and season Exposure assessment method: no data on external exposure; study of Cys34 adducts in serum samples, including for crotonaldehyde by untargeted adductomics. questionnaire for data on diet, BMI, and lifestyle factors	Colon and rectum, incidence	Seven Cys34 adducts to albumin were statistically significantly associated with colorectal cancer. One of the five adducts found to be more abundant in cases than in controls was identified as a crotonaldehyde adduct and clustered with the *s*-methanethiol adduct.			Age, sex	*Exposure assessment critique*: No external exposure assessment, such as on smoking status. Serum samples collected at baseline, before disease occurrence. Crotonaldehyde may have been produced endogenously. *Strengths*: cancer of the colon or rectum confirmed by colonoscopy and biopsy; data in various lifestyle factors collected by questionnaire. *Limitations*: small sample size.

BMI, body mass index; CI, confidence interval; HMPMA, *N*-acetyl-*S*-(3-hydroxy-1-methylpropyl)-L-cysteine; mo, month; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NR, not reported; OR, odds ratio; PheT, r-1,t-2,3,c-4-tetrahydroxy1,2,3,4-tetrahydrophenanthrene; RR, relative risk; yr, year.

Yuan et al. (2012) and Yuan et al. (2014) published results from two nested case–control studies from the Shanghai Cohort Study, which included 18 244 men residing in one of four small communities in Shanghai and aged 45–65 years at enrolment (1986–1989). The methodology of the two nested case–control studies was very similar. Besides in-person interviews, a spot urine sample was taken from each participant at baseline and stored until laboratory analysis. Lung cancer incidence and mortality data were obtained from annual in-person interviews of all surviving participants, the local cancer registry, and the vital statistics office. Exposure to crotonaldehyde was represented by its urine metabolite HMPMA at enrolment. [The Working Group noted that both studies used a nested case–control design, with a long follow-up and few losses to follow-up. As a measure of exposure, tobacco-specific biomarkers were determined in urine samples. Urine biomarkers were based on single urine samples at enrolment, and smoking status was also collected at enrolment. The rate of histopathological confirmation of lung cancer was moderate, at 65% and 74% of cases for each study respectively. Otherwise, the classification was based on clinical diagnosis.]

In the first study (Yuan et al., 2012), the cohort was followed for 20 years through 2006; loss during follow-up was 4.6%. The aim of the study was to examine the relationship between some volatile carcinogens and toxicants from tobacco smoke and lung cancer development in smokers. A total of 706 cases of lung cancer were identified, of which 574 were in current smokers at baseline. For each case in a smoker, one control was selected, also a smoker, who was alive and free of cancer at the time of cancer diagnosis and matched on age at enrolment, date of urine sample collection, and neighbourhood of residence. After excluding cases and controls whose urine samples were depleted and had missing values for one or more mercapturic acid metabolites, 343 lung cancer cases and 392 controls were included in the analysis (all current smokers at baseline). Urine samples were analysed for mercapturic acids, including a metabolite of crotonaldehyde (HMPMA), as well as for metabolites of polycyclic aromatic hydrocarbons (*r*-1,t-2,3,c-4-tetrahydroxy1,2,3,4-tetrahydrophenanthrene; PheT), tobacco-specific nitrosamines (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNAL), and nicotine (total cotinine). Smoking duration was 34.4 years for cases and 30.8 years for controls. Lung cancer cases had significantly higher concentrations of HMPMA than did controls ($P < 0.001$), and HMPMA concentration was positively associated with the daily number of cigarettes smoked and duration of smoking ($P < 0.001$). Comparing the highest with the lowest quartiles of HMPMA concentration, risk of lung cancer was almost doubled in models adjusting for matching factors and number of cigarettes smoked per day and years of cigarette smoking at baseline. In models with further adjustment for metabolites of polycyclic aromatic hydrocarbons (PheT) and tobacco-specific nitrosamines (NNAL) and/or cotinine, no association was found between HMPMA concentration and lung cancer. [The Working Group noted that there were multiple correlated exposures as measured by biomarkers. The strengths of the study included a relatively large sample size, a sufficiently long follow-up (20 years), few losses to follow-up, that the urinary biomarker HMPMA was collected before disease occurrence, and that smoking status was verified by total cotinine. The nearly two-fold increase in risk of lung cancer associated with the highest quartile of HMPMA concentration when adjusting only for intensity and duration of smoking disappeared with further adjustment for other smoking biomarkers such as cotinine. HMPMA is likely to be a biomarker of smoking. Overall, the study was not informative regarding the carcinogenicity of crotonaldehyde.]

In the second study (Yuan et al., 2014), a similar design as in the paper published in

2012 was applied. Male never-smokers at baseline were included to examine the relationship between environmental exposure to air pollutants, including secondhand smoke, and lung cancer. The follow-up was extended through 2008 (22 years). Loss to follow-up was 5.4%. A total of 80 cases of lung cancer and 82 controls (all never-smokers at baseline) were included in the analysis, after excluding cases with urinary cotinine concentrations above 18 ng/mL (indicating that they may have been smokers) and missing values for cotinine and mercapturic acids. The same biomarkers as in the previous paper were measured, including HMPMA for crotonaldehyde; PheT; 3-OH-Phe (3-hydroxyphenanthrene) and total OH-Phe (total hydroxyphenanthrenes, the sum of 1-, 2-, 3- and 4-OH-Phe) for polycyclic aromatic hydrocarbons; and cotinine for nicotine. Urinary concentrations of HMPMA were similar in both cases and controls. After adjustment for matching factors and urinary cotinine concentration, HMPMA was not associated with elevated risk of lung cancer (fourth quartile versus first quartile OR, 1.00; 95% CI, 0.41–2.41). [The Working Group noted that only internal exposure to crotonaldehyde was assessed. In addition, urinary cotinine represents a short-term biomarker of passive smoking and therefore there may not have been full adjustment for long-term secondhand smoke exposure.]

Grigoryan et al. (2019) published results of a nested case–control study on cancer of the colon or rectum within the cohort study European Prospective Investigation into Cancer and Nutrition, in Italy (EPIC-Italy), with participants recruited from 1993 through 1997. Serum samples were obtained at baseline to detect Cys34 adducts of albumin, as an exposure marker. Cases of colorectal cancer were confirmed by colonoscopy and biopsy. Healthy controls were selected from the cohort, and matched on age, sex, and enrolment year and season. Data on different lifestyle factors were obtained by questionnaire at baseline. After excluding gelled serum samples and samples from two subjects with a high percentage of missing adducts, 57 cases and 72 controls were included in analyses (including 47 matched case–control pairs). Seven Cys34 adducts were associated in a statistically significant manner with colorectal cancer. Five adducts were found to be more abundant in the cases than in controls. One of these was identified as a crotonaldehyde adduct and clustered with the *s*-methanethiol adduct. These adduct findings may have resulted from the infiltration of gut microbes into the intestinal mucosa and subsequent inflammatory response. [The Working Group noted the small sample size and the lack of information regarding external exposure to crotonaldehyde as limitations of this study.]

2.2 Evidence synthesis for cancer in humans

Epidemiological evidence available on crotonaldehyde in relation to cancer in humans comprised one occupational cohort study (Bittersohl, 1975) and three nested case–control studies in population-based cohorts (Yuan et al., 2012, 2014; Grigoryan et al., 2019). Regarding cancer sites evaluated across these studies, three of the four studies examined lung cancer (Bittersohl, 1975; Yuan et al., 2012, 2014), while the occupational cohort study (Bittersohl, 1975) also reported on cancers of the oral cavity, stomach, and colon. One nested case–control study (Grigoryan et al., 2019) reported on cancers of the colon or rectum.

2.2.1 Exposure assessment

The quality of the exposure assessment carried out within the available studies was of concern, as detailed in Section 1.6. One study considered external occupational exposure to crotonaldehyde (Bittersohl, 1975), but provided no quantitative exposure assessment, and

therefore no exposure–response analyses could be carried out. In addition, study participants were simultaneously exposed to multiple, undifferentiated chemical agents, and the potential associations between individual chemicals and cancer risk could not be evaluated.

The two other studies (Yuan et al., 2012, 2014) considered crotonaldehyde exposure in two nested case–control studies of smokers and non-smokers, respectively, as determined by urinary metabolites. These studies did not consider external exposure to crotonaldehyde explicitly. Although information on smoking was available and may have been an important source of crotonaldehyde exposure, these studies adjusted for smoking through restriction or statistical adjustment.

2.2.2 Cancers of the lung and other sites

Two case–control studies (Yuan et al., 2012, 2014) nested in a population-based cohort studied several biomarkers in relation to lung cancer (one study among current smokers, and one among never-smokers at baseline). Analyses conducted among the smokers (Yuan et al., 2012) revealed a two-fold risk of lung cancer for the highest compared with the lowest quartile of the crotonaldehyde biomarker HMPMA adjusted for intensity and duration of smoking. Further adjustment for markers of smoking (NNAL, PheT, cotinine) diminished the association between crotonaldehyde and lung cancer, which suggested that crotonaldehyde represents a biomarker of smoking. The study examined the relationship between some volatile carcinogens and toxicants from tobacco smoke and lung cancer development in smokers and was considered uninformative regarding the carcinogenicity of crotonaldehyde as such.

One cohort study (Bittersohl, 1975) among workers currently employed in an aldehyde factory and exposed to multiple chemicals, including aldehydes, reported four different types of cancer among nine male workers (lung, oral cavity, stomach, colon). The study was considered uninformative due to the poorly defined external exposure, small number of cases, and flaws in study design.

One nested case–control study (Grigoryan et al., 2019) found an association between cancers of the colon or rectum and an albumin adduct of crotonaldehyde, interpreted as the effect of an inflammatory response to the gut microbiota infiltrating the colon mucosa.

Taken together, these studies provide little evidence of a positive association between crotonaldehyde exposure and cancer in humans. Some of the available studies were of a mechanistic nature, i.e. they investigated a crotonaldehyde metabolite with null results after controlling for smoking-related biomarkers. In other studies, the design including external exposure assessment was poor.

3. Cancer in Experimental Animals

In a previous evaluation, the *IARC Monographs* programme concluded that there was *inadequate evidence* in experimental animals for the carcinogenicity of crotonaldehyde (IARC, 1995).

Studies on the carcinogenicity of crotonaldehyde in experimental animals are summarized in Table 3.1.

3.1 Mouse

3.1.1 Inhalation

In a study that complied with Good Laboratory Practice (GLP), groups of 50 male and 50 female Crj:BDF_1 [$B6D2F_1$/Crlj] mice (age, 6 weeks) were treated by inhalation with crotonaldehyde (purity, > 99.9%; CAS No., 123-73-9) by whole-body exposure for 6 hours per day, 5 days per week, for 104 weeks (JBRC, 2001a, b, c). The

Table 3.1 Studies of carcinogenicity with crotonaldehyde in experimental animals

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Results	Significance	Comments
Full carcinogenicity Mouse, B6D2F$_1$/Crlj (M) 6 wk 104 wk JBRC (2001a)	Inhalation (whole-body) > 99.9% Clean air 0, 3, 6, 12 ppm 6 h/day, 5 days/wk 50, 50, 50, 50 33, 30, 38, 43	*All sites*: no significant increase in the incidence of tumours		Principal strengths: multiple-dose study; use of males and females; study complied with GLP. Other comments: the incidence of hyperplasia of the respiratory tract was significantly increased in treated animals compared with controls; the Working Group considered hyperplasia of the respiratory tract to be a pre-neoplastic lesion.
Full carcinogenicity Mouse, B6D2F$_1$/Crlj (F) 6 wk 104 wk JBRC (2001a)	Inhalation (whole-body) > 99.9% Clean air 0, 3, 6, 12 ppm 6 h/day, 5 days/wk 50, 50, 50, 50 30, 25, 30, 34	*All sites*: no significant increase in the incidence of tumours		Principal strengths: multiple-dose study; use of males and females; study complied with GLP. Other comments: the incidence of hyperplasia of the respiratory tract was significantly increased in treated animals compared with controls; the Working Group considered hyperplasia of the respiratory tract to be a pre-neoplastic lesion.
Full carcinogenicity Mouse, B6C3F$_1$ (M) Neonatal (age 8 days) 12 mo Von Tungeln et al. (2002)	Intraperitoneal injection NR DMSO 0, 3000 nmol Injections with 1/3 and 2/3 of the total dose in 30 µL DMSO at age 8 and 15 days, respectively 24, 24 24, 24	*Liver* Adenoma Incidence: 0/24, 4/24 Carcinoma Incidence: 0/24, 1/24 Adenoma or carcinoma (combined) Incidence: 0/24, 4/24 Multiplicity: 0, 1.3	 NS NS NS NR	Principal strengths: use of males and females. Principal limitations: use of single dose; lack of body-weight data; rationale for dose not given; only data regarding liver tumours were reported.
Full carcinogenicity Mouse, B6C3F$_1$ (F) Neonatal (age 8 days) 12 mo Von Tungeln et al. (2002)	Intraperitoneal injection NR DMSO 0, 3000 nmol Injections with 1/3 and 2/3 of the total dose in 30 µL DMSO at age 8 and 15 days, respectively 24, 24 23, 24	*Liver* Adenoma Incidence: 0/23, 0/24 Carcinoma Incidence: 0/23, 0/24 Adenoma or carcinoma (combined) Incidence: 0/23, 0/24 Multiplicity: 0, 0	 NA NA NA NA	Principal strengths: use of males and females. Principal limitations: use of single dose; lack of body-weight data; rationale for dose not given; only data regarding liver tumours were reported.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Results	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (M) Neonatal (age 8 days) 15 mo Von Tungeln et al. (2002)	Intraperitoneal injection NR DMSO 0, 1500 nmol Injections with 1/3 and 2/3 of the total dose in 30 µL DMSO at age 8 and 15 days, respectively 24, 24 24, 23	*Liver* Adenoma Incidence: 4/24, 4/23 Carcinoma Incidence: 0/24, 0/23 Adenoma or carcinoma (combined) Incidence: 4/24, 4/23 Multiplicity: 1.0, 1.3	 NS NA NS NR	Principal strengths: use of males and females. Principal limitations: use of single dose; lack of body-weight data; rationale for dose not given; only data regarding liver tumours were reported.
Full carcinogenicity Mouse, B6C3F$_1$ (F) Neonatal (age 8 days) 15 mo Von Tungeln et al. (2002)	Intraperitoneal injection NR DMSO 0, 1500 nmol Injections with 1/3 and 2/3 of the total dose in 30 µL DMSO at age 8 and 15 days, respectively 24, 24 24, 24	*Liver* Adenoma Incidence: 0/24, 0/24 Carcinoma Incidence: 0/24, 0/24 Adenoma or carcinoma (combined) Incidence: 0/24, 0/24 Multiplicity: 0, 0	 NA NA NA NA	Principal strengths: use of males and females. Principal limitations: use of single dose; lack of body-weight data; rationale for dose not given; only data regarding liver tumours were reported.
Full carcinogenicity Rat, F344/DuCrj (M) 6 wk 104 wk JBRC (2001d)	Inhalation (whole-body) > 99.9% Clean air 0, 3, 6, 12 ppm 6 h/day, 5 days/wk 50, 50, 50, 50 39, 39, 45, 38	*Nasal cavity* Adenoma Incidence: 0/50, 1/50 (2%), 1/50 (2%), 2/50 (4%) Rhabdomyosarcoma Incidence: 0/50, 0/50, 0/50, 1/50	 NS NS	Principal strengths: multiple-dose study; use of males and females; study complied with GLP. Other comments: historical control data in F344 male rats: nasal cavity adenoma, 1/1199 (0.08%); nasal cavity rhabdomyosarcoma, 0/1199; the incidence of hyperplasia of the respiratory tract was significantly increased in treated animals compared with controls; the Working Group considered hyperplasia of the respiratory tract to be a pre-neoplastic lesion.
Full carcinogenicity Rat, F344/DuCrj (F) 6 wk 104 wk JBRC (2001d)	Inhalation (whole-body) > 99.9% Clean air 0, 3, 6, 12 ppm 6 h/day, 5 days/wk 50, 50, 50, 50 39, 38, 40, 40	*Nasal cavity:* adenoma Incidence: 0/50, 0/50, 0/50, 1/50	NS	Principal strengths: multiple-dose study; use of males and females; study complied with GLP. Other comments: historical control data for nasal cavity adenoma in F344 female rats, 0/1097; the incidence of hyperplasia of the respiratory tract was significantly increased in treated animals compared with controls; the Working Group considered hyperplasia of the respiratory tract to be a pre-neoplastic lesion.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Results	Significance	Comments
Full carcinogenicity Rat, F344 (M) 6 wk 113 wk Chung et al. (1986a)	Oral administration (drinking-water) > 99% Distilled water 0, 0.6, 6.0 mmol/L in drinking-water 23, 27, 23 16 (at 110 wk), 17 (at 110 wk), 13 (at 110 wk)	*Liver* Hepatocellular adenoma Incidence: 0/23, 9/27*, 1/23 Hepatocellular carcinoma Incidence: 0/23, 2/27, 0/23 Hepatocellular adenoma or carcinoma (combined) Incidence: 0/23, 9/27*, 1/23 *Urinary bladder:* transitional cell papilloma Incidence: 0/23, 2/27, 0/23	*[$P = 0.0022$, two-tailed Fisher exact test] [NS] *[$P = 0.0022$, two-tailed Fisher exact test] [NS]	Principal strengths: long-term study (> 2 yr). Principal limitations: small number of rats per group; use of males only, increased mortality and lower body weight observed at the highest dose; rationale for doses not given.

DMSO, dimethyl sulfoxide; F, female; GLP, Good Laboratory Practice; h, hour; M, male; mo, month; NA, not applicable; NR, not reported; NS, not significant; ppm, parts per million; wk, week; yr, year.

concentration in the exposure chamber was set to 0 (clean air, control), 3, 6 or 12 ppm for males and females. The mean ± SD values monitored every 15 minutes for the groups at 3, 6, and 12 ppm were 3.0 ± 0.0, 6.0 ± 0.0, and 12.0 ± 0.1 ppm, respectively. Survival in males and females was not affected by exposure. Survival in the groups at 0, 3, 6, and 12 ppm, respectively, was: 33/50, 30/50, 38/50, and 43/50 in males, and 30/50, 25/50, 30/50, and 34/50 in females. Male mice at 6 and 12 ppm showed a significant decrease in body-weight gain compared with the control value from week 7 to week 78, and from the first week to the end of exposure, respectively. The relative final body weight in males at 3, 6, and 12 ppm was 101%, 90%, and 66%, respectively, of the value for controls. There was a significant decrease in body-weight gain in female mice at 12 ppm from the first week to the end of the exposure when compared with the control value. The relative final body weight in females at 3, 6, and 12 ppm was 103%, 101%, and 79%, respectively, of the value for controls. All mice underwent complete necropsy, and all organs and tissues were examined microscopically. In all groups of treated male and female mice, there was no significant increase in the incidence of any tumours.

Regarding non-neoplastic lesions in the respiratory tract (see also Section 4 of this monograph), a significant increase in the incidence and/or severity of necrosis, atrophy, cuboidal change, and squamous cell metaplasia in the respiratory epithelium; atrophy and respiratory metaplasia in the olfactory epithelium; exudate; oedema of lamina propria; and hyperplasia and respiratory metaplasia of the nasal glands was observed in the nasal cavity in mice at 12 ppm. The incidence of cuboidal change in the respiratory epithelium was also significantly increased in male mice at 6 ppm. A significant increase in the incidence and/or severity of necrosis, atrophy, inflammation, hyperplasia, cuboidal change, and squamous cell metaplasia in the respiratory epithelium; atrophy and respiratory metaplasia in the olfactory epithelium; exudate; and respiratory metaplasia of the glands was observed in the nasal cavity of female mice at 12 ppm. The incidence of cuboidal change in the respiratory epithelium was also significantly increased in female mice at 6 ppm. [The Working Group considered the hyperplasias of the respiratory tract observed in both males and females to be pre-neoplastic lesions.]

[The Working Group noted this was a GLP study conducted with multiple doses and used males and females.]

3.1.2 Intraperitoneal injection

In the first experiment in a study of carcinogenicity focused on the induction of liver and lung tumours in mice (Von Tungeln et al., 2002), groups of 24 male and 24 female B6C3F$_1$ mice (age, 8 days) were given crotonaldehyde [purity, not reported; assumed to be predominantly *trans*-2-butenal] by intraperitoneal injection in 30 µL of dimethyl sulfoxide (DMSO) at a dose of 3000 nmol, with one third of the total dose [1000 nmol] given at age 8 days and two thirds [2000 nmol] at age 15 days. Control groups of 24 males and 24 females were given 30 µL of DMSO by intraperitoneal injection. There was no significant effect on survival. Mice were killed at age 12 months and underwent complete necropsy. The livers, lungs, and all gross lesions of all mice were examined microscopically. In treated males, a non-statistically significant increase in the incidence of liver adenoma (controls, 0/24, controls; treated, 4/24), and liver adenoma or carcinoma (combined) (controls, 0/24, controls; treated, 4/24) was observed. No liver tumours were observed in treated or control females. In a second experiment in the study by Von Tungeln et al. (2002), groups of 24 male and 24 female B6C3F$_1$ mice (age, 8 days) were given crotonaldehyde at a dose of 1500 nmol by intraperitoneal injection in 30 µL of DMSO, with one

third [500 nmol] of the total dose given at age 8 days and two thirds [1000 nmol] at age 15 days. Control groups of 24 males and 24 females were given 30 μL of DMSO by intraperitoneal injection. There was no significant effect on survival. Mice were killed at age 15 months. No statistically significant differences in the incidence of liver adenoma or liver carcinoma were observed in treated male animals compared with controls. No liver tumours were observed in treated or control females. [The Working Group noted that the principal strength of the study was the use of males and females. The principal limitations were the use of a single dose, that justification for the dose used was not provided, only data regarding liver tumours were reported, and no data on body weight were reported.]

3.2 Rat

3.2.1 Inhalation

In a study that complied with GLP, groups of 50 male and 50 female F344/DuCrj rats (age, 6 weeks) were treated by inhalation with crotonaldehyde (purity, > 99.9%; CAS No., 123-73-9) by whole-body exposure for 6 hours per day, 5 days per week, for 104 weeks (JBRC, 2001d, e, f). The concentration in the exposure chamber was set to 0 (clean air, control), 3, 6, or 12 ppm for males and females. The mean ± SD values monitored every 15 minutes for the groups at 3, 6, and 12 ppm were 3.0 ± 0.0, 6.0 ± 0.0, and 12.0 ± 0.1 ppm, respectively. Survival in males and females was not affected by exposure. Survival in the groups at 0, 3, 6, and 12 ppm was 39/50, 39/50, 45/50, and 38/50 in males, respectively; and 39/50, 38/50, 40/50, and 40/50 in females, respectively. Male rats at 12 ppm showed a significant decrease in body-weight gain compared with the value for controls throughout the exposure period. The relative final body weight in males at 3, 6, and 12 ppm was 99%, 96%, and 91% of the value for controls, respectively. Female rats at 12 ppm showed a significant decrease in body-weight gain from week 2 to the end of exposure compared with the value for controls. The relative final body weight in females at 3, 6, and 12 ppm was 100%, 99%, and 91% of the value for controls, respectively. All rats underwent complete necropsy, and all organs and tissues were examined microscopically.

In treated male rats, there was no significant increase in the incidence of any tumours. The incidence of nasal cavity adenoma was 0/50 (control), 1/50 (2%, 3 ppm), 1/50 (2%, 6 ppm) and 2/50 (4%, 12 ppm). [The Working Group noted an apparent dose–response relationship, although it was not statistically significant.] Although it was not statistically significant, the value for males at 12 ppm (4%) was in excess of the incidence in historical controls (1/1199, 0.08%). One (1/50, 2%) rhabdomyosarcoma of the nasal cavity was observed in a male rat at 12 ppm; this tumour was not observed in 1199 male historical controls. [The Working Group considered that the adenomas of the nasal cavity were exposure-related, and that the rhabdomyosarcoma of the nasal cavity may have been exposure-related.]

In treated female rats, there was no significant increase in the incidence of any tumours. One (1/50, 2%) adenoma of the nasal cavity, never reported in historical controls (incidence, 0/1097), was observed in a female rat at 12 ppm. [The Working Group considered that this rare adenoma of the nasal cavity may have been be related to exposure.]

Regarding non-neoplastic lesions in the respiratory tract (see also Section 4 of this monograph), a significant increase in the incidence and/or severity of: inflammation (at ≥ 3 ppm), hyperplasia (at ≥ 6 ppm), squamous cell metaplasia (at ≥ 3 ppm), and squamous cell hyperplasia (at 12 ppm) in the respiratory epithelium; atrophy (at 12 ppm) and respiratory metaplasia (at ≥ 3 ppm) in the olfactory epithelium; and inflammation with foreign body (at ≥ 6 ppm)

was observed in the nasal cavity of treated males. A significant increase in the incidence and/or severity of: inflammation (at ≥ 6 ppm), hyperplasia (at ≥ 6 ppm), and squamous cell metaplasia (at ≥ 3 ppm) in the respiratory epithelium; atrophy (at 12 ppm) and respiratory metaplasia (at 12 ppm) in the olfactory epithelium; and inflammation with foreign body (at 12 ppm) was observed in the nasal cavity of treated females. [The Working Group considered that the hyperplasias of the respiratory tract observed in both males and females were pre-neoplastic lesions.]

[The Working Group noted this was a GLP study conducted with multiple doses and used males and females.]

3.2.2 Oral administration (drinking-water)

Groups of 23–27 male Fischer 344 rats (age, 6 weeks) were given drinking-water containing crotonaldehyde (purity, > 99%; *trans*-2-butenal) at a dose of 0 (control, distilled water only), 0.6, or 6.0 mmol/L for 113 weeks (Chung et al., 1986a). In rats at the highest dose of crotonaldehyde, increased mortality (survival at 110 weeks: controls, 16/23; 0.6 mmol/L, 17/27; and 6.0 mmol/L, 13/23) and lower body weight [no statistics provided, but approximately –12% read from graph] were observed. Gross lesions and representative samples from all major organs [not further specified] were taken for microscopic examination. In treated rats, a significant increase in the incidence of liver neoplastic nodules [hepatocellular adenoma] was observed at the lower dose compared with controls, with an incidence of 0/23 (control), 9/27 [$P = 0.0022$], and 1/23, respectively. In treated rats, a non-statistically significant increase in the incidence of hepatocellular carcinoma was also observed at the lower dose, with an incidence of 0/23 (control), 2/27, and 0/23, respectively. Overall, there was a significant increase in the incidence of hepatocellular adenoma or carcinoma (combined) at the lower dose, with an incidence of 0/23 (control), 9/27 [$P = 0.0022$; Fisher exact test], and 1/23, respectively. The unusual dose–response relationship was attributed to extensive hepatotoxicity (fatty metamorphosis, focal liver necrosis, fibrosis, cholestasis, and mononuclear cell infiltration) in the group at the higher dose. [The increased mortality and lower body weight of the rats at the higher dose might at least in part explain the lack of a dose–response relationship for the induction of hepatocellular adenoma and hepatocellular carcinoma. The small number of animals used might at least in part explain why the increase in the incidence of hepatocellular carcinoma was not statistically significant.] In treated rats, a non-statistically significant increase in the incidence of urinary bladder transitional cell papilloma was also observed at the lower dose, with an incidence of 0/23 (control), 2/27, and 0/23, respectively. Regarding pre-neoplastic lesions, a significant increase in the incidence of altered liver foci was observed at the lower and higher doses. [The Working Group noted that the principal strength of the study was that it was a long-term study (> 2 years). The principal limitations were the small number of animals per group, the use of males only, that the rationale for the doses used was not provided, and that increased mortality and lower body weight were observed at the higher dose.]

3.3 Evidence synthesis for cancer in experimental animals

The carcinogenicity of crotonaldehyde has been assessed in one GLP study in male and female mice and one GLP study in male and female rats treated by inhalation with whole-body exposure. The other available studies included two studies in newborn male and female mice treated by intraperitoneal administration, and one study in male rats treated by oral administration (in the drinking-water).

The GLP inhalation study with crotonaldehyde in F344/DuCrj rats reported a low incidence of nasal cavity adenoma and a single nasal cavity rhabdomyosarcoma in exposed male rats. The incidence of nasal cavity adenoma had an apparent dose-related positive trend, and the nasal cavity rhabdomyosarcoma was observed at the highest dose. A single nasal cavity adenoma was also reported in females at the highest dose. Both nasal cavity adenoma and nasal cavity rhabdomyosarcoma are very rare in the rat strain used in the study (JBRC, 2001d, e, f).

The GLP inhalation study in B6D2F$_1$/Crlj mice did not report a significant increase in the incidence of any tumours in male or female mice exposed to crotonaldehyde (JBRC, 2001a, b, c).

Crotonaldehyde administered in the drinking-water of male Fischer 344 rats caused a significant increase in the incidence of hepatocellular adenoma and of hepatocellular adenoma or carcinoma (combined) at the lowest but not the highest dose tested. The lack of a dose–response relationship was attributed to extensive hepatotoxicity at the highest dose of crotonaldehyde (Chung et al., 1986a).

Treating neonatal B6C3F$_1$ mice by intraperitoneal injection did not result in an increased incidence of tumours (Von Tungeln et al., 2002).

4. Mechanistic Evidence

4.1 Absorption, distribution, metabolism, and excretion

The information below pertains to mixtures of the *trans*- (*E*-) and *cis*- (*Z*-) isomers of crotonaldehyde, unless stated otherwise.

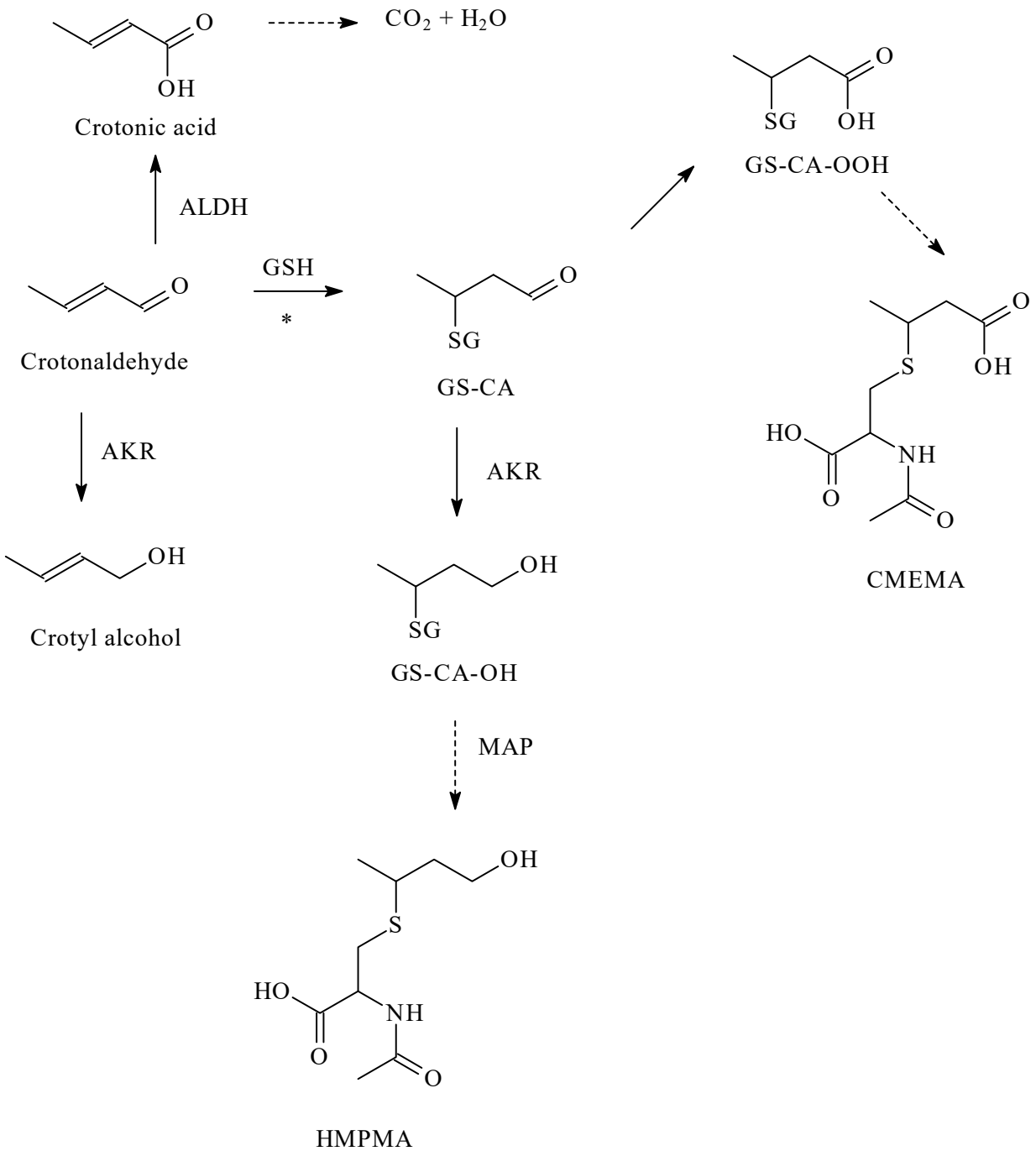
4.1.1 Humans

(a) Exposed humans

Sparse information was available to the Working Group on the absorption and distribution of crotonaldehyde in humans.

The most extensive data on the fate of crotonaldehyde in humans are related to the detection and quantification of urinary crotonaldehyde-specific mercapturates (Fig. 4.1). Numerous studies have reported the use of sensitive analytical methods, primarily based on LC-MS/MS, to assess urinary biomarkers of human exposure to mixtures of volatile organic compounds, including crotonaldehyde (Scherer et al., 2006, 2007; Carmella et al., 2009; Hecht et al., 2010; Alwis et al., 2012; Carmella et al., 2013; Zhang et al., 2014; Hecht et al., 2015; Pluym et al., 2015; Frigerio et al., 2019). These studies have consistently demonstrated the ubiquitous presence of HMPMA in human urine, at statistically significant higher concentrations (3- to 7-fold) in smokers than in non-smokers. Smoking cessation or switching to cigarettes with lower crotonaldehyde delivery resulted in significant reductions in urinary HMPMA concentrations (Scherer et al., 2006, 2007). When measured up to 56 days after smoking cessation, urinary HMPMA concentrations rapidly decreased, from a baseline value of 1965 ± 1001 (mean ± SD) to 265 ± 113 nmol/24 hours after 3 days, and remained approximately constant thereafter (Carmella et al., 2009). Some of these studies (Scherer et al., 2007; Pluym et al., 2015; Frigerio et al., 2019) also reported the detection and quantification of a second crotonaldehyde-derived mercapturate, CMEMA, in human urine. In contrast to rats (see Section 4.1.2), in which CMEMA was found to be a minor urinary metabolite, urinary concentrations of CMEMA in humans were comparable to, or even higher than, those of HMPMA. However, whereas HMPMA concentrations were significantly correlated with smoking status, this was not the case

Fig. 4.1 Major pathways of crotonaldehyde metabolism



ALDH, aldehyde dehydrogenase; AKR, aldo-keto reductase; CMEMA, *N*-acetyl-*S*-(3-carboxy-1-methylpropyl)-L-cysteine; GS–CA, glutathione–crotonaldehyde adduct; GS–CA-OH, reduced glutathione–crotonaldehyde adduct; GS–CA-OOH, oxidized glutathione–crotonaldehyde adduct; GSH, glutathione; HMPMA, *N*-acetyl-*S*-(3-hydroxy-1-methylpropyl)-L-cysteine; MAP, mercapturic acid pathway; *, with and/or without catalysis by glutathione *S*-transferase.
Compiled by the Working Group.

for CMEMA (Scherer et al., 2007; Pluym et al., 2015). Conversely, concentrations of CMEMA (but not HMPMA) were significantly higher in non-smoking gasoline-station attendants than in unexposed workers (Frigerio et al., 2019). [The Working Group noted that the reasons for these discrepancies are not clear. Both HMPMA and CMEMA may also be formed from exposure to crotonaldehyde present in food and ambient air, or formed endogenously. Elevated concentrations of CMEMA might reflect exposure to crotonic acid or crotonates in humans.]

A genome-wide association study conducted in samples from more than 2200 smokers from five ethnic groups reported a significant association between urinary HMPMA concentration and a variant on chromosome 12 near the *TBX3* gene, which is involved in encoding transcription factors, but the implications of this association with regard to crotonaldehyde metabolism and excretion were not clear (Park et al., 2015). Moreover, no association was detected with chromosome 11, which contains the glutathione *S*-transferase pi *1 (GSTP1)* gene. [These observations suggest that glutathione conjugation with crotonaldehyde, ultimately leading to formation of HMPMA, is mainly a non-enzyme-catalysed process in humans.]

(b) Human cells in vitro

Although crotonaldehyde reacts rapidly with glutathione in vitro (see Section 4.1.2), some degree of enzyme-catalysed conjugation has been demonstrated in vitro with several allelic variants of human GSTP1-1, with catalytic efficiencies (k_{cat}/K_m) in the range of 12–17 mM^{-1} s^{-1} (Pal et al., 2000). Consistent with glutathione conjugation, human polymorphonuclear leukocytes had a dose-related decrease in surface and soluble sulfhydryl (SH) groups after treatment with crotonaldehyde in vitro (Witz et al., 1987).

In studies with purified recombinant aldo-keto reductase family 1 B10 (AKRB10), which is expressed in the human colon and small intestine, the enzyme was demonstrated to catalyse the reduction of crotonaldehyde to crotyl alcohol at 0.9 μM, with K_m = 86.7 ± 14.3 μM and V_{max} = 2647.5 ± 132.2 nmol/mg protein per min, and also the carbonyl reduction of the glutathione–crotonaldehyde conjugate at 0.5 μM, with K_m = 245.7 ± 21.2 μM and V_{max} = 1900.7 ± 90.9 nmol/mg protein per min (Yan et al., 2007; Zhong et al., 2009). *AKRB10* downregulation enhanced the susceptibility of colorectal cancer HCT-8 cells to crotonaldehyde, resulting in rapid cell death (Yan et al., 2007). In a subsequent study, catalytic efficiency for the reduction of crotonaldehyde was 400 times lower for purified recombinant aldo-keto reductase family 1 B1 (AKRB1) (ubiquitously expressed in humans) than for AKRB10. Although AKRB1 appeared to have higher specificity than AKRB10 for glutathione-conjugated carbonyls, data for the glutathione–crotonaldehyde conjugate were not presented (Shen et al., 2011).

4.1.2 Experimental systems

(a) Non-human mammals in vivo

The available data on the absorption, distribution, metabolism, and excretion of crotonaldehyde in experimental animals are few. Nonetheless, protein and DNA adducts of crotonaldehyde have been detected in multiple tissues from rats and mice (see Section 4.2.1), demonstrating that crotonaldehyde undergoes systemic distribution.

In a study with groups of three or four adult male Fischer 344 rats given a single dose of [^{14}C]-crotonaldehyde (radiochemical purity, > 96%) at 2.6–2.9 mg/kg body weight (bw) in aqueous ethanol by intravenous injection, approximately 31% of the administered radiolabel was excreted as [^{14}C]-labelled carbon dioxide in the expired air and 37% in the urine within 6 hours after dosing. At the same time-point, the excretion of other volatiles in the breath accounted for approximately 1%

of the total radiolabel, whereas the amount of radiolabel associated with blood and selected tissues (skin, muscle, adipose tissue, and liver) accounted for 10% of the total dose administered. At 72 hours after dosing, the elimination of crotonaldehyde as [^{14}C]-labelled carbon dioxide had increased to approximately 41%, and urinary metabolites accounted for 48% of the administered radiolabel, with negligible (< 0.5%) faecal elimination and low accumulation of [^{14}C] (< 5% radioactivity) detected in the analysed tissues. The parent crotonaldehyde accounted for > 1% of the urinary excretion of [^{14}C] and its oxidation product, crotonic acid, for < 2%. The elimination of [^{14}C] in the breath and urine appeared to be biphasic, with similar half-lives of approximately 2 hours for the initial phase and 13 hours for the second phase estimated for both routes (NTP, 1985).

In a concomitant study, adult male Fischer 344 rats were given [^{14}C]-labelled crotonaldehyde by gavage as a single dose at 0.7, 3, or 35 mg/kg bw. Absorption from the gastrointestinal tract occurred readily. By 12 hours after dosing, elimination in exhaled air and urine combined accounted for 78% and 60% of the administered radiolabel at the lowest and highest dose, respectively. By 72 hours, 44–49% of the administered dose was excreted in the breath as [^{14}C]-labelled carbon dioxide, 38–39% in the urine, and 6–7% in the faeces, indicating that the absorption of [^{14}C]-labelled crotonaldehyde from oral doses was > 93%. Elimination of [^{14}C] from the tissues and blood was biphasic; there was an initially rapid elimination stage, with half-lives of approximately 1 hour or less, followed by a much slower elimination of the last 10% of the dose, with terminal half-lives of 2.5 days or longer (NTP, 1985).

In an earlier study, groups of male albino and black hooded rats were given a single subcutaneous injection of crotonaldehyde at 0.75 mmol/kg bw [approximately 53 mg/kg bw] in olive oil. Two mercapturate metabolites were identified in urine collected in the 24 hours after dosing. The major metabolites, which accounted for 6–15% of the administered dose, was characterized as HMPMA by hydrolytic conversion to *S*-(3-hydroxy-1-methylpropyl)-L-cysteine and comparison with a synthetic standard of the latter. The minor urinary metabolite, which was detected occasionally but not quantified, was characterized as CMEMA (Gray & Barnsley, 1971). HMPMA was also detected in the urine of adult male C57BL6/J mice after whole-body exposure to mainstream cigarette smoke (equivalent to 12 cigarettes over 6 hours) but not in the urine of mice exposed to electronic cigarette aerosols (Conklin et al., 2018) or smokeless tobacco extracts in tap water (Malovichko et al., 2019).

The structures of the urinary mercapturates are indicative of Michael-type addition of glutathione to the α,β-unsaturated carbonyl of crotonaldehyde, followed by either reduction or oxidation of the aldehyde group and subsequent catabolism (Fig. 4.1). When given by intraperitoneal injection at a dose of 2 mmol/kg bw [140 mg/kg bw] to male Wistar rats, crotonaldehyde did cause an early decrease in the hepatic glutathione concentrations, as measured 3 hours after dosing. However, the approximate liver glutathione content in rats treated with crotonaldehyde at 0.75 mmol/kg bw was comparable to that of control rats when measured 12 hours after dosing (Oguro et al., 1990).

(b) Non-human mammalian cells in vitro

Upon in vitro incubation with stomach content homogenate from an untreated rat, at an amount equivalent to a dose of 1.8 mg/kg bw, [^{14}C]-labelled crotonaldehyde remained essentially intact after 2 hours, with 94% of the radioalabel being recovered as the parent compound (assessed by HPLC) and approximately 5% found to be bound to particulate matter (NTP, 1985). In contrast, incubation of [^{14}C]-labelled crotonaldehyde (approximately 7.33 µg/g) with rat plasma at

37 °C demonstrated that the compound was not stable under these conditions. After 5 minutes, only 42% of the radiolabelled parent compound remained intact, and this had decreased to 15% after 30 minutes. The initial degradation of crotonaldehyde subsequently became much slower, with 8% of the parent compound still present after 20 hours. The reaction products were not identified (NTP, 1985).

Crotonaldehyde reacts readily with SH groups in vitro. It undergoes spontaneous reaction with glutathione, although some degree of enzyme catalysis has also been documented after incubation with rat liver preparations (Boyland & Chasseaud, 1967; Gray & Barnsley, 1971) or with purified glutathione *S*-transferases (Stenberg et al., 1992; Eisenbrand et al., 1995). In an additional study, rat pulmonary alveolar macrophages exhibited a dose-related decrease in surface and soluble SH groups after treatment with crotonaldehyde in vitro (Witz et al., 1987).

Upon incubation of adult rat lung alveolar cells with crotonaldehyde at 100, 200, or 500 µM for 20 minutes, the effective concentration (EC_{50}) for 50% intracellular glutathione depletion was estimated to be 130 µM. At the crotonaldehyde concentrations used in the study, the rate of glutathione depletion was characteristic of a non-enzymatic second-order reaction for adduct formation (Meacher & Menzel, 1999). [The Working Group noted that the data from this study indicated a key role for molecular reactivity in the process.]

While the reaction between crotonaldehyde and glutathione in buffer solution yields the expected 1,4-addition product (glutathione–crotonaldehyde adduct; GS–CA, Fig. 4.1), this species is only detected at very low levels in cell media. In contrast, a glutathione–crotonaldehyde adduct resulting from subsequent reduction of the aldehyde carbonyl (GS–CA-OH, Fig. 4.1) was clearly identified after a 30-minute incubation of B16-BL6 mouse melanoma cells with crotonaldehyde at 10 µM (Horiyama et al., 2016). The same crotonaldehyde-specific adduct was readily detected ($t \leq 1$ minute) in sheep erythrocytes exposed to cigarette smoke extract (Horiyama et al., 2018), indicating that the initially formed glutathione–crotonaldehyde adduct is a substrate for mammalian intracellular carbonyl reductases.

Several studies have also addressed the oxidative metabolism of crotonaldehyde to crotonic acid in rat hepatocytes and rat liver mitochondrial, cytosolic, and microsomal fractions. Crotonaldehyde was consistently found to be both a poor substrate for the liver aldehyde dehydrogenases (ALDHs), with a K_m of 515 µM calculated for the microsomal ALDH, and was a potent inhibitor of the high-affinity mitochondrial and cytosolic ALDH isoforms (Cederbaum & Dicker, 1982; Dicker & Cederbaum, 1984; Mitchell & Petersen, 1993).

4.2 Evidence relevant to key characteristics of carcinogens

This section summarizes the evidence for the key characteristics of carcinogens (Smith et al., 2016), including whether crotonaldehyde is electrophilic or can be metabolically activated to an electrophile; is genotoxic; induces oxidative stress; induces chronic inflammation; or is immunosuppressive. Insufficient data were available for the evaluation of other key characteristics of carcinogens.

4.2.1 Is electrophilic or can be metabolically activated to an electrophile

(a) Human

(i) Exposed humans

See Table 4.1.

Crotonaldehyde forms α-methyl-γ-hydroxy-1,N^2-propano-2′-deoxyguanosine adducts in DNA, of which there are two identified diastereoisomeric forms – 8*R*,6*R* and 8*S*,6*S* (see Fig. 4.2

Table 4.1 Crotonaldehyde-derived DNA adducts in exposed humans

Biosample	Location, setting	No. of subjects	Adduct frequency (analytical method) Response (significance)	Comments	Reference
Liver	Autopsy samples from Columbia University (NY), USA	2 M, 3 F	0.13–1.0 adducts/10^6 G (^{32}P-postlabelling)		Nath & Chung (1994)
Peripheral blood	Healthy volunteers	2 M (1 smoker), 2 F (1 smoker)	0.003–0.025 µmol/mol G (^{32}P-postlabelling) No difference between smokers and non-smokers	Smoking	Nath et al. (1996)
Mammary tissue	Breast-reduction surgery samples from Anderson Cancer Center, Houston (TX), USA	3 F	0.004–0.077 µmol/mol G (^{32}P-postlabelling)		
Gingival tissue	Samples from surgery at a periodontal clinic at New York University Dental Center (NY), USA	11 smokers (4 M, 7 F); 12 non-smokers (8 M, 4 F)	Adduct levels significantly higher in smokers ($P = 0.003$) (^{32}P-postlabelling) CdG1 adduct: 0.53 ± 0.44 µmol/mol G in smokers; 0.06 ± 0.07 µmol/mol G in non-smokers ($P = 0.0015$) CdG2 adduct: 1.72 ± 1.26 µmol/mol G in smokers; 0.31 ± 0.40 µmol/mol G in non-smokers ($P = 0.0014$)	Smoking	Nath et al. (1998)
Liver	Surgical samples obtained from the Cancer Center Tissue Procurement Facility, University of Minnesota, USA	23	4/23 positive 6S,8S adduct: 6.70 ± 2.92 fmol/µmol dG (mass spectrometry) 6R,8R adduct: 7.87 ± 4.47 fmol/µmol dG	Smoking status of donors unknown.	Zhang et al. (2006)
Lung	Surgical samples obtained from the Cancer Center Tissue Procurement Facility, University of Minnesota, USA	45	16/45 positive 6S,8S adduct: 7.19 ± 4.14 fmol/µmol dG (mass spectrometry) 6R,8R adduct: 12.8 ± 7.6 fmol/µmol dG	Samples were from self-reported smokers (but not clear whether past or present).	
Peripheral blood	9 buffy-coat samples from the University of Minnesota Transdisciplinary Tobacco Use Research Center, and 2 from Mid-South Regional Blood Center, Memphis (TN), USA	11	0/11 positive LOQ, 4 fmol/µmol dG (mass spectrometry)	Smoking status not reported.	
Peripheral blood	Healthy volunteers, Taiwan, China	9	6.2 ± 3.8 adducts/10^8 nucl (mass spectrometry)		Chen & Lin (2009)
Placenta	Commercial DNA sample	1	26 adducts/10^8 nucl		

Table 4.1 (continued)

Biosample	Location, setting	No. of subjects	Adduct frequency (analytical method) Response (significance)	Comments	Reference
Saliva	Healthy volunteers, Taiwan, China	27	22/27 individuals positive Mean, 7.5 ± 12 (range, 0–48.5) adducts/10^8 nucl (mass spectrometry)	Smoking status not reported. Study included simultaneous detection of other adducts potentially derived from products of lipid peroxidation.	Chen & Lin (2011)
Urinary samples	Urban (São Paulo City) and rural (São João da Boa Vista) dwellers, Brazil	47 urban, 35 rural	Urban: median 20.8 (range, ND–330.0) fmol/mg creatinine (mass spectrometry) Rural: median, 7.9 (range, 2.6–53.1) fmol/mg creatinine ($P < 0.05$)	Publication is a short communication, lacking details on study subjects or sources of exposure.	Garcia et al. (2013)
Urinary samples	China	13	6S,8S adduct: 1.01 ± 0.85 nmol/mol creatinine 6R8R adduct: 0.89 ± 0.67 nmol/mol creatinine		Zhang et al. (2016a)
Lung	Lung Tissue Research Consortium of the National Heart Lung and Blood Institute (NIH), USA	41 lung cancer patients (smokers); 13 non-lung cancer patients (non-smokers)	Significantly higher levels of CdG in smokers than non-smokers (immunoassay and ^{32}P-postlabelling)	*P* value not reported; adduct levels shown graphically (range, 0 to ~40 adducts/10^7 dG).	Weng et al. (2018)
Buccal cells		33 smokers; 17 non-smokers	PdG adducts (derived from acrolein and crotonaldehyde combined) significantly higher in smokers ($P < 0.0001$)	Adduct levels shown graphically (range, 0 to ~2.5 adducts/10^5 dG).	
Sputum		22 smokers; 8 non-smokers	PdG adducts (derived from acrolein and crotonaldehyde combined) significantly higher in smokers ($P < 0.0193$)	Immunoassay method only was used. Adduct levels shown graphically (range, 0 to ~2.5 adducts/10^5 dG).	
Peripheral blood		1 smoker, 1 non-smoker	Smoker: 28.3 adducts/10^7 nucl (mass spectrometry) Non-smoker: 3.5 adducts/10^7 nucl		Alamil et al. (2020)

CdG, crotonaldehyde-derived l,N^2-propano-deoxyguanosine; dG, deoxyguanosine; F, female; G, guanine; LOQ, limit of quantification; M, male; nucl, nucleotides, ND, not detected; PdG, cyclic 1,N^2-propano-deoxyguanosine.

Fig. 4.2 Diastereoisomeric adducts, 8*R*,6*R* and 8*S*,6*S*

CdG 1
(6*S*, 8*S*-isomer)

CdG 2
(6*R*, 8*R*-isomer)

CdG, crotonaldehyde-derived 1,N^2-propano-deoxyguanosine.
Adapted from Nath et al. (1996).

and Section 4.2.1.b). These crotonaldehyde adducts were detected in normal human liver at levels ranging from 0.13 to 1.0 adducts/10^6 deoxyguanosine (Nath & Chung, 1994). In subsequent studies, these adducts were detected in other normal tissues, including in peripheral blood and mammary tissue (Nath et al., 1996); in oral (gingival) tissue (Nath et al., 1998); in liver, lung, and blood cells (Zhang et al., 2006); in placenta, blood cells, and saliva (Chen & Lin, 2009, 2011); in urine samples (Garcia et al., 2013; Zhang et al., 2016a); and in peripheral blood (Alamil et al., 2020). [The Working Group noted that different methods were used in these studies, which may account for differences in levels detected.]

In studies comparing smokers and non-smokers, adduct levels were significantly elevated in smokers, indicating their formation by crotonaldehyde from tobacco smoke; the presence of adducts in tissues of non-smokers is widely interpreted as being indicative of formation from endogenous sources such as lipid peroxidation (Nath et al., 1996). In a comparison of residents in two areas of Brazil, adduct levels in urine samples were significantly higher in the urban population than in rural residents (Garcia et al., 2013). This was attributed to differences in levels of air pollution as the source of exposure to crotonaldehyde.

In a study from the EPIC-Italy colon cancer cohort, Cys34 adducts of crotonaldehyde in serum albumin were more abundant in cases than in controls, suggesting an inflammatory response involving the generation of crotonaldehyde via lipid peroxidation (Grigoryan et al., 2019). Interestingly, this adduct, along with several other adducts that can result from lipid peroxidation, was also present at significantly higher concentrations in the serum albumin of workers exposed to benzene than in unexposed controls (Grigoryan et al., 2018).

In a study on various smoking-related DNA adducts in different human tissues, crotonaldehyde-derived 1,N^2-propano-2′-deoxyguanosine adducts were the most common adducts detected in buccal cells from smokers and in normal lung tissue from lung cancer patients who were smokers, but not in lung tissues of non-smokers (Weng et al., 2018).

(ii) Human cells in vitro

See Table 4.2.

Several studies have demonstrated the formation of crotonaldehyde-derived DNA adducts or DNA damage in human cells treated in vitro with crotonaldehyde. Adducts characteristic of

Table 4.2 Genetic and related effects of crotonaldehyde in human cells in vitro

End-point	Tissue, cell line	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
DNA adducts (^{32}P-postlabelling)	Human xeroderma pigmentosum fibroblasts (GM 5509)	+	NT	1 μM		Wilson et al. (1991)
DNA adducts (^{32}P-postlabelling)	Human primary normal bronchial fibroblasts	+	NT	100 μM	Only one concentration tested.	Wilson et al. (1991)
DNA adducts (^{32}P-postlabelling)	Human skin fibroblasts from a cystic fibrosis patient (GM 4539)	+	NT	100 μM	Only one concentration tested.	Wilson et al. (1991)
DNA adducts (mass spectrometry)	MRC5 fibroblast cell line	+	NT	1 μM	6S,8S and 6R,8R adducts were detected in untreated cells; levels were enhanced by crotonaldehyde treatment across the range 1–100 μM.	Zhang et al. (2016b)
DNA interstrand crosslinks (comet assay, thermal denaturation, circular dichroism)	Lymphocytes	+	NT	50 mM	High concentration tested. Lack of positive control.	Ul Islam et al. (2014)
DNA interstrand crosslinks (dynamic light scattering)	Placental DNA	+	NT	50 mM	Not a standard genotoxicity assay. High concentration tested. Lack of positive control.	Ul Islam et al. (2016)

GM, geometric mean; HIC, highest ineffective concentration; LEC, lowest effective concentration, NT, not tested.

[a] +, positive.

1,N^2-propano-2′-deoxyguanosine were detected by ^{32}P-postlabelling in the DNA of human xeroderma pigmentosum (XP) fibroblasts treated with crotonaldehyde at 1–100 μM (Wilson et al., 1991). The same range of crotonaldehyde concentrations increased the levels of these adducts in MRC5 cells above the levels already present in untreated cells (Zhang et al., 2016b).

DNA interstrand crosslinks were detected in human lymphocytes and placental DNA treated with crotonaldehyde at 50 mM (Ul Islam et al., 2014, 2016).

Treatment of human HepG2 liver cells with the carcinogen aflatoxin B_1 resulted in the formation of aflatoxin–DNA adducts, and also crotonaldehyde-derived DNA adducts (at a 30-fold higher level) induced by lipid peroxide generation of crotonaldehyde (Weng et al., 2017). Both types of adducts were preferentially formed at codon 249 of the *TP53* gene, a hotspot for mutation in hepatocellular carcinoma associated with aflatoxin exposure.

(b) Experimental systems

(i) DNA and protein binding in chemical reactions

Crotonaldehyde is a bifunctional α,β-unsaturated aldehyde (enal) that can form cyclic adducts in DNA, DNA interstrand crosslinks, and DNA–protein crosslinks.

Michael addition of the N^2-amino group of deoxyguanosine and of deoxyguanosine residues in DNA, to C3 of crotonaldehyde, followed by ring closure between N1 of deoxyguanosine and C1 of crotonaldehyde forms α-methyl-γ-hydoxy-1,N^2-propano-2′-deoxyguanosine adducts, frequently referred to as crotonaldehyde-derived 1,N^2-propano-2′-guanosine adducts (Eder et al., 1982; Chung & Hecht, 1983; Chung et al., 1984; Chung et al., 1986b). These are guanine positions that are involved in base pairing in DNA. Chirality at the methyl-bearing carbon atom in the 1,N^2-propano ring results in a pair of diastereoisomeric adducts, 8*R*,6*R* and 8*S*,6*S* (see Fig. 4.2).

Monoclonal antibodies specific for the 8*R*,6*R* and 8*S*,6*S* stereoisomers have been produced (Foiles et al., 1987). Methods for detecting crotonaldehyde derived DNA adducts using ^{32}P-postlabelling analysis (Chung et al., 1989, Foiles et al., 1990, Nath et al., 1994, Pan et al., 2006) and mass spectrometry (Doerge et al., 1998, Zhang et al., 2006, Chen & Lin, 2009, Garcia et al., 2013, Zhang et al., 2016b, Alamil et al., 2020) have also been reported.

Reaction of deoxyguanosine with an excess of crotonaldehyde at 80 °C gave rise not only to 1,N^2-propano adducts but also to *N7*,C8 cyclic adducts and 1,N^2,7,8 bicyclic adducts (Eder & Hoffman, 1992). Reaction of crotonaldehyde with deoxyadenosine produces 1,N^6-propano-2′-deoxyadenosine adducts equivalent to the deoxyguanosine adducts (Chen & Chung, 1994).

Crotonaldehyde is a metabolite of *N*-nitrosopyrrolidine (NPYR), a carcinogenic environmental nitrosamine. α-Acetoxy-NPYR, a synthetic stable precursor to the proposed proximate carcinogen α-hydroxy-NPYR, reacts with DNA to form crotonaldehyde-derived 1,N^2-propano-2′-deoxyguanosine and cyclic *N7*,C8 guanine adducts (Wang et al., 1989, 1998).

Crotonaldehyde-derived 1,N^2-propano-2′-deoxyguanosine may also be generated by endogenous processes. Their formation by ω-3 polyunsaturated fatty acids, including docosahexaenoic acid, linoleic acid, and eicosapentaenoic acid (Pan & Chung, 2002), suggests a possible source, as products of lipid peroxidation, of adducts detected in human and animal tissues not knowingly exposed to crotonaldehyde.

The ability of crotonaldehyde to form interstrand crosslinks in DNA depends on the stereochemistry at the C6 position of the 1,N^2-propano-2′-deoxyguanosine adduct. It requires a 5′-CpG-3′ (cytosine-phosphate-guanine) sequence where the orientation of the aldehyde within the minor groove favours reaction of

the 6*R* configuration relative to the 6*S* (Kozekov et al., 2003; Stone et al., 2008; Minko et al., 2009). Molecular modelling studies predict less disruption of the duplex structure, and greater thermodynamic stability for the crosslink formed by the *R* adduct (Cho et al., 2006a, b, 2007).

Histones, which are rich in basic amino acids such as arginine and lysine, accelerate the reaction of crotonaldehyde with deoxyguanosine and DNA under physiological conditions (Sako et al., 2003; Inagaki et al., 2004). Crotonaldehyde reacts with lysine and histidine in bovine serum albumin (Ichihashi et al., 2001) and can also form DNA–protein crosslinks (Kuykendall & Bogdanffy, 1992). Crotonaldehyde-derived 1,N^2-propano-2′-deoxyguanosine adducts crosslink to peptides via Schiff base linkage (Kurtz & Lloyd, 2003).

Several studies have indicated that crotonaldehyde, and crotonaldehyde-derived DNA adducts, can arise from acetaldehyde, a metabolite of alcohol, under physiological conditions or at biologically relevant concentrations of acetaldehyde (Stornetta, et al., 2018), indicating that alcohol exposure is confounding when performing studies of crotonaldehyde–DNA binding. Micromolar concentrations of acetaldehyde in the presence of spermidine led to formation of α-methyl-γ-hydroxy-1,N^2-propano-2′-deoxyguanosine adducts in DNA (Theruvathu et al., 2005). Crotonaldehyde can be produced in aqueous solutions of acetaldehyde by aldol condensation. Enzymatic or neutral hydrolysis of DNA in the presence of crotonaldehyde produces paraldol, the dimer of 3-hydroxy-butanal (aldol) that, when it reacts with DNA, generates a class of adducts described by Wang et al. (2000). Base treatment of acetaldehyde results in the formation of its trimer, aldoxane, which is in equilibrium with crotonaldehyde in solution. This too can lead to the formation of adducts in DNA (Wang et al., 2001), although it is not known whether aldoxane or paraldol are produced from acetaldehyde in vivo.

(ii) DNA adducts in experimental systems

See Table 4.3 and Table 4.4.

After treatment of Fischer 344 rats by gavage with a single dose of crotonaldehyde (200 mg/kg bw), 2.9 adducts/10^8 nucleotides were detected in the liver; treatment with repeated doses (1 mg/kg bw, five times per week for 6 weeks) resulted in a similar level of adduct formation (2.0 adducts/10^8 nucleotides) (Eder et al., 1999; Budiawan et al., 2000). No adducts were detected in the livers of untreated rats in these studies (limit of detection, 3 adducts/10^9 nucleotides), in contrast to studies by other investigators who reported the presence of adducts in the livers of both untreated and treated mice and rats (Chung et al., 1989; Nath & Chung, 1994; Nath et al., 1996; Pan et al., 2006). [The Working Group noted that in one of these studies treatment of rats with *N*-nitrosopyrrolidine (NPYR) also gave rise to crotonaldehyde-derived 1,N^2-propano-2′-deoxyguanosine adducts in liver (Chung et al., 1989).] DNA adducts have also been detected in mouse skin after topical treatment with crotonaldehyde (Chung et al., 1989) and in multiple tissues (lung, kidney, colonic mucosa, prostate, mammary tissue, brain, and leukocytes) of untreated rats and also in the skin of untreated mice (Nath et al., 1996).

Tissues of mice exposed to mainstream tobacco smoke (5 days per week for 12 weeks) were analysed for multiple DNA adducts, including those derived from benzo[*a*]pyrene, 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK), acrolein, and crotonaldehyde (Weng et al., 2018). Adducts derived from crotonaldehyde were detected in the lung and urinary bladder, but not in the heart and liver.

Male Wistar rats were exposed via inhalation to exhaust from either ultra-low sulfur diesel (ULSD) or biodiesel containing 30% rapeseed methyl ester in ULSD. No significant increases in the frequency of lung crotonaldehyde–DNA adducts were observed in either treatment group

Table 4.3 Detection of crotonaldehyde-derived DNA adducts in non-human mammals in vivo

Species, strain (sex)	Tissue	Exposure	Results (LED or HID)	Comments[a]	Reference
Rat, F344 (F)	Liver, lung, kidney, colon	Gavage; 200 mg/kg single dose; 1 mg/kg, 5×/wk for 6 wk	+ 200 mg/kg single dose; 30 mg/kg (total) multiple dose. No adducts detected in livers of untreated animals; LOD, 3 adducts/10^9 nucl	Purity, NR.	Eder et al. (1999); Budiawan et al. (2000)
Mouse, A/J (M) Rat, F344 (M)	Liver Liver	None (untreated aimals) None	+	Mice, 4. Rats, 4.	Nath & Chung (1994)
Mouse, A/J (F)	Skin	None (untreated animals)	+	Mice, 5	Nath et al. (1996)
Rat, F344 (M, F)	Lung, kidney, colonic mucosa, prostate, mammary tissue, brain, and leukocytes	None (untreated animals)	+	Up to 6 rats studied.	Nath et al. (1996)
Mouse, Sencar (F)	Skin	Topical, 6.7 mg 5×/wk for 3 wk	+ 100 mg		Chung et al. (1989)
Rat, F344 (M)	Liver	None (untreated animals)	+		
Rat, F344 (M)	Liver	6 mM NPYR in drinking-water for 14 days	+		
Rat, Long Evans	Liver	None (untreated animals)	+ LOD, 9 adducts/10^9 nucl	One rat analysed.	Pan et al. (2006)
Mouse, FVBN (M)	Lung	Tobacco smoke, ~75 mg/m^3 for 12 wk	+		Weng et al. (2018)

F, female; HID, highest ineffective dose; LED, lowest effective dose; LOD, limit of detection; M, male; nucl, nucleotides; NPYR, *N*-nitrosopyrrolidine; NR, not reported; wk, week.

[a] +, positive.

Table 4.4 Detection of crotonaldehyde-derived adducts with oligonucleotides and DNA

Test system (species, strain)	Results[a]	Concentration (LEC or HIC)	Comments	Reference
	Without metabolic activation			
Calf thymus DNA (acellular)	+	21 mg/mL [300 mM]		Chung et al. (1984)
T/C 25-mer (acellular)	+	1.25 M	Only one concentration tested.	Borys-Brzywczy et al. (2005)
Nucleosides and 5′-mononucleotides (acellular)	+	70 mg/mL [1 M]		Eder & Hoffman (1992)
Deoxycytidine (acellular)	+	292 mM	Only one concentration tested.	Borys-Brzywczy et al. (2005)
Deoxythymidine (acellular)	–	292 mM	Only one concentration tested.	Borys-Brzywczy et al. (2005)

HIC, highest effective concentration; LEC, lowest effective concentration; NT, not tested.
[a] +, positive; –, negative.

when compared with rats exposed to filtered air (Douki et al., 2018).

It has also been reported that crotonaldehyde forms 1,N^2-propano-2′-deoxyguanosine adducts, as detected by ^{32}P-postlabelling analysis in Chinese hamster ovary cells (Foiles et al., 1990).

In acellular studies, crotonaldehyde induced the formation of DNA adducts in calf thymus DNA (Chung et al., 1984; Kailasam & Rogers, 2007), as well as in oligonucleosides and mononucleotides (Eder & Hoffman, 1992; Borys-Brzywczy et al., 2005; see Table 4.4).

4.2.2 Is genotoxic

[The information below pertains to mixtures of the *trans*- (*E*-) and *cis*- (*Z*-) isomers of crotonaldehyde, unless stated otherwise.]

(a) Humans

(i) Exposed humans

No data were available to the Working Group.

(ii) Human cells in vitro

See Table 4.5.

Crotonaldehyde-induced DNA single-strand breaks were observed in human lymphoblastoid (Namalwa) cells (Eisenbrand et al., 1995). Dittberner et al. (1995) obtained a positive result for sister-chromatid exchange, structural chromosomal aberration, and micronucleus formation in both human primary lymphocytes and Namalwa cells treated with crotonaldehyde. However, a negative result was obtained for centromere-positive micronuclei, as detected by fluorescence in situ hybridization (FISH), in both cell lines. [The Working Group noted that this indicates a clastogenic effect.] Additionally, the lymphocytes were only examined for the number of aneuploid metaphases; no significant increase was found (Dittberner et al., 1995).

In three experiments, plasmids containing the *supF* gene were reacted with crotonaldehyde and then transfected into various human cell types to allow for repair and replication; the *supF* mutant frequency was subsequently assessed in *Escherichia coli* and found to be significantly increased in a dose-dependent manner in all cases (Czerny et al., 1998; Kawanishi et al., 1998; Weng et al., 2017). In one study in which the exposed plasmid was transfected into human hepatocellular carcinoma cells (HepG2), crotonaldehyde induced G→C transversions (41%), G→T transversions (37%), deletions (16%), and G→A

Table 4.5 Genetic and related effects of crotonaldehyde in human cells in vitro

End-point	Tissue, cell line	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
DNA single-strand breaks (alkaline elution)	Lymphoblastoid Namalwa cells	+	NT	0.2 mM		Eisenbrand et al. (1995)
Chromosomal aberrations – structural; sister-chromatid exchanges	Primary lymphocytes	+	NT	10 µM		Dittberner et al. (1995)
Chromosomal aberrations – structural	Lymphoblastoid Namalwa cells	+	NT	100 µM		Dittberner et al. (1995)
Chromosomal aberrations – numerical	Primary lymphocytes	–	NT	250 µM		Dittberner et al. (1995)
Micronucleus formation	Lymphoblastoid Namalwa cells, primary lymphocytes	+	NT	40 µM		Dittberner et al. (1995)
Micronuclei – centromere positive	Lymphoblastoid Namalwa cells, primary lymphocytes	–	NT	150 µM		Dittberner et al. (1995)
Sister-chromatid exchange	Lymphoblastoid Namalwa cells	+	NT	20 µM		Dittberner et al. (1995)
Plasmid pZ189 (exposed acellularly); transfected into transformed human normal lymphoblasts (GM0621)	Forward mutation (*supF*)	+	NT	10 mM	Plasmids exposed to crotonaldehyde then transfected into human cells to allow for repair and replication.	Czerny et al. (1998)
Plasmid pMY189 (exposed acellularly); transfected into transformed normal human fibroblasts (W138-VA13)	Forward mutation (*supF*)	+	NT	1.2 M	Plasmids exposed to crotonaldehyde then transfected into human cells to allow for repair and replication.	Kawanishi et al. (1998)
Plasmid pSP189 (exposed acellularly); transfected into human hepatocellular carcinoma cells (HepG2)	Forward mutation (*supF*)	+	NT	5 mM	Plasmids exposed to crotonaldehyde then transfected into human cells to allow for repair and replication.	Weng et al. (2017)

HIC, highest ineffective concentration; LEC, lowest effective concentration; NT, not tested.

[a] +, positive; –, negative.

transitions (6%) (Weng et al., 2017). In another *supF* shuttle-vector study using normal human fibroblasts (W138-VA13), 85% of the crotonaldehyde-induced mutations were base substitutions (single substitutions, 47%; tandem or multiple substitutions, 38%), 14% were deletions, and 1% were insertions; of the base substitutions, they found that G→T transversions predominated (50%), followed by G→A transitions (23%), and G→C transversions (13%) (Kawanishi et al., 1998). In a study in which the exposed plasmid was transfected into transformed human normal lymphoblasts (GM0621), crotonaldehyde induced primarily deletions (46%), as well as base-pair substitutions (39%), insertions (12%), and inversions (3%); two hot spot deletions were identified, which represented 62% of all deletions (Czerny et al., 1998).

In another study, a DNA vector containing either the 8*R*,6*R* or 8*S*,6*S* adducts was introduced into human xeroderma pigmentosum A (XPA) cells; both adduct isomers were found to inhibit DNA synthesis, with the 8*S*,6*S* adduct being more mutagenic than the 8*R*,6*R* isomer (10% versus 5%, respectively). Additionally, for the 8*S*,6*S* adduct, G→T transversions were the most common, followed by G→C transversions, and G→A transitions, whereas with the 8*R*,6*R* isomer, G→T and G→A were induced at almost the same frequency, followed by G→C (Stein et al., 2006).

(b) Experimental systems

(i) Non-human mammals in vivo

See Table 4.6.

Chromosomal aberrations in the bone marrow were observed in male and female Swiss albino mice exposed to crotonaldehyde as a single intraperitoneal injection, with a significant response seen at sampling times of 6, 12, and 24 hours (Jha et al., 2007). Chromosomal aberrations were observed in spermatozoa analysed 24 hours after exposure to crotonaldehyde (Jha et al., 2007). A significant increase in abnormal sperm head morphology (an end-point used as an indicator of mammalian germ cell mutagens) was observed in male Swiss albino mice in samples obtained 1, 3, and 5 weeks after a single intraperitoneal dose of crotonaldehyde (Jha & Kumar, 2006). Male Swiss albino mice exposed by intraperitoneal injection to crotonaldehyde once daily for 5 days were mated with untreated females during the post-exposure periods in weeks 1, 2, 3, 4, or 5. An increase in the number of dominant lethal mutations (DLMs) and the number of dead implants per female was reported (Jha et al., 2007). From mating week 1, a significant increase in DLMs was induced by the highest dose; for mating weeks 2 and 3, DLMs were induced by all three doses; for mating week 4, DLMs were induced by the highest dose, and from mating week 5, there was a small but non-significant dose-related increase in DLMs (Jha et al., 2007). [The Working Group noted that the examination of these end-points after different post-exposure mating schedules allows for analysis of the sensitivity of the male germ cells at different developmental stages, and that these results indicated that male mouse germ cells appear to be most sensitive to the mutagenic effects of crotonaldehyde when exposed during the repair-proficient spermatid and late spermatocyte stages.]

(ii) Non-human mammalian cells in vitro

See Table 4.7.

In crotonaldehyde-treated primary rat hepatocytes, a significant increase in the frequency of DNA single-strand breaks was observed at 1 mM, as assessed by the alkaline elution assay (Eisenbrand et al., 1995). Crotonaldehyde treatment of primary rat colon and stomach mucosa cells induced DNA damage at 0.4 mM, as assessed by the alkaline comet assay (Gölzer et al., 1996). Higher doses (up to 71.3 mM) failed to elicit a significant increase in the amount of DNA in the comet tail in primary rat hepatocytes when assessed by the comet assay; however, condensed comet heads characteristic of DNA cross links

Table 4.6 Genetic and related effects of crotonaldehyde in non-human mammals in vivo

End-point	Species, strain, (sex)	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Chromosomal aberrations	Mouse, Swiss albino, (M, F)	Bone marrow	+	8 µL/kg bw [7 mg/kg bw]	Intraperitoneal, 1×; 6 h sampling time		Jha et al. (2007)
Chromosomal aberrations	Mouse, Swiss albino, (M, F)	Bone marrow	+	8 µL/kg bw [7 mg/kg bw]	Intraperitoneal, 1×; 12 h sampling time		Jha et al. (2007)
Chromosomal aberrations	Mouse, Swiss albino, (M, F)	Bone marrow	+	8 µL/kg bw [7 mg/kg bw]	Intraperitoneal, 1×; 24 h sampling time		Jha et al. (2007)
Chromosomal aberrations	Mouse, Swiss albino, (M)	Spermatozoa	+	16 µL/kg bw [14 mg/kg bw]	Intraperitoneal, 1×; 24 h sampling time		Jha et al. (2007)
Sperm head morphology	Mouse, Swiss albino (M)	Spermatozoa	+	16 µL/kg bw [14 mg/kg bw]	Intraperitoneal 1×, 1-wk sampling time	Treated cells were spermatozoa.	Jha & Kumar (2006)
Sperm head morphology	Mouse, Swiss albino (M)	Spermatozoa	+	16 µL/kg bw [14 mg/kg bw]	Intraperitoneal 1×, 3-wk sampling time	Treated cells were spermatids (repair-proficient).	Jha & Kumar (2006)
Sperm head morphology	Mouse, Swiss albino (M)	Spermatozoa	+	27 mg/kg bw	Intraperitoneal 1×, 5-wk sampling time	Treated cells were preleptotene spermatocytes.	Jha & Kumar (2006)
Dominant lethal	Mouse, Swiss albino, (M)	Embryos in non-exposed pregnant females	+	27 mg/kg bw	Intraperitoneal, 1×/day for 5 days, mating schedule 1–7 days	Treated cells were spermatids (repair-deficient due to highly condensed chromatin).	Jha et al. (2007)
Dominant lethal	Mouse, Swiss albino, (M)	Embryos in non-exposed pregnant females	+	7 mg/kg bw	Intraperitoneal, 1×/day for 5 days, mating schedule 8–14 days	Treated cells were spermatids (repair-proficient).	Jha et al. (2007)
Dominant lethal	Mouse, Swiss albino, (M)	Embryos in non-exposed pregnant females	+	7 mg/kg bw	Intraperitoneal, 1×/day for 5 days, mating schedule 15–21 days	Treated cells were spermatocytes.	Jha et al. (2007)
Dominant lethal	Mouse, Swiss albino (M)	Embryos in non-exposed pregnant females	+	27 mg/kg bw	Intraperitoneal, 1×/day for 5 days, mating schedule 22–28 days	Treated cells were preleptotene spermatocytes and spermatocytes.	Jha et al. (2007)

Table 4.6 (continued)

End-point	Species, strain, (sex)	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Dominant lethal	Mouse, Swiss albino (M)	Embryos in non-exposed pregnant females	–	27 mg/kg bw	Intraperitoneal, 1×/day for 5 days, mating schedule 29–35 days	Treated cells were spermatogonia.	Jha et al. (2007)

bw, body weight; F, female; h, hour; HID, highest ineffective dose; LED, lowest effective dose; M, male; wk, week.
[a] +, positive; –, negative.

Table 4.7 Genetic related effects of crotonaldehyde in non-human mammalian cells in vitro

End-point	Species, tissue, cell line	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
DNA single-strand breaks (alkaline elution assay)	Wistar rat, primary hepatocytes	+	NT	1 mM		Eisenbrand et al. (1995)
DNA damage (comet, alkaline)	Sprague-Dawley rat, primary colon mucosa cells	+	NT	0.4 mM	Comets were classified into three size classes depending on tail length.	Gölzer et al. (1996)
DNA damage (comet, alkaline)	Sprague-Dawley rat, primary colon mucosa cells	+	NT	0.4 mM	Comets were classified into three size classes depending on tail length.	Gölzer et al. (1996)
DNA damage (comet, alkaline)	Wistar rat, primary hepatocytes	–	NT	71.3 mM	94% of cells had a central condensed spot characteristic of DNA and/or protein crosslinks. High concentrations used.	Kuchenmeister et al. (1998)
DNA and/or protein cross-links (comet, alkaline)	Wistar rat, primary hepatocytes	+	NT	28.5 mM		Kuchenmeister et al. (1998)
Unscheduled DNA synthesis	Rat, primary rat hepatocytes	–	NT	125 µM		Williams et al. (1989)
Gene mutation (*Tk*)	Mouse, lymphoma L5178Y/*Tk*$^{+/-}$ -3.7.2C cells	+	NT	25 µM		Demir et al. (2011)
Gene mutation (*Hgprt*)	Chinese hamster, fibroblasts, V79-4	+	NT	10 µM	Only tested concentration.	Li et al. (2012)
Gene mutation (*Hgprt*)	Chinese hamster, fibroblasts, V79-4, expressing human AKR7A2	+	NT	10 µM	Only tested concentration.	Li et al. (2012)

AKR, aldo-keto reductase; HIC, highest ineffective concentration; Hgprt, hypoxanthine-guanine phosphoribosyltransferase; LEC, lowest effective concentration; NT, not tested; Tk, thymidine kinase.

[a] +, positive; –, negative.

were observed at 28.5 mM (Kuchenmeister et al., 1998). [The Working Group noted that similar comet responses for other cross-linking chemicals have been reported elsewhere (Pfuhler & Wolf, 1996; Merk & Speit, 1999). The Working Group also noted the high concentrations used.] A negative response was obtained for unscheduled DNA synthesis in crotonaldehyde-treated primary rat hepatocytes (Williams et al., 1989). Crotonaldehyde treatment resulted in a significant increase in the frequency of mutations of the *Tk* gene in mouse lymphoma cells (L5178Y) (Demir et al., 2011), and the *Hgprt* gene in standard Chinese hamster fibroblasts (V79-4), as well as in V79-4 cells expressing the human aldo-keto reductase enzyme AKR7A2 (Li et al., 2012).

Using shuttle vectors containing either adduct isomer, the 8*R*,6*R* and 8*S*,6*S* crotonaldehyde-derived 1,N^2-propano-2′-guanosine adducts were found to be mutagenic in African green monkey kidney (COS-7) cells, with similar percentage mutagenicity observed for both isomers (i.e. 4.7% and 6.2%, respectively) (Fernandes et al., 2005).

(iii) Non-mammalian experimental systems

See Table 4.8.

In *Drosophila melanogaster*, a negative result was obtained for sex-linked recessive lethal mutation when crotonaldehyde was administered in the feed, but the result was positive for both sex-linked recessive lethal mutations and heritable translocations when crotonaldehyde was administered by injection (Woodruff et al., 1985). A positive response was observed in the somatic mutation and recombination test (SMART) wing spot mutation assay with crotonaldehyde in the feed (Demir et al., 2013).

Crotonaldehyde has been evaluated in several *Salmonella typhimurium* strains that are sensitive to base-pair substitutions (i.e. strains TA1535, TA100, and TA104) and frameshift mutations (i.e. strains TA1537, TA1538, and TA98). However, no strains specific to the detection of cross-linking agents (e.g. TA102) were employed. In some cases, tests were only carried out without metabolic activation (–S9); with metabolic activation (+S9), the number of revertants was lowered. In the base-pair substitution strains, crotonaldehyde gave negative results with and without metabolic activation in several plate-incorporation assays with strain TA1535 (Lijinsky & Andrews, 1980; Neudecker et al., 1981; Haworth et al., 1983) and TA100 (Lijinsky & Andrews, 1980; Neudecker et al., 1981); however, in strain TA100 two positive results with and without metabolic activation were also observed (Haworth et al., 1983; Neudecker et al., 1989). When the more sensitive preincubation version of the assay was employed, a negative result was still obtained in strain TA1535 (Grúz et al., 2018). However, in strain TA100 the result was positive with and (when tested) without metabolic activation in five of the six preincubation assays (Lijinsky & Andrews, 1980; Neudecker et al., 1981, 1989; Cooper et al., 1987; Eder et al., 1992; Grúz et al., 2018). A positive response was obtained in strain TA104 without metabolic activation (Marnett et al., 1985). Crotonaldehyde gave negative results with and without metabolic activation in the frameshift strains TA1537, TA1538, and TA98 (Lijinsky & Andrews, 1980; Neudecker et al., 1981, 1989; Haworth et al., 1983; Eder et al., 1992; Grúz et al., 2018). Positive results without metabolic activation were observed in several YG test strains engineered with different polymerases (Grúz et al., 2018). A weak positive response for SOS induction was observed in strain TA1535 (Benamira & Marnett, 1992), and two negatives and a weak positive result were obtained in the SOS chromotest in *Escherichia coli* when DMSO was used as the solvent (Eder et al., 1992, 1993; Eder & Deininger, 2002); however, when ethanol was used as the solvent in two additional assays, robust positive responses were observed (Eder et al., 1993; Eder & Deininger, 2002). Negative results were obtained for both forward and reverse mutation in *Salmonella typhimurium* BA9 when the plate-incorporation version was

Table 4.8 Genetic and related effects of crotonaldehyde in non-mammalian experimental systems

Test system (species, strain)	End-point	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Drosophila melanogaster	Sex-linked recessive lethal mutation	–	NA	4000 ppm [57 mM] (feed)		Woodruff et al. (1985)
Drosophila melanogaster	Sex-linked recessive lethal mutation	+	NA	3500 ppm [50 mM] (injection)		Woodruff et al. (1985)
Drosophila melanogaster	Heritable translocation	+	NA	3500 ppm [50 mM] (injection)		Woodruff et al. (1985)
Drosophila melanogaster	SMART wing spot mutation	+	NA	25 mM (feed)	Small spots only, mwh/flr3 only.	Demir et al. (2013)
Salmonella typhimurium TA1535 pSK 1002	SOS (*umu*) induction assay, DNA damage	(+)	NT	21 µg/mL		Benamira & Marnett (1992)
Salmonella typhimurium TA1535	Reverse mutation	–	–	167 µg/plate	Purity, 83%.	Haworth et al. (1983)
Salmonella typhimurium TA1535	Reverse mutation	–	–	1000 µg/plate		Lijinsky & Andrews (1980)
Salmonella typhimurium TA1535	Reverse mutation	–	–	NR		Neudecker et al. (1981)
Salmonella typhimurium TA1535, preincubation assay	Reverse mutation	–	NT	3 µg/plate		Grúz et al. (2018)
Salmonella typhimurium TA100	Reverse mutation	+	+	21 µg/mL		Neudecker et al. (1989)
Salmonella typhimurium TA100	Reverse mutation	+	+	100 µg/plate	Purity, 83%.	Haworth et al. (1983)
Salmonella typhimurium TA100	Reverse mutation	–	–	1000 µg/plate		Lijinsky & Andrews (1980)

Table 4.8 (continued)

Test system (species, strain)	End-point	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Salmonella typhimurium TA100	Reverse mutation	–	–	NR		Neudecker et al. (1981)
Salmonella typhimurium TA100, preincubation assay	Reverse mutation	+	+	10 µg/plate		Lijinsky & Andrews (1980)
Salmonella typhimurium TA100, preincubation assay	Reverse mutation	–	NT	0.54 mM [37.8 µg/mL]	Purity, 85%.	Cooper et al. (1987)
Salmonella typhimurium TA100, preincubation assay	Reverse mutation	+	NT	70 µg/plate		Neudecker et al. (1989)
Salmonella typhimurium TA100, preincubation assay	Reverse mutation	+	NT	2 µg/plate		Grúz et al. (2018)
Salmonella typhimurium TA100, preincubation assay	Reverse mutation	+	+	NR		Eder et al. (1992)
Salmonella typhimurium TA100, liquid suspension	Reverse mutation	+	+	0.025 µl/mL [21 µg/mL] (-S9), 0.075 µl/mL [64 µg/mL] (+S9)	Modified pre-incubation assay was performed suspended in either 0.1 M phosphate buffer or nutrient broth. Both were positive, but a more sensitive result was obtained with phosphate buffer.	Neudecker et al. (1981)
Salmonella typhimurium TA104	Reverse mutation	+	NT	20 µg/plate		Marnett et al. (1985)
Salmonella typhimurium TA1538	Reverse mutation	–	–	1000 µg/plate		Lijinsky & Andrews (1980)

Table 4.8 (continued)

Test system (species, strain)	End-point	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Salmonella typhimurium TA1538	Reverse mutation	–	–	NR		Neudecker et al. (1981)
Salmonella typhimurium TA98	Reverse mutation	–	–	1000 µg/plate		Lijinsky & Andrews (1980)
Salmonella typhimurium TA98	Reverse mutation	–	–	167 µg/plate	Purity, 83%.	Haworth et al. (1983)
Salmonella typhimurium TA98	Reverse mutation	–	–	NR		Neudecker et al. (1981)
Salmonella typhimurium TA1537	Reverse mutation	–	–	167 µg/plate	Purity, 83%.	Haworth et al. (1983)
Salmonella typhimurium TA1537	Reverse mutation	–	–	500 µg/plate		Lijinsky & Andrews (1980)
Salmonella typhimurium TA1537	Reverse mutation	–	–	NR		Neudecker et al. (1981)
Salmonella typhimurium YG6248, preincubation assay	Reverse mutation	+	NT	2 µg/plate		Grúz et al. (2018)
Salmonella typhimurium YG5197, YG9060 preincubation assay	Reverse mutation	+	NT	1 µg/plate		Grúz et al. (2018)
Salmonella typhimurium YG9028, YG6251, YG9135 preincubation assay	Reverse mutation	+	NT	2 µg/plate		Grúz et al. (2018)
Salmonella typhimurium YG5196, preincubation assay	Reverse mutation	–	NT	3 µg/plate		Grúz et al. (2018)

Table 4.8 (continued)

Test system (species, strain)	End-point	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Salmonella typhimurium BA9	Forward mutation	–	NT	1836 nM		Ruiz-Rubio et al. (1984)
Salmonella typhimurium BA9	Reverse mutation	–	NT	1836 nM		Ruiz-Rubio et al. (1984)
Salmonella typhimurium BA9, preincubation assay	Forward mutation	+	NT	612 nmol/plate [43 µg/plate]		Ruiz-Rubio et al. (1984)
Salmonella typhimurium BA9, preincubation assay	Reverse mutation	+	NT	612 nmol/plate [43 µg/plate]		Ruiz-Rubio et al. (1984)
Escherichia coli PQ37, SOS chromotest	DNA damage	–	NT	NR		Eder et al. (1992)
Escherichia coli PQ37, SOS chromotest	DNA damage	–	NT	NR		Eder et al. (1993)
Escherichia coli PQ37, SOS chromotest	DNA damage	+	NT	NR	Ethanol used as solvent in place of DMSO.	Eder et al. (1993)
Escherichia coli PQ37, SOS chromotest	DNA damage	(+)	NT	NR	Weak positive when tested with DMSO (no SOSIP; I_{max}, < 1.5).	Eder & Deininger (2002)
Escherichia coli PQ37, SOS chromotest	DNA damage	+	NT	NR	Positive when ethanol used as solvent.	Eder & Deininger (2002)
Calf thymus DNA (acellular)	DNA damage (fluorescence screening for changes in DNA melting and annealing behaviour)	+	NT	100 mM		Kailasam & Rogers (2007)

DMSO, dimethyl sulfoxide; flr, flare; HIC, highest effective concentration; I_{max}, maximal concentration for induction; LEC, lowest effective concentration; mwh, multiple wing hairs; NA, not applicable; NR, not reported; NT, not tested; ppm, parts per million; SMART, somatic mutation and recombination test; SOSIP, SOS-inducing potency.

[a] +, positive; –, negative; (+), positive in a study of limited quality.

carried out, but robust increases in forward and reverse mutation were observed with the more sensitive preincubation version (Ruiz-Rubio et al., 1984).

An increase in DNA damage, assessed via a fluorescence-based screen quantifying changes in DNA melting/annealing behaviour, was observed in calf thymus DNA reacted with crotonaldehyde in an acellular study (Kailasam & Rogers, 2007).

4.2.3 Alters DNA repair

A single study on the ability of crotonaldehyde to alter DNA repair was available. Using the host cell reactivation assay, crotonaldehyde was found to inhibit both nucleotide excision repair and base excision repair capacity in human hepatocellular carcinoma cells (HepG2) (Weng et al., 2017). In a subsequent experiment, nucleotide excision repair was instantaneously inhibited when crotonaldehyde was added to cell lysates, indicating that crotonaldehyde reacts with and inhibits proteins that are critical for nucleotide excision repair (Weng et al., 2017).

4.2.4 Induces oxidative stress

(a) Humans

No data in exposed humans were available to the Working Group.

In vitro studies in human vein endothelial cells demonstrated that crotonaldehyde (50 μM; 1 hour) increases the formation of reactive oxygen species (Ryu et al., 2013). Crotonaldehyde treatment also increased gene expression and protein levels of haem oxygenase 1 in a dose-dependent manner, consistent with a cellular response to oxidative stress (Lee et al., 2011). In human bronchial epithelial cells, crotonaldehyde decreased concentrations of intracellular glutathione (at up to 10 μM) and increased the formation of reactive oxygen species (at 40 μM) (Liu et al., 2010).

(b) Experimental systems

See Table 4.9.

In rats, depletion of hepatic glutathione (a marker of oxidative stress) occurs after acute intraperitoneal administration of crotonaldehyde (Cooper et al., 1992). In male Wistar rats, subchronic oral administration of crotonaldehyde increased production of proinflammatory cytokines and elevated serum malondialdehyde concentrations, indicative of increased lipid peroxidation (Zhang et al., 2019b). In another study in male Wistar rats, subchronic (up to 120 days) oral exposure to crotonaldehyde decreased serum glutathione peroxidase and superoxide dismutase activity and elevated malondialdehyde concentration (Li et al., 2020).

In vitro studies have shown that crotonaldehyde exposure can inhibit glutathione *S*-transferase activity, resulting in depletion of intracellular glutathione (van Iersel et al., 1996). Crotonaldehyde exposure for 4 hours decreased intracellular glutathione concentration (at 25 μM) and increased reactive oxygen species formation (at ≥ 25 μM) in a rat alveolar macrophage cell line (Yang et al., 2013a).

4.2.5 Induces chronic inflammation

(a) Humans

No data were available to the Working Group.

(b) Experimental systems

See Table 4.9.

In a study of subchronic toxicity (120 days) in rats treated by gavage, crotonaldehyde was associated with myocardial necrosis, cardiac fibrosis, renal tubular epithelial cell oedema, and renal lymphocyte infiltration, suggestive of an inflammatory response (Zhang et al., 2019b). In a study of chronic toxicity in male rats treated by inhalation, crotonaldehyde was associated with a dose-dependent increase in the incidence and severity of inflammation in the nasal respiratory

Table 4.9 Effects of crotonaldehyde on markers of oxidative stress or chronic inflammation in non-human mammals in vivo

End-point	Species, strain (sex)	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Reference
Oxidative stress markers						
GSH	Rat, F344 (M)	Liver	↓	450 µmol/kg [31.5 mg/kg]	Intraperitoneal, 1×	Cooper et al. (1992)
MDA	Rat, Wistar (M)	Serum	↑	8.5 mg/kg per day	Oral (gavage), 120 days	Zhang et al. (2019b)
GPx, SOD	Rat, Wistar (M)	Serum	↓	8.5 mg/kg per day	Oral (gavage), 120 days	Zhang et al. (2019b)
GPx, MDA, SOD	Rat, Wistar (M)	Lung	↓	4.5 mg/kg per day	Oral (gavage), 120 days	Li et al. (2020)
Inflammation markers						
Inflammatory cell infiltration, oedema, or inflammatory markers	Rat, Wistar (M)	Heart Kidney	↑ ↑	4.5 mg/kg per day	Oral (gavage), 120 days	Zhang et al. (2019b)
Respiratory epithelial inflammation	Rat, F344 (M, F)	Nasal cavity	↑	3 ppm (M), 6 ppm (F)	Inhalation, 6 h/day, 5 days/wk, 104 wk	JBRC (2001e)
Respiratory epithelial inflammation	Mouse, Crj:BDF1 (M,F)	Nasal cavity	↑	12 ppm (F only; no effect in M)	Inhalation, 6 h/day, 5 days/wk, 104 wk	JBRC (2001b)
Inflammatory cell infiltration, macrophage phagocytic ability and number, shifts in T lymphocyte populations	Rat, Wistar (M)	Lung (BALF)	↑	4 µL/kg [3.4 mg/kg]	Intratracheal instillation, 1×	Wang et al. (2018)
Inflammatory cell infiltration	Rat, Wistar (M)	Lung	↑	4.5 mg/kg per day	Oral (gavage), 120 days	Li et al. (2020)

BALF, bronchoalveolar lavage fluid; F, female; GPx, glutathione peroxidase; GSH, glutathione; h, hour; HID, highest ineffective dose; LED, lowest effective dose; M, male; MDA, malondialdehyde; ppm, parts per million; SOD, superoxide dismutase; wk, week.
[a] ↑, increase; ↓, decrease.

epithelium (JBRC, 2001b). In female mice and rats exposed to crotonaldehyde by inhalation in a study of chronic toxicity, inflammation was seen at 12 and 6 ppm, respectively (JBRC, 2001e, b); see also Section 3. [The Working Group noted that changes in cell proliferation in response to crotonaldehyde exposure has not been evaluated in experimental systems.] Intratracheal instillation of crotonaldehyde resulted in inflammatory cell infiltration, shift in the number of CD4+ and CD8+ T lymphocytes, decreased numbers of mononuclear phagocytes in bronchoalveolar lavage fluid in male Wistar rats (Wang et al., 2018). In a study of subchronic toxicity (up to 120 days) in male Wistar rats, oral exposure to crotonaldehyde (at 4.5 mg/kg bw) resulted in increased inflammatory cell infiltration, as well as increased lung concentrations of TNFα, interleukin 6 (IL6), and IL1β (Li et al., 2020).

4.2.6 Other key characteristics

(a) Is immunosuppressive

No data in exposed humans were available to the Working Group.

In cultured human monocytic U937 cells differentiated along the macrophagic line, crotonaldehyde increased the release of IL8 and TNFα (Facchinetti et al., 2007). In cultured human macrophages, human lung fibroblasts, and small

airway epithelial cells, crotonaldehyde increased the release of IL8, and this response was mediated via p38 MAPK- and ERK1/2-dependent pathways (Moretto et al., 2009).

Shifts in T-lymphocyte populations, decreased numbers of mononuclear phagocytes in bronchoalveolar lavage fluid, and decreased lung macrophage function were reported in male Wistar rats after intratracheal instillation of crotonaldehyde in the study of Wang et al. (2018) referenced above (see Table 4.9). Crotonaldehyde was found to suppress phagocytic function in cultured rat alveolar macrophages and was associated with a dose-dependent decrease in cell viability (Yang et al., 2013b).

(b) Modulates receptor-mediated effects

No data in exposed humans were available to the Working Group.

Crotonaldehyde activated peroxisome proliferator-activated receptor PPARγ and PPARβ/δ luciferase reporter activity in a dose-dependent manner in cultured TSA201 cells derived from human embryonic kidney cells (HEK293) (Matsushita et al., 2019). Crotonaldehyde enhanced thyroid hormone action by modulating thyroid hormone binding to thyroid hormone receptors (TR) resulting in upregulation of gene transcription in cultured human embryonal kidney (TSA 201) cells (Hayashi et al., 2018). In TSA201 cells transfected with the ligand-binding domain of TRα1 or TRβ1 coupled to a luciferase reporter system, it was demonstrated that, in the presence of thyroid hormone, crotonaldehyde induced TRα1-mediated transcription activity while not affecting TRβ1 (Hayashi et al., 2018).

(c) Multiple characteristics

Transcript profiling has been performed in a human monocytic leukaemia THP-1 cell line exposed to crotonaldehyde (Yang et al., 2014). In this system, 342 or 663 genes were statistically significantly differentially expressed after either a 6- or 12-hour exposure, respectively, to crotonaldehyde at 80 µM (Yang et al., 2014). Crotonaldehyde affected the expression of genes related to oxidative stress, including several involved in glutathione metabolism. Haeme oxygenase 1 (*HO-1*) was also upregulated after crotonaldehyde exposure (Yang et al., 2014). Other pathways dysregulated by crotonaldehyde exposure included those involved in apoptosis and regulating cellular responses to DNA damage (Yang et al., 2014).

Liu et al. (2010) evaluated transcript profiles in human bronchial epithelial cells exposed to crotonaldehyde. Multiple inflammatory responsive genes (e.g. *XCL1, CXCL2, CXCL3, CCL2, CSF1, CSF2, NFKBIA, NFKBIZ*) were downregulated by crotonaldehyde, whereas fewer genes (*CMTM, PAG1,* and *PTX3)* were upregulated. Some genes involved in cytokine production and inflammation (*IL6, IL8*) were downregulated, whereas *HOX-1* was upregulated after treatment with crotonaldehyde (Liu et al., 2010).

4.3 Other relevant evidence

4.3.1 Humans

No data were available to the Working Group.

4.3.2 Experimental systems

See Table 4.9.

Two-year studies have been performed in F344/DuCrj rats and Crj:BDF1 mice treated with crotonaldehyde by inhalation with whole-body exposure (JBRC, 2001b, e). Rats and mice (in groups of 50 per species, sex, and dose group) were exposed at 0, 3, 6, or 12 ppm (6 hours per day, 5 days per week for 104 weeks). In male and female rats, chronic inhalation of crotonaldehyde was associated with a dose-dependent increase in the incidence and severity of inflammation and squamous cell hyperplasia and metaplasia in the nasal respiratory epithelium, and necrosis and atrophy in the olfactory epithelium (JBRC,

2001e). In male and female mice, chronic inhalation of crotonaldehyde of 12 ppm was associated with an increased incidence of squamous cell metaplasia of the nasal respiratory epithelium (JBRC, 2001b). Evidence of inflammation in the nasal respiratory epithelium was only seen in female mice at 12 ppm. Atrophy and metaplasia of the olfactory epithelium was seen in male and female mice at 12 ppm (JBRC, 2001b).

5. Summary of Data Reported

5.1 Exposure characterization

Crotonaldehyde is a High Production Volume chemical that is produced by the aldolization reaction of acetaldehyde. It is a reactive chemical and is widely used for synthesizing other chemicals, including the food preservative sorbic acid and vitamin E (two major products), but also for the production of intermediates such as crotonic acid, crotyl alcohol, *n*-butanal, and *n*-butanol in different industries such as pharmaceuticals, rubber, chemicals, leather, and food and agriculture.

Crotonaldehyde occurs naturally in a ubiquitous fashion. It is produced endogenously by plants and animals including humans as part of lipid peroxidation and metabolism. It is found in many foods and beverages.

Tobacco smoke is a major exposure source in the general population, followed by gasoline and diesel engine exhaust, indoor cooking on wood-burning stoves, heating by coal and coal briquette fuels, and heated cooking oil. The urinary metabolites *N*-acetyl-*S*-(3-hydroxy-1-methylpropyl)-L-cysteine (HMPMA) and *N*-acetyl-*S*-(3-carboxy-1-methylpropyl)-L-cysteine (CMEMA) have been studied as markers to assess exposure, but no accepted reference values are available for these metabolites.

Occupational exposure to crotonaldehyde occurs through its application in industry and wherever organic material is burned; however, no data were found on workers' exposure during these processes. Air concentrations of crotonaldehyde were reported in studies among workers in a plant producing aldehydes, garage workers, workers in toll booths, firefighters, as well as coke-oven workers.

Occupational exposure reference values exist for crotonaldehyde and acute environmental exposure values are also available.

5.2 Cancer in humans

One occupational cohort study and three nested case–control studies in population-based cohorts were available. The study in an occupational cohort was uninformative due to small numbers, poor external exposure assessment and flaws in design. Two nested case–control studies in a population-based cohort studied several biomarkers (including metabolites of crotonaldehyde) in relation to lung cancer among current smokers and non-smokers respectively, without demonstrating an etiological association with crotonaldehyde exposure. The third nested case–control study reported on colorectal cancers in relation to crotonaldehyde adducts. In summary, all studies were judged to be uninformative in terms of providing evidence on a causal relationship between crotonaldehyde exposure and cancer in humans. The studies were either of poor quality regarding design or exposure assessment, or they were of a mechanistic nature.

5.3 Cancer in experimental animals

Exposure to crotonaldehyde caused an increase in the incidence of an appropriate combination of benign and malignant neoplasms in a single sex and species in one study, and an increase in the incidence of a very rare benign neoplasm in a second study.

In the first study, there was a significant increase in the incidence of hepatocellular adenoma and of hepatocellular adenoma or carcinoma (combined) in male Fischer 344 rats given drinking-water containing crotonaldehyde.

In the second study, there was a low incidence of nasal cavity adenoma in male F344/DuCrj rats exposed to crotonaldehyde by inhalation. Nasal cavity adenoma is a very rare tumour in the rat strain used in this study.

5.4 Mechanistic evidence

The available data on the absorption and distribution of crotonaldehyde in humans are scarce. Nonetheless, increased concentrations of crotonaldehyde metabolites in the urine of tobacco smokers are consistent with absorption. Crotonaldehyde is efficiently conjugated with glutathione, ultimately yielding HMPMA and CMEMA as urinary metabolites in humans and in rats. Other metabolic pathways are reduction to crotyl alcohol, catalysed by aldo-keto reductases, and oxidation to crotonic acid, catalysed by aldehyde dehydrogenases. In rats treated intraperitoneally or by oral gavage the primary routes of elimination are through the urine (as mercapturates) and the breath (as exhaled carbon dioxide).

There is consistent and coherent evidence that crotonaldehyde exhibits multiple key characteristics of carcinogens. Crotonaldehyde is an electrophilic bifunctional α,β-unsaturated aldehyde (enal) that can form cyclic adducts in DNA, DNA interstrand crosslinks and DNA–protein crosslinks. It forms DNA adducts in vivo and in vitro. The identified adducts formed in vivo are two diastereoisomeric forms of α-methyl-γ-hydroxy-1,N^2-propano-2′-deoxyguanosine. These crotonaldehyde adducts have been detected in normal human liver and in other normal tissues including peripheral blood, mammary tissue, oral (gingival) tissue, liver, and placenta, and in saliva and urine. In studies in which smokers and non-smokers were compared, adduct levels were significantly elevated in tobacco smokers, indicating their formation by crotonaldehyde in tobacco smoke; their presence in tissues of non-smokers which may be indicative of crotonaldehyde formation by endogenous processes, including lipid peroxidation, or from other external sources. In human cells treated in vitro with the agent, several studies have demonstrated the formation of crotonaldehyde-derived DNA adducts. In rats treated with crotonaldehyde by gavage, DNA adducts were detected in the liver. In some studies, but not all, the presence of crotonaldehyde-derived adducts has been reported in various tissues, including the liver, of untreated rodents. In mice chronically exposed to mainstream tobacco smoke, DNA adducts derived from crotonaldehyde were detected in the lung and bladder, but not in the heart and liver. Crotonaldehyde and crotonaldehyde-derived DNA adducts can also be formed in the presence of biologically relevant concentrations of acetaldehyde, a metabolite of ethanol, under physiological conditions. Crotonaldehyde is also a metabolite of *N*-nitrosopyrrolidine, a carcinogenic environmental nitrosamine.

Crotonaldehyde is genotoxic. No data in exposed humans were available to the Working Group. In human primary cells and human cell lines, crotonaldehyde was clastogenic. In Swiss albino mice, crotonaldehyde induced dominant lethality in embryos, and induced chromosomal aberrations in bone marrow and spermatozoa. In cultured rodent cells, crotonaldehyde induced DNA damage and gene mutations at *Tk* and *Hprt* loci. Crotonaldehyde induced mutations in *Drosophila melanogaster*, and induced base-pair substitution mutations in the absence of metabolic activation in *Salmonella typhimurium*. Crotonaldehyde induced *supF* mutations in exposed plasmids.

Crotonaldehyde induces oxidative stress. No data in exposed humans were available. In vitro exposure of human endothelial cells or bronchial

epithelial cells to crotonaldehyde resulted in increased production of reactive oxygen species. Crotonaldehyde also decreased intracellular glutathione concentration in human bronchial epithelial cells. Depletion of hepatic glutathione occurs in rats after acute intraperitoneal administration of crotonaldehyde. Subchronic oral administration of crotonaldehyde to rats increased proinflammatory cytokine concentrations and elevated serum malondialdehyde concentration, indicating increased lipid peroxidation. Subchronic oral administration of crotonaldehyde to rats increased lung malondialdehyde concentration. In vitro studies in rodent cells showed that crotonaldehyde inhibits glutathione *S*-transferase activity, depletes intracellular glutathione concentrations, and increases the formation of reactive oxygen species.

Crotonaldehyde induces chronic inflammation, with mild increases in inflammation in the nasal respiratory epithelium reported in rats and mice in studies of chronic toxicity. In studies of subchronic toxicity in rodents, crotonaldehyde showed either renal lymphocyte infiltration after oral exposure or a dose-dependent increase in the incidence and severity of inflammation in the nasal respiratory epithelium after inhalation.

Few data were available regarding other key characteristics of carcinogens. Regarding whether crotonaldehyde is immunosuppressive, crotonaldehyde exposure altered cytokine release in human cells in vitro. Shifts in T-lymphocyte populations, decreased numbers of mononuclear phagocytes in bronchoalveolar lavage fluid, and decreased lung macrophage function have been observed in rats after intratracheal instillation of crotonaldehyde. Crotonaldehyde also suppressed phagocytic function in cultured rat alveolar macrophages.

6. Evaluation and Rationale

6.1 Cancer in humans

There is *inadequate evidence* in humans regarding the carcinogenicity of crotonaldehyde.

6.2 Cancer in experimental animals

There is *limited evidence* in experimental animals for the carcinogenicity of crotonaldehyde.

6.3 Mechanistic evidence

There is *strong evidence* that crotonaldehyde exhibits multiple key characteristics of carcinogens from studies in human primary cells and in various experimental systems, supported by studies in humans for DNA adducts.

6.4 Overall evaluation

Crotonaldehyde is *possibly carcinogenic to humans (Group 2B).*

6.5 Rationale

The *Group 2B* evaluation for crotonaldehyde is based on *strong* mechanistic evidence. There is *strong evidence* in human primary cells that crotonaldehyde exhibits key characteristics of carcinogens; crotonaldehyde is electrophilic and genotoxic. It also induces oxidative stress and induces chronic inflammation in experimental systems. In addition, there is supporting evidence from studies in humans for DNA adducts.

There is also *limited evidence* for cancer in experimental animals, based on an increase in the incidence of an appropriate combination of benign and malignant neoplasms in a single sex and species in one study, and an increase in the incidence of a very rare benign neoplasm in a

second study. The evidence regarding cancer in humans is *inadequate*. The few available studies were small, and/or had major design limitations, and/or could not distinguish the effects of crotonaldehyde exposure from other constituents of cigarette smoking.

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ARECOLINE

1. Exposure Characterization

1.1 Identification of the agent

1.1.1 Nomenclature

Chem. Abstr. Serv. Reg. No.: 63-75-2 (free base); 300-08-3 (hydrobromide); 61-94-9 (hydrochloride)

EC/List No.: 200-565-5 (free base); 206-087-3 (hydrobromide); 200-523-6 (hydrochloride)

Deleted CAS Reg. Nos: 1398-01-2 (free base)

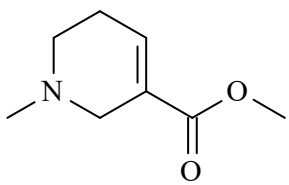
Chem. Abstr. Serv. name: 3-pyridinecarboxylic acid, 1,2,5,6-tetrahydro-1-methyl-, methyl ester (free base); 3-pyridinecarboxylic acid, 1,2,5,6-tetrahydro-1-methyl-, methyl ester, hydrobromide (1:1); 3-pyridinecarboxylic acid, 1,2,5,6-tetrahydro-1-methyl-, methyl ester, hydrochloride (1:1)

IUPAC systematic name: methyl 1-methyl-3,6-dihydro-2*H*-pyridine-5-carboxylate (free base); methyl 1-methyl-3,6-dihydro-2*H*-pyridinium-5-carboxylate hydrobromide; methyl 1-methyl-3,6-dihydro-2*H*-pyridinium-5-carboxylate hydrochloride

Synonyms: arecoline (6CI); nicotinic acid, 1,2,5,6-tetrahydro-1-methyl-, methyl ester (8CI); 1-methyl-1,2,5,6-tetrahydro-pydine-3-carboxylic acid methyl ester; 3-methoxycarbonyl-1-methyl-1,2,5,6-tetrahydropyridine; arecaidine methyl ester; arecolin; arecoline base; methyl 1,2,5,6-tetrahydro-1-methyl-nicotinate; methyl *N*-methyl-1,2,5,6-tetrahydronicotinate; methyl arecaidine; methylarecaidine; NSC 56321 (free base); arecoline hydrobromide; methyl 1,2,5,6-tetrahydro-1-methylnicotinate hydrobromide; methyl *N*-methyl-1,2,5,6-tetrahydronicotinate hydrobromide; nicotinic acid, 1,2,5,6-tetrahydro-1-methyl-, methyl ester, hydrobromide; taeniolin (hydrobromide); nicotinic acid, 1,2,5,6-tetrahydro-1-meth-yl, methyl ester, hydrochloride (O'Neil, 2013; ECHA, 2020a, b, c; NCBI, 2020a, b, c; SciFinder, 2020a, b, c).

1.1.2 Structural and molecular formulae, and relative molecular mass

Structural formula of the free base:



Molecular formula: $C_8H_{13}NO_2$ (free base); $C_8H_{13}NO_2$·HBr (hydrobromide); $C_8H_{13}NO_2$·HCl (hydrochloride)

Relative molecular mass: 155.20 (free base); 236.11 (hydrobromide); 191.65 (hydro-

chloride) (O'Neil, 2013; NCBI, 2020a, b, c; SciFinder, 2020a, b, c).

1.1.3 Chemical and physical properties

(a) Free base

Description: oily liquid (O'Neil, 2013)

Boiling point: 209 °C (O'Neil, 2013)

Melting point: < 25 °C (SciFinder, 2020a)

Density: 1.0495 g/cm^3 at 20 °C (SciFinder, 2020a)

Solubility: soluble in chloroform; miscible with water, alcohol, ether (O'Neil, 2013)

Acid dissociation constant: pK_a 6.84 (O'Neil, 2013)

Reactivity: arecoline is a strong base that forms salts with acids, e.g. it may be crystallized as hydrobromide (CAS No. 300-08-3) or hydrochloride (CAS No. 61-94-9)

(b) Hydrobromide

Melting point: 169–171 °C (SciFinder, 2020b)

(c) Hydrochloride

Melting point: 157–158 °C (SciFinder, 2020c).

1.1.4 Technical products and impurities

Commercial qualities with purities in the range of 90–98% for the free base and hydrochloride are available, while the hydrobromide is also available in purities > 99% (SciFinder, 2020a, b, c). No information about impurities of technical products was available.

1.2 Production and use

1.2.1 Production process

The isolation of arecoline from nuts of the areca palm *Areca catechu* was first described by Jahns (1888). According to a historical 1911 version of the German Pharmacopoeia, arecoline (as its hydrobromide) was produced from areca nuts using extraction with acidified water followed by several clean-up steps (Anselmino & Gilg, 1911). The first industrial-scale extraction, reported in 1927, was based on extraction of arecoline with diethyl ether (Chemnitius, 1927). There are various approaches for the synthetic production of arecoline starting from nicotinic acid and iodomethane; methylamine hydrochloride, formaldehyde and acetaldehyde; or ethyl acrylate and methylamine. The most modern approach involves nicotinic acid methyl ester and methyl iodide (Volgin et al., 2019). [The Working Group was unable to obtain information regarding which process is currently preferred in industrial practice.]

Arecoline salts such as arecoline hydrochloride or arecoline hydrobromide may be obtained by dissolving arecoline in an alcohol of low relative molecular mass (such as methanol, ethanol, isopropanol, butanol, or amyl alcohol) and adding sufficient amounts of acid (hydrochloric or bromic acid) to give a weakly acidic solution. The crystallized salts may be separated from the alcohol by filtration (Howland-Knox, 1950).

1.2.2 Production volume

The international database Chem Sources lists 5 companies worldwide that manufacture arecoline (freebase) (USA, Canada, China, France, and the UK), 3 companies that manufacture arecoline hydrochloride (USA, Germany, and the UK), and 26 companies that manufacture arecoline hydrobromide (USA, Germany, China, Switzerland, UK, France, Japan, and Ukraine) (Chem Sources, 2020). The Scifinder database

lists 37 companies worldwide that manufacture arecoline (free base) (USA, UK, Canada, China, France, and Hong Kong Special Administrative Region (SAR) China), 18 companies that manufacture arecoline hydrochloride (USA, China, France, Germany, and the UK), and 94 companies that manufacture arecoline hydrobromide (USA, Belgium, Singapore, India, Republic of Korea, Canada, China, France, Germany, Japan, UK, and Hong Kong SAR China) (SciFinder, 2020a, b, c).

[No data on production volume were available to the Working Group. Arecoline is not included on national or international lists of High Production Volume chemicals. Extrapolating from the number of manufacturing companies, the Working Group noted that arecoline appears to be most commonly traded and used in the form of its hydrobromide.]

1.2.3 Uses

Historically, arecoline was used as an antiparasitic drug and included in several pharmacopoeias, but it has been replaced by other drugs and is rarely administered directly at the present time (Ahuja et al., 2016; Zhao et al., 2018). Arecoline is, however, still applied indirectly in the form of patent medicines or crude preparations of the areca nut (seed) in traditional Chinese medicine (see Section 1.4.1; Yi et al., 2012; He et al., 2018; Zhao et al., 2018). Three types of crude herbal preparation of areca are used: raw Arecae semen, Arecae semen tostum and Arecae semen carbonisata. Raw Arecae semen is prepared by collecting the mature seeds of *Areca catechu* and following a series of processing steps involving purifying, soaking, nourishing, slicing, and drying. Arecae semen tostum and Arecae semen carbonisata are generally prepared by a stir-fry method at high temperature until the seed surface turns light brown to black-brown (Sun et al., 2017). Areca nut is also an integral part of traditional Indian Ayurveda medicine. Global consumption and production of areca nut are shown in Fig. 1.1 (Volgin et al., 2019).

Arecoline stimulates both muscarinic and nicotinic acetylcholine receptors (Brown et al., 2018). It has been studied in model systems as a treatment for Alzheimer disease (Asthana et al., 1995, 1996; Liu et al., 2016).

In veterinary medicine, arecoline has been used as an anthelmintic (for cestodes, a type of parasitic flatworm that includes tapeworm), cathartic, and cholinergic agent (Mascavage et al., 2010; O'Neil, 2013). The anthelmintic action is assumed to cause the cestode muscles to relax and the host to purge so that the detached worms are removed. Oral administration of 40 mg of arecoline hydrobromide for 5 days in dogs completely controlled tapeworm, but had a low efficiency against ascaris (a common parasitic roundworm) (Tang & Eisenbrand, 1992).

1.3 Methods of detection and quantification

[The Working Group noted that analytical methods specified for arecoline are applicable to arecoline hydrochloride and arecoline hydrobromide dissolved in suitable solvents.]

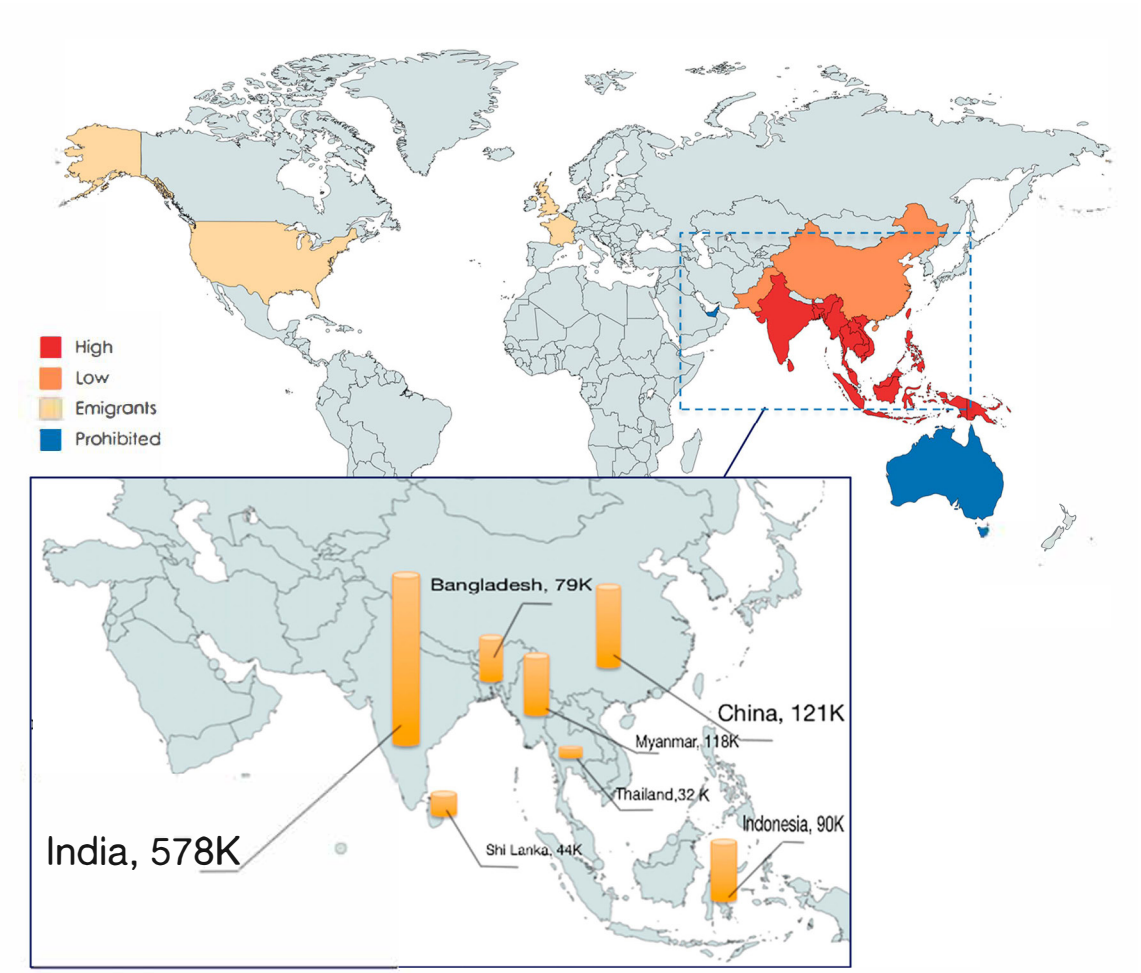
No methods for analysing arecoline in air, water, or soil were available to the Working Group.

1.3.1 Food, beverages, and consumer products

Historical methods for the identification of arecoline using various colour reactions, as well as quantitative determinations using paper chromatography, titrimetric methods, or colorimetric assays were reviewed by Arjungi (1976).

Recent analytical methods for arecoline have primarily focused on its detection in areca nuts as well as in areca nut-containing products such as *pan masala* (a powdered chewing mixture of areca nut with slaked lime, catechu, and other

Fig. 1.1 Global consumption and production of areca nuts



Global consumption (top panel) and production (inset) of areca nuts (red, high; orange, low; yellow, immigrants only). Blue denotes countries (Australia and United Arab Emirates) that currently ban areca-nut consumption. As shown in the inset, in 2015, India produced the majority of areca-nut products (578 000 tonnes/year), followed by China (121 000 tonnes/year), Indonesia (90 000 tonnes/year), and other countries in the region. For references to colour in this figure, please see the online version of this paper.
Reprinted with permission from Volgin et al. (2019). DARK Classics in Chemical Neuroscience: Arecoline. *ACS Chem Neurosci.* 10: 2176-85.

flavouring agents) (Table 1.1). After thin-layer or liquid chromatographic separation, arecoline can be detected using ultraviolet detection with absorption in the range of 200–250 nm owing to its chromophoric conjugated system of two double bonds (α,β-unsaturated carbonyl system) (Huang & McLeish, 1989; Lin et al., 1992; Sun et al., 2017). Mass spectrometry has been applied as a more specific and sensitive detection methodology allowing accurate detection down to the picogram range (Jain et al., 2017). Near-infrared spectroscopy has allowed rapid analysis of arecoline and process control during parching (Xue et al., 2011).

Table 1.1 Representative methods for the detection and quantification of arecoline in areca nut and products derived from areca nut

Sample matrix	Assay procedure	Limit of detection	Reference
Areca nut decoction pieces	MCE-CCD	5 μM	Cai et al. (2012)
Areca nut	HPLC	12.2 ng/mL	Huang & McLeish (1989)
Areca nut	MALDI-TOF-MS	0.2 μM	Feng & Lu (2009)
Areca nut	CE-ECL	5×10^{-9}mol/L	Xiang et al. (2013)
Areca nut (dried seed powder)	HPLC	2.86 μg/mL	Jantarat et al. (2013)
Areca nut	CZE	NR	Lord et al. (2002)
Areca nut and Indian nontobacco pan masala	HPTLC	3.25 ng	Adhikari et al. (2015)
Areca nut	PEC	30 pM	Dai et al. (2014)
Areca nut (raw, roasted, and boiled) and pan masala	HPTLC	35 ng/spot	Dutta et al. (2017)
Areca nut	HPLC-ES-MS	0.4 μg/mL	Ding & Shulian (2008)
Fruits of 11 *Arecaceae* species	UPLC-MS	NR	Wu et al. (2019)
Raw Arecae semen and its processed drugs	HPLC	0.89 ng	Sun et al. (2017)
Areca nut and different manufactured areca nut-containing products	LC-MS/MS	0.1 pg (on column) LOQ 0.5 pg (on column)	Jain et al. (2017)
Areca nut	Capillary electrophoresis	0.25 mg/L	Zhao et al. (2009)
Areca nut	NIR technology, HPLC	NR	Xue et al. (2011)
Areca nut	TLC-densitometric method	NR	Lin et al. (1992)
Areca nut	RP-HPLC	NR	He et al. (2011)

CE-ECL, capillary electrophoresis-electrochemiluminescence; CZE, capillary zone electrophoresis; HPLC, high-performance liquid chromatography; HPLC-ES-MS, high-performance liquid chromatography-electrospray mass spectrometry; HPTLC, high-performance thin-layer liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LOQ, limit of quantification; MALDI-TOF-MS, matrix-assisted laser desorption ionization mass spectrometry with a time-of-flight analyser; MCE-CCD, microchip capillary electrophoresis with contactless conductivity detection; NIR, near infrared; NR, not reported; PEC, photoelectrochemical detection; RP, reversed phase; TLC, thin-layer chromatography; UPLC, ultra-performance liquid chromatography.

1.3.2 Biological specimens

Several methods were available for the direct analysis of arecoline in various biological matrices, including saliva, urine, and serum, and typically combining liquid chromatography with mass spectrometry (Table 1.2). Chou et al. (2012) suggested a method for the determination of arecoline–protein adducts in human plasma.

[The Working Group was unable to identify an internationally accepted and validated biomarker for arecoline exposure.]

1.4 Occurrence and exposure

1.4.1 Occurrence

Arecoline occurs naturally in areca nut, the seed of the fruit of the areca palm (*Areca catechu* L.), which is widespread in south and south-east Asia (Volgin et al., 2019). For further information on the areca nut, see *IARC Monographs* Volumes 85 and 100E (IARC, 2004; 2012). Arecoline concentrations in areca nuts and various products derived from areca nut are summarized in Table 1.3.

Cai et al. (2012) reported the arecoline concentration in "semen Arecae" samples collected from Sri Lanka, and in Hainan and Guangzhou,

Table 1.2 Representative methods for the detection and quantification of arecoline in biological matrices

Sample matrix	Assay procedure	Limit of detection	Limit of quantification	Reference
Humans				
Plasma	GC-MS	0.5 ng/mL	1 ng/mL	Hayes et al. (1989)
Cord serum	LC-ESI-MS	0.001 µg/g	0.004 µg/g	Pichini et al. (2003)
Blood	LC-MS/MS	0.02 ng/mL	0.5 ng/mL	Wu et al. (2010)
Hair	LC-ESI-MS	0.09 ng/mg	0.30 ng/mL	Marchei et al. (2005)
Saliva	HPLC	50 pg	NR	Cox et al. (2004)
Saliva	HPLC-MS	NR	NR	Cox et al. (2010)
Saliva	LC-MS/MS	0.156 ng/mL	1.25 ng/mL	Lee et al. (2015)
Saliva	HPLC-MS/MS	NR	NR	Venkatesh et al. (2018)
Urine, newborn	LC-ESI-MS	0.0004 µg/g	0.001 µg/g	Pichini et al. (2003)
Urine	Online SPE LC-MS/MS	0.016 ng/mL	0.05 ng/mL	Hu et al. (2010)
Breast milk	LC-MS/MS	16 µg/L	50 µg/L (using 1 mL of human milk per assay)	Pellegrini et al. (2007)
Meconium	LC-ESI-MS	0.001 µg/g	0.005 µg/g	Pichini et al. (2003)
Typical stained tooth from an Iron Age skeleton	LC-MS/MS and LC-HR-ToF-MS	NR	NR	Krais et al. (2017)
Arecoline–protein adducts	Nanoscale LC-MS	NR	NR	Chou et al. (2012)
Experimental systems				
Rat urine	LC-MS and LC-MS/MS	< 8 ng/mL	NR	Zhu et al. (2006)
Rat plasma	LC-MS/MS	1 ng/mL (LLQ)	0.5 ng/mL	Pan et al. (2018)

GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; HPLC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry; LC-ESI-MS, liquid chromatography-electrospray quadrupole mass spectrometry; LC-HR-ToF-MS, liquid chromatography-high-resolution time-of-flight mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLQ, lower limit of quantification; NR, not reported; SPE LC-MS/MS, solid-phase extraction liquid chromatography-tandem mass spectrometry.

China. The maximum average arecoline concentration was measured in semen Arecae from Sri Lanka (0.43%), followed by Guangzhou (0.41%) and Hainan (0.24%). Concentrations of arecoline were higher in unripe than in ripe areca nuts from Thailand (0.14% versus 0.09% w/w of dried seed powder, respectively) (Jantarat et al., 2013). Adhikari et al. (2015) analysed arecoline concentrations in various areca-nut products available on the Indian market, including pan masala, mouth freshener, scented *supari* (the colloquial name for areca nut in Hindi and other Indian languages), and areca nut. The average concentration of arecoline in areca-nut products was reported to be in the range of 132.7 to 415.6 ng/mg, while areca nut itself had a concentration of 434.4 ng/mg.

Wu et al. (2019) reported average concentrations of arecoline in the range of 0.06 to 0.77 mg/g dry weight in unripe (stages 1–3) and ripe fruits, spadices, female flowers, male flowers, tender leaves, and ripe leaves of *Areca triandra* from Hainan province in China. In another study from China by Sun et al. (2017), average concentrations of arecoline measured in raw, tostum, and carbonisata Arecae semen were 3.38, 2.23, and 1.36 mg/g, respectively. Similarly, concentrations of arecoline were measured at 0.64–2.22 mg/g in bulk areca nut, pan masala, and *gutkha* from India, China, and Minneapolis, USA, by Jain et al. (2017). *Gutkha*, or betel quid

Table 1.3 Concentrations of arecoline in products derived from areca nut

Product	Average concentration	Standard deviation or range	Unit	Country or region	Reference
Areca nut	0.2191	± 0.0070	%	Import (NR)	Lin et al. (1992)
Areca nut	0.1896	± 0.0072		Hainan, China	
Areca nut	0.5607	± 0.0125		Guangzhou, China	
Areca nut	0.4310	± 0.0067	%	Sri Lanka	Cai et al. (2012)
Areca nut	0.2396	± 0.0042		Hainan, China	
Areca nut	0.4078	± 0.0085		Guangzhou, China	
Areca nut	6.6	NR	mg/g	NR	Dai et al. (2014)
Areca nut	434.4	± 4.42	ng/mg sample	India	Adhikari et al. (2015)
Sundried areca nut	0.5	0–1.4	%	Mumbai, India	Awang (1986)
Areca nuts from boiled varieties	0.8	0.4–1.3			
Roasted areca nuts	0.9	0.4–1.3			
Unripe areca nut Ripe areca nut	0.1434 0.0944	± 0.0016 ± 0.0002	%w/w of dried seed powder	Thailand	Jantarat et al. (2013)
Raw areca nut	1.15	± 0.008	%	NR	Dutta et al. (2017)
Pan masala areca nut	0.94	± 0.006			
Boiled areca nut	0.79	± 0.009			
Roasted areca nut	0.85	± 0.007			
Chinese areca nut	[1.27]	1.07–1.44	mg/g product dry weight	Changsha City, Hunan Province, China	Jain et al. (2017)
Bulk areca nuts	[1.27]	0.91–2.22		Mumbai, India	
Raw Arecae semen	3.38	± 0.157	mg/g	China	Sun et al. (2017)
Arecae semen tostum	2.23	± 0.223			
Arecae semen carbonisata	1.36	± 0.219			
Rajnigandha flavoured pan masala	376.9	± 7.71	ng/mg sample	India	Adhikari et al. (2015)
Parag pan masala	362.5	± 4.63			
Paras premium pan masala	349.2	± 6.36			
Dilruba sahi pan masala	255.2	± 4.01			
Pan parag pan masala	386.1	± 2.90			
Shikhar pan masala	415.6	± 3.83			
Chutki mouth freshener	132.7	± 5.92			
Tiranga pan masala	384.7	± 2.65			
Sir g finest pan masala	294.9	± 4.06			
Bahar heritage pan masala	322.7	± 5.94			.
Sweety *supari* kesar scented	167.4	± 3.55			
Pan masala *Gutkha*	[0.93] [0.95]	0.64–1.25 0.74–1.16	mg/g product dry weight	Indore, Madhya Pradesh, India, and Minneapolis	Jain et al. (2017)
Areca triandra (unripe fruits stage 1)	0.271	± 0.00773	mg/g dry weight	Hainan Province, China	Wu et al. (2019)
Areca triandra (pericarp of unripe fruits stage 2)	0.402	± 0.00698			
Areca triandra (endosperm of unripe fruits stage 2)	0.772	± 0.0419			

Table 1.3 (continued)

Product	Average concentration	Standard deviation or range	Unit	Country or region	Reference
Areca triandra (pericarp of unripe fruits stage 3)	0.180	± 0.00202	mg/g dry weight	Hainan Province, China	Wu et al. (2019) (cont.)
Areca triandra (endosperm of unripe fruits stage 3)	0.332	± 0.00570			
Areca triandra (pericarp of ripe fruits)	0.0621	± 0.00388			
Areca triandra (endosperm of ripe fruits)	0.191	± 0.00307			
Areca triandra (spadices)	0.164	± 0.00130			
Areca triandra (female flowers)	0.186	± 0.00650			
Areca triandra (male flowers)	0.190	± 0.00720			
Areca triandra (tender leaves)	0.660	± 0.00497			
Areca triandra (ripe leaves)	0.177	± 0.00362			

NR, not reported; w/w, weight per weight.

with tobacco, is a form of chewing tobacco containing a mixture of tobacco, crushed areca nut (also called betel nut), spices, and other ingredients (IARC, 2004). The average concentration of arecoline in sundried (0.5%), boiled (0.8%), and roasted areca nuts (0.9%) from Mumbai, India, was measured by Awang (1986). Lin et al. (1992) recorded average arecoline concentrations of 0.19% and 0.56% in areca nuts from Hainan and Guangzhou, respectively, in China.

Several researchers estimated arecoline concentrations in areca nut and in products containing areca nut. Recently, Dutta et al. (2017) found that raw areca nut and pan masala contained the highest concentrations of arecoline at 1.15% and 0.94%, respectively. Boiled areca nut contained the lowest concentration (0.79%) and roasted areca nut showed an intermediate concentration of arecoline (0.85%).

Adhikari et al. estimated the concentrations of arecoline in 11 brands of pan masala from Kolkata, India. Arecoline concentrations ranging from 130 to 415 µg/g of dried pan masala were detected (Adhikari et al., 2015).

Areca nut is an integral part of traditional Indian Ayurveda and Chinese medicines. Arecoline, an alkaloid, is mainly responsible for the areca nut's pharmaceutical properties (Arjungi, 1976). Yi et al. (2012) determined the arecoline content of the Chinese patent medicine Si-Mo-Tang, a liquid preparation that is taken orally and that is used in the treatment of gastrointestinal dyspeptic disease. The observed mean arecoline concentration was 29 ± 7 µg/mL, and concentrations ranged from 19 to 43 µg/mL. Another Chinese traditional medicine, Simo decoction, which is widely used to treat gastrointestinal dysmotility, contains arecoline as one of 94 ingredients (He et al., 2018).

1.4.2 Exposure in the general population

No information on direct exposure to arecoline as an isolated chemical was available to the Working Group. Exposure of the general population to arecoline is generally indirect via the use of areca nut and areca nut-derived

products. For further information on areca nut see IARC (2004, 2012).

Areca nut, sometimes called "betel nut" (although the latter is not botanically correct), is reportedly consumed by ~10–20% of the global population, making arecoline the fourth most frequently consumed psychoactive substance in the world after alcohol, nicotine, and caffeine (Volgin et al., 2019). The use of areca nut has been an integral part of various social customs and ceremonies in many Asian countries for thousands of years (Volgin et al., 2019). Its consumption is socially accepted in many Asian countries due to its pharmacological properties, which have been described historically (Volgin et al., 2019). Areca nut is mainly consumed in Asian countries (e.g. south China, Malaysia, tropical India, Sri Lanka, Pakistan, Myanmar, Indonesia, the Philippines), but also in countries of Oceania (Micronesia, Polynesia, and South Pacific islands), as well as parts of east Africa. The nuts are used either fresh or processed by sun drying, baking, boiling, or roasting to alter their flavour (Sinor et al., 1990; Leghari et al., 2016). A variety of products containing areca nut are available in other parts of the world where there are Asian immigrants or habitual users, but with limited availability due to local regulations (see Section 1.5; Warnakulasuriya, 2002; Blank et al., 2008). For example, betel products were collected from Richmond, Virginia, USA, between March and May 2006 and included pure areca nut, areca nut with tobacco, and areca nut with additives. Most packaging labels did not contain health warnings specific to arecoline.

The manner in which areca nut is consumed varies around the world. Most commonly it is placed in the mouth as small pieces wrapped in betel leaf and slaked lime. Sometimes additives, spices, sweeteners, and tobacco are added to this preparation (Blank et al., 2008).

Betel quid is a combination of areca nut, betel leaf, slaked lime, and flavouring ingredients (varying according to region) and is the common form in which areca nut is consumed in Asia. An interviewer-administered survey followed by an examination for oral mucosal disorders was conducted by the Asian Betel-Quid Consortium to investigate the population burden of betel-quid use and its effect on oral premalignant disorders in south, south-eastern, and east Asia. A total of 8922 participants from Taiwan and mainland China, Malaysia, Indonesia, Nepal, and Sri Lanka, were recruited. The prevalence of betel-quid use varied from 0.8% to 46.3% across the six populations studied (Lee et al., 2012).

According to a narrative review, the highest prevalence of betel-quid use was in Papua New Guinea, followed by Bangladesh, India, Pakistan, Myanmar, and Sri Lanka, whereas prevalence was relatively lower in Cambodia, Malaysia, Indonesia, and Taiwan, China (Gunjal et al., 2020).

[These studies mainly described betel-quid use/abuse and did not specifically mention consumption of areca nut or arecoline.]

Areca nut and smokeless tobacco are widely consumed across Myanmar (Papke et al., 2020) and by Palauans (one of the largest immigrant groups) in Hawaii, USA (Quinn Griffin et al., 2014). Yoganathan (2002) found that different types of product containing areca nut were available in New Zealand and Australia, mainly in Asian groceries. They were primarily consumed by Indian immigrants to these countries.

Areca nut is consumed in many forms by south-east Asians: raw, mixed with some additives, and as commercially available preparations. The most popular product in India is pan masala, which is a blend of areca nut powder and additives. Another commonly consumed areca-nut product is *gutkha*, which contains tobacco as a major constituent. These products are readily available with attractive packaging at affordable prices and are therefore popular among young people (Dutta et al., 2017).

Javed et al. (2008, 2010) interviewed 1000 adults (aged 45–64 years) from Karachi, Pakistan, to study the reasons why people used the areca-nut product *gutkha*. Of the study participants, 24% of those with type 2 diabetes and 8% of those without type 2 diabetes reported that they chewed *gutkha* to control hunger. A cross-sectional study conducted by Leghari et al. (2016) among schoolchildren of Karachi, Pakistan, found a high frequency of areca nut (78%) and *gutkha* chewing (60%).

A significant number of studies in humans have identified the presence of arecoline in samples of saliva, blood, urine, hair, and breast milk (Table 1.4); [these data might be useful for qualitative exposure assessments].

Salivary concentrations of arecoline in areca-nut chewers were measured during chewing and at different post-chewing intervals. Arecoline was undetectable at baseline. During chewing, mean arecoline concentrations were 77 ng/mL (regular users of areca nut) and 65 ng/mL (control group of occasional users) in areca-nut chewers and 130 ng/mL in the placebo group (regular users of areca nut who were given rubber base to chew). After chewing, mean arecoline concentrations were 196 ng/mL, 321 ng/mL, and 44 ng/mL, respectively. Arecoline concentrations were higher after chewing areca nut than during chewing. Arecoline concentrations were significantly higher in the areca-nut chewers than in the placebo group. During chewing, the highest arecoline concentrations were reached during the first minute. After chewing, arecoline concentrations were high until 10 minutes post-chewing, after which they started to decline in both groups of areca-nut chewers (Venkatesh et al., 2018). A study conducted by Franke et al. (2016) looked for the presence of arecoline in the hair, saliva, and urine of areca-nut chewers. No arecoline was detected in the hair samples tested. Arecoline was detected in the saliva and urine within 2–8 hours post-chewing; concentrations were highest up to 2 hours post-chewing, declined with time, and returned to baseline 8 hours post-chewing. Marchei et al. (2005) reported mean arecoline concentrations of 0.61–1.27 ng/mg in the hair of long-term areca-nut consumers.

High concentrations of salivary arecoline (up to 140 µg/mL) in betel-quid chewers (mean values, 52 µg/mL and 30 µg/mL with and without tobacco, respectively) were reported by Nair et al. (1985). García-Algar et al. (2005) reported the detection of arecoline in the meconium of six Asian newborn babies whose mothers consumed areca nut during pregnancy. Placental tissue from these mothers also showed the presence of arecoline. The observed ranges of arecoline concentrations measured in this study were 0.006–0.022 µg/g in meconium and not detected to 0.015 µg/g in placenta. [The Working Group noted that due to the lack of a baseline arecoline range in mothers who were not areca-nut chewers at the time, it was not possible to arrive at any conclusion.]

Wu et al. (2010) reported a statistically significant correlation between betel-quid consumption and blood arecoline concentrations (Spearman correlation coefficient, $r = 0.81$; $P < 0.01$). [Therefore, serum arecoline is a promising short-term indicator of betel-quid consumption.] Franke et al. (2020) studied the presence of arecoline in the saliva and hair of areca-nut chewers. Arecoline was only detected in hair samples from men. Conversely, sex did not influence salivary arecoline concentrations determined within 5–24 hours of chewing areca nut. These alkaloids were found to be present in hair months after the cessation of areca-nut chewing. A study conducted by Pellegrini et al. (2007) in Italy reported the presence of arecoline (together with other substances such as nicotine, caffeine, and cotinine) in human breast milk. Cox et al. (2010) estimated arecoline concentrations in the saliva of areca-nut chewers ($n = 32$) and non-chewers/controls ($n = 6$). Arecoline was detected in all areca-nut chewers. Maximum concentrations of arecoline were measured in the

Table 1.4 Concentrations of arecoline in biological samples from humans

Sample	Location	Concentration (mean, median, or range)	Method of estimation	Comments	References
Plasma	USA	Range, ND–5 ng/mL	GC-MS	Healthy volunteers, transdermal administration of arecoline 3 mg/h, measurements during 30 h after dose.	Hayes et al. (1989)
Plasma	USA	LOQ, 0.16 ng/mL C_{max} mean ± SD, 27.8 ± 20.5 ng/mL C_{max} range, 7.8–83.3 ng/mL	GC-MS	Pharmacological use of intravenous arecoline 5 mg/30 min; up to 40 mg/day for memory enhancement in 15 patients with Alzheimer disease.	Asthana et al. (1996)
Blood	Taiwan, China	Betel quid chewers: mean ± SD, 7.0 ± 10.7 ng/mL Never-chewers: mean ± SD, 0.3 ± 0.2 ng/mL; range, 0–0.63 ng/mL	LC-MS/MS	Blood from 13 betel-quid chewers and 5 never-chewers.	Wu et al. (2010)
Hair	Italy, India, Spain	Women: mean ± SD, 1.27 ± 0.20 ng/mg hair Men: mean ± SD, 0.61 ± 0.52 ng/mg hair Range reported 300 pg/mg to 1.70 ng/mg hair	HPLC-MS	First report measuring arecoline in hair samples from 11 long-term areca-nut users (2–35 years); arecoline can be used as a non-invasive biomarker for areca-nut use.	Marchei et al. (2005)
Hair	Papua New Guinea	Mean, 3.56 ng/mg hair; median, 2.24 ng/mg hair; range, 60 pg/mg to 18 ng/mg hair	UPLC-MS/MS and UPLC-Q-Tof-MS	Included 11 men and 8 women who had been abusing areca nut for more than 6 mo before hair sampling. Hair seems to be a promising marker of long-term exposure to areca nut.	Gheddar et al. (2020)
Saliva	Australia	Maximum concentration during chewing: range, 5.6–97 µg/mL	HPLC-MS	Samples collected from 32 habitual areca-nut chewers before, during and after chewing.	Cox et al. (2010)
Saliva	Taiwan, China	Range, 0–80 µg/mL C_{max} mean ± SD, 44 ± 32 µg/mL	LC-MS/MS	Saliva samples of 5 men after chewing one 5 g of areca nut. The highest concentrations were measured after 5 min of chewing.	Lee et al. (2015)
Saliva	India	Areca-nut chewers during chewing: C_{max} mean, 77 ng/mL C_{max} range, 49–280 ng/mL Areca-nut chewers after chewing: C_{max} mean, 196 ng/mL C_{max} range, 154–333 ng/mL Placebo group after chewing: C_{max} mean, 321 ng/mL	HPLC-MS	Salivary arecoline concentrations for 20 individuals before and after during chewing of 0.5 g of fresh areca nut. Baseline levels were ND. The placebo group had higher arecoline levels than the areca nut-chewing group.	Venkatesh et al. (2018)
Neonatal urine	Spain	Urine: range, ND–0.01 µg/mL		Neonatal urine samples from 2 babies born at the Hospital de Mar of Barcelona, Spain.	Pichini et al. (2003)

Table 1.4 (continued)

Sample	Location	Concentration (mean, median, or range)	Method of estimation	Comments	References
Urine	Taiwan, China	Mean ± SD, 23.9 ± 39.3 ng/mg creatinine; range, ND–142 ng/mg creatinine	Solid-phase extraction LC-TMS	Included 33 regular areca-nut chewers who were also cigarette smokers. First study to report the presence of *N*-methylnipecotic acid and arecaidine besides arecoline in urine of areca-nut chewers.	Hu et al. (2010)
Breast milk	Spain, Italy, India	Range, 18–150 µg/L	LC-MS/MS	Included 4 betel-quid-consuming breastfeeding mothers. Levels of arecoline in breast-fed infants and relationship with clinical outcomes.	Pellegrini et al. (2007)
Meconium and placenta	Spain	Meconium: range, 0.006–0.022 µg/g Placenta: range, ND–0.015 µg/g	HPLC/electrospray quadrupole-MS	First study to detect arecoline in meconium of newborns and placental tissue of 6 Asian mothers who were areca-nut chewers during pregnancy.	García-Algar et al. (2005)
Meconium	Spain	Meconium: range, 0.006–0.008 µg/g	HPLC/electrospray quadrupole-MS	Arecoline measured in biological samples from 2 newborns whose mothers consumed areca nuts; attending Hospital del Mar, Barcelona, Spain.	Pichini et al. (2003)

C_{max}, maximum concentration; GC-MS, gas chromatography-mass spectrometry; h, hour; HPLC-MS, high-performance liquid chromatography-mass spectrometry; LC-MS, liquid-chromatography-mass spectrometry; LC-MS/MS, liquid-chromatography-tandem mass spectrometry; LOQ, limit of quantification; min, minute; ND, not detected; SD, standard deviation; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry; UPLC-Q-Tof-MS, ultraperformance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry.

range of 6 to 97 µg/mL. Salivary arecoline could not be detected in controls.

[The Working Group noted that, considering the available data, arecoline levels in blood and saliva may act as short-term markers, while arecoline levels in hair may act as a long-term marker of arecoline exposure. After successful validation, these markers may have potential applications in future exposure assessments.]

1.4.3 Occupational exposure

No information was available on occupational exposure to arecoline, either directly (e.g. during the purification processes) or indirectly (due to areca-nut exposure). However, it has been suggested that people of lower socioeconomic status, specifically women in India engaged in intensive labour, use smokeless tobacco products including areca nut to suppress their hunger during working hours (Government of India, 2020a). A report from Sri Lanka showed that approximately 53% of the rural population, particularly drivers and labourers, consume areca nut as it is believed to reduce hunger and tiredness (Selvananthan et al., 2018). [The Working Group noted that the areca nut may be consumed to suppress hunger or as a stimulant during long work shifts, but the consumption of areca nut is not related to a particular occupation.]

1.5 Regulations and guidelines

1.5.1 Exposure limits and guidelines

For arecoline itself, only a few regulations are available worldwide. The European Chemicals Agency (ECHA) within its Registration, Evaluation, Authorization and Restriction of Chemicals Regulation (REACH) assessment classified arecoline and arecoline hydrochloride as "1–10 tonne registered substances" likely to meet criteria for category 1A or 1B carcinogenicity, mutagenicity, or reproductive toxicity (ECHA, 2020a, b). Since 2010, arecoline is also included in the List of Substances Prohibited in Cosmetic Products in the European Union (European Commission, 2020).

For areca nut, few regulations are in place worldwide, especially when compared with tobacco and alcohol. An urgent need for regional and global policies to mitigate the misuse of areca nut has been highlighted by various researchers (Mehrtash et al., 2017; Thakur et al., 2020). The import and inter-state transport of areca nut has been restricted by the United States Food and Drug Administration (US FDA). It is also designated as a toxic and carcinogenic substance by the California Environmental Protection Agency (CalEPA, 2006). The sale of products containing areca nut has been banned in Canada. In the European Union, areca nut is treated as an unauthorized novel food, which may not be placed on the market (European Commission, 2019).

In 2018, the Indian government made a strong move to regulate the food safety and import of areca nut through the Food Safety and Standards Authority of India (FSSAI). Previous Indian health policies were based on warnings on the packaging of areca-nut products (FSSAI, 2004), whereas the latest policies enforce the ban on the manufacturing and sale of areca-nut products containing tobacco or nicotine (FSSAI, 2011). Many Indian states have banned areca-nut products under the FSSAI (2006).

Recently, the Indian Council of Medical Research, and the Department of Health Research, Ministry of Health and Family Welfare (Government of India, 2020b) appealed to the public not to consume or spit smokeless tobacco, areca nut, or betel quid in public places in order to contain the spread of SARS-CoV-2, the cause of novel coronavirus disease (COVID-19), under the Epidemic Disease Act, 1897, the Disaster Management Act, 2005 and the Indian Penal Code 1860 and Code of Criminal Procedure. The appeal states that: "Chewing smokeless tobacco products, paan masala and areca nut (*supari*)

increases the production of saliva followed by a very strong urge to spit. Spitting in public places could enhance the spread of the COVID19 virus."

Various other Asian countries, including Bhutan, Myanmar, Taiwan, China, and Sri Lanka also have policies and regulations regarding use of betel quid and areca nut. These include bans on chewing, spitting in public places, and the sale of areca nut (Gunjal et al., 2020).

[These policies are not specific to areca nut/arecoline, but common to smokeless tobacco products and areca nut or betel quid. The Working Group expected that policies regarding areca nut/betel quid would also reduce exposure to arecoline.]

1.5.2 Reference values for biological monitoring of exposure

No validated reference values for arecoline or arecoline biomarkers to quantitatively monitor exposure were available to the Working Group.

2. Cancer in Humans

No data were available to the Working Group.

3. Cancer in Experimental Animals

In previous evaluations, the *IARC Monographs* programme concluded that there was *limited evidence* in experimental animals for the carcinogenicity of arecoline (IARC, 2004).

Studies of carcinogenicity with arecoline and its metabolite arecaidine in experimental animals are summarized in Table 3.1.

3.1 Mouse

3.1.1 Oral administration (gavage)

In the first experiment in a study by Bhide et al. (1984), two groups of male (n = 20–35) and female (n = 18–20) Swiss mice (age, 6 weeks) were treated with vehicle (control) or 1 mg of arecoline hydrochloride [purity not reported; rationale for dose not specified] dissolved in distilled water (adjusted to pH 7) per animal, by gavage, five times per week "throughout the lifespan." Additional groups of male (n = 16–19) and female (n = 8–14) mice were treated with 1 mg of arecoline hydrochloride and 1 mg of laboratory-grade potassium nitrate (KNO_3) [purity not reported; rationale for dose not reported] in distilled water per animal, with 1 mg of arecoline hydrochloride, 1 mg of KNO_3, and 1 mg of slaked lime (commercial brand used to prepare betel quid) in distilled water per animal, or with 1 mg of KNO_3 and 1 mg of slaked lime in distilled water per animal. [Data on body weight or survival were not reported.] Histopathological examination was performed on the liver, lungs, and stomach, and any other abnormal tissue of all animals.

When assessed during 25 months [it was unclear whether this was 25 months of age or 25 months of treatment], male mice treated with arecoline hydrochloride only showed a significant increase [P = 0.0023; Fisher exact test] in the incidence of total tumours compared with the vehicle control group. In the group (n = 35) treated with arecoline hydrochloride, eight mice developed liver haemangioma, four developed lung adenocarcinoma, and three developed squamous cell carcinoma of the stomach. The total tumour incidence in the other treated groups of males did not differ significantly from that in the vehicle control group. No tumours were observed in female mice (Bhide et al., 1984). [The Working Group noted the limited histopathology, the single dose used, the small

Table 3.1 Studies of carcinogenicity with arecoline or its metabolite, arecaidine, in experimental animals

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Mouse, Swiss (M) 6 wk 25 mo Bhide et al. (1984)	Oral administration (gavage) Arecoline hydrochloride, KNO_3, and slaked lime; purity, NR Distilled water (adjusted to pH 7) Control, 1 mg arecoline hydrochloride, 1 mg arecoline hydrochloride + 1 mg KNO_3, 1 mg arecoline hydrochloride + 1 mg KNO_3 + 1 mg lime, 1 mg KNO_3 + 1 mg lime (mg/application per mouse) 5×/wk for 25 mo 20, 35, 19, 16, 17 NR	*All sites* (including liver, lung, and stomach): Tumour incidence: 1/20 (5%), 15/35 (43%)*, 3/19 (16%), 1/16 (6%), 2/17 (12%) *Liver*: haemangioma Tumour incidence: NR, 8/35 (23%), NR, NR, NR *Lung*: adenocarcinoma Tumour incidence: NR, 4/35 (11%), NR, NR, NR *Stomach*: squamous cell carcinoma Tumour incidence: NR, 3/35 (9%), NR, NR, NR	*[$P = 0.0023$, 1 mg arecoline hydrochloride vs control; one-tailed Fisher exact test; all other groups, NS vs control] NA NA NA	Principal limitations: body-weight changes, NR; lack of KNO_3-only control group; survival, NR; rationale for doses, NR; tumours observed in the vehicle control group not further specified; limited histopathology; use of a single dose; small number of mice per group; purity of test articles, NR. Other comments: it was unclear whether 25 mo (see duration and dosing regimen) was the age of the mice or the duration of treatment; KNO_3, laboratory grade; slaked lime, commercial brand used to prepare betel quid.
Full carcinogenicity Mouse, Swiss (F) 6 wk 25 mo Bhide et al. (1984)	Oral administration (gavage) Arecoline hydrochloride (+/– KNO_3 and slaked lime); purity, NR Distilled water Control, 1 mg arecoline hydrochloride, 1 mg arecoline hydrochloride + 1 mg KNO_3, 1 mg arecoline hydrochloride + 1 mg KNO_3 + 1 mg lime, 1 mg KNO_3 + 1 mg lime (mg/application/mouse) 5×/wk for 25 mo 20, 18, 14, 12, 8 NR	*All sites* (including liver, lung, and stomach): Tumour incidence: 0/20, 0/18, 0/14, 0/12, 0/8	NA	Principal limitations: body-weight changes, NR; lack of KNO_3-only control group; survival, NR; rationale for doses, NR; limited histopathology; use of a single dose; small number of mice per group; purity of test articles, NR. Other comments: it was unclear whether 25 mo (see duration and dosing regimen) was the age of the mice or the duration of treatment; KNO_3, laboratory grade; slaked lime, commercial brand used to prepare betel quid.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Mouse, Swiss (M) 8 wk 25 mo Bhide et al. (1984)	Oral administration (gavage) Arecoline hydrochloride (+/– KNO_3 and slaked lime), in mice fed a vitamin B complex-deficient diet; purity, NR Distilled water Control, 1 mg arecoline hydrochloride, 1 mg arecoline hydrochloride + 1 mg KNO_3, 1 mg arecoline hydrochloride + 1 mg KNO_3 + 1 mg lime (mg/application per mouse) 5×/wk for 25 mo 21, 21, 16, 18 NR	*All sites* (including liver, lung, and stomach): Tumour incidence: 2/21 (10%), 7/21 (33%), 1/16 (6%), 7/18 (39%)*	*[$P = 0.0361$, 1 mg arecoline hydrochloride + 1 mg KNO_3 + 1 mg lime vs control; one-tailed Fisher exact test; all other groups, NS vs control]	Principal limitations: body-weight changes, NR; lack of KNO_3-only control group; survival, NR; rationale for doses, NR; lack of KNO_3 + lime control group; tumours observed in the vehicle control group not further specified; limited histopathology; use of a single dose; small number of mice per group; purity of test articles, NR. Other comments: it was unclear whether 25 mo (see duration and dosing regimen) was the age of the mice or the duration of treatment; KNO_3, laboratory grade; slaked lime, commercial brand used to prepare betel quid.
		Liver: haemangioma Tumour incidence: NR, 3/21 (14%), NR, 2/18 (11%)	NA	
		Lung: adenocarcinoma Tumour incidence: NR, 3/21 (14%), NR, 3/18 (17%)	NA	
		Stomach: squamous cell carcinoma Tumour incidence: NR, 0/21, NR, 2/18 (11%)	NA	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Mouse, Swiss (F) 8 wk 25 mo Bhide et al. (1984)	Oral administration (gavage) Arecoline hydrochloride (+/– KNO_3 and slaked lime), in mice fed a vitamin B complex-deficient diet; purity, NR Distilled water Control, 1 mg arecoline hydrochloride, 1 mg arecoline hydrochloride + 1 mg KNO_3, 1 mg arecoline hydrochloride + 1 mg KNO_3 + 1 mg lime (mg/application/mouse) 5×/wk for 25 mo 16, 12, 16, 18 NR	*All sites* (including liver, lung, and stomach): Tumour incidence: 1/16 (6%), 6/12 (50%)*, 2/16 (13%), 8/18 (44%)**	*[P = 0.0132, 1 mg arecoline hydrochloride vs control; **P = 0.0143, 1 mg arecoline hydrochloride + 1 mg KNO_3 + 1 mg lime vs control; one-tailed Fisher exact test; other group NS vs control]	Principal limitations: body-weight changes, NR; lack of KNO_3-only control group; rationale for doses, NR; lack of KNO_3 + lime control group; tumours observed in the vehicle control group not further specified; limited histopathology; use of a single dose; small number of mice per group; purity of test articles, NR. Other comments: it was unclear whether 25 mo (see duration and dosing regimen) was the age of the mice or the duration of treatment; KNO_3, laboratory grade; slaked lime, commercial brand used to prepare betel quid.
		Liver Haemangioma Tumour incidence: NR, 2/12 (17%), NR, 2/18 (11%)	NA	
		Cholangiocarcinoma Tumour incidence: NR, 0/12, NR, 1/18 (6%)	NA	
		Lung: adenocarcinoma Tumour incidence: NR, 4/12 (33%), NR, 3/18 (17%)	NA	
		Stomach: squamous cell carcinoma Tumour incidence: NR, 0/12, NR, 2/18 (11%)	NA	
Full carcinogenicity Mouse, Swiss (M) NR Lifetime Shivapurkar et al. (1980)	Intraperitoneal injection Arecoline, analytical reagent grade; purity, NR 0 (0.1 mL distilled water), 1.5 mg 1×/wk for 13 wk 10, 10 NR	*All organs*: Tumour incidence: 0/10, 0/10	NA	Principal limitations: body-weight changes, NR; survival, NR; vehicle and volume, NR; starting age, NR; precise duration of experiment, NR; use of a single dose; small number of mice per group; short duration of treatment; use of one sex only; justification for the dose, NR.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Mouse, Swiss (M) NR Lifetime Shivapurkar et al. (1980)	Subcutaneous injection Arecoline, analytical reagent grade; purity, NR 0 (0.1 mL distilled water), 1.5 mg 1×/wk for 13 wk 20, 10 NR	*At injection site or of other organs*: Tumour incidence: 0/20, 0/10	NA	Principal limitations: starting age, NR; body-weight changes, NR; survival, NR; vehicle and volume, NR; precise duration of experiment, NR; use of a single dose; small number of mice per group; short duration of treatment; use of one sex only; justification of the dose, NR.
Co-carcinogenicity Mouse, C57BL/6JNarl (M) 6 wk 28 wk Chang et al. (2010)	Oral administration (drinking-water) Arecoline hydrobromide and 4-NQO; purity, NR Water 0 (control), 100 (4-NQO), 200 (4-NQO), 250 (arecoline hydrobromide), 500 (arecoline hydrobromide), 100 (4-NQO) + 250 (arecoline hydrobromide), 100 (4-NQO) + 500 (arecoline hydrobromide), 200 (4-NQO) + 250 (arecoline hydrobromide), 200 (4-NQO) + 500 (arecoline hydrobromide) µg/mL drinking-water for 8 wk, followed by drinking-water only for 20 wk 10, 10, 10, 10, 10, 11, 11, 11, 11 7, 7, 7, 7, 7, 8, 8, 8, 8	*Tongue*: hyperplasia, dysplasia, papilloma, or invasive squamous cell carcinoma (combined) Lesion incidence: 0/7, 2/7 (29%), 4/7 (57%), 0/7, 0/7, 4/8 (50%), 3/8 (38%), 4/8 (50%), 8/8 (100%) *Oesophagus*: hyperplasia, dysplasia, papilloma, or invasive squamous cell carcinoma (combined) Lesion incidence: 0/7, 1/7 (14%), 1/7 (14%), 0/7, 0/7, 4/8 (50%), 1/8 (13%), 1/8 (13%), 0/8	[No significant effect of arecoline hydrobromide] [No significant effect of arecoline hydrobromide]	Principal limitations: short duration of exposure; short duration of follow-up; rationale for doses, NR; small number of mice per group; pre-neoplastic and neoplastic lesions combined. Other comments: three mice per group were killed at 8 wk for histopathological examination, and no lesions were observed.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Co-carcinogenicity Mouse, C57BL/6 (M) 6 wk 28 wk Chen et al. (2017)	Oral administration (drinking-water) Arecoline hydrobromide and 4-NQO; purity, NR Water 0 (control), 100 (4-NQO) for 8 wk, 100 (4-NQO) for 16 wk, 500 (arecoline hydrobromide) for 16 wk, 100 (4-NQO) + 500 (arecoline hydrobromide) for 8 wk, 100 (4-NQO) + 500 (arecoline hydrobromide) µg/mL drinking-water for 16 wk followed by drinking-water up to experimental wk 28 8, 16, 16, 8, 16, 16 8, 16, 14, 8, 15, 13	*Oesophagus* Invasive squamous cell carcinoma Tumour incidence: 0/8, 1/16 (6%), 7/14 (50%)†, 0/8, 6/15 (40%)*,††, 9/13 (69%)‡ Papilloma Tumour incidence: 0/8, 5/16 (31%), 11/14 (79%)†, 0/8, 10/15 (67%)††,‡, 12/13 (92%)‡‡	*[$P = 0.0329$, vs 100 µg/mL 4-NQO for 8 wk; †$P = 0.0201$, vs control; ††$P = 0.0496$, vs control; ‡$P = 0.0024$, vs control; one-tailed Fisher test] †[$P = 0.0005$, vs control; one-tailed Fisher exact test];††(NS; $P = 0.049$, vs 100 µg/mL 4-NQO; one-tailed χ^2 test [but $P = 0.0528$, one-tailed Fisher exact test]); ‡[$P = 0.0026$, vs control; one-tailed Fisher test]; ‡‡[$P < 0.0001$, vs control; one-tailed Fisher exact test]	Principal limitations: body-weight changes, NR; rationale for doses, NR.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Co-carcinogenicity Rat, F344 (M) 6 wk 25 wk Wu et al. (2016)	Oral administration (drinking-water) Arecoline and NMBA; purity, NR Water 0 (control), 500 (NMBA) + 0 (arecoline), 0 (NMBA) + 500 (arecoline), 500 (NMBA) + 500 (arecoline) [μg/kg bw (for NMBA) or μg/mL (for arecoline)] Subcutaneous injection of NMBA, 3×/wk for 5 wk; arecoline in drinking-water for 25 wk 7, 7, 7, 7 7, 7, 7, 7	*Oesophagus*: papilloma Tumour incidence: 0/7, 0/7, 0/7, 7/7 (100%)* Tumour multiplicity: 0, 0, 0, 1.86 ± 0.10* Total tumours: 0, 0, 0, 13 *Tongue*: papilloma Tumour incidence: 0/7, 1/7 (14%), 0/7, 3/7 (43%) Tumour multiplicity: 0, 0.29 ± 0.17, 0, 0.43 ± 0.17 Total tumours: 0, 2, 0, 3	*[$P = 0.0003$, vs other three groups; one-tailed Fisher exact test] *$P < 0.0001$, vs other three groups; one-way ANOVA, followed by Fisher LSD test NR NA NA NR	Principal limitations: rationale for doses, NR; small number of rats per group; short duration of the carcinogenicity study. Other comments: NMBA in 0.2 mL 20% DMSO; body-weight variation was studied, but data NR.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Co-carcinogenicity Rat, F344 (M) 6 wk 30 wk Wu et al. (2016)	Oral administration (drinking-water) Arecoline and NMBA; purity, NR Water 0 (control), 500 (NMBA) + 0 (arecoline), 0 (NMBA) + 500 (arecoline), 500 (NMBA) + 500 (arecoline) [μg/kg bw (for NMBA) or μg/mL (for arecoline)] Subcutaneous injection of NMBA, 3×/wk for 5 wk; arecoline in drinking-water for 30 wk 15, 15, 15, 15 15, 15, 15, 15	*Oesophagus*: papilloma Tumour incidence: 0/15, 8/15 (53%), 2/15 (13%), 11/15 (73%) Tumour multiplicity: 0, 1.07 ± 0.11, 0.13 ± 0.02, 2.27 ± 0.16* Total tumours: 0, 16, 2, 34 *Tongue*: papilloma Tumour incidence: 0/15, 5/15 (33%), 1/15 (7%), 10/15 (67%) Tumour multiplicity: 0, 0.53 ± 0.22, 0.07 ± 0.07, 1.07 ± 0.30* Total tumours: 0, 8, 1, 16	NA *P = 0.0260, vs NMBA group; one-way ANOVA, followed by Fisher LSD test NR NS *P = 0.0494, vs NMBA group; one-way ANOVA, followed by Fisher LSD test NR	Principal limitations: rationale for doses, NR; small number of rats per group; short duration of the carcinogenicity study. Other comments: NMBA in 0.2 mL 20% DMSO; body-weight variation was studied, but data NR.
Full carcinogenicity Hamster, Syrian golden (M+F) (combined) 6–7 wk NR (lifetime) Dunham et al. (1974)	Topical application in the cheek pouch Arecoline, NR Distilled water 0 (control), ~1 mg/day 5×/wk for life 8, 9 NR	*Oesophagus*: papilloma Tumour incidence: 0/8, 1/9 (11%) *Cheek pouch*: Tumour incidence: 0/8, 0/9	[NS] NA	Principal limitations: body-weight changes, NR; survival, NR; small number of hamsters per group; purity of test articles, NR. Other comments: 3–4 h before application of the 1.5% solution of arecoline or the vehicle, 30 mg of calcium hydroxide, as a 0.5% solution in DMSO [6 mL] was administered to the cheek pouch.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Hamster, Syrian golden (M+F) (combined) 6–7 wk NR [lifetime, assumed] Dunham et al. (1974)	Topical application in the cheek pouch Arecoline, NR DMSO 0 (control), ~1 mg/day 3×/wk for 5 mo 8, 8 NR	*Oesophagus or cheek pouch*: Tumour incidence: 0/8, 0/8	NA	Principal limitations: body-weight changes, NR; survival, NR; small number of hamsters per group; purity of test articles, NR. Other comments: 3–4 h before application of the 1.5% solution of arecoline or the vehicle, 30 mg of calcium hydroxide, as a 0.5% solution in DMSO [6 mL] was administered to the cheek pouch.
Full carcinogenicity Hamster, Syrian golden (M+F) (combined) 6–7 wk NR (lifetime) Dunham et al. (1974)	Oral administration (topical application to base of tongue) Arecoline, NR Distilled water 0 (control), ~2 mg/day drops, 2×/day, 5 days/wk for life 4, 8 NR	*Oesophagus or cheek pouch*: Tumour incidence: 0/4, 0/8	NA	Principal limitations: body-weight changes, NR; survival, NR; small number of hamsters per group; purity of test articles, NR. Other comments: application of 2% solution of arecoline; controls received 0.5% calcium hydroxide in distilled water to base of tongue.
Full carcinogenicity Hamster, Syrian golden (M+F) (combined) 6–7 wk NR [lifetime, assumed] Dunham et al. (1974)	Oral administration (feed) Arecoline, NR Feed 0 (control), ~6 mg/day 5 days/wk for 16 mo 4, 4 NR	*Oesophagus or cheek pouch*: Tumour incidence: 0/4, 0/4	NA	Principal limitations: body-weight changes, NR; survival, NR; very small number of hamsters per group; purity of test articles, NR. Other comments: controls and treated group were fed 2.5% calcium hydroxide in the feed (approximately 150 mg/day).

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Hamster, Syrian golden (NR) 1.5 mo 24 mo Dunham et al. (1975)	Oral administration (feed) Arecoline, NR Feed 0 (control), ~6 mg/day 5 days/wk for 12 mo 5, 4 0, 0	*Glandular stomach*: argyrophilic carcinoid Tumour incidence: 0/5, 1/4 (25%)	[NS]	Principal limitations: body-weight changes, NR; very small number of hamsters per group; purity of test articles, NR; control group given feed containing calcium hydroxide for 16 mo (and not 12 mo). Other comments: controls and treated group were given feed containing 2.5% calcium hydroxide (approximately 150 mg/day).
Full carcinogenicity Hamster, Syrian golden (M) 8 wk 22 wk MacDonald (1987)	Topical application in the cheek pouch Arecaidine, NR Distilled water 25 µg/mL 3×/wk for 12 wk 24 13	*Cheek pouch*: Tumour incidence: 0%	NA	Principal limitations: short duration of exposure; short duration of follow-up; body-weight changes, NR; lack of control group; volume applied, NR. Other comments: applications to a 1 cm^2 area of the anterior part of the medial wall of the cheek pouch.
Initiation–promotion (tested as initiator) Hamster, Syrian golden (M) 8 wk 59 wk MacDonald (1987)	Topical application in the cheek pouch Arecaidine, NR Distilled water 25 µg/mL Arecaidine 3×/wk for 12 wk, observed for 10 wk, then croton oil (1% in acetone) 3×/wk for 3 wk 13 3	*Cheek pouch*: Tumour incidence: 0%	NA	Principal limitations: body-weight changes, NR; poor survival; lack of control group; large time gap between initiation and promotion; volume applied, NR; purity of test articles, NR. Other comments: applications to a 1 cm^2 area of the anterior part of the medial wall of the cheek pouch.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Hamster, Syrian golden (M) 11–13 wk 12 wk Lin et al. (1996)	Topical application in the cheek pouch Arecaidine, NR Polyethylene glycol 0 (control), 1000, 2000, 3000 µg/mL Arecaidine, 6×/wk for 12 wk 7, 7, 7, 7 NR	*Cheek pouch*: exophytic squamous cell papilloma or carcinoma (combined) Tumour incidence: 0/7, 0/7, 0/7, 0/7	NA	Principal limitations: body-weight changes, NR; survival, NR; short duration of exposure; short duration of follow-up; rationale for doses, NR; small number of hamsters per group; volumes applied, NR. Other comments: painting using a no. 4 camel-hair brush.
Initiation–promotion (tested as promoter) Hamster, Syrian golden (M) 11–13 wk 12 wk Lin et al. (1996)	Topical application on the cheek pouch Arecaidine, NR Polyethylene glycol 0 (control), 200, 300, 400, 500 µg/mL 0.5% DMBA (in mineral oil), 3×/wk for 8 wk, then arecaidine, 6×/wk for 4 wk 7, 7, 7, 7, 7 NR	*Cheek pouch*: exophytic squamous cell papilloma or carcinoma (combined) Tumour incidence: 5/7 (71%), 5/7 (71%), 5/7 (71%), 7/7 (100%), 7/7 (100%)	[NS; one-tailed Fisher exact test vs control]	Principal limitations: body-weight changes, NR; survival, NR; rationale for doses, NR; volumes applied, NR. Other comments: painting using a no. 4 camel-hair brush.
		Tumour multiplicity: 1.00 ± 0.76, 1.09 ± 1.02, 1.14 ± 0.99, 1.86 ± 0.63, 1.86 ± 0.93	[NS; one-way ANOVA test]	
		Total tumours: 7, 9, 8, 13, 13	NR	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Initiation-promotion (tested as promoter) Hamster, Syrian golden (M) 11–13 wk 12 wk Lin et al. (1996)	Topical application in the cheek pouch Arecaidine, NR Polyethylene glycol 0 (control), 600, 700, 800, 900, 1000 µg/mL 0.5% DMBA (in mineral oil), 3×/wk for 4 wk, then arecaidine, 6×/wk for 8 wk 7, 7, 7, 7, 7, 7 NR	*Cheek pouch*: exophytic squamous cell papilloma or carcinoma (combined) Tumour incidence: 0/7, 5/7 (71%)*, 5/7 (71%)*, 4/7 (57%)*, 7/7 (100%)*, 7/7 (100%)* Tumour multiplicity: 0.00 ± 0.00, 1.00 ± 0.75, 1.14 ± 0.99*, 1.00 ± 0.65, 1.86 ± 0.82*, 2.14 ± 1.09* Total tumours: 0, 7, 8, 7, 13, 15	*[P = 0.0350–0.0003]; one-tailed Fisher exact test, vs control] *[$P < 0.05$; one-way ANOVA followed by Dunnett's test, vs control] NR	Principal limitations: body-weight changes, NR; survival, NR; rationale for doses, NR; volumes applied, NR. Other comments: painting using a no. 4 camel-hair brush.

4-NQO, 4-nitroquinoline 1-oxide; ANOVA, analysis of variance; bw, body weight; DMBA, 7,12-dimethylbenz[*a*]anthracene; DMSO, dimethyl sulfoxide; F, female; KNO_3, potassium nitrate; LSD, least significant difference; M, male; mo, month; NMBA, *N*-benzyl-*N*-methylnitrosamine; NA, not applicable; NR, not reported; NS, not significant; vs, versus; wk, week.

number of animals per group, the fact that the tumours observed in the vehicle control groups were not otherwise specified, and the lack of a KNO_3-only control group.]

In a second experiment in the same study (Bhide et al., 1984), groups of male (n = 16–21) and female (n = 12–18) Swiss mice (age, 6 weeks) were placed on a diet deficient in vitamin B complex. After 2 weeks, they were treated by gavage five times per week "throughout the life-span" with vehicle (control) or 1 mg of arecoline hydrochloride dissolved in distilled water per animal, with 1 mg arecoline hydrochloride and 1 mg KNO_3 in distilled water per animal, or with 1 mg arecoline hydrochloride, 1 mg KNO_3, and 1 mg slaked lime in distilled water per animal.

When assessed over 25 months [it was unclear whether this was 25 months of age or 25 months of treatment], male mice treated with arecoline hydrochloride, KNO_3, and slaked lime showed a significant increase [P = 0.0361] in the incidence of total tumours of the liver, stomach, and lung (two mice developed liver haemangioma, three developed lung adenocarcinoma, and two developed stomach squamous cell carcinoma) compared with the vehicle control group. The total tumour incidence in the other treated groups of males did not differ significantly from that in the vehicle control group. Female mice treated with arecoline hydrochloride showed a significant increase [P = 0.0132] in the incidence of total tumours (two mice developed liver haemangioma and four developed lung adenocarcinoma) compared with the vehicle control group. Likewise, female mice treated with arecoline hydrochloride, KNO_3, and slaked lime showed a significant increase [P = 0.0143] in the incidence of total tumours of the liver, stomach, and lung (two mice developed liver haemangioma, three developed lung adenocarcinoma, two developed stomach squamous cell carcinoma, and one developed a cholangiocarcinoma) compared with the vehicle control group. The total tumour incidence in the other treated group of females (arecoline hydrochloride plus KNO_3) did not differ significantly from that in the vehicle control group (Bhide et al., 1984). [The Working Group noted the limited histopathology, the single dose used, the small number of animals per group, the fact that tumours observed in the vehicle control groups were not further specified, and the lack of a KNO_3-only control group and a KNO_3 plus lime control group.]

3.1.2 Intraperitoneal injection

A group of 10 male Swiss mice [age not reported] was treated with 1.5 mg of arecoline (analytical reagent grade) per animal [rationale for dose not reported; vehicle and volume not reported] by intraperitoneal injection once per week for 13 weeks. A control group of 10 mice was treated with 0.1 mL of distilled water by intraperitoneal injection once per week for 13 weeks. Both groups were monitored for their "lifetime" [the precise duration was not reported]. Neither the control group nor the group treated with arecoline developed any tumours (Shivapurkar et al., 1980). [The Working Group noted the small number of animals per group, the use of male animals only, the single dose used, the lack of survival and body-weight data, and the short duration of treatment. The study was considered inadequate for the evaluation of the carcinogenicity of arecoline in experimental animals.]

3.1.3 Subcutaneous injection

A group of 10 male Swiss mice [age not reported] was treated with 1.5 mg of arecoline (analytical reagent grade) per animal [rationale for dose not reported; vehicle and volume not reported] dorsally by subcutaneous injection once per week for 13 weeks. A control group of 20 mice was treated with 0.1 mL of distilled water by subcutaneous injection once per week for 13 weeks. Both groups were monitored for their "lifetime" [the precise duration was not

reported]. Neither the control group nor the group treated with arecoline developed tumours at the site of injection or in any other organs (Shivapurkar et al., 1980). [The Working Group noted the small number of animals per group, the use of male animals only, the single dose used, the lack of survival and body-weight data, and the short duration of treatment. The study was considered inadequate for the evaluation of the carcinogenicity of arecoline in experimental animals.]

3.1.4 Co-administration with known carcinogens

In a study by Chang et al. (2010), two groups of 10 male C57BL/6JNarl mice (age, 6 weeks) were given drinking-water containing 4-nitroquinoline 1-oxide (4-NQO) [purity not reported] at a concentration of 100 or 200 µg/mL [rationale for the doses not reported] for 8 weeks. Two additional groups of 10 mice received drinking-water containing arecoline hydrobromide [purity not reported] at a concentration of 250 or 500 µg/mL [rationale for the doses not reported] for 8 weeks. Four additional groups of 11 mice received drinking-water containing 4-NQO at 100 µg/mL and arecoline hydrobromide at 250 or 500 µg/mL; or 4-NQO at 200 µg/mL and arecoline hydrobromide at 250 or 500 µg/mL. A control drinking-water group of 10 mice was included. After 8 weeks of treatment, no histopathological evidence of disease was reported in mice (three per group) that were killed to assess lesions of the tongue, oesophagus, liver, colon, kidney, spleen, or stomach. The remaining mice continued to receive control drinking-water for an additional 20 weeks. Mice receiving 4-NQO showed a dose-related decrease in water consumption. Drinking-water consumption was not affected by treatment with arecoline hydrobromide. When assessed at 28 weeks, mice receiving drinking-water containing 4-NQO at 200 µg/mL, or 4-NQO at 100 µg/mL plus arecoline hydrobromide at 500 µg/mL, or 4-NQO at 200 µg/mL plus arecoline hydrobromide at 250 µg/mL, showed significant decreases in body weight [body-weight values being 75–85% of those of the control group]. Histopathological examination was performed and reported for the tongue and the oesophagus.

There were no lesions of the tongue or oesophagus (hyperplasia, dysplasia, papilloma, or invasive squamous cell carcinoma, combined) in mice treated only with drinking-water containing arecoline hydrobromide at 250 or 500 µg/mL for 28 weeks. Arecoline hydrobromide had no significant effect on the incidence of lesions of the tongue or oesophagus (hyperplasia, dysplasia, papilloma, or invasive squamous cell carcinoma, combined) (Chang et al., 2010). [The Working Group noted the small number of animals per group, the short durations of exposure and follow-up, and the combination of pre-neoplastic and neoplastic lesions. The study was considered inadequate for the evaluation of the carcinogenicity of arecoline in experimental animals.]

In a study by Chen et al. (2017), two groups of 16 male C57BL/6 mice (age, 6 weeks) received drinking-water containing 4-NQO at a concentration of 100 µg/mL [purity not reported; rationale for dose not reported] for 8 or 16 weeks. A group of 8 mice received drinking-water containing arecoline hydrobromide at 500 µg/mL [purity not reported; rationale for dose not reported] for 16 weeks. Two groups of 16 mice received drinking-water containing 4-NQO at 100 µg/mL plus arecoline hydrobromide at 500 µg/mL for 8 or 16 weeks. A control drinking-water group of 8 mice was included. After 8 or 16 weeks of treatment, mice were placed on control drinking-water. All surviving mice were killed 28 weeks after the initiation of treatment. Up to three mice per group exposed to 4-NQO died before the end of the experiment. [Treatment-related changes in body weight, and data on drinking-water consumption were not

reported.] Histopathological examination was performed and reported for the oesophagus. The incidence of papilloma and invasive squamous cell carcinoma of the oesophagus was only reported for mice alive at 28 weeks.

In mice given drinking-water containing 4-NQO at 100 μg/mL plus arecoline hydrobromide at 500 μg/mL for 8 weeks, there was a significant increase [$P = 0.0329$] in the incidence of invasive squamous cell carcinoma of the oesophagus compared with mice given only 4-NQO for 8 weeks. In mice given drinking-water containing 4-NQO at 100 μg/mL for 16 weeks or 4-NQO at 100 μg/mL plus arecoline hydrobromide at 500 μg/mL for 8 or 16 weeks, there was a significant increase [$P < 0.05$] in the incidence of invasive squamous cell carcinoma of the oesophagus compared with the control group.

In mice given drinking-water containing 4-NQO at 100 μg/mL for 16 weeks or 4-NQO at 100 μg/mL plus arecoline hydrobromide at 500 μg/mL for 8 or 16 weeks, there was a significant increase [$P < 0.003$] in the incidence of oesophageal papilloma compared with the control group. In mice given drinking-water containing 4-NQO at 100 μg/mL plus arecoline hydrobromide at 500 μg/mL for 8 weeks, there was a non-significant increase [$P = 0.0528$, one-tailed Fisher exact test] in the incidence of oesophageal papilloma compared with mice receiving 4-NQO at 100 μg/mL for 8 weeks. No tumours were observed in mice given only arecoline hydrobromide (Chen et al., 2017).

3.2 Rat

Oral administration (drinking-water)

In a carcinogenicity and co-carcinogenicity study by Wu et al. (2016), male F344 rats (age, 6 weeks) were allocated to one of four groups of 22 rats. A first group received a subcutaneous injection of 0.2 mL of 20% dimethyl sulfoxide (DMSO) three times per week for 5 weeks. This group received control drinking-water for up to 30 weeks. A second group received a subcutaneous injection of *N*-benzyl-*N*-methylnitrosamine (NMBA) [purity not reported] at 500 μg/kg body weight (bw) in 0.2 mL of 20% DMSO three times per week for 5 weeks. This dose was selected based on data from the literature. This group also received control drinking-water for up to 30 weeks. A third group received a subcutaneous injection of 0.2 mL of 20% DMSO three times per week for 5 weeks. This group received drinking-water containing arecoline [purity not reported; rationale for dose not reported] at 500 μg/mL for up to 30 weeks. A fourth group received a subcutaneous injection of NMBA at 500 μg/kg bw in 0.2 mL of 20% DMSO three times per week for 5 weeks. This group received drinking-water containing arecoline at 500 μg/mL for up to 30 weeks. Papillomas of the oesophagus and of the tongue were assessed by macroscopic examination, and confirmed by microscopic examination for all tongue lesions, and only half of the oesophageal lesions.

Twenty-five weeks after the initiation of the study, the incidence of papilloma of the oesophagus and of the tongue was assessed in seven rats from each group. Oesophageal papilloma only occurred in the group treated with NMBA and arecoline, with the incidence (7/7) [$P = 0.0003$] and multiplicity (1.86 ± 0.10) ($P < 0.0001$) significantly increased compared with that in the group treated only with NMBA (incidence, 0/7). Tongue papilloma occurred in rats treated with NMBA and with NMBA plus arecoline; but neither incidence nor multiplicity differed significantly between the two groups.

Thirty weeks after the initiation of the study, the incidence of papilloma of the oesophagus and of the tongue was assessed in the remaining 15 rats from each group. Oesophageal papilloma occurred in groups treated with NMBA, arecoline, and NMBA plus

arecoline; multiplicity was significantly increased (P = 0.0260) in rats treated with NMBA plus arecoline compared with those treated with only NMBA. Tongue papilloma occurred in groups treated with NMBA, arecoline, and NMBA plus arecoline; multiplicity was significantly increased (P = 0.0494) in rats treated with NMBA plus arecoline compared with those treated with only NMBA (Wu et al., 2016). [The Working Group noted the small number of animals per group, and the short duration of the carcinogenicity study.]

3.3 Hamster

Topical application in the cheek pouch or to the base of the tongue

In a study by Dunham et al. (1974), an approximately equal number of male and female Syrian hamsters (age, 6–7 weeks; 9 hamsters total) were treated topically in the right cheek pouch with a 1.5% solution of arecoline [purity not reported] in distilled water five times per week for their lifespan [the duration was not specified]. This dose (approximately 1 mg/day) was anticipated to produce some physiological effects but not kill the animals. Three to four hours before application of the arecoline, 30 mg of calcium hydroxide [purity not reported], as a 0.5% solution in DMSO [6 mL] was applied to the cheek pouch. A second group of eight hamsters (with an approximately equal number of males and females) was treated similarly with 1.5% arecoline (approximately 1 mg/day) in DMSO three times per week for 5 months. This group was also pre-treated with calcium hydroxide. A control group for these two treated groups consisted of eight hamsters given 0.5% calcium hydroxide applied in DMSO.

A third group of eight hamsters (with an approximately equal number of males and females) was treated by oral administration with drops of 2% arecoline (approximately 2 mg/day) applied in distilled water to the base of the tongue twice daily, 5 days per week, for the lifespan. A fourth group of eight hamsters (with an approximately equal number of males and females) was treated with 2% arecoline and 0.5% calcium hydroxide in distilled water in a similar manner. A control group for these two groups consisted of four hamsters and was treated with only 0.5% calcium hydroxide applied in distilled water.

A fifth group of four hamsters (with an approximately equal number of males and females) was given feed containing arecoline at approximately 6 mg/day (prepared by adding a 0.1% aqueous solution to the feed) and 2.5% calcium hydroxide [approximately 150 mg/day] 5 days per week for 16 months. A control group for this treatment group consisted of four hamsters fed only 2.5% calcium hydroxide.

Microscopic examination was performed, and neoplastic lesions were only reported for the cheek pouch or the oesophagus. One female hamster treated with arecoline (in distilled water) in the cheek pouch for life developed an oesophageal papilloma. No hamsters in the control groups or the other arecoline-treated groups developed neoplasms in the cheek pouch or oesophagus (Dunham et al., 1974). [The Working Group noted the small number of hamsters per group and lack of survival and body-weight data. The study was considered inadequate for the evaluation of the carcinogenicity of arecoline in experimental animals.]

Four Syrian golden hamsters (age, 1.5 months) [sex not reported] were given feed containing arecoline [purity not reported] at approximately 6 mg/day (prepared by adding a 0.1% aqueous solution to the feed) and 2.5% calcium hydroxide [purity not reported; approximately 150 mg calcium hydroxide/day], 5 days per week for 12 months. This dose was not anticipated to affect the hamsters' health or survival. A control group consisted of five hamsters given only feed containing 2.5% calcium hydroxide for 16 months. The experiment ended when the last hamster died at age 25.5 months, and

microscopic examination was performed. The only tumour reported was an argyrophilic carcinoid of the glandular stomach, which occurred in one hamster fed arecoline. This is a very rare tumour in Syrian golden hamsters (Dunham et al., 1975). [The Working Group noted the very small number of hamsters per group and lack of body-weight data. The study was considered inadequate for the evaluation of the carcinogenicity of arecoline in experimental animals.]

3.4 Carcinogenicity of metabolites

The available studies on the carcinogenicity of arecoline metabolites concerned topical appplication in the cheek pouch of arecaidine to Syrian golden hamsters.

Twenty-four male Syrian golden hamsters (age, 8 weeks) were treated with arecaidine at 25 µg/mL in distilled water (the pH of the solution was approximately 3) [purity not reported; volume administered not reported] applied topically in one cheek pouch, three times per week for 12 weeks. This dose was based upon the results obtained from an "in vitro" carcinogenicity study with arecaidine. At the end of 12 weeks, 13 hamsters were still alive. These animals were observed for 10 additional weeks. After the 10-week observation period, the 13 hamsters were treated topically in the same cheek pouch with a 1% solution of croton oil [purity, characterization, and stability, not reported; volume administered, not reported; rationale for dose, not specified] three times per week for 3 weeks. The hamsters were monitored for 34 weeks after completion of the croton-oil treatment, at which time three hamsters were still alive. There were no tumours evident upon macroscopic and microscopic examination of the cheek pouch of hamsters treated with only arecaidine or with arecaidine followed by croton oil (MacDonald, 1987). [The Working Group noted the lack of control groups and body-weight data, and the short durations of exposure and follow-up in the carcinogenicity experiment; and the large time gap between initiation and promotion, lack of body-weight data, and poor survival in the initiation–promotion experiment. The Working Group considered the study inadequate for the evaluation of the carcinogenicity of arecaidine.]

In a first initiation–promotion experiment in the study by Lin et al. (1996), groups of seven male Syrian golden hamsters (age, 11–13-weeks) were treated topically in the right cheek pouch with 0.5% 7,12-dimethylbenz[*a*]anthracene (DMBA) [purity not reported; rationale for doses not specified] in heavy mineral oil [volume administered not reported], three times per week for 8 weeks. At the end of the 8-week period, one group was left untreated (control) while the remaining groups were treated topically in the right cheek pouch with arecaidine at 200, 300, 400, or 500 µg/mL [purity not reported; rationale for doses not specified] in polyethylene glycol [volume administered not reported], six times per week for 4 weeks. In another initiation–promotion experiment in the same study, additional groups of seven male Syrian golden hamsters were treated topically in the right cheek pouch with 0.5% DMBA in heavy mineral oil, three times per week for 4 weeks. At the end of the 4-week period, one group was left untreated (control) while the remaining groups were treated topically in their right cheek pouch with arecaidine at 600, 700, 800, 900, or 1000 µg/mL, six times per week for 8 weeks. In a full carcinogenicity experiment in the same study, further groups were treated in the right cheek pouch with arecaidine at 0 (control), 1000, 2000, or 3000 µg/mL, six times per week for 12 weeks. Twelve weeks after the initiation of dosing, the extent of tumorigenesis was assessed in the buccal pouches.

In the first initiation–promotion experiment, the incidence of tumours (exophytic squamous cell papilloma or carcinoma, combined) in the cheek pouch in the group of hamsters treated with DMBA for 8 weeks and then held for an additional 4 weeks was 71%. Neither the tumour

incidence nor the average number of tumours was significantly increased by the subsequent administration of arecaidine for 4 weeks. In the other initiation–promotion experiment, control hamsters treated with DMBA for 4 weeks did not develop cheek pouch tumours when assessed at 12 weeks. The subsequent administration of arecaidine for 8 weeks resulted in a significant increase in the incidence of tumours [range, 57–100%; P = 0.0350–0.0003, versus control] in all treatment groups, coupled with a significant increase in the average number of tumours [$P < 0.05$, versus control] in the groups at 700, 900, and 1000 µg/mL. In the full carcinogenicity experiment, there were no cheek pouch tumours in hamsters treated only with arecaidine (Lin et al., 1996). [The Working Group noted the short durations of exposure and follow-up for the full carcinogenicity experiment, the lack of survival and body-weight data, and the small number of hamsters per group for the full carcinogenicity experiment. The full carcinogenicity experiment was considered inadequate for the evaluation of the carcinogenicity of arecaidine in experimental animals.]

3.5 Evidence synthesis for cancer in experimental animals

The carcinogenicity of arecoline has been assessed in two studies in male and female mice treated by oral administration (gavage). One study in male mice treated by intraperitoneal injection and one study in male mice treated by subcutaneous injection were considered inadequate for assessing the carcinogenicity of arecoline in experimental animals. The carcinogenicity of arecoline has also been assessed in male mice upon co-administration with known carcinogens in two studies, one of which was considered inadequate for the evaluation of the carcinogenicity of arecoline in experimental animals. Additionally, arecoline was assessed in one study in male rats upon administration with known carcinogens. The carcinogenicity of arecoline has been investigated in hamsters, in two studies of oral administration and in studies of topical application to the cheek pouch that were considered inadequate for the evaluation of the carcinogenicity of arecoline in experimental animals. Other studies in hamsters evaluated the carcinogenicity of the arecoline metabolite arecaidine.

In one study, male Swiss mice treated with arecoline hydrochloride by oral administration (gavage) showed a significant increase in the incidence of total tumours. In another study, female Swiss mice fed a vitamin B complex-deficient diet and treated with arecoline hydrochloride by gavage showed a significant increase in the incidence of total tumours (Bhide et al., 1984). No tumours were observed in male Swiss mice treated with arecoline by intraperitoneal or subcutaneous injection in studies considered inadequate for assessing the carcinogenicity of arecoline in experimental animals (Shivapurkar et al., 1980).

In a co-carcinogenicity study in which arecoline hydrobromide was administered by oral administration (in the drinking-water) in combination with the carcinogen 4-NQO to male C57BL/6 mice, arecoline hydrobromide plus 4-NQO increased the incidence of invasive squamous cell carcinoma of the oesophagus compared with mice receiving only 4-NQO (Chen et al., 2017). In a second co-carcinogenicity study that was considered inadequate for the evaluation of the carcinogenicity of arecoline in experimental animals, arecoline hydrobromide was administered in combination with the carcinogen 4-NQO by oral administration (in the drinking-water) to male C57BL/6JNarl mice and had no significant effect upon the incidence of tongue or oesophageal lesions (hyperplasia, dysplasia, papilloma, or invasive squamous cell carcinoma, combined) compared with mice receiving only 4-NQO (Chang et al., 2010).

In one co-carcinogenicity study, male F344 rats given NMBA by subcutaneous injection and drinking-water containing arecoline showed a significant increase in the incidence and multiplicity of oesophageal papilloma and a significant increase in multiplicity of tongue papilloma, compared with rats receiving only NMBA (Wu et al., 2016).

One study was conducted in which male and female Syrian golden hamsters were treated with arecoline applied to the cheek pouch or to the base of the tongue (Dunham et al., 1974). In this study and another, male and female Syrian golden hamsters were also fed a diet containing arecoline (Dunham et al., 1974, 1975). Both studies were judged to be inadequate for assessing the carcinogenicity of arecoline in experimental animals.

Other studies in male Syrian golden hamsters evaluated the carcinogenicity of the arecoline metabolite arecaidine as a complete carcinogen, as a tumour initiator in one experiment, and as a tumour promoter in two experiments. Male Syrian golden hamsters were used in a study to assess the ability of arecaidine to act as a complete carcinogen in one experiment or as a tumour promoter in two experiments. In one experiment, treating hamsters in the cheek pouch with the carcinogen DMBA in the initiation phase followed by arecaidine in the promotion phase resulted in a significant increase in the incidence and multiplicity of exophytic squamous cell papilloma or carcinoma (combined) compared with hamsters treated with DMBA only; hamsters administered only arecaidine did not develop tumours (Lin et al., 1996). In a separate study judged to be inadequate for assessing the carcinogenicity of arecaidine in experimental animals, the ability of arecaidine to act as a complete carcinogen or as a tumour initiator was assessed by applying arecaidine by itself or with croton oil (in the promotion phase) to the cheek pouch of male Syrian golden hamsters (MacDonald, 1987).

4. Mechanistic Evidence

4.1 Absorption, distribution, metabolism, and excretion

4.1.1 Humans

(a) Exposed humans

Arecoline is readily absorbed in the oral cavity. It has been found in saliva (Nair et al., 1985; Cox et al., 2010; Lee et al., 2015; Franke et al., 2016; Venkatesh et al., 2018) as well as in urine (Franke et al., 2016) from areca-nut and betel-quid chewers. In habitual chewers, arecoline was detected in saliva not only during 25 minutes of chewing areca nut but also before and 15 minutes after removing nut particles from the mouth. In 20 out of 22 chewers, arecoline concentrations in saliva were above 0.1 µg/mL, and in 11 chewers (50%) a salivary concentration of 10 µg/mL was exceeded during chewing and after spitting out the areca nut (Cox et al., 2010). Somewhat lower salivary arecoline concentrations (mean during chewing, 77 ng/mL) were detected under similar conditions by Venkatesh et al. (2018).

Pharmacokinetic parameters of arecoline were measured in a study of 15 Alzheimer patients (9 women and 6 men) who received 5 mg of arecoline each by intravenous infusion during 30 minutes. Plasma concentrations quickly decreased following two-phase (biexponential) kinetics. In the plasma, the half-lives of arecoline were 0.95 ± 0.54 minutes and 9.33 ± 4.5 minutes for the first and second phase, respectively. Other kinetic parameters were as follows: maximum plasma concentration, C_{max}, 27.8 ± 20.5 ng/mL; clearance, 13.6 ± 5.8 L/kg; and steady-state apparent volume of distribution, V_d, 2.55 ± 2.05 L/kg (Asthana et al., 1996).

Lee et al. (2015) identified *N*-methylnipecotic acid (MNPA) (see Fig. 4.1) in saliva during and shortly after chewing areca nut. Its concentration and ratio to both arecoline and arecaidine (which

were also detected in saliva) rose in the course of and after chewing, indicating that MNPA is a metabolite of arecoline. MNPA, together with arecaidine and arecoline, was also found in the urine after ingestion of an aqueous extract of areca nut as well as after areca-nut chewing. The major urinary metabolite was arecaidine, with an elimination half-life of 4.3 hours, followed by MNPA, with an elimination half-life of 7.9 hours, and very low levels of arecoline, with an elimination half-life of 0.97 hours. Mean urinary concentrations of arecoline, arecaidine, and MNPA in regular areca-nut chewers (who were also smokers) were 23.9, 5816, and 1298 ng/mg creatinine, respectively (Hu et al., 2010).

Arecoline and its metabolite arecaidine were also found in the plasma of betel-quid chewers. Amounts correlated significantly with self-reported amounts of betel quid chewed the day before blood sampling (Wu et al., 2010).

In a pilot pharmacokinetic study, arecoline, together with three other areca alkaloids, was detected in the saliva of four occasional betel-nut chewers. Arecoline concentrations in saliva peaked within the first 2 hours post-chewing before returning to baseline levels after 8 hours and paralleled urinary excretion in one volunteer (Franke et al., 2016).

Arecoline was detected at concentrations ranging from 18 to 159.9 µg/L in the breast milk of four betel-quid chewers (Pellegrini et al., 2007), as well as in meconium and urine of infants of mothers who used betel nut during pregnancy (Pichini et al., 2003).

Two nitrosamines, namely *N*-nitrosoguvacoline and *N*-nitrosoguvacine, were found in the saliva but not in the urine of betel-quid chewers (Fig. 4.2; Nair et al., 1985). [The Working Group noted that these nitrosamines may have been formed from arecoline as well as from other alkaloids of betel quid, namely, guvacoline and guvacine.]

(b) Human enzymes in acellular systems in vitro

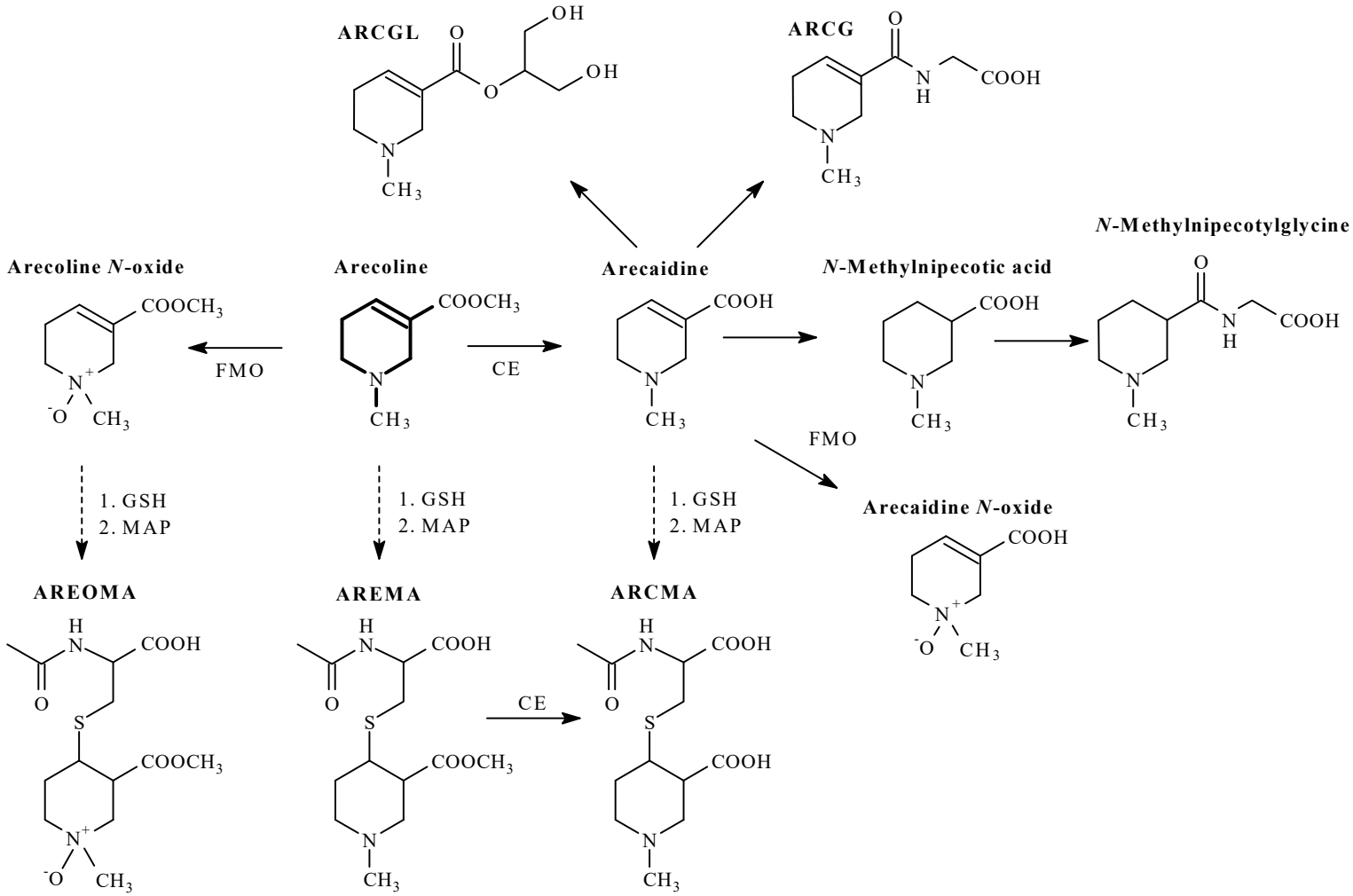
Arecoline was converted to arecoline *N*-oxide in vitro by recombinant human flavin-containing monooxygenases FMO1 (K_m, 13.6 ± 4.9 µM; V_{max}, 0.114 ± 0.01 nmol/min per µg protein) and FMO3 (K_m, 44.5 ± 8.0 µM; V_{max}, 0.014 ± 0.001 nmol/min per µg protein) but not by FMO5 or any of 11 recombinant human cytochrome P450s (CYPs) (Giri et al., 2007).

4.1.2 Experimental systems

(a) Absorption and distribution

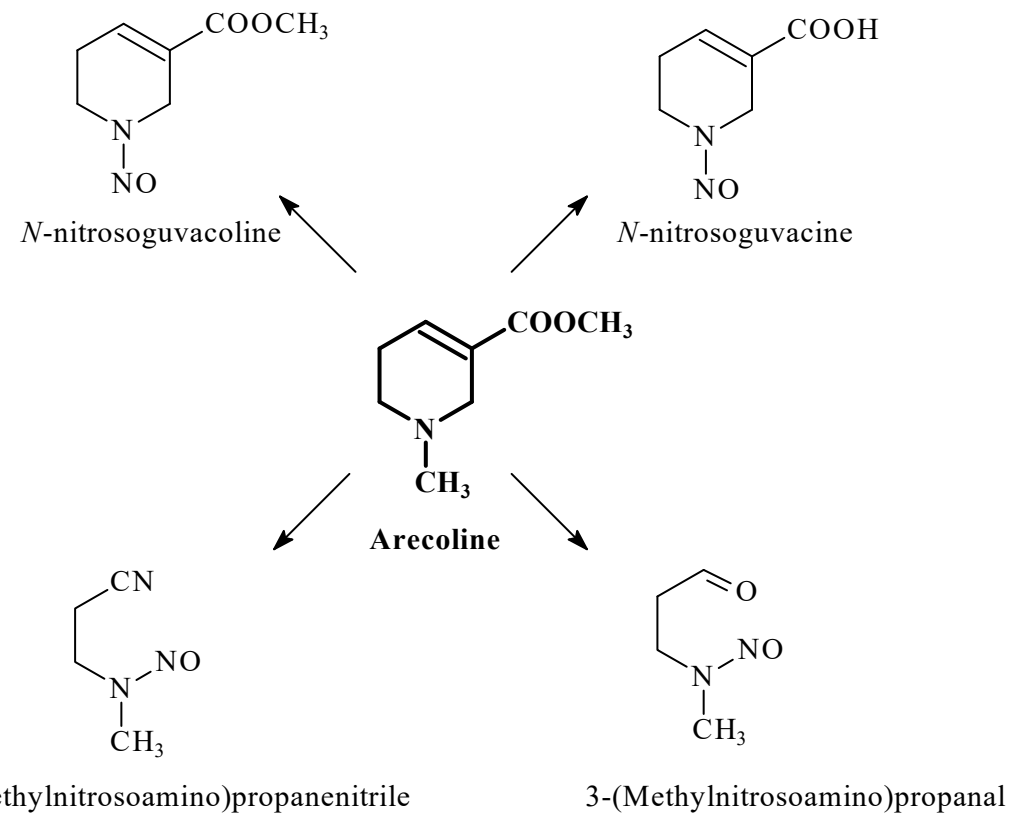
Arecoline was rapidly absorbed in dogs after oral administration (Li et al., 2014), in rats after oral (Pan et al., 2017, 2018), intraperitoneal (Soncrant et al., 1989), intranasal, or intramuscular application (Hussain & Mollica, 1991), and in mice after intraperitoneal (Patterson & Kosh, 1994), intravenous, or oral administration (Sethy & Francis, 1990). When dogs were dosed orally with arecoline hydrobromide at 3 mg/kg bw, the concentration of arecoline in the plasma peaked after 120 minutes, reaching 60.6 ng/mL. Absorption and elimination half-lives were 50 and 69 minutes, respectively, and plasma clearance was 0.19 L/minute per kg (Li et al., 2014). A much shorter elimination time of arecoline ($t_{1/2}$, 6.5 ± 0.4 minutes; n = 5) was found in rats dosed orally with arecoline hydrobromide at 150 mg/kg bw. In this case, plasma concentration peaked 5 minutes after dosing and reached approximately 175 ng/mL (Pan et al., 2017). At a lower oral dose (20 mg/kg bw) the maximum plasma concentration was 15 ng/mL at 0.25 hour. The sensitivity of the analytical method used was not sufficient to determine pharmacokinetic parameters for arecoline, therefore the pharmacokinetics of its abundant metabolites, arecaidine and arecoline *N*-oxide, were followed. For arecaidine and arecoline *N*-oxide, respectively, maximum plasma concentrations

Fig. 4.1 Metabolism of arecoline



ARCG, *O*-arecaidinylglycine; ARCGL, *O*-arecaidinylglycerol; AREMA, *N*-acetyl-*S*-(3-methoxycarbonyl-1-methylpiperid-4-yl)-L-cysteine (arecoline mercapturic acid); ARCMA, *N*-acetyl-*S*-(3-carboxy-1-methylpiperid-4-yl)-L-cysteine (arecaidine mercapturic acid); AREOMA, arecoline *N*-oxide mercapturic acid; CE, carboxylesterases, FMO, flavine-monooxygenases; GSH, glutathione; MAP, mercapturic acid pathway.
Compiled by the Working Group with data from Giri et al. (2006), Boyland & Nery (1969) and Patterson & Kosh (1993).

Fig. 4.2 Nitrosation and cleavage of arecoline



Created by the Working Group.

were 2130 ± 611 and 2761 ± 138 ng/mL at 1.83 ± 0.29 and 1.33 ± 0.58 hours, plasma clearance was 2.02 ± 0.21 and 1.6 ± 0.25 L/hour per kg and apparent volume of distribution was 6.75 ± 2.0 and 6.01 ± 1.85 L/kg, respectively (Pan et al., 2018). Because of its cholinergic activity via binding to muscarine receptors, distribution of arecoline into the brain is of special interest. In a study in Fischer 344 rats (aged 3 and 24 months) given a single intraperitoneal injection of arecoline hydrobromide at 5 mg/kg bw, peak plasma concentrations of 1142 ± 554 ng/mL and 923 ± 368 ng/mL (mean ± standard error; $n \geq 3$), respectively, were measured after 1 minute. Thereafter, arecoline was eliminated with $t_{1/2}$ values of 5.8 and 3.5 minutes, respectively. Arecoline rapidly entered the brain where it reached peak concentrations of 1558 ± 588 ng/g and 1830 ± 317 ng/g (mean ± standard error; $n \geq 3$) in 3 minutes in the cerebral cortex of rats aged 3 and 24 months, respectively. Thereafter, the brain concentrations in all rats declined rapidly, with $t_{1/2}$ values of 3.6 and 2.9 minutes, respectively. Hence, small but statistically significant differences in pharmacokinetic parameters were found between young and old rats (Soncrant et al., 1989). When mice were given a single intraperitoneal dose of arecoline at 15 mg/kg bw, the concentration of arecoline in the brain reached a maximum of 7.9 nmol/g after 3 minutes and decreased to 1 nmol/g after 30 minutes. The highest arecoline concentration was found in

the cortex followed by the subcortex and cerebellum (Patterson & Kosh, 1994). Somewhat slower distribution into and elimination from the brain was reported in mice after an intravenous injection of 10 μmol/kg as indicated indirectly by an ex vivo [^{3}H]-oxotremorine binding assay. The inhibition of oxotremorine binding reached a maximum 10 minutes after injection (Sethy & Francis, 1990). [The Working Group noted that the slower time course observed after intravenous injection might be explained by the much lower dose and/or methodological issues.]

(b) Metabolism and excretion

Primary metabolic pathways of arecoline are ester hydrolysis to arecadine and oxidation to arecoline *N*-oxide, glutathione conjugation to yield *N*-acetyl-*S*-(3-methoxycarbonyl-1-methylpiperid-4-yl)-L-cysteine (arecoline mercapturic acid; AREMA) (Boyland & Nery, 1969; Nery, 1971; Patterson & Kosh, 1993; Giri et al., 2006). Double-bond reduction eventually yielding MNPA, a major arecoline metabolite in mice, was found to be another important metabolic pathway (Giri et al., 2006).

In an early study in female rats dosed with arecoline hydrochloride at 20 mg/kg bw per day by intraperitoneal injection for 3 weeks, three urinary metabolites were identified as arecaidine, AREMA, and *N*-acetyl-*S*-(3-carboxy-1-methylpiperid-4-yl)-L-cysteine (arecaidine mercapturic acid; ARCMA) (Boyland & Nery, 1969). The same metabolites in different relative amounts were also obtained when arecoline hydrochloride was administered orally (0.2% arecoline hydrochloride w/v in water ad libitum for 10 days) (Boyland & Nery, 1969).

In a study in mice dosed with either arecoline hydrobromide or arecaidine (both at 20 mg/kg bw orally and by intraperitoneal injection), 10 arecoline metabolites were identified in the urine, namely: arecaidine, arecoline *N*-oxide, arecaidine *N*-oxide, MNPA, *N*-methylnipecotylglycine, *O*-arecaidinylglycine, *O*-arecaidinylglycerol, ARCMA, AREMA, and arecoline *N*-oxide mercapturic acid (AREOMA) (Giri et al., 2006). Six of these metabolites were formed from arecaidine, namely, arecaidine *N*-oxide, MNPA, *N*-methylnipecotylglycine, ARCMA, *O*-arecaidinylglycine, and *O*-arecaidinylglycerol. Unchanged arecoline excreted in the urine within 12 hours after dosing comprised 0.3–0.4% of the administered dose, major metabolites being arecaidine (7.1–13.1%), arecoline *N*-oxide (7.4–19.0%), and MNPA (13.5–30. 3% of the administered dose) (Giri et al., 2006).

(c) Metabolism in vitro

Arecoline was hydrolysed with mouse liver and kidney homogenates, and a supernatant obtained by centrifugation of the liver homogenate at 10 000 g. In the supernatant, a V_{max} of 4.7 nmol/min per mg protein and K_m of 9.6 mM were obtained. Experiments with several enzyme inhibitors indicated that carboxylesterase EC 3.1.1.1 was primarily responsible for the rapid metabolism of arecoline in the mouse (Patterson & Kosh, 1993).

Arecoline reacted readily with glutathione in a pH-dependent manner (Boyland & Nery, 1969). At pH 7.4 and 37 °C, the reaction of arecoline with glutathione and L-cysteine proceeded following second-order kinetics with the apparent rates of glutathione depletion of 0.0619 ± 0.009 μM^{-1} min^{-1} and 0.2834 ± 0.0637 μM^{-1} min^{-1} for glutathione and L-cysteine, respectively (Hoang et al., 2020). An addition of rat liver homogenate to the incubation mixture of glutathione and arecoline significantly decreased the yield (Boyland & Nery, 1969). [The Working Group noted that this effect can be explained by enzymatic hydrolysis of the methyl ester moiety.]

Induction of CYP2E1 was observed when rats were dosed orally with arecoline at 20 and 100 mg/kg bw per day for 7 days. The induction was attenuated with increasing dose (Huang et al., 2016a). [The Working Group noted that this

suggests possible metabolic interactions between numerous CYP2E1 substrates and arecoline in betel-quid chewers.]

In model experiments of nitrosation under conditions similar to those prevailing in the oral cavity or stomach of betel chewers (37 °C, pH ranging from 2 to 7), three nitrosamines were identified: *N*-nitrosoguvacoline, 3-(methylnitrosoamino)propanenitrile, and 3-(methylnitrosoamino)propanal (Fig. 4.2; Wenke & Hoffmann, 1983).

4.2 Evidence relevant to key characteristics of carcinogens

This section summarizes the evidence for the key characteristics of carcinogens (Smith et al., 2016), including whether arecoline is electrophilic or can be metabolically activated to an electrophile; is genotoxic; alters DNA repair or causes genomic instability; induces epigenetic alterations; induces oxidative stress; is immunosuppressive; modulates receptor-mediated effects; and alters cell proliferation, cell death, or nutrient supply.

4.2.1 Is electrophilic or can be metabolically activated to an electrophile

Arecoline contains an α,β-unsaturated carbonyl moiety in its molecule. This moiety is known to undergo Michael addition reactions by nucleophilic attack at the electrophilic β-carbon. Arecoline electrophilicity was manifested by reaction with glutathione in vitro with and without rat liver homogenate (Boyland & Nery, 1969; Hoang et al., 2020) and in vivo by formation of urinary AREMA (Boyland & Nery, 1969; Giri et al., 2006). Carboxylesterase-catalysed hydrolysis of arecoline to arecaidine and subsequent ionization of the latter under physiological pH diminishes the electrophilic reactivity due to the electron-donating carboxylate ion; however, arecaidine is itself electrophilic and reacts with glutathione. This reaction results in the formation of arecaidine mercapturic acid (Giri et al., 2006). Metabolic activation of arecoline to the corresponding epoxide has not been reported but N-oxidation also produces an electrophilic metabolite capable of reaction with glutathione (Giri et al., 2007) and, possibly, with other nucleophilic sites in biomolecules.

No data on DNA adducts were available to the Working Group. Protein adducts of arecoline and arecaidine were detected by LC-MS at cysteine, lysine, histidine, and the N-terminal amino acid of bovine serum albumin in vitro. Analogous adducts were also found in multiple proteins when rat renal proximal tubular cells (NRK-52E) were incubated with arecoline, and when human plasma was incubated with excess arecoline or arecaidine to mimic high exposure (Chou et al., 2012). Protein adducts at cysteine, lysine, and N-terminal amino acids with arecoline *N*-oxide and arecaidine *N*-oxide were found in human oral keratinocytes treated with areca-nut extract (Kuo et al., 2015). [The Working Group noted that, under physiological conditions, arecoline may form *N*-nitrosamines, which are known to yield alkylating agents upon activation.]

4.2.2 Is genotoxic

(a) Humans

(i) Exposed humans

No data were available to the Working Group.

(ii) Human cells in vitro

Arecoline

See Table 4.1.

Arecoline induced a minor increase in the frequency of DNA strand breaks in buccal epithelial cells in the DNA alkaline elution assay (Sundqvist et al., 1989, 1991), but did not induce DNA strand breaks in oral mucosal fibroblasts (Jeng et al., 1994) or buccal fibroblasts (Chang et al., 1998) in the DNA precipitation assay.

It induced phosphorylation of histone H2AX (γH2AX), a biomarker of DNA double-strand breaks, in a human oral cancer cell line (KB), human embryonic kidney cells (HEK293) (Tsai et al., 2008), an oral squamous cell carcinoma cell line (OC2) (Ji et al., 2012), and human gingival epithelial Smulow–Glickman cells and human oral squamous cell carcinoma cell lines (OEC-M1 and SAS) (Tu et al., 2019). Treatment of normal human gingival fibroblast cells (HGF-1) with arecoline hydrobromide resulted in a slight increase in γH2AX (Kuo et al., 2015). Arecoline induced DNA damage in human epithelial squamous carcinoma cells (HEp-2) analysed by the alkaline comet assay (Huang et al., 2016b).

Arecoline induced unscheduled DNA synthesis in HEp-2 cells (Sharan & Wary, 1992) and chromosomal aberration and sister-chromatid exchange in human lymphocytes (Kumpawat et al., 2003). Arecoline hydrobromide induced micronucleus formation in HEp-2 cells and in human lymphocytes (Kevekordes et al., 2001).

Arecoline N-*oxide*

See Table 4.1.

Treatment of normal human gingival fibroblast cells (HGF-1) with arecoline *N*-oxide resulted in an increase in γH2AX (Kuo et al., 2015, 2019).

(b) Experimental systems

(i) Arecoline

Non-human mammals in vivo

See Table 4.2.

Arecoline induced unscheduled DNA synthesis in Swiss albino mouse spermatids after a single intraperitoneal dose (Sinha & Rao, 1985a).

In C57BL/6J mice given drinking-water containing arecoline hydrobromide for 6 weeks, the transgenic rodent gene mutation assay did not show significant positive results. However, in the oral tissues from several individual animals, mutation frequencies were much higher than in untreated mice and mutation spectra exhibited (G:C→T:A transversion mutations) were unique compared with the untreated controls (Wu et al., 2012).

Several cytogenetic studies have been conducted with arecoline, arecoline hydrobromide, or arecoline hydrochloride administered by intraperitoneal or oral routes. The studies gave consistent positive results for chromosomal aberration, micronucleus formation, and sister-chromatid exchange in bone marrow (Panigrahi & Rao, 1982, 1983; Shirname et al., 1984; Deb & Chatterjee, 1998; Chatterjee & Deb, 1999). Arecoline hydrobromide administered as a single intraperitoneal dose to pregnant Swiss albino mice on day 17 of gestation gave positive results for micronucleus formation as measured in fetal blood (Sinha & Rao, 1985b).

Non-human mammalian cells in vitro

See Table 4.3.

Arecoline induced DNA strand breaks in mouse kidney cells as assessed by the alkaline DNA-unwinding assay (Wary & Sharan, 1988). Arecoline in the hydrobromide form induced DNA damage in rat liver clone-9 cells as shown by the alkaline comet assay (Chou et al., 2008), whereas arecoline did not induce DNA damage in rat liver clone-9 cells as shown by the alkaline comet assay (Wang et al., 2018).

Arecoline hydrochloride induced a significant increase in the frequency of hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) gene mutations with and without metabolic activation in Chinese hamster V79 cells (Shirname et al., 1984).

Several in vitro cytogenetics studies on arecoline or arecoline hydrobromide have been conducted without metabolic activation in Chinese hamster ovary (CHO) cells. Most studies gave positive results for chromosomal aberrations, micronucleus formation, and

Table 4.1 Genetic and related effects of arecoline and arecoline *N*-oxide in human cells in vitro

End-point	Tissue, cell line (sex)	Results[a]		Agent, concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Arecoline						
DNA strand breaks (SSBs, DNA alkaline elution assay)	Primary buccal epithelial cells	(+)	NT	Arecoline, 5 mM [776 µg/mL]	Limited reporting of material and methods. Minor increase.	Sundqvist et al. (1989, 1991)
DNA strand breaks (SSBs, DNA precipitation assay)	Primary oral mucosal fibroblasts	(–)	NT	Arecoline, 400 µg/mL, [2.57 mM]	Data not shown for SSBs.	Jeng et al. (1994)
DNA strand breaks (SSBs, DNA precipitation assay)	Primary oral mucosal fibroblasts	(–)	NT	Arecoline, 400 µg/mL [2.57 mM]	Data not shown for SSBs. No positive control.	Chang et al. (1998)
DNA strand breaks (DSBs, γH2AX)	Human oral cancer cell line, KB, and human embryonic kidney cell line, 293	+	NT	Arecoline, 0.3 mM [46.5 µg/mL]		Tsai et al. (2008)
DNA strand breaks (DSBs, γH2AX)	Normal human gingival fibroblast cell line, HGF-1	+	NT	Arecoline hydrobromide, 200 µM [47.2 µg/mL]		Kuo et al. (2015)
DNA strand breaks (DSBs, γH2AX)	Human oral SCC cell line, OC2	+	NT	Arecoline, 0.5 mM [77.6 µg/mL]		Ji et al. (2012)
DNA strand breaks (DSBs, γH2AX)	Human gingival epithelial Smulow–Glickman cells and human oral SCC cell lines OEC-M1 and SAS	+	NT	Arecoline, 200 µg/mL [1.29 mM]		Tu et al. (2019)
DNA damage (alkaline comet assay)	Human laryngeal SCC cell line, HEp-2	+	NT	Arecoline, 0.1 mM [15.5 µg/mL]		Huang et al. (2016b)
Unscheduled DNA synthesis	Human laryngeal SCC cell line, HEp-2	(+)	NT	Arecoline, 0.04 mM [6.2 µg/mL]	Dose-dependent increase; however, not data shown for vehicle control.	Sharan & Wary (1992)
Chromosomal aberration	Primary human lymphocytes (M)	+	NT	Arecoline, 0.43 mM [67 µg/mL]		Kumpawat et al. (2003)
Micronucleus formation	Primary human lymphocytes	+	+	Arecoline hydrobromide, 12.5 µM [3 µg/mL]		Kevekordes et al. (2001)
Micronucleus formation	Human hepatoma cell line, HEp-G2	+	NT	Arecoline hydrobromide, 25 µM [6 µg/mL]		Kevekordes et al. (2001)

Table 4.1 (continued)

End-point	Tissue, cell line (sex)	Results[a]		Agent, concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Sister-chromatid exchange	Primary human lymphocytes (M)	+	NT	Arecoline, 0.43 mM [67 µg/mL]		Kumpawat et al. (2003)
Arecoline N-*oxide*						
DNA strand breaks (DSBs, γH2AX)	Normal human gingival fibroblasts, HGF-1	+	NT	200 µM [34.5 µg/mL]	Greater increase than with arecoline hydrobromide.	Kuo et al. (2015, 2019)

DSB, double-strand breaks; γH2AX, phosphorylated histone H2AX; HIC, highest ineffective concentration; LEC, lowest effective concentration: M, male; NT, not tested; SSBs, single strand breaks; SCC, squamous cell carcinoma.

[a] +, positive; –, negative; (+) or (–), positive/negative in a study of limited quality.

Table 4.2 Genetic and related effects of arecoline and its metabolites in non-human mammals in vivo

End-point	Species, strain (sex)	Tissue	Results[a]	Agent, dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Arecoline							
Unscheduled DNA synthesis	Mice, Swiss albino (M)	Spermatid (early stage)	+	Arecoline, 20 mg/kg bw	Intraperitoneal; 1×; sampled after 16 h		Sinha & Rao (1985a)
Gene mutation	Mice, *Gpt* delta transgenic mice (C57BL/6J) (M)	Oral (gingival, buccal, pharyngeal, and sublingual)/ liver	–	Arecoline hydrobromide 700 µg/mL, 101 mg/kg bw per day	Oral (drinking-water), 6 wk, sampled after 2 wk	Unique mutation spectra (G:C→T:A transversion mutations) compared to spontaneous mutations.	Wu et al. (2012)
Chromosomal aberration	Mice, Swiss albino (M, F)	Bone marrow	+	Arecoline, 0.25 mg/animal	Intraperitoneal; daily for 10, 20 or 30 days; sampled after treatment		Panigrahi & Rao (1982)
Chromosomal aberration	Mice, Swiss albino (M)	Bone marrow	+	Arecoline, 40 mg/kg bw	Intraperitoneal; single administration; sampled after 20 h		Deb & Chatterjee (1998)
Chromosomal aberration	Mice, Swiss albino (M)	Bone marrow	(+)	Arecoline, 40 mg/kg bw (intraperitoneal), 170 µg/mL (oral)	Intraperitoneal and oral (ad libitum drinking-water); Intraperitoneal daily for 1, 5 or 15 days; sampled after 20 h	Only one concentration. No positive control.	Chatterjee & Deb (1999)
Micronucleus formation	Mice, Swiss albino (M)	Bone marrow	(+)	Arecoline hydrochloride, 2 mg/animal	Intraperitoneal; daily for 2 days, sampled after 6 h	Dose levels appear very high (LD_{50} reported, 2 mg/kg). No body weight reported.	Shirname et al. (1984)

Table 4.2 (continued)

End-point	Species, strain (sex)	Tissue	Results[a]	Agent, dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Micronucleus formation	Mice, Swiss albino (F)	Fetal blood	(+)	Arecoline hydrobromide, 20 mg/kg bw	Intraperitoneal; 1× (day 17 of gestation); sampled after 45 h	Limited details reported in materials and methods.	Sinha & Rao (1985b)
Sister-chromatid exchange	Mice, Swiss albino (M, F)	Bone marrow	+	Arecoline, 0.5 mg/animal	Intraperitoneal, daily for 5, 10, or 15 days; sampled after treatment		Panigrahi & Rao (1983)
Sister-chromatid exchange	Mice, Swiss albino, (M, F)	Bone marrow	(+)	2.5 mg/animal	Intraperitoneal; daily for 5, 15, or 20 days; sample after treatment	No positive control. Dose levels not justified. Toxicity and cytotoxicity not reported.	Panigrahi & Rao. (1984)
Sister-chromatid exchange	Mice, Swiss albino (M)	Bone marrow	+	Arecoline, 20 mg/kg bw	Intraperitoneal; 1×; sampled after 20 h		Deb & Chatterjee (1998)
Sister-chromatid exchange	Mice, Swiss albino (M)	Bone marrow	(+)	Arecoline, 40 mg/kg (intraperitoneal), 170 µg/mL (oral)	Intraperitoneal and oral (drinking-water); daily for 1, 5, or 15 days; sampled after 20 h	Only one concentration. No positive control.	Chatterjee & Deb (1999)
Arecaidine							
Micronucleus formation	Mice, Swiss (M)	Bone marrow	(–)	14 mg/animal	Intraperitoneal; daily for 2 days, sampled after 6 h	Dose levels appear very high (LD_{50} reported 14 mg/kg). No body weight reported.	Shirname et al. (1984)
Sister-chromatid exchange	Mice, Swiss albino, (M)	Bone marrow	+	2.5 mg/animal	Intraperitoneal; daily for 1, 5, or 15 days; sampled after 19 h		Panigrahi & Rao (1984)

Table 4.2 (continued)

End-point	Species, strain (sex)	Tissue	Results[a]	Agent, dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Arecoline N-*oxide*							
DNA strand breaks (DSBs, γH2AX)	Mice, NOD. CB17-*Prkdc*^{scid}/NcrCrl (NOD SCID)	Tongue	(+)	500 µg/mL (cotton swab smearing)	Topical; daily for 5 days, sampled after last treatment	One dose level only. Dose level not justified. No positive control. No clinical signs reported.	Kuo et al. (2015)

bw, body weight; DSB, DNA-strand breaks; F, female; Gpt, glutamic pyruvic transaminase; h, hour; γH2AX, phosphorylated histone H2AX; HID, highest ineffective dose; LD_{50}, median lethal dose; LED, lowest effective dose; M, male; wk, week.

[a] +, positive; –, negative; (+), positive in a study of limited quality.

Table 4.3 Genetic and related effects of arecoline and its metabolites in non-human mammals in vitro

End-point	Species, tissue, cell line	Results		Agent, concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Arecoline, arecoline hydrobromide, arecoline hydrochloride						
DNA strand breaks/ alkaline DNA-unwinding assay	Mice kidney primary cell	(+)	NT	Arecoline, [0.065 mM], 10 µg/mL	Source not reported. Only one concentration, no positive control.	Wary & Sharan (1988)
DNA damage/alkaline comet assay	Rat liver epithelial cells, clone-9 (cell line) (CRL-1439)	+	NT	Arecoline hydrobromide, 0.5 mM [118 µg/mL]		Chou et al. (2008)
DNA damage/alkaline comet assay	Rat liver clone-9 cell line	–	NT	Arecoline, 0.5 mM [77. 5 µg/mL]		Wang et al. (2018)
Gene mutation, *Hprt*	Chinese hamster lung V79 cells	+	+	Arecoline hydrochloride, [0.03 mM], 5 µg/mL		Shirname et al. (1984)
Chromosomal aberration	Chinese hamster ovary cells	+	NT	Arecoline hydrobromide, [0.32 mM], 75 µg/mL		Dave et al. (1992)
Chromosomal aberration	Chinese hamster ovary cells	+	NT	Arecoline hydrobromide, 0.85 mM [200 µg/mL]		Stich et al. (1981)
Chromosomal aberration	Chinese hamster ovary cells	(–)	NT	Arecoline hydrobromide, 0.05 mM [12.5 µg/mL]	Number of replicates not reported. Only one concentration. No cytotoxicity measured.	Trivedi et al. (1993)
Micronucleus formation	Chinese hamster ovary cells	+	NT	Arecoline, 0.2 µM [0.031 µg/mL]		Lee et al. (1996)
Sister-chromatid exchange	Chinese hamster ovary cells	+	NT	Arecoline hydrobromide, 0.05 mM, 12.5 µg/mL		Dave et al. (1992)
Sister-chromatid exchange	Chinese hamster ovary cells	(+)	NT	Arecoline hydrobromide, 0.05 mM, 12.5 µg/mL	Number of replicates not reported. Only one concentration. No cytotoxicity measured.	Trivedi et al. (1993)
Arecaidine						
Gene mutation, *Hprt*	Chinese hamster lung V79 cells	–	+	[0.071 mM], 10 µg/mL	Only one dose tested; No positive control.	Shirname et al. (1984)
Arecoline N-*oxide*						
DNA damage/comet assay	Rat liver clone-9 cells	+	NT	Arecoline, 125 µM [19.5 µg/mL]		Wang et al. (2018)

HIC, highest ineffective concentration; Hprt, hypoxanthine-guanine phosphoribosyltransferase; LEC, lowest effective concentration, NT, not tested.

[a] +, positive; –, negative; (+) or (–), positive in a study of limited quality.

sister-chromatid exchange (Stich et al., 1981; Dave et al., 1992; Lee et al., 1996). The study by Trivedi et al. (1993) gave positive results for sister-chromatid exchange and negative results for chromosomal aberration.

Non-mammalian experimental systems

See Table 4.4.

Arecoline hydrobromide induced DNA damage in *Drosophila melanogaster (hsp70-lacZ) Bg*^{9} as assessed by the comet assay (Shakya & Siddique, 2018; Shakya et al., 2019).

Arecoline hydrobromide did not induce DNA damage in the SOS chromotest in *Escherichia coli* PQ37 (Kevekordes et al., 1999).

Several assays for bacterial gene mutation, mainly in *Salmonella typhimurium* TA98 and TA100, have been conducted with arecoline, arecoline hydrobromide, or arecoline hydrochloride. Most studies gave positive results with and without metabolic activation (Shirname et al., 1983; Wang & Peng, 1996; Lin et al., 2011a). The study by Wang et al. (2018) performed only without metabolic activation gave negative results.

(ii) Arecaidine

See Table 4.2; Table 4.3 and Table 4.4.

Arecaidine did not induce micronucleus formation (Shirname et al., 1984) but induced sister-chromatid exchange in bone marrow after repeated intraperitoneal administration in Swiss albino mice (Panigrahi & Rao, 1984).

An in vitro assay for *Hprt* gene mutation with arecaidine gave positive results in the presence, but not the absence, of metabolic activation (Shirname et al., 1984).

In assays for bacterial gene mutation in *Salmonella typhimurium* TA100, TA1535, TA98, and TA1538, positive results were reported with and without metabolic activation (Shirname et al., 1983).

(iii) Arecoline N*-oxide*

See Table 4.2, Table 4.3 and Table 4.4.

Arecoline *N*-oxide induced γH2AX in mouse (NOD SCID) tongue tissues after 5-day topical application (once daily) (Kuo et al., 2015).

In rat liver clone-9 cells, arecoline *N*-oxide induced DNA damage as assessed by the comet assay (Wang et al., 2018).

Assays for bacterial gene mutation in *Salmonella typhimurium* TA98 and TA100 performed only without metabolic activation gave positive results (Lin et al., 2011a; Wang et al., 2018).

(iv) N*-nitrosoguvacoline*

See Table 4.4.

Assays for bacterial gene mutation in *Salmonella typhimurium* TA98 and TA100 with and without metabolic activation gave positive results (Wang & Peng, 1996).

4.2.3 Alters DNA repair or causes genomic instability

(a) Exposed humans

No data were available to the Working Group.

(b) Human cells in vitro

See Table 4.5.

Arecoline increased O^{6}-methylguanine-DNA methyltransferase activity in primary human oral keratinocytes (Lee et al., 2013a).

Arecoline increased DNA repair at 12.5 μg/mL [0.08 mM] but decreased repair at a higher concentration (i.e. 50 μg/mL [0.32 mM]) in two human oral squamous carcinoma cell lines (i.e. OEC-M1 and SAS) according to the host cell reactivation assay (Tu et al., 2019). Arecoline decreased DNA repair in a human oral cancer cell line (KB), a laryngeal squamous carcinoma cell line that may be a HeLa cervical cancer cell-line derivative (HEp-2), and human embryonic kidney cells (HEK293) assessed by means of

Table 4.4 Genetic and related effects of arecoline, arecaidine, arecoline *N*-oxide and *N*-nitrosoguvacoline in non-mammalian experimental systems

Test system (species, strain)	End-point	Results[a]		Agent, concentration (LEC or HIC)	Comments on study quality	Reference
		Without metabolic activation	With metabolic activation			
Arecoline						
Drosophila melanogaster (*hsp70-lacZ*) *Bg*^{9}	DNA damage (comet assay) on midgut cells	+	NA	Arecoline hydrobromide, 20 µM [5 µg/mL] (in feed)		Shakya & Siddique (2018)
Drosophila melanogaster (*hsp70-lacZ*) *Bg*^{9}	DNA damage (comet assay) on midgut cells	(+)	NA	Arecoline hydrobromide, 80 µM [19 µg/mL] (in feed)	Only one concentration, combined effects with geraniol investigated. No positive control.	Shakya et al. (2019)
SOS chromotest on *Escherichia. coli* PQ37	DNA damage (β-galactosidase activity)	–	–	Arecoline hydrobromide, 156.8 µg/assay		Kevekordes et al. (1999)
Salmonella typhimurium TA100, preincubation method	Reverse mutation	–	+	Arecoline hydrobromide, 1 µg/plate		Lin et al. (2011a)
Salmonella typhimurium TA98, preincubation method	Reverse mutation	–	–	Arecoline hydrobromide, 200 µg/plate		Lin et al. (2011a)
Salmonella typhimurium TA98, plate-incorporation method	Reverse mutation	–	–	Arecoline, 39.0 µmol/plate [6046 µg/plate]		Wang & Peng (1996)
Salmonella typhimurium TA98, plate-incorporation method	Reverse mutation	–	NT	Arecoline, 1 mM [155 µg/mL]		Wang et al. (2018)
Salmonella typhimurium TA1535, TA98, and TA1538, plate-incorporation method	Reverse mutation	(+)	(+)	Arecoline hydrochloride (concentration not reported)	Data not shown for TA1535, TA98, and TA1538 (reported to be positive).	Shirname et al. (1983)

Table 4.4 (continued)

Test system (species, strain)	End-point	Results[a]		Agent, concentration (LEC or HIC)	Comments on study quality	Reference
		Without metabolic activation	With metabolic activation			
Salmonella typhimurium TA100, plate-incorporation method	Reverse mutation	+	+	Arecoline hydrochloride, 10 µg/plate (+S9)		Shirname et al. (1983)
Salmonella typhimurium TA100, plate-incorporation method	Reverse mutation	+	+	Arecoline, 6.5 µmol/plate (–S9) [1009 µg/plate]		Wang & Peng (1996)
Salmonella typhimurium TA100, plate-incorporation method	Reverse mutation	–	NT	Arecoline, 1000 µM [155 µg/mL]		Wang et al. (2018)
Arecaidine						
Salmonella typhimurium TA1535, TA98, and TA1538, plate-incorporation method	Reverse mutation	(+)	(+)	NR	Data not shown for TA1535, TA98, and TA1538 (reported to be positive).	Shirname et al. (1983)
Salmonella typhimurium TA100, plate-incorporation method	Reverse mutation	+	+	100 µg/plate (–S9); 10 µg/plate (+S9)		Shirname et al. (1983)
Arecoline N-*oxide*						
Salmonella typhimurium TA98, plate-incorporation method	Reverse mutation	+	NT	125 µM [21 µg/mL]		Wang et al. (2018)
Salmonella typhimurium TA98, preincubation method	Reverse mutation	+	NT	1 µg/plate		Lin et al. (2011a)

Table 4.4 (continued)

Test system (species, strain)	End-point	Results[a]		Agent, concentration (LEC or HIC)	Comments on study quality	Reference
		Without metabolic activation	With metabolic activation			
Salmonella typhimurium TA100, plate-incorporation method	Reverse mutation	+	NT	500 µM [85 µg/mL]		Wang et al. (2018)
Salmonella typhimurium TA100, preincubation method	Reverse mutation	+	NT	50 µg/plate		Lin et al. (2011a)
N-*Nitrosoguvacoline*						
Salmonella typhimurium TA100, plate-incorporation method	Reverse mutation	+	+	24 µmol/plate (± S9) [4084 µg/plate]		Wang & Peng (1996)
Salmonella typhimurium TA98, plate-incorporation method	Reverse mutation	+	+	15 µmol/plate (–S9) [2552 µg/plate]		Wang & Peng (1996)

HIC, highest ineffective concentration; LEC, lowest effective concentration; NA, not applicable; NR, not reported; NT, not tested; S9, 9000 × *g* supernatant.

[a] +, positive; –, negative; (+), positive in a study of limited quality.

Table 4.5 Altered DNA repair and genomic instability in human cells exposed to arecoline in vitro

End-point	Tissue, cell line	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
DNA repair/O^6-methylguanine-DNA methyltransferase (protein activity)	Primary human oral keratinocytes	↑	NT	[0.13 mM], 20 μg/mL		Lee et al. (2013a)
DNA repair/host cell reactivation assay	Human oral squamous SCC, OEC-M1 and SAS (cell lines)	↑/↓	NT	[0.08 mM] 12.5 μg/mL (increase); [0.32 mM] 50 μg/mL (decrease)		Tu et al. (2019)
DNA repair/host cell reactivation assay	Human oral cancer, KB (cell line); Human laryngeal SCC, HEp-2 (cell line) and human embryonic kidney, HEK 293 (cell line)	↓	NT	0.3 mM [46 μg/mL]		Tsai et al. (2008)
DNA repair/host cell reactivation assay	Human laryngeal SCC, HEp-2 (cell line)	↓	NT	0.1 mM [15.5 μg/mL]		Huang et al. (2016b)
Genomic (chromosomal instability)/biomarker (Bub1, Mad2, Mps1, cyclin B1, Aurora A, BubR1, α-tubulin, histone H3)	Human oral cancer, KB (cell line) and human SCC, HEp-2 (cell line)	(+)	NT	0.3 mM [46 μg/mL]	Only one concentration. No positive control. No cytotoxicity measured.	Wang et al. (2010)

HIC, highest ineffective concentration; LEC, lowest effective concentration: NT, not tested; SCC, squamous cell carcinoma.

[a] ↑, increase; ↓, decrease; ↑/↓, no dose–response with two concentrations; (+), positive in a study of limited quality.

the host cell reactivation assay (Tsai et al., 2008; Huang et al., 2016b).

Arecoline treatment of human oral cancer cells (KB) and laryngeal carcinoma cells (HEp-2) stabilized mitotic spindle assembly, which led to distorted organization of mitotic spindles, misalignment of chromosomes, and upregulation of spindle-assembly checkpoint genes (Wang et al., 2010).

4.2.4 Induces epigenetic alterations

(a) Humans

(i) Exposed humans

No data were available to the Working Group.

(ii) Human cells in vitro

The expression of multiple genes catalysing histone methylation (*MLL*, *SETDB1*, and *SUV39H2*), acetylation (*ATF2*), or demethylation (*JMJD6*) were altered in human leukaemia cells (K-562) treated with arecoline (Lin et al., 2011b).

TP53 promoter methylation and expression reduction were associated with arecoline treatment in primary cultures of oral mucosal fibroblasts. Arecoline treatment was also associated with proliferation and epithelial–mesenchymal transition (EMT) of these cells (Zheng et al., 2018).

Several studies investigated the effect of arecoline on microRNAs (miRs) and EMT-related genes in human cells in vitro (see Table 4.6). In two buccal mucosal fibroblast cell lines (BMF-1 and BMF-2, between passages 3 and 8), arecoline was associated with a dose-responsive decrease in *hsa-mir-200c* expression. Overexpression of miR-200c in these cells reduced arecoline-induced collagen gel contraction, migration, invasion, and wound-healing capacities (Lu et al., 2018). [The Working Group noted that the cells were derived from areca-nut chewing subjects with oral submucous fibrosis.]

Arecoline reduced *hsa-mir-203* expression in immortalized oral keratinocyte (HaCaT) cells, and hsa-mir-203 downregulated secreted frizzled-related protein 4 (SFRP4) mRNA and protein expression, and upregulated transmembrane-4 L six family member 1 (TM4SF1) mRNA and protein expression. SFRP4 is involved in EMT and TM4SF1 is involved in motility and proliferation. Increased expression of hsa-mir-203 was associated with reduced cell proliferation and upregulated CK19 and E-cadherin (CDH1) protein expression, and downregulated N-cadherin (CDH2) and vimentin (VIM) protein expression in control untreated cells (Zheng et al., 2015).

Arecoline treatment of two oral epithelial cell lines (Smulow–Glickman normal gingival cells and FaDu oral squamous cell carcinoma cells) was associated with the acquisition of stemness, EMT, migratory and invasive properties, tumour formation potential in vivo, and chemoresistance, and hsa-mir-145 was found to play an arecoline-dependent albeit partial role (Wang et al., 2016). In particular, hsa-mir-145 overexpression inhibited cancer stemness: hsa-mir-145 was found to directly target stemness-related transcription factors OCT4 and SOX2, whereas SOX2 and OCT4 overexpression was associated with the reversal of hsa-mir-145 stemness features.

Four miRs associated with cancer (hsa-mir-26a, hsa-mir-23a, hsa-mir-30a-5p, and hsa-mir-143) were overexpressed in arecoline-treated gingival fibroblast CRL-2014 cells and gingival carcinoma Ca9-22 cells (Tsai et al., 2011).

After treatment with arecoline, hsa-mir-211 was upregulated in oral squamous cell carcinoma cells (SAS) (Chen et al., 2016).

In ORL-48(T) and ORL-136(T) oral squamous cell carcinoma cells treated with arecoline, hsa-mir-22 was reduced and inversely correlated with *IL6* and *STAT3* gene expression and *MYC* gene and protein expression. Arecoline also increased cell viability, proliferation, and G2/M proportion in ORL-48(T) and increased *MYC* promoter activity. In these same cells, oncostatin

Table 4.6 Effect of arecoline on microRNAs and genes related to the epithelial–mesenchymal transition in human cells in vitro

End-point	Tissue, cell line	Result[a]	Concentration (LEC or HIC), duration	Comments	Reference
hsa-miR-200c	Buccal mucosal fibroblasts BMF-1 and BMF-2	↓	5 µg/mL for 48 h	Cells derived from areca-nut chewing OSF subjects.	Lu et al. (2018)
hsa-miR-203	Oral keratinocyte cell line, HaCaT	↓	0.08 mM for 72 h		Zheng et al. (2015)
CK19		↑	0.04 mM for 72 h		
CDH2		↓	0.04 mM for 72 h		
CDH1		↑	0.04 mM for 72 h		
VIM		↓	0.02 mM for 72 h		
SFRP4		↓	0.08 mM for 72 h		
TM4SF1		↑	0.08 mM for 72 h		
hsa-miR-145	Oral epithelial cell lines: SG (Smulow-Glickman normal gingival) and FaDu (oral SCC) cells	↑	10 µg/mL for 90 days		Wang et al. (2016)
Oct4		↑			
Nanog		↑			
Sox2		↑			
Snail		↑			
Twist		↑			
Slug		↑			
VIM		↑			
E-Cadherin		↓			
hsa-miR-26a	Normal gingival fibroblasts (CRL-2014); gingival carcinoma Ca9-22 cells	↑	100 µg/mL for 24 h		Tsai et al. (2011)
hsa-miR-23a		↑			
hsa-miR-30a-5p		↑			
hsa-miR-143		↑			
hsa-miR-211	Oral SCC cell line (SAS)	↑	2.5 µg/mL for 24 h		Chen et al. (2016)
hsa-miR-22	ORL-48(T) and ORL-136(T) oral SCC cells	↓	0.025 µg/mL for 24 h		Chuerduangphui et al. (2018)
hsa-miR-486-3p	Primary oral SCC cells	↓	100 µM for 5 days		Chou et al. (2012)
MEG3 (hsa-miR-329 and hsa-miR-R410)	Human oral keratinocytes (HOK)	↓	100 µmol/L, up to 9 days	Chemical form of arecoline not specified.	Shiah et al. (2014)

CDH1, E-cadherin; CDH2, N-cadherin; CK19, cytokeratin 19; EMT, epithelial mesenchymal transition; h, hour; HIC, higher ineffective concentration; LEC, lowest effective concentration; miR, microRNA; OSF, oral submucous fibrosis; SCC, squamous cell carcinoma; TM4SF1, transmembrane-4 L six family member 1; VIM, vimentin.

[a] ↑, increase; ↓, decrease.

M (*OSM*) was upregulated and inversely correlated with hsa-mir-22, which was found to be directly targeted and suppressed in hsa-mir-22-transfected oral squamous cell carcinoma and 293FT cells, via a luciferase reporter assay (Chuerduangphui et al., 2018).

Arecoline treatment was associated with recruitment of DNA methyltransferase 3B (DNMT3B) to the ANK1 promoter, and reduction in the expression of hsa-mir-486-3p in oral squamous cell carcinoma cells. miR-486-3p was shown to decrease discoidin domain receptor-1 (DDR1) expression in vitro by directly targeting its 3′-UTR. Overexpression of hsa-mir-486-3p resulted in growth inhibition and apoptosis by knockdown of DDR1 via MTT and annexin V assays (Chou et al., 2019).

Arecoline reduced the expression of MEG3, and of the 14q32.2 miRs hsa-mir-329 and hsa-mir-R410 (silenced by arecoline-induced DNA methylation of the MEG3 DMR) in a time-dependent manner in human oral keratinocyte (HOK) cells. Arecoline upregulated *WNT7B* and increased the phosphorylation of GSK3β and active-β-catenin in oral squamous cell carcinoma (HOK) cells, with a concomitant upregulation of cyclin D and Myc proteins (Shiah et al., 2014). [The Working Group noted that the chemical form of arecoline was not specified.]

(b) Experimental systems

Histone proteins H1 in spleen cells and H2B in bone-marrow cells of male Swiss albino mice exhibited an increase in poly-ADP-ribosylation after treatment with arecoline hydrobromide (10 μg/mL in drinking-water for up to 5 weeks), while all other tested proteins exhibited a reduction. The chromatin of spleen and bone-marrow cells was also increasingly open with increasing arecoline doses (Saikia et al., 1999). [The Working Group noted that the data were sparse and showed no clear overall pattern.]

4.2.5 Induces oxidative stress

(a) Humans

See Table 4.7.

(i) Exposed humans

No data were available to the Working Group.

(ii) Human cells in vitro

Nine reports have provided direct evidence for production of reactive oxygen species (ROS) after treatment with arecoline (see Table 4.7) in normal cells, such as BMF cell cultures (Lee et al., 2016; Hsieh et al., 2018), primary oral keratinocytes and the oral epithelial cell line OECM-1 (Lee et al., 2013b), the spontaneously immortalized keratinocyte cell line HaCaT (Thangjam & Kondaiah, 2009), and human umbilical vein endothelial cells (HUVEC, passages 3 to 5) (Hung et al., 2011) [the Working Group noted that the chemical form of arecoline was not specified]; and from studies in cancer cells, such as two oral squamous cell carcinoma cell lines (Ji et al., 2012; Shih et al., 2020) and two oesophageal squamous cell carcinoma cell lines (Wang et al., 2019). In the study by Shih et al. (2020), the antioxidant-responsive element (ARE)-containing genes *NRF2*, *HO1*, *SOD1*, and *NQO1* were upregulated.

N-acetyl-L-cysteine (NAC) or epigallocatechin-3-gallate was used to support findings in six of these studies. (Hung et al., 2011; Ji et al., 2012; Lee et al., 2013b, 2016; Hsieh et al., 2018; Wang et al., 2019). [The Working Group noted that the chemical form of arecoline was not specified by Hung et al. (2011).] One study found that arecoline exposure and ROS production are inversely correlated in human polymorphonuclear lymphocytes (Lai et al., 2007).

In addition, one study reported that the arecoline metabolite arecoline *N*-oxide induced ROS production in the liver WRL 68 cell line (Wang et al., 2018). [The Working Group noted that WRL 68 may be a HeLa cervical cancer cell line derivative with hepatocyte morphology

Table 4.7 Effect of arecoline and arecoline *N*-oxide on production of reactive oxygen species in human cells in vitro

Cell line	Result[a]	Concentration (LEC or HIC), duration	Comments	Reference
Arecoline				
Normal buccal mucosal fibroblasts	↑	20 µg/mL for 8 h		Lee et al. (2016)
Normal buccal mucosal fibroblasts	↑	0.2 mM for 1 h	Single concentration.	Hsieh et al. (2018)
Oral keratinocytes (HOK)	↑	20 µg/mL for 24 h		Lee et al. (2013b)
Oral SCC cell line (OECM-1)	↑	40 µg/mL for 24 h		
Keratinocyte cell line (HaCaT)	↑	50 µg/mL for 48 h		Thangjam & Kondaiah (2009)
HUVECs (passages 3 to 5)	↑	10 µg/mL for 2 h	Chemical form of arecoline not stated.	Hung et al. (2011)
Oral SCC cell line (OC2)	↑	0.3 mM [46.8 µg/mL] for 2 h		Ji et al. (2012)
Oral SCC cell line (OEC-M1)	↑	100 µg/mL for 24 h		Shih et al. (2020)
Oesophageal cell lines (OE21 and CE81T)	↑	15.6 µM for 24 h	Single concentration.	Wang et al. (2019)
Arecoline N-*oxide*				
Liver cell line (WRL 68)	↑	31.25 µM for 24 h	WRL 68 may be a HeLa cervical cancer cell line derivative.	Wang et al. (2018)

h, hour; HIC, highest ineffective concentration; HUVECs, human umbilical vein endothelial cells; LEC, lowest effective concentration; SCC, squamous cell carcinoma.

[a] ↑, increase.

and liver-enzyme expression profile, as noted by the European Collection of Authenticated Cell Cultures.]

Regarding indirect evidence, based on ROS-induced changes in gene expression, the available data concerned heat shock protein 70 (HSP70) and haem oxygenase-1 (HO1). Regarding HSP70, it was found to be expressed via Western-blot analysis in a time- and dose-dependent manner in response to arecoline treatment in an oral epithelial cell line of gingival carcinoma provenance (GNM). NAC protected the cells from this effect, as did curcumin, PD98059, and staurosporine (Lee et al., 2008). Regarding HO1, mRNA and protein levels were found to be correlated with arecoline concentration in human umbilical vein endothelial cells (HUVECs) and only partly due to increased ROS levels. Arecoline treatment also increased intercellular adhesion molecule-1 (ICAM1) and vascular cell adhesion molecule-1 (VCAM1) protein expression. Pre-treatment with glutathione prevented ROS production and VCAM1 expression, but not ICAM1 expression (Hung et al., 2011) [The Working Group noted that the chemical form of arecoline was not specified.] HO1, as well as other genes including glucose-6-phosphate dehydrogenase (*G6PDH*), and glutathione reductase (*GRD*), were found to be upregulated at the mRNA level in HaCaT cells after arecoline treatment, and co-treatment with NAC prevented this upregulation. ROS production and cell-cycle arrest at the G1/G0 phase, and reduced catalase activity, were found to be associated with arecoline treatment (Thangjam & Kondaiah, 2009).

(b) Experimental systems

See Table 4.8.

Experiments in at least three species of experimental animal support a link between arecoline

treatment and the induction of ROS: the rat (Run-mei et al., 2014; You et al., 2019); the mouse (Dasgupta et al., 2006; Laskar et al., 2019) [the Working Group noted that the chemical form of arecoline was not specified. The Working Group also noted that only female mice were tested]; and the fruit fly (Shakya & Siddique, 2018).

Male Sprague-Dawley rats exposed daily to drinking-water containing arecoline hydrobromide at 10 mg/mL n for 49 days displayed increased protein levels of α-collagen-1 (COL1A), α-smooth muscle actin (ASMA), NADPH oxidase 4 (NOX4), nucleotide-binding domain leucine-rich repeat containing pathway 3 (NLRP3), and interleukin-1β (IL1B) in oral submucosa tissues (You et al., 2019). [The Working Group noted that only one dose level of arecoline was tested.] The levels of superoxide dismutase (SOD), CAT, GSH-Px, and glutathione were decreased in male Wistar rat liver after oral administration of arecoline hydrobromide at 100 mg/kg bw per day for 7 days (Run-mei et al., 2014).

One report of a study in Swiss albino mice [sex not reported] showed that reductions in liver enzyme levels of SOD, glutathione, and glutathione *S*-transferase were all dose-responsive after treatment with arecoline doses inferior or equal to 20 mg/kg bw per day for 14 days (Dasgupta et al., 2006). Increased levels of glutathione *S*-transferase, CYPB5, CYP450, and malondialdehyde were seen in Swiss albino mice given arecoline intraperitoneally (10, 20, or 40 mg/kg per day) for 10 or 30 days. Reduced sulfhydryl content was seen after administration of arecoline at 40 mg/kg per day for 10 or 30 days (Singh & Rao, 1993).

A study in Swiss albino mice exposed daily to drinking-water containing arecoline hydrobromide at 10 μg/mL for 24 weeks showed that arecoline treatment was associated with pre-neoplastic nodulation in the liver and white patches in the lungs of the mice, along with increased lipid peroxidation in the liver, lungs, and intestines, increased thiobarbituric acid-reactive substances in the liver, lungs, and intestines, and reduced protein carbonylation (Laskar et al., 2019). [The Working Group noted that the chemical form of arecoline was not specified, and that only one dose level of arecoline was tested.]

The metabolite arecoline *N*-oxide (31.25 μM, 2 hours) induced ROS production in rat liver clone-9 cells and this increase was relieved by NAC (Wang et al., 2018)

4.2.6 Modulates receptor-mediated effects

(a) Humans

No data were available to the Working Group.

(b) Experimental systems

Reduced T3 and T4 thyroid hormones and increased thyroid-stimulating hormone, increased pineal and seral serotonin, reduced pineal and seral *N*-acetyl serotonin, reduced pineal and seral melatonin and increased testosterone, fructose, and sialic acid, were observed in male Wistar rats injected with arecoline intraperitoneally twice per day for 10 days (10 mg/kg bw per day) (Saha et al., 2018, 2020). [The Working Group noted that only one dose was tested.]

4.2.7 Alters cell proliferation, cell death, or nutrient supply

(a) Humans

No data in exposed humans were available to the Working Group.

A significant decrease in TP53 and the downstream p21 (CDKN1) protein levels was observed in primary cultured oral mucosal fibroblasts treated with arecoline, indicating a loss of tumour-suppression activity (Zheng et al., 2018). Arecoline treatment also decreased the expression of E-cadherin protein but increased the expression of *N*-cadherin and vimentin, overall suggesting an arecoline-mediated cellular proliferative and EMT response in oral mucosal fibroblasts (Zheng et al., 2018). Arecoline treatment

Table 4.8 Effect of arecoline and arecoline *N*-oxide on production of reactive oxygen species in experimental systems

End-point	Species, strain (sex)	Tissue	Results[a]	Dose (LED, HID)	Route, duration, dosing regimen	Comments	Reference
Arecoline							
COL1A, αSMA, CTGF, NOX4, NLRP3, and IL1β protein levels	Rats, Sprague-Dawley (M)	Oral submucous tissues	↑	10 mg/mL	Injection into oral mucosa, 1×	Single exposure; Males only.	You et al. (2019)
SOD, CAT, GSH-Px, and GSH	Rats, Wistar (M)	Liver	↓	100 mg/kg per day for 7 days	Oral		Run-mei et al. (2014)
SOD GSH GST	Mice, Swiss albino (NR)	Liver	↓ ↓ ↓	10 mg/kg bw per day for SOD 5 mg/kg bw per day for GSH 10 mg/kg bw per day for GST	Intraperitoneal, 1×/day for 14 days	Sex of mice not stated.	Dasgupta et al. (2006)
GST, CYPB5, CYP450, and MDA SH content	Mice, Swiss albino (M, F)	Liver	↑ ↓	10 mg/kg per day for 10 or 30 days 40 mg/kg per day for 10 or 30 days	Intraperitoneal, 1×/day for 10 or 30 days	The chemical form of arecoline was not stated.	Singh & Rao (1993)
TBARS Protein carbonylation	Mice, LACA Swiss albino (F)	Liver, lung, and intestine	↑ ↑	10 µg/mL	Oral (drinking-water), 24 weeks	The amount of drinking-water ingested was not reported.	Laskar et al. (2019)
GSH content GST activity, lipid peroxidation, protein carbonyl content	Fruit fly *Drosophila melanogaster* (hsp70-lacZ) Bg third instar larvae)	Fruit fly homogenate	↓ ↑	20 µM	Oral (feed), 24 h		Shakya & Siddique (2018)
Arecoline N-*oxide*							
ROS	Rat liver clone-9 cells			31.25 µM	2 h		Wang et al. (2018)

bw, body weight; COL1A, α-collagen 1A; CTGF, connective tissue growth factor; CYP450, cytochrome P450; F, female; GSH, glutathione; GST, glutathione *S*-transferase; h, hour; HID, highest ineffective dose; IL, interleukin; LED, lowest effective dose; M, male; MDA, malondialdehyde; NOX4, nicotinamide adenine dinucleotide phosphate oxidase 4; NLRP3, NOD (nucleotide oligomerization domain)-like receptor family pyrin domain containing 3 inflammasome complex; NR, not reported; ROS, reactive oxygen species; SH, sulfhydryl; α-SMA, α-smooth muscle actin; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

[a] ↑, increase; ↓, decrease.

in Smulow–Glickman human gingival epithelial cells and FaDu pharynx epithelial squamous cell carcinoma cells led to a dose-dependent elevation in expression of the zinc-finger transcription factor ZEB1, which was associated with migration ability, cell invasiveness, and anchorage-dependent growth (Ho et al., 2015). Lin et al. (2015) also demonstrated arecoline-induced proliferation, migration, invasiveness, and anchorage-independent growth in Smulow–Glickman and FaDu cells, citing Lin28B, an RNA-binding protein that was dose-dependently expressed, as a key player.

Arecoline alone, and in combination with nicotine, activated epidermal growth factor receptor/protein kinase B (EGFR/AKT) signalling in head and neck squamous cell carcinoma cells, which resulted in enhanced anti-apoptotic and pro-EMT signalling (Yang et al., 2018). Furthermore, reverse transcription-polymerase chain reaction analysis confirmed by Western blotting showed increased expression of transcripts of mesenchymal markers, including vimentin and E-cadherin (Wang et al., 2016). Bronchial smooth-muscle cells were shown to proliferate and migrate after exposure to cytokines in cultured media taken from human bronchial epithelial cells treated with arecoline (Kuo et al., 2011).

Oral fibroblasts treated with arecoline and its metabolite, arecoline *N*-oxide, showed an increase in the expression of the fibrotic-related genes transforming growth factor β 1 (*TGFB1*), S100 calcium-binding protein A4 (*S100A4*), matrix metallopeptidase-9 (*MMP-9*), interleukin 6 (*IL6*) and fibronectin, and a decrease in the expression of E-cadherin (Kuo et al., 2015). Arecoline *N*-oxide elicited a significantly stronger response than did the parent compound (Kuo et al., 2015).

Fibroblasts cultured from human gingival, normal buccal mucosa, and oral submucous fibrosis showed dose-dependent increases in cell proliferation with arecoline treatment (≤ 10 μg/mL) as measured by 5-bromo-2′-deoxyuridine labelling (Chen et al., 1995). [The Working Group noted the inhibition of cell proliferation with arecoline at 100 μg/mL.] These findings are consistent with arecoline-induced cell proliferation in oesophageal squamous cell carcinoma cells (arecoline concentration, ≤ 31.2 μM) (Wang et al., 2019) and oral squamous cell carcinoma cells (arecoline concentration, ≤ 0.25 μg/mL) (Chuerduangphui et al., 2018), shown to be activated through AKT and extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation or induction of c-MYC and reduction of miR-22, respectively.

(b) Experimental systems

Early work showed that treatment of mouse kidney cells with arecoline at 10 μg/mL increased cell proliferation (Wary & Sharan, 1988). In a nude mouse assay, SCC-9 xenografts showed increased Ki-67 staining after arecoline treatment (Zheng et al., 2018).

4.3 Data relevant to comparisons across agents and end-points

Arecoline is one of the approximately 1000 chemicals tested across the full assay battery of the Toxicity Testing in the 21st Century (Tox21) and Toxicity Forecaster (ToxCast) programmes supported by the United States Environmental Protection Agency (US EPA, 2020). In vitro assay descriptions to map Tox21 and ToxCast screening data in the context of the 10 key characteristics were previously summarized by Chiu et al. (2018). Results in this data set only show 7 active hits out of 235 assays. Six out of the 7 assays with active hits tested for cell viability after 0, 8, 16, 24, 32, and 40 hours of exposure in human liver cells using a dead-cell DNA stain. Results indicated that treatment with arecoline led to a loss of cell viability. [The Working Group noted the flag for less than 50% efficiency with this assay

at 0, 24, 32, and 40 hours; only one concentration above baseline was active in the assay tested after 8 hours of exposure.] Arecoline was active in the thyrotropin-releasing hormone receptor (TRHR)-Hek293 cell-based assay. Intracellular calcium is measured using fluorescence detection as an indicator of Gq-protein-coupled TRHR activation. These results suggested arecoline involvement in modulating receptor-mediated effects through potential agonism of the TRHR. [The Working Group noted one flag for < 50% efficiency with this assay.]

5. Summary of Data Reported

5.1 Exposure characterization

Arecoline is the primary active ingredient of the areca nut, which has been previously classified by the *IARC Monographs* programme as *carcinogenic to humans (Group 1)*. Areca nut is widely cultivated in Asia. It has been estimated that more than 10% of the world's population, primarily in south-eastern Asia, chew areca nut for its mild psychoactive effects. Arecoline is commonly traded and used in the hydrobromide form, but the hydrochloride form is also commercially available. Arecoline is now rarely used medicinally as an antiparasitic drug, but it is still applied indirectly in the form of areca nut as an ingredient in traditional Chinese and Ayurveda medicines. Exposure to arecoline in occupational settings is expected from the use of areca nut to suppress hunger during intensive labour and as a stimulant, but no exposure data were found for workers directly handling arecoline or areca nut. After exposure, arecoline can be detected in saliva, blood, urine, hair, and breast milk from humans. However, short-term markers in blood and saliva provide only qualitative information.

5.2 Cancer in humans

No data were available to the Working Group.

5.3 Cancer in experimental animals

5.3.1 Arecoline

Arecoline increased the incidence of tumours in one co-carcinogenicity study, increased the incidence and multiplicity of tumours in another co-carcinogenicity study, and caused an increase in the incidence of total tumours in two studies.

In one co-carcinogenicity study in which arecoline hydrobromide was administered orally (in the drinking-water) to male C57BL/6 mice in combination with the carcinogen 4-nitroquinoline 1-oxide (4-NQO), arecoline in combination with 4-NQO increased the incidence of invasive squamous cell carcinoma of the oesophagus compared with mice receiving only 4-NQO.

In another co-carcinogenicity study, male F344 rats given *N*-benzyl-*N*-methylnitrosamine (NMBA) by subcutaneous injection and arecoline by oral administration (in the drinking-water) showed a significant increase in the incidence and multiplicity of papilloma of the oesophagus and a significant increase in multiplicity of papilloma of the tongue, compared with rats receiving only NMBA.

Male Swiss mice treated with arecoline hydrochloride by oral administration (gavage) had a significant increase in the incidence of total tumours in one study. In another study, female Swiss mice fed a vitamin B complex-deficient diet and treated with arecoline hydrochloride by oral administration (gavage) showed a significant increase in the incidence of total tumours.

5.3.2 Arecaidine

Arecaidine caused an increase in the incidence of tumours in one initiation–promotion study.

Male Syrian golden hamsters treated in the cheek pouch with the carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) in the initiation phase followed by arecaidine in the promotion phase showed a significant increase in the incidence and multiplicity of exophytic squamous cell papilloma or carcinoma (combined) compared with hamsters treated with DMBA only.

5.4 Mechanistic evidence

Arecoline is readily absorbed in the oral cavity. It has been found in the saliva, blood, and urine of areca-nut chewers and can readily enter the brain of rats. It is rapidly metabolized. The main metabolic pathways are hydrolysis to arecaidine, oxidation to arecoline *N*-oxide catalysed by human flavin-containing monooxygenases, and conjugation with glutathione leading eventually to arecoline and arecaidine mercapturic acids. In mice, the metabolic pathways are similar and altogether 10 metabolites have been identified. Under physiological conditions, arecoline can react with sodium nitrite to form *N*-nitrosamines, a class of carcinogenic agents that are known to be metabolically activated to alkylating agents. Two nitrosamines, namely *N*-nitrosoguvacoline and *N*-nitrosoguvacine, were found in the saliva of betel-quid chewers.

There is consistent and coherent evidence that arecoline exhibits multiple key characteristics of carcinogens. Arecoline is an electrophilic α,β-unsaturated ester that can undergo Michael addition with cellular nucleophiles. No data were available regarding DNA-adduct formation by arecoline. Arecoline reacts with glutathione, ultimately yielding mercapturic acids, and forms adducts at multiple sites within proteins in human plasma and in human primary cells in vitro.

Arecoline is genotoxic. Data in humans were not available. Arecoline showed mixed results for DNA strand breaks in human primary cells and in several human cell lines in vitro using different assays, but there were consistently positive results for unscheduled DNA synthesis, micronucleus formation, chromosomal aberration, and sister-chromatid exchange. Similarly, arecoline showed consistent positive results for chromosomal aberration, micronucleus formation, and sister-chromatid exchange in experimental systems both in vitro and in vivo. Arecoline induced gene mutations in most assays for bacterial gene mutation and in mammalian cells in vitro. Arecoline altered the mutation spectrum in a transgenic mouse mutation assay. Results from the few available genotoxicity tests on *N*-nitrosoguvacoline, and the arecoline metabolites arecaidine and arecoline *N*-oxide, were positive.

Arecoline alters DNA repair and causes genomic instability. No data were available in exposed humans or in non-human experimental systems, and data in human primary cells were scarce. In human cell lines in vitro, arecoline altered DNA-repair enzyme activity. Arecoline increased O^6-methylguanine-DNA methyltransferase activity in primary human oral keratinocytes. In human cell lines, arecoline altered DNA repair and stabilized mitotic spindle assembly, which led to distorted organization of mitotic spindles, misalignment of chromosomes, and upregulation of spindle assembly checkpoint genes.

Arecoline induces oxidative stress. No data in humans were available. In several studies in various human primary cells, arecoline increased production of reactive oxygen species. Various reports of production of reactive oxygen species in vivo were also available in different experimental species (mouse, rat, and fruit fly) and in rat liver clone-9 cells.

There is suggestive evidence that arecoline alters cell proliferation in experimental systems. In vitro studies using human-derived cells have shown that arecoline is capable of inducing proliferation, migration, invasiveness, and anchorage-independent growth. In one study

in primary cultured oral mucosal fibroblasts, arecoline decreased TP53 and the downstream CDKN1A protein levels, indicating a loss of tumour-suppression activity.

There is sparse evidence regarding other key characteristics of carcinogens. The available data that arecoline induces epigenetic alterations mainly concerned microRNAs (miRs). Arecoline treatment reduced the expression of four miRs (hsa-mir-200c, hsa-mir-203, hsa-mir-329, and hsa-mir-410), and increased the expression of six miRs (hsa-mir-145, hsa-mir-26a, hsa-mir-23a, hsa-mir-30a-5p, hsa-mir-143, and hsa-mir-211) in human cells.

Arecoline was essentially without effects in the assay battery of the Toxicity Testing in the 21st Century and Toxicity Forecaster research programmes.

6. Evaluation and Rationale

6.1 Cancer in humans

There is *inadequate evidence* in humans regarding the carcinogenicity of arecoline.

6.2 Cancer in experimental animals

There is *limited evidence* in experimental animals for the carcinogenicity of arecoline.

6.3 Mechanistic evidence

There is *strong* evidence in human primary cells and in various experimental systems that arecoline exhibits multiple key characteristics of carcinogens.

6.4 Overall evaluation

Arecoline is *possibly carcinogenic to humans (Group 2B).*

6.5 Rationale

The *Group 2B* evaluation for arecoline is based on *strong* mechanistic evidence. There is *strong* evidence in human primary cells that arecoline exhibits multiple key characteristics of carcinogens; arecoline is electrophilic; it is genotoxic; and it induces oxidative stress. It also alters DNA repair or causes genomic instability in experimental systems.

There is also *limited evidence* for cancer in experimental animals, on the basis of increased incidence of tumours in one co-carcinogenicity study, increased incidence and multiplicity of tumours in another co-carcinogenicity study, and increased incidence of total tumours in two studies. The evidence regarding cancer in humans is *inadequate*, as no studies were available.

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LIST OF ABBREVIATIONS

ACGIH	American Conference of Governmental Industrial Hygienists
AEGL	acute exposure guideline levels
AKR	aldo-keto reductases
ALDH	aldehyde dehydrogenase
ARE	antioxidant-responsive element
AREMA	*N*-acetyl-*S*-(3-methoxycarbonyl-1-methylpiperid-4-yl)-L-cysteine (arecoline mercapturic acid)
AREO	arecoline *N*-oxide
ATSDR	Agency for Toxic Substances and Disease Registry
B[*a*]P	benzo[*a*]pyrene
BAR	biological reference value for workplace substances
BCNU	1,3-bis-(2-chloroethyl)-1-nitrosourea
BrdU	5-bromo-2′-deoxyuridine
CEMA	*N*-acetyl-*S*-(carboxyethyl)-L-cysteine (2-carboxyethylmercapturic acid)
CESG	*S*-(2-carboxyethyl)glutathione
CHEMA	*N*-acetyl-*S*-(2-carboxy-2-hydroxyethyl)-L-cysteine (2-carboxy-2-hydroxyethylmercapturic acid)
Cinc1	chemokine-induced neutrophil chemoattractant-1
C_{max}	maximum plasma concentration
CMEMA	*N*-acetyl-*S*-(3-carboxy-1-methylpropyl)-L-cysteine
CYP	cytochrome P450
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DLM	dominant lethal mutation
DMBA	7,12-dimethylbenz[*a*]anthracene
DNEL	derived no-effect level
DNPH	2,4-dinitrophenylhydrazine
dNTP	deoxynucleoside triphosphate
DSSTox	Distributed Structure-Searchable Toxicity
dTTP	deoxythymidine triphosphate
EC	European Community
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial–mesenchymal transition
EPIC	European Prospective Investigation into Cancer and Nutrition
E-PRTR	European Pollutant Release and Transfer Register

EU	European Union
FANFT	*N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide
FDP	3-formyl-3,4-dehydropiperidine
Gclc	glutamyl-cysteine ligase catalytic subunit
GC-MS	gas chromatography-mass spectrometry
GLA	glycidaldehyde
GM	geometric mean
GSH	glutathione
GST	glutathione *S*-transferase
GSTM1	glutathione *S*-transferase mu 1
GSTT1	glutathione *S*-transferase theta 1
Hmox1	haem oxygenase 1
HMPMA	*N*-acetyl-*S*-(3-hydroxy-1-methylpropyl)-L-cysteine
hOGG1	human 8-oxoguanine DNA glycosylase
HPLC	high-performance liquid chromatography
HPMA	*N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine (3-hydroxypropylmercapturic acid)
HPSG	*S*-(3-hydroxypropyl)glutathione
IC_{50}	half maximal inhibitory concentration
IL	interleukin
ICSC	International Chemical Safety Card
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MGMT	O^6-methylguanine-DNA methyltransferase
miR	microRNA
MLH1	mutL homolog 1
MNPA	*N*-methylnipecotic acid
MS	mass spectrometry
NAC	*N*-acetyl-L-cysteine
NDEA	*N*-nitrosodiethylamine
NHANES	National Health and Nutrition Examination Survey
NHBEs	primary normal human bronchial epithelial cells
NHL	non-Hodgkin lymphoma
NHLF	normal human lung fibroblast
NIOSH	National Institute for Occupational Safety and Health
NMBA	*N*-benzyl-*N*-methylnitrosamine
1,N^6-PdA	1,N^6-propano-deoxyadenosine derivative
NPRI	National Pollutant Release Inventory
NPYR	*N*-nitrosopyrrolidine
4-NQO	4-nitroquinoline 1-oxide
Nqo1	NADPH quinone oxidoreductase 1
NRF2	nuclear factor erythroid 2-related factor 2 [NFE2L2, nuclear factor, erythroid 2-like 2]
OEL	occupational exposure limit
8-OHdG	8-hydroxy-2′-deoxyguanosine
α-OH-PdG	α-1,N^2-propano-deoxyguanosine
γ-OH-PdG	γ-1,N^2-propano-deoxyguanosine
OPSG	*S*-(3-oxopropyl)glutathione
OSHA	Occupational Safety and Health Administration
PCNA	proliferating cell nuclear antigen
PI3K	phosphatidylinositol 3-kinase

PKC	protein kinase C
PMS2	PMS1 homolog 2, mismatch repair system component
PPAR	peroxisome proliferator-activated receptor
PRTR	Pollutant Release and Transfer Register
PTEN	phosphatase and tensin homologue
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RIOPA	Relationship of Indoor, Outdoor and Personal Air
ROS	reactive oxygen species
RTECS	Registry of Toxic Effects of Chemical Substances
SAR	Special Administrative Region
SOD	superoxide dismutase
STEL	short-term exposure limit
TLC	thin-layer chromatography
TRHR	thyrotropin-releasing hormone receptor
TSCA	Toxic Substances Control Act
TWA	time-weighted average
ULSD	ultra-low sulfur diesel fuel
US EPA	United States Environmental Protection Agency
USGS	United States Geological Survey
UV	ultraviolet
V_d	steady-state apparent volume of distribution
v/v	volume per volume
w/w	weight per weight
XPA	xeroderma pigmentosum group A
XPV	xeroderma pigmentosum group V

ANNEX 1. SUPPLEMENTARY MATERIAL FOR ACROLEIN, SECTION 1, EXPOSURE CHARACTERIZATION

These supplementary web-only tables are available from: https://publications.iarc.fr/602.

Tables S1.1, S1.2, S1.4, and S1.5 were produced in draft form by the Working Group and were subsequently fact-checked and edited.

Table S1.3 was produced in draft form by the Working Group and was not subsequently fact-checked or edited.

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ANNEX 2. SUPPLEMENTARY MATERIAL FOR CROTONALDEHYDE, SECTION 1, EXPOSURE CHARACTERIZATION

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Tables S1.6 and S1.7 were produced in draft form by the Working Group and were not subsequently fact-checked or edited.

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IARC MONOGRAPHS

This volume of the *IARC Monographs* provides evaluations of the carcinogenicity of acrolein, crotonaldehyde, and arecoline.

Acrolein is a High Production Volume chemical used to manufacture numerous chemical products, and as a herbicide in recirculating water systems. Found in emissions from combustion of fuels, wood, and plastics, and in ambient air pollution and electronic cigarette vapour, acrolein is also generated in kitchens during high-temperature roasting and deep-fat frying. Crotonaldehyde is a High Production Volume chemical that is widely used for synthesizing chemical agents used in the pharmaceutical, rubber, chemical, and leather industries, as well as in food production and agriculture. Crotonaldehyde is also formed during combustion of vehicle fuels and wood, and in thermal treatment of food, and is also found in cooking fires, ambient air pollution, electronic cigarette vapour, and some foods and heated cooking oils. Tobacco smoke is a major source of exposure to crotonaldehyde and acrolein in the general population. Occupational exposures may occur among firefighters, coke-oven workers, and workers in aldehyde manufacture, garages, and toll booths. Both acrolein and crotonaldehyde are also formed endogenously.

Arecoline is the primary active ingredient of the areca nut, which is *carcinogenic to humans (Group 1)*. At least 10% of the global population, primarily in south-eastern Asia, chews areca nut for its mild psychoactive effects. Arecoline has been used medicinally as an anthelmintic and is still applied in the form of areca-nut preparations in traditional Chinese and Ayurveda medicines.

An *IARC Monographs* Working Group reviewed evidence from cancer bioassays in experimental animals and mechanistic studies to assess the carcinogenic hazard to humans of exposure to these agents and concluded that:

- **Acrolein is *probably carcinogenic to humans (Group 2A)***
- **Crotonaldehyde and arecoline are *possibly carcinogenic to humans (Group 2B).***