

**EVALUATION OF QUALITY OF CARCINOGENICITY STUDIES
CONDUCTED BY THE RAMAZZINI FOUNDATION**

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EXECUTIVE SUMMARY

Since Dr. Cesare Maltoni and colleagues, starting working in chemical carcinogenesis in 1970 at the “F. Addarii” Institute of Oncology in Bologna, and subsequently at the European Foundation of Oncology and Environmental Sciences “B. Ramazzini” (the Ramazzini Foundation), which was founded in 1992, this group of researchers has reportedly performed 398 cancer bioassays on 200 compounds/agents (Soffritti et al. 2002a). Since published reports exist for far fewer than 200 substances (see References), however, many of these studies have apparently never been published.

This represents a significant body of work in the area of investigation of carcinogenicity, second in quantity only, perhaps, to that of the U.S. National Toxicology Program and the National Cancer Institute. Unlike those institutions, however, the Ramazzini Foundation has displayed a lack of openness regarding the conduct and details of the results of its studies. Certain aspects of the design, conduct, and reporting of studies carried out by the Ramazzini Foundation raise questions regarding their appropriateness for use in assessing risk. In particular, the combination of allowing the animals to live until spontaneous death, gang caging, and the high incidence of chronic infections in the animals may tend to produce a combination of autolysis, cannibalism, and severe age-related, and infectious disease-related conditions that may confound accurate pathological diagnosis.

Questions also arise regarding whether the Ramazzini Foundation studies follow appropriate good laboratory practice procedures. Although some of the more recent Ramazzini Foundation publications refer to GLP, older reports do not, and the nature of the laboratory practices referred to in the more recent studies is unclear.

Further confounding interpretation is the practice of the Ramazzini Foundation scientists of not reporting information on non-neoplastic lesions in their published reports. Such data would help in assessing the overall health of the animals, and determine whether there were underlying disease processes that might interfere with interpretation of the study data.

Additional factors that make interpretation of these studies difficult include a high frequency of infections, some of which may contribute to the development of lymphomas associated with the respiratory system seen in some studies. These tumors, reported in the Ramazzini Foundation bioassays of MTBE, methanol, formaldehyde, and aspartame, but not seen in other studies of these chemicals, are of questionable value in assessing the carcinogenic potential of these, or other chemicals. In the case of most chemicals to which the Ramazzini Foundation has attributed the ability to cause hemolymphoreticular neoplasms, the tissue location of these neoplasms is not identified in the published reports; hence the role of respiratory infections cannot be evaluated. Variability in the extent and impact of respiratory

infection may also, in part, explain the very wide range in the incidence of lymphomas and leukemias reported in different Ramazzini Foundation studies.

In addition to issues regarding study design and conduct, issues arise regarding the methods of statistical analysis used in the interpretation of these studies. Because of their unusual design, particular attention needs to be paid to the time of identification of tumors, using methods such as time-to-tumor modeling. At least in the case of MTBE, there is some indication that the apparent increase in Leydig cell tumors was influenced by the longer average lifespan of the treated animals than the controls.

Overall, limitations in the details of study methods and results reported by the Ramazzini Foundation in its published study reports, and questions raised by some of its unusual practices make use of data from these studies of questionable value for regulatory purposes. The recent release of some individual animal neoplastic lesion data by the Ramazzini Foundation represents a positive step, but raises additional questions because of discrepancies between the recently released data and previously published data for some chemicals.

We recommend that, before relying upon data from the Ramazzini Foundation, regulatory agencies:

- (i) seek full study documentation of the type available for NTP studies;
- (ii) evaluate the studies using NTP criteria;
- (iii) give little weight to any study for which adequate documentation of conduct and results is absent;
- (iv) thoroughly evaluate the role of infection in tumor production in the SD rats used by the Ramazzini Foundation.

In addition, the unusual protocol design used by the Ramazzini Foundation is difficult to evaluate. Some type of peer-review of the design, perhaps by a committee of the National Academy of Sciences, would be highly valuable.

I. INTRODUCTION

In light of concerns that have been raised in the scientific community regarding the utility of carcinogenicity studies conducted by the Ramazzini Foundation, ENVIRON International Corporation was asked by the National Petrochemical and Refiners Association and the Methanol Institute to perform a critical evaluation of the scientific quality of these studies, and to comment on what would be needed to ensure scientific rigor before any of these studies were used as a basis for regulatory action.

A. The Ramazzini Foundation

What is now formally called the European Foundation of Oncology and Environmental Sciences “B. Ramazzini” was founded in 1992, built on the work performed at the “F. Addarii” Institute of Oncology in Bologna by Dr. Cesare Maltoni and colleagues, starting in 1970 (Soffritti et al. 2002a). The “Ramazzini Foundation” as it is now commonly known, is a non-profit, private institution located in Bentivoglio, in the province of Bologna, Italy. Its facilities include the Cesare Maltoni Cancer Research Center with more than 10,000 square meters of laboratories (where the carcinogenicity studies under consideration here were conducted).

This group of researchers first gained international notice when it announced confirmation of the findings of another group of Italian cancer researchers who were the first to demonstrate the carcinogenicity of vinyl chloride in rats exposed by inhalation (Viola et al. 1971). Maltoni and colleagues initiated a series of experiments in 1971 to investigate the carcinogenicity of vinyl chloride. This work was funded by European chemical companies (Montedison, ICI, Solvay, and Rhone-Progil). The results of that work were presented in several meetings and publications, including a Workshop sponsored by the New York Academy of Sciences in 1974 and subsequently published in the *Annals of the New York Academy of Sciences* (Maltoni and Lefemine 1975).

Subsequently, the Ramazzini Foundation has reportedly performed 398 cancer bioassays on 200 compounds/agents (Soffritti et al. 2002a). Since published reports exist for much fewer than 200 substances (see References), many of these studies have apparently never been published. More recently, in an expert report prepared in a litigation case, Fiorella Belpoggi, one of the senior scientists at the Ramazzini Foundation, identified 205 agents (including ionizing and non-ionizing radiation) the Ramazzini Foundation has studied, some in multiple studies via several routes of exposure.

The aims and methods of the work performed by the Ramazzini Foundation have been discussed in several publications (Maltoni et al. 1999; Soffritti et al. 1999, 2002a). The paper by Maltoni et al. (1999) in particular lays out the philosophical approach espoused by these

researchers. Maltoni et al. (1999) endorsed five prerequisites for “protecting this branch of research from the amateur or anecdotal approach.” These are:

1. “Use of animal species and strains whose basic tumorigram and kind of response to cancer stimuli is not too remote from the human counterpart.”
2. “Continuing bioassays until the end of the life of an animal.”
3. “Following the rules of Good Laboratory Practice as a minimum standard in experiment management.”
4. “Choosing precise parameters to assess neoplastic response. In our opinion, such parameters are: total number and percentage of animals carrying benign and malignant tumors and the various kinds of tumor; total number of benign and malignant tumors, and number of the various kinds per 100 animals (in view of the fact that one and the same animal may develop multiple tumors of various kinds at various sites); latency time for all specific benign and malignant tumors; and incidence of malignancy precursors.” and
5. “Standardizing the experimental conditions for conducting experiments, parameter assessment, and data presentation.”

While expert bodies and regulatory agencies would agree with items 1, 3, and 5, as discussed below, items 2 and 4 are not consistent with the approach taken by national and international expert bodies (The U.S. National Toxicology Program [NTP], the Organization for Economic Cooperation and Development [OECD], the International Agency for Research on Cancer [IARC]) and regulatory authorities (the U.S. Environmental Protection Agency [EPA], the U.S. Food and Drug Administration [FDA], the European Food Safety Authority [EFSA], etc.).

The primary difference in design between the studies performed by the Ramazzini Foundation and the design endorsed by regulatory agencies and carcinogenicity testing groups world wide (EPA, FDA, OECD, NTP, Japanese Ministry of Health, Labour and Welfare, etc.) is in continuing the bioassays until the death of all of the animals (item 2 in Maltoni et al. (1999)’s five prerequisites). This procedure, together with the Ramazzini Foundation’s choice of housing several animals in one cage, raises potential problems associated with autolysis, cannibalism, and severe age-related pathological changes that may render interpretation of carcinogenic lesions very difficult, or impossible. This is discussed in greater detail in Section II.

B. Approach to Our Review

To perform this review, we examined publications by Ramazzini Foundation scientists, including several papers discussing their experimental philosophy and methods (Maltoni et al.

1999; Soffritti et al. 1999, 2002a), and many papers reporting the results of their studies (see References). In reviewing reports on individual chemicals, we focused in particular on four chemicals that share certain aspects of metabolism, and are all reported by the Ramazzini Foundation (but not by other researchers) to induce hemolymphoreticular neoplasms¹. These chemicals are methyl *tertiary*-butyl ether (MTBE), aspartame, methanol, and formaldehyde. MTBE metabolism yields formaldehyde, aspartame metabolism yields methanol, and methanol is itself partly metabolized to formaldehyde. The four chemicals thus share the capacity to create various degrees of exposure to formaldehyde.

An important limitation of this review is that we could rely only on the published journal articles describing the Ramazzini Foundation studies, and did not have access to the underlying data. Because the published articles include few details of the study results, this limits what we can conclude from them².

¹ Hemolymphoreticular neoplasms include all forms of leukemia and lymphoma – malignancies derived from blood cell precursors.

² The Ramazzini Foundation recently made available on its web site tables listing individual animal tumor pathology diagnoses and statistical analyses of tumor incidence for the studies of aspartame, methanol, MTBE, and *tertiary*-amyl methyl ether (TAME). No data were made available regarding non-tumor pathology, however, and no data were made available regarding any other chemicals.

II. ASSESSMENT OF INFORMATION RELATED TO THE QUALITY OF RAMAZZINI FOUNDATION STUDIES

A. What is Quality?

The term, “quality” is somewhat amorphous with a variety of meanings. One useful definition of quality is:

“Conformance to requirements or fitness for use. Quality can be defined through five principal approaches: (1) Transcendent quality is an ideal, a condition of excellence. (2) Product-based quality is based on a product attribute. (3) User-based quality is fitness for use. (4) Manufacturing-based quality is conformance to requirements. (5) Value-based quality is the degree of excellence at an acceptable price. Also, quality has two major components: (1) quality of conformance—quality is defined by the absence of defects, and (2) quality of design—quality is measured by the degree of customer satisfaction with a product’s characteristics and features.” (<http://src.ncsu.edu/public/DEFINITIONS/P%20-%20R.html>)

In the context of toxicology studies, quality is a combination of two distinct factors: (1) the appropriateness of the study design to answer the question of interest, and (2) the care with which a study was performed, including freedom from uncontrolled variables that might interfere with its conduct and interpretation. These factors determine the reliability and utility of the study results. In the case of studies intended for regulatory purposes these two primary determinants of quality are addressed in two ways. To address the study design question, regulatory agencies such as EPA

(http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/index.html), and FDA (<http://www.cfsan.fda.gov/~redbook/red-toca.html>), and

organizations such as the OECD

(http://www.oecd.org/document/40/0,2340,en_2649_34377_37051368_1_1_1_1,00.html) have developed guidelines for study protocols that should be followed in designing studies to address questions of toxicity and safety. Similarly, in the case of cancer bioassays, the US National Toxicology Program has developed procedures designed to optimize the detection of animal carcinogens. These procedures have evolved over the past 35 years to become “a highly complicated endeavor” (Bucher 2002)

In addition, to ensure that well designed studies are conducted appropriately, guidelines of good laboratory practice have been developed.

B. Good Laboratory Practice (GLP) Regulations

Good Laboratory Practice (GLP) requirements were first implemented in the mid-to-late 1970s in the United States in response to incidents of study misconduct and even fraud (Spindler and Seiler 2002; OECD 2007). GLP implementation expanded regulatory attempts to ensure the quality of the toxicology data supporting use of products such as drugs, pesticides, and industrial chemicals. The principles of GLP are important because application of these principles assure “reliable and traceable data”, although GLP requirements do not “influence the scientific aspects of the study planning and conduct” (Spindler and Seiler 2002). GLP requirements (Lilly *et al.* 1995; OECD 2007), whatever their origin or the industry targeted, stress the importance of resources (e.g., qualified personnel), protocols, standard operating procedures (SOPs), test substance and test system characterization, documentation, including raw data, final report and archives, and a quality assurance (QA) unit independent of the individuals performing the study.

Because toxicity studies, especially those involving long-term dosing, are logistically complex and yield enormous amounts of data, the opportunities for error are substantial. GLP requirements are designed to minimize error by ensuring that fully experienced scientists and technicians are involved in study conduct, and that all of the procedures used are fully documented. Highly detailed protocols are required, and any deviations from those protocols that are necessary during the conduct of the study need to be fully justified and documented. Records involving observations and measurements made during the conduct of a study are required to be complete in all respects. Animal health must be monitored, and instances of disease outbreaks unrelated to treatment must be recorded. These and many other aspects of study conduct and documentation are now regulatory requirements, throughout the world, for studies to be submitted to regulatory agencies to support product approvals (e.g., for drugs and pesticides). As a result, in developing their evaluations of risk, regulatory agencies now rely heavily on studies that have been conducted under GLP regulations. GLP compliance is required for all studies submitted to FDA (21 CFR 58), or to EPA for studies conducted in support of pesticide regulation (40 CFR 160) or for testing required to be conducted under Section 4 of the Toxic Substances Control Act (TSCA; 40 CFR 792).

GLP requirements are not mandatory for toxicology testing and research that is not intended to support product approvals. Thus, scientists in academic and other settings are not required to adhere to all of the GLP requirements (though they generally follow the general principles of GLP), and are free to publish their work in the scientific literature as long as it passes peer review (which does not generally focus on many of the quality and data recording issues that are central to GLPs). Regulatory agencies may choose to rely upon studies published in the peer-reviewed literature, even in the absence of GLP documentation, provided the studies are of high quality. The regulatory use of data for which GLP documentation is not available generally arises for substances for which there are no specific pre-market testing and approval

requirements. Where agencies rely on non-GLP data, they often ask study authors for additional details of the study that do not appear in the published version, and may reasonably refrain from using studies for which such additional data are not forthcoming.

C. Quality Issues Related to Ramazzini Foundation Studies

ENVIRON reviewed many journal articles authored by Ramazzini Foundation scientists (see References), relevant regulatory documents, and published reviews of the Ramazzini Foundation studies to determine whether there were indications that the Quality Assurance (QA) program of the Ramazzini Foundation follows GLP procedures equivalent to those of the OECD or other authoritative bodies. Because journal articles are in essence summaries of the study reports, the quality of the study design and results has to be inferred from available pertinent information, or the lack thereof. The aspartame study information (Belpoggi et al. 2006; Soffritti et al. 2005, 2006a; EFSA 2006) is the most recent and permits the most thorough evaluation of the quality of Ramazzini Foundation study designs. Moreover, the European Food Safety Agency (EFSA 2006) was able to acquire raw study data from the Ramazzini Foundation. Because the aspartame study was conducted more recently than the studies of the other three chemicals of particular interest (MTBE, methanol, and formaldehyde), it is possible that the quality of the study design and data from the aspartame study is comparable to or better than the quality of the study design and data of the earlier studies (Belpoggi *et al.* 1995, 1997; Soffritti et al. 1989, 1998, 2002b,c). In the absence of complete study data, of course, it is not possible to verify this supposition.

For all four of these substances, the Ramazzini Foundation authors stated that the studies followed good laboratory practices, but there was no specific GLP guideline reference cited, such as those of the OECD. Consequently, this lack of information raises the issue of how completely the Ramazzini Foundation scientists followed any specific GLP requirements. In its review of the Ramazzini Foundation study of aspartame, the European Food Safety Authority (EFSA 2006) indicated that there appeared to be no independent verification that the Ramazzini Foundation studies followed any specific GLP guidelines. In general, the information presented in the published Ramazzini Foundation articles indicated that at least some OECD GLP requirements were followed, such as the presence of a protocol and SOPs. Also, as noted in the Introduction, Maltoni et al. (1999) espoused the need for following “the rules of Good Laboratory Practice” to ensure high quality work. The precise nature of the procedures followed, however, is not described in any of the Ramazzini Foundation publications.

1. Test Design

Some reviewers of studies performed by the Ramazzini Foundation (Bucher 2002; EFSA 2006; Gradient 2006; Hardisty 2006³; McGregor 2006) indicated that problems with the study design and test system may have affected the quality or integrity of the data. In an unpublished review, Hardisty (2006) noted that “the study design is not considered the standard for carcinogenicity bioassay studies.” EFSA (2006) also indicated that the Ramazzini Foundation did not follow OECD guideline 451 (carcinogenicity) for the aspartame study.

There are several major differences between the Ramazzini Foundation carcinogenicity study design and that used by other laboratories: 1) The SD rats used have been isolated and inbred at the Ramazzini Foundation for decades, and are not specific pathogen free (SPF), which could make this particular colony susceptible to medical disorders and disease states not commonly seen in commercially outbred SD colonies; 2) animals are kept on study until they die, and are not sacrificed after a maximum of two years; and 3) the animals were housed with several animals in a single cage.

The health of the animals used in a toxicology study is very important for the validity of the study, particularly for long-term studies like cancer studies, because disease processes may enhance or obscure the development of toxic and carcinogenic effects (Semler et al. 1992; White 2001; Bucher 2002). This in turn may lead to a false positive (or false negative) result. The general health of the Ramazzini Foundation SD rat colony has been questioned by a number of reviewers (Bucher 2002; EFSA 2006; Gradient 2006; Hardisty 2006; McGregor 2006; McConnell, personal communication). Only in one of the aspartame study articles (Soffritti et al. 2006a), did the authors state that the “experiment was conducted according to Italian law regulating the use of animals for scientific purposes” and that the “health status of the animals was regularly checked by the veterinarians”. There were, however, no additional details given, such as whether the colony was healthy or not. EFSA (2006), which obtained access to the full study report and raw data for the aspartame study, not just the published data, reported a “high incidence of [background] infection seen in the [aspartame] study,” including pleuritis, hepatitis, pericarditis, meningitis, and pyelonephritis. In an unpublished review of the Ramazzini Foundation’s methanol study, Hardisty (2006) also noted that there was “high background incidence of chronic inflammatory changes in the lungs,” consistent with a chronic respiratory system infection.

Other study design issues that could have affected the quality or integrity of the data were caging and monitoring of study animals and that the animals are on study until death.

³ This is an unpublished manuscript, not a peer-reviewed journal article.

EFSA (2006) noted that for the aspartame study, the size of the cages may not have been sufficient for adult animals housed with several animals in each cage and may have contributed to the infection observed in animals. In addition, Hardisty (2006) and EFSA (2006) noted that autolytic changes (post mortem deterioration of the tissues) could compromise pathological diagnoses and sub-classification of neoplasms. That autolysis was observed raises the question whether the study animals were monitored regularly and frequently for moribundity or mortality (as would be required under OECD Guideline 451). Autolysis would develop in carcasses when they are not processed immediately after death. Autolysis may even begin in severely moribund animals prior to death (as in the case of gangrene). Because the animals were housed several to a cage, the presence of autolysis indicates that the carcasses were probably also cannibalized, which would result in incomplete pathological data for that cannibalized rat. In addition, because the animals were left on study until they died, age-related pathology may have interfered with the interpretation of the study data. The OECD Guideline 451 for carcinogenicity studies states:

“It is necessary that the duration of a carcinogenicity test comprise the majority of the normal life span of the animals to be used. It has been suggested that the duration of the study should be for the entire lifetime of all animals. However, a few animals may greatly exceed the average lifetime, and the duration of the study may be unnecessarily extended and complicate the conduct and evaluation of the study. Rather, a finite period covering the majority of the expected life span of the strain is preferred since the probability is high that, for the great majority of chemicals, induced tumours will occur within such an observation period.”

<http://lysander.sourceoecd.org/vl=1302165/cl=37/nw=1/rpsv/ij/oecdjournals/1607310x/v1n4/s32/p1>

Other expert bodies have also recommended a fixed duration of study rather than the open-ended “lifetime” duration used by the Ramazzini Foundation. The use of a 24-month study duration by the National Toxicology Program is well known (Bucher et al. 2002). Such a fixed study duration is also recommended by the World Health Organization (WHO 1978), who states:

“Some research workers have adopted a duration for carcinogenicity tests of 2 years in rats and 18 months in mice, while others have preferred an observation period extending over the entire life span of the animals. A long finite period – that is, not less than 2 years – is recommended in preference to the entire life span of the animals, for the following reasons: (a) induced tumours usually occur within this observation period; (b) “spontaneous” tumours appear with highest frequency late in life and their appearance may make it more difficult to evaluate the carcinogenicity of a compound, particularly if of low potency; (c) a few animals may far exceed the normal life span of the species and extend

unnecessarily the duration of the experiment; (d) tests are very expensive and any justifiable abbreviation means good economy” (references omitted).

Similarly, the International Agency for Research on Cancer (IARC 1986) stated:

For carcinogenicity testing, some investigators have advocated a duration of 24 months for rats and 18 months for mice and hamsters, while others have preferred an observation period extending over the entire life span of the animals. For economic reasons and confounding factors such as changes in background tumour incidences with age, the latter procedure is not very suitable for carcinogenicity screening.... Today, an acceptable duration would be of the order of 24 months for rats and mice, and 18 to 20 months for hamsters, unless there are appreciable losses from compound-related fatal tumours” (references omitted).

Another important difference in test design between that used by the Ramazzini Foundation and that used by most other scientists relates to how animals are assigned to individual dose groups. The standard procedure endorsed by international study guidelines and endorsed by the National Toxicology Program is to assign weanling animals randomly to different dose groups following stratification by weight, with very light or very heavy animals excluded from study to ensure that all dose groups are comparable (Haseman 1984; Gart et al. 1986; Bucher 2002;

http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Series/870-4200.pdf; <http://www.cfsan.fda.gov/~redbook/red-ivc6.html>; <http://massetto.sourceoecd.org/vl=4228374/cl=19/nw=1/rpsv/cgi-bin/fulltextew.pl?prpsv=/ij/oecdjournals/1607310x/v1n4/s32/p1.idx>). According to Bucher (2002), however, the Ramazzini Foundation assigns all animals in a litter to the same dose group and uses all animals, irrespective of body weight. This can introduce bias into the study if a particular litter of animals has a genetic predisposition to tumor development. If all such animals are in the same dose group, this may give the appearance of a treatment-related effect when none actually exists.

There is also some question about the sharing of control groups among different test substances by the Ramazzini Foundation. While they say they have conducted cancer studies on more than 200 substances, they report historical control data for only 2,265 males and 2,274 females, of which 1,934 males and 1,945 females are reportedly from studies with more than 100 rats/sex/dose (Soffritti et al. 2006a). In a recent deposition for a litigation case, Belpoggi (2007) lists 409 individual bioassays on 205 substances/agents that have been conducted by the Ramazzini Foundation, using a total of 151,507 animals. While a small fraction of these were mice and hamsters, the majority (more than 100,000) were rats. With less than 5,000 controls (total) accounted for by Soffritti et al (2006a), this corresponds to a ratio of dosed to control rats of more than 20 to 1. The majority of

published Ramazzini Foundation studies have less than 5 dose groups (including control). This suggests that either the Ramazzini Foundation has not made available the majority of its historical control data, or several studies shared the same control group. Confirmation of this (at least in a few cases) comes from the footnote to Table 1 in Maltoni et al. (1999), where it is noted that “(e)xperiments 3, 5, 7, 10 and 11, experiments 8 and 9, and experiments 12 and 13 have control groups in common....” Also, in Table IV-1 (below), shared control groups are indicated for Ramazzini Foundation studies of fluorocarbons FC11 and FC12, for two zeolites, and for toluene and xylene. Also, though not specifically identified in the published papers, the coincidence of identical control incidences of lymphomas/leukemias among both male (12/158) and female (1/149) offspring in the studies of vinyl chloride (Maltoni and Cotti 1988) vinylidene chloride (Cotti et al. 1988) and acrylonitrile (Maltoni et al. 1988c) strongly suggests that those three chemicals all shared the same control group.

2. Data Interpretation, Including Statistical Analyses and Methods for Control of Bias

The Ramazzini Foundation authors generally did not report any endpoints other than tumor incidences and did not mention the overall health of the rat colony in their published articles (Hardisty 2006; WHO 2002; cited in EFSA 2006). EFSA (2006), however, reported a “high incidence of [background] infection” and reported that non-neoplastic observations were recorded but other typical endpoints for a chronic study (e.g., OECD Guideline 451) were not reported.

In addition, the Ramazzini Foundation researchers did not justify combining tumor types for statistical analyses, especially those tumor types having different cellular origin (EFSA 2006; Hardisty 2006; WHO 2002, cited in EFSA 2006). Chemical carcinogens cause increases in different types of cancer in experimental animals. The tissues affected, and even the cell type within a tissue, are specific for a specific chemical (DeVita et al. 2005). Chemical carcinogens do not simply increase the incidence of all types of cancer (McConnell et al. 1986). Conversely, the specificity of chemical carcinogens is such that cells in different tissues that share the same developmental origin often respond similarly to a carcinogen. For example, a carcinogen that increases the incidence of tumors of the cells lining the urinary bladder often will also increase the incidence of tumors of cells lining other parts of the upper urinary tract, such as the transitional cells of the kidney and the ureter (the tube linking the kidney to the urinary bladder). Combining data on tumors in tissues that are not related by cellular developmental origin may give misleading results in assessing whether a chemical is a carcinogen.

The National Toxicology Program has identified tumor types/sites that are appropriate to group together for this purpose and those that are not (McConnell et al. 1986). Importantly, these NTP criteria include consideration of different types of leukemia and lymphomas, some of which are considered appropriate to combine, and some are not (McConnell et al. 1986). In the past, publications from the Ramazzini Foundation routinely combined all forms of hemolymphoreticular neoplasms, including types that NTP and other expert bodies consider inappropriate to combine. More recently, since the 1998 update of the MTBE study (Soffritti et al. 1998), Ramazzini Foundation publications have sometimes (e.g., Soffritti et al. 2002b, 2006a), but not always (e.g., Soffritti et al. 2002c, 2005) reported the incidence of specific types of hemolymphoreticular neoplasms in addition to the total combined incidence. They do not, however report combined incidences following the NTP guidance on combining such types of neoplasms. With the very recent release of individual animal data for a limited number of substances – representing about 1% of the cancer bioassays the Ramazzini Foundation has reportedly conducted (Soffritti et al. 2002a) – it is possible to perform independently the combination of appropriate tumor types.

The potential bias introduced by the non-random distribution of animals into treatment groups identified by Bucher (2002) is not addressed by the Ramazzini Foundation in any of its publications, however.

D. Conclusion

Part of the reasoning behind quality assurance programs and GLP compliance is to achieve consistent, reliable data that, if necessary, could generally be reproduced by or compared to the results from other laboratories. There are many factors that may have affected the quality and reliability of the data presented in the Ramazzini Foundation publications. These factors include:

- the unique test design, including lifetime duration, gang caging, and lack of randomization;
- incomplete data collection (e.g., not following OECD guideline 451);
- selective data reporting, such as not including non-neoplastic histopathology data, clinical observations, and background chronic pulmonary disease information; and
- lack of justification for some methods of data analysis, such as criteria for combining incidences of different tumor types.

Because of these factors, the results presented by the Ramazzini Foundation authors would be difficult to compare with data from other laboratories or reproduce in other laboratories, and it is difficult to judge the quality of the studies or the reliability of their findings.

III. CONSISTENCY WITH OTHER LABORATORIES

A. General Considerations

Issues have been raised related to the consistency of the results from the Ramazzini Foundation bioassays with those reported by other laboratories. One question is whether the differences in responses observed are due to the different protocol employed by the Ramazzini Foundation, or, as discussed previously, issues related to GLP. A comparison between the results of the Ramazzini Foundation and the National Toxicology Program (NTP) has been reported (Huff 2002). In addition, as part of the current analysis, a comparison of the Ramazzini Foundation results with results from bioassays available in the published literature for four selected compounds, MTBE, methanol, aspartame, and formaldehyde, was performed to evaluate the consistency of the Ramazzini Foundation results with those reported in other laboratories or institutions conducting chronic bioassays. The purpose of reviewing the Huff (2002) evaluation and comparing the Ramazzini Foundation results with those from other laboratories was to determine if, in general, there is consistency between the carcinogenic endpoints reported in rats by the Ramazzini Foundation to be associated with chemical exposure and those reported by other laboratories.

Huff (2002) compared the results of bioassays for the 14 chemicals that have been tested by both the Ramazzini Foundation and the NTP (Table III-1). The route of administration was the same across laboratories for 8 chemicals, while 6 chemicals were tested by different routes of exposure. In Huff's analysis, results were classified as "positive" if a chemical-related carcinogenic response was reported in one or more target organs, and "negative" if there was no evidence of carcinogenic activity related to chemical exposure. Site concordance was reported if there was a correlation of similar positive responses between laboratories or no evidence of carcinogenic responses from both laboratories. If there was no correlation of responses between laboratories, Huff (2002) reported no site concordance.

For those compounds (xylenes, vinylidene chloride, and toluene) for which there was a difference in classification as positive (showing a carcinogenic response of some type) or negative between NTP and the Ramazzini Foundation, protocol differences were noted by Huff (2002). In the case of xylenes, while classified as positive by Ramazzini Foundation, no tumor incidences were increased until after week 112 of the study, and increases in the incidence of hemolymphoreticular neoplasms were not observed until week 144 of the study. This is later than the standard study termination of NTP of approximately 104 weeks and Huff suggested that this may explain the differences in classification, though a tumor that killed an animal at 112 or even 144 weeks might well have been detectable microscopically at 104 weeks. In addition,

while increases in several tumor responses were reported by the Ramazzini Foundation, Huff (2002) noted that none of the increases was dose-related. Therefore, while classified by the Ramazzini Foundation as “positive,” this classification is questionable due to the lack of a dose-related increase in the response.

For vinylidene chloride, increases in total malignant tumors, an endpoint not typically considered by NTP, was part of the basis for a positive classification by the Ramazzini Foundation, with an increase in the incidence of leukemia also providing support. The incidence of leukemia was noted only in animals with exposure initiating *in utero* and continuing for two years after birth. In the breeding animals, only exposed for two years of their lifespan, no increase in the incidence of this endpoint was noted. Therefore, this difference in protocol, as well as the difference in tumor combinations, contributed to a difference in response between laboratories.

In considering more closely the results for those compounds classified as positive by both laboratories, site concordance for at least one target organ was reported by Huff (2002) for 8/11 compounds. These differences between results across laboratories do not appear to be related to route of exposure, since site concordance was observed for selected chemicals with the same or different exposure routes. For two of the eight compounds (chlorine and styrene), Huff (2002) noted that the association of the response with chemical exposure in the NTP study is questionable (Huff 2002). For styrene in particular, Huff (2002) reported a potential site concordance for lung tumors between the NTP and the Ramazzini Foundation; however, this appears to be an error. The lung tumors reported by NTP were observed in the mouse while the Ramazzini Foundation did not conduct a bioassay in mice for styrene. While the Ramazzini Foundation reported an increase in mammary gland tumors in rats exposed to styrene by inhalation, but not gavage, no treatment-related tumors were reported in rats by NTP. Therefore, this would decrease the number of bioassays with site concordance to seven. Overall, these results suggest that the consistency in response between these two laboratories is approximately 50%, with similar responses observed in only 7/14 studies conducted using in the same compound.

B. MTBE, Methanol, Aspartame, and Formaldehyde

We conducted an additional analysis to compare results from Ramazzini Foundation chronic bioassays for a selected set of compounds to those results reported for laboratories other than NTP. This analysis focused on MTBE, methanol, aspartame, and formaldehyde. Tables III-2 through III-5 show the tumor incidences reported in bioassays in rats for these compounds. The entries in each table for studies conducted by Sofritti et al. or Belpoggi et al. are the results from bioassays conducted by the Ramazzini Foundation.

One observation for all four compounds is the identification in the Ramazzini Foundation studies of an increase in the incidence of hemolymphoreticular leukemia and lymphomas. The incidence of these tumors is not reported in any other chronic bioassay for these compounds. It is also important to note that there is no evidence of site concordance in the available bioassays for these four compounds, with the possible exception of one target organ site in the MTBE bioassays (Table III-2). Leydig cell tumors are reported by Ramazzini Foundation following oral exposure, as well as in the inhalation bioassay conducted by Chun et al. (1992; later published by Bird et al. (1997)). The validity of the identification of Leydig cell tumors as an endpoint in the Ramazzini Foundation study, however, is questionable since the apparent increase in tumor incidence with increasing dose was accompanied by increasing survival in the MTBE-dosed animals (two-year survival was just 8.3% in control males, 16.7% in low-dose males, and 33% in high-dose males). Since Leydig cell tumors are common age-related tumors in rats, a greater incidence in older animals is expected, and the apparent increase is not statistically significant when appropriate time-to-tumor statistical analysis methods are used (Goodman et al. 2007; Beck 2007). The Chun et al. (1992) study used Fischer 344 rats, which have an extremely high and variable spontaneous incidence of Leydig cell tumors; indeed the incidence in the control group (32/50) was unusually “low” for this strain of rats, and the incidence in all dose groups was within the historical control range at the laboratory where the study was performed of 86% to 91% (Chun et al. 1992), suggesting that the apparent dose-related increase in incidence was spurious.

There was no observation of increased incidence of leukemia/lymphoma in studies of these chemicals other than the Ramazzini Foundation studies. In addition, renal tubular cell tumors were noted by Chun et al. (1992; later published by Bird et al. (1997)) but not in the MTBE study conducted by the Ramazzini Foundation. No examples of site concordance in carcinogenic response are available for methanol, aspartame, or formaldehyde. In the case of aspartame, both the European Food Safety Agency (EFSA 2006) and the U.S. Food and Drug Administration (FDA 2007) have concluded that the Ramazzini Foundation study of aspartame does not provide evidence of carcinogenicity. FDA (2007) notes that “Based on our review, pathological changes were incidental and appeared spontaneously in the study animals, and none of the histopathological changes reported appear to be related to treatment with aspartame.” A slightly different scattering of spontaneous tumors was recently reported by Soffritti et al. (2007) in the second Ramazzini Foundation carcinogenicity study of aspartame (See Table III-4). Both Ramazzini Foundation studies reported an increase in lymphoma/leukemia, but as noted in Section IV, there is considerable uncertainty regarding the validity of those findings. As noted in Table III-4, the first study reported an increase in kidney tumors, but no such finding was reported in the second study, while the increase in mammary carcinoma reported in the second study (Soffritti et al. 2007) is not mentioned in the reports of the first study (Soffritti et al. 2006a;

Belpoggi et al. 2006a). The individual animal data for the first study that was recently released on the Ramazzini Foundation website reports fluctuations in mammary adenocarcinoma⁴ incidence with the various doses of aspartame administered, but no indication of a dose-related response. The individual animal data released by the Ramazzini Foundation for MTBE and methanol reports that only 28/60 female MTBE controls and only 54/100 female methanol controls had their mammary glands examined microscopically (suggesting widespread post-mortem autolysis or cannibalism). Among those that did, the incidence of mammary adenocarcinoma was 4/28 in the MTBE control females, and 8/54 in the methanol control females. These incidences are comparable to the highest fluctuation in incidence in any of the aspartame groups, strongly suggesting that the interpretation of the mammary tumor response by Soffritti et al. (2007) is incorrect.

C. Conclusions

There does not appear to be consistency between the carcinogenic responses reported by the Ramazzini Foundation and those for other laboratories conducting carcinogenicity research. For the limited number of compounds evaluated by both the Ramazzini Foundation and the NTP, target organ concordance is only observed in about 50% of the studies. In addition, in evaluating a selected group of compounds (MTBE, methanol, aspartame, and formaldehyde), target site concordance was not observed. The causes of these differences are not known, although the differences in protocol design, study quality (see below regarding the role of test animal infection), and interpretations of tumor pathology are probably the major contributors.

⁴ The individual animal data report “adenocarcinoma” but do not mention mammary “carcinoma” as the tumors are called by Soffritti et al. (2007). Mammary adenocarcinoma are very common spontaneous tumors in Sprague-Dawley rats (Giknis and Clifford 2001, 2004)

Table III-1.

Chemicals Tested in Both the Ramazzini Foundation and NTP Programs (Huff 2002)

Chemical	Route of Exposure	
	Ramazzini Foundation	NTP
Acrylonitrile	inhalation, gavage	Inhalation
Benzene	inhalation, gavage	Gavage
Chlorine	drinking water	drinking water
Diesel fuel	gavage	Skin
Ethylbenzene	gavage	inhalation
Methylene chloride	inhalation, gavage	inhalation
Propylene	inhalation	inhalation
Styrene	inhalation, gavage, injection	gavage
Styrene oxide	gavage	gavage
Toluene	gavage	inhalation
Trichloroethylene	inhalation	gavage
Trichlorofluoromethane	inhalation	gavage
Vinylidene chloride	inhalation	gavage
Xylenes	gavage	gavage

Table III-2. MTBE

Reference	Route	Dosing Duration	Strain	Dose	Leydig Cell Adenoma	Lymphoma/Leukemia	Renal Tubular Cell Tumors
Belpoggi <i>et al.</i> 1995; 1998 ^a	gavage in olive oil	4 d/wk, 104 weeks	SD rats	0, 250, 1000 mg/kg BW	2/60 ^b , 2/60, 11/60	2/60, 6/60, 12/60 (females) ^c	
Chun <i>et al.</i> 1992; Bird <i>et al.</i> 1997 ^d	inhalation	6 hrs/d, 5 d/wk, 24 months	Fischer 344 rats	0, 400, 3000, 8000 ppm (M: 0, 40, 310, 830 mg/kg BW; F: 0, 60, 450, 1190 mg/kg BW)	32/50, 35/50, 41/50, 47/50		1/50, 0/50, 8/50, 3/50 ^e (males)

^a Reevaluation

^b One control animal had autolysis of the testes; hence the presence of Leydig cell tumors could not be evaluated. The incidences reported here are those reported by Belpoggi *et al.* (1995); Belpoggi *et al.* (1998) reports the incidences as 3/60, 5/60, and 11/60 in the control, low, and high dose groups, respectively.

^c The incidences reported here are those reported by Belpoggi *et al.* (1995); Belpoggi *et al.* (1998) reports the incidences as 2/60, 7/60, and 12/60.

^d Pancreatic islet cell adenomas/carcinomas, mammary adenomas, fibroadenoma or carcinomas, large granular lymphocytic leukemia noted in McGregor, but not treatment related.

^e Increased mortality in two highest concentration groups

Table III-3. Methanol

Reference	Route	Dosing Duration	Strain	Dose	Ear Duct Carcinoma	Lymphoma/Leukemia	Comments
Soffritti <i>et al.</i> 2002b	drinking water	104 weeks	SD rats	M: 0, 25, 250, 1000 mg/kg BW F: 0, 29, 285, 1140 mg/kg BW	M: 9/100, 13/100, 17/100, 24/100 F: 9/100, 8/100, 16/100, 19/100	M: 28/100, 35/100, 36/1000, 40/200 F: 13/100, 24/100, 24/100, 28/100	
New Energy Development Organization (NEDO) 1987 ^a	inhalation	19.5 hrs/d, 5 d/week, 104 weeks	Fischer 344 rats	0, 10, 100, 1000 ppm (M: 0, 2, 22, 225 mg/kg BW; F: 0, 4, 34, 340 mg/kg BW)			No dose-related increases in any tumor type

^a Renal tubule adenoma/carcinoma, Leydig-cell adenoma, mononuclear cell leukemia, mammary fibroadenoma, pancreatic islet adenoma/carcinoma noted in McGregor, but not treatment related.

Table III-4. Aspartame (SD rats dosed in diet)

Reference	Dosing Duration	Dose	Brain Tumors	Lymphoma/Leukemia	Preneoplastic/Neoplastic Lesions of the Renal Pelvis and Ureter	Mammary Carcinoma	Comments
Soffritti <i>et al.</i> 2006a; Belpoggi <i>et al.</i> 2006a	until natural death	0, 80, 400, 2,000, 10,000, 50,000, 1000,000 ppm in diet (~0, 4, 20, 100, 500, 2,500, 5,000 mg/kg BW/day)		F: 13/150, 22/150, 30/150, 28/150, 19/100, 25/100, 25/100	F: 2/150, 6/150, 9/150, 10/150, 10/100, 10/100, 15/100	Not reported	
Soffritti <i>et al.</i> 2007	Prenatal until natural death	0, 400, 2000 ppm in diet (~0, 20, 100 mg/kg/day)		F: 12/95, 12/70, 22/70	Not reported	F: 5/95, 5/70, 11/70 M: 0/95, 0/95, 2/95	
FDA 1981	104 wks	0, 1, 2, 4, or 6-8 g/kg BW/day					No dose-related increases in any tumor type
FDA 1981	0, 2, or 4 g/kg BW/day through mother's diet both <i>in utero</i> and lactation, followed by 104 wks in diet	0, 10, 100, 1000 ppm (M: 0, 2, 22, 225 mg/kg BW/day; F: 0, 4, 34, 340 mg/kg BW/day)	M: 3/60, 2/40, 1/40 F: 1/60, 1/40, 1/40				No dose-related increases in any tumor type

Table III-5. Formaldehyde

Reference	Route	Dosing Duration	Strain	Dose	Lymphoma/Leukemia	Gastrointestinal Tumors	Malignant Nasal Tumors	Comments
Soffritti <i>et al.</i> 2002c	Drinking water	104 weeks	SD	0, 10, 50, 100, 500, 1000, or 1500 mg/L	M: 8/100, 4/50, 10/50, 13/50, 12/50, 11/50, 23/50 F: 7/100, 5/50, 7/50, 8/50, 7/50, 11/50, 10/50			Soffritti <i>et al.</i> 1989 and 2002c appear to be reporting the results of the same study. The reason for the differences in reported incidence of lymphoma/leukemia is unknown. There are also unexplained discrepancies between the two publications for g.i. tumor incidence.
Soffritti <i>et al.</i> 1989	Drinking water	104 weeks	SD	0, 10, 50, 100, 500, 1000, or 1500 mg/L	M: 4/100, 2/50, 10/50, 10/50, 16/50, 12/50, 22/50 F: 3/50, 4/50, 8/50, 8/50, 8/50, 14/50, 14/50	M: 0/100, 4/50, 0/50, 0/50, 0/50, 2/50, 10/50 F: 0/100, 2/50, 4/50, 0/50, 0/50, 2/50, 6/50		All animals in the 500 ppm group died prior to 24 months and had degenerative lesions of the forestomach. No tumors were reported.
Tobe <i>et al.</i> 1989	Drinking water	24 months	Wistar	0, 200, 1000, or 5000 ppm				Severe damage to the gastric mucosa, but did not result in gastric tumors or tumors at other sites.
Til <i>et al.</i> 1989	Drinking water	24 months	Wistar	0, 20, 260, or 1900 mg/L				
Kamata <i>et al.</i> 1997	inhalation	6 hrs/day, 5 days/week, 28 months	Fischer 344	0, 0.3, 2, or 15 ppm			0/32, 0/32, 0/32, 13/32	
Kerns <i>et al.</i> 1983	inhalation	6 hrs/day, 5 days/week, 24 months	Fischer 344	0, 2, 5.6, or 14.3 ppm			0/232, 0/236, 2/235, 106/232	
Monticello <i>et al.</i> 1996	inhalation	6 hrs/day, 5 days/week, 24 months	Fischer 344	0, 0.7, 2, 6, 10, 14 ppm			0/90, 0/90, 0/96, 1/90, 20/90, 69/147	

IV. VARIABILITY IN LYMPHOMA/LEUKEMIA RATES

A. Introduction

The interpretation of a cancer bioassay requires an understanding of the response of the test system in the absence of exposure to the substance under test. Of primary importance in this regard is a control group of animals carefully matched to the experimental groups in every respect other than the chemical exposure. The control and treated groups should be equivalent in terms of strain, source, age, body weight, diet, water supply, and housing. Such a matched control group provides the primary point of comparison to assess whether the treatment caused a significant carcinogenic effect. Also of importance, however, is an understanding of the range of responses that may occur in similar groups of control animals. Such historical controls can provide insight into the biological significance of any response that is seen in a particular experiment, particularly for tumor types that show a high and variable spontaneous incidence. With such tumors, a statistically “significant” response may be seen if the matched control group in an experiment is at the lower end of the range of spontaneous incidence for that tumor type in that strain at that laboratory. With a full understanding of the range of historical controls, it may be possible to infer that such a response does not represent a biologically significant response, but may be a result of random variability, or poor matching of control and treated groups.

B. Historical Controls

One tumor endpoint that the Ramazzini Foundation researchers highlight as being increased in incidence in their studies of MTBE, methanol, aspartame, and formaldehyde is “lymphomas and leukemias.” With MTBE, methanol, and aspartame, most of the increase in the treated animals was in what were described as “lymphoblastic lymphoma” or “lymphoimmunoblastic lymphoma” (Belpoggi et al. 1998; Soffritti et al. 2002a; Soffritti et al. 2006). With formaldehyde, the increase was largely in “lymphoblastic leukemias and lymphosarcomas” (Soffritti et al. 1989). It is unclear whether these different descriptions reflect real differences in pathology, or simply reflect an evolution in pathological nomenclature used by scientists at the Ramazzini Foundation.

Because of this confusion regarding pathological nomenclature, and because the published Ramazzini Foundation studies do not consistently report the incidence of different subtypes of lymphomas and leukemias, this discussion is largely limited to the combination of all “lymphomas and leukemias,” which is what is reported in most Ramazzini Foundation studies.

Table IV-1 presents a summary of the incidence of “lymphomas and leukemias” in 3,062 male and 3,241 female Sprague-Dawley rats that formed the control groups reported in Ramazzini Foundation studies published between 1980 and 2006. As indicated there, the overall

average incidence in these studies was 12.2% in males and 7.0% in females, but the range was broad, from 0% (0/90) to 31.8% (35/110) in males, and from 0% (0/50) to 23.3% (14/60) in females. Some information on historical control rates is also presented in Soffritti et al. (2006a). There, a substantially higher incidence is presented. Soffritti et al (2006a) state that among 2,265 untreated males and 2,274 untreated females, the average incidence of “lymphomas/leukemias” was 20.6% (range, 8.0 – 30.9%) in males, and 13.3% (range, 4.0 – 25.0%) in females. The reason for this discrepancy is unclear, but Soffritti et al. (2006a) appear to have omitted the several control groups identified in Table IV-1 in which no lymphomas or leukemias were reported in the original publications.

Table IV-1 “Lymphomas and Leukemias” in Control Groups of Sprague-Dawley Rats in Ramazzini Foundation Studies*					
Chemical	Control Male		Control Female		Reference
	Incidence	%	Incidence	%	
MTBE	10/60	16.67%	2/60	3.3%	Belpoggi et al. 1995, 1997; Soffritti et al. 1998
Methanol	28/100	28.00%	13/100	13.00%	Soffritti et al. 2002a
Aspartame	31/150	20.67%	13/150	8.67%	Soffritti et al. 2005, 2006a; Belpoggi et al. 2006a
Formaldehyde	8/100	8.00%	7/100	7.00%	Soffritti et al. 1989, 2002b
Ethanol breeders	35/110	31.82%	17/110	15.45%	Soffritti et al. 2002a
Ethanol offspring	8/49	16.33%	39/277	14.08%	Soffritti et al. 2002a
Coca-Cola breeders	51/235	13.19%	11/55	20.00%	Belpoggi et al. 2006b
Coca-Cola offspring	62/291	21.31%	39/277	14.08%	Belpoggi et al. 2006b
Acetaldehyde	6/50	12.00%	2/50	4.00%	Soffritti et al. 2002b
Mancozeb	16/75	21.3%	11/75	14.67%	Belpoggi et al. 2002a
TAME & DIPE	17/100	17%	7/100	7%	Belpoggi et al. 2002b
ETBE	3/60	5%	3/60	5%	Maltoni et al. 1999
Vinyl acetate breeders	0/14	0.00%	8/37	21.62%	Maltoni et al. 1997a; Minardi et al. 2002
Vinyl acetate offspring	13/107	12.15%	11/99	11.11%	Maltoni et al. 1997a; Minardi et al. 2002
Vinyl chloride breeders			2/60	3.33%	Maltoni & Cotti 1988
Vinyl chloride offspring	12/158	7.59%	1/149	0.67%	Maltoni & Cotti 1988
Vinylidene chloride breeders			2/60	3.33%	Cotti et al. 1988
Vinylidene chloride offspring	12/158	7.59%	1/149	0.67%	Cotti et al. 1988

Table IV-1
“Lymphomas and Leukemias” in Control Groups of Sprague-Dawley Rats
in Ramazzini Foundation Studies*

	Control Male		Control Female		
Styrene (inhal)	3/60	5.00%	3/60	5.00%	Conti et al. 1988
Styrene (gavage)	0/40	0.00%	1/40	2.50%	Conti et al. 1988
Styrene (ip)	3/40	7.50%	0/40	0.00%	Conti et al. 1988
Styrene (sc)	1/40	2.50%	1/40	2.50%	Conti et al. 1988
Styrene oxide (gavage)	2/40	5.00%	1/40	2.50%	Conti et al. 1988
p-Methylstyrene	2/30	6.67%	2/30	6.67%	Conti et al. 1988
p-Methylstyrene	4/60	6.67%	14/60	23.33%	Conti et al. 1988
Propylene (inhal)	2/120	1.67%	4/120	3.33%	Ciliberti et al. 1988
FC11 & FC12	9/150	6.00%	8/150	5.33%	Maltoni et al. 1988
FC22	5/60	8.33%	1/60	1.67%	Maltoni et al. 1988
Trichloroethylene (8 wk)	17/90	18.89%	8/90	8.89%	Maltoni et al. 1988b
Trichloroethylene (104 wk)	9/135	6.67%	7/145	4.83%	Maltoni et al. 1988b
Acrylonitrile (BT 203)	4/75	5.3%	3/75	4%	Maltoni et al. 1988c
Acrylonitrile (BT 201)	0/30	0%	0/30	0%	Maltoni et al. 1988c
Acrylonitrile (breeders)			2/60	3.3%	Maltoni et al. 1988c
Acrylonitrile (offspring)	12/158	7.6%	1/149	0.7%	Maltoni et al. 1988c
Methylene chloride (olive oil)	3/50	6%	1/50	2%	Maltoni et al. 1988d
Methylene chloride (none)	2/20	10%	0/26	0%	Maltoni et al. 1988d
Zeolites (ip)	3/20	15.00%	2/20	10.00%	Maltoni & Minardi 1988
Ethylene dichloride	0/90	0.00%	3/90	3.33%	Maltoni et al. 1980
Benzene (BT 901)	0/30	0.00%	1/30	3.33%	Maltoni et al. 1989
Benzene (BT 902)	3/50	6.00%	1/50	2.00%	Maltoni et al. 1989
Chlorine	4/50	8.00%	0/50	0.00%	Soffritti et al. 1997
Toluene & Xylene	5/50	10.00%	3/50	6.00%	Maltoni et al. 1997b
Toluene & Xylene	3/50	6.00%	1/50	2.00%	Maltoni et al. 1997b
Tamoxifen	14/100	14.00%	9/100	9.00%	Maltoni et al. 1997c
Tamoxifen			13/150	8.67%	Maltoni et al. 1997c
Tamoxifen			12/139	8.63%	Maltoni et al. 1997c
Weighted Mean:		12.2%		7.4%	

* Data are reported only for published studies in which data for lymphoma/leukemia were reported. Some (e.g., the Soffritti et al. (2006b) study of sodium arsenite) did not report lymphoma/leukemia incidence. TAME = *tert*-amyl methyl ether; DIPE = di-isopropyl ether; ETBE = ethyl-*tert*-butyl ether

It is also worth noting that the range of incidence in control females reported by Soffritti et al. (2006a) does not include the incidence (3.3%) found in the female control group in the MTBE

study (Belpoggi et al. 1995, 1997; Soffritti et al. 1998), raising additional questions regarding the validity of that study. The control range does, however, include the entire range of incidence seen in the treated groups in both the MTBE and aspartame studies, raising questions as to whether the apparent increases in incidence for these chemicals were truly treatment-related.

C. Inconsistent Dose-Response Relationships

In addition to variability in lymphoma and leukemia incidence in control groups in different studies, there is a lack of consistency in dose response relationships in males and females for the four chemicals of primary interest here. This is summarized in Table IV-2 which lists tumor incidence and percent animals with tumors for the Ramazzini Foundation studies of MTBE, methanol, aspartame, and formaldehyde. As shown there, there was an apparent dose-related increase in incidence of lymphomas and leukemias in female, but not in male rats receiving MTBE or aspartame.

In the case of MTBE, the apparent dose-related increase in lymphomas and leukemias in females was mirrored by an apparent dose-related *decrease* in males. In both sexes and at all dose levels the incidence was within the range of historical controls (Figure IV-1), with the female MTBE controls at the extreme low end of the historical control range, strongly suggesting that there was no causal relationship between MTBE dosing and the observed lymphomas and leukemias.

With methanol and formaldehyde, there appeared to be an increase in both sexes, with perhaps a slightly greater effect in males than females. This inconsistency argues against a common mechanism of action involving metabolic generation of methanol and formaldehyde from MTBE and aspartame as Soffritti et al (2006a) have proposed.

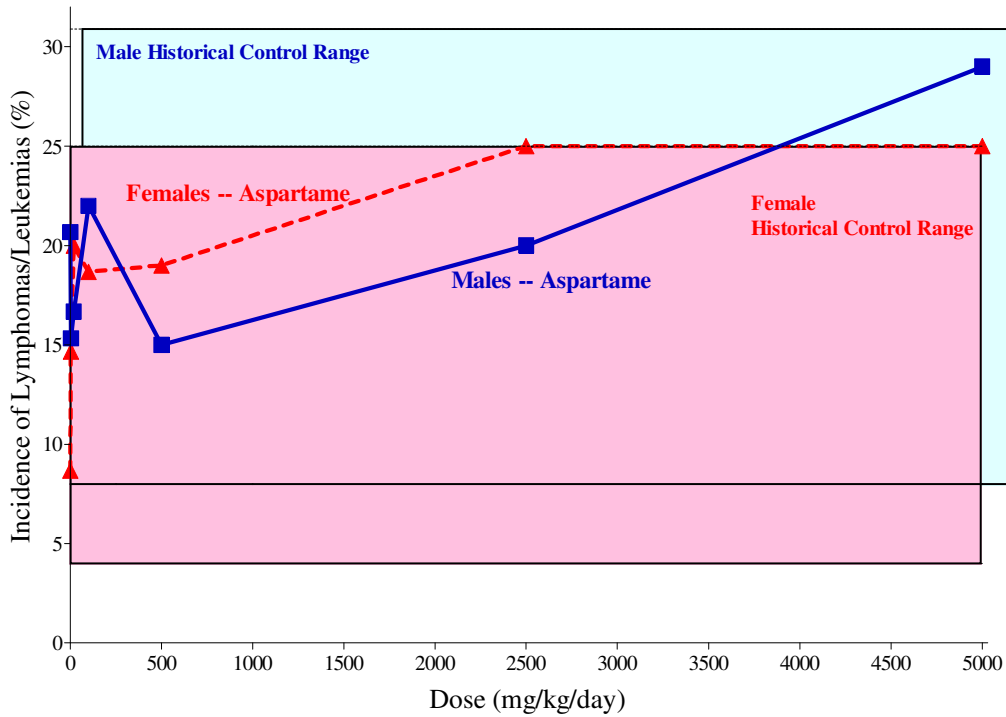
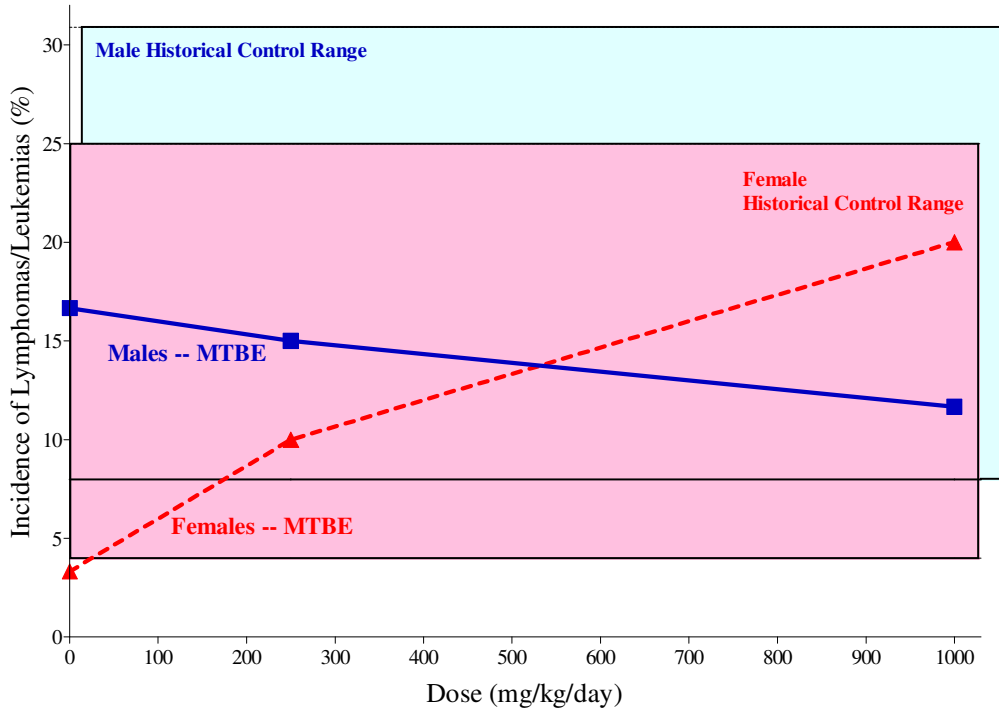
D. Conclusions

Wide variations in the incidence of neoplasms in control groups in different studies, and a lack of consistency in dose-response relationships for chemicals that are proposed to have a common mechanism of action add to concerns regarding the validity of the reported results.

Table IV-2
"Lymphomas/Leukemias" in Sprague-Dawley Rats in Ramazzini Foundation Studies of
MTBE, Methanol, Aspartame, and Formaldehyde

		MTBE		Methanol		Aspartame		Formaldehyde	
		Male	Female	Male	Female	Male	Female	Male	Female
Control	Incidence	10/60	2/60	28/100	13/100	31/150	13/150	8/100	7/100
	%	16.67%	3.33%	28.00%	13.00%	20.67%	8.67%	8.00%	7.00%
Dose 1	Incidence	9/60	6/60	35/100	24/100	23/150	22/150	4/50	5/50
	%	15%	10%	35.00%	24.00%	15.33%	14.67%	8.00%	10.00%
Dose 2	Incidence	7/60	12/60	36/100	24/100	25/150	30/150	10/50	7/50
	%	11.67%	20%	36.00%	24.00%	16.67%	20.00%	20.00%	14.00%
Dose 3	Incidence			40/100	28/100	33/150	28/150	13/50	8/50
	%			40.00%	28.00%	22.00%	18.67%	26.00%	16.00%
Dose 4	Incidence					15/100	19/100	12/50	7/50
	%					15.00%	19.00%	24.00%	14.00%
Dose 5	Incidence					20/100	25/100	11/50	11/50
	%					20.00%	25.00%	22.00%	22.00%
Dose 6	Incidence					29/100	25/100	23/50	10/50
	%					29.00%	25.00%	46.00%	20.00%

Figure IV-1
Comparison of Incidence of Lymphomas/Leukemias in Ramazzini
Foundation Studies of MTBE and Aspartame with Ramazzini Foundation
Historical Controls



V. POTENTIAL ROLE OF RESPIRATORY INFECTION IN DEVELOPMENT OF LYMPHOMA/LEUKEMIA

Another aspect of the lymphomas and leukemias in rats dosed with MTBE, methanol, and aspartame is their location (data on location of lymphomas and leukemias in the formaldehyde study are not present in the published reports or on the Ramazzini Foundation website).

Lymphomas and leukemias are most commonly found in the blood (leukemia), or in the spleen or lymph nodes (lymphomas) (IARC 1993). In the Ramazzini Foundation studies of MTBE, methanol, and aspartame, however, the increase in “haemolymphoreticular neoplasias” was preferentially associated with the lung (Belpoggi et al. 1998; Soffritti et al. 2006a; Ramazzini Foundation website).

With MTBE, 11 of 12 high-dose female rats with “haemolymphoreticular neoplasias” had such tumors located in the lung (though not all exclusively in the lung), as did 5 of 7 low-dose females, but zero of 2 control females (Belpoggi et al. 1998). Note, the individual animal tumor pathology tables made available recently for download from the Ramazzini Foundation website identifies one of these two female controls with hemolymphoreticular neoplasia as having a lymphoimmunoblastic lymphoma in the lung. This discrepancy between the individual animal tumor pathology tables and the published report of Belpoggi et al. (1998) is not explained, but adds an additional note of caution in relying on these data. These web-site data also show that of the males in the MTBE study with some form of hemolymphoreticular neoplasia, 6/7 high-dose males, 8/9 low-dose males, and 9/10 control males had them diagnosed in the lung.

With aspartame also, the lung was identified as one of the major locations of these tumors, though the specific breakdown of locations was not identified in the published reports (Soffritti et al. 2005, 2006a; Belpoggi et al. 2006a). The individual animal tumor pathology tables made available recently for download from the Ramazzini Foundation website identifies this breakdown. This is summarized in Table V-1. Overall, of 338 animals in the aspartame study diagnosed with some form of hemolymphoreticular neoplasia, 243 (72%) had them diagnosed in the lung, and 149 of 164 (91%) of animals diagnosed with lymphoimmunoblastic lymphoma had them diagnosed in the lung. While some of these may represent metastases, it is unusual to see so many lung metastases.

A similar pattern is seen in the recently released individual animal tumor pathology tables for methanol from the Ramazzini Foundation. Overall, of 172 animals diagnosed with lymphoimmunoblastic lymphoma, 156 (91%) had them diagnosed in the lung.

Table V-1				
Animals with "Lymphomas/Leukemias" Diagnosed in the Lung in the Ramazzini Foundation Study of Aspartame				
Dietary Concentration (ppm)	Males		Females	
	Total	Lung	Total	Lung
0	31	23	13	7
80	23	21	22	12
400	25	23	30	17
2,000	33	28	28	15
10,000	15	12	19	9
50,000	20	15	25	17
100,000	29	27	25	17

As reported by EFSA (2006) lung-associated lymphoreticular tumors of this type may occur as a consequence of severe chronic respiratory disease (Innes *et al.* 1967, Nelson 1967, Swaen and van Heerde 1973). EFSA noted that their evaluation of the non-tumor pathological findings in the aspartame study indicated a very high rate of infections in treated and control groups that is “unusual for toxicology studies according to current standards.” Evidence for infections included high incidences of brain abscesses, pyelonephritis, pleuritis, bronchopneumonia, peritonitis, liver abscesses, pericarditis, and meningitis.

Nelson (1967) showed that the elimination of such chronic respiratory disease from rats reduced the incidence of lymphosarcomas almost to zero. Similarly, Paget and Lemon (1965) reported a 3.2% incidence of lymphosarcomas in conventional Wistar-derived rats, but no such tumors in specific pathogen-free (SPF) animals. Likewise, Sinkeldam et al. (1991) found a high incidence of lymphoreticular tumors of the peribronchial tissue in high-dose male Wistar rats treated with acesulfame-K, but when this chemical was retested in the same rat strain, but now cleaned of mycoplasma infection and bred under specific-pathogen-free (SPF) conditions, chronic respiratory disease was absent and no lymphoreticular tumors were seen. Thus, the tumors found in the first experiment appeared to be unrelated to treatment with acesulfame-K, but were associated with the occurrence of peribronchial and perivascular lymphoid accumulations in response to chronic inflammation of the lungs (Feron et al. 1990).

Hyperplasia of bronchus-associated lymphoid tissue is characteristic of murine respiratory mycoplasmosis caused by *Mycoplasma pulmonis* (an organism that preferentially colonizes the luminal surface of the respiratory epithelium in rats); it is documented that mycoplasmas produce lymphokine-like substances that are mitogenic for B and T lymphocytes *in vitro* (Naot et al.

1979; Davis et al. 1980; Lindsey et al. 1985; Comparative Pathology Laboratory Disease Data Sheets, University of California (at Davis), <http://ccm.ucdavis.edu/CPL/index1.htm#MYCO>). Such a chronic mitogenic stimulus likely contributes to the development of these neoplasms.

As EFSA (2006) concluded, “[t]he most plausible explanation of the findings in this study with respect to lymphomas/leukaemias is that they have developed in a population of rats suffering from chronic respiratory disease. The slight increase in incidence in rats fed aspartame is considered to be an incidental finding and should therefore be dismissed.”

An alternative hypothesis that is worthy of evaluation has been proposed by Dr. Ernest E. McConnell, former Director of the Toxicology and Research Program at the National Toxicology Program. Early in his career, before the routine use of specific pathogen free (SPF) animals and barrier facilities in testing laboratories, mycoplasma infections occurred occasionally in rodent colonies, and the standard response to such an infection (at least in the US) was to immediately destroy all of the animals in the facility and thoroughly decontaminate it because many of the results obtained with such infected animals were considered uninterpretable. In the past 20 or 30 years, such infections have been essentially eliminated in the U.S., and younger pathologists may be unfamiliar with the characteristics of lesions associated with such infections. He has suggested (personal communication) that some of the lesions diagnosed by the Ramazzini Foundation scientists as lymphoma may be inflammatory lesions, not neoplasms, that develop in response to mycoplasma infection. Indeed, when examining the figures labeled by Belpoggi et al. (1999) as representing “immunoblastic lymphoma”, Dr. McConnell questioned these diagnoses because of the apparent heterogeneity of the cells composing the lesions, which he considered to represent inflammatory lesions, not neoplasms. Confirmation of these diagnoses would require examination of the original slides, not just the low-resolution black-and-white micrographs in the published paper. Dr. McConnell also suggested that it may be possible to confirm the presence of mycoplasma infection if paraffin-embedded tissue is still available from these studies using polymer chain reaction (PCR) techniques to identify the DNA “fingerprint” of mycoplasma.

Conclusions

Because of the incomplete reporting of data and the absence of truly independent pathology review, the role of infection in the reported incidence of lymphomas and leukemias, and indeed the accuracy of the diagnoses, cannot currently be determined. Sufficient questions have been raised, however, regarding the nature and etiology of the lesions in question to merit caution in using these data for any regulatory purpose without a comprehensive peer review of the studies, including the histopathology.

VI. STATISTICAL ISSUES

For the majority of the published Ramazzini Foundation bioassays, limited information is available on the statistical methods employed in analyzing results of their chronic bioassays. In early studies, no statistical methods were reported (Soffritti et al. 1989) or tests noted were limited to prevalence tests, with different methods applied depending on whether the tumor was assumed to be lethal or nonlethal (Belpoggi et al. 1995). In later published Ramazzini Foundation reports, statistical tests were noted to include the χ^2 test to evaluate differences in tumor incidence between treated and control groups, and the Cochran Armitage test to evaluate for dose-related trend in response (Soffritti et al. 2002a). It is only in recent bioassays conducted by the Ramazzini Foundation (Soffritti et al. 2006), that the statistical methods applied included tests, such as the Poly-K, to incorporate information on survival.

In the NTP standard study protocol (NTP 2007), which is a chronic study design that has been evolving since the 1970s, statistical significance is a critical consideration (Bucher 2002). Statistical significance, as well as biological significance is also considered, with cases of non-significant increases in tumors determined to be biologically relevant, as well as statistically significant increases determined to be not relevant to human health. The standard NTP statistical protocol includes analyses for dose-related effects, incidence rates, and quantal-response methods (e.g. Poly-K) that consider not only treatment-related effects, but also the impact of survival on the observed responses. In contrast, the standard methods reported by the Ramazzini Foundation (Soffritti et al. 2002c; Maltoni et al. 1999; Soffritti et al. 1999), do not consider statistical evaluation a critical factor in evaluating bioassay results.

The protocol for bioassays at the Ramazzini Foundation is different from many laboratories in that it allows the animals to live until their natural death. This difference in protocol has been suggested to be the reason for the lack of observations in bioassays by other laboratories. It is possible that the incidence of some endpoints reported in the Ramazzini Foundation studies are increased simply because the animals lived longer and had more opportunity to develop tumors. Therefore, it is critical that when conducting dose-response modeling or applying similar statistical methods to the Ramazzini Foundation results, the age of the animals is taken into account using methods such as with time-to-tumor modeling.

Goodman et al. (2007) and Beck (2007) conducted statistical analyses based on the incidence of Leydig cell tumors reported in rats following administration of MTBE (Belpoggi et al. 1995, 1998) and survival information reported on the Ramazzini Foundation website

(www.ramazzini.it)⁵. The statistical analyses conducted considered differences in longevity (prevalence analysis), as well as differences in survival patterns across dose groups (Poly-K analysis). The statistical procedures currently used by the Ramazzini Foundation do not consider the age of the animal or consider survival-related effects, which in the case of the Ramazzini Foundation MTBE bioassay is important because the high-dose rats lived longer than control or low-dose animals. It is also critical, because of differences in protocol, that this information be considered in comparing the results of Ramazzini Foundation studies to those obtained from laboratories applying a protocol with sacrifices prior to natural death.

In evaluating the prevalence of Leydig cell adenomas following administration of MTBE, the Ramazzini Foundation (Belpoggi et al. 1995, 1998) considered only a single time interval, which is the incidence of tumors observed from the time of first tumor until the death of the last animal. Because of the increased survival rates in the high-dose animals, this type of analysis can skew the results. Although the study authors (Belpoggi et al. 1995, 1998) reported a dose-related increase in Leydig cell adenomas, additional statistical analysis conducted by Goodman et al. (2007) (F test) indicated a non-significant trend, with pair-wise tests indicating marginal statistical significance only in the high-dose group ($p=0.04$). These statistical procedures still do not enable the researcher to determine whether the tumors were treatment-related or a result of having a longer time period for tumors to develop.

When age of the animal is a critical consideration in tumor development, statistical tests, such as the Poly-K test should be employed. The Poly-K test accounts for differences in survival rates and can account for lesions that occur as a power of age (Goodman et al. 2007). It is important to note, that these types of analyses require knowledge of the time of death of each animal, which is not provided in the published Ramazzini Foundation bioassays. Therefore, any attempts to account for the impact of age on the outcome of the Ramazzini Foundation bioassays have to be based on assumptions.

In the case of MTBE, Belpoggi et al. (1995, 1998) reported that the first Leydig cell tumor occurred at 96 weeks of age and that the last animal died at 174 weeks of age. Based on this information, Goodman et al. (2007) applied Poly-K analyses to three extreme scenarios related to the possible occurrence of Leydig cell tumors to obtain a range of all possible outcomes. These included a minimum dose-response trend in which tumors occur first in treated animals and later in the control animals, as well as a maximum dose response trend in which tumors occur first in control animals and later in treated animals. The authors concluded that,

⁵ Although the tabulated information available on the website indicates the number of days on test for each animal, the display is limited to three digits. For animals that survived on test for more than 1,000 days, the reported “day on test” value is apparently truncated, so the actual number of days on test is not known for several animals. For example, in the MTBE study, Belpoggi et al. (1995, 1998) reported that the last animal died at 174 weeks of age (1,162 days on test), and in the aspartame study, Soffritti et al. 2006a report that the last animal died at 159 weeks of age (1057 days on test).

once age of the animal at the time of tumor observation was considered, even in biologically implausible situations, pair-wise statistical significance or a significant dose-related trend was not demonstrated.

With the recent release of the individual animal data from selected bioassays on the Ramazzini Foundation website (www.ramazzini.it), Beck (2007) conducted an additional Poly-K analysis relying upon the actual raw data, rather than the assumptions used in the Goodman et al. (2007) analysis. The results of the Beck (2007) analysis were consistent with those reported by Goodman et al. (2007) in that there was no statistically significant increase in the incidence of Leydig cell tumors in animals administered MTBE when survival was appropriately taken into account. These results demonstrate the importance of time-to-tumor information in statistically evaluating the results from the Ramazzini Foundation bioassays.

Conclusions

Limitations in the methods of statistical analysis routinely applied by the Ramazzini Foundation call into question the significance of some of their reported results, and warrants caution in the use of data from these studies unless and until they have undergone rigorous independent peer review.

VII. CONCLUSIONS AND RECOMMENDATIONS

Aspects of the design, conduct, and reporting of studies carried out by the Ramazzini Foundation raise questions regarding their appropriateness for use in assessing risk. In particular, the combination of allowing the animals to live until spontaneous death, gang caging, and the high incidence of chronic infections in the animals may tend to produce a combination of autolysis, cannibalism, and severe age-related conditions that confound accurate pathological diagnosis. Questions also arise regarding whether the Ramazzini Foundation studies follow appropriate good laboratory practice procedures. Although some of the more recent Ramazzini Foundation publications refer to GLP, older reports do not, and the nature of the laboratory practices referred to in the more recent studies is unclear. Further confounding interpretation is the practice of the Ramazzini Foundation scientists of not reporting information on non-neoplastic lesions in their published reports. Such data would help in assessing the overall health of the animals, and determine whether there were underlying disease processes that might interfere with interpretation of the study data.

Additional factors that make interpretation of these studies difficult include a high frequency of infections, some of which may contribute to the development of lymphomas associated with the respiratory system seen in some studies. These tumors, reported in the Ramazzini Foundation bioassays of MTBE, aspartame, and methanol, but not seen in other studies of these chemicals, are of questionable value in assessing the carcinogenic potential of these, or other chemicals. In the case of other chemicals to which the Ramazzini Foundation has attributed the ability to cause hemolymphoreticular neoplasms, the tissue location of these neoplasms is not identified in the published reports; hence the role of respiratory infections cannot be evaluated. Variability in the extent and impact of respiratory infection may also, in part, explain the very wide range in the incidence of lymphomas and leukemias reported in different Ramazzini Foundation studies.

In addition to issues regarding study design and conduct, issues arise regarding the methods of statistical analysis used in the interpretation of these studies. Because of their unusual design, particular attention needs to be paid to the time of identification of tumors, using methods such as time-to-tumor modeling. At least in the case of MTBE, there is some indication that the apparent increase in Leydig cell tumors was influenced by the longer average lifespan of the treated animals than the controls.

Overall, limitations in the details of study methods and results reported by the Ramazzini Foundation in its published study reports, and questions raised by some of its unusual practices make use of data from these studies of questionable value for regulatory purposes. The recent release of some individual animal neoplastic lesion data by the Ramazzini Foundation represents

a positive step, but raises additional questions because of discrepancies between the recently released data and previously published data for some chemicals.

We recommend that, before relying upon data from the Ramazzini Foundation, regulatory agencies:

- (i) seek full study documentation of the type available for NTP studies;
- (ii) evaluate the studies using NTP criteria;
- (iii) give little weight to any study for which adequate documentation of conduct and results is absent;
- (iv) thoroughly evaluate the role of infection in tumor production in the SD rats used by the Ramazzini Foundation, preferably including histopathology peer review by pathologists experienced in diagnosing mycoplasmosis, and if possible, perform PCR analysis of retained lung tissue samples to identify mycoplasma.

In addition, the unusual protocol design used by the Ramazzini Foundation is difficult to evaluate. Some type of peer-review of the design, perhaps by a committee of the National Academy of Sciences, would be highly valuable.

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