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Report on the Peer Review of the Methanol Bioassays: Soffritti et al. (2002) and Apaja (1980)

Expert Review Organized by Toxicology Excellence for Risk Assessment (*TERA*)

www.tera.org

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INTRODUCTION

The Methanol Institute asked Toxicology Excellence for Risk Assessment (*TERA*) to conduct an independent letter peer review of two key studies on methanol, a substance which is currently being assessed by the US EPA. The two studies include the European Ramazzini Foundation's drinking water bioassay of methanol, published as Soffritti et al. (2002) and Apaja (1980), an unpublished PhD thesis that includes methanol as a positive control in a cancer bioassay. This report describes the independent peer review and presents the reviewers' written comments on the studies.

The U.S. Environmental Protection Agency (US EPA) is currently developing a human health assessment of methanol (CASRN 67-56-1) for its Integrated Risk Information System (IRIS) database that will be reviewed by the US EPA Science Advisory Board (SAB) in early 2010. Only a few animal studies are available in the published literature that shed light on the carcinogenicity of methanol. Because the US EPA IRIS process has a strong preference for use of peer-reviewed studies, US EPA arranged for the Eastern Research Group (ERG) to conduct an external letter peer review of several study reports from the New Energy Development Organization (NEDO) in June 2009. However, US EPA did not ask ERG to conduct a peer review of another key methanol study by Soffritti et al. (2002). Because this is a key study for assessing methanol's carcinogenicity, the Methanol Institute thought it should also have a peer review and contracted to *TERA* to conduct an independent letter peer review of this study, in a fashion similar to the EPA-sponsored reviews of the NEDO studies, and make the results publicly available. In addition, on January 12, 2010, US EPA released its External Review Draft of the IRIS Toxicological Review of Methanol. In their draft assessment, US EPA relies on an unpublished PhD thesis, Apaja (1980), for weight-of-evidence conclusions regarding the carcinogenicity of methanol. Apaja (1980) evaluated the toxicity and carcinogenicity of malonaldehyde in two separate studies: a skin painting study and a drinking water study. Both studies included several doses of methanol as a positive control. Therefore, the Methanol Institute asked TERA to include the Apaja (1980) study in the peer review.

The Methanol Institute requested that *TERA* conduct the review with total independence and with no input or influence from the Methanol Institute or those working with the Institute. To this end, the agreement between *TERA* and the Methanol Institute (see Appendix A) specifically required that *TERA* have sole discretion to select and secure the panel members. The Methanol Institute and their agents have not been consulted about, nor informed about, the identity of the peer reviewers. The agreement also stated that *TERA* will release the peer review report to the public before it delivers the report to the

Methanol Institute. Finally, in subsequent communication, the Methanol Institute has asked that *TERA* submit the final peer review report directly to the EPA docket.

TERA used its standard procedures to identify a list of review candidates with the necessary expertise to conduct this review. These candidates were then evaluated for conflict of interest to ensure that the public and others can have confidence that the peer reviewers do not have financial or other interests that would interfere with their ability to carry out their duties objectively. *TERA* asked each promising candidate to complete a questionnaire designed to identify financial and other relationships with the Methanol Institute, the member companies of the Methanol Institute, the European Ramazzini Foundation, and the US EPA's Science Advisory Board. The completed questionnaires were reviewed by *TERA* staff and discussed further with panel candidates as needed. (See www.tera.org/peer/COI.html for *TERA* conflict of interest and bias policy and procedures for panelist selection.)

TERA has determined that the selected peer reviewers have no conflicts of interest and are able to objectively participate in this peer review. None of the panel members has a financial or other interest that would interfere with his or her abilities to objectively participate on the panel. None of the panel members is employed by the Methanol Institute or its member companies, or the European Ramazzini Foundation. Nor do the panel members have any financial interests in these organizations or in the outcome of the review. None of the panel members was involved in the studies under review.

The panel includes five scientists who have expertise in the key disciplines and areas of concern to review the methanol bioassays. Each panelist is a well-respected scientist in his or her field. Collectively, the panel has expertise in toxicity of alcohols, design and conduct of carcinogenicity bioassays, biostatistics, use of bioassay data in risk assessment, and U.S. EPA risk assessment methods. See Appendix B for more information about these panel members:

- Judy Buelke-Sam, MA; Toxicology Consultant
- David Dorman, PhD, DABT, FATS; North Carolina State University
- Janis Eells, PhD; University of Wisconsin-Milwaukee
- David Gaylor, PhD, FATS; Gaylor and Associates Consultants
- D. Allan Warren, PhD; University of South Carolina-Beaufort

The reviewers were asked to answer to provide written answers to five charge questions for each study (See Appendix C). *TERA* reviewed the responses for completeness and clarity, and has collated the reviewer responses for each of the charge questions. The individual reviewers' comments are presented anonymously below (although the same number is used to identify each reviewer throughout). Due to unforeseen circumstances,

one reviewer was not able to complete the review of the Apaja (1980) study, so the comments from only four reviewers are presented in that section of the report. The reviewers' comments on the Soffritti et al. (2002) study are presented first followed by the reviewers' comments on the Apaja (1980) study.

REVIEW OF SOFFRITTI ET AL. (2002)

1. Study Design - ***Based on your knowledge of toxicological study protocols, please comment on the experimental design of the Soffritti et al (2002) study:***

- ***Do you see any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?***
- ***In light of the chemical and toxicological profile for methanol, comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation.***
- ***The study was conducted using the full lifespan of the animals. Please comment on if or how this study design affects the interpretation of the results.***

Reviewer 1

- **Do you see any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?**

Employment of 100 animals per dose group provided more precise results than cancer bioassays that typically employ 50 animals. However, only one species (rats) were used.

I conducted benchmark dose analyses for the lesions considered related to methanol exposure. For all of these lesions, the lower 95% confidence limit for the benchmark concentration estimated to produce an extra 10% incidence of the lesion ($BMCL_{10}$) fell within the range of the experimental concentrations (500-20,000 ppm). Hence, the spacing of the three experimental concentrations provided adequate information on tumor incidences for calculating $BMCL_{10}$'s.

The statistical analyses employed did not take into account differences in survival among dose groups. Since tumor incidence increases with age, dose groups that survive longer tend to have higher incidences of tumors. Survival was similar among dose groups in the methanol study. The National Toxicology Program utilizes the Poly-k technique to adjust tumor incidence rates for differences in survival. Subsequent statistical analyses using the Poly-k technique, as reported by the Methanol Institute, generally provided results similar to Soffritti *et al.* (2002).

The high doses used in the study do not provide any direct information on the shape of the dose response below 500 ppm. In hindsight, it would have been useful to have one or more concentrations below 500 ppm for risk assessment.

- **In light of the chemical and toxicological profile for methanol, comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation.**

It would have been useful to have measures of cell proliferation rates in order to determine to what extent tumors may have been a result of cytotoxicity and increased cell regeneration at the high concentrations employed.

- **The study was conducted using the full lifespan of the animals. Please comment on if or how this study design affects the interpretation of the results.**

Utilization of a full lifespan study did not appear to present any particular difficulties in the methanol study. Survival was similar across methanol concentration groups.

Reviewer 2

- **Do you see any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?**

A number of significant deficiencies exist within the Soffritti study including:

1. Animals being observed until they died – this is a significant deviation from standard rodent carcinogenicity studies conducted under USEPA or NTP study guidelines. One advantage of this study design is that so-called "late life stage" tumors could emerge after the traditional 24 month endpoint used in most studies. The main disadvantages are that the statistical analyses may be more complicated, background tumor incidence rates could be higher, and animals could die while in a moribund condition. It is also more difficult to compare the incidence data for this experiments cohorts versus historical norms published for others (with 24 month old Sprague-Dawley rats).
2. Data for survival rates, body weights, body weight gain, or feed and water consumption were only presented in summary form (the authors state: "decrease in water consumption in females treated with the highest dose between 8 and 56 weeks of age. A slight increase was observed in the body weight of males and, to a lesser extent, of females treated with the highest dose. No substantial changes in survival or

behavioral changes were observed among the groups. No treatment-related non-oncologic pathological changes were detected by gross inspection or histopathologic examination). This is important with respect to the US EPA Guidelines for Carcinogenicity testing that state: "Other signs of treatment-related toxicity associated with an excessive high dose may include (a) significant reduction of body weight gain (e.g., greater than 10%), (b) significant increases in abnormal behavioral and clinical signs, (c) significant changes in hematology or clinical chemistry, (d) saturation of absorption and detoxification mechanisms, or (e) marked changes in organ weight, morphology, and histopathology." Insufficient data is presented to determine whether or not the high dose group was associated with overt toxicity.

3. Histopathologic data did not include the incidence or severity of non-tumor lesions. Importantly, the authors did not indicate whether any background (non-tumor) lesions were present in the animals used. The concern of whether the colony used by the investigators has a background incidence of respiratory tract infection has been raised by others and is addressed later in this review.
4. Organ weight and gross necropsy findings were not reported.
5. Soffritti reports that "Methyl alcohol is produced by J.T. Baker, Deventer, Holland, and has a purity grade of 99.8%" – however no mention of impurities is mentioned.
6. The manuscript states that "The experiment on ethyl alcohol started in January 1986 and ended with the death of the last offspring at 179 weeks of age. Experiments were performed according to Good Laboratory Practices (GLP) and Standard Operating Procedure (SOP) of the CRC/RF." – it should be noted that the authors don't explicitly state whether so-called GLPs were used for the methanol study. It is also important to note that the GLPs cited in the manuscript represent the performing laboratories best practices and they do not adhere to an external agency's GLP requirements. No mention was made whether any significant deviations occurred.
7. The authors state the following: "During the experiment, both compounds were stored at a temperature of 4°C. Methyl alcohol was administered in drinking water at concentrations of 20,000, 5000, 500, or 0 ppm supplied ad libitum for 104 weeks to groups of male and female Sprague-Dawley rats beginning at 8 weeks of age. Control animals received tap water."… and "Each morning, residual liquids from the previous day were removed, and the glass drinking bottles were washed and filled with fresh solution." – It is not clear whether fresh solution was produced each day or that the drinking water was replaced – this draws into concern the stability of the dosing solution(s) used.
8. Animal groups and assignment of animals to treatment groups was poorly described. Cruzan (2009) has also pointed out concerns as to whether a concurrent control group was used in this methanol bioassay. The data presented by Soffritti for the controls in the methanol and ethanol experiments have different values suggesting that different control groups were used. However, there is insufficient information in the Soffritti manuscript to refute the claim posed by Cruzan.

9. Statistical methods used are described by the study authors ("Statistical analysis was performed using the $\chi 2$ test to evaluate differences in tumor incidence between treated and control groups. The Cochrane Armitage test was used to evaluate dose-response relations."). However, the specific statistical test used (e.g., Pearson's Chi Square test) was not explicitly stated. The Chi Square test assumes that the observations are independent of each other – it is that this is true for all of the reported tumors and is likely inappropriate when the incidence data were pooled across tumor types. Statistical methods used to assess non-cancer endpoints were not specified.

    - **In light of the chemical and toxicological profile for methanol, comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation.**

1. Blood methanol concentrations would have provided additional useful information.
2. Clinical chemistry data should have been collected on a satellite group.
3. Descriptions of all non-cancer endpoints are lacking. It is unclear whether animals were subjected to only cage side observations. Soffritti reports that "Status and behavior of animals were examined 3 times daily, and they were submitted to clinical examination for gross changes every 2 weeks." – however behavioral and clinical assessment methods were not described.
4. A modern study might include an assessment of formaldehyde adducts, DNA-protein cross links etc. However, this is not a sufficient concern to dismiss the Soffritti study.

    - **The study was conducted using the full lifespan of the animals. Please comment on if or how this study design affects the interpretation of the results.**

1. One advantage of this study design is that so-called "late life stage" tumors could emerge after the traditional 24 month endpoint used in most studies.
2. The main disadvantages are that the statistical analyses may be more complicated, background tumor incidence rates could be higher, and animals could die while in a moribund condition.
3. It is also more difficult to compare the incidence data for this experiments cohorts versus historical norms published for others (with 24 month old Sprague-Dawley rats).
4. Statistical analysis of certain data (e.g., survival rates) may be affected by this study design. In my opinion this is a minor concern.
5. There is no indication of whether the pathology samples were optimal in that animals that died during the course of the experiment may have undergone cannibalization and autolysis prior to preservation.

Reviewer 3

- **Do you see any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?**

Numerous significant problems associated with study design are apparent in the European Ramazzini Foundation (ERF) study conducted by Soffritti et al (2002).

A. Animals and Animal Husbandry

1. The ERF study was not conducted according to established Good laboratory practices (GLP). This may adversely affect the quality and reliability of the study (1).

2. Animals not Maintained in Barrier-maintained, specific pathogen free facility. According to published studies (1, 2, 7) the rat colony used for the ERF methanol carcinogenesis study was conventionally maintained and not subject to the rigorous health assurance and disease control measures necessary to exclude pathogens. Barrier maintained pathogen-free conditions are required for a reliable long-term carcinogenesis study.

3. No documentation of sanitation and animal hygiene practices including sterilization of food, water filtration and sterilization, sterilization of cages and other equipment. Regular replacement of cages, feeders and water replacement not documented or discussed.

4. Wood shavings used for bedding. The rodent bedding used in the ERF studies consisted of white wood shavings. Wood shavings, including white woods like pine, contain volatile organic compounds primarily terpenes. Volatile organics from wood shaving bedding has been documented to alter liver function and xenobiotic metabolism(13). Wire bottom cages or the use of commercially available laboratory animal bedding does not present this potential confounding factor.

5. No evidence of disease surveillance in the rat colony or over the course of the study. For appropriate disease surveillance, blood must be collected from study and sentinel animals every 4-6 months and checked for serum antibodies to rodent viruses (hepatitis, adenovirus, Sendai virus) and bacteria (*Mycoplasma pulmonis, Bacillus piliformis, Salmonella typhimurium, Corynebacterium kutscerihi*.)

B. Study Design and Protocol

1. ERF uses non-conventional study design that does not conform to EPA or NTP guidelines for chronic carcinogenicity studies thus making data comparison difficult if not impossible.

2. Animals were not randomly assigned to treatment groups. Instead animals from a given litter were all assigned to the same treatment group. Because there is no way to evaluate litter effects, there is no way to differentiate between congenital effects and treatment-related effects. Moreover, since *Mycoplasma pulmonis* infection is passed from mother to offspring, this could be a factor in the higher degree of respiratory neoplasms in treatment litters (1).

3. Use of historical controls: Based on evidence for the use of historical controls

4. Lifetime study –as discussed by Goodman et al (7) full-lifespan carcinogenicity studies can lead to erroneous conclusions *"because older animals are: (1) more heterogeneous, (2) are more susceptible to illness and spontaneous tumors, (3) have an increased background pathology and (4) have a higher probability of autolysis than do younger animals". (7, 8).*

5. Dose of methanol administered: No confirmation of correct dosage of methanol. HPLC determination of concentration of methanol in the water required for reliable study.

6. Methanol Dose Range: The highest dose of methanol used in this study (20,000 ppm or approximately 2000 mg/kg/day) is at least twice the lethal dose of methanol in humans. The 5000 ppm dose of methanol is estimated to be 542 and 630 mg/kg/day in male and female rats respectively (my calculations and Cruzan, 2009). The 20,000 ppm dose is estimated to be 1840 and 2250 mg/kg/day in male and female rats respectively (my calculations and Cruzan, 2009). The lethal dose of methanol in humans is estimated to be between 300 and 1000 mg/kg (9, 11, 12). Although carcinogenesis studies are designed to examine toxic actions at doses greater than those anticipated to be encountered in an environmental exposure these doses far exceed any scenario for environmental methanol exposure.

The 5000 ppm and 20,000 ppm doses selected for the Soffritti study are 80 –300 x greater that the highest estimated exposure to methanol in a transportation setting. The highest estimated exposure to methanol vapors in a "hot soak" personal garage situation is 240 mg/m^3. This exposure is calculated to increase the body burden of methanol by 0.6

mg/kg (assuming 100% absorption across the lung epithelium) for a 15 minute exposure (14). A 24 hour exposure would be predicted produce a body burden of 57 mg/kg which is 1/80th of the 5000 ppm dose and 1/300th of the 20,000 ppm dose.

C. Endpoints Recorded:

1. Methanol and Formic Acid: The Soffritti study did not determine blood methanol, or blood formic acid concentrations. Measurement of blood methanol concentrations is an essential endpoint for an appropriately designed study. Measurement of formaldehyde concentrations would be unlikely to provide useful information since numerous studies have shown that an increase above basal concentrations of formaldehyde [basal concentrations] is undetectable in blood samples (due to its rapid half-life of less than one minute in the blood). However, with chronic methanol administration, small concentrations (1.8 – 2 mM) of formic acid does accumulate above basal concentrations (0.5 mM) to concentrations ranging between 1.8 – 2 mM. This occurs in folate-competent Long Evans rats despite the ability of this species to rapidly detoxify formate by tetrahydrofolate-dependent oxidation These concentrations produce moderate reductions in retinal function in this animal model. (15).

2. Indices of oxidative stress and oxidative damage: Investigations by Parthsarthy et al (a) have shown that treatment of Wistar rats with 2.4 g/kg of methanol (HPLC grade) (similar to the 20,000 ppm exposure) per day for 15 and 30 days profoundly increases oxidative stress in rat lymphoid organs. The dose of methanol administered per day in the Parthsarthy study (10) (2.4 g/kg) is approximately equivalent to the 20,000 ppm (calculated dose of 1.84 (male) and 2.25 (female) g/kg per day) treatment paradigm in the ERF study.

Appropriate endpoints for the ERF study include: assessment of oxidative and nitoxidative damage by measuring – oxidative damage to lipids, proteins, and DNA. lipid peroxidation, nitrotyrosine concentrations, 6-OH deoxyguanosine (2) assessment of enzymatic antioxidant activity catalase activity, superoxide dismutase activity and assessment of non-enzymatic antioxidants including reduced and oxidized glutathione, vitamin E and vitamin C.

D. Terminal Procedures:

1. Potential for autolysis confounding pathological evaluation: Study guidelines for chronic carcinogenicity studies require that moribund animals be immediately euthanized and tissues harvested to minimize tissue autolysis. Tissue autolysis is known to confound pathological evaluation. Since the EMF study allowed animals to die rather than be

euthanized at given time intervals the potential for autolysis prior to necropsy is much greater in this study than in studies conducted by approved EPA and FDA protocols.

2. No Testing for pathogens: given the high degree of lung pathology reported in these studies in control and treatment groups and the lack of a barrier pathogen-free animal colony, postmortem testing for pathogens should have been conducted.

3. No comparison of animals at same age for pathology: The EMF protocol does not terminate the study until the death of each animal, thus there are no groups of animals terminated at the same time to allow an accurate comparison of pathology.

4. Statistical Analysis: This reviewer has limited expertise in statistical analysis of carcinogenesis studies, however the statistics applied in this study are consistent with those used in other similar studies and thus presumed to be appropriate.

- **In light of the chemical and toxicological profile for methanol, comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation.**

1. Methanol has been shown to be metabolized to formaldehyde then to formate and finally to CO_2 by sequential oxidative steps in all species which have been investigated. However, only primate species are susceptible to the neurotoxicity associated with methanol intoxication. The reason for the species-specific neurotoxicity toxicity of methanol is due to species differences in tetrahydrofolate-dependent formate oxidation (detoxification) to CO_2. Formic acid is the toxic metabolite responsible for the neurotoxic and lethal actions associated with methanol intoxication. Formate accumulates in species susceptible to methanol toxicity (humans and non-human primates) and not in species resistant to methanol toxicity (rodents) The differential accumulation of formate (formic acid) is due to species-specific differences in hepatic tetrahydrofolate (THF) concentrations and the activity of formyl-THF dehydrogenase, the rate-limiting enzyme in the conversion of 10-formylTHF to CO_2 and water. Hepatic concentrations of THF in rats are nearly double those in humans and 5-formyl THF reductase four-times greater (Black et al, Jochlin et al). As a consequence the lethal dose of methanol in humans is between 300 mg/kg and 1000 mg/kg, whereas the lethal dose in rats is between 7400 and 13,000 mg/kg. Moreover, rodents die of the CNS depression induced by methanol itself whereas humans die from the neurotoxic actions of the metabolic poison, formic acid (11, 12).

2. The use of a rodent model to investigate the carcinogenic actions of a chemical which exhibits an entirely different metabolic profile and toxicity profile in humans is thus

inherently flawed. This reviewer recognizes the difficulty in examining carcinogenic endpoints in primate species and suggests that a more appropriate model may have been a folate-depleted rodent model controlling for the increased susceptibility to tumor formation in folate-depleted states.

3. In addition to this concern, the metabolism of methanol also generates a highly reactive molecule, formaldehyde. Despite the fact that formaldehyde has a half-life of less than one minute (11, 12), this molecule is likely to react with tissue components, particularly peptides and proteins in the mitochondrion upon its formation. Formaldehyde is known to be a potent cross-linking agent that has been documented to inactivate proteins. Formaldehyde was shown to react with the amino group of the N-terminal amino acid residue and the side-chains of arginine, cysteine, histidine, and lysine residues (4). A comprehensive examination of the mode of action of methanol toxicity would have included assessment of formaldehyde cross linked proteins.

- **The study was conducted using the full lifespan of the animals. Please comment on if or how this study design affects the interpretation of the results.**

There are pros and cons of treatment for full life-span of animals.

> Pro: As articulated by Caldwell et al (5, 6) lifespan observation increases the sensitivity of the assessment of a chemical's cancer potency. They note that the ERF study protocol using full-lifespan studies *"has been credited as the primary reason that the ERF was the first laboratory to associate carcinogenic responses with chemicals that are now recognized as known human carcinogens, including vinyl chloride, acrylonitrile and benzene."* (5, 6)
>
> Con: However, as discussed by Goodman et al (7) full-lifespan carcinogenicity studies can lead to erroneous conclusions *"because older animals are: (1) more heterogeneous, (2) are more susceptible to illness and spontaneous tumors, (3) have an increased background pathology and (4) have a higher probability of autolysis than do younger animals"*. (7, 8)

Taking both arguments into account, this reviewer concludes that increased heterogeneity, increased susceptibility, increased background pathology and higher probability of autolysis in full-lifespan carcinogenicity studies vastly increases the difficulty in causally linking tumors in treated animals to treatment rather than to one of these intervening factors.

Reviewer 4

- **Do you see any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?**

Issues of significance with the Ramazzini methanol study of some 20 years ago abound, many of which have been belabored by industry-sponsored academicians and consultants. While many of these issues stem from differences in the study's design, conduct, and analysis compared to studies conducted in the U.S. and elsewhere under more current bioassay guidelines, such differences in and of themselves are insufficient to warrant its summary dismissal. However, patterns of "tumor" responses in multiple Ramazzini studies, including that of methanol, suggest that such differences may have resulted in issues that severely threaten the validity of causal inferences, principal among them, unrecognized *Mycoplasma pulmonis* infection. This possibility has been the subject of a substantive debate that, to this point, has defied resolution. As such, the study's utility is rightfully limited, particularly in a regulatory context. That is, use of the Soffritti et al. (2000) study for cancer slope factor derivation would imply a degree of certainty in the study's validity that does not exist, owing partly to a lack of quality control testing to detect infection. While application of the precautionary principle has its merits, adoption of Soffritti et al. (2000) as a critical study should, at a minimum, be contingent upon resolution of the *M. pulmonis* infection issue. Short of that, its use by USEPA would introduce a degree of uncertainty capable of eroding the recent gains made by the Agency in allowing sound science to drive the risk assessment process.

- **In light of the chemical and toxicological profile for methanol, comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation.**

As for additional endpoints that should have been assessed, organ weight and hematological and clinical chemistry parameters would likely have informed the *M. pulmonis* issue. In addition, given the considerable interspecies differences in methanol metabolism, blood methanol concentrations might have been of value in the interspecies extrapolation of tumor data.

- **The study was conducted using the full lifespan of the animals. Please comment on if or how this study design affects the interpretation of the results.**

Concerning the full lifespan design of the Ramazzini methanol study, the argument can be made that it potentially increases the sensitivity to detect solid and disseminated tumors. This is likely the case, as tumor incidence, background or otherwise, is a function of age. Some argue that this potential benefit is outweighed by the inability to compare tumor rates and other toxicological or physiological parameters among control and treated animals of the same age. Granted, the original publication by Soffritti et al. (2000) gave no indication that any statistical means of adjusting for differential survival had been undertaken. However, supplemental data tables supplied by the European Ramazzini Foundation (ERF) indicate that such adjustment has been made, allaying my concerns for the full lifetime design.

Reviewer 5

- **Do you see any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?**

It appears this study, initiated in 1990, was intended to make a statement concerning risks associated with high levels of consumption of methyl alcohol (MA) and ultimately provide data for risk assessments. Apparently, the basic design of allowing animals to live past termination of exposure at 24 months until their death has been used by this laboratory since at least the mid-1970s. However, the overall design and information included poses several evaluation issues from my perspective. The control group is unclearly a study-specific concurrent control and no inclusion of historical control data is available to use for either a formal or informal comparison of tumor incidence within a "non-standard" design. Additionally, while the study was initiated prior to issue of relatively current testing guidelines, it was not so early as to pre-date GLPs, or include GLP principles that are apparently lacking in the report, e.g., verification of stability of MA in the exposure solution, reporting of actual water consumption values and/or actual external "dose" consumed, etc. While the statistical analyses used may be "standard" for environmental studies (and even the US EPA guidelines, which are usually often very specific to a fault, leave these analyses open "as appropriate") they do not follow the standard approaches I am used to seeing in such studies of potential new pharmaceutical agents. Finally, I am always a bit leery of published results that make sweeping statements (i.e., no effects or slight effects on . . .) without presentation of those results in either tabular or graphic form, and in this case with no historical control data on any endpoints for reference, I find this even more troubling.

- **In light of the chemical and toxicological profile for methanol, comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation.**

Even within standard guideline hazard identification studies, there is always additional information that would be useful in profiling toxicity if data were collected as part of the study. The most obvious of these is measurement of internal exposure to the agent in question (toxicokinetics) and measures of potential mechanisms of toxicity. The latter is rarely including in a bioassay or screening study. The former is much easier to measure, analyses were available during the time of study conduct, and biological metabolites had been identified. As a naturally-occurring compound, the background range MA internal exposure in humans, even in high-exposure situations, can be and have been identified or estimated. While the major risk assessment procedures used by US EPA, e.g., benchmark dose calculations, are based solely on "external dose," the availability of "internal dose" information at such remarkably high external doses of the agent in question provides a much more direct basis of evaluation of relative risk. I find the results of the MA study to be very unusual, in that the applied statistics and the "eye ball" evaluation of overall tumor incidence suggests such an effect in the apparent absence of remarkable changes in other standard indications of measured toxicity, e.g., survival, body weight changes, etc., at such a high external concentration range.

- **The study was conducted using the full lifespan of the animals. Please comment on if or how this study design affects the interpretation of the results.**

As noted by the authors, this basic design element had been in use within this laboratory for many years, yet it is an unusual element within the hazard identification research community. As such, a minimal addition to this report should have included some data reference to survival rates, spontaneous tumor rates, standard pathological findings normally obtained under these laboratory non-barrier conditions in studies using this design, i.e., basic historical control information as well as data from the current study on basic background toxicity. The more unusual the design, the more important actual background data becomes in evaluating treatment-related results.

2. Study Results - ***Please comment on the strength, credibility, and relevance of the toxicological results of the Soffritti et al (2002) study, supplemented by the study data tables from ERF:***

- ***Were the individual animal data correctly summarized?***
- ***Are there nomenclature issues that need clarification?***
- ***Was adequate statistical information provided for quantitative dose-response analyses?***
- ***The study reported the following results:***

  "The occurrence of benign and malignant tumors is shown in TABLE 1. Differences observed between treated and control animals were: (1) a dose-related increase of total malignant tumors in males and females of treated groups (TABLE 2); (2) a dose-related increase of carcinomas of the head and neck, mainly in the ear ducts, in males of treated groups and in females treated with 20,000 and 5,000 ppm (TABLE 3); (3) a statistically significant increase (P <0.01) of testicular interstitial cell hyperplasias and adenomas in the group treated with the highest dose; (4) an increase in sarcomas of the uterus at the highest dose; (5) a dose-related increase in osteosarcomas of the head in males and females of the treated groups (TABLE 4); and (6) a dose-related increase in hemolymphoreticular neoplasias in males and females of the treated groups (TABLE 5)." Page 58.

For each lesion listed above (total malignant tumors, carcinormas and osteosarcomas of head and neck, testicular hyperplasia/adenoma, uterine sarcoma, hemolymphoreticular neoplasias), please comment on the strength of the evidence supporting the authors' conclusions that the lesion is treatment-related.

- ***Soffritti et al (2002) reported an increased incidence of total hemolymphoreticular neoplasms that was statistically significant in females at all doses and dose-related in males (see Table 5, page 59). Lymphoimmunoblastic lymphomas were the primary tumor type observed. Other studies conducted by the ERF, most notably the bioassays for MTBE and aspartame, also reported increased incidences of lymphomas, leading to debate in the scientific literature on whether the animal colony at ERF may have been suffering respiratory infection due to Mycoplasma pulmonis and whether the lymphomas are an immunologic response to this infection. For more information, see Caldwell et al. (2008, 2009), Schoeb et al. (2009), Goodman et al (2009), and Soffritti (2008). Please comment on whether evidence for a Mycoplasma pulmonis infection exists in this study. If so,***

how would such an infection affect the results of the study or affect the interpretation of the study results.

Reviewer 1

- **Were the individual animal data correctly summarized?**

Individual animal data appear to be properly summarized.

- **Are there nomenclature issues that need clarification?**

Histopathological nomenclature is outside my area of expertise.

- **Was adequate statistical information provided for quantitative dose-response analyses?**

There were sufficient results presented for tumor incidence rates for dose groups in order to conduct quantitative dose response analyses, including the calculation of benchmark concentrations. In order to conduct dose response analyses adjusted for survival, the time on study for each individual animal is required.

- **Analysis of Reported results**

I agree that a statistically significant dose response was demonstrated for total malignant tumors in males and females. These results are amenable for the calculation of benchmark concentrations.

I agree that statistically significant dose responses were obtained for carcinomas of the head and neck, mainly in the ear ducts, for males and females. These results are amenable for the calculation of benchmark concentrations.

No data were presented to support the purported statistically significant increase in testicular interstitial cell hyperplasia/adenoma at the highest concentration. The incidence of adenoma was not statistically significant.

No data were presented that indicated an increase in sarcomas of the uterus at the highest concentration.

Using the Cochran-Armitage trend test, I obtained a statistically significant ($P<0.05$) dose-related increase for osteosarcomas of the head in females, but not in males.

A statistically significant trend P<0.03 was obtained for males and females combined. The data for females and for males and females combined are amenable for benchmark concentration calculations.

A statistically significant dose-related increase (P<0.05) in hemolymphoreticular neoplasia , amenable for benchmark dose analysis, was obtained for females.
For males, the level of statistical significance was P<0.08 for a dose-related trend.

- **Respiratory Infection**

If hemolymphomareticular neoplasms induced by methanol and respiratory infection are independent, the slope of the dose response would not be altered. If there is synergism between methanol and respiratory infection, the incidence of tumors with increasing dose would be increased above the dose response with methanol alone.

Reviewer 2

- **Were the individual animal data correctly summarized?**

YES.

- **Are there nomenclature issues that need clarification?**

1. Soffritti uses a variety of terms to describe the neoplasia(s) observed in the study. Diagnoses include lymphoblastic lymphoma, lymphocytic lymphoma, and lympho-immunoblastic lymphoma. The histologic descriptions used to support the Soffritti diagnoses are not provided thus it is difficult to discern whether these diagnoses are consistent with other attempts at tumor nomenclature. For example, Firth (1988) describes common morphological classification of hematopoetic neoplasms in Sprague-Dawley rats. Tumor types include: lymphomas (lymphoblastic lymphoma, immunoblastic lymphoma, follicular center cell (FCC) lymphoma, plasma cell lymphoma, and large granular lymphocyte lymphoma).
2. Note also that Firth (1988) states: "Autolytic change may complicate the diagnosis of LGL lymphoma…Severe autolysis may necessitate the diagnosis of lymphoma, NOS (not otherwise specified)." This illustrates my previous concern about possible autolysis of the samples from animals that died on study.

- **Was adequate statistical information provided for quantitative dose-response analyses?**

NO:

1. The authors have pooled certain tumor types for statistical evaluation (e.g., "total tumors) – this is a significant deviation from the US EPA Guidelines. For example the EPA's Guideline explicitly states the following: "Statistical analysis of a long-term study should be performed for each tumor type separately. The incidence of benign and malignant lesions of the same cell type, usually within a single tissue or organ, are considered separately but may be combined when scientifically defensible (McConnell et al., 1986). "
2. The trend test used (Cochran-Armitage test) examines whether the results in all dose groups together increase as dose increases. A pair-wise comparison test such as the Fisher exact test could also have been considered to evaluate whether an incidence in one dose group was increased over that of the control group. It appears that certain pair-wise tests may have been performed although these have not been consistently described.
3. Also see my earlier concerns raised about the author's statistical methods.

- **Analysis of Reported Results**

Strength of the evidence supporting the authors' conclusions that the lesion is treatment-related:

1. Total malignant tumors (data presented below for total number of tumor bearing animals – brackets provide number of tumors):

Gender	Control	500 ppm	5000 ppm	20000 ppm
Male	50 [66]	55 [78]	64 [97]	70 [104]
Female	43 [60]	48 [72]	48 [73]	63 [95]

It is inappropriate to combine the incidences of all cancers for statistical evaluation because different cancers are derived from different cell types and do not share a common derivation. Likewise, Soffritti also combined tumor incidence data for male and female rats – this is also inappropriate since several tumor types demonstrate strict gender differences.

2. Carcinomas and osteosarcomas of head and neck (data presented below for 'head tumors'– brackets provide data for other sites): Note study authors state: "a dose-related increase in osteosarcomas of the head in males and females of the treated groups"

Gender	Control	500 ppm	5000 ppm	20000 ppm
Male	6 [2]	6 [1]	13 [0]	11 [1]
Female	1 [0]	4 [1]	3 [0]	6 [0]

Data presented do not appear to strongly support the author's conclusions. The incidence of head tumors in male rats exposed to 500 ppm methanol are identical to that seen in the control animals. Interestingly, the incidence seen in females from the highest dose group is similar to that seen in control male rats drawing into question whether the observed incidence is similar to "background". It's also unclear whether the analysis was performed on the pooled (male and female) data. The tumor type seen in the head is primarily defined as an osteosarcoma – it's curious to note that osteosarcoma incidence at other sites did not appear to be affected by methanol treatment.

3. Testicular hyperplasia/adenoma (data presented below for % of male rats with interstitial cell adenoma): Note study authors state: "a statistically significant increase ($P < 0.01$) of testicular interstitial cell hyperplasias and adenomas in the group treated with the highest dose"

Gender	Control	500 ppm	5000 ppm	20000 ppm
Male	12	9	13	17

Data presented appear to support the author's conclusions histological descriptions of hyperplasia versus a "true adenoma" are lacking.

4. Uterine sarcoma. Note study authors state: "an increase in sarcomas of the uterus at the highest dose"

The authors refer to Table 1 in support of this conclusion; however, there are at least seven distinct diagnoses provided for uterine tumors. It is not clear whether these tumor types have been pooled to create a composite data analysis for "sarcomas". No individual tumor type appears to have reached statistical significance – pooling of the data is inappropriate.

5. Hemolymphoreticular neoplasias: (data presented below for # of rats with lymphoimmunoblastic lymphoma – numbers in brackets are for total number with a hemolymphoreticular neoplasia): Note study authors state: "a dose-related increase in hemolymphoreticular neoplasias in males and females of the treated groups"

Gender	Control	500 ppm	5000 ppm	20000 ppm
Male	16 [28]	24 [35]	28 [36]	37 [40]
Female	9 [13]	17 [24]	19 [24]	21 [28]

The incidences of so-called hemolympho-reticular tumors have been pooled for analysis. This analysis is inappropriate since the cell types for the tumor types differ and no mode of action data are identified to suggest that pooling this data is appropriate. The relatively high incidence of this tumor type may occur with a higher incidence than that seen with historical control data published by other investigators. Importantly, no increase in the incidence of hemolymphoreticular neoplasms was found in the NEDO carcinogenicity study with methanol.

- **Respiratory Infection**

1. The Soffritti study does not provide any independent data to support whether or not a *Mycoplasma pulmonis* respiratory infection (or other) exists in this study. Some (Cruzan, 2008) have suggested that the increased early mortality in the Soffritti study may suggest that an infection was present, however, this remains speculative.
2. One clinical effect associated with *Mycoplasma* infection in rats is chronic otitis interna. Whether this could contribute to ear duct carcinomas is of possible concern. For example, chronic otitis media can lead to bony proliferation and other changes that may confound a diagnosis of ear duct carcinoma.
3. Concerns raised by some of the cited authors remain controversial. Several distinct "camps" have emerged that question the underlying premise that a respiratory tract infection could be a significant confounder. Personally, I believe that lung infection could affect chemical-induced tumor induction.
4. This issue may be resolved using immunohistochemical approaches on archived tissue samples (Liang et al., 2004).
5. Qualifications of the study pathologist is poorly defined in the study – was the individual experienced with veterinary toxicological pathology remains an open question.

Reviewer 3

- **Were the individual animal data correctly summarized?**

The information in the original publication was extremely limited and incomplete, however, the animal data in the detailed tables supplied by the ERF appears to be complete and correctly summarized with the exception of any indication of autolysis.

Significant autolysis would be anticipated in a study design that allows the animals to die before necropsy.

- **Are there nomenclature issues that need clarification?**

This reviewer is not an expert in the pathological classification of lesions in a chronic carcinogenicity study. However, based on the concerns of the Pathology working group regarding the classification of ear duct carcinomas and the concerns of other reviewers with respect to the combination of all leukemias and lymphomas into one category (hemolymphoreticular tumors) complicates the interpretation of the data presented and confounds any comparison of this study with similar studies.

- **Was adequate statistical information provided for quantitative dose-response analyses?**

This is not my area of expertise.

- **Reported Study Results**

1. Total malignant tumors –
The study reports a statistically significant (Cochrane-Armitage test for dose-response relationship) dose-dependent increase in the number of animals (separated into male & female but not total) exhibiting tumors. However analysis of the actual numbers of animals is indicative of increases only at 5000 and 20,000 ppm. Statistically significant increases in tumor numbers are documented in males at 20.000 ppm for tumor bearing animals and 5000 ppm and 20,000 ppm for total tumors. The lack of significant increases in female animals and total animals does not support the authors' conclusions.

2. Similar concerns exist for tumors in the specific divisions of head and neck, testicular, uterine and hemolymphoreticular neoplasia.

3. There is particular concern regarding the reliability of the data for both ear-duct carcinomas and hemolymphooreticular neoplasms. The concern regarding the reliability of the data for ear-duct carcinomas is predicated on the Pathology Working Group (PWG) which agreed with the ERF pathological classification in only 50% of the diagnoses (3). The concern regarding hemolymphoreticular neoplasms is based on the lung pathology discussed below.

- **Respiratory Infection**

There is convincing evidence for some type of lung pathology in the animals used in the ERF study. The details provided indicate that the animals in this study were from a colony that was not maintained under barrier-pathogen-free conditions. Chronic respiratory infections are common in conventionally maintained rodent colonies. It is thus very likely that these animals suffer from some chronic lung infection. Moreover, a high background incidence of chronic inflammatory changes in the lung have been reported in this study and in several other ERF studies (1, 2). In the ERF methanol study the vast majority of early deaths showed evidence of lung pathology and there was an extremely high percentage of lung pathology in both the control and treatment groups. In the ERF study, lympho-immunoblastic lymphoma was the most frequently reported hematopoietic neoplasm and the lung was the most frequently affected organ.

The nature of the infection produced by *M. pulmonis*, as well as, the incidence of this organism in rodent respiratory infections make it likely that the ERF colony suffered from a respiratory infection caused by M. pulmonis. However, the only way to be certain of an *M. pulmonis* infection would be to test for it. It is possible to resolve this question by testing the tissue collected from this study for the presence of *M. pulmonis* using published methods (1). In addition, testing animals in the ERF colony by standard serological tests for M. pulmonis would also assist in resolving this question.

Schoeb et al make a very convincing and well-documented argument that the results are the ERF methanol study were confounded by the presence of *M. pulmonis* disease and that *M. pulmonis* lesions were interpreted as lymphoma. They base this argument on (1) the fact that the morphology and organ distribution of the lymphomas reported in this study are atypical of lymphoma in rats. (2) Lymphocyte and plasma cell accumulation in the lung is characteristic of M. pulmonis infection and (3) *M. pulmonis* disease can be exacerbated by chemical treatment.

Reviewer 4

The paucity of experimental methanol studies, particularly those related to carcinogenicity, may result in the acceptance of data that would otherwise be rejected for regulatory purposes if a robust database existed. While the ERF has made several noteworthy contributions to cancer risk assessment, the strength and credibility of its methanol study are diminished by the lack of rigor to ensure, to the extent possible, the absence of factors that could potentially confound the causal relationships its authors espouse. In the case of the methanol study, this should preclude its consideration for quantitative risk assessment or a weight-of-evidence evaluation until the health of

experimental animals is confirmed. Quite simply, those wishing to use the study for regulatory purposes should bear the burden of restoring the study's credibility.

- **Were the individual animal data correctly summarized?**

The Soffritti et al. (2000) publication has been supplemented with individual animal data for several parameters (e.g., neoplasms, individual animal pathology, body weight, animal history) that appear complete and well organized. In particular, neoplasms by individual animal revealed few cases of insufficient tissue, missing tissue, autolysis precluding evaluation, or a failure to examine microscopically. The accompanying summary tables or tables of average values supplied by ERF likewise appear complete and well organized, but a systematic effort to determine whether they accurately represent the aggregate of all individual animal data was not made. The supplementary data do increase the strength and credibility of the Ramazzini methanol study considerably, but are insufficient to resolve the issue of whether the bioassay was conducted in healthy animals. It appears as though a very large proportion of rats, regardless of dose group, exhibited inflammatory responses, particularly in the lung. For example, the data on non-neoplastic lesions indicate mild to moderate inflammation of the bronchus in 52, 43, 28 and 24% of male rats and 66, 54, 43 and 35% of females at 0, 500, 5000 and 20,000 ppm methanol, respectively. Based on these data, the incidence of inflammation was inversely related to methanol concentration, suggesting an alternative causal factor.

- **Are there nomenclature issues that need clarification?**

As for nomenclature, the only issue recognized as potentially problematic is the use of the term "lympho-immunoblastic lymphoma". Its use in the case of the Ramazzini methanol study is important in that it accounts for the vast majority of hemolymphoreticular lymphomas or leukemias in males and females regardless of dose (i.e., 61-85% of all hemolymphoreticular neoplasms for both sexes combined). Others have argued that this terminology is problematic since its use is confined to ERF studies and thus affords little or no opportunity to compare rates of this specific type of lymphoma to those of historical controls. As it is the dominant tumor type in the methanol study in terms of the total number of animals affected, defining its exact meaning would be a logical early step in resolving the issue of whether lesions secondary to *M. pulmonis* were mistaken for this neoplasm type.

- **Was adequate statistical information provided for quantitative dose-response analyses?**

Concerning the adequacy of statistical information for dose-response analysis, the original publication of Soffritti et al. (2002) was limited to use of the chi-square test to evaluate differences in tumor incidence between treated and control groups and the Cochran-Armitage test to evaluate the dose-dependency of tumor rates. These statistical tests have been supplemented by the ERF to include detailed statistical analysis of survival data, non-neoplastic lesions and primary tumors, including several poly-*k* tests (essentially survival-adjusted Cochran-Armitage tests) which were necessitated by the full lifetime design of the study and the resulting differential mortality patterns observed across dose groups. With these supplemental data supplied by ERF, adequate information is available to support quantitative dose-response analysis. Concerns for data credibility remain, however, which no amount of statistical testing can alleviate.

- **Reported Study Results**

The following are brief evaluations of the strength of evidence supporting Soffritti et al.'s conclusions that various lesion types are methanol related:

1) Total malignant tumors: Table 2, p. 56 of the Ramazzini methanol study presents data on total malignant tumors that are supplemented with ERF-supplied data entitled, "Statistical Analysis of Primary Tumors in Rats" (see p. 120 for male data and p. 242 for female data). For both sexes, a statistically significant dose-response relationship is seen, with tumor rates statistically significantly differing from that of controls at one or more methanol concentrations. Even so, as a rule, I do not consider total malignant tumor data from a variety of sites as evidence of a treatment-related carcinogenic effect. That is, tumors with different target organs, originating in different cell types, and potentially occurring by different mechanisms of action should not be combined to generate incidence data. [Note that Table 2 of the Soffritti et al. (2000) publication indicates 50 tumor-bearing males in the control group, whereas the supplemental table on p. 120 indicates 49].

2) Carcinomas and osteosarcomas of head and neck: Table 3, p. 57 of the Ramazzini methanol study presents data on carcinomas of the head and neck, of which only those in ear ducts warrant attention. As with total malignant tumors, ear duct carcinomas exhibit a statistically significant dose-response relationship in both sexes, with tumor rates statistically significantly differing from that of controls at one or more methanol concentrations (see "Statistical Analysis of Primary Tumors in Rats," p. 21 for male data and p. 138 for female data). Contrary to that reported in a commentary prepared for the Methanol Institute, ear duct carcinoma in rats is not a tumor type specific to the ERF. Most importantly, the NTP limited pathology working group of the Ramazzini aspartame

study felt as though many lesions diagnosed as ear duct carcinomas were not tumors at all. This alone is sufficient to cast doubt upon the reported ear duct carcinoma increase in the methanol study. In addition, in the ERF supplemental table detailing statistical analyses of non-neoplastic lesions, 57 to 80% of females and 60-71% of males reportedly had ear inflammation. The occurrence of ear inflammation exhibited a *reverse* dose trend in both sexes (statistically significant in females), implicating a causal factor other than methanol. This is of interest considering that the middle ear is a typical site of colonization with *M. pulmonis* and inflammation is commonplace under such conditions. [Note that Table 3 of the Soffritti et al. (2000) publication indicates 17 and 13 animals with ear duct carcinomas in the 5000 and 500 ppm dose groups, whereas the supplemental table on p. 21 indicates 16 and 12].

As for osteosarcomas of the head as detailed in Table 4, p. 58 of Soffritti et al. (2000), the dose-response data are not robust enough to be considered evidence of a treatment-related effect. This is supported by the lack of a statistically significant dose trend in both sexes and no statistically significant difference in tumor rates between treated and control rats at any methanol concentration (see "Statistical Analysis of Primary Tumors in Rats", p. 15 for male data and p. 132 for female data). [Note that Table 4 of the Soffritti et al. (2000) publication indicates 6 females with osteosarcomas in the 20,000 ppm methanol group, whereas the supplemental table on p. 132 indicates 5].

3) Testicular hyperplasia/adenoma: It is recognized that in the rat testes, focal hyperplasias tend to progress to adenomas, making both of interest. Page 87 of the ERF supplement entitled, "Statistical Analysis of Primary Tumors in Rats," indicates no statistically significant dose trend (12, 9, 13 and 17% at 0, 500, 5000 and 20,000 ppm, respectively) and no statistically significant differences in the rates of testicular adenoma between any methanol treated group and controls. According to p. 109 of the ERF supplement entitled, "Statistical Analysis of Non-Neoplastic Lesions in Rats," testicular hyperplasia demonstrated a statistically significant dose trend with an increase in the rate of hyperplasia being a high-dose phenomenon (10, 3, 6 and 23% at 0, 500, 5000 and 20,000 ppm, respectively). Thus, testicular hyperplasia may well be methanol related, but clear progression to benign tumor was not observed under the experimental conditions.

4) Uterine sarcoma: Data for tumors classified specifically as "uterine sarcomas" could not be located in Table 1 of Soffritti et al. (2000) or in the ERF supplement entitled, "Statistical Analysis of Primary Tumors in Rats." However, the two main types of uterine sarcomas are leiomyosarcoma (cancer that begins in smooth muscle cells) and endometrial stromal sarcoma (cancer that begins in connective tissue cells). According to Table 1 of Soffritti et al. (2000), only one rat developed a leiomyosarcoma and that was

in the 5000 ppm dose group. As uterine leiomyomas can rarely become malignant leiomyosarcomas, also of interest is that the ERF supplemental data indicate statistically significant *reverse* dose trends for leiomyomas (p. 222 of "Statistical Analysis of Primary Tumors in Rats") and stromal polyps of the uterus (p. 225), suggesting that neither are methanol related.

5) Hemolymphoreticular neoplasias: As discussed previously, according to Table 5 of Soffritti et al. (2000), lymphoimmunoblastic lymphoma is the only histocytotype of hemolymphoreticular neoplasm that warrants attention, as it accounts for the lion's share of such neoplasms and is the only one to demonstrate a positive dose-response relationship. Due to possible confounding by *M. pulmonis* infection, the validity of the treatment related differences in reported lymphoimmunoblastic lymphoma is suspect. Such treatment related differences may be due to methanol itself, *M. pulmonis* infection being mistaken for lymphoma, or the two acting in concert. A comparison of Table 5 in the Soffritti et al. (2000) publication and p. 44 (males) and p. 160 (females) of the ERF supplement entitled, "Statistical Analysis of Primary Tumors," indicates that all but four of the lymphoimmunoblastic lymphomas in males and all but twelve in females were in the lung. Thus, it is of interest that lymphoimmunoblastic lymphomas in the lungs of both sexes demonstrated a statistically significant dose trend and statistically significant differences between at least one methanol treatment group and controls. Should *M. pulmonis* infection be ruled out, it would therefore be prudent to carefully consider the lymphimmunoblastic lymphoma data for risk assessment purposes. Until that time, I must agree with Schoeb et al. (2009) that *M. pulmonis* is the most plausible explanation for the lymphomas.

- **Respiratory Infection**

I have reviewed the tumor patterns in the Ramazzini methanol study, commentaries by others on tumor patterns in the Ramazzini studies of aspartame and MTBE, several publications on *M. pulmonis* infection and its relationship to cancer, and the advocacy pieces mentioned in the question above. Commendable arguments for and against involvement of *M. pulmonis* infection have been made, but I believe evidence for infection exists, albeit circumstantial. I am most impressed with the effort of Schoeb et al. (2009) that argues infection is a plausible explanation for the lymphoma excesses reported. These authors appear to have a level of expertise on the issue of *M. pulmonis* not shared by others participating in what has seemingly become a counterpoint argument. Nonetheless, not unlike others who have examined the issue, I have no direct evidence with which to resolve the *M. pulmonis* issue with any degree of scientific certainty. Under such circumstances, I believe the issue rightfully becomes a philosophical one. In a recent publication, USEPA employees (Caldwell et al., 2008) evaluated evidence for infection as a mode of action for rat lymphomas and conclude

with a paragraph that suggest the absence of *direct* evidence supporting the assertion of *M. pulmonis* involvement justifies concluding otherwise. I disagree and argue essentially the opposite – studies which fail to take reasonable steps to ensure the validity of so-called causal relationships (by the application of serological testing and other widely accepted QA procedures) should be viewed as suspect and deemed unfit for regulatory purposes. After all, it is the USEPA that is considering Soffritti et al. (2000) for the purpose of cancer risk assessment, and thus the Agency should bear the burden of proving its validity prior to use. Such validation would come with evidence that animals used in the Ramazzini methanol study (or perhaps animals used in the study of aspartame and/or MTBE) were *M. pulmonis* free. I encourage the USEPA to bear in mind the old adage that "the absence of proof is not the proof of absence" when it comes to *M. pulmonis* infection.

Reviewer 5

- **Were the individual animal data correctly summarized?**

Individual data seem to be adequately listed in supplemental tables provided to me for evaluation. Means of summarizing the tumor data are stated, but the analytical approach is less than desirable. In my experience, individual tumor types are analyzed separately, and if warranted, additional summary analyses may also be conducted. I find it difficult to evaluate "statistical findings" of one without the other, much the same as absolute and relative organ weight data in standard toxicity studies.

- **Are there nomenclature issues that need clarification?**

Tumor and pathological nomenclature is not within my specialty area regarding carcinogenicity studies. I did find the nomenclature used in the individual findings and summary evaluations to not always be clear and straight-forward.

- **Was adequate statistical information provided for quantitative dose-response analyses?**

Apparently "adequate" statistical information was provided for "standard" quantitative external concentration-response analyses. What I do not see is in this report is any evaluation of exposure-related patterns of tumors over time of exposure, an especially important component of evaluating risk in this study design, and no distinction of occurrence within this laboratory/design of "rare" versus "common" tumors in the analyses - again in part supporting the importance of being able to reference available historical control information.

- **Reported Study Results**

(1) a dose-related increase of total malignant tumors in males and females of treated groups (TABLE 2);

The data presented as summarized and analyzed appear to support this statement.

(2) a dose-related increase of carcinomas of the head and neck, mainly in the ear ducts, in males of treated groups and in females treated with 20,000 and 5,000 ppm (TABLE 3);

The data presented as summarized and analyzed appear to support this statement

(3) a statistically significant increase (P <0.01) of testicular interstitial cell hyperplasias and adenomas in the group treated with the highest dose;

The data presented in Table 1 do not include an incidence of hyperplasia, only adenomas, and the summary data on which the stated statistically significant increase was found is not presented.

(4) an increase in sarcomas of the uterus at the highest dose;

The data presented in Table 1 do **not** support this statement.

(5) a dose-related increase in osteosarcomas of the head in males and females of the treated groups (TABLE 4); and

The data presented as summarized and analyzed appear to support this statement.

(6) a dose-related increase in hemolymphoreticular neoplasias in males and females of the treated groups (TABLE 5).” Page 58.

The data presented as summarized and analyzed appear to support this statement.

- **Respiratory Infection**

The above referenced studies suggest that there may be a health issue in the laboratory environment in which standard studies are conducted and/or the preponderance of such "life-time" neoplasms in the animal source used in these studies under the study design employed. Without testing or reporting basic health screening results (that have been standard components for long-term regulatory studies for well more than 2 decades) from test subjects in the current study, the laboratory leaves itself open to such criticism - and thus, provides another reason, in addition to those others mentioned above, for questioning the bottom line findings of the current study (and perhaps all of these studies).

3. Study Conclusions

- ***Were there critical results or issues that were not addressed? Were there any contradictory statements or observations made?***
- ***Do you agree with the authors' conclusions of the study?***

Reviewer 1

- **Were there critical results or issues that were not addressed? Were there any contradictory statements or observations made?**

As discussed above, the authors did not utilize age adjusted statistical tests that address the effect of animal survival on tumor incidence rates. Subsequent age adjusted analyses did not indicate important differences from the results reported by the authors.

- **Do you agree with the authors' conclusions of the study?**

As discussed above in Section 2d, I agree with some, but not all of the conclusions presented by the authors.

Reviewer 2

- **Were there critical results or issues that were not addressed?**

1. The experimental design remains poorly/incompletely described. For example, Soffritti does not explicitly state whether or not a concurrent control group was used. Supplemental data show that animals were not randomly assigned to treatment groups – this is a significant deviation from accepted study designs.
2. Most importantly, histological descriptions of the tumors are not provided. In addition, the tumors diagnoses were not subjected to external peer review by qualified toxicologic (veterinary) pathologists – this is a significant weakness in the study.
3. Sentinel animal programs (bacteriology, virology, etc) were not incorporated into the study design.

- **Were there any contradictory statements or observations made?**

None noted

- **Do you agree with the authors' conclusions of the study?**

1. Based on the data presented the author's conclusions appear largely supported. However, the overall quality of the study description raises a number of significant

concerns and the incomplete data reporting (e.g., histologic descriptions of tumors, etc) preclude my ability to independently support the author's conclusions.
2. The lack of data concerning non-treatment related findings is also troubling in this study.

Reviewer 3

- **Were there critical results or issues that were not addressed? Were there any contradictory statements or observations made?**

The conclusions section of the EMF study relating to methanol was 9 lines in length. It generalized the results obtained with methanol using sweeping statements stating that that methanol was a multipotenial carcinogenic agent with no discussion of the supporting data. It also skimmed over the potential carcinogenic metabolites generated in the metabolism of methanol suggesting that formaldehyde was a possible agent or that methanol enhanced the effects of unnamed endogenous or exogenous carcinogenic factors. Essentially there was no discussion of the results obtained in this study or their relationship to the findings of other comparable studies. The lack of a detailed and thoughtful discussion section further undermines the credibility of this research.

- **Do you agree with the authors' conclusions of the study?**

The evidence presented in the ERF study suffer from numerous limitations, most importantly the documented pulmonary disease in the control and treatment groups confounding any reliable interpretation of the results.

Reviewer 4

As stated earlier, issues with the study of Soffritti et al. (2000) abound, many of which in a practical sense have little influence on the validity of the bioassay results. One obvious exception is the issue of *M. pulmonis* infection which cast severe doubt upon the validity of data used to support the authors' label of methanol as a multipotential carcinogen. Based on my strength-of-evidence evaluation for several lesion types (see my response to question number 2), I obviously disagree with the authors that their data are robust enough to support such a classification. In my opinion, the singular issue of *M. pulmonis* infection is sufficient to not only call into question lymphomas that occurred mainly in the lung, but also other tumor types reported in excess in different organ systems, as well as non-neoplastic lesions. Not surprisingly, issues such as potential confounding by *M. pulmonis* were not mentioned in the original manuscript, but have been addressed in subsequent publications including those in which Soffritti himself defended the work of the ERF. Several issues that have been raised by detractors of the ERF can admittedly be

resolved by the ERF-furnished supplementary data tables that provide details beyond what a typical journal article can accommodate. Unfortunately, the one issue that cast the most doubt as to the study's validity (*M. pulmonis* infection) is the most difficult (if not impossible) to resolve. Of interest would be whether USEPA or ERF scientists have attempted to apply state-of-the-science techniques to paraffin embedded or frozen tissues to once and for all lay the issue to rest. If not, why not?

Reviewer 5

- **Were there critical results or issues that were not addressed? Were there any contradictory statements or observations made?**

The majority of my critical concerns have been touched on in the above responses: 1) no historical information; 2) no internal exposure information; 3) no evaluation of time-frame of exposure and occurrence of apparently treatment-related tumors; 4) lack of standard GLP components in the study, e.g. health status, external dose consumption, agent stability in drinking water, etc.; and 5) some strong concluding statements without data supporting those statements (uterine and testicular conclusions).

- **Do you agree with the authors' conclusions of the study?**

For the reasons mentioned immediately above, it's difficult for me to fully agree with the conclusions and implications the authors draw from their study. It's truly unfortunate that such deficiencies occurred in the conduct of this oral 2-year bioassay. The conclusions and implications of the findings from this study are different from the earlier NEDO 2-year study conducted in a different strain of rats, using a smaller sample size/group, round-the-clock inhalation exposure of apparently lower external "dose" levels, as well as a different profile of findings being reported in the two rat studies.

4. Study Reliability – *Describe the reliability of the study for consideration in the derivation of EPA IRIS quantitative health benchmarks and the qualitative characterization of cancer risk. Describe any major strengths or uncertainties with this study that might preclude it from being used as consideration for:*

- ***derivation of a noncancer reference concentration,***
- ***determination of the weight-of evidence for methanol's cancer risk***
- ***derivation of a cancer slope factor***

Reviewer 1

- **Derivation of a non-cancer reference concentration**

The authors addressed only tumors. No data or discussion of non-cancer endpoints was presented. The study certainly provided results for non-cancer endpoints for which benchmark dose analyses could be conducted.

- **Determination of a weight-of-evidence for methanol cancer risk**

Statistical significance levels for dose response trend tests were provided by the authors and summarized above in Section 2d based on some additional statistical analyses conducted by this reviewer. Further, it is indicated in Section 2d if dose response trends were observed in only one or both sexes.

- **Derivation of cancer slope factors**

Those endpoints amenable for the calculation of benchmark doses were in indicated above in the discussion for Question 2.

Reviewer 2

- **Derivation of a noncancer reference concentration**

As noted earlier, the study does not provide an adequate description of non-tumor endpoints. Thus, the Soffritti study is inadequate for derivation of a noncancer reference concentration.

- **Determination of the weight-of evidence for methanol's cancer risk**

1. The Soffritti study does not provide any useful mechanistic data to support a cancer mode of action for methanol. Likewise, it did not develop any data concerning cell proliferation or other pre-neoplastic changes that could support the reported carcinogenicity data.
2. The study quality does not meet contemporary standards for rodent carcinogenicity studies.
3. Study lacks responses in a second species.
4. Study is not supported by other animal carcinogenicity studies (e.g., NEDO study).

- **Derivation of a cancer slope factor**

Note quantitative cancer risk assessment approaches is beyond my expertise

Reviewer 3

The following are uncertainties that preclude this study from being used in consideration for all of endpoints listed above:

- Lack of barrier maintained pathogen free animal facility
- Animals not randomly assigned to treatment groups
- Lack of characterization of disease surveillance
- Sacrifice of animals at end of lifetime
- Absence of age-matched control and treatment pathology
- Possible use of historical controls for study
- Methanol concentrations in drinking water not measured making it impossible to determine the dose administered to each animal
- Limited data on water intake confounding assessment of dose administered.
- No measurement of blood methanol concentrations in control and treatment group
- Evidence of lung pathology in all treatment groups
- Degree of lung pathology indicative of chronic respiratory infection in the colony
- likelihood that studies were confounded by *M. pulmonis* infection and that the lesions of this disease were incorrectly interpreted as lymphoma
- Absence of external pathology evaluation

Reviewer 4

The original Soffritti et al. (2000) study failed to present incidence data for non-neoplastic lesions, but such data were furnished subsequent to publication by the ERF with lesions graded as minimal, mild, moderate or marked. I reviewed the ERF-furnished supplementary table entitled, "Statistical Analysis of Non-Neoplastic Lesions in Rats" for those lesions having both a statistically significant positive trend test and a significant elevation in lesion rate compared to controls. The lesions listed below met these two criteria. Lesion rates in the 0, 500, 5000 and 20,000 ppm groups are shown in parentheses. I was struck by the extraordinarily large number of non-neoplastic lesions exhibiting statistically significant *reverse* dose trends, something that cast doubt on the health of control animals and suggest causal factors other than methanol. Not a single lesion listed below in either sex increased in severity with increasing dose, but all lesion types at all doses were deemed "moderate" with the exception of fatty liver degeneration in females that was "marked" in all dose groups, including controls. Based on these findings and considering sex concordance, robustness of the dose-response function and

lesion severity, I remain unconvinced that any non-neoplastic lesion identified in the Ramazzini methanol study is suitable to serve as the basis for a non-cancer reference dose. This is particularly the case as I recently reviewed the NEDO methanol studies for US EPA and consider them more credible than that of Soffritti et al. (2000) for use in this context. For reasons discussed in response to other questions, use of the Ramazzini methanol study for cancer slope factor derivation would also be inappropriate. Until the *M. pulmonis* infection issue is resolved, I believe its use in a weight-of-evidence cancer classification also runs counter to good science and policy.

Males

- Kidney, bilateral renal tubule inflammation (2, 1, 2 and 9%) – severity did not increase with dose
- Kidney, renal tubule inflammation (2, 1, 2 and 9%) – severity did not increase with dose
- Lung dysplasia (8, 12, 11 and 17%) –severity did not increase with dose
- Lymph node, mediastinal dysplasia (0, 1, 2 and 8%) – severity did not increase with dose
- Stomach, forestomach dysplasia (1, 0, 0 and7%) – severity did not increase with dose
- Testes degeneration (1, 4, 2 and 9%) – severity did not increase with dose
- Testes hyperplasia (10, 3, 6 and 23%) – severity did not increase with dose

Females

- Liver, fatty degeneration (27, 17, 23 and 53%) – severity did not change with dose, but was marked in all dose groups including controls
- Lung, dysplasia (4, 7, 14 and14%) – severity did not increase with dose
- Lung, bronchus chronic inflammation (0, 5, 1 and 13%) – severity did not increase with dose
- Lymph node, axillary, inguinal, mesenteric dysplasia (1, 5, 1 and 7%) – severity did not increase with dose
- Spleen, pigmentation hemosiderin (18, 26, 32 and 35%) – severity did not increase with dose
- Stomach, forestomach dysplasia (0, 0, 0 and 7%) – severity did not increase with dose

Reviewer 5

- **Derivation of a noncancer reference concentration**

While derivations are not my area of expertise, data are not presented in this publication/study for such a derivation, and in fact suggest that even at the highest external concentration used (which is apparently a very high "dose") there was little or no

standard toxicity observed ("slight" increases in body weights, no change in survival rates, etc), incomplete reporting of overall histopathological findings, in the endpoints collected.

- **Determination of the weight-of evidence for methanol's cancer risk**

Reliability, or confidence, in hazard identification to me means general replicability of findings. It seems that the only truly repeatable suggestion of findings from this laboratory is a treatment-related increase in lymphomas, as also noted in more recent publications of studies using other agents than MA. It's hard to dismiss the overall potential for an increase in tumors from the data provided, but it is difficult for me to be confident in that potential when basing it on data from a study using this overall design and especially the lack of a variety of information as I've noted above.

- **Derivation of a cancer slope factor**

This is not my area of expertise. Apparently there is enough data contained in the report and in the accompanying individual data for some standard risk assessment calculations. From my perspective, I'm not sure what such calculations mean relative to human risk when using data from a study using this overall design and especially the lack of reference information as I've noted above.

5. Other Issues - *Please identify and discuss any other relevant scientific issues or comments not addressed by the above questions.*

Reviewer 1

High concentrations of methanol (≥500 ppm) were used in the study. The question always remains whether pathways and reactions were altered at high concentrations that would not prevail at human exposure levels.

Reviewer 2

None Identified

Reviewer 3

The questions above articulate the scientific issues that concern this reviewer

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Reviewer 4

None Identified.

Reviewer 5

None Identified.

REVIEW OF APAJA (1980)

1. Study Design - ***Based on your knowledge of toxicological study protocols, please comment on the experimental design of the Apaja (180) study:***

- ***Do you see any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?***
- ***In light of the chemical and toxicological profile for methanol, comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation.***
- ***The study was conducted using the full lifespan of the animals. Please comment on if or how this study design affects the interpretation of the results.***

Reviewer 1

- **Do you see any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?**

Employment of 25 animals per methanol dose group is less than bioassays that typically employ 50 animals per dose group. Only one species (mice) were used.

I conducted benchmark dose analyses for the lesions related to oral methanol exposure. For all of these lesions, the lower 95% confidence limit for the benchmark concentration estimated to produce an extra 10% incidence of the lesion ($BMCL_{10}$) fell within or near the range of experimental methanol concentrations (2222-8889 ppm). Hence, the spacing of the three concentrations provided adequate information on the incidences of lesions for calculating $BMCL_{10}$'s for oral exposures to methanol.

The statistical analyses employed did not take into account differences in survival among dose groups. Visual examination of Figures 11-13 do not appear to show large differences in survival among the three methanol concentration groups.

The high methanol concentrations used in the control groups do not provide any direct information on the shape of the dose response below 2222 ppm.

- **In light of the chemical and toxicological profile for methanol, comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation.**

None suggested.

- **The study was conducted using the full lifespan of the animals. Please comment on if or how this study design affects the interpretation of the results.**

The full lifespan group with 2222 ppm methanol did not appear, upon visual inspection, to present serious difficulties. Survival appeared visually to be similar across the three methanol concentration groups.

Reviewer 2

- **Do you see any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?**

A number of significant deficiencies exist within the study including:

1. Animals being observed until they died – this is a significant deviation from standard rodent carcinogenicity studies conducted under USEPA or NTP study guidelines.
2. *No concurrent control group existed in either the dermal or drinking water study.* This is a fatal flaw for this study. The reported incidences for the methanol-exposed mice were reported to be within the normal incidence for this strain of mice at the Institute thus there is no compelling data from the study to demonstrate a carcinogenic response to methanol.
3. Organ weight data were not reported.
4. Histopathologic data did not include adequate histologic descriptions of either the tumor or non-tumor lesions. Qualification of the pathologist(s) reading the study is not provided. No peer review of the pathology data occurred.
5. Purity of methanol used and analytical methods used to confirm dosing solutions are not provided. Stability of chemical dosing solutions is not described.
6. Research standards (GLP or otherwise) are not provided.
7. Statistical methods used are incompletely described. The specific statistical test used (e.g., Pearson's Chi Square test) was not explicitly stated. The Chi Square test assumes that the observations are independent of each other – it is not clear whether this applies for all tumor types). Statistical methods used to assess non-

cancer endpoints were not specified. No control group was provided so the only statistical comparisons available were to malonaldehyde-exposed treatment groups or historical control data.

8. Sentinel animal programs are not included – background viral or bacterial infections cannot be ruled out.
9. It appears that increased late mortality may have occurred with the high dose oral methanol group – however no explanation for this observation is provided by the study author.

- **In light of the chemical and toxicological profile for methanol, comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation.**

1. Blood methanol concentrations would have provided additional useful information.
2. A modern study might include an assessment of formaldehyde adducts, DNA-protein cross links etc.
3. No mechanistic data are provided.
4. Clinical chemistry data are lacking.

- **The study was conducted using the full lifespan of the animals. Please comment on if or how this study design affects the interpretation of the results.**

There is no indication of whether the pathology samples were optimal in that animals that died during the course of the experiment may have undergone cannibalization and/or autolysis prior to preservation.

Reviewer 3

- **Do you see any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?**

A. Animals and Animal Husbandry:

1. Animals not Maintained in Barrier-maintained, specific pathogen free facility.
According to method presented the mouse colony used for the Apaja studies was conventionally maintained and not subject to the rigorous health assurance and disease control measures necessary to exclude pathogens. Barrier maintained pathogen-free conditions are required for a reliable long-term carcinogenesis study.

2. No documentation of sanitation and animal hygiene practices including sterilization of food, water filtration and sterilization, sterilization of cages and other equipment. Regular replacement of cages, feeders and water replacement not documented or discussed.

3. No evidence of disease surveillance in the mouse colony or over the course of the study. For appropriate disease surveillance, blood must be collected from study and sentinel animals every 4-6 months and checked for serum antibodies to rodent viruses (hepatitis, adenovirus, Sendai virus) and bacteria (*Mycoplasma pulmonis, Bacillus piliformis, Salmonella typhimurium, Corynebacterium kutscerihi*.)

B. Study Design and Protocol:

1. Animals were randomly assigned to treatment groups – appropriate experimental design

2. There were 25 animals in each treatment group. The small "n" in this study limits the power of the statistical analysis. To define a statistically ($p<0.05$) difference between 40% and 18% would require approximately 80 animals in each treatment group.

3. This study used historical untreated controls for methanol exposure. It is important for untreated control animals to be included in the study. The use of historical controls confounds the interpretation of the data. Moreover historical controls are not matched to the experimental group in each aspect of treatment except methanol exposure.

4. Dose of methanol administered: No confirmation of correct dosage of methanol. HPLC determination of concentration of methanol in the water required for reliable study.

5. Methanol Dose Range: Similar to the ERF study the calculated doses of methanol were 500, 1000 and 2000 mg/kg per day, six days per week for the life of the animals. Despite the fact that carcinogenesis studies are designed to examine toxic actions at doses greater than those anticipated to be encountered in an environmental exposure, these doses far exceed any scenario for environmental methanol exposure.

C. Endpoints Recorded:

1. Methanol and Formic Acid: The Apaja study did not determine blood methanol, or blood formic acid concentrations.

D. Terminal Procedures:

Animals were euthanized when moribund, thus greatly reducing the potential for tissue autolysis which was not controlled for in the ERF study.

Statistical Analysis: Sample size too small for reliable statistical analysis

Reviewer 4

See below.

2. Study Results - *Please comment on the strength, credibility, and relevance of the toxicological results of the Apaja (1980) study, supplemented by the study data tables from ERF:*

- ***Were the individual animal data correctly summarized?***
- ***Are there nomenclature issues that need clarification?***
- ***Was adequate statistical information provided for quantitative dose-response analyses?***
- ***For each lesion identified by Apaja (1980), please comment on the strength of the evidence supporting the authors' conclusions that the lesion is treatment-related.***

Reviewer 1

- **Were the individual animal data correctly summarized?**

Appropriate data summaries are presented.

- **Are there nomenclature issues that need clarification?**

Histopathological nomenclature is outside of my area of expertise.

- **Was adequate statistical information provided for quantitative dose-response analyses?**

Sufficient results were presented for neoplastic and pathological lesions for concentration groups in order to conduct quantitative dose response analyses and estimation of benchmark concentrations.

- **For each lesion identified by Apaja (1980), please comment on the strength of the evidence supporting the authors' conclusions that the lesion is treatment-related.**

The author did not examine the results of methanol exposure. Statistical analyses performed by this reviewer indicated a statistically significant increase in the incidence of malignant lymphoma (lymphocytic well differentiated) with increasing methanol concentration in females, based on the Cochran-Armitage trend test yielding a P-value < 0.05. Using the U.S. EPA Benchmark Dose software (BMDS), the multistage cancer model provided a $BMCL_{10}$ = 5400 ppm of methanol. Also, a statistically significant (P<0.025) increasing trend for the incidence of pancreatitis with increasing concentrations of methanol was present in males. Using the logistic model, a $BMCL_{10}$ = 7400 ppm of methanol was obtained for pancreatitis in males.

Reviewer 2

- **Are there nomenclature issues that need clarification?**

1. Individual animal results are not available. Tumor incidence data and time of death are not provided.
2. Tumor nomenclature is poorly defined. Histological descriptions of tumor types are lacking.
3. Apaja uses a variety of terms to describe the neoplasia observed in the study. Diagnoses include well differentiated lymphocytic lymphoma, moderately differentiated lymphocytic lymphoma, poorly differentiated lymphocytic lymphoma, mixed cell type lymphoma, histiocytic lymphomas, and unclassified lymphomas. The histologic descriptions used to support these diagnoses are not provided thus it is difficult to discern whether these diagnoses are consistent with others. For example, Firth (1988) describes common morphological classification of hematopoetic neoplasms in Sprague-Dawley rats. These include: lymphomas (lymphoblastic lymphoma, immunoblastic lymphoma, follicular center cell (FCC) lymphoma, plasma cell lymphoma, and large granular lymphocyte lymphoma).
4. Note also that Firth (1988) states: "Autolytic change may complicate the diagnosis of LGL lymphoma…Severe autolysis may necessitate the diagnosis of lymphoma, NOS (not otherwise specified)." This illustrates my previous concern about possible autolysis of the samples from animals that died on study.

- **Was adequate statistical information provided for quantitative dose-response analyses?**

No.

- **Were there critical results or issues that were not addressed?**

1. The experimental design is flawed since a concurrent control group was not used.
2. Most importantly, histological descriptions of the tumors are not provided. In addition, the tumors diagnoses were not subjected to external peer review by qualified toxicologic (veterinary) pathologists – this is a significant weakness in the study.
3. Sentinel animal programs (bacteriology, virology, etc) were not incorporated into the study design.
4. Data for observed tumors appear to fall within the lab's historical controls and therefore does not provide evidence in favor of methanol being carcinogenic in mice.
5. Clinical chemistry data are not reported.
6. Clinical signs data and cage side observations (if any) were not reported.

Reviewer 3

1. The small size of each treatment group limits the reliability of the study

2. The observation that the incidence of lymphoma was greater in the methanol control group than in the malonaldehyde treatment groups limits the reliability of the study

3. The lack of an untreated control group limits the reliability of the study

Reviewer 4

See below.

3. Study Conclusions

- ***Were there critical results or issues that were not addressed? Were there any contradictory statements or observations made?***
- ***Do you agree with the authors' conclusions of the study?***

Reviewer 1

The effects of methanol were not examined by the author. Conclusions based on the analyses conducted by this reviewer are presented above in the response to Question 2.

Reviewer 2

- **Were there any contradictory statements or observations made?**

Of note, Apaja reports incidences of malignant lymphoma in females of 16%, 36%, and 40% for the low, mid, and high dose groups, respectively. Males from the drinking water study had incidences of malignant lymphoma of 4, 24, and 16% for the low, mid, and high dose groups, respectively. Reportedly, the incidence rates for the mid and high dose exposure groups were higher than the home laboratories historical controls. Despite that observation, the authors report that the "percentages are still within normal ranges of malignant lymphomas in Eppley Swiss mice." Also note that Apaja's control data for malignant lymphomas are presented in two different Tables (Table 9 and 11). Table 11 presents an overall incidence rate for malignant lymphoma of 18% in 100 untreated control female Swiss mice (note age at evaluation is not specified). Table 9 seemingly reports an overall incidence rate for malignant lymphoma of 20% in 100 untreated control female Swiss mice (age at death varied from 31 to 116 weeks). Table 9 also reports an overall incidence rate for malignant lymphoma of 8% in 100 untreated control male Swiss mice (age at death varied from 28 to 112 weeks). Neither Table appears to support the author's conclusion that "percentages are still within normal ranges of malignant lymphomas in Eppley Swiss mice".

- **Do you agree with the authors' conclusions of the study?**

1. The authors did not provide a definitive conclusion regarding the carcinogenicity of methanol.
2. The lack of data concerning non-treatment related findings is also troubling in this study.

Reviewer 3

- **Were there critical results or issues that were not addressed? Were there any contradictory statements or observations made?**

Yes. -- In the Apaja dissertation study the author concludes that there was a statistically significant increase in the occurrence of malignant lymphoma in both males (24%) and females (40%) [each treatment group n=25 animals] compared to historical data (18%) [n=100 animals], However, he later states that that "the percentages (assumed to be the percentages reported for the methanol control group in the present study) are within the normal range of occurrence of malignant lymphomas in Eppley Swiss mice.

- **Do you agree with the authors' conclusions of the study?**

The Apaja study does not make a case supporting methanol induced carcinogenicity in Eppley Swiss mice following chronic oral administration of methanol at 500, 1000 and 2000 mg/kg. This assessment is based on (1) the small sample size of the present experiment [25 mice in each experimental group]; (2) the observation that the female methanol control group (40% incidence of lymphoma) had a significantly greater incidence of respiratory disease than untreated historical controls and (3) the observation that the incidence of lymphoma was greater in methanol control groups than in malonaldehyde exposed groups.

Reviewer 4

See below.

4. Study Reliability – *Describe the reliability of the study for consideration in the derivation of EPA IRIS quantitative health benchmarks and the qualitative characterization of cancer risk. Describe any major strengths or uncertainties with this study that might preclude it from being used as consideration for:*

- ***derivation of a noncancer reference concentration,***
- ***determination of the weight-of evidence for methanol's cancer risk***
- ***derivation of a cancer slope factor***

Reviewer 1

- **Derivation of non-cancer reference concentration**

This study provides data for the estimation of $BMCL_{10}$'s for potential use as points of departure in establishing a non-cancer reference concentration for oral exposure to methanol.

- **Determination of weight-of-evidence for methanol cancer risk**

The study by Apaja (1980) provides evidence of a carcinogenic effect of methanol in a second species (mice), which reinforces the carcinogenic effects as observed in rats by Soffritti *et al.* (2002).

- **Derivation of cancer slope factor**

The study by Apaja (1980) provides additional data for the estimation of a cancer slope factor.

Reviewer 2

- **Derivation of a noncancer reference concentration**

As noted earlier, the study provides some useful information of non-tumor pathology – however, the histologic descriptions for the observed changes are lacking. Moreover, no concurrent control group was used thus this study is inadequate for derivation of a noncancer reference concentration.

- **Determination of the weight-of evidence for methanol's cancer risk**

1. The study does not provide any useful mechanistic data to support a cancer mode of action for methanol. Likewise, it did not develop any data concerning cell proliferation or other pre-neoplastic changes.
2. The study quality does not meet contemporary standards for rodent carcinogenicity studies.
3. Study lacks responses in a second species.
4. Study is not supported by other animal carcinogenicity studies (e.g., NEDO study).

- **Derivation of a cancer slope factor**

This study cannot be used for this purpose.

Reviewer 3

The following are uncertainties that preclude this study from being used in consideration for all of endpoints listed above:

- ❖ Lack of barrier maintained pathogen free animal facility
- ❖ Sacrifice of animals at end of lifetime
- ❖ Use of historical controls for study
- ❖ Methanol concentrations in drinking water not measured making it impossible to determine the dose administered to each animal
- ❖ No measurement of blood methanol concentrations in control and treatment group
- ❖ Number of animals in each treatment group (n=25) insufficient for confidence in assessment of treatment differences.

Reviewer 4

See below.

5. Other Issues - *Please identify and discuss any other relevant scientific issues or comments not addressed by the above questions.*

Reviewer 1

High concentrations of methanol (≥2222 ppm) were used in the study. The question always remains whether pathways and reactions were altered at these high concentrations that would not prevail at much lower human exposure levels.

Reviewer 2

Drinking water pilot study:
Apaja (1980) performed subchronic (41 week) oral study with methanol (0.25 and 1%) in drinking water in female Swiss Webster mice (5 mice/group). It is assumed that the mice were from the Eppley Institute breeding colony. No data other than it appears that the animals survived were provided for this study.

NOTE: This study is not suitable as a carcinogenicity bioassay.

Dermal (skin painting) carcinogenicity studies:

Apaja (1980) also performed a dermal (skin painting) carcinogenicity studies on methanol (as a solvent control for the author's work with malonaldehyde). This study was performed using random-bred female Eppley Swiss mice (8 weeks old at study initiation, 40 mice/ treatment group). Mice were housed five/plastic cage and fed Wayne Lab-Blox pelleted diet (Allied Mills Inc). Water was available a*d libitum*. Animal room temperature was maintained at 21–23°C and the humidity at 45–55%. Mice were shaved and treated with 0.05 ml of methanol containing 0.625% water 3 times weekly for life. The daily methanol dose in the dermal study was ~21.3 mg or ~550 mg/kg. Animals were checked daily, weighed weekly, and killed when moribund. Dermal irritation was also assessed.

Organs evaluated included skin, lungs, liver, spleen, pancreas, kidneys, adrenal glands, esophagus, stomach, small and large intestines, rectum, urinary bladder, uterus, ovaries, tumors, and other pathological lesions. Tumor incidence data were analyzed using a Chi square test. A continuity correction was used since tumor frequencies were usually small.

NOTE: The thesis states that testes, prostate gland, vesicular glands were also evaluated however the methods explicitly state that female mice were used.

Endpoint	Finding
Skin irritation	Negative
Survival	@25% loss of animals by end of year 1 of the study @55% loss of animals by end of week 76 of the study @90% loss of animals by the end of year 2 of the study

NOTE: The mouse survival rate in this study was comparable to that reported by Prejean et al., 1973 for this strain of mice. Apaja also reported non-tumor lesion incidence for a variety of organs. Of importance, Apaja describes an overall incidence rate of @ pneumonia at ~ 22.5%. This rate is higher than that observed in other mouse strains (acidophilic macrophage pneumonia in C57BL/6J usually occurs at <10% incidence [Zurcher et al. 1982; Murray et al. 1990; Ernst et al. 1996]). Other incidental lesions (e.g., glomerulopathy, amyloidosis, ovarian atrophy, etc) appear to occur at rates similar to those seen by others in other mouse strains (see Brayton, Spontaneous Diseases in Commonly Used Mouse Strains / Stocks, available at http://www.cldavis.org/cgi-bin/download.cgi?pid=52).

NOTE: Tumor incidence rates seen in the Apaja dermal study are also similar to those reported by Prejean et al., (1973) and are consistent with the historical control data .

Selected tumor	Incidence (%)	Historical incidence (%)[a]
Lung adenoma	15	18.4
Lung adenocarcinoma	2.5	37
Mammary gland adenocarcinoma	5	6.3
Liver hemangioma	2.5	0
Ovary (granulosa cell tumor)	10	7.9

[a]from Prejean et al (1973)

Oral (drinking water) carcinogenicity study:

Apaja (1980) also performed an oral (drinking water) carcinogenicity studies on methanol (as a solvent control for the author's work with malonaldehyde). This study was performed using random-bred Eppley Swiss mice (8 weeks old at study initiation, 25 mice/gender/treatment group)). Mice were housed five/plastic cage and fed Wayne Lab-Blox pelleted diet (Allied Mills Inc). Water was available a*d libitum*. Animal room temperature was maintained at 21–23°C and the humidity at 45–55%. Methanol concentrations were 0.222, 0.444, and 0.889% in drinking water. Drinking water with methanol was given 6 days a week for life. Animals were checked daily, weighed weekly, and killed when moribund. Liquid consumption was assessed 3 times a week. Average liquid consumption was evaluated for the three methanol exposure groups and ranged from 9.2 to 11.1 ml/day/animal.

Organs evaluated included skin, lungs, liver, spleen pancreas, kidneys, adrenal glands, esophagus, stomach, small and large intestines, rectum, urinary bladder, uterus, ovaries, and testes, prostate gland, vesicular glands, tumors and other pathological lesions. Tumor incidence data were analyzed using a Chi square test. A continuity correction was used since tumor frequencies were usually small.

As with the dermal study, the mouse survival rate in this drinking water study for the low and mid-methanol dose groups were comparable to that reported by Prejean et al., 1973 for this strain of mice and mimics data seen in the dermal study conducted by Apaja. The mice in the high dose group had a much higher fatality rate by week 84 of the study when compared with the lower exposure groups. In general, background non-tumor lesions appear to have occurred at a rate similar to that seen in Swiss mice (Apaja's conclusion). Apaja does note that animals with pneumonic infiltrations were found quite frequently (overall incidence rate was 8-28%). Apaja also notes that pancreatitis may have been a

treatment related effect seen in the methanol-exposed mice. The incidence rate for pancreatitis (Males/Females) in the Apaja oral study is summarized below.

Lesion	Low Dose Incidence (%)	Low Dose Incidence (%)	Low Dose Incidence (%)
Pancreatitis	0/8	0/20	12.5/0

The incidence rate does not appear to demonstrate a clear dose dependency in female mice and has questionable dose-dependency in the male mice. The only incidence rate I could find for aged mice suggest that the observed incidence for "pancreatitis" (note: this reflects my concern that the nature of the inflammatory infiltrate was not identified) may be in the expected range seen with other mouse strains (Hayashi et al., 1989). Note: part of my uncertainty rests with the lack of a clear histologic diagnosis for the observed pancreatic change. Amyloidosis was also more commonly seen in the methanol-treated mice (when compared with mice given malonaldehyde). This is also a common background lesion seen in some strains of mice.

Incidence rates (Males/Females) for select tumors in the Apaja oral study are summarized below:

Selected tumor	Low Dose Incidence (%)	Mid Dose Incidence (%)	High Dose Incidence (%)
Lung adenoma	24/16	32/8	17/24
Malignant lymphoma (total)	4/16	24/36	17/40

Another concern with the reported historical control data for the laboratory is an inadequate description of the diet fed to the reference control groups – for example diet is known to influence the incidence of tumor and non-tumor lesions in rodents (e.g., .see Keenan et al., 1996; Maronpot et al., 2004)

References:

Hayashi Y, Utsuyama M, Kurashima C, Hirokawa K. Spontaneous development of organ-specific autoimmune lesions in aged C57BL/6 mice. Clin Exp Immunol. 1989 Oct;78(1):120-6.

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Maronpot RR, Flake G, Huff J. Relevance of animal carcinogenesis findings to human cancer predictions and prevention. Toxicol Pathol. 2004 Mar-Apr;32 Suppl 1:40-8.

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Reviewer 3

None Identified.

Reviewer 4

I wish to take this opportunity to discuss the Apaja dissertation entitled, "Evaluation of toxicity and carcinogenicity of malonaldehyde (MA)." It was published in 1980, 10 years prior to the conduct of the Ramazzini methanol study and 5 years prior to the publication of the NEDO bioassays. The skin painting study fails to significantly inform the issue of the neoplastic or non-neoplastic hazard potential of methanol. It consists of only two lifelong treatment groups (a methanol control group and the high concentration MA group), mice of one sex (females), and failed to employ a concurrent, untreated control group forcing reliance on historical control data that lacked sufficient detail to make rate comparisons for most reported outcomes. The two treatment groups suffered no dermal irritation, developed no skin tumors, and did not differ in terms of survival or average body weight. Most importantly, 15% of methanol-treated animals developed malignant lymphoma (seemingly the only neoplastic lesion at issue in the female Swiss mice), a rate indicated by the author to be within normal range based on historical controls. Indeed, historical control data in Table 9 indicate that 20% of untreated female Eppley Swiss mice develop malignant lymphomas in "other tissues", with the percentage being considerably higher when occurrences in the lung and blood vessels are also considered. Historical control data in Table 11 also suggest that the malignant

lymphomas observed in methanol-treated mice were not excessive. Indeed, the author characterizes the malignant lymphomas as spontaneous. As for non-neoplastic lesions, several were noted to be in excess of historical untreated controls. However, the inability to examine dose-response, the lack of a concurrent, untreated control group, and no detailed information on background rates of non-neoplastic lesions make it impossible to ascribe them to methanol.

In the feeding study, three control groups were given varied concentrations of methanol in water to account for methanol liberated from the acetal in MA test solutions. The consumption of MA-containing water was considerably lower than that of methanol-containing water, which makes the three methanol groups suspect as valid controls (see average dose of methanol per day in Table 5). Differences in average body weight between MA-treated and methanol control mice further contribute to this concern. While these issues obviously impact the interpretation of MA data, they have little bearing on the methanol data when examined relative to historical untreated controls. Along these lines, the author notes that while malignant lymphoma is elevated relative to historical untreated control rates presented in Table 9, observed rates are still within normal ranges. Given the absence of concurrent untreated controls, the failure to observe malignant lymphoma at rates beyond the upper bound of historical untreated controls precludes one from ascribing such tumors to methanol. Therefore, like tumor data from the skin painting study, tumor data from the feeding study are of little to no value for quantitative risk assessment. Finally, the author points out that survival in the three methanol groups was decreased relative to that of untreated historical controls. However, this does not appear methanol related, as survival was inversely proportional to methanol concentration at 40, 60, 80 and 100 weeks of age. Therefore, for the purposes of cancer risk assessment, including a weight-of-evidence evaluation, the Apaja dissertation provides no convincing evidence for methanol's carcinogenicity and is thus consistent with the two NEDO bioassays in this regard. Any conclusion to the contrary is apt to be the product of a superficial analysis.

As for non-neoplastic changes related to methanol in drinking water, a thorough review of Table 7 provides no convincing evidence for any methanol-related effect. For example, the rate of liver cell necrosis is rather low (8%) and was observed in only 2 of 25 female mice (only 1 male mouse exhibited liver cell necrosis); amyloidosis of the liver and spleen in methanol-treated mice was inversely proportional to methanol concentration in both sexes; amyloidosis in the kidney involved extremely small numbers (1 or 2 mice regardless of dose); pancreatitis and amyloidosis of the pancreas were sex-specific conditions among all three dose groups; and nephropathy of the kidney occurred at equal (females) or lower rates (males) at the high dose of methanol compared to the low dose. Such findings, coupled with the absence of a concurrent control group and the

high probability that many of the non-neoplastic lesions are simply age-related pathological changes, suggest that the Apaja dissertation is of little or no value in establishing a reference dose.

APPENDIX A - *TERA*'s Agreement With the Methanol Institute

TERA Proposal for Independent Letter Peer Review
November 9, 2009

Toxicology Excellence for Risk Assessment (*TERA*) is pleased to provide this proposal to the Methanol Institute for an independent letter peer review of the Soffritti et al. (2002) publication. This proposal outlines the approach and cost estimate for this independent letter review. *TERA* is a non-profit, 501(c)(3) corporation organized for scientific and educational purposes. Our mission is to protect public health by developing and communicating risk assessment information, organizing peer reviews and consultations, improving risk methods through research, and educating the public on risk assessment issues. As an independent non-profit we have the needed independence and experience to organize and coordinate this peer review. More information about *TERA*'s review program can be found at www.tera.org/peer.

Introduction

The U.S. EPA is currently developing a human health assessment of Methanol (CASRN 67-56-1) for IRIS that will be reviewed by the Science Advisory Board (SAB) in early 2010. There are only a few animal studies available in the published literature that shed light on the carcinogenicity of methanol. Because the EPA IRIS process has a strong preference for use of peer-reviewed studies, EPA arranged for the Eastern Research Group (ERG) to conduct an external letter peer review of several study reports from the New Energy Development Organization (NEDO) in June 2009. However, EPA did not ask ERG to conduct a peer review of another key methanol study by Soffritti et al. (Soffritti, M., Belpoggi, F., Cevolani, D., Guarino, M., Padovani, M. and Maltoni, C. 2002. Results of Long-Term Experimental Studies on the Carcinogenicity of Methyl Alcohol and Ethyl Alcohol in Rats, Ann. N.Y. Acad. Sci. 982: 46–69). Because this is a key study for assessing methanol's carcinogenicity, The Methanol Institute has asked *TERA* to conduct an independent letter peer review of this third study, in a fashion similar to the EPA reviews of the NEDO studies, and make the results publicly available.

The objective of this task is to conduct an independent letter peer review of Soffritti et al. (2002), similar to the review conducted by ERG on the NEDO studies for EPA.

Technical Approach

This section presents *TERA*'s technical approach to performing the tasks required to conduct an independent peer review. In order to establish the independence of the peer review, *TERA* will be solely responsible for all aspects of organizing the review including selection of peer review panel members, development of the charge to reviewers, and

preparation of the peer review report. The Methanol Institute will not participate in any way in the organization of the review and has agreed that *TERA* will not share the reviewer's comments on the Soffritti study with them, nor allow the Methanol Institute to review the peer review report prior to its release to the public.

Task 1: Select Reviewers/COI Screening

The Project Manager will become familiar with the Soffritti study to identify scientific issues and needed areas of expertise. The Project Manager will select five to eight technical experts with the necessary background and expertise to serve as peer reviewers. In order to facilitate the rapid turnaround needed for this review, *TERA* will contact EPA's chemical manager for the methanol assessment and request suggestions for appropriate expertise and charge questions. In addition, *TERA* will consider the five experts who reviewed the NEDO studies for ERG, evaluating if they have the appropriate qualifications to review the Soffritti study. If needed, *TERA* will select additional experts to address the key issues for the Soffritti study (e.g., respiratory infections and lymphomas).

An important part of conducting an independent peer review is selecting a panel that is free from conflict of interest and bias. *TERA* defines situations that constitute a conflict of interest early in the selection process then utilizes a multi-step process to identify potential conflicts or biases. In initial conversations with each candidate, *TERA* will discuss the nature of the review, the sponsor, and other interested parties, and stakeholders (if appropriate). *TERA* will ask the candidates questions regarding their work and relationships with these parties. Each potential peer reviewer is given a copy of *TERA*'s COI policy statement and asked to complete a questionnaire which is used to determine whether the candidate's involvement in certain activities could pose a conflict of interest or could create the appearance that the panel member might lack impartiality. *TERA*'s questionnaire includes generic questions regarding employment, consulting, funding, investments, etc., as well as specific questions tailored to the particular review and work product, including financial and/or professional relationships with the Methanol Institute. *TERA* staff carefully review these forms and discuss the responses with the reviewers to ascertain whether conflicts of interest or unacceptable bias might exist.

TERA's conflict of interest policy is found at http://www.tera.org/peer/COI.html. It identifies the typical types of situations that could create a real or perceived conflict of interest. Because perceptions are so important in this area, *TERA* is sensitive to identifying and avoiding situations that may hinder the credibility of a review and carefully considers prior to panel selection what situations would preclude an expert from participating. Transparency is a key attribute of a high quality review and *TERA* will

prepare biographical sketches and conflict of interest statements for each reviewer. These will be part of the final meeting report.

Task 2: Develop Charge and Review Package

After becoming familiar with the study in question and speaking with the EPA chemical manager, the project manager will identify the key scientific issues and questions for the review. These issues form the basis for the *Charge to Reviewers* – the instructions provided the panel to guide their review. To facilitate a rapid review, *TERA* will consider the charge questions used by ERG for the review of the NEDO studies and will consider questions that the Methanol Institute suggested to EPA. However, *TERA* has the sole responsibility to independently develop the appropriate charge questions and the Methanol Institute will not review the charge before it is sent to the reviewers.

TERA will prepare a review package and distribute it to the reviewers. The review package will include the Soffritti et al. (2002) publication, data tables from the study that were provided to EPA from the authors, the charge, additional references (e.g.., publications by Caldwell et al. (2007) and Schoeb et al. (2009), and others), and instructions to the reviewers. The reviewer biographical sketch and COI statements will also be distributed with the review package. The review package will be distributed electronically. Reviewers will be given 2-3 weeks to return their comments.

Task 3: Prepare Letter Peer Review Report

TERA will compile the written comments of the experts into a single report. *TERA* will collate the reviewers' comments by question and will also provide an appendix with each reviewer's full comments. In addition, the report will include documentation of the process we used to organize the review, reviewer biographical sketches, charge questions, and any other information deemed necessary for a fully transparent report. The experts will review the draft final report to insure that their comments are accurate and complete. Upon finalization this report will be posted on the *TERA* website for full public access. *TERA* will not share or discuss the reviewers' comments with the Methanol Institute or any other outside party prior to the report being finalized and released on the *TERA* website.

Timeline

November 2009

- *TERA* identifies key scientific issues, and identifies specific types of expertise needed for the review. *TERA* anticipates that 5-8 reviewers will be selected

for the panel, but will make final decision after identifying the key scientific issues,

- *TERA* prepares Charge to Peer Reviewers.

December 2009

- *TERA* distributes packages to reviewers for their review.
- Reviewers deliver written comments to *TERA*.
- *TERA* reviews comments for completeness and coherence, requesting clarification from reviewers as needed.

January 2010

- *TERA* compiles written comments into a preliminary peer review report.
- *TERA* distributes to panel for one week review period.
- *TERA* finalizes meeting report and posts report on web (January 15).

Budget

A cost proposal outlining an estimate of *TERA* labor hours and honoraria is attached.

Project Management

A *TERA* senior scientist (Ms. Joan Strawson) with experience in toxicology, risk assessment and independent peer review, will serve as the Project Manager for this contract. She will be assisted by Dr. Michael Dourson, DABT, ATS, and Ms Jacqueline Patterson, *TERA* Peer Review Program Manager.

TERA proposes to do this work on a time and materials basis, charging the Methanol Institute only for the labor hours necessary to accomplish the tasks. Direct expenses, such as honoraria, photocopying, teleconferencing and express mail will be billed without an additional fee. The cost estimate attached is an estimate, the actual costs for the peer review may vary. *TERA* will notify the sponsor immediately if it appears that the actual costs may exceed the estimate.

TERA will provide the Sponsor with monthly invoices summarizing work accomplished and listing the hours worked for that month by labor category. *TERA* will provide monthly progress reports with each invoice. Payment is due 30 days after issue with 1.5% interest accruing each month thereafter.

TERA understands that the authority to grant funds on projects rests with the sponsor personnel. The role of *TERA* is to administer these funds as described in specific proposals and notify the Sponsor of any and all deviations. Any benefit achieved is based on both Sponsor funding and recommendations and *TERA* decisions.

The Sponsor will hold *TERA* harmless for any and all loss, damages, costs, legal fees, and expenses on account of any and all claims or actions brought against the Sponsor by any person, firm, corporation, or other entity as a result of or otherwise arising out of the advice, analysis, consultation or testimony rendered by *TERA* or its personnel for or on behalf of specific projects of the Sponsor.

This Agreement is subject to, and is to be construed under, the laws of the State of Ohio, United States of America. Actions brought under this Agreement shall be brought in any court of competent jurisdiction in the State of Ohio.

Signature

This Proposal is submitted on behalf of Toxicology Excellence for Risk Assessment, this 9th day of November 2009.

Submitted by:

_____

Michael L, Dourson, President
Toxicology Excellence for Risk Assessment
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November 20, 2009

Dr. Michael L. Dourson
President
Toxicology Excellence for Risk Assessment
2300 Montana Ave., Suite 409
Cincinnati, OH 45211

Dear Dr. Dourson:

Thank you for your November 9th submittal of the project proposal "Independent Letter Peer Review of Soffritti et al. (2002)" to conduct a letter peer review of the methanol study conducted by the Ramazzini Foundation. I am pleased to inform you that your proposal has been accepted for funding by the Methanol Foundation, the 501 (c)(3) research arm of the Methanol Institute.

We have reviewed TERA's Conflict of Interest policy statement on your web site, as well as the email correspondence from Joan Strawson to Chuck Elkins discussing TERA's internal COI search regarding methanol and related chemicals. We agree that there are no COI issues that would be an impediment to TERA's performance of the proposed work for the Methanol Foundation.

[Confidential Business Information related to costs has been deleted]

We look forward to the final product of this letter peer review, and greatly appreciate your efforts to expedite this project.

Sincerely,

Gregory Dolan
Program Director
Methanol Foundation

From: Chuck Elkins [mailto:elkinsenv@aol.com]
Sent: Tuesday, January 12, 2010 3:37 PM
To: Joan Strawson
Cc: gdolan@methanol.org
Subject: Methanol review

At a meeting of a committee of the Methanol Institute today I was asked to request that TERA submit its peer review document DIRECTLY to EPA's docket for the methanol IRIS review at the same time you post it to the TERA website. In this way, it will go to EPA before the methanol industry has had a chance to see it. This will be one more step in keeping the development of TERA's document separate from any involvement whatsoever by the Methanol industry, consistent with the other safeguard provisions of your contract with the Methanol Institute for the funding of this peer review.

Chuck Elkins

APPENDIX B - Conflict of Interest Statement and Panel Biographical Information

Conflict of Interest

An essential part of an independent expert review is the identification of conflicts of interest and biases that would disqualify a candidate, as well as identification and disclosure of situations which may appear to be a conflict or bias. *TERA* was selected by the Methanol Institute to independently organize and conduct this expert panel review and is solely responsible for the selection of the panel. Prior to selecting *TERA* to conduct this expert review, the Methanol Institute reviewed information regarding *TERA* past and current work necessary to evaluate *'TERA's* independence. *TERA* searched its records to identify past work done on methanol and on chemicals with related issues including formaldehyde, formalin, methylene oxide, methanal, methaldehyde, MTBE. *TERA* has no current, ongoing tasks on any of these chemicals; we have no past tasks on methylene oxide, methanol, or methaldehyde. *TERA* had completed past tasks on methanol, formaldehyde, and MTBE. None of these issues constitute a COI because none of the projects are ongoing and none were conducted on behalf of the Methanol Institute. As outlined in the contract between *TERA* and the Methanol Institute (See Appendix A), *TERA* has independently selected the panel, organized this review, and prepared the final report. The Methanol Institute has had no influence on the selection of the panel or implementation of the process. In addition, the Methanol Institute has not been informed about the identity of the reviewers and has not received the final report until after it was submitted to EPA.

The purpose for evaluating conflict of interest is to ensure that the public and others can have confidence that the peer reviewers do not have financial or other interests that would interfere with their ability to carry out their duties objectively. *TERA* asked each promising candidate to report on his or her financial and other relationships with the Methanol Institute, the member companies of the Methanol Institute, the European Ramazzini Foundation, and the US EPA's Science Advisory Board.

The evaluation of real and perceived bias or conflict of interest is an important consideration in panel selection. *TERA* follows the U.S. National Academy of Sciences (NAS) guidance on selection of panel members to create panels that have a balance of scientific viewpoints on the issues to be discussed. As a result, the expert panels have a broad and diverse range of knowledge, experience, and perspective, including diversity of scientific expertise and affiliation. Panel members serve as *individuals*, representing their own personal scientific opinions. They do not serve as representatives of their companies, agencies, funding organizations, or other entities with which they are associated. Their opinions should not be construed to represent the opinions of their employers or those with whom they are affiliated.

Prior to selection, the candidates completed a questionnaire, which *TERA* used to determine whether their activities, financial holdings, or affiliations could pose a real or perceived conflict of interest or bias. The completed questionnaires were reviewed by *TERA* staff and discussed further with panel candidates as needed. (See www.tera.org/peer/COI.html for *TERA* conflict of interest and bias policy and procedures for panelist selection.)

TERA has determined that the selected peer reviewers have no conflicts of interest and are able to objectively participate in this peer consultation. None of the panel members has a financial or other interest that would interfere with his or her abilities to objectively participate on the panel. None of the panel members is employed by the Methanol Institute or its member companies, or the European Ramazzini Foundation. Nor do the panel members have any financial interests in these organizations or in the outcome of the review. None of the panel members was involved in the studies under review. A brief biographical sketch of each panel member is provided below. To promote transparency, a short statement describing situations which might appear to present a conflict of interest or bias are included, as appropriate.

The Methanol Review Panel

The Methanol panel includes five scientists who have expertise in the key disciplines and areas of concern to review the methanol bioassays. Each panelist is a well-respected scientist in his or her field. Collectively, the panel has expertise in toxicity of alcohols, design and conduct of carcinogenicity bioassays, biostatistics, use of bioassay data in risk assessment, and U.S. EPA risk assessment methods. *TERA* was solely responsible for the selection of the panel members.

Each panel member has disclosed information pertinent to evaluating potential conflicts of interest and biases related to methanol as well as the sponsors of the peer review, and the original study authors. *TERA* carefully evaluated this information when selecting panel members. Short biographical sketches and disclosure statements for panel members are provided below.

Judy Buelke-Sam, MA
Toxicology Services

Ms. Buelke-Sam is currently a toxicology consultant specializing in non-clinical subchronic/chronic, carcinogenicity, reproductive, developmental, neurobehavioral, and juvenile toxicology/pharmacokinetic consultations for drugs, chemicals, and pesticides. She is also experienced in ICH toxicology requirements for drug/biologics development; regulatory reporting; IND/CIB preparation/updating; drug development program planning and reviews. She holds a MA in Experimental Psychology from Western Michigan University. Ms. Buelke-Sam has extensive experience as a toxicology Study Director, particularly in the area of neurodevelopmental toxicity testing. She has served on advisory panels for the National Academy of Sciences and the National Toxicology Program. Ms. Buelke-Sam is the author of numerous peer-reviewed journal articles.

Ms. Buelke-Sam was selected for this panel for her expertise in the design, conduct and interpretation issues for carcinogenicity, multi-generation and developmental and adult neurotoxicity studies of chemicals and pesticides and for her experience in serving on advisory panels.

Disclosure: None.

David Dorman, PhD, DABT, FATS
North Carolina State University

Dr. David Dorman is currently Associate Dean for Research and Graduate Studies and Professor of Toxicology at the College of Veterinary Medicine, North Carolina State University (NCSU). He also holds adjunct faculty appointments with the Integrated Toxicology Programs at Duke University and the University of North Carolina-Chapel Hill. Dr. Dorman holds a D.V.M. from Colorado State University, and a Ph.D. in Veterinary Biosciences/Toxicology from the University of Illinois at Urbana-Champaign. While at the University of Illinois he also completed a residency in clinical veterinary toxicology. Prior to joining NCSU in 2007, Dr. Dorman was a Senior Scientist and Director of the Biological Sciences Division with the Centers for Health Research at The Hamner Institutes (previously known as the Chemical Industry Institute of Toxicology, CIIT) in Research Triangle Park, NC. After completing a postdoctoral fellowship at CIIT, he was appointed to the Institute's senior scientific staff to lead the neurotoxicology research program. Dr. Dorman's research interests include neurotoxicology, nasal toxicology, and pharmacokinetics. His chemicals of interest include manganese, hydrogen sulfide, methanol, tungsten, and acetaldehyde - amongst others. Dr. Dorman is certified by the American Board of Veterinary Toxicology and the American Board of Toxicology and is also a Fellow of the Academy of Toxicological Sciences. He has served on the editorial board of several journals, as well as on government, National Academy of Sciences, and National Research Council advisory panels. Dr. Dorman is an author or co-author of more than 50 book chapters and monographs, 120 peer-reviewed manuscripts, and 10 technical reports.

Dr. Dorman was selected for this panel for his expertise in methanol toxicity and clinical veterinary toxicology as well as for his experience in serving on panels of expert scientists in peer review of scientific assessments.

Disclosure: None.

Janis Eells, PhD
University of Wisconsin- Milwaukee

Dr. Janis T. Eells is a Wisconsin Distinguished Professor of Pharmacology and Toxicology at the University of Wisconsin- Milwaukee. She holds a Ph.D. in Pharmacology from the University of Iowa. Dr. Eells conducted postdoctoral research in Neurotoxicology at the University of Iowa and at Northwestern University. She has over twenty years of academic research experience in Pharmacology and Toxicology at the Medical College of Wisconsin and the University of Wisconsin-Milwaukee. Dr. Eells' expertise is in neurotoxicology and she is widely recognized as an expert in the

mechanisms of retinal and optic nerve toxicity having served as an advisor and consultant to pharmaceutical companies, government agencies, and the World Health Organization in the area of methanol-induced ocular toxicity. She has a broad-based knowledge of physiology, pharmacology, and neurotoxicology with particular expertise in methanol-induced neurotoxicity. Dr. Eells' research program is focused on the mechanisms of retinal and optic nerve toxicity with an emphasis on the role of mitochondrial dysfunction and reactive oxygen species in retinal and optic nerve disease processes. Mitochondrial dysfunction plays a pivotal role in the mechanism of methanol-induced neurotoxicity and is also a key feature in neurodegenerative diseases and cellular aging. Research in Dr. Eells' laboratory is directed at understanding mitochondrial signaling mechanisms involved in mediating cellular toxicity and protection. One component of her research program focuses on the molecular mechanisms of toxicity associated with the actions of environmental chemicals that act as mitochondrial poisons and disease states that produce mitochondrial dysfunction. Dr. Eells' laboratory has developed and characterized a rodent model of methanol-induced toxicity that recapitulates the clinical, biochemical and neurotoxic features of human methanol intoxication. Using this animal model, the Eells' laboratory has tested several therapeutic interventions for the treatment of mitochondrial dysfunction caused by acute and chronic methanol intoxication.

Dr. Eells was selected for this panel for her extensive expertise in methanol toxicity and mechanisms of action, and for her experience in serving on panels of expert scientists in review of risk assessments.

Disclosure: None.

David Gaylor, PhD, FATS
Gaylor and Associates

Dr. Gaylor is retired from the National Center for Toxicological Research (NCTR) of the Food and Drug Administration (FDA), where he was Director of the Division of Biometry and Risk Assessment. Currently, he is a consultant in the area of quantitative health risk assessment. Dr. Gaylor is also an Adjunct Professor of Biostatistics, University of Arkansas for Medical Sciences. He obtained a Ph.D. in Statistics from North Carolina State University in 1960 followed by employment with the Research Triangle Institute and the National Institute of Environmental Health Sciences. Dr. Gaylor's research has focused on the statistical design and analysis of toxicological experiments and the development of techniques for quantitative health risk assessment. He has published more than 180 journal articles, 25 book chapters, and made over 100 presentations at scientific conferences. Dr. Gaylor has served on more than 70 national

and international committees on aspects of biometry, toxicology, and risk assessment for the FDA, U.S. EPA, CDC, World Health Organization, Health Canada, International Life Sciences Institute, and the National Research Council. He is a Fellow of the Academy of Toxicological Sciences, American Statistical Association, and Society for Risk Analysis. He currently is a member of the editorial board for Risk Analysis, Human and Ecological Risk Assessment, Regulatory Toxicology and Pharmacology, and Toxicology and Industrial Health.

Dr Gaylor was selected for this panel for his expertise in biostatistics, dose-response assessment, and for his experience in serving on panels of expert scientists in review of risk assessments.

Disclosure: None.

D. Allan Warren, PhD
University of South Carolina Beaufort

Dr. Warren is currently the Program Director for Environmental Health Science and the University of South Carolina Beaufort. He holds a PhD from the Department of Pharmacology and Toxicology, College of Pharmacy, University of Georgia. At the University of South Carolina Beaufort, Dr. Warren has developed an undergraduate education program in environmental health science and conducts field- and laboratory-based research in environmental and human health risk assessment. He directs the University's water quality laboratory and is a funded consultant on toxicological issues to the South Carolina Department of Health and Environmental Control. He also serves as a technical resource to local, state and federal governments on environmental health-related matters and remains active in private consultation on a variety of toxicological issues. Dr Warren's research programs have focused on assessing the pharmacokinetic profile and toxicity of solvents and vapors. He is well versed in risk assessment procedures and has developed numerous site-specific exposure and risk assessments. He created comprehensive toxicity profiles for numerous chemicals, assessed the scientific merits of toxicity constants (cancer slope factors and reference doses) established by state and federal agencies and in some cases, generated alternative toxicity constants to those of the U.S. EPA. Dr. Warren is the author of numerous papers on risk assessment and solvent toxicity; he has served on prior peer review panels for U.S. EPA.

Dr. Warren was selected for this panel for his expertise in risk assessment, solvent toxicity, and for his experience in serving on panels of expert scientists in review of risk assessments.

Disclosure: None.

APPENDIX C - Instructions to Reviewers and Charge

Dear Methanol Peer Review Panel:

Enclosed are the following materials for your review of Soffritti et al (2002):

- Soffritti, M., Belpoggi, F., Cevolani, D., Guarino, M., Padovani, M. and Maltoni, C. 2002. Results of Long-Term Experimental Studies on the Carcinogenicity of Methyl Alcohol and Ethyl Alcohol in Rats, Ann. N.Y. Acad. Sci. 982: 46–69
- Charge to Reviewers
- Additional publications:
    - Caldwell et al. (2008, 2009)
    - Schoeb et al. (2009)
    - Goodman et al (2009)
    - Soffritti (2008)

In addition, you will receive a CD with study tables from the European Ramazzini Foundation's Methanol study by regular mail, because these are too large to email. If you wish to examine study tables before you receive the CD, you can download some of the tables from the Methanol Institute website at http://www.methanol.org/contentIndex.cfm?section=hse&topic=specialReports&title=Ramazzini. Note, however, that some of the links do not work. If during the course of your review, you identify additional publications that you would like retrieved to assist with your review, please let me know and *TERA* can obtain them for you.

Please review the enclosed materials carefully and consider the issues and questions identified in the charge. Please provide specific comments on any issues that affect interpretation of the Soffritti et al (2002) study. If you consider a particular question to be outside your area of expertise, please indicate this in your written comments.

We ask that you provide us with your written comments by Friday, January 22, 2010 (sooner, if possible). After receiving all of the written comments, *TERA* will evaluate them for completeness or unresolved issues. We will then collate the written comments into a single report and distribute to the panel for members to consider the comments made by other panel members.

Charge to Reviewers

Peer Review of Methanol Cancer Bioassay

Soffritti, M., Belpoggi, F., Cevolani, D., Guarino, M., Padovani, M. and Maltoni, C. 2002. Results of Long-Term Experimental Studies on the Carcinogenicity of Methyl Alcohol and Ethyl Alcohol in Rats, Ann. N.Y. Acad. Sci. 982: 46–69

The Methanol Institute has asked Toxicology Excellence for Risk Assessment (*TERA*) to conduct an independent letter peer review of the European Ramazzini Foundation's (ERF) drinking water bioassay of methanol, published as Soffritti et al. (2002). The U.S. Environmental Protection Agency (EPA) is currently developing a human health assessment of Methanol (CASRN 67-56-1) for IRIS that will be reviewed by EPA's Science Advisory Board (SAB) in early 2010. Only a few animal studies are available in the published literature that shed light on the carcinogenicity of methanol. Because the EPA IRIS process has a strong preference for use of peer-reviewed studies, EPA arranged for the Eastern Research Group (ERG) to conduct an external letter peer review of several study reports from the New Energy Development Organization (NEDO) in June 2009. However, EPA did not ask ERG to conduct a peer review of another key methanol study by Soffritti et al. Because this is a key study for assessing methanol's carcinogenicity, the Methanol Institute has contracted to *TERA* to conduct an independent letter peer review of this third study, in a fashion similar to the EPA reviews of the NEDO studies, and make the results publicly available. Note that Soffritti et al. (2002) reports the results of cancer bioassays on both methanol and ethanol. **This peer review is asking panel members to review *only* the study on methanol.** Peer reviewers are asked to comment on the following questions, evaluating the scientific and technical merit of the study. Peer reviewers are asked to provide specific comments and describe any issues that affect interpretation of the Soffritti et al (2002) study. If a reviewer considers a particular question to be outside his or her area of expertise, the reviewer should indicate this in his or her written comments.

1. Study design - Based on your knowledge of toxicological study protocols, please comment on the experimental design of the Soffritti et al (2002) study:

- Do you see any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?
- In light of the chemical and toxicological profile for methanol, comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation.
- The study was conducted using the full lifespan of the animals. Please comment on if or how this study design affects the interpretation of the results.

2. Study Results - Please comment on the strength, credibility, and relevance of the toxicological results of the Soffritti et al (2002) study, supplemented by the study data tables from ERF:

- Were the individual animal data correctly summarized?
- Are there nomenclature issues that need clarification?

- Was adequate statistical information provided for quantitative dose-response analyses?
- The study reported the following results:

  "The occurrence of benign and malignant tumors is shown in TABLE 1. Differences observed between treated and control animals were: (1) a dose-related increase of total malignant tumors in males and females of treated groups (TABLE 2); (2) a dose-related increase of carcinomas of the head and neck, mainly in the ear ducts, in males of treated groups and in females treated with 20,000 and 5,000 ppm (TABLE 3); (3) a statistically significant increase (P <0.01) of testicular interstitial cell hyperplasias and adenomas in the group treated with the highest dose; (4) an increase in sarcomas of the uterus at the highest dose; (5) a dose-related increase in osteosarcomas of the head in males and females of the treated groups (TABLE 4); and (6) a dose-related increase in hemolymphoreticular neoplasias in males and females of the treated groups (TABLE 5)." Page 58.

For each lesion listed above (total malignant tumors, carcinormas and osteosarcomas of head and neck, testicular hyperplasia/adenoma, uterine sarcoma, hemolymphoreticular neoplasias), please comment on the strength of the evidence supporting the authors' conclusions that the lesion is treatment-related.

- Soffritti et al (2002) reported an increased incidence of total hemolymphoreticular neoplasms that was statistically significant in females at all doses and dose-related in males (see Table 5, page 59). Lymphoimmunoblastic lymphomas were the primary tumor type observed. Other studies conducted by the ERF, most notably the bioassays for MTBE and aspartame, also reported increased incidences of lymphomas, leading to debate in the scientific literature on whether the animal colony at ERF may have been suffering respiratory infection due to *Mycoplasma pulmonis* and whether the lymphomas are an immunologic response to this infection. For more information, see Caldwell et al. (2008, 2009), Schoeb et al. (2009), Goodman et al (2009), and Soffritti (2008). Please comment on whether evidence for a *Mycoplasma pulmonis* infection exists in this study. If so, how would such an infection affect the results of the study or affect the interpretation of the study results.

3. Study Conclusions:
   - Were there critical results or issues that were not addressed? Were there any contradictory statements or observations made?
   - Do you agree with the authors' conclusions of the study?

4. Study Reliability – Describe the reliability of the study for consideration in the derivation of EPA IRIS quantitative health benchmarks and the qualitative characterization of cancer risk. Describe any major strengths or uncertainties with this study that might preclude it from being used as consideration for:
   - derivation of a noncancer reference concentration,

- determination of the weight-of evidence for methanol's cancer risk
- derivation of a cancer slope factor

5. Please identify and discuss any other relevant scientific issues or comments not addressed by the above questions.

Review of Apaja (1980)

Apaja (1980) evaluated the toxicity and carcinogenicity of malonaldehyde in both a skin painting study and a drinking water study. Both studies included several doses of methanol as a positive control. **This peer review is asking panel members to review *only* the aspects of the study that pertain to methanol.** Peer reviewers are asked to comment on the following questions, evaluating the scientific and technical merit of the study. Peer reviewers are asked to provide specific comments and describe any issues that affect interpretation of the Apaja (1980) study. If a reviewer considers a particular question to be outside his or her area of expertise, the reviewer should indicate this in his or her written comments.

1. Study design - Based on your knowledge of toxicological study protocols, please comment on the experimental design of the Apaja (180) study:

- Do you see any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?
- In light of the chemical and toxicological profile for methanol, comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation.
- The study was conducted using the full lifespan of the animals. Please comment on if or how this study design affects the interpretation of the results.

2. Study Results - Please comment on the strength, credibility, and relevance of the toxicological results of the Apaja (1980) study, supplemented by the study data tables from ERF:

- Were the individual animal data correctly summarized?
- Are there nomenclature issues that need clarification?
- Was adequate statistical information provided for quantitative dose-response analyses?
- For each lesion identified by Apaja (1980), please comment on the strength of the evidence supporting the authors' conclusions that the lesion is treatment-related.

3. Study Conclusions:

- Were there critical results or issues that were not addressed? Were there any contradictory statements or observations made?
- Do you agree with the authors' conclusions of the study?

4. Study Reliability – Describe the reliability of the study for consideration in the derivation of EPA IRIS quantitative health benchmarks and the qualitative characterization of cancer risk. Describe any major strengths or uncertainties with this study that might preclude it from being used as consideration for:

- derivation of a noncancer reference concentration,
- determination of the weight-of evidence for methanol's cancer risk
- derivation of a cancer slope factord

5. Other Issues - Please identify and discuss any other relevant scientific issues or comments not addressed by the above questions.