Report on Carcinogen Wood Smoke and Wildfire Cancer Hazard Evaluation Protocol Part 2

Animal Cancer and Mechanistic Studies, Sufficient Similarity Assessment, and Evidence Integration

National Institute of Environmental Health Sciences
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Background and Objectives

Wood smoke is a complex mixture consisting of particulate matter, gases, and hundreds of different chemicals, including U.S. Environmental Protection Agency EPA defined hazardous pollutants and carcinogens (e.g., polycyclic aromatic hydrocarbons (PAHs), benzene). In the United States, wood smoke is emitted primarily from wood stoves, fireplaces, and boilers used for heating although some restaurants use wood for cooking. Over 2 million U.S. households use wood as their primary heating fuel. Biomass and coal together comprise solid fuel. Biomass fuels include wood, charcoal, animal dung, and agricultural residues. Recently, concerns about wood stoves use in the United States has attracted attention (Kruzman 2022).

The International Agency for Research on Cancer (IARC) (working group met in 2006, monograph published in the 2010 monograph) has characterized indoor emissions from household combustion of biomass fuel (primary wood) as *probably carcinogenic to humans* (2A). The IARC working group concluded there was limited evidence for a causal association with lung cancer.

Another important source of exposure to wood smoke is from wildfires; this is an emerging concern, as the frequency of these fires is increasing due to weather related climate change. Wildland fire fighters are an occupational group of concern and studies have found that over 46 million people living in Western United States have been exposed to at least one smoke wave (concentration of $PM_{2.5}$ is at least 20 mg/m^3 for 2 or more consecutive days) between 2004 and 2008 (Climate Matters 2018).

Because exposure to wood smoke and wildfire poses a potential carcinogenic hazard for people living in the United States, NIEHS is conducting a cancer hazard evaluation of wood smoke and wildfire for potential listing in the Report on Carcinogens, a congressionally mandated, science-based public health document. The overall cancer hazard evaluation will (1) assess and integrate the evidence from human and animal cancer studies and mechanistic studies, and (2) apply the RoC listing criteria to the assessment to reach a listing recommendation. For wildfires, we will also integrate findings from a sufficient similarity analysis (see Section 3). This document is the protocol for the cancer hazard evaluation of the animal cancer and mechanistic studies, and evidence integration. The protocol for evaluating human cancer studies is available on the RoC website.

Scope of the Evidence

The literature or evidence is defined by the EECO statement (evidence stream type, exposure, comparison group, and outcome) described below. Note the EECO is adapted from PECO statements used in systematic reviews of human evidence, and the evidence stream has replaced population because our evaluation is multidisciplinary (e.g., human and animal cancer studies and mechanistic evidence). We did not identify animal cancer studies for wildfire exposure. Because wildfire smoke is largely composed of wood smoke, we plan to conduct a sufficient similarity assessment by comparing reported chemical components of wildfire mixtures to those of wood smoke mixtures as part of the cancer hazard evaluation.

The table below outlines the general literature included in the evaluation. Details on the animal cancer and mechanistic studies are in the protocols following this introduction.

Table A: Overall evidence type, exposure, comparison, and outcome

Evidence Type	Exposure	Comparison	Outcome
Experimental animals	Woodsmoke (whole or extracts)	No exposure to woodsmoke	Tumors (malignant and benign)
Exposed humans	Wood smoke or wildfire	No or low exposure to woodsmoke or wildfire	All cancers types
Experimental animals	Wood smoke or wildfire (whole or extracts)	No or low exposure to woodsmoke or wildfire	Biological effects related to carcinogenicity, such as KCC
Exposed humans	Wood smoke or wildfire	No or low exposure to woodsmoke or wildfire	Biological effects related to carcinogenicity, such as KCC
In vitro, ex vivo, or cell free	Wood smoke or wildfire (whole or extracts)	No or low exposure to wood smoke or wildfire	Biological effects related to carcinogenicity, such as KCC
Sufficient similarity Chemistry analysis	Chemicals in wildfire samples	Chemicals in wood smoke samples	Extent of chemical similarity between wood smoke and wildfire mixtures

KCC = key characteristic of carcinogens

Protocol Objective and Components

Objective: To provide methods for assessing the evidence from human cancer studies (published on the RoC website), the animal cancer studies (Section 2), the mechanistic evidence (Section 3), conducting a sufficient similarity analysis of reported chemical components of wildfire and wood smoke samples (Section 4), and integrating the evidence across disciplines to reach a recommendation for listing in the RoC (Section 5). Section 6 briefly discusses public health information. Methods for conducting a sufficient similarity of chemical components analysis in wood smoke and wildfire samples will be added in a later update.

Appendix A provides the literature search terms and the evaluation team and responsibilities, and Appendix B provides background information on biomarkers or indicators for the key characteristics of carcinogens biomarkers and indicators.

1. Evaluating Human Cancer Studies of Exposure to Wood Smoke

Published Human Cancer Protocol

The protocol for evaluating wood smoke exposure and human cancer studies was published in April 2022 and is available here. This protocol included detailed evaluation and evidence integration methods for three types of cancer: lung cancer, nasopharyngeal carcinoma (NPC), and esophageal cancer. The protocol included methods for conducting a meta-analysis of the lung cancer studies.

In November 2023 breast cancer was added as a fourth cancer endpoint after new articles were published, and a protocol addendum was published. The evaluation and informativeness assessment of the human cancer studies has been completed.

Wood smoke and NPC meta-analysis

We will also conduct a meta-analysis for NPC following the same criteria and methods that were written for lung cancer.

2. Evaluating Animal Cancer Studies of Exposure to Wood Smoke

Overall Objective and Aims

Overall Objective

To reach conclusions about the level of evidence of the carcinogenicity of wood smoke provided by animal cancer studies based on the RoC listing criteria (see Section 2.3).

Key questions

- Which animal cancer studies should be included in the review?
- What are key issues for evaluation of the studies?
  - How is exposure characterized in the studies and what is the most relevant exposure for humans?
  - What are the most sensitive animal models?
- How informative (e.g., risk of bias, study sensitivity) are the studies for the evaluation?
- What is the level of evidence (i.e., sufficient or not sufficient) for carcinogenicity of wood smoke from studies in experimental animals?
  - What tumor sites are related to exposure?

Protocol Contents and Evaluation Process

This document describes the (1) completed scoping and problem formulation steps used to develop the framework (Section 2.1) and (2) proposed methods used to conduct the cancer hazard evaluation, including the study evaluation (Section 2.2) and evidence integration (Section 2.3). The methods are based on applying the specific issues relevant to wood smoke to the procedures outlined in the RoC handbook. The roles of the researchers and the literature search terms are described in Appendix A.

Figure 2-1 provides a schematic of how the protocol (Step 2) fits into the cancer hazard evaluation process. The protocol is informed by the scoping and problem formulation (i.e., developing the framework) done in Step 1, and the methods in the protocol are then used to conduct the cancer hazard evaluation and write the RoC monograph (Step 3). Note that Steps 1 and 2 are iterative.

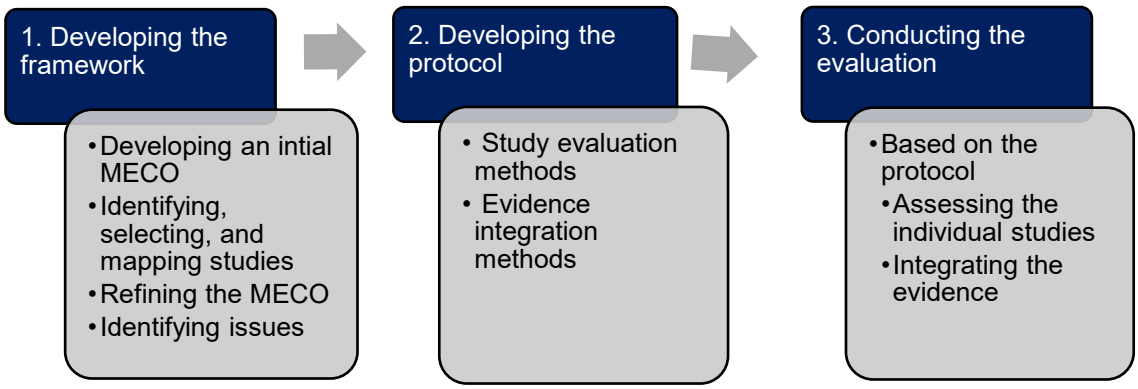

Figure 2-1. Cancer hazard evaluation process

Figure 2.1 depicts the cancer hazard evaluation process. Scoping, problem formulation, and evidence mapping lead to the development of the framework (Step 1), which includes the overall objective and aims, MECO statements (i.e., body of evidence) to address the study objective(s), and identification of hazard specific issues to be explored in the evaluation. This step has been completed and the findings are reported in Section 2.1. This step also informed the methods, i.e., the protocol (Step 2) for conducting the cancer hazard evaluation (Step 3), the results of which will be captured in the RoC monograph. The methods focus on study evaluation (bias and study sensitivity, Section 2.2) and evidence integration (Section 2.3). **MECO** = **M**odel, **E**xposure, **C**omparison group, and **O**utcome.

2.1. Developing the Framework

Preliminary scoping and problem formulation activities informed the evaluation framework for the entire cancer hazard evaluation for wood smoke, which includes the evaluation of animal cancer studies using the methods described in this protocol, as well as evaluation of human cancer studies (methods described in a separate protocol [NTP 2022b]) and mechanistic studies in humans, animals, and cells.

These activities informed the research questions and the body of evidence to answer the research questions. The body of evidence is defined by the MECO (**M**odel, **E**xposure, **C**omparison Group, **O**utcome) Statements.

2.1.1. Identifying and Selecting the Literature

Biomedical citation databases, namely PubMed, Scopus, and Web of Science, were searched for animal cancer studies and exposure to wood smoke by combining search terms for exposure to wood smoke (see Appendix A), cancer (see RoC Search String Document), and animal cancer studies (see RoC Search String Document) using the procedures outlined in the RoC Handbook. We also searched cited references in the International Agency for Cancer Research (IARC) Monographs on Household Use of Solid Fuels and High Temperature Frying (2010) and Occupational Exposure as a Fire Fighter (2023).

Search results were processed in Endnote and imported into a content management system [e.g., Health Assessment Workplace Collaborative (HAWC)] software to select relevant literature

(Shapiro et al. 2018). Studies are included on the initial MECO. In addition, we include supporting studies that have non-cancer data that is informative for a cancer assessment, such as reporting preneoplastic lesions, or describe non-neoplastic lesions that are considered part of a morphologic continuum to neoplasia.

2.1.2. Mapping the Evidence

No animal studies were identified on exposure to wildfires. Citations of animal cancer studies from wood smoke exposure were characterized by study design, type of exposure, animal model, and exposure route (Table 2-1) based on the initial MECO (see Table 2-2). Studies included exposure to wood smoke and studies involving exposure to extracts or particles from wood smoke or soot samples.

Table 2-1. Characteristics of experimental animal studies

Study Design	# of Studies	Exposure	Route	Species
Cancer bioassays	3	Smoke/experimental	Inhalation	Mice (M & F)[a,b] Rats (M & F)[b]
	1	WS extract/real world	Subcutaneous	Mice (M)[c]
	1	WS extract/real world	Dermal	Mice (F)[d]
	1	Soot particles/real world	Subcutaneous implants	Rats (M & F)[e]
	2	Soot extract/real world	Subcutaneous	Mice (M & F)[e,f]
Epidemiology study	1	Smoke/real world	Inhalation	Dogs (M & F)[g]
Initiation promotion	4	WS extract/real world	Dermal	Mice (F & not specified)[d, h, i, j],

[a](Reed et al. 2006), [b](Liang et al. 1988), [c](Liang et al. 1984), [d](Mumford et al. 1990), [e](Sulman and Sulman 1946), [f](Khesina et al. 1977), [g](Bukowski et al. 1998), [h](Liang and Wang 1987), [i](Lewtas 2007), [j](Cupitt et al. 1994)
WS = Wood smoke

The initiation promotion studies were conducted in Sencar or Kunming mice reporting on skin papilloma (benign tumors) and generally considered less informative for evaluating causality. However, Walaszek et al. (2007) stated that skin painting studies may be informative for evaluating complex mixture (such as tobacco smoking) and human lung cancer. These studies will be summarized in the monograph as supporting studies but are not included in the final MECO.

Table 2-2. Initial and final MECO

	Initial MECO	Final MECO
Models	All animal cancer models and species	Complete carcinogenicity models; all species
Exposure	Wood smoke or wood smoke extracts	Wood smoke or wood smoke extracts; no exposure to promoting agents
Comparison	No or lower exposure to wood smoke	No or lower exposure to wood smoke
Outcome	Tumors	Tumors

2.2. Study Informativeness Evaluation of Individual Studies

The methods are adapted from the RoC Handbook (update in progress). Each primary study is systematically evaluated for its ability to inform the cancer hazard evaluation using five domains related to bias – selection and attrition bias, exposure assessment, outcome assessment, potential confounding, and analysis – and one domain related to study sensitivity [or the ability of the study to detect a true effect (Cooper et al. 2016)] that includes elements related to study design and exposure conditions. These questions highlight concerns toxicologists usually consider when evaluating study informativeness and are used to increase transparency but are not meant to be a checklist. The potential for a given bias in a study does not necessarily or automatically mean that the findings of the study should be disregarded. When adequate information is available, the direction of the bias (away or towards the null) should be considered. In answering each question on whether there is a potential bias or limitation, reviewers provide their judgment by comparing the study elements with those of an *ideal* study for a specific endpoint. *Ideal* study elements have no to minimal concern for potential bias and are sensitive enough to detect an effect if present (See Tables 2-3 to 2-8 for guidelines on rating biases for each question). In some cases, a rating may not be possible due to the complexity of the issues and will be captured by narrative text. Differences in reviewer judgments are resolved by discussion between the reviewers. A small subset of studies may be used in a "pilot" phase to discuss and resolve any ambiguity before proceeding with evaluation of the full set of studies. Study authors may be contacted by reviewers to obtain additional information needed for our evaluation. Reporting quality may also be noted (e.g., missing information).

Response to signaling questions

- No/Minimal concerns: Study design or methodologies are ideal or very close to the ideal study characteristics and potential for bias is unlikely or minor. These studies are generally considered informative for the cancer hazard evaluation.
- Some concerns: Study design or methodologies indicate a possible low to moderate risk of bias. These studies are generally considered informative for the cancer hazard evaluation.
- Major concerns: Study designs or methodologies suggest that the potential for a specific type of bias is high. However, depending on the direction and distortion of the potential bias, the study may still be informative for cancer hazard evaluation, but should be viewed with caution.

- Critical concerns: Study design or methodologies suggest that the bias would likely make study findings unreliable for hazard identification. This category is rare.
- No information in the study: The information in the study is inadequate to evaluate the level of concern.
- ***Direction of bias:*** ↑Away from the null or overestimate of the effect (e.g., false positive); ↓towards the null or underestimate of the effect (e.g., false negative); not known (unable to determine).

2.2.1. Study Design

The study design domain for experimental studies evaluates two bias questions, one on randomization and the other on controls (Table 2-3). Concurrent controls are the most relevant comparison group for evaluating potential exposure-related tumor effects. Study sensitivity integrates study model, statistical power, and study duration. The selection bias question is for a veterinary cancer epidemiological study in dogs. This study was a case-control study in pet dogs, with a questionnaire on exposure, demographic, and other lifestyle factors completed by the owner.

Table 2-3. Study design: Questions and responses

Signaling questions	Guidance	Response options
Bias Questions (Experimental Studies)		
Randomization		
Is there concern that the methods of randomization of animals were inadequate?	Ideally the method of randomization was reported and based on all animals having the same probability of being in a dose group. If not reported (which is common in older studies), we do not provide a judgement.	***Minor concerns*** Animals are randomized to control and experimental groups. ***Some/Major concerns*** Randomization was conducted but there are concerns about the adequacy of the methods. ***Critical concerns*** There is evidence that randomization of animals to dose groups did not occur.

Signaling questions	Guidance	Response options
Controls		
Is there concern that the concurrent control group is not adequate for evaluating effects across treatment groups? If no concurrent controls were used, are historical controls that could be used in place of concurrent controls reported?	Concurrent controls are the most relevant comparison group for evaluating potential exposure-related tumor effects. Ideally the concurrent control group has the same or greater number of animals as those in each treatment group. In some cases, historical controls of the same animal strain/stock and from the same laboratory may serve in place of concurrent controls.	***Minor concerns*** Controls are treated as similar as possible to the exposed animals but without the test substance, e.g., appropriate vehicle controls. ***Some concerns*** Concurrent controls but some concerns about similarity with (or have substantially fewer animals than) exposed animals. ***Major concerns*** No concurrent controls were used; historical controls are available. ***Critical concerns*** No concurrent or relevant historical controls (that could serve as concurrent controls) are available.
Bias Questions (Epidemiological Studies)		
Selection bias		***Minor concerns***
Is there a concern that selection into the study was related to both exposure (e.g., wood smoke) and cancer? Are there concerns that the selection methods are not adequate? Do the eligibility criteria or recruitment strategies differ for study participants (pet owners and dogs) such as for cases and controls?	Participation should not be an effect of both cancer and wood smoke status and should be similar in all respects except disease status. Controls (identified from disease registries) should not have diseases that are linked to wood smoke.	Cases and controls were selected from the same population by similar methods and criteria. There is no evidence that selection of the participants was related to both wood smoke exposure and cancer. ***Some/Major concerns*** There is some evidence that study selection may be related to both exposure and outcome. ***Critical concerns*** There is substantial evidence that selection or attrition of participants was clearly related to wood smoke exposure and outcome.

2.2.2. Exposure Conditions

The bias questions assess the quality of the type of exposure and dose level (Table 2-4). Dose selection is considered as both a bias issue and sensitivity issue (see Section 2.2.3). The bias questions for the epidemiological studies are related to exposure assessment (measurement/misclassification).

Table 2-4. Exposure: Questions and responses

Signaling questions	Guidance	Response options
Bias Questions (Experimental Studies)		
Exposure proxy Is there concern the test article (exposure proxy) does not represent human exposure to wood smoke?	Ideally, animals should be exposed to wood smoke (aerosol including gaseous/volatile components and solid particles) rather than extracts as extracts may not contain all of wood smoke components. Wood smoke extracts may be a better proxy for the exposure than soot extracts.	***Minor concerns*** The exposure is representative of human exposure to wood smoke. ***Some/Major concerns*** The testing agent is not representative of the complex wood smoke exposure mixture, e.g., extracts on just a subfraction of the mixture. ***Critical concerns*** The exposure is a mixture that contains wood smoke and other non-wood smoke substances (such as coal).
Dose selection Is there concern that the dose level was too high (e.g., exceeds the maximum tolerated dose)?	Ideally, the authors should state their rationale for dose selection and severe toxicity should not be observed. For example, the dose for animals exposed to smoke from wood burning stoves was not considered to be too high.	***Minor concerns*** Minimal treatment-related survival effects (unless mortality is related to tumors) were seen. ***Some concerns*** Reduced survival due to toxicity, but not substantially reduced. ***Major to critical concerns*** Severe toxicity in all treatment groups is seen. Toxicity is so high that survival is substantially reduced. Reduced survival due to tumors is not a concern.

Signaling questions	Guidance	Response options
Bias Questions (Epidemiological Studies)		
Is there concern that the exposure assessment did not distinguish between exposed and non-exposed animals or among exposure categories at a relevant time window of exposure? Is any misclassification differential or nondifferential, and what is the predicted direction or distortion of the effect estimate (if there is adequate information)?	The exposure assessment methods should consider whether the exposure proxy represents the exposure of interest and how well the exposure was measured. The ideal measurement is cumulative exposure to wood smoke, thus information on intensity (e.g., measurement) and frequency and duration (e.g., detailed questionnaire data) should be assessed.	***Minor concerns*** Cumulative exposure to wood smoke is assessed. ***Some concerns*** Some but not all measurements of cumulative exposure are assessed; however, exposed and non-exposed groups can be distinguished. Any exposure misclassification is non-differential. ***Major concerns*** Exposure misclassification between exposed groups is likely and non-differential but there is some ability to separate exposed from non-exposed. Differential recall bias (e.g., among pet owners completing a questionnaire) is possible but not substantial. ***Critical concerns*** Strong evidence of differential recall bias or unable to separate exposed from unexposed.

2.2.3. Sensitivity: Animal Model and Exposure Conditions

The available experimental studies were conducted in five strains/stocks of mice (Kunming, Beijing, SENCAR, C57BL X CBA F1 Hybrid, and A/J), one unspecified strain/stock of mice, and one rat stock (Wistar). Kunming mice are commonly used in China and are an outbred stock derived from Swiss mice. Because A/J mice have a high background of spontaneous lung tumors, tumor multiplicity is considered more informative than tumor incidence. The F1 (C57BL x CBA) hybrid may be of intermediate sensitivity. The epidemiological study included all dog breeds. The cancer studies were conducted in males and females, although not all studies tested both sexes.

Animal studies of cigarette/tobacco smoke (e.g., similar complex mixture) may help inform the evaluation of wood smoke studies recognizing the differences in exposure conditions (actively inhaling cigarettes vs environmental exposure to wood smoke). Cigarette smoke is a weak lung carcinogen in rodents: rodents are obligate nasal breathers and may change their breathing habits in response to smoke exposure (Hecht 2005). Moreover, the portal of entry of pollutants in rodents is the nasopharynx, which may remove 50% of inhaled toxicants (Enomoto et al. 2008). In traditional rodent models, modest increases in lung and nasal cavity tumors (rats only) have been detected in studies using large numbers of rats (Mauderly et al. 2004) and mice (Hutt et al. 2005), exposed daily to high levels of cigarette smoke (~3 to 4 packs or ~250 mg/m3) for a long duration (~2.5 years). Wood smoke has higher concentrations of polycyclic aromatic hydrocarbons than cigarettes (Naeher et al. 2007).

A/J mice are commonly used in cigarette smoke carcinogenicity studies to evaluate lung adenoma multiplicity (Witschi 2005; 2007; Witschi et al. 2002). The typical short-duration (nine months) protocol (developed by Witschi) exposes mice (approximately 20 to 30 animals) daily to cigarette smoke for five months and then to air (recovery period) for another four months. Based on a review of several studies using the Witschi A/J model, exposure to tobacco smoke (ranging from 133 to 735 mg/m^3; average chamber concentration 53 to 147 mg/m3) significantly increased the incidence and multiplicity of lung adenomas in A/J mice (Witschi 2005; Witschi et al. 2002). Studies conducted in other murine strains using this model were negative except for A/HeJ mice (Gordon and Bosland 2009). Female mice were more sensitive to cigarette smoke than males. Malignant lung tumors were not increased in multiple studies using the short-term model but were significantly increased in an 18-month study with either continuous exposure or a recovery period (Stinn et al. 2013).

Common models to study air pollutant extracts are skin painting studies or subcutaneous injection of pollutants; these models require high doses and longer durations and generally have low tumor yields (Epstein 1966). Neonatal murine models are more sensitive. For cigarette smoke condensates, the SENCAR mouse is a sensitive model for the initiation/promotion of skin tumors and considered informative for predicting human lung cancer risk from respiratory carcinogens (Walaszek et al. 2007).

This information was used to develop the guidance for evaluating study sensitivity in Table 2-5.

Table 2-5. Sensitivity: Questions and response

Signaling questions	Guidance	Response options
	Sensitivity Questions (Experimental Studies)	
Is there concern that the study design (i.e., animal model, number of animals/dose group and control group, study duration, dose(s) and exposure conditions) is sensitive enough to adequately detect a neoplastic effect if present? This question considers • Animal model • Statistical power (number of animals/group) • Study duration • Exposure regimen: duration and dose level	The sensitivity rating integrates the animal model, statistical power, study design, and statistical analysis. In some cases, one factor may compensate for limitations in another factor (e.g., when a short study duration is compensated by a highly sensitive animal model that develops tumors within that duration). **Wood smoke inhalation studies**: The ideal study would expose large numbers of rodents (50 to 100/group) daily at high doses (e.g., ~250 mg/m^3 or typical stove conditions) for ~2.5 years. Mouse strains should have at least intermediate sensitivity to lung carcinogens without high background rates. Studies in A/J mice to detect lung adenoma multiplicity should expose mice (20 to 30 mice/group) for reasonable durations and doses (e.g., 5 months at doses of at least 130 mg/m^3) followed by a recovery period (e.g., four months). Studies of longer duration are needed to detect malignant tumors in A/J mice. **Extracts or particles**: The ideal study would be conducted in neonatal mice. Studies in adults may also be sensitive, especially if more than one strain/source of mouse is used. Dermal or s.c. injection are adequate exposure routes. **All experimental studies:** Models that can evaluate malignant tumors at any organ site are more informative than those designed to evaluate only organ-specific (e.g., lung, skin) or benign tumors.	***Minor concerns*** Wood smoke inhalation or extract studies in strains/stocks of at least intermediate sensitivity using conditions close to the ideal conditions (defined in guidance). ***Some concerns*** Models designed to detect only adenomas (primarily one cancer site) and conducted using close to the ideal study. Other studies may have less than ideal conditions for some but not all aspects of the study design or exposure conditions (e.g., duration, number of animals) ***Major concerns*** Insensitive model or inadequate conditions for the animal model (see guidance).

Signaling questions	Guidance	Response options
Sensitivity Question (Epidemiology Studies)		
Does the study have adequate sensitivity to detect an effect from exposure (if present)?	Sensitivity considers statistical power, exposure contrast, latency, and relevance of the exposure metric.	***Minimal concerns*** The study had an adequate number of exposed animals, with substantial exposure (level, duration, or range) and with adequate duration of follow-up for latency status. ***Some concerns*** The study has adequate sensitivity for some but not all elements**.** ***Critical or major concerns*** The study was modest or small, with few exposed animals, and/or the exposure range was minimal.

2.2.4. Outcome Assessment and Measurement

The outcome domain consists of one signaling question (and a related follow-up question) on the quality of the methods to assess tumor outcome in exposed and controls animals (Table 2-6). This question includes both bias and sensitivity concerns. Evaluation of only a few organs for tumors instead of all organs and tissues can limit the sensitivity of the study.

Although blinding is generally considered an important aspect of subjective outcome assessments (such as behavior) to reduce risk of bias, for cancer outcomes non-blinding may be preferred to determine normal background histology. The NTP uses an informed approach to histopathological evaluation in its toxicity and carcinogenicity studies (Sills et al. 2019). This principle applies to non-NTP studies providing that the necropsy and histology methods used are adequate and consistent.

Case-control veterinary epidemiology studies typically report on only one tumor type.

Table 2-6. Outcome: Question and responses

Signaling questions Follow-up question	Guidance	Response options
	Bias (Experimental Studies)	
Is there concern that the methods used to assess tumor outcome (necropsy, gross pathology, histology, or diagnosis) are not adequate to attribute the effects to the exposure?	Ideally, each study should include full gross necropsies of all tissues and histopathological examination of most tissues. At a minimum tumor type (and whether benign or malignant) should be reported. For a study model (strain/route of exposure) designed to detect a specific type of tumor, then it may not be necessary for the study to evaluate all tissues, especially if at least one target organ is known (e.g., lungs are the target organs for wood smoke). For wood smoke, these models include the SENCAR dermal studies and the A/J mice for lung adenomas. Controls and all the treatment groups are treated the same.	***Minor concerns*** Traditional models: Complete necropsies and gross pathology are reported for all tissues and histopathology examination done on most tissues for both controls and treated groups. Models for one tumor type: Histopathology conducted for tumor site of interest. ***Some concerns*** Benign and malignant tumors but not cell type reported**.** ***Major concerns*** Histopathology is not done or reported on tumors. ***Critical concerns*** Controls and treatment groups analyzed differently to the extent it would comprise the study interpretation
	Bias (Epidemiology Studies)	
Is there a concern that the outcome measure does not reliably distinguish between the presence or absence of the cancer under study?	Ideally, cases of cancer should be histologically confirmed and/or undergo independent pathology review (e.g., on a subset of the cases) by the study investigator.	***Minor concerns*** Cancers are histologically/cytologically verified. ***Some concerns*** Cancer diagnoses are reported in record databases but not histologically/cytologically verified. ***Moderate/Major concerns*** Cancer diagnosis and type are self-reported by pet owners, and neither are verified by cancer registry or medical/veterinary hospital records. ***Critical concerns*** There is strong evidence that cancer diagnoses are likely related to exposure status

2.2.5. Potential for Confounding

The confounding domain consists of one signaling and related follow-up question and addresses any potential sources that can influence the study outcome other than the substance under evaluation (Table 2-7).

Potential confounders include consideration of both canine factors and their home environment. Risk factors in dogs include dog breed (e.g., long-nosed, male sex, and older age [Hayes et al. 1982; Reif et al. 1998]).

Most environmental risk factors are occupational agents or formed in the diet from food (e.g., nitrosamines). Some occupational agents may also be present in the home because of pet owner's hobby, off-gassing from consumer projects, or environmental exposures (e.g., formaldehyde, wood dust, 2,3,7,8-tetrachlorodibenzo-p-dioxin, and radon) but are not likely to correlate with indoor exposure to wood smoke or are most likely present in low concentrations. Dogs may be exposed to nickel from collars, but it is unlikely that wearing a nickel collar is related to the home use of wood for heating/cooking. Some data suggest that living in an urban environment and exposure to environmental tobacco smoke (ETS) may be risk factors for nasal cancer in dogs (Llera et al. 2023; Reif et al. 1998). Urban living could be associated with exposure to wood smoke, but it is unclear if ETS correlates with exposure to wood smoke and may depend on the population.

Table 2-7. Potential confounding: Question and responses

Signaling questions Follow-up question	Guidance	Responses options
Confounding		
Is there concern for potential confounding? • What is the relative impact of the confounding?	Experimental studies: Sources of potential confounding in wood smoke animal studies are inadequate animal husbandry conditions, and lack of monitoring for pathogens. Epidemiological studies: Ideally, studies should consider sex, breed, and nasal length of the dog and socioeconomic status of the pet owner. Dogs should not have been exposed to coal.	***Minor concerns*** Experimental studies: The study uses adequate animal husbandry conditions and animals are not co-exposed to other substances (other than wood smoke) in their chambers. Epidemiological studies: the study measured all major potential confounders and used appropriate statistical analysis or designs. ***Some, Moderate/Major concerns*** Experimental studies: Potential for bias depends on documentation of poor husbandry and co-exposures, noting that most studies do not report on husbandry conditions. Animals are exposed to other substances (present in the testing chambers) in addition to wood smoke; could be critical if substances are carcinogens. Epidemiological studies: Potential for bias depends on the degree by which the study addressed the major concerns, including how thoroughly they were addressed (statistical analysis vs. external information), and the number and importance of the confounders. ***Critical concerns*** Experimental studies: Strong evidence that poor animal husbandry conditions will substantially compromise interpretation of the findings and there are no data to evaluate the extent of the confounding. Epidemiological studies: There is strong evidence that the effects of the exposure cannot be distinguished from the effects of the potential confounders.

Analysis

The analysis domain evaluates statistical methods and combining of tumor incidences and consists of two bias questions (Table 2-8). These questions address the methods for grouping the outcome (i.e., tumors) and statistical methods to evaluate the findings. If statistical analysis was not performed, but tumor incidences were reported in enough detail, NIEHS can perform pair-wise statistical calculations. Trend analysis across treatment groups (e.g., Cochran-Armitage trend test) can also be performed if there are three or more dose groups. It shall be noted if statistical analyses were performed by NIEHS.

Table 2-8. Analysis: Questions and responses

Signaling questions	Guidance	Response options
Combined tumors Is there concern that different types of tumors were inappropriately combined in the analysis?	Analyses of benign and malignant tumors from the same tissue type should be reported both separately and combined; tumors of the same cellular origin, which may appear at different organ sites (seen with metastasis) should be combined. Organs that are of the same cellular origin, subjected to similar environmental exposure, and part of the same organ system can be combined e.g., squamous carcinomas of the upper respiratory tract (nasal cavity, pharynx (throat), larynx (voice box), and bronchitrachea) (McConnell et al. 1986).	***Minor concerns*** Tumors of the same cellular origin are reported both individually and combined in the analysis. ***Some concerns*** Tumors of different cellular origin are reported individually but may also be inappropriately combined. However, it may be possible to conduct appropriate ad hoc analysis. ***Major concerns*** Tumor types of different cellular origins are combined, or tumors are only specified for a particular organ, as benign or malignant without reporting their cellular origin.
Statistical analysis Is there concern that statistical analyses are inadequate or were not conducted for evaluating the results? • If statistical analyses are not conducted, are the results reported in sufficient detail for *ad hoc* analysis?	If statistical analyses are not reported, the study should at a minimum present incidence data for specific tumors, so that statistical tests (e.g., Fisher's exact test for pairwise comparisons) can be conducted. If there is evidence of a decreased survival effect, the studies should use adequate statistical methods, such as the poly-3 test (Bailer and Portier 1988), to control for decreased survival. Epidemiology study: The study used relevant data and appropriate assumptions and analysis methods.	***Minor concerns*** The study reports appropriate methods of analysis using relevant data. Analyses are adjusted for survival when relevant. ***Some concerns*** Appropriate analyses are conducted but are not adjusted for survival and there are survival concerns. ***Major to critical concerns*** There is strong evidence that reporting of data and analytical methods are so limited that the findings are not interpretable.

2.2.6. Study Informativeness

The overall study informativeness of a study considers both bias (e.g., systematic flaws or limitations that may compromise the interpretation of the results), and study sensitivity. Studies having elements with major concerns may still be considered for cancer hazard assessment, but the findings should be interpreted with caution. It should also be noted that some concerns about a study element (such as inadequate observation and/or exposure period or statistical power) would decrease the study's sensitivity to detect an effect. If positive findings were described despite these limitations, these studies would inform a cancer hazard assessment. Studies with critical concerns about important issues are generally inadequate to inform the evaluation.

If a study has inadequate information for a reviewer to answer a specific question, the impact on overall study quality evaluation depends on the extent and importance of the missing information and is evaluated on a case-by-case basis.

Study informativeness-level judgment

- ***High*** (no/minor concerns about most potential biases, high or moderate sensitivity)
- ***Moderate*** (some concerns about most potential biases)
- ***Low*** (major concerns about several biases, sensitivity rating varies); depending on the direction and distortion of the potential biases, the study may still be informative for cancer hazard evaluation but should be viewed with caution
- ***Inadequate*** (critical concerns about any bias, sensitivity rating varies)

2.3. Evidence evaluation and integration

2.3.1. Interpretation of the Evidence from Individual Studies

Study findings are interpreted considering study sensitivity and the direction of any potential biases. In determining whether a tumor site is treatment-related (e.g., biological significance), we evaluate several factors, including statistical significance, dose-related trends, the presence of pre-neoplastic lesions, lesion at the tumor site of interest, decreased latency, tumor multiplicity, tumor incidence, animal survival, species, sex, strain, and the rarity of the tumor.

External Validity

External validity addresses the extent to which conclusions from one study can be generalized to other situations (i.e., the relevance of experimental animal data to humans). Neoplasms observed in experimental animals are considered relevant to humans unless there is *compelling* evidence indicating that they occur by a mechanism that does not operate in humans. We considered the following in assessing the relevance of an experimental animal cancer study for evaluating the potential for human carcinogenicity:

- Relevance of the route of exposure.
- Relevance of the species, sex, or animals' age.

- Relevance of the mechanism of tumor formation.

The veterinary epidemiology study helps strengthen external validity with respect to exposure route. All exposure routes of studies identified to date are relevant for wood smoke exposure, e.g., the dermal skin painting studies are thought to inform human lung cancer as they have been shown to cause this type of cancer (Walaszek et al. 2007). Findings of tumors at a similar tissue site by different routes of exposure strengthen the evidence for carcinogenicity. The mechanism section will address biological plausibility.

2.3.2. Evidence Integration Across Animal Cancer Studies

The final steps in evaluating evidence from experimental animal cancer studies are integrating the evidence for treatment-related tumors across studies, applying the RoC listing criteria, and reaching a level-of-evidence conclusion from studies in experimental animals. The conclusion will be based on integration of findings from the veterinary epidemiology study and experimental studies.

RoC listing criteria for evaluating carcinogenicity from studies in experimental animals

Sufficient evidence of carcinogenicity from studies in experimental animals:
An increased incidence of malignant and/or a combination of malignant and benign tumors

- in multiple species, or
- at multiple tissue sites, or
- by multiple routes of exposure, or
- to an unusual degree with regard to incidence, site, or type of tumor or age at onset.

The first step in evidence integration is to evaluate the evidence across studies for each cancer site of interest. The conclusion will be based on integration of findings from the veterinary study and experimental studies of wood smoke extracts and inhalation exposure to smoke. For most databases, heterogeneity in findings is often explained by differences in experimental conditions (e.g., species, sex, strain, doses, duration, route), and few studies have been conducted using the same experimental conditions. As mentioned above, the most informative studies (highest quality and sensitivity) are given the most weight, and positive findings from these studies are considered to provide evidence of treatment-related tumor effects. Moderate- and low-quality studies can also be used in the assessment, especially when it is unlikely that biases (moderate) in the studies would cause false-positive results. Replication of findings across several studies also increases confidence in treatment-related effects.

In general, the RoC criteria for sufficient evidence of carcinogenicity from studies in experimental animals are fulfilled by (1) two studies (by different exposure routes or in different species) reporting positive findings of malignant or combined malignant and benign tumors or (2) one study reporting positive findings at multiple tissue sites. In addition, positive findings from one robust study can fulfill the criteria if the tumors are rare, have an early onset, or have a high incidence. The spectrum of neoplastic responses,

from pre-neoplastic lesions and benign tumors to malignant neoplasms of a specific tumor type, is relevant for the evaluation of whether benign tumors observed at increased incidences are likely to progress to malignancy.

3. Evaluating Mechanistic Evidence

Overall Objective and Aims

Overall Objective

To reach conclusions about the level of evidence of the carcinogenicity to wood smoke and wildfire provided by mechanistic studies based on the RoC listing criteria.

Protocol Contents and Evaluation Process

The methods and strategies in this protocol come from the updated RoC Mechanism Handbook, currently in press. Briefly, the new methods use a fit-for-purpose approach (Figure 3-1) that uses scoping activities (e.g., broad-based literature searches, iterative scoping questions, and mapping the evidence) to develop the framework (see Section 3.1, this step has been completed) and identify influential questions—scientific issues that (1) are critical for understanding cancer mechanisms and biological and human relevance, (2) will most likely impact the LoE conclusions that are specific for the substance under the evaluation—and the literature to answer the questions. For each question, the rigor of the study informativeness assessment depends on how impactful the different groups of studies are to the overall evaluation (see Section 3.2 for methods). The overall LoE is reached by integrating confidence in the evidence across the questions (see Section 3.3 for methods).

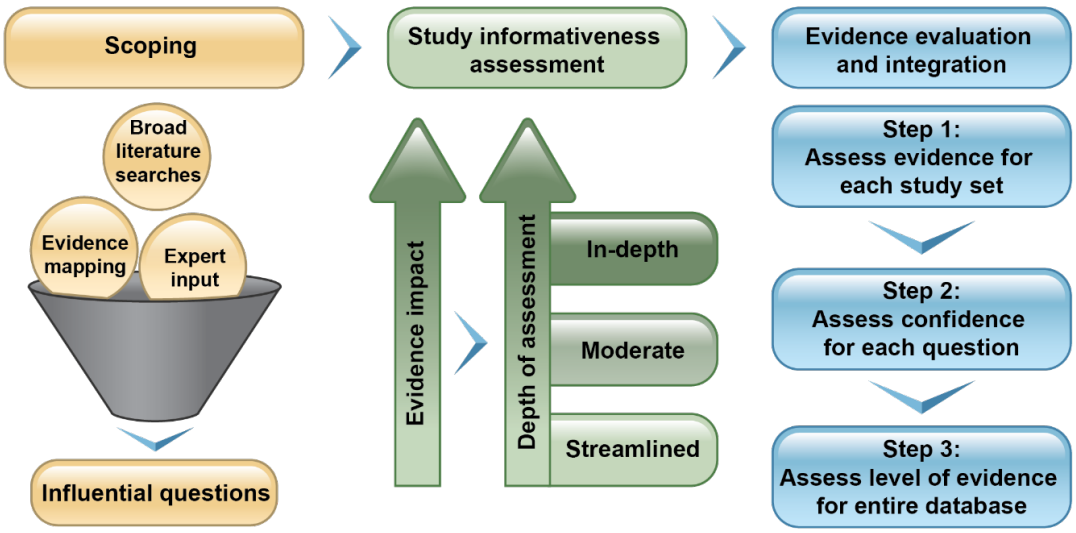

Figure 3-1. Fit-for-purpose evaluations

Influential mechanistic questions are identified from broad, unbiased literature searches. For each question, the relevant literature (e.g., multiple study sets) is identified and evaluated; the rigor (streamlined to in-depth) of the study informativeness and evidence assessment depends on how impactful (e.g., low to high) a study set is to the overall evaluation (see Sections 3.1 and 3.2). Each study set is defined by the type of evidence (e.g., exposed humans or animals, in vitro), exposure, comparison, group, and endpoint (e.g., a specific biomarker or similar biomarkers). Lastly,

we integrate the evidence across study sets to reach a confidence judgment for each question and across questions for an LoE conclusion.

3.1. Developing the Framework

Preliminary scoping and problem formulation activities informed the evaluation framework for the entire cancer hazard evaluation for wood smoke and wildfire exposure (see Background).

Scoping activities for wildfire and wood smoke began with a review of authoritative reviews and other scientific information sources (e.g., CompTox), and literature searches to gather mechanistic information for the substance and develop the initial EECO (**E**vidence stream, **E**xposure, **C**omparison Group, **O**utcome) Statements for determining the level of evidence of carcinogenicity from mechanistic studies. Next, broad-based systematic literature searches, mapping the evidence from literature searches and review of authoritative reports helped to identify the most influential mechanistic questions and literature.

3.1.1. Identifying and Selecting the Literature

Biomedical citation databases, namely PubMed, Scopus, and Web of Science, were searched for mechanistic studies of exposure to wood smoke or wildfire by combining search terms for the exposure (see Appendix B) and KCC or general mechanistic terms (see RoC Handbook). We use the KCCs (Box 3-1) to conduct broad literature searches for mechanistic data because they represent multiple carcinogenic mechanisms and provide an unbiased framework to identify relevant mechanistic literature without relying on prespecified modes of action in isolation (Guyton et al. 2018; Smith et al. 2016; Smith et al. 2020). Note that the RoC has broadened the KCC 'induces chronic inflammation" to include "or immune activation". We also searched cited references.

Box 3-1. Key characteristics of carcinogens

Is electrophilic or can be metabolically activated to electrophiles
Is genotoxic
Alters DNA repair and causes genomic instability
Induces epigenetic alterations
Induces oxidative stress
Induces chronic inflammation or immune activation
Is immunosuppressive
Modulates receptor-mediated effects
Causes immortalization
Alters cell proliferation, cell death or nutrient supply

(Smith et al. 2016)

Search results were processed in Endnote and imported into a content management system [e.g., Health Assessment Workplace Collaborative (HAWC)] software to select relevant literature (Shapiro et al. 2018). Studies are selected for possible inclusion in the evaluation if they meet the following initial EECO statement (Table 3-1). The comparison group was no or low exposure to wood smoke or wildfire based on the initial review (high level) of the study.

Table 3-1. Initial EECO statement

Evidence type	Exposure	Comparison group	Outcome
Exposed humans (includes wildfire firefighters, but not firefighters in general)	Wood smoke (not biomass in general, not coal) or wildfire	Low or no exposure to wood smoke (no coal) or wildfire	KCCs Other: Respiratory, polymorphism cancer studies
Experimental animals	Wood smoke (not biomass in general, not coal) or wildfire	Low or no exposure to wood smoke or wildfire	KCCs Other: Respiratory
In vitro (or cell free)	Wood smoke (not biomass in general, not coal) or wildfire	Low or no exposure to wood smoke or wildfire	KCCs Other: Respiratory

3.1.2. Mapping and Characterizing the Evidence

Based on initial review of the selected studies and tagging, and review of authoritative literature, and of the human and animal cancer data, we developed iterative guiding questions to identify the influential agent-specific mechanistic questions and studies for evaluations and to inform protocol development.

Primary Scoping Questions

1. What are the proposed mechanisms of wood smoke and wildfire?
2. What biological effects have an adequate database for review?

   Most of the selected studies (using HAWC to visualize and sort the findings) evaluated biomarkers of genotoxicity, oxidative stress, and/or immune effects. Next, we identified additional scoping questions and extracted selected data to characterize the studies and answer the questions below and visualize the data using Tableau.

3. What types of studies (design, exposure variables, biomarkers) are available?
   a. Which type of evidence is more influential for the review?
4. What are the results, e.g., are exposure to wood smoke and wildfire positively associated with genotoxicity, oxidative stress, and/or immune biomarkers?

provides an overview of the interactive evidence map for the wood smoke and wildfire studies. The rows depict the types of biomarkers for KCCs related to genotoxicity (also includes adducts), oxidative stress, and immune biomarkers (includes immunomodulation and inflammation). Data are also shown for wood smoke or wildfire components (e.g., PAHs, metals, volatile organic compounds, metals, carbonyls) and exposure variables (e.g., combustion conditions, wood stove type, wood type, natural wildfires, prescribed burns) and for human studies, the population and study design (e.g., cross-sectional, randomized control trials [RCT]). We also extracted the reported findings (e.g., negative, positive, unclear, or non-significant) for each study and endpoint.

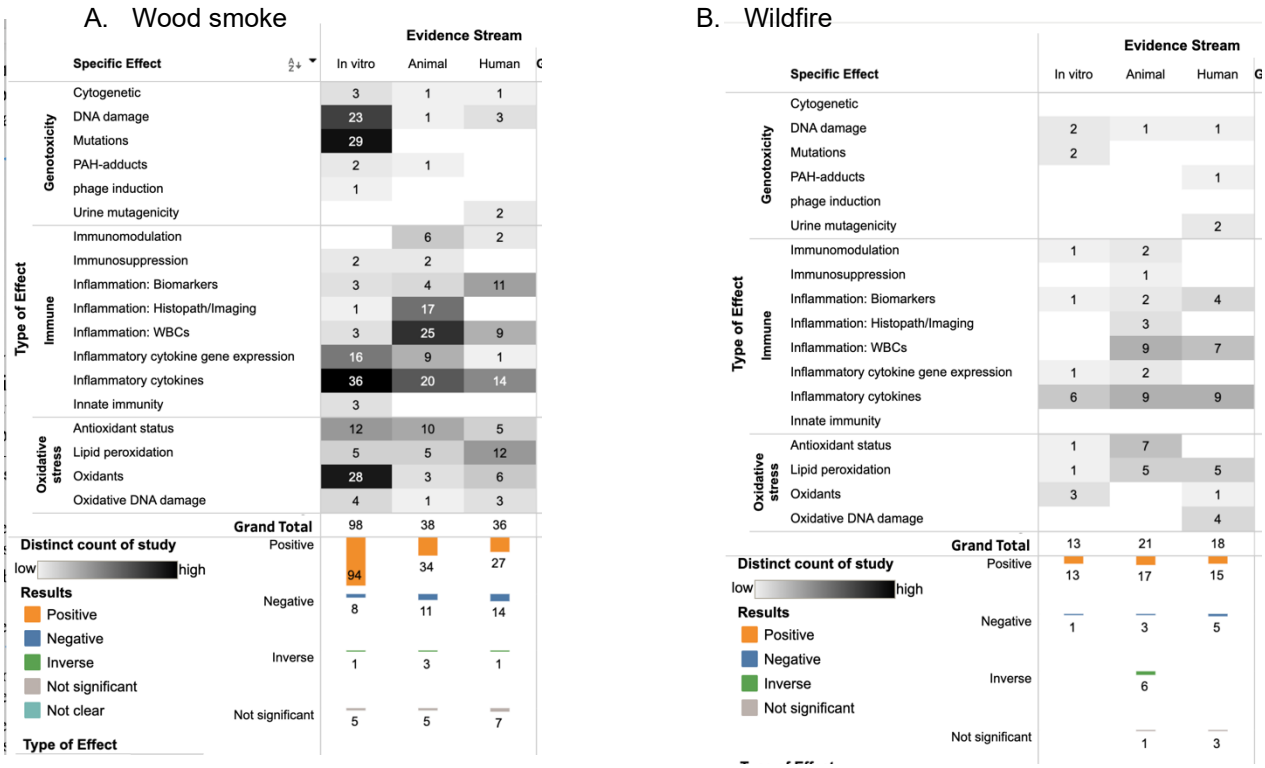

Figure 3-1. Systematic evidence maps (SEMs) of wood smoke and wildfire exposure

Figure 3-2 depicts the SEM of the mechanistic data (i.e., number of studies) reporting on exposure to (A) wood smoke and (B) wildfire. The rows depict the different types of effects, and the columns the evidence stream or types. The cells are the number of studies for each biomarker and evidence type combination. Study finding (positive, null, inverse, or not significant) are reported of all evidence types are reported for each evidence type or by biomarkers (not shown). The SEM allows the user to explore data by various topics, e.g., biomarker evidence, type, exposure (wood smoke or wildfire), study design (human studies), population (humans), exposure variable (e.g., type of stove, or combustion) by selecting on table elements or using filters (not shown).

Wood smoke studies

As of January 2024, 166 studies have evaluated exposure to wood smoke and the endpoints of interest. Over half (~60%, N = 98) of the studies were conducted in *vitro* and almost all reported positive associations for exposure to wood smoke and biomarkers of genotoxicity, oxidative stress, and inflammation/immunomodulation. The most common biomarkers were mutations, DNA damage, oxidants, and proinflammatory cytokines. Studies conducted in exposed animals and humans often evaluated multiple biomarkers; the most common biomarkers or indicators were WBC or subsets, proinflammatory cytokines, inflammation biomarkers, and lipid peroxidation; few studies reported on genotoxicity biomarkers. Although most studies reported positive findings, there was greater heterogeneity in the findings compared to the in vitro studies. Animal studies also reported inflammation using histopathology. A more in-depth assessment of

the studies in exposed animals and humans is needed to reach conclusions on whether wood smoke causes biological effects associated with known carcinogens (e.g., KCCs).

Wildfire studies

As of January 2024, 51 studies have evaluated exposure to wildfires and the biological biomarkers of interest. Most studies reported on immune (e.g., pro-inflammatory cytokines and WBC or subsets) and oxidative stress biomarkers (e.g., lipid peroxidation, antioxidant status) and there were few studies on genotoxicity. Almost all studies conducted in cells or experimental animals reported positive findings for the three groups of biomarkers; studies in exposed humans were also mainly (>80%) positive for any biomarker in each of the three categories (immune, oxidative stress, and genotoxicity); however, findings were more mixed when looking at specific biomarkers of KCCs (e.g., WBC). Because there are no cancer studies in experimental animals and few studies in humans, a detailed assessment of mechanistic data of all evidence types will be conducted.

Box 3-2. Influential questions for wood smoke and wildfire

1. What is the confidence that exposure to wood smoke/wildfire causes genotoxicity?
    a. Which genotoxic biomarkers have the strongest evidence?
2. What is the confidence that exposure to wood smoke/wildfire causes oxidative stress?
    a. What oxidative stress (or sets of) biomarkers have the strongest evidence?
3. What is the confidence that exposure to wood smoke/wildfire causes chronic inflammation or immunosuppression?
    a. What immune (or sets of) biomarkers have the strongest evidence?
    b. Is the evidence consistent with the hypothesis that wood smoke alters pulmonary immune defense via effects on macrophage-mediated immunity?
4. What is the confidence that at least two KCCs are associated with each other (e.g., are oxidative stress biomarkers linked to inflammation biomarkers)?

Secondary Scoping Questions

1. Do the results vary by differences in wood stove types, wood types, combustion factors, and/or other factors related to burning wood or wildfire smoke?
2. What components of wood smoke and/or wildfire were measured in the studies?

Data related to addressing these questions are depicted in the evidence map. This information may be useful for interventions and monitoring. Because it is not critical for the cancer hazard evaluations conclusions, the tableau database is adequate for describing this information.

3.1.3. Influential Questions and Literature

We identified the same influential questions for woodsmoke and wildfire: three questions are confidence levels for specific KCCs, and one is for confidence for connections between multiple KCCs (Box 3-2). The studies associated with each specific question are available in the interactive map by filtering on the specific KCC (e.g., genotoxicity) and evidence stream (exposed human, in vivo).

The study sets or EECO for each question are similar to the initial EECO (Table 3-2 and Table 3-3) except for the outcome, which is specific for the three KCCs above.

Table 3-2. Final EECO statement: Wood smoke

Evidence stream	Exposure	Outcome
Exposed humans (influential) Volunteers Wood smoke impacted communities (workers, women and others who used wood regularly)	Controlled exposure to wood smoke Wood use for cooking or heating Outdoor wood smoke	Q1. Genotoxicity: Primarily mutations and DNA damage; few studies in experimental animals or exposed humans
Experimental animals (influential)	Wood smoke specific (not biomass in general)	Q2. Oxidative stress: Oxidants, lipid peroxidation, antioxidant status, oxidative damage to DNA
In vitro (or cell-free) Streamline data extraction	Wood smoke specific (not biomass in general)	Q3. Immune effects: inflammation biomarkers like pro-inflammatory cytokines, WBC and subsets; histopathology/inflammation

Table 3-3. Final EECO statement: Wildfire

Evidence stream	Exposure	Outcome
Exposed humans (influential) Wildfire firefighters Communities	Prescribed burns Natural wildfires	Q1. Genotoxicity[a]: Primarily mutations and DNA damage; few studies in experimental animals or exposed humans
Experimental animals (excludes invertebrates) (influential)	Wildfire samples	Q2. Oxidative stress: Oxidants, lipid peroxidation, antioxidant status, oxidative damage to DNA
In vitro (or cell-free)	Wildfire samples	Q3. Immune effects: inflammation biomarkers like pro-inflammatory cytokines, WBC and subsets; histopathology/inflammation

[a] Few studies

3.2. Study Overview and Study Informativeness

As discussed in Section 3.1 and Table 3-2 and Table 3-3, the study informativeness assessment primarily focuses on the wood smoke and wildfire studies in exposed humans and experimental animals.

We evaluate the informativeness of mechanistic studies using a domain-based approach consisting of questions and guidance appropriate for the evidence type. For each question, we evaluate the potential, direction, and magnitude of the bias by comparing the study to an "ideal" study element specific to that bias (e.g., selection of participants).

"Ideal" is defined as a study design condition resulting in low concern for bias or sufficient sensitivity to detect an effect if present. Outcome specific judgements, e.g., a study reporting on multiple outcomes, will have multiple judgements of that domain.

Two reviewers assess the studies and resolve differences through mutual discussion and reference to the original data source. To facilitate the review process and reduce ambiguity, we conduct a pilot phase using a small set of studies before proceeding with the complete evaluation. If reporting of the studies is too incomplete to evaluate bias, we will contact the study authors for additional details.

While in vitro wood smoke and wildfire studies are not identified as influential literature, they will still be reviewed as part of the mechanistic evaluation. Findings and general limitations of these studies will be reviewed, and a comparison of the results will be made between the in vitro studies and the studies in exposed humans and animals.

3.2.1. Studies in Exposed Humans

Based on the scoping and mapping activities, wood smoke and wildfire studies in exposed humans could be binned into groups, which share similar study designs, populations, and strengths and limitations. Consistent with recommendations by Savitz et al. (2019), our approach is to identify the most influential factors (as opposed to using all questions) affecting informativeness (biases/sensitivity) and evaluate whether those factors impact the results. We apply this approach across study sets with similar designs rather than on individual studies. Table 3-4 shows the influential questions and issues for the informativeness domains in exposed humans.

Wood smoke exposed humans can be broadly grouped into two categories based on study design (observational vs. experimental related) and population (unexposed volunteers vs. participation based on wood use).

- Wood smoke impacted communities: observational studies of workers, women and other people who use wood fuel daily (e.g., chronically exposed)
- Volunteers: randomized cross-over studies, randomized control trials, or experimental studies of volunteers exposed to wood smoke for a short duration. Several of the volunteer studies were conducted by the same authors using the same design but different volunteers

Wildfire studies in exposed humans were mainly of wildland firefighters and can be categorized based on the type of wildfire exposure (e.g., prescribed burns or natural wildfires). The controls in most of these studies were "self," e.g., the participant before or after exposure.

- Natural wildfires: cross-sectional studies (firefighters and patients) and pre-/post-shift or exposure studies, e.g., studies evaluated biological endpoints in the same person before or after a shift or for longer time periods before or after a wildfire
- Prescribed burns: pre-/post-burn follow-up studies

Table 3-4. Study informativeness of human studies: Influential questions and issues

Type of bias/sensitivity issues	Question	Comments
Exposure assessment	Are the controls exposed to wood smoke or wildfire (e.g., same individual, pre-shift)? Does the exposure assessment (e.g., proxy, measurements) adequately distinguish exposure groups?	In the studies using self as a control, the control samples may represent effects from previous exposure (depending on time since last exposure). This would most likely bias the findings towards the null
Potential confounding	Did the study design or statistical methods address the major potential confounders?	Major confounders (see Human Cancer Protocol) include socioeconomic status, coal, smoking, age, and gender In general, confounding is not a concern in studies using self as the control
Outcome and exposure	Was the biomarker measured at a relevant time frame and is it informative for predicting cancer?	Tissue: local more informative than circulating, especially in sites concordant with cancer sties. Informative biomarkers Genotoxicity: mutations, DNA, and chromosomal damage Oxidative stress: oxidants, reactive species modifications (e.g., lipid peroxidation, oxidative damage to DNA), and antioxidants Cytokines and WBC: IL-6, IL-8, and TNF-alpha and WBC subsets have been shown to be linked to cancer in prospective studies (not specific for wood smoke)
Sensitivity	Is exposure chronic or acute? Is the study sized adequately to detect biological effects?	Although most biomarkers are short-lived, chronic exposure (e.g., wood smoke impacted communities, workers, firefighters) likely reflects the cumulative effects of repeat (acute) exposures

3.2.2. Studies in Exposed Animals

Overview of studies

Wildfire studies: These include (1) observational studies of animals that lived in regions (e.g., farms, zoos, water) where natural wildfires occurred, or (2) experimental studies of animals (rodents or aquatic) exposed to wildfire samples of natural, prescribed burn, or simulated wildfires (see Table 3-5 for an overview of study characteristics). Animals in the observational studies were exposed via inhalation and/or dermally, usually from a single wildfire event (varying in duration from one week to over a month) but some

studies were on animals chronically exposed to multiple fires over several months (e.g., Pace et al. 2023).

Table 3-5. Overview of wildfire studies in exposed animals

Study design[a]	Species	Route	Exposure conditions
Observational	Non-human primates: Monkey Mammals: Cow, dog Aquatic mammals: Dolphin, sea otter Reptiles: Lizard	Inhalation, dermal, prenatal	Natural wildfires
Experimental	Rodents: Mouse, rat Aquatic: Fish, frog	Intratracheal instillation, oropharyngeal aspiration, inhalation (nose-only and whole-body), water (aquatic respiration)	Natural wildfires (real-time) samples, water samples from areas affected by wildfire events (e.g., streams, rivers, lakes)] prescribed burns, simulated wildfires (e.g., components such as wood, plants, biomass smoke extract concentrate)

Wood smoke studies in animals included all experimental studies with variations in the species studied and the exposure materials. Wood smoke was created in experimental conditions through various methods of combustion and wood types. The wood smoke studies can be grouped into two categories by exposure length: acute/short-term and subchronic/chronic. The route of exposure was via the respiratory tract/lungs for all studies, through either inhalation of the whole smoke, or instillation/aspiration of smoke constituents from condensates or filter extracts (varying also in extraction methods). Table 3-6 shows an overview of study characteristics.

Table 3-6. Overview of woodsmoke studies in exposed animals

Study design	Species	Route	Exposure
Acute/short-term [Evaluated respiratory tract and systemic effects]	Rodents: Guinea pig, mouse, rat Rabbits	Inhalation, intratracheal instillation, oropharyngeal aspiration, oral gavage	Wood smoke, smoke extracts, fractionated smoke constituents; wood smoke conditions (e.g., wood type, combustion method, wood stoves)
Subchronic/chronic [Evaluated respiratory tract and systemic effects]	Rodents: Guinea pig, mouse, rat Other mammals: Pigs, sheep	Inhalation	Wood smoke

Study Informativeness

Table 3-7 provides the relevant study questions and guidance, organized by study domain, for evaluating mechanistic studies of wood smoke and wildfire exposure in experimental animals.

Studies in experimental animals should have adequate reporting. For example, inhalation studies should describe atmosphere generation method, exposure chamber type (whole-body, nose-only, head-only), and ideally include information on wildfire or wood smoke characteristics (chemical composition and particle size distribution). Ideally, observational studies should describe the exposure atmosphere concentrations measured at the site of exposure, and verification of smoke components in the air (e.g., smoke plume maps, chemical tracers of biomass burning such as measuring levoglucosan).

Table 3-7. Study informativeness of exposure studies: Influential questions and issues

Question	Comments	Judgements
	Study Design	
Is there concern that the methods by which animals were randomized to groups were inadequate or that animals were not randomized?	Ideally, the randomization method should be reported and based on ensuring that all animals have an equal probability of being assigned to any given control or experimental group.	***Minor concern*** Animals were randomized and adequate vehicle concurrent controls were used for experimental studies, or the animal served as its own control at a previous time point for observational studies
Is there concern that the concurrent control group/sample is not adequate for evaluating effects across treatment groups?	Concurrent controls are the most relevant comparison group for evaluating potential exposure-related biological effects. Ideally, the concurrent control group included at least as many animals as did each treatment group. In the case of instillation or aspiration exposures, if a vehicle other than water is used, a negative control group (vehicle control) should also be included to evaluate the effect of the vehicle on normal lung processes such as inflammation and clearance. In the case of observational studies, where concurrent controls are not used, ideally the exposed animals serve as their own control at a previous time point. The findings may be biased if a later timepoint is used as control samples as they may represent effects from previous exposure (depending on time since last exposure).	***Some concern*** For experimental studies, controls may not be vehicle or may have underlying exposure to wood smoke or wildfire. For observation study, the separate group serves as control at a later time point. ***Major concern*** For experimental studies, there is evidence suggestion that the animals may not be randomized or non-concurrent controls. In observation studies, the unexposed controls may differ from the exposed controls for factors other than exposures. ***Critical concern*** Critical concerns about any bias.

Question	Comments	Judgements
	Exposure Conditions	
Is there concern that the testing exposure or exposure proxy does not reflect the exposure of interest?	The exposure method used should be appropriate for assessing inhalation exposure which is the most physiologically relevant exposure route in humans and animals. Wildfire and wood smoke are complex mixtures consisting of gases, vapors, and particulate components which interact with the respiratory tract system during inhalation exposure occurring naturally (e.g., wildfire). Ideally the exposure should model realistic inhalation exposures of whole smoke (aerosol including gaseous/volatile components and solid particulate matter), with normal exposure/deposition patterns throughout the respiratory tract. Extraction of smoke in a liquid lowers the sensitivity in the exposure to only soluble fractions. In water-based exposures (e.g., aqueous environments or filter extractions), the chemistry is altered biasing towards water-soluble fractions leading to lower sensitivity of negative results where lack of an effect may be due to lack of certain exposure constituents. However, positive findings in these studies may be informative. For observational studies, ideally the exposure atmosphere concentrations should be measured at the site of exposure, and verification of smoke components in the air (e.g., smoke plume maps, chemical tracers of biomass burning such as measuring levoglucosan).	***Minor concern*** Inhalation exposure of wood smoke generated in laboratory settings or from a natural exposure during a wildfire event. No or minimal concerns of excessive toxicity as indicated by significant body weight or survival. ***Some concern*** Inhalation of extracted filter-collected wildfire or wood smoke particulate matter. Resuspension and instillation or aspiration of extracted filter-collected wildfire or wood smoke particulate matter following ideal exposure conditions. Inhalation exposures following less than ideal methods resulting in some concern for bias. Significant treatment related decreases in body weight or survival or other toxic effects ***Major concern*** Low confidence that the exposure contains wood smoke or wildfire smoke. Includes major concerns in sample preparations or filter extraction methods. This study may still be informative, but caution should be taken in interpretation. Substantial treatment related decreases in body weight survival or non-neoplastic lesions.

Question	Comments	Judgements
	Exposure Conditions (cont'd)	
Is there concern that the dose levels were too high to attribute specific effects to the substance?	The high dose but not excess toxicity, for the duration of the study. For intratracheal instillation or oropharyngeal aspiration exposures, the dose volume should be less than 1 mL/kg-bw to limit lung burden of test article. Dose concentration is less concerning in inhalation and water exposures as they reflect the levels found in real-world scenarios of wood smoke and wildfire exposure.	
Is there concern that the exposure conditions may have altered the exposure leading to bias or decreased sensitivity?	For experimental studies, concentration of exposure atmosphere should be sampled and measured, ideally within the animals breathing zone. Ideally, isokinetic sampling methods will be used to preserve the characteristics of the aerosol that the animals are exposed to. The exposure atmosphere concentration should be stable to within 20% variability for aerosols and 10% variability for gases/vapors, following the Organisation for Economic Co-operation and Development (OECD) guidelines.	

Question	Comments	Judgements
	Sensitivity: Model and exposure	
Is there concern that the study design and exposure is sensitive enough to adequately detect a neoplastic effect if present? This question considers • • Animal model • • Statistical power (number of animals/group) • • Study duration • • Exposure regimen: duration and dose	Sensitivity rating integrates the animal model, statistical power, study design, and exposure conditions. In some cases, one factor may compensate for limitations in (e.g., a sensitive animal model may offset a smaller sample size). Ideally, studies would include 5 animals of each sex. However, single sex studies may be acceptable if there are no sex-specific differences for the endpoint being measured. Ideally, exposure levels should induce tolerable toxicity (e.g., slightly decreased survival and/or body weight gain) at the high dose.	***Minor concerns*** Experimental studies: Animal strains/stocks of at least intermediate sensitivity using conditions close to the ideal conditions (defined in guidance), adequate numbers of animals with appropriate exposure dose and duration. Ideally, there should be data from positive controls confirming the dose is adequate. Observational studies: Sufficient statistical power to detect an effect, and good exposure contrast and duration.
Observational Does the study have adequate sensitivity to detect an effect from exposure (if present)?	Sensitivity considers statistical power, exposure contrast, and relevance of the exposure metric.	***Some concerns*** Studies may have less than ideal conditions for some aspects of the study design or exposure conditions (e.g., duration, dose, number of animals) but not all. ***Major concerns*** Insensitive model or inadequate conditions for the animal model (see guidance).

Question	Comments	Judgements
Outcome		
Is there concern that the endpoint proxy (e.g., biomarker) did not represent the effect of interest? Is there concern that the biomarker was not measured in a relevant tissue or cells?	Ideally studies should measure the most relevant biomarkers related to the biological effect or KCCs in tissue or cells. See Appendix B for information on assays and biomarkers. Biomarkers in local tissues are more informative that systematic biomarkers. Examples of relevant endpoint proxies for inflammation evaluate infiltration of immune/inflammatory cells into the target tissue(s), as well as related mediators including cytokines and chemokines responsible for immune cell activation and migration. A variety of biomarkers can indicate oxidative stress including the products (i.e., oxidative damage to DNA, lipid peroxidation) and changes to antioxidant capacity (i.e., levels of glutathione and associated enzymes, catalase).	***Minor concern*** Appropriate biomarkers are relevant measures of the KCCs and are assessed using ideal methods in the appropriate target tissue(s) and using the most relevant assays. ***Some concern*** Less than ideal methods are used to assess the biomarker of interest. Assay not as sensitive for the biomarker of interest. ***Major concern*** Biomarkers assessed do not represent the effect of interest and/or are not measured in a relevant tissue/cell type. Measurement methods are not blinded/randomized or are inappropriate for the biomarker being assessed or are measured using an assay that is not specific or sensitive for the biomarker.

Question	Comments	Judgements
	Outcome (cont'd)	
Measurement methods Is there concern that measurement methods (e.g., timepoints, accuracy, precision) are not adequate to generate valid and reliable data? The measurement methods consider consistency, replication, and sampling (collection methods and timing).	Ideally, each study should use accurate and reliable methods for measuring each endpoint at the appropriate time point and should follow applicable protocols and guidelines. For some biological effects, there are OECD guidelines. Methods of blood collection should be performed to minimize contamination or infection as these would bias interpretation of immune responses. The timing of sample collection is important to determine if an observed effect is transient or persistent. Oxidative stress biomarkers can be transient with persistence from minutes to days. The length of the exposure period as well as the timing of sample collection should both be considered in the determination of chronic responses. The study should include enough replicates or repetitions to generate reliable results for the endpoint of interest and without any serious uncertainties or limitations in the sampling process. *See Appendix B for endpoint/KCC specific guidance*	Judgements same as above
Differential measurement error Is there concern for differential measurement error, e.g., the treated and control groups were assessed differently?	All treatment and control groups should follow the same protocols for endpoint assessment and should be clearly reported to properly interpret results. Blinding and randomization should be used during endpoint assessment.	
Sensitivity questions Is there concern that the assay or methods were not sensitive enough to detect an effect?	Ideally, appropriate and sensitive assays (e.g., current state-of-the-science assays with sufficient counts, such as the number of cells) should be used to measure biological effects (e.g., KCCs) in the target tissue or tissue of interest.	

3.3. Evidence Evaluation and Integration

As mentioned in Section 3.1, the influential questions in our evaluation of wood smoke and wildfire relate to evaluating the confidence in the evidence for the following biological effects related to KCCs 2, 5, and 6. We will also evaluate whether there is evidence for associations between the KCCs.

1. Genotoxicity
2. Oxidative stress
3. Chronic inflammation and immunomodulation

3.3.1. Evidence Evaluation

Study findings are interpreted considering study sensitivity and the direction of any potential biases and external validity when relevant.

External Validity

External validity relates to the generalizability of a study to populations and situations other than those included in the original study. External validity includes the relevance of the chosen animal model and outcome endpoints to human exposures and responses. Studies using mammalian models have minimal concern of issues regarding external validity of exposure and mechanistic outcomes. Selection of non-mammalian animal models may have limited applicability to conclusions related to human health. For example, invertebrates, such as bivalves, lack adaptive immune systems and have different mechanisms of innate immunity than seen in humans and other mammals, and therefore have been excluded due to this lack of external validity (Allam and Raftos 2015). Fish, however, do have more comparable immune systems to mammals to support their use as a model for inflammatory responses (Magadan et al. 2015), however these studies are indirect evidence of the responses observed from inhalation as their exposure route differs greatly.

The route of exposure can affect external validity. As wood smoke exposure in humans is mainly through inhalation into the respiratory tract/lungs, model organisms whose main exposure route is through water and gills (i.e., not through the inhalation of air) such as fish, have limited generalizability as differences in their anatomy alter exposure.

- ***No/minor concern***: Mammalian model is used for experiments.
- ***Some concern:*** Fish model is used for experiments.
- ***Major concern:*** Invertebrate models are used for experiments.

Study Level Judgment

For the animal studies (and human if the data permit) measuring multiple biomarkers, we plan to provide a study level judgment for the evidence of chronic inflammation (KCC 6) using the following guidance. This approach prioritizes biomarkers that measure direct effects in target respiratory tract tissues. Direct evidence includes measuring primary inflammatory mediators and observing histopathological changes directly within the

affected tissues. In contrast, indirect evidence represents downstream effects and secondary responses to the inflammatory process, including systemic effects.

Examples of direct evidence

- Immune cell function (phagocytosis, antigen presentation, antibody production, bacterial clearance)
- Local infiltration of acute inflammatory cells (macrophages, neutrophils, lymphocytes, and eosinophils) AND increases in local cytokines (e.g., IL-1 beta, TNF-alpha, IL-6, IL-8, IFN-gamma) OR chemokines (e.g., IL-8, MCP1, MIP1α, KC) at the protein or gene level
- MCP1/MIP1α AND increased macrophage number
- KC AND increased neutrophil or macrophage number

Examples of indirect evidence

- Local MMPs AND local increase in immune cells
- Local COX-2 AND local increase in immune cells

3.3.2. Evidence Integration

We use a three-step process for evaluating the evidence and reaching a conclusion regarding the level of evidence for carcinogenicity from mechanistic studies.

Step 1 Assess the evidence from each study set for each biological effect

Step 2 Assess the confidence of the evidence for each biological effect

- Integrate the evidence across each study/data set assessment

Step 3 Assess the level of evidence (convincing, supportive)

Step 1: Evaluating the Evidence of Each Study Set

For each study set, we plan to consider the following factors:

- Consistency of the evidence across studies for individual biomarkers and study level judgments (see below)
- Strength of the association, such as magnitude of the effect and exposure-response relationships
- Study informativeness (i.e., internal validity, sensitivity)
    - The target tissues of concern from wood smoke/wildfire exposure are the respiratory tract/lungs. Therefore, the endpoints are ideally assessed for local effects to the lung tissue or resident/recruited cells. Systemic effects as measured in the circulating blood can be informative, however they may be altered by changes in the recruitment of immune cells out of the circulation and into target tissues rather than as a biomarker of systemic inflammation
    - Relevance (e.g., exposure dose and duration, timing of sample collection) of the exposure and measurement of the biological effect

- The assessment uses a triangulation approach, which integrates evidence from different study designs and methods, as well as different sources of potential biases, to reach conclusions about consistency

An advantage of the animal mechanistic studies is that they measured multiple oxidative stress and immune/inflammation biomarkers in respiratory tract (local) and systemic tissues, whereas only a few studies in exposed humans (primarily the volunteer studies of exposure to wood smoke) measured biomarkers in respiratory tract tissues.

Step 2: Assess the Confidence of the Evidence: Biological Effects

For each influential question (Box 3-2), we integrate the assessments across study sets, using triangulation approaches, to reach a confidence judgment based on the guidance outline in Table 3-8. KCC-specific informativeness guidance for genotoxicity (KCC 2), oxidative stress (KCC 5), chronic inflammation and immune activation (KCC 6), and immunosuppression (KCC 7) are presented in Table 3-9. Key considerations are the consistency and coherence for both the effect and the biomarkers used to measure the effect/KCC, the overall quality of the collective evidence across study sets, and the informativeness of the biomarkers.

Table 3-8. Confidence judgment for biological effect

Descriptor	Biological Effect (Including Connection between Effects)
High	*Individual biological effect (KCC)*
	Consistent evidence of informative KCC biomarkers/indicators across studies of sufficient quality and ideally in more than one evidence type
	Coherence across individual KCC biomarkers/indicators
	Relationships between KCCs or or multi-omics data
	Consistent evidence across studies for an association between two or more biological effect biomarkers/indicators
Moderate/Limited	Less than consistent evidence across studies, KCC biomarkers/indicators, and evidence types
	Consistent evidence for less relevant KCC biomarkers/indicators or less biological or human-relevant evidence[a] types, or fewer number of studies
Inadequate	Negative or unexplained inconsistent findings
	Few data or limited study informativeness
	Evidence mainly from evidence types with low biological or human relevance for the substance and specific biological effect under evaluation[a]
	KCC biomarkers/indicators are of limited informativeness for the biological effect and cancer development

[a]Biological and human relevance are evaluated on a case-by-case basis, e.g., some non-mammalian in vivo assays may be relevant for some but not other KCCs, and some species may be more relevant than others.

Table 3-9. KCC specific informativeness guidance

KCC	Ideal evidence for high confidence	Other evidence for high confidence: Combination of biomarkers
KCC2: Is genotoxic	Positive findings for ≥1 endpoint in exposed animals or humans • Mutations • Chromosomal damage: CA or MN (indicative of CA and changes in chromosome number)	DNA damage: strand breaks in target tissues of exposed humans or animals Mutations or chromosomal damage: in vitro studies using bacteria or eukaryotic cells
KCC5: Induces oxidative stress	Several oxidative stress biomarkers (pro- and anti-oxidative stress) • Integrative score • Pro-and anti-oxidative stress biomarkers in the same studies	One or more clinically relevant oxidative stress biomarker(s), such as F2-isoP and oxidative damage to DNA *And* Link with other KCCs (e.g., KCC2, KCC6)
KCC6: Induces chronic inflammation or immune activation	Evidence of chronic inflammatory (e.g., autoimmune) diseases in exposed humans or animals Histological evidence of chronic cellular inflammation in target tissues (WBC- e.g., lymphocytes) with local increases in proinflammatory biomarkers (e.g., specific cytokines or chemokines) in exposed humans or animals • Ideally, the biomarkers are associated with cancer and are identified in the context of evidence of chronic exposure (or repeated acute exposures)	Histological evidence of chronic cellular inflammation in non-target tissues with local increases in proinflammatory biomarkers in exposed humans or animals Systemic increases in acute (with evidence of chronic exposure) or chronic inflammatory cells or proinflammatory biomarkers (e.g., cytokines, chemokines, or acute phase proteins) in circulation • Ideally, the biomarkers are associated with cancer and are identified in the context of evidence of chronic exposure (or repeated acute exposures) • Systemic inflammation may be more informative for blood cancers compared to solid tumors
KCC7: Is immunosuppressive	Evidence of increased viral infections in exposed humans or animals Impaired immune function, such as decreased antibody responses (e.g., vaccine-induced), NK, CTL, and T cell activation/activity in exposed humans or animals	Evidence of increased non-viral infections in exposed humans or animals and supporting evidence (e.g., alterations in immune components or organs) Severe decreases in WBC/leukocyte subsets and supporting evidence (e.g., decreased cytokines)

Step 3: Assessing the Level of Evidence

The LoE conclusion of carcinogenicity from mechanistic evidence is reached by applying the RoC listing criteria to the body of literature. The final step integrates the confidence judgments across the influential mechanistic questions on genotoxicity, oxidative stress, and immune/inflammation-related changes. The review also summarizes the data that were not considered to be influential. The LoE of carcinogenicity from mechanistic studies is integrated with the LoE of carcinogenicity from cancer studies in humans and

animals to reach a cancer hazard conclusion (i.e., listing recommendation) (see Section 3).

Information on connections, sequence, and timing between genotoxicity, oxidative stress, and immune changes may help identify substance-specific biological pathways and increase the certainty of the evidence. Guidance for reaching LoE conclusions is available in Table 3-10.

Table 3-10. Level of evidence guidelines

RoC criterion	Convincing	Supporting
Plausibility: Biological effects KCCs or other relevant effects, including metabolism Connectivity between KCCs	Consistency and coherence in the database (including mechanistic data, toxicokinetics) AND High confidence for one or more *informative* biological effects (KCCs) considering the following: • Number, sequence, connections between KCCs leading to the development of a biological pathway • Specificity and informativeness of KCC biomarkers • Supporting cancer-related data • Suppression of biological effect led to suppression of tumor development • Biological effect measured in cancer studies for the substance • Biological effect measured in target tissue(s)/cancer site(s) of interest	High or moderate confidence for *informative* KCCs • High confidence requires a greater number and specificity of the KCCs • Moderate confidence may be reached by a single specific KCC or several less specific KCCs

LoE of carcinogenicity from mechanistic studies is integrated with the LoE of carcinogenicity from cancer studies in humans and animals to reach a cancer hazard conclusion (i.e., listing recommendation) (see Section 3).

4. Sufficient similarity comparison between wood smoke and wildfire smoke

Introduction

As discussed in other sections of the protocol (see Section 2 for animal cancer studies, Section 3 for mechanistic studies, and the published protocol for human cancer studies [NTP 2022c]), there is an adequate database to evaluate the potential carcinogenicity of woodsmoke; however, the database of cancer studies for wildfires is limited. The wildfire database mainly consists of mechanistic studies for limited biological effects; no animal cancer studies were found, and the human cancer studies were inadequate to evaluate a specific type of cancer. Since wood is the main component of most wildfires, we plan to conduct a sufficient similarity comparison of the chemical components of wood smoke and wildfire. Complex mixtures are sufficiently similar when they display limited differences in chemical composition and biological activity, so that data from the reference mixture(s) can be used to make conclusions about the mixture(s) lacking toxicity data (Catlin et al. 2018; EPA 2000; Marshall et al. 2013; Rager and Rider 2023). We aim to explore whether specific chemical levels of wood smoke mixtures (adequate database for a cancer assessment) are similar enough to those in wildfire (limited database for a cancer assessment). If so, the cancer hazard conclusion for wood smoke could serve as a proxy for wildfire, considering any uncertainty for less direct evidence. We expect the chemical composition of each type of exposure to vary by source (different types of wood stoves, different wildfires). Thus, we are hypothesizing that the constituent variability across different wood smoke samples will be greater than the variability between wood smoke and wildfire samples. Wood smoke samples will serve as the reference mixtures used in this analysis; however, we will also use other known toxic mixtures that do not involve wood burning, diesel exhaust and traffic-related air pollution (TRAP). We posit that the chemical composition in wildfire mixtures will be more like that in wood smoke mixtures than diesel and traffic related air pollution (TRAP) mixtures.

4.1. Developing the Framework

The planned strategy for the sufficient similarity analysis consists of the following steps:

1. Identify studies reporting on chemical components for the four mixtures: wildfire, wood, TRAP, and diesel
2. Extract data (e.g., concentrations) on selected chemical components
3. Conduct chemical similarity analyses using multiple statistical approaches (see Section 4.3)
4. Integrate the findings with mechanistic studies of wildfire (see Section 4)

Figure 4-1 depicts the interpretation of various scenarios from the analyses.

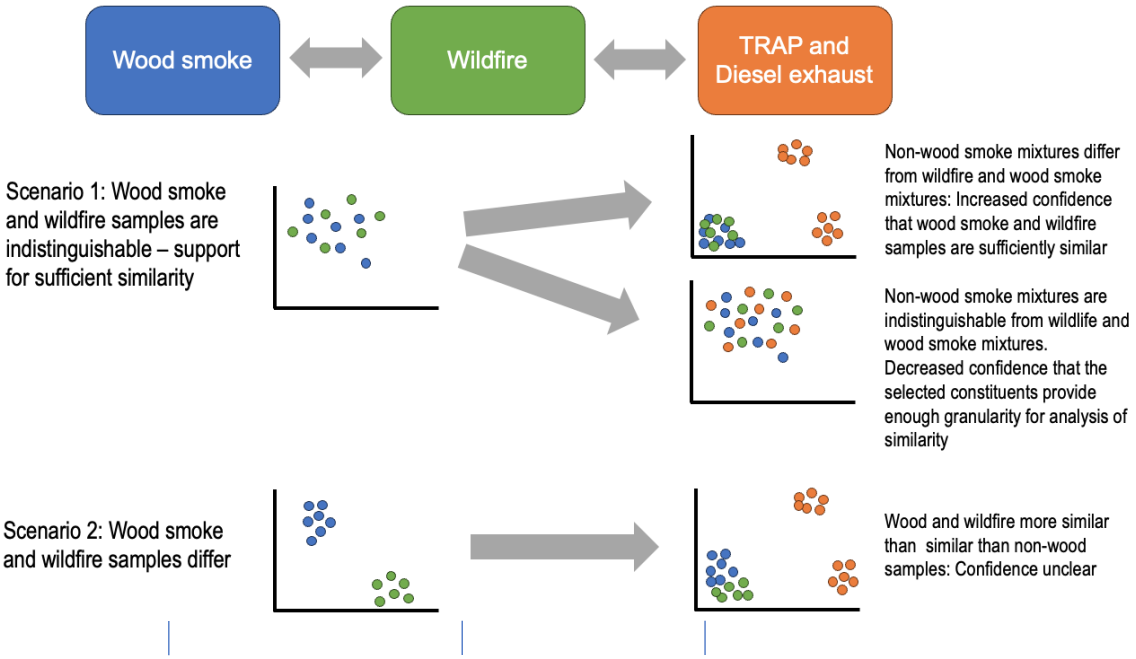

Figure 4-1. Interpretation of sufficient similarity analysis

This schematic shows different scenarios for the chemical sufficient-similarity approach. Chemical component data from wildfire, wood smoke, and non-wood smoke studies will be analyzed to determine if there are sufficient similarities across wood-related exposure groups but not with non-wood mixtures. If so, the wood smoke hazard cancer hazard conclusion can be used in the wildfire hazard assessment.

4.1.1. Identifying the Literature

Chemical component studies for wood smoke and wildfire were initially identified from the preliminary data extraction of the mechanistic articles (Figure 4-1). Sections 3.1.1 and 3.1.2 describe the literatures searches and evidence mapping for mechanistic studies of wood smoke and wildfire. Briefly, biomedical citation databases (PubMed, Scopus, and Web of Science) were searched for mechanistic literature as described in section 3.1.1 of the protocol using terms for exposure (wood smoke or wildfire) and key characteristics of carcinogens (See also Appendix A and the RoC search terms document). As part of the evidence mapping, information on whether components of wood smoke or wildfire (PAHs, metals, volatile organic compounds [VOCs], and carbonyls [compounds with a C-O functional group)) were measured or reported in a study was extracted (Figure 4-2).

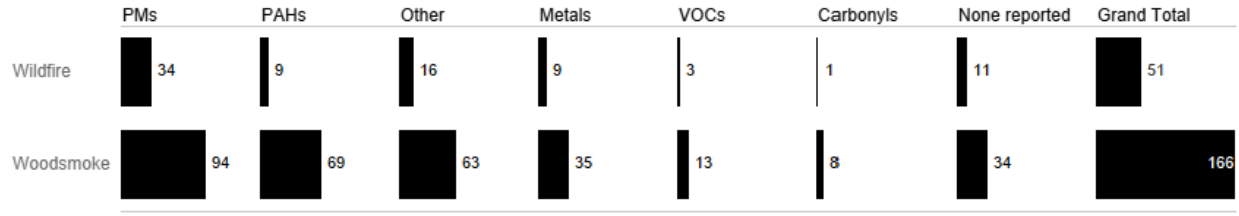

Figure 4-2. Number of articles with chemical components measured or reported in wood smoke or wildfire mechanistic studies

PM = particulate matter, PAHs = polycyclic aromatic hydrocarbons, VOCs = volatile organic chemicals.
The figure shows the number of studies reporting on specific chemical components or particles (columns) for each type of exposure, wildfire and woodsmoke (rows). The availability of component data was extracted from mechanistic studies of wood smoke and wildfire, and is included as part of the evidence mapping in tableau. Not all studies that reported components provided concentration data.

The most commonly reported woodsmoke components were PAHs, metals, VOCs, and carbonyls. The other components column in Figure 4-2 includes the number of articles reporting on all other chemical classes. No wildfire studies were found that reported individual carbonyls, so only studies reporting PAHs, metals, and VOCs will be considered for the analysis.

Enough wood smoke studies (n = 45) reporting concentration data for chemical components were identified from the mechanistic studies searches, however, only a few of the wildfire studies (n=7) reporting on the presence of chemical components also provided concentration data. Therefore, targeted searches for studies on wildfire components were conducted. These searches and the searches for component concentration data for the other comparison groups are described in below.

Targeted wildfire component study searches

Supplemental searches for wildfire component articles combined chemical and component terms for PAHs, metals, and VOCs with our wildfire exposure terms (See Table 4-1, and appendix A). Targeted searches were conducted in PubMed and Web of Science.

Table 4-1. Targeted search for wildfire component studies

Wildfire exposure terms	Chemical component and composition terms
((wetland*) and (fire*)) OR ((wildland*) and (fire*)) OR (wildfire*) OR (forest AND fire*) OR (landscape AND fire*)	((chemical AND (component* OR characteristic* OR mixture* OR composition*)) OR ((((PAH OR PAHs OR metal* OR "trace elements" OR "polycyclic aromatic hydrocarbon*"))) OR ("volatile organic chemical*" OR VOC OR VOCs)))

Wildfire exposure terms and chemical component terms were combined with "AND"

Non-wood comparison group component searches

To identify concentration data for TRAP and diesel exhaust, we searched a combination of authoritative data and published articles. Two authoritative sources were identified, the first was TRAP data from air monitoring stations near or on major roads in several US states identified

from EPA (https://aqs.epa.gov/aqsweb/airdata/download_files.html). These data reported metal concentrations over time. Diesel exhaust concentration data was identified for PAHs from the reports on the standard diesel samples available from the National Institute of Standards and Technology (NIST).

To supplement these sources, searches were done using the chemical component and composition terms in table 4-1combined with the terms "diesel" and "traffic related".

4.1.2. Selection of studies for consideration and review

Wildfire and wood smoke

Studies were selected based on the following inclusion and exclusion criteria:

Inclusion criteria

- Study reported concentrations of components either by mass (e.g., mg/kg) or by volume (e.g., $\mu g/m^3$) or reports data that can be converted into similar units.
- Reports component concentration information on at least 5 PAHs, 5 metals, or 5 VOCs
    - Five chemicals or elements per report was chosen to ensure there would be enough variety and overlap between articles in the reported chemicals or elements to do a comparison
- Reports component concentration information from direct sources of smoke (ash, smoke, or PM)
- Wildfire studies report components for a defined wildfire event

Exclusion criteria

- Report component information from indirect sources of smoke (e.g. water runoff)
- Report components from the breathing zone of wildfire fighters (e.g., in the equipment or respirator air)
- Report smoke or components transported during a wildfire season over a large area, or an undefined period of time after a wildfire
- Report component information as fractionation of particulate matter extracts
- Report peat as the fuel being burned

After applying these inclusion and exclusion criteria, a total of 104 unique articles for wood smoke and wildfire components were selected for data extraction (see Table 4-2).

TRAP and Diesel Articles

Articles were screened and included if they met the inclusion criteria below. The goal was to identify at least three articles or data sources for each exposure/chemical/unit combination. See table 4-2.

Inclusion criteria

- Study reported concentrations of components either by mass (e.g., mg/kg) or by volume (e.g., $\mu g/m^3$) or reports data that can be converted into similar units.

- Reports component concentration information on at least 5 PAHs, 5 metals, or 5 VOCs

Exclusion criteria

- Report component information as fractionation of particulate matter extracts

After applying these inclusion and exclusion criteria, a total of 37 unique TRAP and diesel exhaust articles/reports were selected for data extraction (see Table 4-2).

Table 4-2. Total articles by component type

Exposure	PAH articles	Metal articles	VOC articles
Wood Smoke (59 unique articles)	32	30	6
Wildfire (45 unique articles)	19	28	9
Traffic-related air pollution (18 unique articles)	11	7	5
Diesel exhaust (19 unique articles)	9	11	2

Studies were included for data extraction if they reported on at least 5 PAHs, metals, or VOCs

4.2. Quantitative Data Extraction

Data will be extracted from each identified wood smoke or wildfire component study and include general characteristics of the wood smoke or wildfire smoke reported, the units reported for each component type, and the concentrations of each reported component. The type of data extracted is detailed in Table 4-3. A second researcher will review the data entry and QA the data extraction. Any discrepancies will be resolved through discussion. Prior to data analysis, all component concentration values will be converted to common units for analysis.

Table 4-3. Type of information and quantitative data extracted

WS or WF	Type of information extracted
Wood smoke	• Source of wood smoke • Type of fuel burned • Combustion conditions • Sample matrix and origin (e.g., PM from smoke) • PM size (if applicable) • Units and concentrations of individual PAHs, metals, or VOCs

WS or WF	Type of information extracted
Wildfire	• Type of wildfire (natural or prescribed) • Urban or wooded wildfire • Sample matrix and origin • Units and concentrations of individual PAHs, metals, or VOCs
TRAP	• Source of TRAP data • Country • Units and concentrations of individual PAHs, metals, or VOCs
Diesel	• Source of diesel data • Country • Units and concentrations of individual PAHs, metals, or VOCs

4.3. Data Analysis

A comparison of the complex mixtures of chemicals/elements from wood smoke and wildfires will be conducted using quantitative concentration data for PAHs, metals, and VOCs extracted from the literature. Analyses on the TRAP and diesel exhaust data will be conducted in the same manner. Values for individual mixture constituents will be input into JMP software (version 16.1.0, SAS, Raleigh, NC).

Multiple clustering approaches will be conducted to add redundancy. Multiple analytical methods providing the same answers will provide additional confidence in the determination of similarity, whereas different answers from each method will decrease confidence in conclusions. These methods vary in the completeness of the data required. In the first method, data will be analyzed using the Ward method for hierarchical clustering using JMP 16.1.0 software (Cary, NC), which organizes samples by minimizing the variance within clusters (Ward 1963). The hierarchical clustering results will be visualized using a constellation diagram of the dendrogram to show the relative distances between samples. We will be inspecting the data to understand whether samples derived from wood smoke cluster together and separately from wildfire samples or if wood smoke and wildfire samples are distributed across clusters. The latter would indicate that wildfire samples are sufficiently similar to wood smoke samples. This method requires a relatively complete dataset for each sample. In effect, only chemicals with approximately 80%of samples reporting values can be included in the analysis. Therefore, analysis will be limited to data sets that are relatively complete.

In the second method, Bayesian principal component analysis (PCA) or projection pursuit PCA, all available extracted data (including incomplete data sets with missing values) will be analyzed. PCA methods reduce the dimensionality of large datasets by identifying the components that capture the most variation in the data. Bayesian PCA optimizes the method by incorporating priors derived from the data (Nounou et al. 2002). The third method, k-means clustering, requires that missing data be imputed. A random forest plot method will be used to provide values for missing data by observing patterns in complete data sets and applying those patterns to fill in

incomplete data sets (Pantanowitz and Marwala 2009). The k-means clustering algorithm forms a designated number of clusters by minimizing within cluster variance (Sinaga and Yang 2020). For each of the methods described above, group comparison statistics (multi-group ANOVA) will be used to evaluate which clusters are significantly different from one another.

5. Evidence Integration

The last step in the cancer hazard evaluation process (Figure 5-1) is to integrate the evidence from the cancer studies in humans and animals with the evidence from mechanistic and other relevant data and apply the Report on Carcinogens (RoC) listing criteria to reach a preliminary listing recommendation. Because wood smoke is part of wildfires, we also integrate information comparing the chemical components across wood smoke and wildfire mixtures (under development).

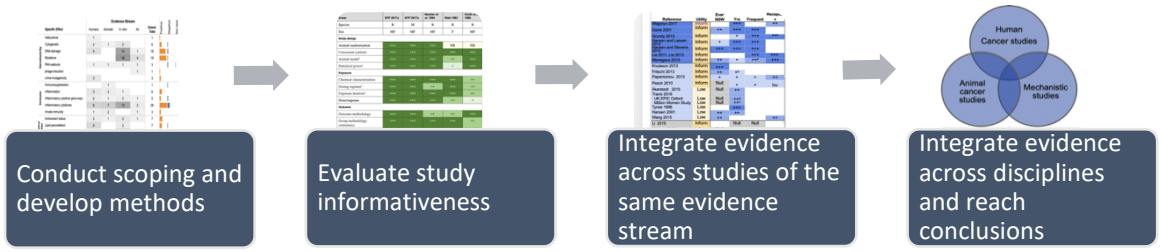

Figure 5-1. Cancer hazard evaluation process

A substance is listed in the RoC as either *known or reasonably anticipated to be a human carcinogen*. The listing recommendation is reached by applying the RoC criteria to the cancer hazard assessment (see Box 5-1). Conclusions regarding the carcinogenicity of a substance are based on scientific judgment, considering all relevant data. The listing categories reflect the strength of confidence in the evidence.

Box 5-1. Summary of RoC listing criteria

Known to be a human carcinogen

Sufficient evidence of carcinogenicity from studies in humans*

Reasonably anticipated to be a human carcinogen
Meets one of the following:

Limited evidence of carcinogenicity from studies in humans*

Sufficient evidence of carcinogenicity from studies in experimental animals

The substance belongs to a structurally related class of substances that are listed in the RoC

Convincing relevant information that the agent acts through a mechanism indicating that it would likely cause cancer in humans

* Evidence from studies in humans includes cancer epidemiology studies and mechanistic studies in exposed humans

Source: (NTP 2022a).

In reaching our listing decisions, we use triangulation approaches, considering Hill's factors to integrate the evidence across evidence streams. Triangulation refers to integrating evidence from different research or methodological approaches, each of which have different but unrelated sources of potential bias (Lawlor et al. 2016). For evidence integration, we collectively consider and adopt the following Hill factors: strength and consistency across evidence streams, biological plausibility and coherence, and temporality. Strong LoE conclusions (e.g., sufficient from human or animal cancer studies, convincing from mechanistic studies) in more than one evidence stream (consistency) increases the strength of the hazard evaluation classification.

5.1. Evaluating the Cohesiveness of the Evidence

To increase transparency and facilitate the overall cancer hazard evaluation, we use the following stepwise approach to integrate the evidence across evidence streams and provide the information in evidence-based tables.

Step 1: Summarize the Assessments of the Collective Evidence for Each Data Stream

The first step in the evidence-integration process brings forward the assessments from each evidence stream, for example, each relevant entry (referred to as study set) in the overall cancer hazard EECO (evidence, exposure, comparison group, outcome/endpoint). Example templates for wood smoke and wildfires are presented below in Table 5-1 and Table 5-2.

Wood smoke

Table 5-1. Template example for summarizing the assessment of each evidence stream

Outcome	Evidence Streams	Strength and Limitations	Assessment
Cancers: lung, nasopharyngeal, esophageal, female breast	Number and type of **human cancer studies** Cohort studies Case-control studies Pooled or meta-analyses	Summary of most influential biases (direction, magnitude, impact) across studies by study design or other relevant grouping	Consistency of findings and patterns for factors, such as exposure matrices and levels, cancer subtypes, effect modifiers
Cancer type or across multiple cancers	Number and type of **animal cancer studies** Animal models (e.g., route, species)	Summary of most influential biases (direction, magnitude, impact) across studies by model, route, or other relevant grouping	Exposure-related cancer sites, common cancer sites across groups of chemicals Information relevant to evidence integration
Biological effect Genotoxicity, oxidative stress, chronic inflammation, immunosuppression	Number and type of **mechanistic studies** Model (e.g., in vitro, in vivo) Exposed humans	Summary of potential biases, types of biomarkers, and relevance of the evidence type	Confidence judgment for influential question(s) Information relevant to evidence integration

Table 5-2. Template example for summarizing the assessment of each evidence stream

Outcome	Evidence Streams	Strength and Limitations	Assessment
Biological effect Genotoxicity, oxidative stress, chronic inflammation, immunosuppression	Number and type of **mechanistic studies** Model (e.g., in vitro, in vivo) Exposed humans	Highlight of most influential biases and strengths across studies	Confidence judgment for influential question(s) Information relevant to evidence integration
Exposure comparison of smoke components	Wildfire and wood smoke mixtures	N/A	Sufficient similarity conclusions

N/A = not applicable

Step 2: Integrate the Evidence Across All Streams

The final step in the assessment is to integrate all the relevant evidence and apply the RoC listing criteria to this assessment to reach a listing decision. Here we present the evidence integration assessment, and Section 5.2 discusses applying the RoC listing criteria to that assessment. The overall cancer hazard evaluation uses triangulation approaches for integrating and assessing the coherence of the cancer (human and animal) and mechanistic assessments. Triangulation approaches for the overall evidence evaluation consider biases for a collective body of evidence. For example, human cancer studies are the most relevant studies but can be subjected to biases both toward and away from the null due to their observational nature. Animal cancer studies are controlled exposure but are less human relevant, and mechanistic studies are not on the apical endpoint of interest (e.g., tumors). The wood smoke and wildfire assessments are captured in evidence-based tables.

5.2. Integrating Level of Evidence Conclusions

Table 5-3 delineates how LoE from each evidence stream relates to each RoC criterion. The overall listing recommendation also considers the cohesiveness of the body of evidence and all relevant information, as discussed in Section 5.1.

Table 5-3. Evidence integration guidance table[a]

RoC Criterion	Human Cancer Epidemiology	Animal Cancer	Mechanisms: Overall	Mechanisms: Exposed Humans	Listing[b]
Sufficient Evidence from Studies in Humans	Sufficient	Any[c]	Any[c]	Any[c]	Known
	Limited	Any[c]	Supporting[d]	Robust	Known
Limited Evidence from Studies in Humans	Limited	Any[c]	Any[c]	Not robust	RAHC
	Inadequate	Not sufficient	Convincing	Robust	RAHC

RoC Criterion	Human Cancer Epidemiology	Animal Cancer	Mechanisms: Overall	Mechanisms: Exposed Humans	Listing[b]
Sufficient Evidence from Studies in Experimental Animals	Inadequate	Sufficient	Any[c]	Any[c]	RAHC
Biological Plausibility[e] or Member of a Listed Class	Inadequate	Not sufficient	Convincing	Any[c]	RAHC

RAHC = reasonably anticipated to be a human carcinogen.
[a]Descriptors based on the RoC listing criteria and convention. Human cancer studies: sufficient, limited, inadequate animal cancer studies: sufficient, not sufficient; mechanism: convincing, supporting.
[b]Also considers the coherence of the database.
[c]Any indicates that the LoE for the evidence stream does not affect the cancer hazard conclusion (e.g., for animal cancer studies, it could be sufficient or not sufficient).
[d]The LoE from mechanistic data depends in part on the human cancer epidemiology studies (e.g., limited evidence can range from bordering inadequate to bordering sufficient, and a similar range could be made for the strength of evidence from human mechanistic studies).
[e]Convincing can be from a mode of action, biological effects, or cancer predictions from clustering/read-across approaches.

For wildfires, we will also integrate with the mechanistic evidence the sufficient similarity analyses comparing chemical components of wildfire mixtures (exposure of interest) to wood smoke mixtures (reference mixtures with an adequate database to evaluate potential carcinogenicity) (see Section 3). If there is sufficient similarity, i.e., the specific chemical levels and composition of wood smoke mixtures (adequate database for a cancer assessment) are similar enough to those in wildfire (limited database for a cancer assessment) then the cancer hazard conclusion for wood smoke can serve as a proxy for wildfire, considering uncertainty for less direct evidence. The level of evidence conclusions of carcinogenicity from the mechanistic evidence can help define uncertainty.

The monograph will provide the preliminary listing recommendation (known, reasonably anticipated or not to list) for wood smoke and wildfire and the rationale to support the recommendation.

6. Public Health Information

The monograph will provide information on regulations, interventions, health disparities, and sensitive populations (such as life stages) as part of the overall cancer hazard evaluation and other relevant media (Lunn et al. 2022).

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Appendix A. Search Terms and Evaluation Team Responsibilities

A.1. Evaluation team:

Evaluation teams are composed of federal staff and contractor staff. Procedures are in place to avoid actual or perceived conflicts of interest. Members of the evaluation team have experience or training in conducting literature searches and/or evaluating occupational and environmental epidemiology studies.

A.1.1. Project Leader

Develops research concept, rationale, and framework; serves as a researcher

- Ruth M. Lunn, DrPH, NIEHS
- Whitney Arroyave, PhD, ILS – an Inotiv Company

A.1.2. Team Members

Develop search terms, conduct literature searches, and manage literature (e.g., endnote libraries, HAWC uploads)

NIEHS	ILS – an Inotiv Company
Suril Mehta, PhD; Mechanistic and human cancer studies	Danila, Cuomo, PhD, Mechanistic studies
Cynthia Rider PhD: Sufficient similarity	Rachael Kalsch, MLIS, Librarian
	Alton Peters, MS, Sufficient similarity, human exposure
	Mona Sethi, PhD, mechanistic and animal studies
	Tracy Saunders, BS, document preparation and data visualization

Former members: Stanly Atwood, MS, Andrew Evens, PhD, Rebecca Arechavala

A.1.3. Protocol Peer Reviewers

William Gwinn, PhD., NIEHS

Esra Mutlu, PhD, EPA

Cynthia Rider, PhD, NIEHS (did not review sufficient similarity)

Kirsten Ryan, PhD, NIEHS (sufficient similarity)

Wood Smoke Animal Cancer and Mechanism Studies Search Terms

Database	Search String
Pubmed	("wood carboniz*"[Title/Abstract] OR "carbonized wood"[Title/Abstract] OR "collier*"[Title/Abstract] OR ("fires"[MeSH Terms] OR ("wood smoke*"[Title/Abstract] OR "woodsmoke"[Title/Abstract] OR "wood fired"[Title/Abstract] OR "wood burning*"[Title/Abstract] OR "burning wood"[Title/Abstract] OR "wood stove*"[Title/Abstract] OR "woodstove*"[Title/Abstract]))) OR ("biomass fired"[Title/Abstract] OR "biomass stove*"[Title/Abstract] OR "burn biomass"[Title/Abstract] OR "burning biomass"[Title/Abstract] OR "biomass fuel*"[Title/Abstract] OR "biomass cook*"[Title/Abstract])) OR ("cookstove*"[Title/Abstract] OR "cooking/instrumentation"[MeSH Terms] OR "cooking stove*"[Title/Abstract] OR "cook stove*"[Title/Abstract]) OR ("cooking"[MeSH Terms] OR "cook*"[Title/Abstract] OR ("heating"[MeSH Terms] OR "heat*"[Title/Abstract]))) AND ("air pollut*"[Title/Abstract] OR "air pollutants/adverse effects"[MeSH Terms] OR ("smoke"[Title/Abstract] OR "smoky"[Title/Abstract] OR "smoke"[MeSH Terms]) OR ("wood"[Title/Abstract] OR "biomass"[Title/Abstract] OR "fuel*"[Title/Abstract])) OR (("charcoal"[All Fields] OR "charcoal"[All Fields] OR "charcoals"[All Fields]) NOT ("coal"[All Fields] OR "coal"[All Fields])) OR (((wetland*) and (fire*)) OR ((wildland*) and (fire*)) OR (wildfire*) OR (forest AND fire*) OR (landscape AND fire*))
Scopus	TITLE-ABS-KEY(wood-smoke* OR woodsmoke OR wood-fired OR wood-burning* OR burn-wood OR burning-wood OR wood-stove* OR woodstove* OR Wood-carbonis* OR carbonising-wood OR carbonised-wood OR Wood-carboniz* OR carbonizing-wood OR carbonized-wood OR collier* OR biomass-fired OR biomass-stove* OR burn-biomass OR burning-biomass OR biomass-fuel* OR biomass-cook* OR cook-biomass OR cooking-biomass OR cookstove* OR cooking-stove* OR cook-stove*) OR (TITLE-ABS-KEY(cook* OR heat*) AND TITLE-ABS-KEY(air-pollut* OR smoke OR smoky OR wood OR biomass OR fuel*)) OR TITLE-ABS-KEY(charcoal* NOT Coal*) OR TITLE-ABS-KEY (fire* AND wetland*) OR TITLE-ABS-KEY (fire* AND wildland*) OR TITLE-ABS-KEY(wildfire*) OR TITLE-ABS-KEY(forest AND fire*) OR TITLE-ABS-KEY(landscape AND fire*)
Web of Science	TS=(wood-smoke* OR woodsmoke OR wood-fired OR wood-burning* OR burn- wood OR burning-wood OR wood-stove* OR woodstove* OR Wood-carbonis* OR carbonising-wood OR carbonised-wood OR Wood-carboniz* OR carbonizing-wood OR carbonized-wood OR collier* OR biomass-fired OR biomass-stove* OR burn-biomass OR burning-biomass OR biomass-fuel* OR biomass-cook* OR cook-biomass OR cooking-biomass OR cookstove* OR cooking-stove* OR cook-stove*) OR ((TS=(cook* OR heat*)) AND (TS=(air-pollut* OR smoke OR smoky OR wood OR biomass OR fuel*))) OR TS=(charcoal* NOT coal*) OR TS=(Fire* AND wetland*) OR TS=(Fire* AND wildland*) OR TS=(Wildfire*) OR TS=(forest AND fire*) OR TS=(landscape AND fire*)

For animal cancer searches, the wood smoke specific terms were combined using "AND" with the RoC Animal Terms and RoC Cancer terms found in the standard search term document.

For mechanism searches, the wood smoke specific terms were combined using "AND" with the RoC KCC and RoC general mechanism search terms found in the standard search term document.

Appendix B. Background information on Key Characteristics of Carcinogens (KCC) Biomarkers and Indicators

Tables in this appendix provides background information on the biomarkers and indicators for evaluating study informativeness (Table 3-4 and Table 3-7) and reaching a confidence of the evidence of selected KCCs for wood smoke and wildfire studies (See Table 3-8).

B.1. Is genotoxic (KCC2)

Genotoxicity typically refers to a substance's ability to cause gene mutations, DNA damage, structural chromosome aberrations, and aneuploidy (numerical chromosome aberrations) and is directly linked to carcinogenicity. OECD provides guidance for testing many of these endpoints. Genotoxicity overlaps with KCC1 (is electrophilic [e.g., DNA adducts]); KCC3 (alters DNA repair or causes genomic instability); and KCC5 (induces oxidative stress [e.g., oxidative damage to DNA]).

Table B-1. Background information on common biomarkers or indicators of genotoxicity (KCC2)

Subtype	Biomarkers/indicators	Evidence type: biospecimen	Persistence/induction	Comments or guidance
DNA damage	DNA damage • In vivo and in vitro comet assays [including specialized lesions] • Activation of transcription factor p53 • Phosphorylation of H2AX (γH2AX) • TGx-DNA Damage Induced Transcriptomic Biomarker	Exposed humans or animals: Lymphocytes, exfoliated cells (exposed humans), target tissues In vitro: Various cell lines (e.g., human lymphoblastoid TK6 cells)	Hours	OECD Test No. 489: In Vivo Mammalian Alkaline Comet Assay • Timing for in vivo comet assay depends on substance-specific metabolism and DNA repair kinetics Multiplexed fluorescence staining assays for DNA damage
Mutations	-	-	-	Ames-positive and in vivo MN-positive chemicals are strong predictors of carcinogenicity. Chemical-specific mutational spectra observed in cancers
	Bacterial reverse mutation tests • Base-pair substitution/frame shifts	In vitro: Bacteria (Ames) In vitro: Panel of *Salmonella* and some *E. coli* strains; positive result in any strain is relevant Exposed humans: Can use urine to test mutagenicity	Persistent (cell life)	OECD Test No. 471: Bacterial Reverse Mutation Test

Subtype	Biomarkers/indicators	Evidence type: biospecimen	Persistence/induction	Comments or guidance
Mutations (cont'd)	Forward gene mutations: Reporter locus • HPRT • XPRT • tk (broader range)	In vitro Various cell lines (e.g., Chinese hamster As52)	Persistent (cell life) Days to weeks	OECD Test No. 476: In Vitro Mammalian Cell Gene Mutation Tests Using the *HPRT* and *XPRT* Genes
		In vitro: Mouse lymphoma assay; TK6 cells	Days	OECD Test No. 490: In Vitro Mammalian Cell Gene Mutation Tests Using the *Thymidine Kinase* Gene
	Somatic or germ cell transgenic rodent assays (e.g., Big Blue mouse or rat)	Exposed rodents: almost every organ or tissue	Days (fast dividing) to weeks (slow dividing)	OECD Test No. 488: Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays
	Pig-a gene mutation assay	Exposed humans or animals: Blood (erythrocytes)	Persistent (cell life)	OECD Test No. 470: Mammalian Erythrocyte Pig-a Gene Mutation Assay
	Glycophorin A *HPRT* mutational frequency	Exposed humans: blood (erythrocytes) Exposed humans (usually): lymphocytes	-	-
	Ultra-accurate, error-corrected DNA sequencing approaches (not locus-dependent)	In vitro, exposed animals or humans (blood, cells from urine)	Persistent	Duplex sequencing, PacBio HiFi sequencing
Chromosomal damage	Structural chromosomal aberration [CA] test (with or without FISH)	In vitro or ex vivo: Primary cells or cell lines (e.g., lymphocytes)	Persistent (cell life)	MN and CA associated with increased cancer risk in prospective cohort studies OECD Test No. 473: In Vitro Mammalian Chromosomal Aberration Test
	-	Exposed humans or animals: bone marrow, whole blood, lymphocytes, exfoliated cells (humans), target tissues	Days to weeks	OECD Test No. 475: Mammalian Bone Marrow Chromosomal Aberration Test

Subtype	Biomarkers/indicators	Evidence type: biospecimen	Persistence/induction	Comments or guidance
Chromosomal damage (cont'd)	Micronucleus [MN] test: Structural and numerical (CBMN, centromere/kinetochore analysis)	In vitro cells Exposed humans or animals: Erythrocytes and other proliferating cells	Days to weeks	OECD Test No. 487: In Vitro Mammalian Cell Micronucleus Test OECD Test No. 474: Mammalian Erythrocyte Micronucleus Test
Older tests or less common: Mutations	Rodent dominant lethal Sex-linked recessive lethal (drosophila), assays in yeast	Exposed animals Exposed non-mammalian systems	-	OECD Test No. 478 No longer recommended; good indicators of genotoxicity; however, its relevance to humans is unclear
Older tests or less common: Chromatid damage	Sister-chromatid exchanges	In vitro Ex vivo: Cells from exposed humans/animals	-	No longer recommended; findings do not correlate well with rodent carcinogenicity

Sources: (Olsen *et al.* 1996, Kirkland *et al.* 2005, Norppa *et al.* 2006, Battershill *et al.* 2008, European Commission 2008, Eastmond *et al.* 2009, Bonassi *et al.* 2011, Myers and Grant 2014, OECD 2015, 2016b, f, d, g, a, e, c, Ladeira and Smajdova 2017, Li *et al.* 2019, OECD 2020, Smith *et al.* 2020, OECD 2022)
CBMN = Cytokinesis-blocked micronucleus, FISH = Fluorescence in Situ Hybridization, HPRT = Hypoxanthine-guanine Phosphoribosyl Transferase, MN = micronucleus, OECD = Organisation for Economic Co-operation and Development, TK = Thymidine Kinase, XRPT = Xanthine Phosphoribosyl Transferase, - = cell left blank intentionally

B.2. Induces oxidative stress (KCC 5)

Oxidative stress occurs when there is an imbalance in the redox status within target tissues that favors formation of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) at the expense of their detoxification (Smith *et al.* 2020). This imbalance can lead to oxidative damage to DNA, proteins, and lipids and other effects that are directly related to several other KCCs. These include genotoxicity (KCC2), altered DNA repair (KCC3), chronic inflammation (KCC6), and altered cell proliferation, cell death, or nutrient supply (KCC10). Although oxidative stress is a KCC, it is not specific to carcinogens as many non-carcinogens can also induce oxidative stress.

Table B-2. Background information on common biomarkers or indicators of oxidative stress (KCC5)

Subtype	Biomarkers/indicators	Evidence type: biospecimen	Persistence/ induction	Comments or guidance
Oxidants: ROS, RNS, ROM	ROS: H_20_2, OH^-, ROO^-, or O_2^- RNS: $ONOO^-$, NO_2	Cell-free Exposed humans or animals: WBC or other cells, cellular components In vitro (real/time live cells)	Very transient (nsec to sec) to longer lived[a]	Measurement instrumentation: electron spin resonance, fluorescent probes, biosensors Fresh samples are needed; oxidants are unstable Not recommended for ex vivo tissue homogenates Urinary H_2O_2 can be an indicator of whole-body oxidative stress but is confounded by diet.
	ROM: ROOH	Exposed humans or animals: Serum/plasma	-	Criticisms of the reliability of the d-ROM test
ROS Modifications: lipid, DNA, protein	-	-	-	Systemic or tissue-specific oxidative stress
Lipid peroxidation	-	Exposed humans or animals: Body fluid (e.g., urine, serum, plasma), exhaled breath cells, tissues	Minutes to hours	May directly affect the function of target molecules or enzymes or indicate local degrees of oxidative stress
	MDA/TBARs	-	-	MDA/TBARs is an unspecific biomarker and is prone to methodology bias but may have

Subtype	Biomarkers/indicators	Evidence type: biospecimen	Persistence/ induction	Comments or guidance
				clinical relevance (induces IL-7 producing cells). TBARS/MDA is not recommended as the only test of lipid peroxidation. MDA measured by MS is useful.
Lipid peroxidation, cont.	F2-isoP	-	Minutes (serum), hours (urine)	IsoP in serum and urine correlate with in vivo oxidative stress in humans and animals (unaffected by diet). Preferred method and recognized by EFSA Conflicting findings found for breast cancer risk
	Others: HNE, LOOH, oxLDL	-	-	oxLDL recognized by EFSA
Oxidative damage to DNA/RNA	8-OH-dG	Exposed humans or animals: Urine, plasma, serum, tissue	Minutes	Rapidly repaired, usually measured in urine, may serve as an indicator of whole-body oxidative stress Some evidence shows that pre-diagnosis frequencies are associated with increased breast cancer risk in postmenopausal women and lung cancer in non-smokers. Often carries greater weight than other biomarkers
	Thymidine glycol	-	-	Greater specificity than 8-OHdG, sustained in tissues
	Oxidized guanine/guanosine (OxGua)	Exposed humans or animals: Urine	-	Pre-diagnosed levels are associated with increased risks of all cancer in non-smokers and possibly men; colorectal cancer in women, Non-smokers, and non-obese people; and prostate cancer in non-smokers. OxGua molecules are derived from repair products of the oxidatively generated

Subtype	Biomarkers/indicators	Evidence type: biospecimen	Persistence/ induction	Comments or guidance
				DNA/RNA lesions, 8-OH-dGuo from DNA, and 8-OHGuo from RNA.
Oxidative damage to DNA/RNA, cont.	Comet assay modified with lesion-specific repair endonucleases (e.g., OGG1, FPG, Endonuclease III)	Cells (e.g., leukocytes), ex vivo	-	Accepted by EFSA
	Repair enzymes: hOGG APE	-	-	-
Oxidative stress: Proteins	Carbonylated proteins, AOPP	Exposed humans or animals: Plasma/serum	Days	CPs: irreversible; a hallmark of oxidative stress and is biologically significant and clinically relevant
	3-nitrotyrosine	-	-	Circulating levels of the biomarkers are not equivalent to tissue levels.
	s-glutathionylation	-	-	Prone to methodological artifacts
ROS generating enzymes	MPO, XO	Exposed humans or animals: serum, urine, tissues Ex vivo: neutrophils	Hours	MPO released from neutrophils can also be an indicator of inflammation (KCC6). MPO is associated with cancer progression.
Inflammation/oxidative stress biomarkers	COX-2	Exposed humans or animals: tissues, serum In vitro	Hours to days	COX-2 inhibitors can prevent the carcinogenesis of colorectal cancer. It can also be considered as a pro-inflammatory biomarker (KCC6).
Antioxidant status	Enzymes: SOD, catalase, GST, GPx	Exposed humans or animals: Serum, erythrocytes (catalase)	Minutes	-

Subtype	Biomarkers/indicators	Evidence type: biospecimen	Persistence/ induction	Comments or guidance
Antioxidant status, cont.	GSH; Vitamin A, C, E	-	Minutes	Unstable, difficult to analyze
	Nrf2-ARE response pathway	Exposed humans or animals: Leukocytes, tissue	-	Difficult to analyze
	Total antioxidant capacity	-	-	-
Indices	GSH/GSSG ratio	-	-	-
	Oxy score (damage – protection)			

Sources: (Basu 1998, Loft *et al.* 2006, Harris *et al.* 2008, Dai *et al.* 2009, Ho *et al.* 2013, Lim and Thomas 2013, Loft *et al.* 2013, Brenner *et al.* 2014, Frijhoff *et al.* 2015, Gryszczynska *et al.* 2017, Ito *et al.* 2017, Lee *et al.* 2017, Marrocco *et al.* 2017, Tas and Erturk 2017, Gao *et al.* 2019, Katerji *et al.* 2019, Smith *et al.* 2020, Andries *et al.* 2021, Menzel *et al.* 2021, Murphy *et al.* 2022, Valadez-Cosmes *et al.* 2022)

8-OH-dG = 8-oxoguanine DNA glycosylase, APE = apurinic/apyrimidinic endonuclease, AOPP = advanced oxidation protein products, COX-2 = cyclooxygenase-2, FRG = formamidopyrimidine (fapy)-DNA glycosylase, GPx = glutathione peroxidase GSH = glutathione (reduced), GSSG = oxidized glutathione, GST = glutathione S-Transferase, ,HNE = 4-hydroxy-2-nonenal, hOGG = 8-oxoguanine-DNA-glycosylase, Iso-P = isoprostanes, LOOH = lipid hydroperoxides, oxLDL = oxidized low density lipoproteins, MDA = malondialdehyde, MPO = myeloperoxidase, OGG1 = 8-oxoguanine DNA glycosylase, ROS = reactive oxygen species, RNS= reactive nitrogen species, SOD = superoxide dismutase, XO = xanthine oxidase, TBARS = thiobarbituric acid reactive substances, WBC = white blood cells, H_20_2, = hydrogen peroxide, OH^- = hydroxyl radical, NO_2 = nitrogen dioxide radical, $ONOO^-$ = peroxynitrite, O_2^- = superoxide, ROOH = hydroperoxides, ROO = peroxyl radicals

[a]Radical electrons/ionization charge species are very transient, others such as H_2O_2 are longer lived, - = cell left blank intentionally

B.3. Induces chronic inflammation (KCC6) or immune activation

Many protein biomarkers (e.g., cytokines) can indicate chronic or acute inflammation depending on the exposure conditions. Thus, evidence of chronic or persistent/repeated exposure and/or the timing/duration of response is critical in determining whether the study is measuring chronic inflammation. The RoC review also considers immune activation (e.g., by B-cell antigens) which may be linked to chronic inflammation.

Table B-3. Background information on common biomarkers or indicators of chronic inflammation (KCC6) or immune activation

Subtype	Biomarkers/indicators	Evidence type: biospecimen	Persistence	Comments or guidance
Chronic inflammatory diseases (e.g., autoimmune diseases)	Increased risk or incidence of autoimmune diseases that have been linked to cancer	Exposed humans Animal models of autoimmune disease	Months to years	-
Chronic inflammation with WBC infiltration	Histology Local evidence of infiltration of acute (with evidence of acute exacerbations from repeated exposures) or chronic inflammatory cells	Exposed animals or (possibly) humans: tissue	Timing/persistence can vary	Specific cell types can indicate chronic inflammation. Granulocytes (including neutrophils) in tissue indicate acute inflammation. Lymphocytes, plasma cells, and monocytes/macrophages in tissue indicate chronic inflammation. Pathologists can diagnose acute vs. chronic Histological evaluation is limited in exposed humans.
Some key pro-inflammatory cytokines and chemokines	Interleukins[a]: IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-12, IL-15, IL-23 Interferon: IFNγ Tumor necrosis factor: TNFα Transforming growth factor: TGFβ Chemokines: MCP-1, MIP-2	Exposed humans or animals: serum/plasma, tissue, body fluids, exhaled breath Ex vivo, in vitro	Minutes: IL-1β, IL-8, TNFα Hours: IL-6	IL-6 is involved in inflammation, autoimmunity, and B-cell malignancies. Pre-diagnosed elevated circulating (systemic) levels of IL-6 and IL-8 are associated with increased lung cancer risk. Increased IL-6 levels are also associated with all cancers combined and CRC in several studies or meta-analyses.

Subtype	Biomarkers/indicators	Evidence type: biospecimen	Persistence	Comments or guidance
				Experimental (rodent) models for the role of TNFα, TGFβ, IL-1, IL-6, and IL-23 in cancer development or progression The associated cytokine/chemokine receptors are also critical and ideally should be considered together with ligands.
Acute phase proteins	CRP	Exposed humans or animals: serum/plasma, tissue	Hours to days	CRP is non-specific and the most sensitive acute phase protein in humans.
	Serum amyloid A	Ex vivo, in vitro	24 to 48 hr	Pre-diagnosed elevated circulating CRP is associated with increased cancer incidence/mortality for all cancers combined, and several cancer types, such as lung, CRC, breast, and ovarian. SAA is associated with an increased risk of several cancers, such as lung and colon, and is correlated with CRP.
	ESR	-	Weeks	ESR is the most widely used laboratory test for evaluating inflammation status in clinical practice, including infection, autoimmunity, and cancer.
Transcription factors	NF-κβ JAK/STAT	Exposed humans or animals: cells/tissue	Minutes	NF-κβ activation is essential for inflammation and is activated in several types of cancer.
		Ex vivo, in vitro	Hours	
Prostaglandin endoperoxide synthase	COX-2	Exposed humans or animals: tissue, serum	Hours to days	COX-2 inhibitors can prevent the carcinogenesis of colorectal cancer.
		In vitro		It can also be considered as an oxidative stress biomarker (KCC5).
Circulating WBC	Increases in total WBC or leukocyte subsets: lymphocytes, monocytes, granulocytes	Exposed humans or animals: blood	Days to weeks	Decreased systemic WBC can also indicate increased inflammation via extravasation into tissue (local inflammation).

Subtype	Biomarkers/indicators	Evidence type: biospecimen	Persistence	Comments or guidance
	Ratios (NLR, PLR, LMR) and SII Increased bone marrow hematopoiesis			In general, lymphocytes are chronic inflammation indicators, and granulocytes are acute inflammation indicators. Pre-diagnosed elevated lymphocytes, monocytes, neutrophils, basophils, and NLR are associated with increased lung cancer risk. NLR represents the imbalance between the innate and adaptive immune response. Pre-diagnosed elevated leukocytes are associated with an increased risk of all cancers combined, lung cancer, or CRC. SII is based on peripheral lymphocyte, neutrophil, monocyte, and platelet counts.
Immune cell activation	Macrophage and granulocyte phagocytosis, ROS production	Exposed humans or animals: cells/tissue Ex vivo, in vitro	-	ROS from immune cells contribute to oxidative stress (immune-regulated ROS can be considered under inflammation or oxidative stress- KCC5).
	-	-	-	Persistent immune cell activation can be a driver of chronic proinflammatory responses. It can be difficult to definitively determine if evidence/endpoints of immune activation are linked to chronic inflammation.
	B cell stimulation/antigens (antibody production)	Exposed humans or animals: cells/tissue Ex vivo	-	B-cell stimulation (by self due to autoimmunity or foreign antigens due to immunosuppression) leads to DNA damage from genomic recombination and mutation during class/isotype switching and somatic hypermutation and possibly increased B-cell lymphoma.

Sources: (Germano *et al.* 2008, Van Hemelrijck *et al.* 2011, Zhou *et al.* 2012, Brenner *et al.* 2014, Zhou *et al.* 2014, Kakourou *et al.* 2015, Allin *et al.* 2016, Brenner *et al.* 2017, Puar *et al.* 2018, Kang *et al.* 2019, Qian *et al.* 2019, Chauhan and Trivedi 2020, Smith *et al.* 2020, Wong *et al.* 2020, Hirano 2021, Liu *et al.* 2021, Michels *et al.* 2021, He *et al.* 2022, Ji *et al.* 2022, Zhu *et al.* 2022)

COX-2 = cyclooxygenase-2, CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, JAK/STAT = Janus kinase/Signal transducers and activators of transcription, INFγ = interferon gamma, LMR= lymphocyte to monocyte ratio, MCP-1 = macrophage chemoattractant protein-1, MCP=2 = macrophage chemoattractant protein-2, NF-κβ = nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ), NLR = neutrophil to lymphocyte ratio, PLR = platelet to lymphocyte ratio, ROS = reactive oxygen species, SAA = serum amyloid A, SII = systemic immune-inflammation index, TGFβ = transforming growth factor beta, TNFα = tumor necrosis factor alpha, WBC = white blood cell, - = cell left blank intentionally

[a]Some interleukins (like IL-8) are considered to be chemokines.

B.4. Is immunosuppressive (KCC7)

Immunosuppression is characterized by a reduction in the capacity of the immune system to respond effectively to foreign antigens, including surface antigens on tumor cells. Potentially neoplastic cells may escape immune surveillance which facilitates the survival of tumor cells. T cells and natural killer cells are critical components in anti-tumor immunity.

Table B-4. Background information on common biomarkers or indicators of immunosuppression (KCC7)

Subtype or assay	Biomarkers/indicators	Evidence type: biospecimen	Comments or guidance
Increased infections	Increases in the incidence of opportunistic infections (especially viral)	Exposed humans (observational studies) or animals	Strong evidence for immunosuppression
Immune function (challenge to foreign antigen)	Decreases in primary or secondary antibody response to vaccinations or natural antigens	Exposed humans (controlled clinical or observational studies) or animals	Strong evidence for immunosuppression but indicator may not be relevant for immunosurveillance and cancer risk. Not usually conducted in humans because of ethical reasons Observational studies may evaluate whether environmental exposure affects vaccination antibody response.
	Decreases in NK function, phagocytosis/bacterial killing by PMNLs, antigen presentation	Exposed humans or animals Ex vivo	-
Immune function (humoral or cell-mediated immunity)	Decreases in antibody production (e.g., T cell dependent, antigen-specific) including specific subclasses/isotypes, NK or CTL activity, T cell activity	Exposed humans or animals Ex vivo	Impact on CTL or NK activity and memory T cells may be most relevant for cancer (e.g., immunosurveillance) with B cell/antibody production less relevant. Low CTL activity is associated with increased cancer risk.
Immune components	Lymphocyte phenotyping (decreased NK, NKT, CD4+	Exposed humans or animals	Not sensitive or predictive alone to predict immunosuppression but may be used to support experimental animal data.

Subtype or assay	Biomarkers/indicators	Evidence type: biospecimen	Comments or guidance
	T, CD8+ T; increased CTLA4+ T, Tregs)		
Immune components, cont.	Cytokines: IL-10, TGFβ	-	-
	Immunoglobulins (T cell-dependent or -independent)	-	-
Immune components (hematology)	Altered WBC and leukocyte subsets	Exposed humans or animals	Only severe changes are sufficient evidence of immunosuppression.
Immune organs (histopathology and organ weights)	Lymph node or splenic germinal centers, bone marrow suppression of hematopoiesis	Exposed humans or animals	Reduced organ weight may be secondary to general toxicity or stress. Extensive histopathology may support the weight of evidence for immunosuppression. Evaluation is limited in exposed humans.

Source: (Imai *et al.* 2000, IPCS 2012, Ponce *et al.* 2014, Lebrec *et al.* 2016, Sharma *et al.* 2017, Smith *et al.* 2020)
CTL = cytotoxic T lymphocyte, NK= natural killer cell, PMNL = polymorphonuclear leukocyte, TGFβ = transforming growth factor beta, WBC = white blood cell, - = cell left blank intentionally

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