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Review Article

Systematic review of the scientific evidence on ethylene oxide as a human carcinogen

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ABSTRACT

Ethylene oxide is a highly reactive chemical primarily used as an intermediate in chemical production and as a sterilant of medical equipment and food products; it also is produced endogenously as a result of physiological processes. We conducted a systematic review of the potential carcinogenicity of inhaled ethylene oxide in humans using methods that adhere to PRISMA guidelines and that incorporate aspects from the Institute of Medicine (IOM) (now the National Academy of Medicine) as well as several US Environmental Protection Agency (EPA) frameworks for systematic reviews. After a comprehensive literature search and selection process, study quality was evaluated following a method adapted from the EPA Toxic Substances Control Act (TSCA) framework. The literature screening and selection process identified 24 primary studies in animals or humans and more than 50 mechanistic studies. Integrating epidemiological, animal, and mechanistic literature on ethylene oxide and cancer according to the IOM framework yielded classifications of suggestive evidence of no association between ethylene oxide and stomach cancer, breast cancer and lymphohematopoietic malignancies at human relevant exposures. However, we acknowledge that there is additional uncertainty in the classification for lymphohematopoietic malignancies owing to a paucity of evidence for specific types of these tumors, each of which is a distinct disease entity of possibly unique etiology.

1. Introduction

Ethylene oxide (EtO; CAS Number 75-21-8) is primarily used as a feedstock for the production of other chemicals, including glycol ethers, polyglycol ethers as well as emulsifiers, detergents and solvents. EtO also is widely used to disinfect medical equipment, especially components that would be damaged if heat-sterilized, and as a fumigant for disinfecting food products including spices [1]. Notably, however, only about 1% of the total production of EtO is used as a sterilant or fumigant [2]. EtO is present in the ambient environment from sources such as automobile exhaust, cigarette smoke and industrial processes [2,3]. Concentrations in ambient air range from 0.15 to 0.22 ppb [4]. EtO also is produced from physiological processes, with estimated endogenous equivalent concentrations of 0.13–6.9 ppb EtO [5,6]. Thus, all humans have background levels of EtO in their bodies.

The International Agency for Research on Cancer (IARC) classified EtO as a Group 1 carcinogen (carcinogenic to humans), based on "limited" evidence of breast and lymphatic and hematopoietic cancers in humans and sufficient evidence in experimental animals. IARC also noted that, "There is strong evidence that the carcinogenicity of EtO, a direct-acting alkylating agent, operates by a genotoxic mechanism", which IARC "relied heavily on" in making its determination [1].

Similarly, in December 2016, the United States Environmental Protection Agency (EPA) classified EtO as a human carcinogen based on

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what it concluded was clear and consistent evidence across epidemiological, animal and mechanistic studies, but with an apparent focus on the positive evidence of genotoxicity; namely, chromosomal damage *in vivo* and *in vitro* and in exposed humans [5].[1] EtO's genotoxic potential is not surprising given its efficacy as a sterilant; however, previous published reviews have not fully assessed the body of epidemiological, animal and mechanistic information according to current systematic review methods to evaluate not only the absolute carcinogenic hazard but more importantly the likelihood of carcinogenicity under human-relevant exposure scenarios. The aim of this comprehensive review is to critically review and integrate these lines of relevant evidence, considering in particular the evidence for key events in the mode of action (MOA) at low exposure levels.

2. Materials and methods

We conducted a systematic review with a focus on the quality of each primary study, synthesizing and integrating evidence both within and across epidemiological, toxicological and mechanistic lines of inquiry. In the scoping and protocol development phases, recent reviews and agency documents were consulted to provide basic understanding of key scientific questions and hypotheses regarding the carcinogenicity of EtO. The review was conducted in accordance with best practices and guidance – incorporating aspects from the National Toxicology Program (NTP) Office of Health Assessment and Translation (OHAT) framework [7], Integrated Risk Information System (IRIS), Toxic Substances Control Act (TSCA), the Office of Pollution Prevention and Toxics (OPPTS) *Application of Systematic Review in TSCA Risk Evaluations* [8], and guidance from the National Academies of Sciences, Engineering (NASEM). This novel "hybrid" approach drew upon the strongest aspects of each framework, while acknowledging the commonality of the basic principles of systematic review across all of the agency approaches. The review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist, provided in the Supplemental Materials.

2.1. Article eligibility criteria

We developed inclusion and exclusion criteria *a priori* to identify the most relevant articles for full review consistent with systematic review principles. The full set of criteria is provided in the protocol in the Supplemental Materials. In brief, selected literature pertained to EtO exposure via inhalation and any cancer endpoints. We included epidemiological studies, experimental animal studies in mammalian species and mechanistic studies in humans, as well as laboratory studies *in vivo* or *in vitro* with mammalian or bacterial cell lines.

2.2. Information sources, search strategy, and selection process

We performed literature searches using PubMed and used existing agency reviews as a basis for cross-referencing critical studies. The preliminary search string was as follows: ("ethylene oxide") AND ("epidemiology" OR epidemiological OR cohort OR "case control" OR animal OR experimental OR rat OR rats OR mice OR mouse) AND (carcinogenicity OR cancer). Additional searches were run using filters for animal/toxicology studies, and for mechanistic/MOA studies, using search terms including but not limited to the following: micronuclei, sister chromatid exchange, chromosome aberrations, DNA adduct, DNA methylation, inflammation, mechanism, "mode of action" and MOA.

Toxicokinetic, experimental animal and mechanistic studies were selected based on overall relevance to the chronic health effects (primarily cancers) and adherence to our Population, Exposure, Comparator, and Outcome (PECO) criteria (see Protocol in Supplementary Materials). Epidemiological studies were selected to include groups or populations exposed to EtO, including employees of EtO production or sterilization plants.

2.3. Data abstraction and study quality evaluation

Each eligible study was reviewed for relevance, and if the full text met PECO criteria, study details were extracted and the study evaluated for methodological and reporting quality. For this review, studies were evaluated for relevance and quality specifically for the purposes of evaluating carcinogenicity. Epidemiological study findings also were displayed in forest plots using R statistical software.

We followed a modified version of the study quality framework used by US EPA for the amended TSCA risk evaluations, as outlined in the Draft Protocol for Systematic Review in TSCA Evaluations [9] (previously, the Application of Systematic Review in TSCA Risk Evaluations [8]). This framework involves reviewing and rating studies according to six quality domains (e.g., outcome assessment and exposure characterization), each of which includes two to seven individual study metrics. Two reviewers independently evaluated the quality of each individual study; any disagreements regarding quality ratings were resolved with the assistance of a third author.

2.3.1. Epidemiological studies

Our evaluation adapted the framework for study quality evaluation used by EPA for risk evaluation of chemicals under the amended Toxic Substances Control Act [8,9: Appendix R] with enhancements from the guidance documents of other professional organizations (specifically, the tiered approach employed by NTP's OHAT systematic review risk of bias evaluation system). Briefly, all studies first were evaluated qualitatively (low, medium, or high quality) based on several metrics within five specific domains (i.e., study participation, exposure characterization, outcome assessment, potential confounding/variability control and analysis) and 15 corresponding metrics within those domains in the TSCA framework (see Supplemental Table S.7 and S.8).

To arrive at an overall quality rating for each study meeting defined inclusion criteria, we developed a tiered system that placed more emphasis on robust exposure characterization methods as well as consideration of co-exposure to chlorohydrin (production facilities only) and other potential confounding factors. A judgment of low-quality based on either exposure characterization or potential confounding resulted in an overall study quality rating of "low." If studies received a high or medium judgment on both of these critical domains, the overall study quality judgment was based on the distribution of relative scores for all domains. The overall study rating process is described in detail below and summarized in Fig. 1.

As exposure characterization was deemed crucial to the validity of study results, Tier I of the quality review process evaluated the robustness of the exposure measures, weighting more heavily quantitative/semi-quantitative measures of exposure such as sampling data, biological verification of exposure through medical records, measurements collected and/or verified by an industrial hygienist and characterization of exposure by work history. Studies with no specific characterization of EtO exposure (e.g., by occupation or job title only, where exposure would be possible but not known for any individual, or qualitatively, such as exposed or unexposed only) were rated as low quality overall. Studies with a high probability of individual or group exposure (e.g., confirmed but limited exposure) or those with robust quantitative or semi-quantitative estimates were rated as either medium or high quality for this domain, depending on their fulfillment of the TSCA criteria for exposure characterization. Temporality between exposure, allowing for

[1] United States Environmental Protection Agency. Evaluation of the Inhalation Carcinogenicity of Ethylene Oxide (CASRN 75- 21–8). 2016. In Support of Summary Information on the Integrated Risk Information System (IRIS). EPA/635/R-16/350Fa. Washington, DC: National Center for Environmental Assessment, Office of Research and Development, U.S. Environmental Protection Agency.

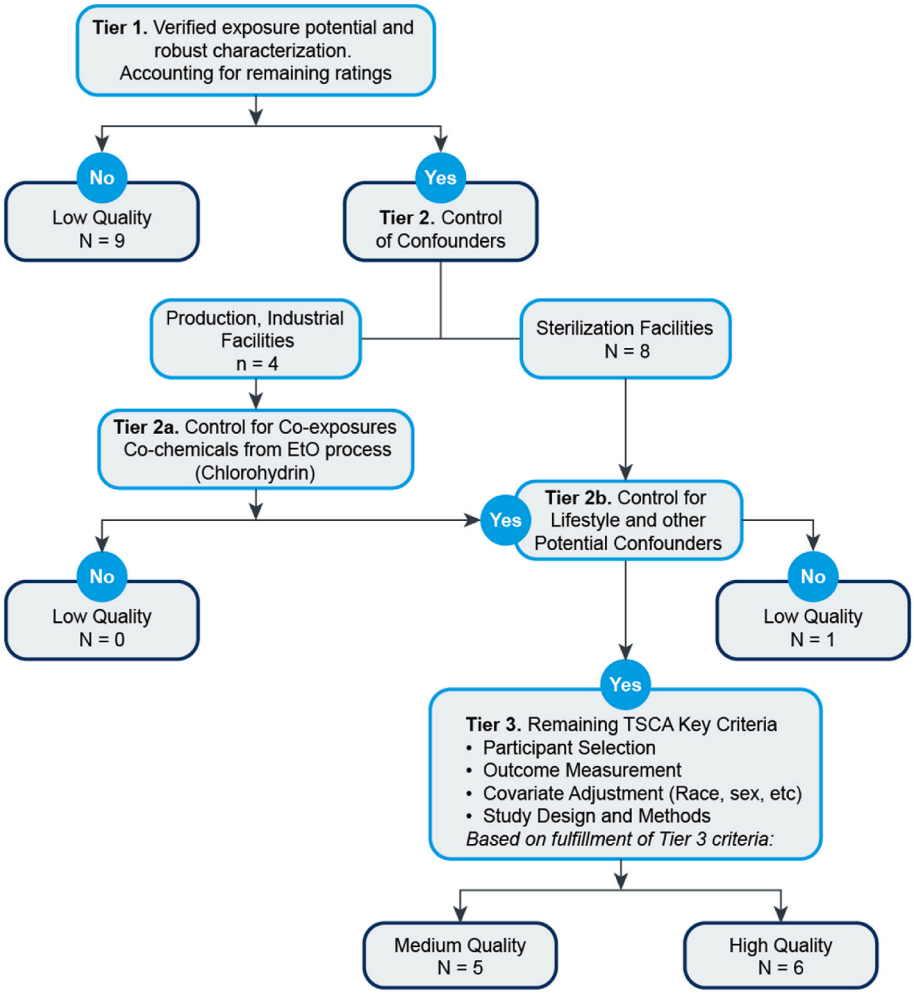

Fig. 1. Study quality evaluation framework for epidemiology studies.

sufficient latency period, and onset of disease also was considered.

In Tier II of the quality review process, all studies rated high- and medium-quality in Tier I were evaluated based on the robustness of consideration of co-exposures and potential confounding factors. Studies of workers in the production of EtO were evaluated separately from those employed in sterilization facilities using EtO. Facilities that synthesized EtO via the chlorohydrin method (common before 1957 [10]) required various intermediates and created several byproducts, such as ethylene dichloride, ethylene/propylene chlorohydrin, bis (2-chloroethyl) ether, some of which may be carcinogenic. Therefore, these studies additionally were evaluated to ensure that proper methods were used to account for potential co-exposure to chlorohydrin process byproducts and if co-exposures were not well controlled, these studies were assigned a low overall quality score. Studies rated as high or medium were evaluated further for other risk factors such as reproductive history, sex, race and calendar year. Sterilization facilities have few co-exposures and were evaluated for potential non-occupational risk factors such as reproductive history, use of hormonal therapies, BMI, life style factors, age and sex.

In Tier III, studies with medium- or high-quality exposure characterization and sufficient handling of potential confounding were rated for overall quality based on the remaining key EPA TSCA framework evaluation domains, including study recruitment/participation methods, outcome ascertainment, other potential confounding/variable control (e.g., smoking) and statistical analysis. Based on the relative quality ratings for each of these, studies were rated as medium or high overall quality. A study qualified as "high" if most of the categories were rated as high (or received the highest possible rating according to the TSCA framework) and no categories were rated as low. Similarly, a study qualified as "medium" if most of the categories (excluding exposure) were rated as medium and only one or two categories were rated as low. While these definitions are arbitrary and require some element of subjective judgment, they were clearly defined and applied uniformly across all studies that remained for Tier III evaluation.

2.3.2. *Experimental animal toxicology and mechanistic studies*

For animal toxicology and selected mechanistic studies, we followed the TSCA study quality evaluation framework and assigned relative numerical ranks to each of the outcomes (1, 2 and 3 corresponding to high, medium and low) for each metric, then averaged the metric scores to arrive at an overall relative score of high, medium, or low quality.

2.4. *Evidence synthesis and hazard characterization*

The totality of evidence first was synthesized within each line of inquiry (i.e., epidemiological, toxicological and mechanistic studies) and then integrated to reach conclusions on human cancer hazards. In addition to the systematic review frameworks identified above, we also drew upon other frameworks focused specifically on causal interference. The within-stream integration of evidence included consideration of consistency, coherence and evidence of exposure-response relationships. Conclusions based on evidence integration for each cancer were based on the IOM classifications for causation: sufficient evidence, limited/suggestive evidence, or inadequate/insufficient evidence of an

association; or limited/suggestive evidence no association [11] (Table 1). These categorizations are based upon the relative strength of evidence, considering the quality and consistency of evidence, levels of exposure, presence of dose-response relationships, and biological plausibility of the potential association.

3. Results

3.1. Literature search and selection

The primary literature search in PubMed (conducted in March 2021) yielded a total of 523 publications. These results were cross-checked with references included in agency reviews (IARC, ATSDR). After abstract review followed by full-text review, which included eliminating duplicate or studies that were subsequently updated, and applying the inclusion and exclusion criteria determined *a priori*, 20 epidemiological and 4 experimental animal studies remained and were selected for full review, data abstraction, and evaluation. The results of the literature search and study selection process for the epidemiological and toxicological literature are summarized in Fig. 2. Mechanistic evidence, including genotoxicity studies, also were identified from the initial search and cross-checked with agency reviews. While the results from some of these studies (e.g., standard guideline genotoxicity assays) were extracted from agency reviews without obtaining the full text of the primary publication, the full-text of 40 *in vitro* and *in vivo* studies providing information pertinent to the understanding of the potential mode of action (MOA) were obtained and reviewed.

Table 1
IOM (2001) categorizations for evaluating strength of evidence.[a]

Classification	Description
Sufficient Evidence of a Causal Relationship	Evidence is sufficient to conclude that a causal relationship exists between the exposure to a specific agent and a health outcome in humans. The evidence fulfills the criteria for sufficient evidence of an association (below) and satisfies several of the criteria used to assess causality: strength of association, dose-response relationship, consistency of association, temporal relationship, specificity of association, and biological plausibility.
Sufficient Evidence of an Association	Evidence is sufficient to conclude that there is a positive association. That is, a positive association has been observed between an exposure to a specific agent and a health outcome in human studies in which chance, bias, and confounding could be ruled out with reasonable confidence.
Limited/Suggestive Evidence of an Association	Evidence is suggestive of an association between exposure to a specific agent and a health outcome in humans, but is limited because chance, bias, and confounding could not be ruled out with confidence.
Inadequate/Insufficient Evidence to Determine Whether an Association Does or Does Not Exist	The available studies are of insufficient quality, consistency, or statistical power to permit a conclusion regarding the presence or absence of an association between an exposure to a specific agent and a health outcome in humans.
Limited/Suggestive Evidence of No Association	There are several adequate studies covering the full range of levels of exposure that humans are known to encounter, that are mutually consistent in not showing a positive association between exposure to a specific agent and a health outcome at any level of exposure. A conclusion of no association is inevitably limited to the conditions, levels of exposure, and length of observation covered by the available studies. In addition, the possibility of a very small elevation in risk at the levels of exposure studied can never be excluded.

Source: IOM [11].
[a] IOM has since updated the classification language, but the same general underlying considerations for reaching each conclusion. The previous classification categories were retained as we believed the previous categories were clearer than the updated categories.

3.2. Toxicokinetics

Inhaled EtO is rapidly and well absorbed via the respiratory tract and systemically circulated (~75–80%) and widely distributed to tissues such as muscle, brain, blood and testis. EtO largely is metabolized via phase I and II enzymes and glutathione S-transferase (GST), mainly in the liver and kidneys. It does not accumulate in tissues and is excreted primarily in urine. The human half-life in the body is estimated to be about 40 min [12,13].

3.3. Experimental animal studies

3.3.1. Carcinogenicity bioassays

Four EtO inhalation carcinogenicity assays were identified, including two in rats [14,15], and one in mice [16]. Lynch et al. [17] also evaluated target organ toxicity in monkeys but because monkeys were only exposed for two years, the study is not informative regarding tumorigenicity. All studies were determined to be high overall quality; however, some deficiencies were noted in each study, as discussed below and in Supplemental Table S.1 and S.2-S.5.

Snellings et al. [15] exposed Fischer F344 rats to 0, 10, 33, or 100 ppm of EtO vapor via whole-body inhalation for 6 h per day, 5 days per week, for approximately 2 years. Mortality was increased in exposed rats, beyond that associated with a non-treatment-related infection in the colony, reaching statistical significance in the 100-ppm group. At 24 months, the incidence of mononuclear cell leukemia (MNCL) was statistically significantly increased ($p < 0.001$) in both the 33- and 100-ppm exposure groups of female rats, relative to controls: there also was a statistically significant dose-related trend. Brain tumors, including gliomas, malignant reticuloses, and granular cell tumors, were statistically significantly increased in both sexes at 33 and 100 ppm. There also was a statistically significant increase in peritoneal mesothelioma tumors (originating in the testicular serosa) in males, relative to controls. The frequency of multiple primary neoplasms (benign or neoplastic) was statistically significantly greater for the male rats in the 100-ppm group and significantly greater ($p < 0.05$) for female rats in all three exposure groups, relative to controls. Although this study was rated as high quality overall, an infection in the colony caused high mortality and an interruption in dosing. Further, a possible exceedance of the maximum tolerated dose (MTD) at 100 ppm was noted.

In a study carried out by NIOSH, male Fischer 344 rats [14,17] and cynomolgus monkeys [14] were exposed to 0, 50, or 100 ppm of EtO vapor via whole-body inhalation for 7 h per day, 5 days per week, for 24 months. The two species were housed together during exposure. There was a statistically significant increase in the incidence of mononuclear cell leukemia (MNCL) ($p = 0.03$) in rats in the 50-ppm but not the 100-ppm group compared to controls (interim and terminal sacrifice): when mortality was adjusted for, the exposure-response trend also was significant. The incidence of peritoneal mesothelioma, arising from the tunica vaginalis of the testis and spreading into the peritoneal cavity, was increased significantly ($p = 0.002$) in rats exposed at 100 ppm, relative to controls. Tests for trends indicated dose-response relationships for mesothelioma and glioma. The incidence of other neoplasms common in F344 rats, including pituitary adenomas, islet cell adenomas of the pancreas, pheochromocytomas of the adrenal gland, and interstitial cell tumors of the testes, were not statistically significantly

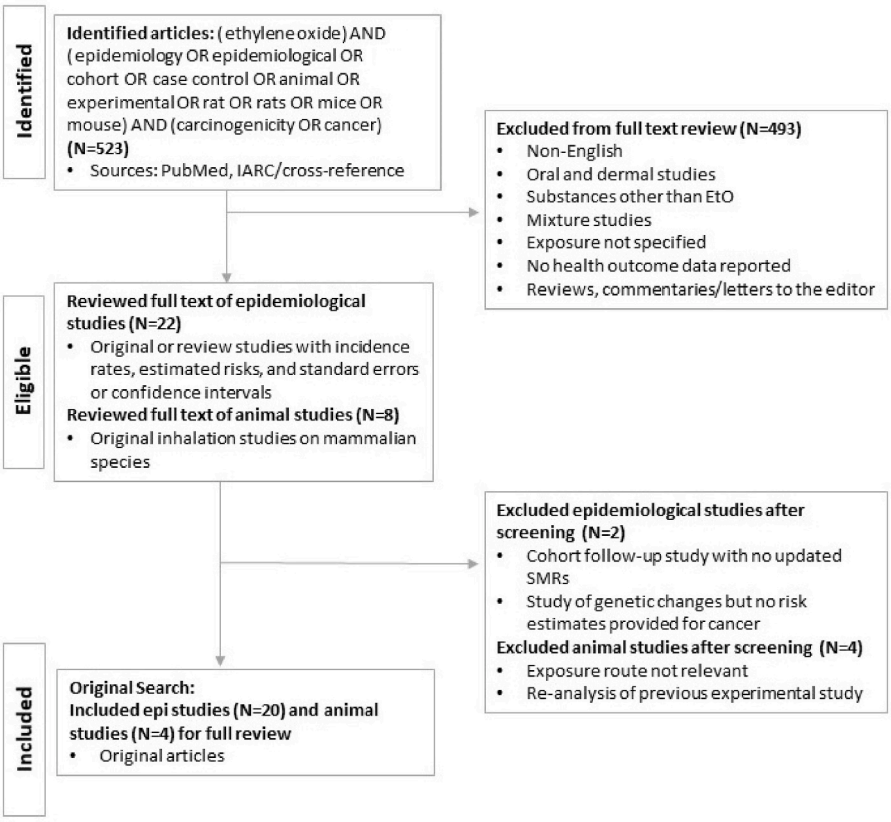

Fig. 2. Literature search and selection process (epidemiology and toxicology).

increased in exposed animals.

Three of the tumor types observed in rats may not have been treatment related. MNCL commonly develops in aging F344 rats (no longer used in cancer bioassays for this reason), occurring in 20–50% of animals at 20–24 months [18,19]. Similarly, spontaneous mesothelioma is also common in aging male F344 rats, a strain no longer used in cancer bioassays [20]. With regard to the brain tumors, a detailed analysis of the original Snellings et al. [15] data by Garman et al. [21] reported that the brain tumors were "similar in appearance to those that develop spontaneously in rats," but larger. The authors compared brain tumor incidence to historical controls and reported that the incidence in the study was higher than historical controls (8% in the high-dose and about 6% in the mid dose groups compared to about 0.6% in historical controls).

NTP [16] exposed male and female B6C3F1 mice to 0, 50, and 100 ppm of EtO vapor via whole-body inhalation for 6 h per day, 5 days per week for 102 weeks. No body weight changes or respiratory effects were observed. Statistically significantly increased incidence of alveolar/-bronchiolar carcinoma in males and of alveolar/bronchiolar adenoma and alveolar/bronchiolar carcinoma (combined) in female mice were observed among animals in the highest exposure group [16]. Incidence of Harderian gland papillary cystadenoma was increased at 50 ppm and at 100 ppm in males and females [16]. Female mice in the 100-ppm group also exhibited a slightly increased incidence of malignant lymphoma and uterine adenocarcinoma. Female mice in the 50-ppm but not 100 ppm group also exhibited increased incidence of hepatocellular adenoma and mammary gland adenocarcinoma or adenosquamous carcinoma combined [16].

Alveolar/bronchiolar adenomas, alveolar/bronchiolar carcinomas, and Harderian gland tumors commonly arise spontaneously in aging mice. In this study, the incidence of these tumors was slightly above the reported upper range of incidence in historical controls (at 100 ppm for the lung tumors and at both doses for the Harderian gland tumors).

3.3.2. *Synthesis of experimental animal evidence*

Overall, experimental animal cancer bioassays indicate that relatively high EtO exposures (33–100 ppm) are associated with increased incidence of several tumor types, including MNCL and brain tumors in rats and alveolar/bronchiolar adenoma or carcinoma, lymphoma, uterus, and mammary gland tumors in mice. Some of these tumors may be spontaneous and within historical control levels (MNCL, mesothelioma), but the remainder were considered treatment related.

3.4. *Mechanistic and mode of action information*

Mode of action (MOA) analysis is an important step in the assessment of the human carcinogenic potential of an agent [22]. As discussed above, in laboratory studies with mice and rats, chronic inhalation exposure to EtO has been associated with tumors of the mammary gland, brain, lung, peritoneum, and uterus, as well as lymphoma [14–17]. Thus, the primary purpose of this analysis is to evaluate the mechanisms underlying carcinogenicity of inhaled EtO in animals that might be relevant to human malignancies, such as those of the lymphohematopoietic system and breast.

Overall, the MOA is expected to be driven by the reactivity of EtO – specifically, EtO is a direct alkylating agent that can bond covalently with DNA. Insufficient repair or misrepair of DNA adducts can lead to mutations, including mutations in proto-oncogenes and tumor suppressor genes, as well as cytogenetic damage. Cellular proliferation and clonal expansion of cells with heritable DNA damage (pre-neoplastic foci) can lead to cancer progression and ultimately tumor formation.

EtO consistently has been shown to produce DNA adducts, mutations, and chromosome-level effects in *in vitro* studies with bacterial and mammalian test systems, *in vivo* studies with laboratory animals, and studies of occupationally exposed humans. Limited to no evidence is available for alternative modes of carcinogenic action [5]. While it is possible that other processes (e.g., oxidative stress) contribute to

EtO-induced tumor formation, mutagenesis and genotoxicity currently are considered the primary modes of action underlying possible carcinogenicity in humans.

3.5. DNA adduct formation

The molecular initiating event in the mutagenic/genotoxic mode of carcinogenic action is DNA adduct formation. EtO forms several different adducts with cellular macromolecules such as proteins (e.g. hemoglobin) and DNA [5]. EtO acts by the SN2 (substitution-nucleophilic-bimolecular) mechanism and has high reactivity (Swain-Scott substrate constant s-value of 0.96) [5]. Due to these properties, the predominant DNA adduct formed by EtO is N7-(2-hydroxyethyl)guanine (N7-HEG); however, other DNA adducts, including N3-hydroxyethyladenine (N3-HEA), and O6-hydroxyethylguanine (O6-HEG), are also formed at lower levels [23–27].

Dose-dependent increases in N7-HEG adduct formation have been reported in several *in vivo* studies with rodent models, following acute and subacute inhalation exposures to EtO, including at levels below and in the range of those used in chronic cancer bioassays; N7-HEG adduct formation occurred non-specifically in all tissues evaluated [24,26–33]. No studies of adduct formation in the stomach were identified: a single intraperitoneal administration study reported no statistically significant increase in N7-HEG in stomach tissue [34]. While formation of O6-HEG and N3-HEA adducts were detected in one *in vivo* study with rats following subacute inhalation exposure to EtO, the exposure level used was several times higher than the highest level used in chronic cancer bioassays (and these adducts were found at levels 250 to 300-times lower than those of N7-HEG) [27]. In occupational exposure studies, increased levels of N7-HEG adducts reportedly were found in white blood cells and granulocytes from EtO-exposed persons relative to those in the reference group; however, considerable interindividual variation was detected, and the differences were not statistically significant ([5, 35]; Van Delft et al., 1994, as cited in EPA [5]). No evidence of EtO-induced O6-HEG or N3-HEA formation in humans was identified.

Interpreting the DNA adduct studies is complicated by the fact that, as discussed above, EtO also is produced endogenously (Bolt 2000 and Tornqvist 1996, as cited in EPA [5]). Thus, all humans have endogenously produced EtO adducts associated in their DNA (as well as hemoglobin), and in exposed populations, any adducts that were quantified would be the result of both endogenous production and background environmental exposures [3]. Background levels of EtO adducts are highly variable in the population; thus, changes in frequencies are small and it is difficult to separate adducts formed by different exogenous exposures and to separate those formed by exogenous relative to endogenous sources (notably, however recent efforts have been made to quantify “background” EtO exposures) [3]. Further, the biological significance of increased DNA adduct formation, particularly if relatively small (and often within the range of overall population background levels) is unclear because adducts may not lead to mutations (or may be present in intergenic sequences) or chromosomal aberrations.

3.6. Mutagenesis

The specific DNA adducts responsible for and mechanisms underlying EtO-induced mutagenicity are not known. While the N7-HEG adduct is the predominant adduct formed by EtO DNA-alkylation, this adduct is not directly mutagenic [5]. The position of the N7-HEG adduct makes it unlikely to interfere with the hydrogen bonding involved in DNA base-pairing and allows it to be rapidly depurinated by DNA glycolase enzymes involved in base excision repair processes (Boysen et al., 2009, as cited in EPA [5]). It has been hypothesized that imidazole ring-opening of N7-HEG may result in stable potentially mutagenic lesions (Solomon et al., 1999, as cited in EPA [5]); however, this has not been demonstrated *in vivo* [36]. Further, it has been hypothesized that depurination of N7-HEG may result in accumulation of apurinic sites, which can result in mutations due to miscoding during cell replication ([5,27]; Walker et al., 1993 as cited in EPA [5]) however, accumulation of apurinic sites was not observed following inhalation exposure to 100 ppm EtO in rats [30].

While present at much lower levels, N3-HEA and O6-HEG adducts may contribute to mutagenesis. Specifically, N3-HEA adducts can interact with the minor groove of the DNA helix, leading to strand scission at the replication fork and inhibition of DNA replication (Mazon et al., 2009, as cited in EPA [5]). N3-HEA also indirectly may lead to mutations through accumulation of apurinic sites or imidazole ring-opening, through mechanisms similar to that for N7-HEG [37]. Further, O6-HEG adducts directly can interfere with nucleotide base-pairing (typically resulting in thymine incorporation) and be highly pro-mutagenic (Mazon et al., 2009, as cited in EPA [5]). However, because these adducts occur at such low levels (~250–300-times lower than N7-HEG adduct levels in *in vivo* studies), they are believed not to be responsible for all observed mutagenicity [5,27,36].

As noted above, *in vivo* studies have demonstrated dose-dependent increases in mutation frequencies (at the *Hprt* and *LacI* genes) following subacute inhalation exposures to EtO at levels in the ranges used in chronic cancer bioassays [31,32,38–40]. *Hprt* and *LacI* genes are used as surrogates for cancer-associated mutagenesis but are not directly involved in cellular transformation. With respect to effects the lymphohematopoietic system, increased mutation frequencies were measured in splenic lymphocytes and T cells of rats [5,31,32], as well as splenic and thymic lymphocytes and bone marrow of mice [38,40]. Increased mutation frequencies also have been reported in other tissues, including the testes and lung [38,41,42]. Further, the tumors from the EtO-exposed mice from the NTP [16] cancer bioassay (mammary gland carcinomas, as well as lung, Harderian gland, and uterine tumors) were found to have increased mutation frequencies as well as distinct mutation spectra (relative to those of spontaneous tumors), including in proto-oncogenes (*Hras* and *Kras*) and tumor-suppressor genes (*Trp53*) [43,44]. This evidence indicates that EtO induced mutations in proto-oncogenes and tumor-suppressor genes, which subsequently contributed to tumor formation in multiple tissues [43,44]. However, as these mutations were found in terminal tumor tissues, it is unclear if mutations resulted from direct EtO genotoxicity or were secondary to other events not related to genotoxicity.

A few studies have evaluated gene mutations in humans occupationally exposed to EtO ([5,45–47]; Major et al., 2001, as cited in EPA [5]). Statistically significantly increased *Hprt* mutation frequencies in peripheral blood lymphocytes were identified among factory workers: *Hprt* frequencies also were reported to be weakly but not statistically significantly increased among hospital workers [47]; however, the factory workers were more likely to be exposed to other chemicals than the hospital workers. In other studies, no significantly increased *Hprt* mutation frequencies were detected in peripheral blood lymphocytes of exposed populations ([5,47]; Major et al., 2001, as cited in EPA [5]). In a cross-sectional study of workers at a sterilization plant in Egypt, gene mutations in *p53* exons were detected in blood DNA of exposed workers; however, they were measured in only 7–13 workers per exposure group. Mutations were not consistently observed across all exons and were somewhat variable across groups with different exposure profiles. No statistical tests were performed, and most importantly, there was no comparison group, i.e., mutations were not measured in an unexposed group [45].

3.7. Cytogenic damage

The specific mechanistic events underlying EtO induced-chromosomal damage are not known [5]. Potential mechanisms via which EtO-induced DNA adducts may ultimately progress to chromosomal damage are described below.

With respect to N-alkylated bases, N7-HEG and N3-HEA DNA adducts are removed by base excision repair ([5,37]; Memisoglu and Samson 2000, as cited in EPA [5]). The base excision repair process involves cutting the DNA on either side of the adducted base, removal of the adducted base creating an abasic site, replacement of the base using the opposite strand as a template, and ligation of the newly inserted base into the DNA strand [37]. Thus, inherently, the base excision repair process can create apurinic sites and single strand breaks (Memisoglu and Samson 2000, as cited in EPA [5]). In general, apurinic sites and DNA single strand breaks can lead to DNA double strand breaks during replication through fork collapse. If not repaired, double strand breaks can lead to DNA mutations or cytogenetic effects, which may then lead to cellular transformation and ultimately potentially tumor formation.

With respect to O6-HEG, if the lesion is not removed prior to DNA replication, replication can still occur past the adduct, but the O-alkylated guanine is mis-paired with T instead of C. The DNA mismatch repair system then attempts to correct this error by excising the T, which creates a strand gap. The strand gap may be repaired and filled with the correct residue. However, if the strand gap persists, it can block the subsequent round of DNA replication, and the stalled replication fork can induce double strand breaks and resulting chromosomal aberrations [37].

As noted above, Rusyn et al. [30] failed to demonstrate accumulation of apurinic sites in rodents. However, in *in vivo* studies with rodents and monkeys, inhalation exposures to EtO were associated with cytogenic damage, including single strand breaks, unscheduled DNA synthesis, chromosome aberrations, sister chromatid exchanges, and micronuclei formation ([5,14,17,32,48–51]; Preston and Abernethy 1993, Lorenti Garcia et al., 2001, Ong et al., 1993, Sega et al., 1988, Vergnes and Pritts 1994, Yager and Benz 1982, 1987, as cited in EPA [5]). However, with respect to sister chromatid exchanges, OECD no longer recommends sister chromatid exchange testing because it is considered an unreliable endpoint to assess genotoxicity [53].

Specifically, subacute to chronic EtO inhalation exposure in the range of concentrations used in rodent cancer bioassays were associated with increases in sister chromatid exchanges in various cell types in the lymphohematopoietic system, including peripheral blood lymphocytes and splenic lymphocytes ([5,31,49,50]; Lorenti Garcia et al., 2001, Ong et al., 1993, Preston and Abernethy 1993, Yager 1982, 1987, as cited in EPA [5]). A few studies provide evidence of micronuclei formation and chromosome aberrations in rodent lymphocytes and bone marrow cells ([5,48,52]; Ribero et al. 1987, as cited in EPA [5]).

In several human studies, chromosomal effects (i.e. strand breaks, cross-links, chromosome aberrations, sister chromatid exchanges, and micronuclei formation) in lymphocytes, as well as buccal cells and nasal mucosa cells, were associated with occupational EtO exposures, and were generally found to be related to exposure level and duration (See Occupational Exposure section of Supplemental Table S.6).

4. Dose and temporal concordance of key effect and outcome events

Understanding the dose and temporal dependency of key events and their relationship to the onset of apical events informs cancer risk conclusions. Herein we evaluate the dose and temporal dependency of selected key events in the proposed mutagenic/genotoxic mode of action for EtO-induced lymphohematopoietic and breast cancers. Specifically, we use the information available from *in vivo* EtO inhalation studies with laboratory animals pertaining to tumor formation (focusing on mononuclear cell leukemia, lymphoma, and mammary gland tumors), as well as induction of DNA adducts, DNA point mutations, and chromosome level effects. The onset doses and timing of carcinogenesis and selected key events are discussed below and presented in Supplemental Table S.6. Dose concordance for key events in leukemia and lymphoma development in experimental animal studies are provided in Figs. 3 and 4, respectively.

4.1. Tumor formation

Increased incidence of lymphohematopoietic malignancies has been reported in chronic inhalation carcinogenicity studies with laboratory animals [14–17]. In one study, increased incidence of MNCL was observed in female F344 rats (but not male rats) exposed to EtO at 33 and 100 ppm (but not 10 ppm), exposure levels associated with statistically significant decrease in body weight [15]. Another study reported statistically significantly increased incidence of MNCL in male F344 rats exposed to EtO at 50 and 100 ppm (all exposure levels tested): high mortality and extramedullary hematopoiesis may have been associated with MNCL [14,17]. A study with B6C3F1 mice observed statistically significantly increased incidence of malignant lymphoma in female mice (but not male mice) exposed to 100 ppm (but not 50 ppm) [16]. In the same study, female mice exposed at 50 ppm (but not 100 ppm) exhibited increased incidence of mammary gland tumors [16].

4.2. Relationship with key events

4.2.1. Evidence from inhalation studies with rats

In chronic inhalation carcinogenicity studies with F344 rats, increased incidence of MNCL was observed at EtO exposures as low as 33–50 ppm [14,15]. The molecular initiating event in the proposed mutagenic/genotoxic mode of action for leukemia is DNA adduct formation. Increased formation of N7-HEG adducts was consistently reported to occur in splenic tissue from F344 rats at the lowest EtO exposure levels evaluated [26,27,29,30,33]. Specifically, N7-HEG adduct formation was associated with exposures as low as 3 ppm in subacute studies [33], as well as in acute studies at slightly higher exposures (as low as 10 ppm) [29]. These are lower exposure levels and shorter exposure durations than those associated with MNCL in chronic carcinogenicity studies [14,15,17], suggesting both dose and temporal concordance. However, N7-HEG adducts are not directly mutagenic and may not contribute to mutation formation.

With respect other DNA adducts, one subacute study reported significantly increased formation of O6-HEG and N3-HEA adducts in spleen tissues from F344 rats at 300 ppm [27]. However, It should be noted that this exposure level is up to nine times higher than the lowest observed onset level for MNCL (33 ppm) in F344 rats and three times higher than the highest level used in the chronic carcinogenicity studies (100 ppm; which was associated with excess mortality, as well as splenic toxicity) [14,15,17]. Thus, the relevance of EtO-induced O6-HEG and N3-HEA adduct formation to cancer development remains unclear.

DNA adducts can be repaired or misrepaired leading to DNA mutations. Increased frequency of *Hprt* mutations were reported to occur in splenic lymphocytes and T-cells from rats in subacute studies [5,31,32,39]. In one study, increased mutation frequencies were observed in splenic lymphocytes from Lewis rats following subacute exposure to 50 ppm [5,39], which is a comparable exposure level and shorter exposure duration than those inducing MNCL in chronic carcinogenicity studies [14,15,17]. Other subacute studies also reported increased frequencies of point mutations in splenic lymphocytes and T cells from Lewis and F344 rats, respectively [31,32]; however, the exposure levels (200 ppm) were above those used in the chronic studies, and thus are not useful for determining dose concordance.

With respect to chromosome level effects, increased sister chromatid exchanges in relevant tissue types were reported in various acute, subacute, and subchronic studies with F344 and Lewis rats ([5,31,50]; Lorenti Garcia et al., 2001, Preston and Abernethy 1993, Ong et al., 1993, as cited in EPA [5]). Of note, in two subacute studies, increased sister chromatid exchanges in splenic lymphocytes were observed in Lewis rats following exposures to EtO at 50 ppm ([5,31]; Lorenti Garcia et al., 2001, as cited in EPA [5]), a similar exposure level to (but shorter exposure duration than) those associated with the development of MNCL in chronic studies with F344 rats (33–50 ppm) [14,15,17]. While sister chromatid exchanges also consistently were found to be positive at

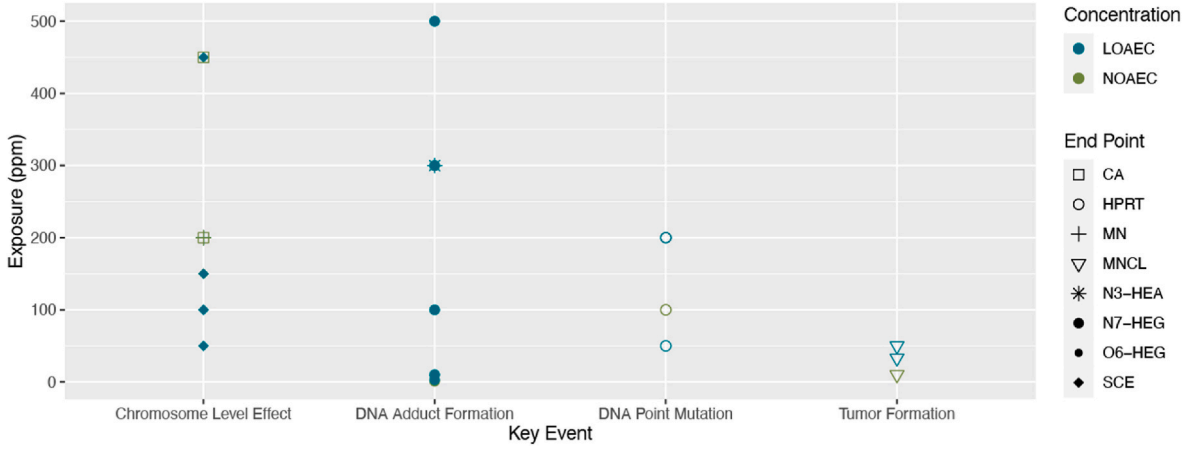

Fig. 3. Dose concordance for key events in leukemia in rats.

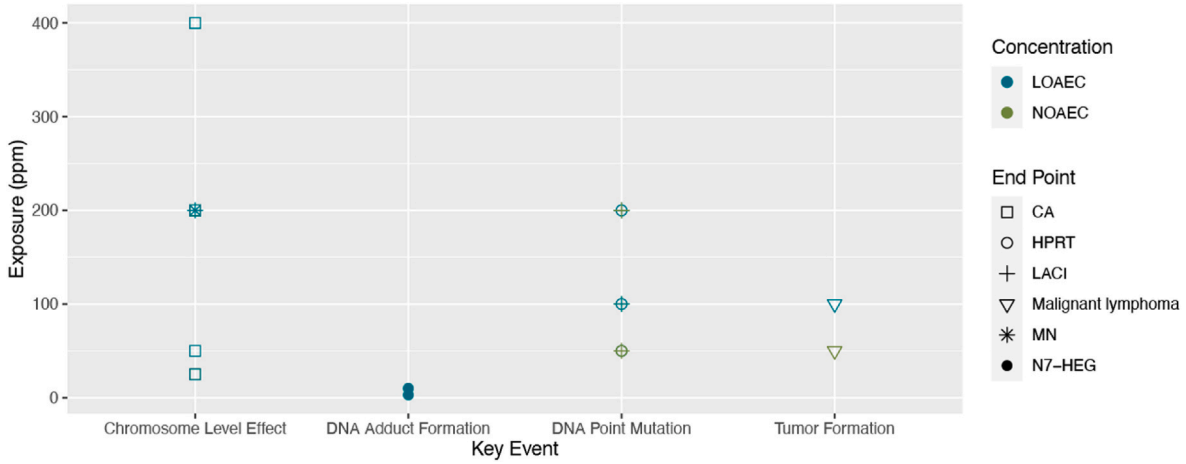

Fig. 4. Dose concordance for key events in lymphoma in mice.

the lowest exposure levels evaluated in various other subacute and subchronic studies, the levels used in these studies (100–150 ppm) were above the threshold for MNCL, as well as general toxicity, in chronic studies [14,15,17] and thus are not useful for determining dose concordance. However, as noted above, SCEs are no longer recommended by OECD for genotoxicity testing [53].

Negative results were obtained for chromosome aberrations in peripheral blood and splenic lymphocytes from F344 and Lewis rats with acute exposures of 450 ppm and subacute exposures of 200 ppm, respectively [31,50], which are higher than those inducing MNCL in chronic carcinogenicity studies [14,15,17]. Thus, the evidence does not indicate that chromosome aberrations would contribute to tumor formation at human-relevant exposures.

The evidence of EtO-induced micronuclei formation was mixed. In one subacute study, exposures of 50, 100 and 200 ppm all were negative for micronuclei formation in splenic lymphocytes from Lewis rats (Vergnes and Pritts 1994, as cited in EPA [5]), suggesting that micronuclei formation does not occur at exposures similar to or greater than those associated with MNCL formation in chronic studies with F344 rats. In another subacute study, statistically significantly increased micronuclei formation was reported in bone marrow cells of F344 rats exposed to 200 ppm EtO [31]; however, this exposure level is higher than the onset dose for MNCL and general chronic toxicity and carcinogenicity [14,15,17], and thus of unclear relevance to lower exposures.

4.2.2. *Evidence from inhalation studies with mice*

In a chronic inhalation carcinogenicity study with B6C3F1 mice, increased incidence of malignant lymphoma of the hematopoietic system was observed in female mice (but not male mice) at exposures of 100 ppm (but not at 50 ppm) [16]. Induction of certain key events has been demonstrated in relevant tissue types in mice following exposure to EtO at similar or lower levels and shorter durations than those associated with lymphoma tumor formation, demonstrating dose and temporal concordance.

Specifically, increased formation of N7-HEG adducts was reported to occur in splenic tissues from B6C3F1 mice following subacute exposures to EtO at the lowest concentrations evaluated (3–10 ppm) [27,33], below those associated with malignant lymphoma in the chronic carcinogenicity study [16]. However, as discussed above, N7-HEG adducts are not directly mutagenic. No studies evaluating formation of other DNA adducts, including the directly mutagenic O6-HEG or N3-HEA, in mice following EtO exposures were identified.

With respect to DNA point mutations, increased frequency of *Hprt* mutations were observed in splenic lymphocytes from B6C3F1 (*LacI* transgenic) mice following subacute exposure to EtO at concentrations as low as 50 ppm (the lowest exposure level evaluated) [40], which is the same exposure level associated with lymphoma in the chronic carcinogenicity study [16], suggesting dose and temporal concordance. Increased chromosomal aberrations were reported in peripheral blood lymphocytes from B6C3F1 mice following chronic EtO exposures of 25 ppm and 50 ppm for 48 weeks and 24 weeks, respectively [48]. Induction of chromosomal aberrations was associated with lower exposure levels and shorter exposure durations (e.g. 25 ppm for 48 weeks) than those seen with lymphoma development (50 ppm for 102 weeks) [16].

While increased micronuclei formation was found in bone marrow from B6C3F1 mice following subacute exposure to 200 ppm EtO (Vergnes and Pritts 1994, as cited in EPA [5]), this exposure level (200 ppm) is four times higher than the lowest concentration shown to induce lymphoma (50 ppm) in the chronic carcinogenicity study with B6C3F1 mice (and two times higher than the highest concentration used (100 ppm). Thus, the contribution of micronuclei formation to lymphoma development in mice cannot be demonstrated based on the available evidence.

Further, in the same chronic inhalation carcinogenicity study with B6C3F1 mice, an increased incidence of mammary gland tumors was observed in female mice at exposures of 50 ppm but not at 100 ppm [16]. Laboratory animal studies informing the key events underlying EtO-induced mammary gland tumor formation are limited. Houle et al. [44] evaluated mammary gland tumors from mice exposed to 50 ppm EtO in the NTP [16] cancer bioassay, and reported increased mutation frequencies in *Hras* (proto-oncogene) and *Trp53* (tumor-suppressor genes) in the tumor tissues, as well as distinct mutation spectra suggestive of EtO-induced mutagenesis. These EtO-induced mutations in proto-oncogenes and tumor-suppressor genes likely contributed to tumor formation in the mammary gland tissue [44]. However, it cannot be determined if the observed mutations resulted from direct EtO genotoxicity or were secondary to other events not related to genotoxicity. No other studies evaluating key events were identified for mammary gland tissues in mice or other species. Thus, the current body of relevant evidence is insufficient for evaluation of dose and temporal concordance for mammary gland tumors.

4.3. Synthesis of MOA information

Overall, the evidence generally supports a mutagenic/genotoxic mode of action for EtO-induced tumor formation. When comparing studies evaluating the same species and tissue types, thresholds for induction of certain key events occur at exposure levels similar to or below the observed thresholds for carcinogenesis, demonstrating dose concordance of early events. Additionally, certain key events occur with shorter exposure duration than those identified as inducing tumorigenesis, further demonstrating temporal concordance. With regard to dose-response relationships, there is some evidence that N7-HEG adducts accumulate more slowly at low doses due to DNA repair, accumulate somewhat linearly at middle doses, and more steeply once DNA repair mechanisms are saturated (See Supplemental Table S.6). However, these adducts are not directly mutagenic; thus, while DNA adduct formation is the molecular initiating event in the MOA, it is not a rate-limiting process or indicator of the point at which carcinogenesis may develop. Formation of mutations or chromosome effects that can directly cause cellular transformation and cancer progression are the rate-limiting processes in carcinogenicity. However, for both of these endpoints, there are no studies at doses below those believed to induce tumors.

DNA mutations and chromosomal aberrations are more biologically significant (relative to DNA adduct formation) because they contribute to cellular transformation and cancer progression. Some evidence suggests that direct-acting DNA reactive agents, including DNA alkylating agents such as EtO may exhibit threshold exposure-response relationships for genotoxicity, specifically DNA mutations and chromosomal aberrations [37]. Several biological mechanisms contribute to threshold responses for genotoxicity, including protective mechanisms such as detoxification of the genotoxic agent, exclusion from the nucleus and repair of DNA damage, and involvement of redundant targets (e.g. spindle fibers). With respect to DNA alkylating agents, several studies suggest that DNA repair may play a key role in potential threshold exposure-response relationships [37].

Specifically, EtO forms adducts that are repairable and dose-response trends in the available studies indicate that these adducts may be cleared effectively at low exposures. Specifically, both N7-alkylguanine and N3-alkyl adenine adducts are repaired by DNA glycolases of the base excision repair (BER) pathway. Further, O6-alkylguanine adducts are repaired by alkylguanine DNA transferases (AGT) in an enzymatic suicide process. At higher exposures (e.g. above 33 ppm in mice and 100 ppm in rats) [27], repair mechanisms may become saturated, resulting in persistence and accumulation of DNA adducts and potential for conversion into biologically significant DNA mutations or chromosomal aberrations. There is some evidence that various N7G, N3A and O6G-alkylating agents exhibit thresholds of activity for mutagenicity and chromosome damage due to saturation of DNA repair [37]. Studies of EtO-induced mutations and chromosomal aberrations are not available in the low dose range; however, it is plausible that EtO also would exhibit such a dose-response relationship for genotoxicity.

In addition to DNA repair, other protective metabolic mechanisms may explain observed threshold responses. EtO is detoxified by two major metabolic pathways, glutathione conjugation and hydrolysis (enzymatic and non-enzymatic) [5,54]. Glutathione may become depleted at high exposures, resulting in dose-disproportionate increased tissue doses of EtO, which may form adducts with DNA and other cellular macromolecules. EtO also can form adducts with other cellular macromolecules, such as proteins [5]. Thus, it is possible that EtO may interact with cellular macromolecules in the cytoplasm, resulting in exclusion of some EtO from the nucleus. It also is possible that EtO damages DNA replication and repair enzymes or spindle fibers involved in chromosome segregation, which could result in thresholds in genotoxic responses due to the involvement of these redundant targets [37].

5. Epidemiological evidence

5.1. Overview

The full text of 22 publications, including 20 occupational cohort studies reporting results from 9 separate occupational cohorts and 2 case-control studies, was reviewed. The cohorts studied consisted of EtO production workers (9 studies) and/or sterilization workers (10 studies) or hospital workers not necessarily involved in sterilization (3 studies). The cohort studies evaluated a variety of cancers, the vast majority of which indicated no association with EtO exposure.

There were five key cohorts with multiple publications, some updating results based on extended follow-up time. The first is a Swedish cohort that included 2170 sterilization workers employed for at least one year in one of two plants that produced disposable medical equipment sterilized with EtO [55–57]. The second, (the “NIOSH cohort”), followed more than 18,000 US workers exposed to EtO at 14 plants producing sterilized medical supplies and spices [58–63]. Park et al. [58] analyzed the association between EtO exposure and job termination but did not include SMR updates and was excluded from further review.

The third cohort consisted of 2174 men employed between 1940 and 1978 by a large chemical company in West Virginia and assigned to the EtO production department [10,64,65]. The fourth cohort consisted of 2876 British men and women with potential exposure to EtO from four companies and eight hospitals that used or produced EtO beginning in the 1950s [66,67]. The fifth cohort included 709 Swedish chemical production workers exposed to EtO [68,69].

Analyses of the remaining four cohorts were reported in one article each. Bisanti et al. [70] followed 1971 Italian chemical workers licensed to handle EtO; no exposure data were available. Norman et al. [71] evaluated a cohort of 1132 sterilization workers with assumed exposure to EtO. Morgan et al. [72] studied a cohort of 812 male chemical plant workers potentially exposed to EtO in Texas. Kardos et al. [73] followed 299 female pediatric hospital workers in Eger, Hungary. All of the cohorts used national mortality rates, death certificates, or cancer registries to calculate SMRs or SIRs.

Additionally, one case-control [74] and one case report [75] were identified in which EtO was included as a potential exposure. Kiran et al.

[74] conducted a population-based case-control study of 2347 lymphoma cases and 2463 controls from 6 European countries. Occupational exposure to 35 different chemicals was evaluated; however, only 31 lymphoma cases and 27 controls were identified as ever having been exposed to EtO based on interview responses evaluated by industrial hygienists. The second study [75] conducted cytogenetic analysis of 61 EtO-exposed nurses and 125 historical, local, and hospital controls in Eger, Hungary. As the study reported mean total aberrations, chromatid aberrations, and chromosome aberrations for women in reproductive age, odds ratios and risk ratios were not reported and the study was excluded from further review.

For the current review, we selected the following as the primary cancer outcomes, all of which were addressed in multiple (primarily cohort) studies: stomach cancer (12 studies), breast cancer (9 studies) and lymphohematopoietic malignancies (15 studies).

5.2. Quality evaluation

Of the 19 cohort study publications, 9 were rated overall as low quality and 10 as high quality. However, there were differences in quality ratings across all domains assessed (See Supplemental Tables S.7 and S.8). The single case-control study [74] was rated as medium quality. The results of the quality evaluation are briefly summarized below.

Tier I: The rating for exposure characterization varied, with studies using surrogates for exposure such as licensure [70] or job classification [66,69]; considering all employees as equally exposed [71]; or qualitatively stratifying exposure into three categories ("low", "medium", or "high") based on job title, manufacturing operations, and verified exposure measurements with industrial hygienists [57,60,64]. Studies using the last method noted were assigned medium or high ratings. Studies rated as low quality either did not quantitatively measure exposure to EtO and other chemicals or did not use an industrial hygienist-validated exposure surrogate such as job exposure matrices or exposure reconstruction based on area and personal samples. In Tier I, eight cohort studies were rated as low quality based on exposure characterization with one or more other low ratings among the remaining evaluation domains. The single case-control study, Kiran et al. [74], was advanced to Tier II due to its overall quality score and method used to characterize potential exposure.

Tier II: Few studies considered co-exposures or potential confounding factors beyond age, sex and calendar year. The Swaen et al. and Steenland et al. cohorts [60,64] either attempted to limit cohort members to individuals with no likely exposure to other potential carcinogens or addressed confounding among the exposed group through additional analysis such as Cox proportional hazard regression models accounting for length of employment. These studies received high overall quality ratings. Studies rated as low quality tended not to account for potential co-exposure (i.e. radiation, other process chemicals), or potential confounding factors including life style factors, BMI, reproductive history or parity (i.e., Wong [76]). Due to statistical control for confounding factors, the case control study by Kiran et al. [74] was rated as medium or high for each metric evaluated, and further evaluated in Tier III.

Tier III: Studies rated medium or high in Tier II were further evaluated and rated for overall quality as follows: four studies were classified as medium quality, generally because of reasonable efforts to control for potential confounding factors, robust characterization of exposure, and few (i.e., only one or two) other domains being rated as low quality [55–57,74]; and seven studies were rated high quality due to their control of potential confounding factors and reasonably robust characterization of exposure, as well as overall medium or high scores in the remaining TSCA criteria (i.e., [10,59–62,64,65]). Full details of the quality analysis are provided in Supplemental Tables S.7 and S.8.

In summary, nine studies were rated low quality, four (including the remaining case-control study) were rated medium quality, and seven were rated high quality overall. There were differences in quality ratings across all five domains assessed (see Supplemental Table S.7 and S.8). Generally, studies were rated medium or high quality in the domains of study participation and analysis. All 20 publications assessed received a high quality rating for the outcome assessment domain, as mortality and cancer incidence were ascertained using death certificates, cancer registries, and verified medical records, lending confidence to the outcome measurements. The remaining two domains (exposure characterization and potential confounding/variability control) represented the metrics that varied most across the studies and ultimately determined the overall quality score.

6. Synthesis of epidemiological evidence

6.1. Stomach cancer

For stomach cancer (and other cancers below) we based our evaluation on the most recent results reported for six cohorts (and one case-control study where appropriate). Primary results are presented by study quality category in a forest plot (Fig. 5). Except for one study rated as low quality, no study reported a statistically significant association between EtO and stomach cancer.

Hogstedt [69] reported an excess of deaths due to stomach cancer based on 10 observed stomach cancer deaths with about two expected deaths (SMR = 5.46, no CI reported). The authors also stratified cause of death by length of employment, with a reported SMR of 4.82 (95% CI calculated as: 2.66–10.22) for those employed 1–9 years and a SMR of 5.77 (6 observed and 1 expected) for those employed ≥10 years.

The remaining two studies rated as low quality [66,70] and the only study rated as medium quality [57] reported no excess risk among those exposed to EtO.

Six studies rated as high quality reported no statistically significantly increased risk of stomach cancer among those exposed to EtO [10,59–61,64,65]. Swaen et al. [64], updating Greenberg et al. [10], reported no increased risk of death from stomach cancer among workers exposed to EtO in a cohort of 2063 male production workers. Proportional hazard modeling for all causes, including stomach cancer, revealed no significant trends or associations with cumulative exposure to EtO. Steenland et al. [60] (updating [59,61,63]) similarly reported no increase in stomach cancer based on 25 total deaths in sterilization workers.

6.2. Breast cancer

The results of the cohort studies of breast cancer are shown in Fig. 6 (note that only the most recent update of cohort is shown in the forest plot).

Two studies rated low quality that provide the most recent updates of two cohorts for breast cancer and EtO reported equivocal results [66,71]. These studies were rated low quality largely due to failing to account for potential confounders such as family history of breast cancer, reproductive history and nulliparity. Wong and Trent [63] also received a low ranking; however, this cohort is captured in a more recent (and higher quality) study by Steenland et al. [60]. Both Wong and Trent [63] and Coggon et al. [66] reported no statistically significantly increased risk of breast cancer in the overall analyses, whereas Norman et al. [71] reported an increased SMR for breast cancer among the workers exposed to EtO, based on 12 observed deaths. The authors noted discrepancies between the follow-up period and number of people contributing to the observed and expected cancers, but the potential impact of this combined with the other deficiencies (e.g., two breast cancer deaths among women employed less than one month) is unclear.

The only study rated medium quality, Mikoczy et al. [57], found no overall increase in breast cancer incidence compared to an external population. Despite the overall deficit of observed breast cancers [57], additional analyses were performed comparing breast cancer rates in the two highest exposure groups to the rate for those in the lowest 50th

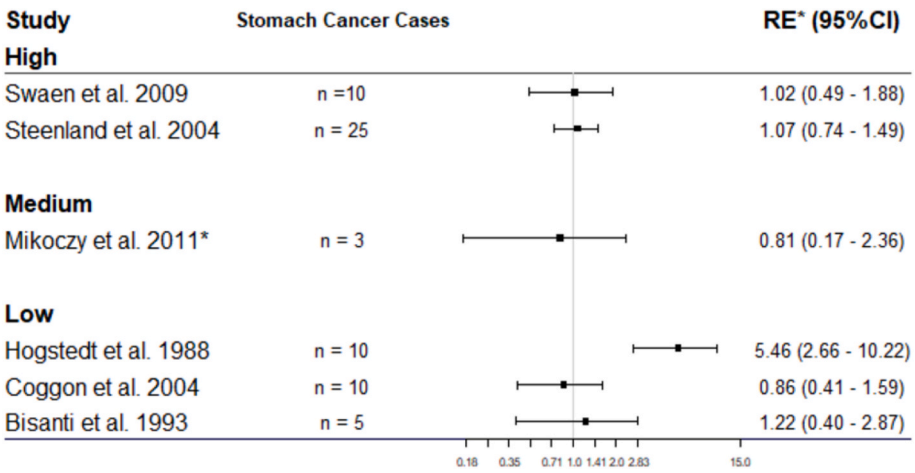

Fig. 5. Forest plot of SIRs and SMRs for stomach cancer by overall study quality rating category RE indicates relative effect size.
*Study reports an SIR.

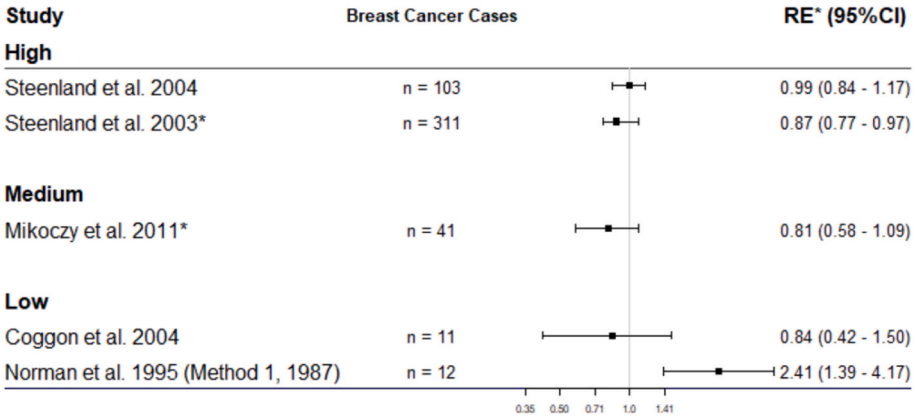

Fig. 6. Forest plot of SIRs and SMRs for breast cancer by overall study quality rating. RE indicates relative effect size.
*Study reports an SIR.

percentile of the exposure distribution. Because the deficit in risk was largest in the *lowest* exposure group, its use as the referent group generated spuriously large relative risk estimates (IRR 2.76, 95% CI; 1.20–6.33 and IRR 3.55, 95% CI; 1.58–7.93, respectively). Because the risk associated with the selected referent group was not a valid representation of the "background" risk, these findings are misleading. For there to be no overall excess occurrence of breast cancer incidence and a truly increased risk at higher exposure levels, one must conclude that EtO is powerfully protective of breast cancer at lower exposures (which is implausible).

Both studies rated high quality [60,62] found no overall increased risk of breast cancer. Steenland et al. (2004) [62] reported results for breast cancer incidence and Steenland et al. (2003) [60] reported breast cancer mortality based on the same cohort. Steenland et al. (2004) [62] reported a statistically significant deficit of incident breast cancer among 7576 women (SIR = 0.87, 95% CI: 0.77–0.97). Nevertheless, despite the clear deficit of incident breast cancer Steenland et al. (2004) [62] conducted additional analysis using the least exposed subgroup as the referent – the subgroup with the clearest deficit of breast cancers. Several analyses examining different exposure categories and periods of latency indicated increased relative risks (primarily driven by the anomalously decreased risk in the referent group); however, one analysis lagging exposures by 15 years generated a statistically significant odds ratio for the highest exposure group with more than 14,620 ppm-days cumulative exposure) (OR = 1.91, 95% CI; 1.22–2.15) [62]. Steenland et al. (2003) [60] found no increased risk of breast cancer mortality with a reported SMR approaching unity. The authors also did find a significant SMR when applying a 20-year lag among the highest exposure group with more than 12,322 ppm-days (SMR = 2.07, 95% CI; 1.10–3.54).

6.3. *Lymphohematopoietic malignancies*

We reviewed 14 cohort studies and 1 case-control study that reported results for all lymphohematopoietic malignancies (LHM) combined. Although some studies reported results for specific LHMs, few reported results for the same malignancy and many were based on small numbers. Though typically justified as a way to avoid the problem of evaluating small numbers of specific LHM, it rarely is appropriate to combine discrete and etiologically unrelated myeloid malignancies, and this likely holds true for the lymphatic malignancies [5,77]. However, in exchange for greater overall statistical stability, associations between risk factors of interest and specific LHMs will be masked. Nevertheless, as shown in Fig. 7, all but one study (again, the same one rated low quality for breast cancer) reported no association between EtO exposure and overall LHM risk.

Studies rated low quality reported mixed results for the combined group of all LHMs. Coggon et al. [63] and Wong and Trent [66] reported no excess of LHM overall or for any LHM subtype (SMR = 1.02, 95% CI: 0.74–1.38). Bisanti et al. [70] reported no excess of all hematopoietic cancers combined but reported a statistically significant excess of lymphosarcoma and reticulosarcoma based on four deaths (SMR = 6.82,

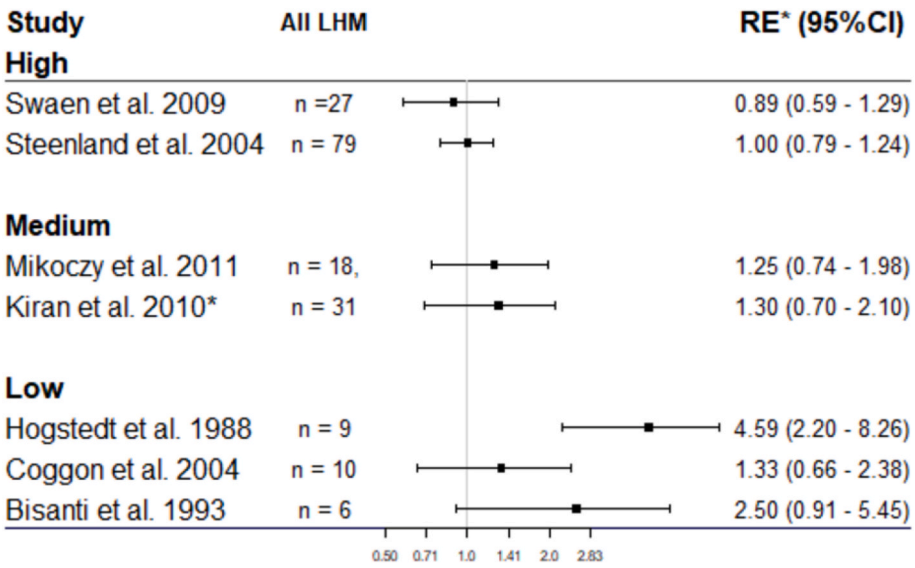

Fig. 7. Forest plot of SIRs and SMRs for LHM by overall study quality rating. RE = relative effect size; LHM = lymphohematopoietic malignancies. For Coggon et al. SMR is based on definite exposure in chemical manufacturers + continual exposure in hospitals.
*Study reports an OR.

95% CI: 1.86–17.45). Hogstedt et al. [69] reported a presumably statistically significant (i.e., no p-values or CIs were presented) excesses of "leukemia" (SMR = 9.21, based on 7 observed) and "blood and lymphatic" cancers (SMR = 4.59, 95% CI calculated as 2.20–8.26) among 709 workers.

Among studies rated medium and high quality, none reported excess mortality for all lymphohematopoietic malignancies [57,60,64]. Kiran et al. [74] conducted a population-based case-control study of 2347 lymphoma cases and 2463 controls from 6 European countries. Occupational exposure to 35 different chemicals was evaluated, and only 31 lymphoma cases and 27 controls were identified as ever having been exposed to EtO based on interview responses evaluated by industrial hygienists. Kiran et al. [74], the only case-control study included in the review, reported no statistically significant association between all "lymphoma" and EtO exposure. Similarly Mikoczy et al. [57], updating Hagmar et al. [55,56], reported no association between all LHMs combined and EtO exposure.

Although reporting no excess occurrence of LHMs – and as done with the breast cancer studies – Steenland et al. [60] reported an apparent positive exposure-response for lymphoid malignancy mortality in males, based on comparisons of more highly exposed groups with the least exposed group. Based on analysis controlling for age, race and date of birth, Steenland et al. [60] reported an odds ratio of 3.76 (95% CI: 1.03–13.64) for men in the highest exposure category and accounting for a 15-year lag; however, a similar association was not observed among women. As noted above in the breast cancer section, use of a reference group with an anomalously low risk will generate the spurious appearance of an increased risk in other exposure categories, even where there is no clear excess occurrence of these cancers. To reach an overall finding of no increased risk, the risk reportedly associated with high EtO exposure must be offset by a comparably protective effect at lower exposure levels. Steenland et al. [60] also presented specific SMRs for NHL, Hodgkin lymphoma and leukemias. For NHL, the SMR was 1.00 (95% CI: 0.72–1.35 based on 31 deaths) overall, but was higher for men and lower for women. The overall and sex-specific results were similar for Hodgkin disease (overall SMR = 1.24, 95% CI: 0.53–2.43), but showed a deficit overall for myeloma (overall SMR = 0.92, 95% CI: 0.54–0.87). The deficit for myeloma was not statistically significant in the sex-specific results. Standardized mortality ratio results for lymphocytic leukemia were not presented in Steenland et al. [60] but leukemia SMRs were near unity overall, and for both men and women. Aside from the statistically significantly deficit of myeloma, no other LHM-specific results were statistically significant.

7. Conclusions for epidemiological evidence of EtO and cancer

In summary, our critical review and synthesis of the epidemiological evidence to date indicates no association between exposure to EtO and risk of stomach cancer. Studies rated as either high or medium quality either reported no association [57,60,64] or observed no cases of stomach cancer [55,56]. Indeed, two of the three medium-to-high quality studies as well as all low quality studies based SMRs on relatively small numbers with reported deaths ranging between five and ten [57,64,66,69,70].

The totality of epidemiological evidence also demonstrates no clear or consistent positive association between exposure to EtO and breast cancer. Two cohort studies evaluating breast cancer rated as high quality [60,62] reported a slightly decreased incidence or mortality of breast cancer compared to the general population, which appeared to be more profound among the lowest-exposed sub-cohort. When this group served as the referent group in subsequent analyses, all other exposure groups spuriously appeared to have increased relative risks of breast cancer. Similarly, Mikoczy et al. [57] (rated medium quality) observed a deficit in breast cancers among the study cohorts overall. Nevertheless, the authors conducted additional analysis, stratifying by exposure level and using the groups with the lowest exposure – but also with strong risk deficits – as the referent group, which yielded artificially increased relative risk estimates for all higher exposure groups. Only one study reported a statistically significant association between EtO and breast cancer; however, this was classified as low quality [71].

The epidemiological evidence consistently demonstrates no clear or consistent excess occurrence of LHMs, whether combined across leukemias, lymphomas or both, or as individual malignancies. The study results addressing specific types of LHMs were few, likely because only three cohorts observed greater than 10 total LHM cases precluding meaningful analyses of individual malignancies [57,60,64]. Nevertheless, the lack of evidence of increased occurrence of LHMs as a group does not suggest that they are occurring in excess among cohorts highly exposed to EtO (e.g., for the NIOSH cohort: average TWAs of 4–6 ppm in the mid-1980s and lower thereafter, but with 90th percentile TWA exposures estimated as high as ~180 ppm in the 1940s–1970s [60,78]).

Based on our application of what we consider to be current, strong

and transparent methods for conducting systematic reviews, we conclude that the body of epidemiological literature on occupational exposure to EtO and risk of stomach and breast cancers – as well as LHMs as a group and the individual malignancies contained in this broad group - does not demonstrate any clear or consistent excesses of these cancers. Therefore, we find no valid epidemiological basis for concluding that EtO causes stomach or breast cancers, or LHMs individually or combined, in humans.

8. Discussion (evidence integration and hazard characterization)

The basis for our conclusions regarding hazard for each cancer type, based on the IOM classification system [11], is described below, in the systematic review protocol provided in the Supplemental Materials. As discussed in the Methods, the IOM categorization scheme includes the following categories:

- Sufficient evidence of a causal relationship
- Sufficient evidence of an association
- Limited/suggestive evidence of an association
- Inadequate/insufficient evidence to determine whether an association does or does not exist
- Limited/suggestive evidence of no association.

The evidence integration process across all streams of evidence is detailed in Supplemental Tables S9-S11.

8.1. Overall evidence and mode of action

Experimental animal studies indicate that relatively high EtO exposures (i.e., approximately 33–100 ppm) are associated with several tumor types, including MNCL and brain tumors in rats and alveolar/bronchiolar adenoma or carcinoma, lymphoma, uterus, and mammary gland tumors in mice. Some of these tumors may be spontaneous and within historical control levels (e.g., MNCL, mesothelioma), but others appear to be related to EtO dosing. However, the same tumor types seen in rodents (e.g., brain, lung, uterine) have not been observed in excess in any of the epidemiological studies of acceptable quality. Furthermore, it is worth noting that animal carcinogenicity studies of ethylene, which is metabolized to EtO, observed no tumors in rats at concentrations equivalent to approximately 5.5 ppm EtO [79,80].

Regarding mechanistic information, human and animal studies report dose-dependent increases in N7-HEG adducts in several tissues, including the blood, brain, lung, spleen and liver following EtO inhalation exposure. There is some evidence that N7-HEG adducts accumulate more slowly at low doses due to DNA repair, accumulate somewhat linearly at middle doses, and more steeply once DNA repair mechanisms are saturated. These adducts are not directly mutagenic; thus, while DNA adduct formation is the molecular initiating event in the MOA, it is not the rate-limiting step. There are no studies on mutations or chromosome effects below doses that also cause cancers in animals. Human studies of mutation frequencies are inconsistent; while some studies reported increase HPRT mutations, other studies reported no significant increases. Without additional empirical evidence for key events in cellular transformation at low/human relevant inhalation exposure levels it is unclear at what exposure level the final turning point for carcinogenesis is reached.

8.2. Stomach cancer

No gastrointestinal tumors were observed in any of the experimental animal studies. No inhalation studies informing the MOA specifically for stomach cancer were identified. Although not often examined, levels of N7-HEG adducts in stomach DNA were not statistically significantly increased in rats exposed to radiolabeled EtO via injection, relative to exogenous production of these adducts. The epidemiological evidence shows no increased risk of stomach cancer among cohorts highly exposed to EtO (average TWAs of 4–6 ppm in the mid-1980s, and lower thereafter; however, 90th percentile TWA exposures have been estimated as high as ~180 ppm in the 1940s–1970s [60,78]). Studies rated either high or medium quality either reported no association or observed no cases of stomach cancer. Integrating all streams of evidence according to the IOM framework yielded classifications of suggestive evidence of no association between EtO and stomach cancer at human relevant exposures, but conclusions are limited to the conditions, levels of exposure, and length of observation covered by the available studies.

8.3. Breast cancer

Experimental animal studies reported no increase in mammary tumors in rodents, with the exception of the mid-dose group (50 ppm) but not the high-dose (100 ppm) group of female mice in the NTP study. Increased mutation frequencies in proto-onco and tumor suppressor genes, well as distinct mutation spectra suggestive of EtO-induced mutagenesis, were observed in the mammary gland tumors from the mice exposed to 50 ppm EtO in the NTP [16] cancer bioassay [44]. However, no other inhalation studies evaluating precursor events to mammary gland tumor formation were identified. As noted above, the positive findings in other tissues are likely relevant across sites. For the purposes of comparison with the occupational epidemiological studies, assuming a regional gas deposition ratio of 1 and adjusting from 6-h to 8-h occupational exposures, the human equivalent concentrations (HECs) are estimated at 38 ppm and 75 ppm for animal exposures of 50 and 100 ppm EtO, respectively. The animal exposure levels are well above air concentrations reported for sterilization and production facilities in most cases (e.g., approximate average TWAs of 7–70 ppm in the 1940–1970s, 4–6 ppm in the mid-1980s, and $\leq$1 ppm in the 1990s and after; [60,64,78]).

The results for breast cancer and EtO in epidemiological studies is complicated by methodological decisions made by the authors. Specifically, several studies of occupationally exposed populations rated as either medium or high quality reported a slightly decreased incidence of breast cancer compared to the general population, which appeared to be more profound among in the lowest-exposed sub-cohort. When the low-exposure groups with the strong deficit in risk were used as the referent group in subsequent analyses, all higher exposure groups appeared to have increased relative risks of breast cancer. Thus, the reported increased relative risk cannot be interpreted as a truly increased risk of breast cancer relative to the risk in the population of unexposed women, unless one accepts that the deficit risk in the lowest exposure group somehow is directly protective against breast cancer, despite the lack of any other evidence to support this speculation. Integrating all streams of evidence according to the IOM framework yielded classifications of suggestive evidence of no association between EtO and breast cancer at human relevant exposures, but conclusions are limited to the conditions, levels of exposure, and length of observation covered by the available studies.

8.4. Lymphohematopoietic cancers

Experimental animal cancer bioassays indicate that relatively high EtO exposures (i.e., approximately 33–100 ppm) are associated with lymphohematopoietic tumor types, including MNCL in rats and lymphoma in mice. There is a high spontaneous rate of MNCL in the rat strain tested, indicating that those findings may not entirely be related to the EtO exposure. However, the lymphomas occurred above the level of that recorded in historical controls and assuming susceptibility has not changed, these observations may be relevant. In mechanistic studies, increased mutation frequencies were observed in splenic lymphocytes and T cells of rats and in splenic lymphocytes, thymic lymphocytes and bone marrow of mice, at doses in the range of those associated with

cancer in rodents. No animal data were available for mutation frequency at lower exposure levels. While one study reported increases in *hprt* mutations in EtO production workers, two other studies reported no statistically significantly increased mutation frequencies in occupational groups exposed to EtO. No studies of mutation frequency in minimally exposed humans were identified.

Among epidemiological studies rated medium and high quality, none reported excess mortality due to lymphohematopoietic malignancies in exposed workers. For specific subtypes, positive findings for "all leukemias" were observed in low-quality studies but were not replicated in other analyses in the same study or in studies of comparable or higher quality. Although reporting no excess occurrence of total LHMs, one high-quality study reported a positive exposure-response for "lymphoid cancer" mortality in males when compared to the least exposed group, which – as with the results for breast cancer – demonstrated a deficit of mortality due to these malignancies. Similar to the breast cancer studies, given the lack of overall excess occurrence of LHM mortality, the apparent "excess" relative risk in the high-exposure groups is a product of analytical methodology and should not be construed as indicating a biological effect or a true excess.

Mechanistic studies indicate that high-level EtO exposure is associated with DNA adducts in lymphocytes, and animal studies also indicate that high-level EtO exposure is associated with lymphoma in mice (100 ppm, HEC of ~75 ppm). Experimental animal and mechanistic data in the low-dose range are lacking. In contrast, the available epidemiological evidence does not demonstrate a clear and consistent association between LHMs and EtO, even at relatively high occupational exposure levels (average 8-h TWA exposures of 4–6 ppm in the mid-1980s and higher in previous decades). Overall, there is suggestive evidence of no association between EtO and any specific lymphohematopoietic cancers at human relevant exposures, but conclusions are limited to the conditions, levels of exposure, and length of observation covered by the available studies. In particular, the confidence in conclusions for specific types of LHMs, however, is only moderate because of the few available studies of the same specific lymphohematopoietic cancers, each of which is a distinct disease entity of possibly unique etiology.

9. Conclusions

Using the IOM classification system for carcinogens [11], our systematic review of EtO and cancer finds suggestive evidence of no association for exposure to EtO and breast and stomach cancer, as well as limited evidence of no association for LHMs. The comprehensive search strategy and detailed methods we followed are documented in this report and the supplemental materials so that others can verify, replicate and constructively comment on our methods, findings and interpretations. Furthermore, as our evaluation indicated that any one framework for performing systematic reviews and integration of the scientific evidence on human cancer risks may not be comprehensive or superior in all aspects, we drew from the strongest aspects of established methodologies of several organizations' guidance in an attempt to provide a full and transparent hybrid evaluation. We recognize, however, that there still may be areas in need of refinement in the approach. Further, because we did not employ any tiering system for the quality evaluation of animal studies, the studies appear largely homogenous, when there may have been individual quality metrics that could be used to further distinguish these studies. We also acknowledge that despite our best efforts, some subjective decisions and interpretations remain unavoidable: wherever possible we attempted to be transparent, allowing the reader to understand the basis for each decision, whether in agreement or not.

The results of this systematic review are consistent with previous reviews including Vincent et al. [81], which concluded that there was a lack of clear and consistent evidence between EtO and cancer. Vincent et al. [81] evaluated study quality using a different methodology, and there are some differences in how specific studies ultimately were rated in the current analysis. Despite these differences the assessment of study quality and relevance of the epidemiological evidence is consistent between the two reviews and revealed no disagreements. Vincent et al. [81] expanded the understanding of the MOA for EtO, and the current analysis provides a robust and more comprehensive evaluation and summary of the evidence underlying the molecular initiating events and key events. Our analysis also identifies areas in which data gaps remain in the MOA (most notably, DNA adduct and mutation frequency below levels causing tumors in animals).

Our conclusions regarding the epidemiological evidence also are similar to those of another systematic review and meta-analysis focused on the epidemiology [82] as well as a pooled analyses of the NIOSH and Union Carbide Corporation (UCC) cohorts [83]. Marsh et al. [82] observed that meta-analyses restricted to early publications (before 2000, but particularly before 1990) yielded positive overall effect estimates for EtO and total LHMs; however, meta-analyses specific to the publications from the 2000s to 2010s yielded null effect estimates. Similar findings were reported for meta-analyses of breast cancer and occupational EtO exposure. Marsh et al. [82] thus concluded that the most meaningful findings were those published after 2000 owing to increased precision in the underlying studies and consequently, *meta*-RRs for this time period. Bogen et al. [78] constructed an exposure model that demonstrated substantial decreases in estimated occupational EtO exposure during the period 1938 to 1986. Such an exposure situation could explain why some early epidemiological studies may have found some increases in cancer risks (i.e., with 8-h TWAs >10 ppm and possibly >100 ppm as a 90th percentile), while studies with more recent reduced levels of occupational exposure (TWAs of about <1–6 ppm) fail to find increases in cancer risk. However, many of the earlier epidemiological studies were lower quality studies, which also lowers the confidence in their findings.

Neither Marsh et al. [82] nor Bogen et al. [78] noted the impact on relative risk estimates when the lowest exposure groups – with striking deficit risks – were used as referent groups in the studies demonstrating no excess of LHM or breast cancers [57,62]. The US EPA's inhalation unit risk (IUR) for EtO was directly derived from models based on these analyses. Specifically, the IUR reflects the sharp upward transition from the deficit risk group back to the risk of the next most highly exposed group, which was close to background [5].

The International Agency for Research on Cancer (IARC) classified EtO as a Group 1 carcinogen (carcinogenic to humans), based on "limited" evidence of breast and lymphatic and hematopoietic cancers in humans and sufficient evidence in experimental animals. IARC stated, "There is strong evidence that the carcinogenicity of EtO, a direct-acting alkylating agent, operates by a genotoxic mechanism" [1]. IARC classifies agents based on hazard, without consideration of exposure-response patterns. Alkylating agents, such as EtO, are clearly associated with DNA adduct formation, which if not entirely or errantly repaired, may cause DNA mutations or chromosome level effects, and ultimately under some circumstances progress to tumorigenesis.

The key question, then, in evaluating the potential risks to human health is, at what exposure levels might EtO increase cancer risks that would be observable in large epidemiological studies such as the NIOSH studies? *In vivo* experimental and *in vivo* and *in vitro* mechanistic studies demonstrate the occurrence of DNA adducts at and below exposures associated with animal tumors, but data on mutations is limited to exposure levels also causing animal tumors, which appear higher than exposures in most if not all occupational studies, where exposures historically were non-trivial and orders of magnitude higher than exposures in any workplace or community today. Studies of rate-limiting key events in the MOA in the low-exposure range were not identified for either animals or humans. However, considering the lack of evidence of increased cancer in the epidemiological studies, particularly the most recent occupational cohort studies (i.e., published after 2000), EtO would not be expected to be associated with cancer at human-relevant exposure levels today. The findings of our systematic review indicate

that recent concerns over extremely low concentration community-level EtO and associated risk evaluations (e.g., ATSDR [2]), may not reflect the best or most current scientific understanding.

Declaration of competing interest

HNL, KAM, WJT, JSK, AJR, RDF, and HRD are employed by Cardno ChemRisk, a consulting firm that provides scientific support to the government, corporations, law firms, and various scientific/professional organizations. KAM and WJT have provided scientific evaluation on behalf of clients in litigation and regulatory settings in which it was alleged that EtO causes various cancers. EJC has no potential conflicts to disclose. The content and the conclusions of the manuscript are exclusively those of the authors. This work was supported by a grant from the Center for Truth in Science, an independent, non-profit organization.

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Appendix A. Supplementary data

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