

**Evaluation of the Ramazzini Foundation
Study of Methanol in Drinking Water
In Sprague-Dawley Rats**



Prepared for the Methanol Institute

Dr. George Cruzan
ToxWorks

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Executive Summary

The Ramazzini Foundation (RF) published a study on the carcinogenicity of methanol in Sprague-Dawley rats in the *Annals of the New York Academy of Science*, a non-peer journal (Soffritti, et al., 2002a). Exposure to methanol was 0, 500, 5,000 or 20,000 ppm in drinking water for 2 years with the animals being observed until they died. In the published paper no data on survival, body weight, feed and water consumption, organ weight, blood methanol, clinical tests, macroscopic data, or non-tumor pathology were reported. Histopathologic evaluation did not include non-tumor pathology, but reported a statistically significant increase in hemolymphoreticular tumors in the high dose (20,000 ppm) females, carcinomas of ear duct in high dose (20,000 ppm) males, and total tumor bearing animals (20,000 ppm both sexes) was also reported. The Ramazzini Foundation has subsequently made more data from the study available on their Website and to the US EPA. The Methanol Institute has obtained much of the data supplied to the EPA. Because the Ramazzini Foundation historically has not made these kinds of data tables available for review, the data release offers an unusual opportunity to evaluate the validity of a Ramazzini Foundation study. This evaluation is based on the data from the paper, the website and tables from the EPA.

The individual pathology record (00117_68_p14.pdf) for each animal was examined and an Excel spreadsheet was developed that recorded all pathologic findings in the lung, all neoplasms (cancers) derived from blood forming cells (leukemias, lymphomas, sarcomas) in lung and other tissues, and number of days on study. Incidences of neoplasms were compared to the individual tumor incidence table (00117_68_p4.pdf) and statistical evaluations in the NTP database (00117_68_p8.pdf). Results from the RF methanol study were compared to control results from other studies at the Ramazzini Foundation and from studies conducted in Sprague-Dawley rats at other facilities.

The Ramazzini Foundation uses a unique methodology and study design that does not conform with OECD, US EPA, or NTP Study Guidelines for chronic/carcinogenicity studies. This complicates any attempt to validate the results of the study, even with the release of some of the data tables, as in the present case. The Ramazzini Foundation administered methanol in the drinking water of groups of 100 male and 100 female Sprague-Dawley rats (5 rats/cage, 8 weeks

old at the start of dosing) from their own breeding colony for 104 weeks at nominal concentrations of 0, 500, 5000, and 20,000 mg/L (Soffritti et al., 2002). The animals were not randomly assigned to treatment groups, but animals from a given litter are all assigned to the same treatment group (Bucher, 2002). It is indicated that they record which litter each animal came from (Bucher, 2002), but in the data released to date, there is no indication of litter. Therefore, at the present time, litter effects cannot be evaluated.

There is no confirmation of correct preparation of drinking water solutions because the concentration of methanol in the drinking water was not measured analytically. The paper indicates that fresh water/methanol solutions were placed in the animal cages each day for 104 weeks and that the drinking water consumed was measured one day each week for the first 13 weeks and one day every two weeks until week 104. This is a very crude estimate of water consumption. Animals were weighed weekly for 13 weeks, biweekly until week 104 and every 8 weeks until death. After death each animal received a necropsy and histopathologic examination of the normal set of tissues.

Approved study guidelines require moribund animals to be sacrificed to minimize autolysis (tissue decay) which would interfere with pathologic evaluation. Laboratories that conduct studies for regulatory submissions examine the animals at least twice each day for moribundity. The Ramazzini Foundation does not sacrifice animals, but allows them to die. Thus, animals could be dead for up to 12 hours before necropsy if observations were conducted twice a day. Such a delay in necropsy will lead to autolysis of tissues. The Ramazzini evaluation and data tables do not indicate any autolysis, which seems unlikely.

Approved protocols call for termination of rat studies at 24 months, so a sufficient number of controls and treated animals can be compared at the same age for pathology. Ramazzini does not terminate the study until the last animal dies. Thus there is no group of animals terminated together after a long period on study in the Ramazzini studies, further complicating the evaluation of the validity of these studies.

The Ramazzini Foundation program started over 30 years ago and has evaluated 200 compounds in 398 studies (averages to a new study every month) (Soffritti et al 2002b). In a paper on Aspartame (Soffritti, et al., 2005), Soffritti indicated that over the last 20 years almost 2000 male and 2000 female were used as controls (basis of the historical control data). This averages out to one control group per year (vs. an average of 12 studies per year). In one of the MTBE paper RF details the use of common controls for several chemicals (Belpoggi et. al 1995). Because the incidence of “hemolymphoreticular” neoplasms varies considerably among studies reported by the Ramazzini Foundation, it is essential to know if the animals of the control group and the test groups are from the same batch of animals.

Examining the survival and pathology data already made available indicates that the animals were not healthy according to current standards of laboratory animal care. There was excessive early mortality (up to 30% by 18 months), although survival at 104 weeks (standard study termination) was within the historic control range for Sprague-Dawley rats. Secondly, lung pathology (inflammation, dysplasia, or neoplasm) was present in 80 to 95% of those dying before Study day 540 (18 months), in 82 to 92% of those dying before Study day 730 (24 months) and in 87 to 94% of those dying anytime in the study (up to Study day 1071). Based on the data, there is no indication that lung pathology was an occurrence of old age because it was present in nearly every animal, regardless of when they died. The contribution of lung pathology to tumor formation, particularly in lungs, cannot be ascertained from the data tables. A review of the pathology slides by an independent pathologist or pathology working group is needed to evaluate the degree of respiratory infection and its potential impact on the outcome of the study.

Pathologists at the Ramazzini Foundation have recently begun using a diagnosis of “lympho-immunoblastic lymphoma”. This diagnosis is not used by any other pathologist evaluating animal cancers. The cancer diagnosis needs verification by other pathologists, including how this cancer differs from lymphoblastic lymphoma. A pathology peer review of all the slides from the methanol study (and potentially other studies) with this diagnosis is needed to define this cancer.

The incidences of total cancers derived from blood forming cells, designated as hemolymphoreticular tumors by Ramazzini pathologists, is consistently about 4 times higher in

control groups than the incidences of such tumors in other laboratories using Sprague-Dawley rats. The Ramazzini uses Sprague-Dawley rats from its own breeding colony, not animals from a commercial supplier. The genetics and health status of this colony compared to commercial colonies is not known. A thorough examination of the animal colony (including genetics and health status) is needed to understand the reasons for differences from other sources of Sprague-Dawley rats in cancer response.

Because different cancers from blood forming cells are derived from different cell types, it is generally considered inappropriate to combine all of them into one evaluation. In the methanol study, the incidences of histiocytic sarcoma, leukemias, and lymphoblastic lymphoma were not higher in methanol treated rats than in the controls. In addition, the incidences of these cancers were within the historic control range from two other databases. The only cancer that was increased in the methanol study was lympho-immunoblastic lymphoma. There are no historic control data to compare this study to. The diagnosis is not used by any other pathologist in animal studies. The significance of observation cannot be determined until the diagnosis is validated by other pathologists. The increase in lympho-immunoblastic lymphoma was not related to allowing each animal to live until death; the percent of animals dying with lympho-immunoblastic lymphoma remained the same throughout the study.

Ear duct carcinomas have apparently been reported only by the Ramazzini Foundation. The NTP limited pathology working group (PWG) of the aspartame study reported that many of the lesions diagnosed as ear duct carcinoma were not tumors.

In a recent article, Caldwell et al. (2008) assert a number of facets to demonstrate the superiority of Ramazzini studies based on an analysis of the MTBE study. Several of their key points do not hold for the Ramazzini methanol study.

1. Caldwell: Any lung infections in rats in the Ramazzini colony occur only near death in old rats and could not be related to lung neoplasms. Methanol: In the Ramazzini methanol study, nearly every rat had lung pathology, even nearly all of the first 10% dying in each group, indicating an ongoing lung infection.

2. Caldwell: If there were a lung infection, it would cause early mortality. Methanol: In the methanol study there were more deaths early in the study than expected; most of them had lung pathology, thus supporting the possibility that there is an ongoing lung infection in the colony that affects the health of the animals.
3. Caldwell: Hemolymphoreticular neoplasms are a late occurring phenomenon (primarily after the usual termination of studies at 104 weeks). Methanol: In the methanol study there was no difference in percentage of animals dying with hemolymphoreticular neoplasms after 104 week or before 104 weeks.
4. Caldwell: Increased hemolymphoreticular neoplasms occur only in females, and if related to a lung infection, they should occur in both sexes. However, in three of the 8 studies cited, hemolymphoreticular neoplasms were increased in both sexes. Methanol: In the methanol study, the total hemolymphoreticular tumors was increased only in females, but lympho-immunoblastic lymphoma was increased in both males and females. Thus, in at least 4 of the 8 studies, their premise does not hold.
5. Caldwell: The incidence of hemolymphoreticular neoplasms in control groups in Ramazzini Foundation studies is “low and stable.” A range of 0-33% or 0-23% is a wide range that would not be considered stable by most reviewers. Further, incidences up to 33% or 23% are not considered to be low. Methanol: The incidences of total hemolymphoreticular neoplasms in the males in the methanol study ranged from 25 to 40%, just barely outside the 33% historical control range. In females the range in the methanol study was 13-28%; also not much outside the historical control range of 23%.

Based on the criteria set forth by Caldwell et al., lung infections probably played a role in the formation of lympho-immunoblastic lymphoma in the Ramazzini methanol study.

Based on a review of the data tables made available thus far, a number of questions remain regarding this study, including the health of the animals, the degree of respiratory infection, and the diagnostic criteria for the finding “lympho-immunoblastic lymphoma”. This diagnosis is not used by any other pathologist examining animal carcinogenicity studies. This diagnosis should not be relied on for cancer classification until it is validated by other pathologists who routinely perform evaluations of this type of study. There is considerable evidence that there was lung

pathology not related to methanol exposure that influenced the outcome of the study. However, an accurate evaluation of the Ramazzini Foundation methanol study cannot be conducted unless the pathology slides and diagnoses are examined by an outside pathologist or group of pathologists.

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Introduction

The Ramazzini Foundation (RF) published a study on the carcinogenicity of methanol in Sprague-Dawley rats in the *Annals of the New York Academy of Science*, a non-peer-reviewed journal (Soffritti, et al., 2002a). Exposure to methanol was 0, 500, 5,000 or 20,000 ppm in drinking water for 2 years with the animals being observed until they died. In the published paper no actual data on survival, body weight, feed and water consumption was presented. No organ weight, blood methanol, clinical tests or macroscopic data were reported. Histopathologic evaluation did not include non-tumor pathology, but reported significant treatment-related effects in the tumor response including a statistically significant increase in hemolymphoreticular tumors in the high dose (20,000 ppm) females, carcinomas of ear duct in high dose (20,000 ppm) males, and total tumor bearing animals (high dose)– 20,000 ppm both sexes was also reported.

The RF has subsequently made more data from the methanol study available on their Website and to the US EPA. The Methanol Institute has obtained much of the data supplied to the EPA. This evaluation is based on the data from the paper, the website and tables from the EPA.

Method of Study Evaluation

The RF methanol study was initiated April 1990 and published in 2002, by Soffritti et al. Normally, the RF only publishes a relatively brief report of their findings in a non peer-reviewed journal. Historically, the RF has resisted providing additional data on the individual studies. However, the RF recently entered into a contract with the U.S. National Institute for Environmental Health Sciences (NIEHS) of NIH to put the data from some of their studies into the National Toxicology Program (NTP) data format and provide it to NIEHS. In addition, the RF has placed a portion of the information on its own website. In turn, the U.S. Environmental Protection Agency (EPA) entered into an agreement with NIEHS to obtain the data from some of these studies, including the data on the methanol study. Subsequently, in response to a Freedom of Information Act request from the Methanol Institute, the EPA made available 11 of the 21 NTP database files they received, plus 2 files available on the RF website. The RF has claimed Confidential Business Information (CBI) on the remaining 8 files, and EPA has not yet released them. The release of these data presents an opportunity for the scientific community to evaluate a

RF study in considerably more detail than is usually possible. This current evaluation is therefore based on the data from the published study, the website and the tables obtained from the EPA.

The files released to date are:

ANIMAL REMOVAL SUMMARY BY TREATMENT GROUP	00117_68_e1.pdf
MEAN BODY WEIGHTS AND SURVIVAL TABLE	00117_68_e4.pdf
CLINICAL OBSERVATIONS SUMMARY	00117_68_e5.pdf
MEAN FEED CONSUMPTION BY TREATMENT GROUP	00117_68_e6.pdf
MEAN WATER CONSUMPTION BY TREATMENT GROUP	00117_68_e7.pdf
ANIMAL HISTORY	00117_68_e12.pdf
INDIVIDUAL BODY WEIGHT TABLE	00117_68_e14.pdf
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STATISTICAL ANALYSIS OF SURVIVAL DATA	00117_68_p11.pdf
INDIVIDUAL ANIMAL PATHOLOGY DATA	00117_68_p14.pdf
INCIDENCE RATES OF NON-NEOPLASTIC LESIONS BY ANATOMIC SITE (a) WITH AVERAGE SEVERITY GRADES[b]	00117_68_p18.pdf

The evaluation of these data was carried out as follows: The individual pathology record (00117_68_p14.pdf) for each animal was examined and an Excel spreadsheet was developed that recorded all pathologic findings in the lung, all neoplasms (cancers) derived from blood forming cells (leukemias, lymphomas, sarcomas) in lung and other tissues, and number of days on study. Incidences of neoplasms were compared to the individual tumor incidence table (00117_68_p4.pdf) and statistical evaluations in the NTP database (00117_68_p8.pdf).

In addition, results from the RF methanol study were compared to control results from other studies at the RF and from studies conducted in Sprague-Dawley rats at other facilities.

Study Design

The RF uses unique methodology and study design. This does not conform with OECD, US EPA, or NTP Study Guidelines for chronic/carcinogenicity studies. This complicates any attempt to validate the results of the study, even with the release of some of the data tables, as in the present case. A comparison of the RF study design and methodology with the NTP guidelines is presented in Table 1.

Table 1. Comparison of Standard NTP Bioassay Methods with the RF Methanol Study

INFORMATION	NTP	RF
Preliminary studies	Yes	No
GLP	Yes	Not described
Peer Reviewed	Yes	Internal only
Sample Characterized and Stability Checked	Yes	No
Water Consumption	Yes	Yes
Active Disease Surveillance Program	Yes	No
Random Assignment of Animals to Treatment Groups	Yes	No
Body Weight	Yes	Yes
Survival Data	Yes	Yes
Sacrifice Animals in Extremis	Yes	No
Terminal Sacrifice	Yes	No
Hematology & Clinical chemistry	Yes	No
Organ Weight	Yes	No
Macroscopic Changes	Yes	Yes
Non-neoplastic Lesions	Yes	Yes
Neoplastic lesions	Yes	Yes
QA Review	Yes	No
Use Historical Neoplastic data	Yes	No
Pathology Working Group	Yes	No

There are a number of aspects to quality studies that are not included in RF studies, including sample characterization, disease surveillance, random assignment of animals, sacrifice of animals in extremis, and external pathology slide examination. These limitations make evaluation of the methanol study difficult.

The Ramazzini Foundation administered methanol in the drinking water of groups of 100 male and 100 female Sprague-Dawley rats (5 rats/cage, 8 weeks old at the start of dosing) from their own breeding colony for 104 weeks at nominal concentrations of 0, 500, 5000, and 20,000 mg/L (Soffritti et al., 2002). The animals were not randomly assigned to treatment groups, but animals from a given litter are all assigned to the same treatment group (Bucher, 2002). It is indicated that they record which litter each animal came from (Bucher, 2002), but in the data released to date, there is no indication of litter. Therefore, at the present time, litter effects cannot be

evaluated. This makes differentiating between congenital effects and chemically-related effects more difficult.

There is no confirmation of the correct preparation of drinking water solutions because the concentration of methanol in the drinking water was not measured analytically. There is no evidence that they actually tested methanol. The paper indicates that fresh water/methanol solutions were placed in the animal cages each day for 104 weeks and that the drinking water consumed was measured one day each week for the first 13 weeks and one day every two weeks until week 104. This provides a very crude estimate of water consumption and therefore methanol intake during the methanol study. Animals were weighed weekly for 13 weeks, biweekly until week 104 and every 8 weeks until death. After death each animal received a necropsy and histopathologic examination of the normal set of tissues.

Approved study guidelines require moribund animals to be sacrificed to minimize autolysis (tissue decay) which would interfere with pathologic evaluation. Laboratories that conduct studies for regulatory submissions examine the animals at least twice each day for moribundity. The Ramazzini Foundation does not sacrifice animals, but allows them to die. Thus, animals could be dead for up to 12 hours before necropsy if observations were conducted twice a day. Such a delay in necropsy would be expected to lead to autolysis of tissues, although the RF data tables do not indicate any autolysis, which seems unlikely.

Approved protocols call for termination of rat studies at 24 months, so a sufficient number of controls and treated animals can be compared at the same age for pathology. Ramazzini does not terminate the study until the last animal dies. Thus there is no group of animals terminated together after a long period on study in the Ramazzini studies.

Control groups

The Ramazzini Foundation program started over 30 years ago and has evaluated 200 compounds in 398 studies (averages to a new study every month) (Soffritti et al 2002b). In a paper on Aspartame (Soffritti, et al., 2005), Soffritti indicated that over the last 20 years almost 2000 male

and 2000 female were used as controls (basis of the historical control data). This averages out to one control group per year (vs. an average of 12 studies per year).). In one of the MTBE paper RF details the use of common controls for several chemicals (Belpoggi et. al 1995). This possible use of common controls for several studies increases the difficulty of interpreting the results of the studies. Normally, a control group of animals is carefully matched to the experimental groups in every respect other than chemical exposure so as provide a point of comparison to assess whether the treatment caused a significant carcinogenic effect. If the controls are not matched to the treated animals, then there is the possibility of extraneous unknown factors influencing the results, leading to a misinterpretation of seeming abnormalities seen in the treated animals.

Data Evaluation

Survival

Treatment with methanol did not decrease survival (Table 2). In fact, males treated with methanol survived better than the controls from 100% to 50% survival. 50% survival occurred on study day 629, 686, 639 and 701 in males at 0, 500, 5000, and 20,000 mg/L, respectively. The survival data raises two issues.

1. How well did the control group compare to the treated groups? There is no indication in the study data whether the control animals were of the same age and studied at the same time as the treated animals. However, the different survival pattern suggests they may have been from different batches of animals.
2. Sprague-Dawley rats, in general, do not survive as long as F344 rats and the survival at 104 weeks is not outside the historical control range for Charles River Sprague-Dawley rats. However, there was considerable early mortality in this study. By 18 months, 30% of the male controls had died. In females, there were no differences in survival between control and treated groups. Although less pronounced than in males, there still is more early mortality than expected.

Table 2. Survival Summary of Rats Treated with Methanol for 104 Weeks

	Males				Females			
Conc.	0	500	5000	20000	0	500	5000	20000
Survival – study days to:								
90%	392	489	401	413	414	403	443	418
80%	476	551	483	476	536	523	544	551
70%	535	603	567	565	600	594	593	612
60%	570	651	619	613	665	647	644	648
50%	629	686	639	701	717	691	678	708
40%	709	733	702	733	779	734	709	737
30%	755	777	760	759	810	787	733	782
20%	798	810	792	832	859	821	782	810
10%	884	878	845	899	922	895	939	913
0%	1034	1012	1008	1033	1047	976	1028	1071

In a recent article regarding the Ramazzini study of MTBE, Caldwell et al.(2008) asserted that if there were an ongoing lung infection in the Ramazzini colony, one would expect early mortality. Then they compared the mortality at 104 weeks in the Ramazzini MTBE study to mortality at the same time point in a few other studies (which were terminated at 104 weeks). They concluded that the mortality in the Ramazzini MTBE study was about the same as in other databases and that there was no lung infection affecting mortality. Such cannot be said of the Ramazzini methanol study. While the survival at 104 weeks was within the normal, but widespread, range for Sprague-Dawley rats, there was significant early mortality among all groups, including the controls. The historical databases do not contain survival percentages at time points other than 104 weeks, but one study in Sprague-Dawley rats can be compared. The control group from an inhalation study (Cruzan et al. 1998) had much better survival through 104 weeks than seen in the Ramazzini methanol study (Table 3).

Table 3. Comparative Survival of Control Sprague-Dawley Rats Through 104 weeks in Two Studies

Males				Females		
% Survival	Ramazzini	Cruzan	Difference	Ramazzini	Cruzan	Difference
90	392 ^a	539	147	414	490	76
80	476	574	98	536	546	10
70	535	630	95	600	637	37
60	570	707	137	665	679	14

^aValues shown are the number of days on study to reach the % survival.

Water Consumption

Water consumption was measured one day each week for the first 13 weeks and one day each two weeks until 104 weeks. It is important to measure water consumption accurately in a study where the test material is administered in the drinking water; test material dose is calculated from the amount of water consumed. Animals do not consume the same amount of water each day. Thus the ideal procedure is to measure water consumption each day. Because this is a lot of work, laboratories typically determine the amount of water consumed by each animal over several days; i.e., weekly water consumption usually means the amount of water consumed during a week, not one day of the week. Having 5 rats/cage results in some averaging of the individual variability, but water consumption should be measured continuously.

The number of animals and cages reported in the drinking water statistical analysis (file 00117 – 68-e7) indicates that water consumption was determined on only half of the cages in each group. The table indicates that water consumption was measured on 50 animals in 10 cages, until mortality reduced the number of animals. It also appears that the water consumption is somewhat higher than one would expect. This suggests that there was some spillage of water by the animals, which was not accounted for.

Based on the limited data from the study, there is no obvious effect of methanol exposure on water consumption.

Body Weight

Males treated with 20,000 ppm methanol in the drinking water weighed more than the controls (up to 14%) throughout the study (file 00117 – 68-e4). The rats treated at 500 and 5000 ppm methanol did not differ from the control weights (Table 4). Females treated with 20,000 ppm methanol weighed more than the controls throughout the study (up to 7%). Females treated at 5000 ppm weighed more (4%) than the controls at 24 months, but not at earlier times. Weights of females treated at 500 ppm did not differ from control body weights (Table 5).

Table 4. Mean Body Weight of Male Rats at Different Times of the Study

	0 ppm	500 ppm	5000 ppm	20,000 ppm
Day 92 (3 mo)	460	449	455	486
Day 176 (6 mo)	495	486	489	526
Day 358 (12 mo)	567	541	559	613
Day 540 (18 mo)	569	558	586	648
Day 736 (24 mo)	537	529	560	583

Table 5. Mean Body Weight of Female Rats at Different Times of the Study

	0 pm	500 ppm	5000 ppm	20,000 ppm
Day 92 (3 mo)	285	279	283	290
Day 176 (6 mo)	310	311	310	319
Day 358 (12 mo)	356	357	360	375
Day 540 (18 mo)	391	392	396	420
Day 736 (24 mo)	405	389	423	435

The body weight data do not indicate methanol toxicity and do not provide any indication that the control group was not concurrent with the treated groups

Lung Pathology

From the data provided, it is difficult to assess the degree of inflammation in the lungs of the rats in methanol study because no other lung information was recorded for rats when a neoplasm in the lung was recorded. The primary characteristic of the pathology is that nearly all rats in all dose groups have some pathology in the lung. The finding of lung pathology was consistent

regardless of the age at death; i.e., lung pathology was not an old age response. There is essentially no difference in the percent of rats with any lung pathology (including neoplasms), lung inflammation, or dysplasia between those dying prior to 18 months (Table 6), or 24 months (normal study termination, Table 7, 8) and those dying later (Table 9, 10). The amount of lung pathology indicates that these were not healthy rats.

Based on a personal communication from Dr. Soffritti without supporting data, Caldwell et al. (2008) asserted that the lung infection in the Ramazzini animal colony is an acute event that happens terminally in old animals and is not present throughout the MTBE study. However, examination of the data in the Ramazzini methanol study does not support this conclusion. Lung pathology was reported in 80% to 96% of the rats that died before 18 months, which was essentially the same as at later periods of the study. Thus in the methanol study, indications of lung infection were present in nearly every animal that died regardless of how young or old. In fact, lung pathology was present in 70 to 100% of the first 10% of deaths in each group (70, 80, 80, 100% in males and 90, 90, 100, 100% in females at 0, 500, 5000, and 20,000 ppm respectively). If lung pathology in rats dying early in the study is an indication of an ongoing lung infection in the colony, then the methanol data indicates an ongoing lung infection.

Table 6. Incidence of Reported Lung Pathology in Rats dying Before 18 Months (Study day 540)

	0 ppm	500 ppm	5000 ppm	20,000 ppm
Percent of males with any lung finding	90	89	89	96
Percent of females with any lung finding	85	80	89	95

Table 7. Incidence of Reported Lung Pathology in Male Rats Dying up to 24 Months (day 730) on Study

	0 ppm	500 ppm	5000 ppm	20,000 ppm
Percent with any lung finding	92	91	92	91
Percent with inflammation or edema	56	49	34	34
Percent with dysplasia	8	14	17	16
Percent with adenoma or metastasis from other organ	5	2	2	0

Table 8. Incidence of Reported Lung Pathology in Female Rats Dying up to 24 Months (day 730) on Study

	0 ppm	500 ppm	5000 ppm	20,000 ppm
Percent with any lung finding	82	86	93	90
Percent with inflammation or edema	62	58	52	58
Percent with dysplasia	4	8	19	8
Percent with adenoma or metastasis from other organ	2	7	1	2

Table 9. Incidence of Reported Lung Pathology in Male Rats (dying up to 148 weeks; day 1034 on study)

	0 ppm	500 ppm	5000 ppm	20,000 ppm
Percent with any lung finding	91	94	94	92
Percent with inflammation or edema	54	49	47	35
Percent with dysplasia	8	11	11	17
Percent with adenoma or metastasis from other organ	5	1	1	2

Table 10. Incidence of Reported Lung Pathology in Female Rats (dying up to 153 weeks; day 1071 on study)

	0 ppm	500 ppm	5000 ppm	20,000 ppm
Percent with any lung finding	87	88	89	94
Percent with inflammation or edema	69	63	53	53
Percent with dysplasia	4	7	15	13
Percent with adenoma or metastasis from other organ	2	4	1	5

Hemolymphoreticular Neoplasms

The RF sometimes uses diagnostic terms for neoplasms that are not in the standard pathologic guidelines. When they do, it is difficult to understand what they are reporting. In the methanol study, they use the diagnoses of lymphoblastic lymphoma and lymphocytic lymphoma; standard pathology guides use the term lymphocytic lymphoma. It is not known how lymphoblastic and

lymphocytic lymphoma differ. They also report a neoplasm called lympho-immunoblastic lymphoma; no other pathologists use this diagnosis. Therefore, the meaning of the diagnosis is unknown without careful examination of the pathology slides by other pathologists.

The most prominent cancer finding reported in the methanol study was lympho-immunoblastic lymphoma (LIL). LIL was reported mostly in the lung; 157 of the 171 rats with LIL had LIL in the lung. In 66 of the 157 rats (42%), LIL was reported only in the lung. Based on standard NTP statistical analysis, the incidence of LIL in the lung was increased in males at 20,000 ppm (Table 11) and in females at 5000 and 20,000 ppm (based on poly3 and lifetable tests; Table 12).

Table 11. Incidence of Lympho-immunoblastic Lymphoma Reported in Male Rats (dying up to 148 weeks; day 1034)

	0 ppm	500 ppm	5000 ppm	20,000 ppm
Percent with LIL in lung	16	23	25 ^a	36
Percent with LIL only in lung	8	10	14	13
Percent with LIL not in lung	0	2	2	1
Percent with LIL in any organ	16	25	27	37*

^aFile 00117_68_p4.pdf indicates there are 27 animals in this group with LIL, but animal Number 365 does not have a diagnosis of LIL in the individual pathology file - 00117_68_p14.pdf.

*Significantly different from control, based on poly3 and lifetable analysis, p<0.05)

Table 12. Incidence of Lympho-immunoblastic Lymphoma Reported in Female Rats (dying up to 153 weeks; day 1071)

	0 ppm	500 ppm	5000 ppm	20,000 ppm
Percent with LIL in lung	9	10	16	19
Percent with LIL only in lung	3	5	6	7
Percent with LIL not in lung	0	7	3	2
Percent with LIL in any organ	9	17*	19*	21*

* Significantly different from control, based on poly3 and lifetable analysis, p<0.05)

The RF often combines the incidences of all cancers derived from blood forming cells for statistical evaluation and calls them “hemolymphoreticular tumors” (Tables 13, 14). This is generally not considered appropriate because different cancers are derived from different cell types and do not share a common derivation. The cancer diagnoses included in the “hemolymphoreticular tumors” were: histiocytic sarcoma, mononuclear leukemia, myeloid

leukemia, lymphoblastic lymphoma and lympho-immunoblastic leukemia. These cancers are often found in many organs, including lungs, but in the methanol study they were very prominent in lungs; 92% of the “hemolymphoreticular” cancers occurred in lungs. This suggests that either they were highly susceptible to “hemolymphoreticular” cancers because of lung inflammation or the observations may not have been cancers. In the methanol study, the incidence of total “hemolymphoreticular” cancers was increased in all female treated groups. The incidences in males were not statistically different from control.

Table 13. Incidence of Hemolymphoreticular Neoplasms Reported in Male Rats (dying up to 148 weeks; day 1034)

Percent with histiocytic sarcoma in lung	2	4	1	1
Percent with histiocytic sarcoma in any organ	2	4	1	1
Percent with leukemia in lung (mononuclear or myeloid)	6	4	6	1
Percent with leukemia in any organ (mononuclear or myelocytic)	9	4	6	1
Percent with lymphoblastic lymphoma in lung	0	2	1	0
Percent with lymphoblastic lymphoma in any organ	1	3	1	0
Percent with any hemolymphoreticular neoplasms in lung	24	33	33	38
Percent with any hemolymphoreticular neoplasms in any organ	28	35	35	40

In a critique of mode of action for MTBE, Caldwell et al., (2008) have asserted that hemolymphoreticular tumors are late occurring and thus the incidence increases dramatically after the usual study termination time at 104 weeks. They stated this as support for the use of the Ramazzini study of MTBE in assessing its carcinogenic potential. The authors cite this as a personal communication from Dr. Soffritti, but show no supporting data. The data from the Ramazzini methanol study do not support this statement. Of the animals that died up to the end of week 104, 33% of the males and 20% of the females were diagnosed with some form of hemolymphoreticular neoplasm, while 38% of the males and 25% of the females dying after week 104 were diagnosed with some form of hemolymphoreticular neoplasm. Thus in the

Ramazzini study of methanol, there was no difference in the percent of animals with hemolymphoreticular neoplasms in animals dying early or late.

Table 14. Incidence of Hemolymphoreticular Neoplasms Reported Female Rats (dying up to 153 weeks; day 1071)

Percent with histiocytic sarcoma in lung	0	2	2	1
Percent with histiocytic sarcoma in any organ	1	2	2	3
Percent with leukemia in lung (mononuclear or myelocytic)	3	1	1	3
Percent with leukemia in any organ (mononuclear or myelocytic)	3	2	2	3
Percent with lymphoblastic lymphoma in lung	0	1	1	1
Percent with lymphoblastic lymphoma in any organ	0	1	1	1
Percent with lymphocytic lymphoma in any organ	0	1	0	0
Percent with any hemolymphoreticular neoplasms in lung	12	14	20	23
Percent with any hemolymphoreticular neoplasms in any organ	13	23*	24*	28*

* Significantly different from control, based on poly3 and lifetable analysis, $p < 0.05$

Ear Duct Carcinomas

The RF reported incidences of ear duct carcinomas in the high dose animals (Table 15). A few selected slides from the RF study on aspartame were reviewed by an NTP pathology working group (PWG). In almost half the cases of lesions classified as ear duct carcinomas by RF pathologists, the NTP PWG did not agree that the lesions were carcinomas (NTP 2004). This leads to an uncertainty on the incidence of ear duct carcinomas in the Ramazzini Foundation study of methanol. The true incidence and relationship to methanol exposure can only be determined by a thorough review by a pathology working group of all the 113 slides diagnosed as ear duct carcinomas.

Table 15. Incidences of Ear Carcinomas Reported

	0 ppm	500 ppm	5000 ppm	20,000 ppm
Males	9	12	16	24*
Females	9	8	16	19*

* Significantly different from control (poly 3, lifetable)

Comparison to Historic Control Data

Comparison of study results with historical controls can help evaluate the degree of variability among groups of animals, whether the results are expected or unusual. Charles River Laboratories (CRL) maintains control datasets on a number of animal models they sell. They encourage users of their animals to submit the data from the control animals to be incorporated into a database. They also incorporate published data using the same strain of animals from different sources. For cancers of the blood forming cells, the CRL database does not specify which organ they were found in, but lists them as “whole body/ multiple organ”. For this category the CRL database for Sprague-Dawley rats includes 2146 males from 30 studies (Table 16) and 2344 females from 31 studies (Table 17). The incidences of histiocytic sarcoma, mononuclear leukemia, myeloid leukemia, and lymphoblastic (lymphocytic) lymphoma in the Ramazzini Foundation methanol study were within the historical control range of the Charles River database except that the incidence of myeloid leukemia in the control males exceeded the Charles River control range.

Table 16. Charles River Historic Control Data for “Hemolymphoreticular tumors” in Male Rats

Diagnosis	% with finding	Minimum % in a study	Maximum % in a study
Histiocytic sarcoma	2.10	0.77	6.00
Mononuclear Leukemia	0.04	0	2.00
Granulocytic Leukemia (Myeloid*)	0.42	0	2.86
Lymphocytic Lymphoma	1.68	0.91	6.00

Lympho-immunoblastic Lymphoma	Not reported		
Leukemia/lymphoma combined**		21.33	21.33

* CRL Uses Granulocytic; it is presumed that the RF term Myeloid is equivalent.

**From a Ramazzini Foundation study; the only study reporting as combined figure.

Table 17. Charles River Historic Control Data for “Hemolymphoreticular tumors” in Female Rats

Diagnosis	% with finding	Minimum % in a study	Maximum % in a study
Histiocytic sarcoma	0.90	0	3.08
Mononuclear Leukemia	0.04	0	0.91
Granulocytic Leukemia (Myeloid)*	0.21	0	2.73
Lymphocytic Lymphoma	1.11	1.11	10.00
Lympho-immunoblastic Lymphoma	Not reported		
Leukemia/lymphoma combined**		14.67	14.67

* CRL Uses Granulocytic; it is presumed that the RF term Myeloid is equivalent.

**From a Ramazzini Foundation study; the only study reporting as combined figure.

The National Toxicology Program conducted a series of 7 carcinogenicity studies in Sprague-Dawley rats (Walker et al., 2006). The control data from those studies included 371 females and 0 males (Table 18). The incidences of total leukemias in the methanol study were within the NTP historical control range, except for the male controls. Comparison of the “all leukemias” is not possible because the NTP database does not include the diagnosis of lympho-immunoblastic lymphoma.

Table 18. National Toxicology Program Control Data for “Hemolymphoreticular tumors” in Female Sprague-Dawley Rats

Diagnosis	% with finding	Minimum % in a study	Maximum % in a study
Leukemias: all combined	0.81	0	1.89
Lymphomas: all combined	1.08	0	3.77

The RF has published many studies, as well as a compilation of historic control data (Table 19). In their publications, they incorrectly combine all leukemias and lymphomas into one category, so the only comparison that can be made to their historic database is to total leukemia/lymphoma. The total hemolymphoreticular tumors of all male groups in the methanol

study exceeded the historical control range, although they were not significantly different from control. The incidence of total hemolymphoreticular tumors of the high dose females was slightly outside the historic control range for Ramazzini foundation studies (27% vs. 23%). The incidences at 500 and 5000 were at the upper bound of the range (24%).

Table 19. Ramazzini Foundation Control Data for total “Hemolymphoreticular tumors”

	# of Studies	# of Animals	Mean %	Minimum%	Maximum %
Males	38	3022	12.2	0	32
Females	43	3301	7.2	0	23

It should be noted that the incidences of “hemolymphoreticular tumors” tumors reported by the Ramazzini Foundation is considerably higher than the incidences reported by other laboratories (males 12%, 0-32% range vs. 4%, 0-16% range for CRL; females 7%, 0-23% range vs. 2%, 0-12% range for CRL and 2%, 0-6% range for NTP).

Caldwell et al. (2008) assert that the incidence of hemolymphoreticular neoplasms in control groups in Ramazzini Foundation studies is “low and stable.” A range of 0-33 or 0-23 is a wide range that would not be considered stable by most reviewers. Further, incidences up to 33% or 23% are not considered to be low.

Historic Control Data for Ear duct carcinomas

The Charles River database indicates that only 1 of 30 studies in males and 1 of 31 in females found ear duct carcinomas. One study in the database is a RF study. The database does not indicate which study reported ear duct carcinomas, but it could have been the RF study. In that one study, 2 males (2.67%) had ear duct carcinomas and 6 (8%) of the females did. In the NTP Sprague-Dawley rat database (Walker et al., 2006), none of the 371 females had ear duct carcinomas. Along with the PWG evaluation of ear duct carcinomas from the RF aspartame study, the historical database for ear duct carcinomas indicates that the ear duct carcinomas in the methanol study should be questioned.

Conclusions

Based on a review of the data tables made available thus far, a number of questions remain regarding this study, including the health of the animals, the degree of respiratory infection, and the diagnostic criteria for the finding “lympho-immunoblastic lymphoma”. An accurate evaluation of the Ramazzini Foundation methanol study cannot be conducted unless the pathology slides and diagnoses are examined by an outside pathologist or group of pathologists.

Examining the survival and pathology data suggests the animals were not healthy according to current standards of laboratory animal care. There was excessive early mortality (up to 30% by 18 months), although survival at 104 weeks (standard study termination) was within the historic control range for Sprague-Dawley rats. Secondly, lung pathology (inflammation, dysplasia, or neoplasm) was present in 80 to 95% of those dying before Study day 540 (18 months), in 82 to 92% of those dying before Study day 730 (24 months) and in 87 to 94% of those dying anytime in the study (up to Study day 1071). Based on the data, there is no indication that lung pathology was an occurrence of old age because it was present in nearly every animal, regardless of when they died. The contribution of lung pathology to tumor formation, particularly in lungs, cannot be ascertained from the data tables. A review of the pathology slides by an independent pathologist or pathology working group is needed to evaluate the degree of respiratory infection and its potential impact on the outcome of the study.

Pathologists at the Ramazzini Foundation have recently begun using a diagnosis of “lympho-immunoblastic lymphoma”. This diagnosis is not used by any other pathologist evaluating animal cancers. The cancer diagnosis needs verification by other pathologists, including how this cancer differs from lymphoblastic lymphoma. A pathology peer review of all the slides from the methanol study (and potentially other studies) with this diagnosis is needed to define this cancer.

The incidences of total cancers derived from blood forming cells, designated as hemolymphoreticular tumors by Ramazzini pathologists, is consistently about 4 times higher than the incidences of such tumors in other laboratories using Sprague-Dawley rats. The RF uses Sprague-Dawley rats from its own breeding colony, not animals from a commercial supplier. The genetics and health status of this colony compared to commercial colonies is not known. A thorough examination of the animal colony (including genetics and health status) is needed to

understand the reasons for differences from other sources of Sprague-Dawley rats in cancer response.

Because different cancers from blood forming are derived from different cell types, it is generally considered inappropriate to combine all of them into one evaluation. In the methanol study, the incidences of histiocytic sarcoma, leukemias, and lymphoblastic lymphoma were not higher in methanol treated rats than in the controls. In addition, the incidences of these cancers were within the historic control range from two other databases. The only cancer that was increased in the methanol study was lympho-immunoblastic lymphoma. There are no historic control data to compare this study to. The diagnosis is not used by any other pathologist in animal studies. The significance of observation cannot be determined until the diagnosis is validated by other pathologists. The increase in lympho-immunoblastic lymphoma was not related to allowing each animal to live until death; the percent of animals dying with lympho-immunoblastic lymphoma remained the same throughout the study.

Ear duct carcinomas have apparently been reported only by the RF. The NTP limited PWG of the aspartame study reported that many of the lesions diagnosed as ear duct carcinoma were not tumors.

In summary, the RF study of methanol reported increased incidences of lympho-immunoblastic lymphoma. This diagnosis is not used by any other pathologist examining animal carcinogenicity studies. This diagnosis should not be relied on for cancer classification until it is validated by other pathologists who routinely perform evaluations of this type of study.

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