Commentary

The Carcinogenic Effects of Aspartame: The Urgent Need for Regulatory Re-Evaluation

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Aspartame (APM) is an artificial sweetener used since the 1980s, now present in >6,000 products, including over 500 pharmaceuticals. Since its discovery in 1965, and its first approval by the US Food and Drugs Administration (FDA) in 1981, the safety of APM, and in particular its carcinogenicity potential, has been controversial.

The present commentary reviews the adequacy of the design and conduct of carcinogenicity bioassays on rodents submitted by G.D. Searle, in the 1970s, to the FDA for market approval. We also review how experimental and epidemiological data on the carcinogenic risks of APM, that became available in 2005 motivated the European Commission (EC) to call the European Food and Safety Authority (EFSA) for urgent re-examination of the available scientific documentation (including the Searle studies). The EC has further requested that, if the results of the evaluation should suggest carcinogenicity, major changes must be made to the current APM specific regulations.

Taken together, the studies performed by G.D. Searle in the 1970s and other chronic bioassays do not provide adequate scientific support for APM safety. In contrast, recent results of life-span carcinogenicity bioassays on rats and mice published in peer-reviewed journals, and a prospective epidemiological study, provide consistent evidence of APM's carcinogenic potential. On the basis of the evidence of the potential carcinogenic effects of APM herein reported, a re-evaluation of the current position of international regulatory agencies must be considered an urgent matter of public health. Am. J. Ind. Med. 9999;1-17, 2014

KEY WORDS: animal models; aspartame; carcinogenicity; long-term experimental bioassays; public health

INTRODUCTION

Non-sugar sweeteners have been used for centuries, mostly in natural forms derived from the Stevia plant family, and especially in sub-tropical regions where these plants grow [Misra et al., 2011]. Commercial production of chemically synthesized artificial sweeteners began with saccharine in the 1890s [De la Peña, 2010].

Until the 1970s, artificial sweeteners were primarily used to make pharmaceuticals more palatable, and as a sugar substitute in foods designed for patients with diabetes [Talbot and Fisher, 1978]. Since then, a huge industry has developed

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that focuses on "diet," "low calorie," and "light" foods and drinks for the general public, and a number of new, artificial sweeteners have been introduced with increasing use in a wide variety of food products.

Aspartame (APM), a widely used artificial sweetener discovered in 1965, has been on the market for >40 years, and is a market leader. The global production is 34,000,000 pounds per year, and it is used in >6,000 products including over 500 pharmaceuticals [FoodNavigator]. Hundreds of millions of people, including children and pregnant women, consume APM on a daily basis. The US Food and Drug Administration (FDA) approved the use of APM in a limited number of dry foods in 1981 [FDA, 1981], in soft drinks in 1983 [FDA, 1983], and in all foods in 1996 [FDA, 1996]. In 1994, the use of APM was approved in Europe [European Parliament and Council, 1994].

Food additives, like many other food ingredients available for human consumption, must be submitted for pre-market regulatory safety evaluation. The additives are subjected to a variety of toxicological tests, including long-term rodent bioassays to test for potential carcinogenic effects. Guidelines containing the major principles governing conduct of rodent carcinogenic bioassays were made available by the US National Cancer Institute in the mid-1960s. In particular, standard minimal group sizes have been set at 50 animals per group, with no restriction on group sizes larger than 50. This measure increases the statistical power of the data and accounts for animals that die early or are examined prior to the end of the exposure [Sontag et al., 1976]. Guideline updating has been performed by the Environmental Protection Agency [1984], the FDA [2000] and the Organization for Economic Cooperation and Development [2009]. Meanwhile, various bioassays have been conducted by academic and independent scientific institutes, not for regulatory purposes, but rather, to see whether, by using a different protocol design and conduct, the sensitivity, and specificity might be improved [Soffritti et al., 1999; Bucher, 2002; Hayes et al., 2011].

Melnick et al. [2008] noted that in order to minimize controversy over the results of different carcinogenic bioassays and to extrapolate the results from bioassays to humans, test protocols must at least: (1) employ animal models sensitive to the study endpoint; (2) thoroughly characterize both the test chemical and the dose administered; (3) use challenging doses and exposure durations; (4) use sufficient numbers of animals per dose group; (5) use multiple dose groups to detect dosage effects; (6) employ complete and peer-reviewed histological evaluations; and (7) evaluate data using pairwise comparisons and analyses of trends that rely on survival-adjusted tumor incidence.

In applying to the FDA for market approval of APM in the 1970s, G.D. Searle, the manufacturer, submitted three 2-year carcinogenicity bioassays codified as: E-33/34, E-70 on rats, and E-75 on mice. The studies were never published in peer-reviewed scientific literature. Only at the end of 2011 were final reports of these studies made available on the website of the European Food Safety Authority (EFSA), following the EFSA call for data on APM (launched on June 1, 2011) [EFSA, 2011]. Other studies available in the scientific literature were published in the early 1980s [Ishii, 1981; Ishii et al., 1981] and after 2005 [National Toxicology Program, 2005; Iwata, 2006]. Between 2005 and 2010 the Ramazzini Institute (RI) published the results of three peer-reviewed, long-term carcinogenicity bioassays, two of which were performed on rats [Soffritti et al., 2005, 2006, 2007; Belpoggi et al., 2006] and one on mice [Soffritti et al., 2010]. In 2005 and 2012 the results of two prospective epidemiological studies conducted in the US were published. Both studies sought to evaluate the carcinogenic effect of APM among people consuming APM-based products [Lim et al., 2006; Schernhammer et al., 2012].

This commentary presents the results of the long-term bioassays performed by G.D. Searle in the 1970s and other chronic/carcinogenicity bioassays that, to this day, are considered adequate by EFSA and FDA to show the safety of APM. We also trace how the experimental and epidemiological data, available after 2005, contributed to the call from the European Commission (EC) for an urgent re-examination of the available scientific documentation on APM and, if necessary, for major changes to the current APM specific regulations to better protect occupational and public health. A summary of the study designs and the analytical evaluation of the results of the main APM carcinogenicity bioassays performed in the 1970s and after 2005, as well as of the two major prospective epidemiological studies, are reported in Tables I–IV.

DISCUSSION

The G.D. Searle Carcinogenicity Bioassays

The carcinogenicity bioassays on rats and mice, E-33/34, E70 and E75, performed for Searle at the Hazleton Laboratories and reported in 1973 (the former) and in 1974 (the latter two), are derived from documentation posted online by EFSA [EFSA, 2011] (Table I, Part 1).

Study E-33/34, 1973

The study encompassed a control group of 60 male and 60 female Charles River Caesarean Derived (CD) Sprague–Dawley rats and four groups of 40 males and 40 females. The study simulated a daily consumption of APM for 104 weeks at dietary dose levels of 1, 2, 4, 8 g/kg b.w./day, respectively. During the biophase it was observed that: (1) feed

TABLE I. Summary of Design and Results of Long-term Carcinogenicity Bioassays on Aspartame [administered orally with feed to male (M) and female (F) mice and rats]

	Animals				Treatment				Biophase results			Major carcinogenic results[a]		
Author	Species/ Strain	Group	Male/ Female	Age at start	Dose (mg/kg/bw) per day	Duration (wks/LS[b])	Histopath. evaluation	Endpoints	Mean feed consumption differences (%) (M/F)	Mean body weight differences (%) (M/F)	Survival (%) at the end of the study (M/F)	Malignant tumors (% of males)	Malignant tumors (% of females)	Mammary cancer (% of females)
GD Searle et al., 1973: *E-33/34*	Rat Charles River (CD) Sprague-Dawley	I	60/60	NA[c]	0	104	Complete	TI[d]	–	–	38.4/46.7	16.7	23.3	11.7
		II	40/40	NA	1000	104	Limited	TI	−1.4/+1.0	0/+4.5	45.0/ 57.5	17.5	20.0	5.0
		III	40/40	NA	2000	104	Limited	TI	+2.6/+3.0	1.8/−3.2	52.5/ 50.0	7.5	35.0	15.0
		IV	40/40	NA	4000	104	Complete	TI	− 3.1/−8.2*	−0.1/−3.5	57.5/35.0	12.5	22.5	17.5
		V	40/40	NA	6000-8000	104	Complete	TI	− 5.7*/−14.6*	−12.3*/−15.1*	52.5/25.0•	7.5	25.0	17.5
											•($P < 0.05$)	Final sacrifice probably at 110 weeks of age No statistically significant differences between controls and tested animals based on Life-table Technique and T test		
GD Searle et al., 1974: E-70	Rat Charles River (CD) Sprague-Dawley	I	60/60	Prenatal	0	104	Complete	TI	–	–	41.7/48.4	18.3	18.3	
		II	40/40	Prenatal	2000	104	Complete	TI	+1.3/−1.7*	+ 2.2/−0.6	50.0/45.0	10.0	20.0	–
		III	40/40	Prenatal	4000	104	Complete	TI	−8.7*/−3.5*	−2.4/−5.5*	57.5/52.5	20.0	27.5	–
												Final sacrifice probably at 106 weeks of age		
GD Searle et al., 1974: *E-75*	Mice CD1 Swiss	I	72/72	4 wks	0	104	Complete (65/66)[e]	TI	–	–	32.5/41.7	16.9	16.7	–
		II	36/36	4 wks	1000	104	Limited (32/31)[e]	TI	−3.5*/−2.6	−0.5*/+0.8	27.8/38.9	9.4	12.9	–
		III	37/35	4 wks	2000	104	Limited (35/31)[e]	TI	−4.7*/−8.3	+0.7*/+0.5	25.8/41.7	8.6	12.9	–
		IV	37/35	4 wks	4000	104	Complete (27/31)[e]	TI	−7.5*/−9.0*	−0.2*/+1.4	25.0/41.7	22.2	16.1	–
												Final sacrifice at 108 weeks of age. Only in rats followed until 104 weeks[f]		

*P value not available.
[a]For any given dose level, the percentage of animals with a specified tumor type represents the number of animals with the specified type divided by the total number of animals (males or females) in the group.
[b]Life-span.
[c]Not Available.
[d]Tumor Incidence.
[e]Total number of (M/F) mice available for histopathological evaluation.

TABLE I. (*Continued*)

	Animals				Treatment				Biophase results			Major carcinogenic results[a]	
Author	Species/ Strain	Group	Male/ Female	Age at start	Dose (Mg/Kg/Bw) per day	Duration	Histopath. evaluation	Endpoints	Mean feed consumption differences (%) (M/F)	Mean body weight differences (%) (M/F)	Survival (%) at the end of the study (M/F)	Brain tumors (% of males)	Brain tumors (% of females)
Ishii [1981]	Rat, Wistar	I	86/86	6 wks	0	26/52/104	For each dose group, 10M+10F were killed at 26 weeks	Brain tumors[g]	Dose dependent decrease at 2 and 4 g/Kg b.w. and at 4 gr/Kg b.w.	A dose-dependent decrease at 2 and 4 gr/Kg b.w. APM and at 4 gr/Kg b.w.	43.3/ 81.7	0	1.7
Ishii et al. [1981]		II	86/86	6 wks	1000	26/52/104		Brain tumors			26.7/ 66.7	1.7	0
		III	86/86	6 wks	2000	26/52/104		Brain tumors			46.7/ 85.0	0	3.3
		IV	86/86	6 wks	4000	26/52/104	For each dose group, 16M+16F were killed at 52 weeks	Brain tumors	APM+DKP in males and in all treated females (numerical data NA)	APM+DKP in males and in all treated females (numerical data NA)	41.7/ 71.7	1.7	
		V	86/86	6 wks	4000 ASP+DKP[h] (3:1)	26/52/104	For each dose group, 60M+60F were killed at 104 weeks	Brain tumors			48.3/ 68.3	0 Final sacrifice at 110 weeks of age	1.7
National Toxicology Program [2005]	Mice Tg-AC Hemizygous[i]	I	15/15	6 wks	0	40	Complete	Papillomas / carcinomas of skin and/or forestomach	Generally similar among control and treated groups	No differences among the groups	-	There were no neoplasms or non-neoplastic lesions relate to exposure to APM	
		II	15/15	6 wks	520	40	Limited				-		
		III	15/15	6 wks	1040	40	Limited				-	Final sacrifice at 46 weeks of age	
		IV	15/15	6 wks	2110	40	Limited				-		
		V	15/15	6 wks	4190	40	Limited				-		
		VI	15/15	6 wks	7920	40	Complete				-		
National Toxicology Program [2005]	Mice p53 Haploinsufficient[j]	I	15/15	7 wks	0	40	Complete	Lymphomas or sarcomas	Generally similar among control and treated groups	No differences among the groups	-	There were no neoplasms or non-neoplastic lesions related to exposure to APM	
		II	15/15	7 wks	520	40	Limited				-		

	III	15/15	7 wks	1040	40	Limited				-	Final sacrifice at 46 weeks of age
	IV	15/15	7 wks	2110	40	Limited				-	
	V	15/15	7 wks	4190	40	Limited				-	
	VI	15/15	7 wks	7920	40	Complete				-	
National Toxicology Program [2005]	Mice Cdkn2a Deficient[k]	I	15/15	7–9 wks	0	40	Complete	Brain tumors lymphomas and fibrosarcomas	Similar among control and treated groups	No differences among the groups	-	There were no neoplasms or non-neoplastic lesions relate to exposure to APM
		II	15/15	7–9 wks	520	40	Limited				-	
		III	15/15	7–9 wks	1040	40	Limited				-	Final sacrifice at 46 weeks of age
		IV	15/15	7–9 wks	2110	40	Limited				-	
		V	15/15	7–9 wks	4190	40	Limited				-	
		VI	15/15	7–9 wks	7920	40	Complete				-	

[f]No brain tumors observed in animals killed at 26 and 52 weeks.
[g]Six sections in each brain.
[h]Diketopiperazine.
[i]Model susceptible for development of high incidence of skin papillomas in response to topical application of TPA (12-O-tetradecanoyl-phorbol-13-acetate).
[j]Model susceptible for development of high incidence of lymphomas or sarcomas.
[k]Model susceptible for development of high incidence of brain tumors, lymphomas and fibrosarcomas.

consumption for males treated at 8 g/kg b.w./day and females at 4 and 8 g/kg b.w./day was significantly lower than controls; (2) significant decreases in body weight occurred in males and females treated at 8 g/kg b.w./day; (3) survival at the end of the study was lower among females at 4 and 8 g/kg b.w./day than among controls (statistically significant at the highest dose).

Histopathology was performed on all gross lesions and on 20–25 organs and tissues of animals from groups treated at 0, 4, 8 g/kg b.w./day and on roughly one-fourth of the animals treated at 1, 2 g/kg b.w./day groups. No statistical differences in the incidence of various types of tumor were observed among animals treated at 4 and 8 g/kg b.w./day compared to controls. Importantly however, at dietary dose levels of 2, 4 and 8 g/kg b.w./day compared to controls, an increased incidence of females bearing mammary cancers was observed. Moreover, it must be noted that the statistically significant decrease in feed consumption (14%), body weight (15%), and survival (25%) among females treated at the highest dose may have limited the full expression of carcinogenic effects.

Study E-70, 1974

APM was administered in feed to male and female Charles River Caesarean Derived (CD) Sprague–Dawley rats. The study encompassed a control group of 60 males and 60 females, and two groups of 40 males and 40 females receiving APM of 2 or 4 g/kg b.w./day from prenatal life and for 104 weeks past weaning. Parents received the same treatment 60 days prior to mating, during mating, gestation and lactation. At 104 weeks of age the experiment ended and any animals still alive were killed. During the biophase a statistically significant decrease in feed consumption was observed among males treated at the highest dose compared to controls. No differences were observed in mean body weight and survival.

Histopathology was performed on all gross lesions and on 20–25 organs and tissues from all control and treated animals. Microscopic evaluation of eight coronal sections of the brain was performed on all control and treated rats. No significant differences in the incidence of the various types of tumor analyzed were reported among treated groups compared to controls, including brain tumors. At 104 weeks the incidence of females bearing malignant tumors was higher among treated groups than controls.

Study E-75, 1974

APM was administered in the feed to groups of 36 male and 36 female ICR Swiss mice at dose levels of 1, 2, and 4 g/kg b.w./day for 104 weeks starting from 4 weeks of age. The control group included 72 males and 72 females. The experiment ended at 108 weeks of age and animals still alive

were killed. Food consumption was significantly reduced among males treated at all doses and in females treated at the highest dose. The mean body weight for male mice at all dose levels was significantly lower than for male controls. At the end of the study, survivals in all treated groups were comparable to controls. Complete histopathology evaluation was performed on controls and high-dose mice of each sex, and limited evaluation was performed on animals treated at medium and low doses. Additionally, all gross lesions from all animals of each group were examined microscopically. No significant differences were reported in incidences of the various types of tumor analysed.

It should be noted that at the end of the experiment the total number of male mice available for histopathology examination was 159 (87.4%) compared to 182 animals when the study started. Concerning the control group and the group treated at the highest dose, the number of mice available for histopathological examination was 65 (90.3%) and 27 (73%), respectively. The total number of female mice available for histopathological evaluation was 159 (89.3%) out of 178; the controls numbered 66 (91.6%), and those treated at the highest dose 31 (88.6%). It is clear that the small number of animals per group limited the sensitivity of the study.

Other Carcinogenicity Bioassays Considered by the EFSA and FDA

An additional chronic toxicity study was conducted on five groups of 86 male and 86 female Wistar rats to test whether or not dietary administration of APM or APM + DKP (diketopiperazine, one of the degradation products of APM that is formed under certain processing and storage conditions) induces brain tumors [Ishii, 1981; Ishii et al., 1981]. Each group was divided into a main group (60 males and 60 females) and a satellite group (26 males and females). In the satellite group, 10 males and 10 females were killed and examined at 26 weeks, and 16 males and 16 females were killed and examined at 52 weeks. The main group was followed up until 104 weeks, when the survivors were killed (Table I, Part 2).

Beginning at 6 weeks of age, three groups were fed with APM and one group was fed with APM + DKA (3:1) at dose levels of 1, 2, and 4 g/kg b.w./day for APM and 4 g/kg/day for APM + DKP, for 104 weeks. A dose-dependent depression of body weight gain was observed at 2 and 4 gr/Kg APM and at 4 g/kg APM + DKP in males, as well as at all dose levels in females, correlating with decreased food consumption. At *interim* or terminal sacrifice the blood taken was examined for clinical biochemistry; organ weights of the brain, heart, spleen, pituitary, adrenal, liver, kidney, testis, and ovarian were recorded, and representative portions of other organs and gross lesions were fixed in 10% formalin. Microscope evaluation of 6 coronal sections of the brain failed to show any significant difference in the incidence of brain tumors between control and test groups [Ishii, 1981]. It must be noted that the main purpose of this study was to evaluate the chronic toxicological effects of APM or APM + DKP and not the potential carcinogenicity of APM or APM + DKP, as indicated by the plan and conduct of the experiment. In 2006 a histopathological re-evaluation of non-neoplastic and neoplastic lesions to the organs and gross lesions collected during the Ishii study was performed by Iwata at the request of Ajinomoto, the largest manufacturing company of APM. No significant differences between controls and treated animals were identified [Iwata, 2006].

In pilot carcinogenesis studies performed by the US National Toxicology Program exposing groups of 15 male and 15 female Tg. AC transgenic mice to diets containing 0, 3, 125, 6,250, 12,500, 25,000, or 50,000 ppm of APM for 40 weeks did not show any carcinogenic responses [National Toxicology Program, 2005]. However, NTP concluded that the negative findings were of uncertain value: "because this is a new model, there is uncertainty whether the aspartame study possessed sufficient sensitivity to detect a carcinogenic effect" [National Toxicology Program, 2005]. The NTP has since discontinued the use of genetically modified transgenic models as an adequate means for identifying carcinogens.

In our opinion, the carcinogenicity studies that G.D. Searle submitted to FDA for approval of APM use as a food additive must be considered not only inadequate by current standards of design and conduct for carcinogenicity bioassays (e.g., Good Laboratory Practices), but poorly designed and poorly executed. The studies are plagued by incomplete pathology examination due to the loss of animals and/or tissues in advanced autolysis, causing multiple statistical problems. Moreover, if Searle's experiments had been designed using more animals per sex and exposure group, and had prolonged observation after 2 years (which is about two-thirds of a rodent's natural life-span thus representing the equivalent for humans at the age of 60–65), the border line carcinogenic effects shown in females exposed at the highest dose in the two rats experiments, may well have been less equivocal. In the same way, the other chronic toxic/carcinogenicity bioassays performed by Ishii et al. [1981] and re-evaluated later [Iwata, 2006], and the studies performed by NTP [2005], do not provide sufficient scientific support to consider APM free from possible long-term carcinogenic effects. It is our opinion that EFSA is unjustified in concluding, based on methodologically problematic studies, that "there is no reasons to revise the previously established Admitted Daily Intake of 40 mg/kg b.w. for people."

The CarcinoGenicity Studies of the Ramazzini Institute on APM

In 2005 and 2006, at the Cesare Maltoni Cancer Research Center of the Ramazzini Institute (RI), it was

shown for the first time that APM administered with feed to Sprague–Dawley rats from 8 weeks of age, throughout their lifespan, causes cancer in both males and females. Results showed a statistically significant dose-related increased incidence of lymphomas/leukemias, pre-neoplastic, and neoplastic lesions of the renal pelvis in females and malignant Schwannomas of peripheral nerves in males [Soffritti et al., 2005, 2006; Belpoggi et al., 2006]. In 2007, the statistically significant dose-related increase in lymphoma/leukemia incidence (diagnosed following the same morphological criteria as the first experiment) in Sprague–Dawley rats treated from prenatal life with APM in the feed was confirmed [Soffritti et al., 2007]. This study also showed that the carcinogenic potential of APM is enhanced when exposure begins during prenatal life. In 2010 the results of a third study conducted by the RI on Swiss mice (starting the treatment prenatally) showed that APM induces a statistically significant dose-related increase in the incidence of cancers of the liver ($P < 0.05$) and lung ($P < 0.05$) in males [Soffritti et al., 2010]. Given these overall findings, APM should be considered a multiple-site trans-species carcinogen in both sexes and most likely carcinogenic to humans, especially workers handling it and users consuming high quantities (Table II).

Criticisms of the Ramazzini Institute Studies

The results of the long-term bioassays on APM conducted by the Laboratory of the Cesare Maltoni Cancer Research Center of the Ramazzini Institute (RI) have come under coordinated and intense criticism by spokespersons for the chemical industry in Europe, Japan, and the U.S. [Magnuson et al., 2007; Goodman et al., 2009; Schoeb et al., 2009], as well as the EFSA (seconded by the FDA). They considered the RI studies to have several methodological "flaws," such as: (1) the inappropriately large number of animals per sex and per group, the numerous doses tested, prenatal exposure, and the life-span treatment and observation of the animals, the claim being that the study design and conduct are in contrast with current guidelines of the Organization for Economic Cooperation and Development (OECD) [2009] and other international protocols that recommend the use of adult animals (6–8 weeks old) at the start of studies, duration of studies limited to 24 months to avoid high background tumors in controls (which may affect the ability to evaluate the significance of small increased incidences of tumors in the tested groups) [EFSA, 2006, 2009]; (2) high background infection in the RI rat colony allegedly affecting survival and tumor rates [EFSA, 2006; FDA, 2007; Hayes et al., 2011]; (3) uncertainty about the "correctness" of diagnosis of some tumor types, in particular lymphomas/leukemias [EFSA, 2006; Schoeb et al., 2009]; and (4) a lack of relevance to human risk assessment in the cases of statistically significant dose-related increased incidences of hepatocellular carcinomas ($P < 0.05$) and lung carcinomas ($P < 0.05$) observed in male Swiss mice that were induced by non-genotoxic agents EFSA, 2011a; EFSA, 2011b].

Responses to some of these criticisms have already been provided in a number of our earlier works [Soffritti et al., 2007, 2008, 2010].

Rebuttal of Criticisms Targeting the RI

Some criticisms of the RI concern its distinctive features in designing and conducting long-term rodent bioassays, namely: the use of a large number of animals per test dose group, the multiple tested doses, and the life-span duration of experiments (which tends to raise the sensitivity of the studies). Some other criticisms have been more specific to APM studies. To better evaluate the worth of the RI methodological approach, it must be considered that the aim of the RI long-term bioassay program is not only to identify exogenous carcinogens with high social and health impact, but also to obtain information regarding the third part of the life in order to allow a better risk assessment of their effects, particularly at low exposure. In this respect, the aim of the RI bioassay program does indeed differ from that of the US NTP bioassay program and other regulatory agencies that are designed to screen for potential carcinogenic hazards, which is certainly important, but not sufficient for full risk assessment. As reported by Swemberg et al. [1991], to investigate the dose-response relationships and the effects of low-dose exposure to carcinogenic agents, it is first crucial to test them in rodent long-term bioassays using a dose range larger than the one commonly used, as well as a large number of animals per sex and per test dose. It is also crucial to prolong observation until extreme old age. Regulatory agencies like FDA and EPA, which demand that chronic animal studies be terminated after 2 years, may lose information that is important for extrapolation of the data from animals to humans, especially in the cases of carcinogenic agents with a long latency time (weak carcinogens) [Swenberg et al., 1991].

Concerning the longer (over 110 weeks of age) or life-span duration of the experiments, it must be considered that neoplastic response depends not only on the chemical–physical characteristics of the agent and its toxicological properties and potency, the mode of exposure, and the type of animals, but also, to a greater extent, on the latency of the type of tumor, which varies and may be longer than 110 weeks [Littlefield et al., 1980; Maltoni et al., 1999].

With regards to the high background of control group tumor pathology in life-span studies, our data from several studies show that the overall incidence of animal bearing malignant tumors in historical controls is very stable, being no >40–50% (5–10% higher in females than in males), which is very similar to humans [Soffritti et al., 2002].

Finally, this study protocol allowed the RI laboratory to be the first to demonstrate the carcinogenicity (in animals) of vinyl chloride and benzene, recognized as human carcinogens, and to provide the data to determine the limits of exposure in the workplace.

Other criticisms, which are more specific to APM studies, are here discussed in separate sections.

High background of infection in the RI colony, affecting tumor rates and survival

Experimental animals that are allowed to die spontaneously are often subject to infectious pathologies; these are part of the natural dying process in both rodents and humans [Soffritti et al., 2007; Mehlman, 2009]. However, when some experiments conducted at the RI laboratory required that animals be killed by way of *interim* or terminal sacrifice, very slight acute/chronic respiratory inflammations were observed, even at the age of 130–140 weeks (authors' statement). Moreover, data from >2,000 male and 2,000 female Sprague–Dawley rats in the control groups of studies published during the past 20 years [Soffritti et al., 2002], together with indicators of animal good health (water and food consumption, body weight, survival, prevalence of animals bearing malignant tumors) proved quite consistent and in accordance with the data from other laboratories [Huff, 2002]. The general animal care procedures followed by the RI are in compliance with Italian law regulating the use and humane treatment of animals for scientific purposes, as periodically certified by national and local inspectors from public administrations [Decreto Legislativo, 1992]. Concerning the survival of the control group animals in the APM experiment, at 110 weeks of age mean rates were 26% in males and 28.6% in females from the first rat experiment; 17.5% and 40.5% in males and females from the prenatal rat experiment; and 33% and 44% in males and females from the mouse study. This is in the range of the expected survival at this age in Sprague–Dawley rats and Swiss mice.

Uncertainty about the "correctness" of diagnosis of some tumor types

The correctness of the RI pathologists' diagnostic interpretation of tumors was questioned by the EFSA [2006] and FDA [2007] on the basis of a report by a Pathology Working Group. The group was convened on November 15 and 16, 2004 at NIEHS (NC) to provide a second opinion on a set of 63 pre-neoplastic/neoplastic lesions of the first study on APM and was vetted by a group of eight pathologists experienced in toxicologic pathology. The lesions were selected by RI pathologists and included preneoplastic/neoplastic lesions of mammary gland, cranial nerves, brain, hematopoietic organs and tissues, renal pelvis, and other organs and tissues. The slides broadly represented the morphological characteristics of the main lesion types observed in the first experiment on 1,800 rats. The essence of the results/discussion of the PWG was reported by Hailey [Pathology Working Group, 2004] as follows: (1) the three cases of malignant Schwannomas of the cranial nerve were generally confirmed, with the recommendation that the lesions would be better characterized with immunohistochemical staining. The malignant Schwannomas proved positive for immunohistochemical staining by S-100 protein, which has proved useful for the diagnosis of peripheral Schwannomas of rats [Mitsumori and Boorman, 1990]. This information was included in the paper [Soffritti et al., 2006]; (2) there was general agreement on the cases of hyperplasia of transitional epithelium of the renal pelvis and two of the lesions were confirmed as neoplastic. In many instances proliferative lesions appeared, associated with (secondary to) inflammatory lesions; and (3) concerning the diagnosis of lymphoid and histiocytic neoplasms in the 8 cases reviewed, they were all confirmed in all hematopoietic organs and tissues examined (including lung) and within specific histological types exactly as found by the RI pathologists.

On April 4–8, 2011 a Pathology Working Group (PWG) vetting RI studies on five compounds (APM was not included) was convened at the RI by NTP [NTP and EPA, 2011] to assess the quality of the pathology data, address any discrepancies and confirm the diagnoses from selected life-span bioassays. The PWG review agreed with tumor diagnoses made by RI pathologists with the exception of the magnitude of lymphomas, squamous cell carcinomas and osteosarcomas of the inner ear: there was qualitative, but not quantitative agreement for these types of tumors. As recognized by the PWG in its conclusions, differences in diagnostic opinions are not unusual for studies of this type. However, to conclude from these different opinions between NTP and RI pathologists, as the EFSA does in its recent draft on scientific opinion on APM [EFSA, 2013], that a negative verdict applied to other studies carried out by the RI, including APM studies, is far from justified.

Infection as a mode of action for inducing lymphomas/leukemias (L/L) in rats [EFSA, 2006]

The EFSA [EFSA, 2006] claims in its conclusive comments that "the relatively high incidences of lymphomas/leukaemias (L/L) found in the APM-treated groups are most likely to be related to chronic inflammatory changes in the lungs." A similar issue was raised in the past regarding the influence of common viral infections of rats and mice on the prevalence of various tumors. The NTP evaluated body weight, survival, and prevalence of liver tumors, lung tumors and lymphoma in B6C3F1 mice with and without viral infection in male and female untreated control groups and in

males and females from low- and high-dose groups. Overall, it was found that viral infections did not cause consistent or reliable increases or decreases in body weight, survival, or tumor prevalence in the control and chemical treated groups [Rao et al., 1989a]. NTP did a similar study on rats. In that case too, none of the tumors' prevalence and survival differences were statistically significant [Rao et al., 1989b].

Concerning the speculation of infection as a mode of action for induction of L/L in rats [EFSA, 2006], Caldwell and other colleagues from the US EPA, in an extensive review of this issue, concluded that "the scientific evidence does not support the assertion that RI animals are inordinately susceptible to L/L as a result of chronic infection. A review of background rates of L/L at the RI facilities shows only a few of their chronic studies have yielded positive results for L/L, suggesting that the findings are not general, but chemical specific" [Caldwell et al., 2008; Gift et al., 2013].

Moreover, in response to criticisms that lymphoma in rats characteristically arises in lymphnodes or in the thymus, spleen and intestine and only occasionally/rarely in other sites, including the bronchus-associated lymphoid tissue of the lung [Goodman et al., 2009], all the L/L in females exposed from prenatal life at 2,000 ppm of APM, affected multiple organs (including the lung) and tissues (Table III).

In conclusion, the two studies conducted using the same species and strain and producing the same results should be considered robust evidence of the leukemogenic effects of APM. The collaborative research that is ongoing between NTP and RI to better characterize these L/L lesions is scientifically important; the research aims to clarify the diagnostic uncertainties on early L/L lesions in rats, not only regarding APM, but also for chemicals with similar metabolism (methanol, MTBE) which leads to formaldehyde.

Liver and lung cancers in mice are irrelevant for human risk assessment

In 2010 we published the results of a study on mice, which showed that APM induced a statistically significant increase in the dose-related incidence rate of liver and lung cancer in males [Soffritti et al., 2010]. In response, the EFSA noted that: (1) the study design (including trasplacental exposure) does not follow any accepted international test guidelines for conduction of carcinogenic studies in rodents, such as OECD test guideline 451; (2) the results of the RI mice study fall within their own historical control ranges for spontaneous tumors; (3) there is general consensus in the scientific community, backed by a considerable body of evidence, that hepatic and lung tumors in mice—when induced by non-genotoxic compounds—can be irrelevant for human risk assessment. For this reason, the EFSA concluded that the results do not provide evidence for any carcinogenic effects by APM [EFSA, 2011a]. However, a significant dose-related increase in liver and lung cancer in APM treated males, compared to concurrent controls, cannot be scientifically disregarded. The assertion by EFSA that the carcinogenesis results in mice are irrelevant for human risk assessment is inconsistent considering that regulatory agencies like EPA and FDA, NTP and IARC still require studies on both rats and mice in order to demonstrate chemical/physical agent exposure safety, even for non-genotoxic agents.

Epidemiological Studies on the Carcinogenicity of APM

Prior to 2005, few studies were conducted to evaluate the carcinogenic potential of APM among people consuming products containing APM. The results of the RI bioassays motivated two epidemiological groups, the first at the US National Cancer Institute [Lim et al., 2006], and the second at the University of Harvard [Schernhammer et al., 2012], to investigate the potential carcinogenic risk among consumers of APM-containing products, in particular diet beverages. The results dealing with the hematopoietic cancers are summarized in Table IV.

The NCI epidemiological study

This study was based on data from the National Institute of Health—American Association of Retired Persons diet surveillance, and included 473,984 individuals aged 50–71 who were surveyed in 1995, and followed until 2000 for signs of gliomas (315 cases) and hematopoietic tumors (1,888 cases). The authors reported that for a daily intake of APM $>$900 mg/day no significant increase in risk of hematopoietic neoplasms (RR 0.98, 95% CI 0.76–1.27) or of gliomas (RR 0.73, 95% CI 0.46–1.10) was observed. They concluded that the data do not support a positive association between APM containing beverages and brain or hematopoietic neoplasms [Lim et al., 2006]. In our opinion, the NCI study's significance is overstated. The limited duration of exposure, the limited follow-up, and the low exposure levels greatly reduce the power to detect an effect. This is in spite of the large cohort size. Because of the overly simple evaluation of the exposure (measured as the consumption of products containing APM during the 1 year immediately prior to the start of the 5 years follow-up), concerns about the validity of the results still remain [Schernhammer et al., 2012]. Therefore, the position taken by the EFSA [2006, 2009] and other industries spokespersons [Marinovich et al., 2013] that the NCI negative epidemiological study removes any cancer risk concerns, in particular L/L resulting from experimental studies, is misguided.

TABLE II. Summary of Design and Results of RI Studies - Long-term Carcinogenicity Bioassays on Aspartame [administered orally with feed to male (M) and female (F) mice and rats]

	Animals				Treatment				Biophase Results			Major carcinogenic results[a] (*P* values)				
Author	Species/ Strain	Group	Male/ Female	Age at Start	Dose (mg/kg/ bw) per day	Duration (wks/LS[b])	Histopath. evaluation	Endpoints	Mean feed consumption differences (%) (M/F)	Mean body weight differences (%) (M/F)	Survival (%) at the end of the study (M/F)	Malignant tumors (% of males)	Malignant tumors (% of females)	Lymphomas/ Leukemias (% of females)	Carcinomas of renal pelvis and ureter + their precursor (% of females)	Peripheral nerve malignant schwannomas (% of males)
Soffritti et al. [2005]	Rat (Sprague Dawley)	I	150 /150	8 wks	0	LS	Complete	TI[c]	-	-	26.0/28.6	35.3$^{\blacklozenge}$	36.7$^{\blacklozenge\blacklozenge}$	8.7$^{\blacklozenge\blacklozenge\#}$	1.3$^{\blacklozenge\blacklozenge\#\#}$	0.7$^{\blacklozenge\#}$
Belpoggi et al. [2006]		II	150 /150	8 wks	4	LS	Complete	TI	−1.2/−1.4	+0.8/+0.7	32.6/32.6	29.3	42.7	14.7	4.0	0.7
Soffritti et al. [2006]		III	150 /150	8 wks	20	LS	Complete	TI	−3.4/−5.5	−0.7/+1.0	25.3/35.6	32.0	46.7	20.0$^{\#\#}$	6.0$^{\#}$	2.0
		IV	150 /150	8 wks	100	LS	Complete	TI	−6.1/−6.8	−1.7/−0.3	26.3/33.0	40.0	44.7	18.7$^{\#}$	6.7$^{\#}$	1.3
		V	100 /100	8 wks	500	LS	Complete	TI	−1.5/−6.9	0/+1.9	25.0/35.0	34.0	40.0	19.0	10.0$^{\#\#}$	2.0
		VI	100 /100	8 wks	2500	LS	Complete	TI	−6.8/−12.3	+0.6/+2.4	26.0/39.0	38.0	58.0$^{\#\#}$	25.0$^{\#\#}$	10.1$^{\#\#}$	3.0
		VII	100 /100	8 wks	5000	LS	Complete	TI	−13.2/−18.6	−0.4/−4.9	33.0/47.5	43.0	51.0	25.0$^{\#\#}$	15.0$^{\#\#}$	4.0
Soffritti et al. [2007]	Rat (Sprague Dawley)											Malignant tumors (% of Males)	Malignant tumors (% of Females)	Lymphomas/ Leukemias (% of Females)	Lymphomas/ Leukemias (% of Males)	Mammary cancer (% of Females)
		I	95 /95	Prenatal	0	LS	Complete	TI	–	–	17.5/40.5	24.2$^{\bullet\bullet}$	44.2	9.5[c]	12.6$^{\bullet}$	5.3$^{\bullet}$
		II	70 /70	Prenatal	20	LS	Complete	TI	+1.0/+1.9	+2.4/+5.9	14.6/39.3	25.7	44.3	15.7	17.1	7.1
		III	70 /70	Prenatal	100	LS	Complete	TI	+1.6/+1.8	+1.8/+5.9	13.3/30.0	40.0$^{\bullet\bullet}$	52.9	17.1$^{\bullet}$	31.4$^{\bullet\bullet}$	15.7$^{\bullet}$
Soffritti et al. [2010]	Mice (Swiss)											Malignant tumors (% of Males)	Malignant tumors (% of Females)	Hepato-carcinomas (% of Males)	Lung carcinomas (% of Males)	
		I	117 /102	Prenatal	0	LS	Complete	TI	-	-	28.2/38.7	56.4	67.6	5.1$^{\bullet}$	6.0$^{\bullet}$	
		II	103 /122	Prenatal	250	LS	Complete	TI	+3.3/+0.6	+2.8/+6.4	28.6/42.2	56.3	73.8	11.7	5.8	
		III	62 /73	Prenatal	1000	LS	Complete	TI	+2.9/+0.4	+3.6/-0.6	39.5/45.2	72.6	64.4	14.5	11.3	
		IV	64 /64	Prenatal	2000	LS	Complete	TI	0/+5.6	+8.4/+6.2	29.7/32.8	60.9	68.8	15.6$^{\bullet}$	12.5	
		V	83 /62	Prenatal	4000	LS	Complete	TI	−3.2/+0.6	+2.0/+1.7	26.5/45.9	68.7	64.5	18.1$^{\bullet\bullet}$	13.3$^{\bullet\bullet}$	

[a]For any given dose level, the percentage of animals with a specified tumor type represents the number of animals with the specified type divided by the total number of animals (males or females) in the group.
[b]Life-span.
[c]Tumor incidence.
$^{\blacklozenge}$Dose-related statistically significant ($P < 0.05$) or $^{\blacklozenge\blacklozenge}$($P < 0.01$) using the Cochran–Armit age test. Dose–response value is near control incidence.
$^{\#}$Statistically significant ($P < 0.05$) or $^{\#\#}$($P < 0.01$) using poli-K-test (K = 3).
$^{\bullet}$Statistically significant ($P < 0.05$) or $^{\bullet\bullet}$($P < 0.01$) using Cox regression model.

TABLE III. Long-Term Carcinogenicity Bioassays on APM, Administered With Feed From Prenatal Life, Until Death, to Female (F) Sprague–Dawley Rats: Statistical Reanalysis of Lymphomas/Leukemias (L/L)

	Animals		Animals bearing HN[a]		Animals bearing L/L excluding those with lymphoma localized only in the lung[b]	
Group concentration (ppm)	Sex	No.	No.	%	No.	%
I (2,000)	F	70	22	31.4*	22	31.4*
II (400)	F	70	12	17.1	10	14,3
III (0) Control	F	95	12	12.6◆	9	9.5◆

[a]Animals with L/L affecting one site or multiple sites.
[b]Animals with L/L affecting multiple sites with the exclusion of those with lymphoma localized only in the lung.
*Statistically significant ($P \leq 0.01$) using Cox Regression Model.
◆Near the control incidence are the *P*-values ($P \leq 0.01$) associated with the Cox Regression Model for analysis of the trend.

The Brigham and Women's Hospital and Harvard Epidemiological Study

Following the publication of the first RI results that showed the carcinogenic effects of APM, Harvard researchers undertook a prospective study on diet soda and APM consumption in relation to cancers with significant increased risks observed in the RI mega-experiment, namely L/L. Schernhammer et al. [2012] examined data from two longitudinal health surveys: the Nurses' Health Study, which began in 1976, and includes 121,701 female registered nurses; and the Health Professionals Follow-up Study, which began in 1986, and includes 51,529 male health professionals. Dietary intake, including detailed diet soda

TABLE IV. Relative risks (RRs) of Hematopoietic Cancers (HPC) in 2 Prospective Epidemiological Studies Conducted Among People Consuming APM in the USA

Authors	Study design			Results	
	No. of people (age range)	Follow up (years)	APM consumption, mean mg/day (% cohort)	HPC (No. of cases)	Relative risks (95% CI)
Linn et al. [2006]	473,984 males and females (50–71 years)	1995–2000[a]	0 (46%)	869	1 (referent)
			47 (25%)	432	0.91 (0.81–1.03)
			147 (13%)	280	1.10 (0.96–1.26)
			267 (7%)	137	1.01 (0.84–1.21)
			441 (5%)	104	1.05 (0.85–1.29)
			986 (4%)	66	0.98 (0.76–1.27)
Schernhammer et al. [2012]	47,810 males (40–75 years)	1986–2006[b]	No. of servings	RRs (95% CI) of HPC (No. of cases per histotype)	
			Diet Coke	NHL[c]	MM[d]
			None	(172)	(40)
				1.00 (–)	1.00 (–)
			1 - 3.9/week	(124)	(23)
				1.06 (0.83–1.34)	1.04 (0.61–1.78)
			≥ 1 serving/day	(100)	(29)
			(70–180 mg/serving)	1.31 (1.01–1.72)	2.02 (1.14–3.05)
					p-trend 0.01

[a]Food Frequency Questionnaire (FFQ) was mailed once to the participants.
[b]FFQ was mailed to the participants at the beginning and subsequently re-assessed every 4 years.
[c]Non-Hodgkin's lymphoma.
[d]Multiple myeloma.

consumption, was assessed as part of the study questionnaire administered in 1984 to the women, and again in 1986 to both genders. Diet was subsequently re-assessed every 4 years until 2006. The APM study excluded participants who had not completed the baseline nutritional survey or who had a history of cancer. The final study population included 77,218 women and 47,810 men.

Because the dietary histories included specific questions about consumption of APM, investigators had exposure data from the time that APM became available in the U.S. market. This study therefore provides the most complete and comprehensive information on human consumption of APM and health outcomes. Schernhammer and colleagues concluded that, in men, one observes a statistically significant increase in the risk of non-Hodgkin's lymphoma in subjects who consumed $\geq$1 serving of diet soda per day [Schernhammer et al., 2012]. Moreover, in men, the risk of multiple myeloma increased linearly with increased consumption and a statistically significant increase was observed in subjects who consumed $\geq$1 diet soda per day. The authors concluded that their data provide some support for findings from recent animal experiments that suggested a positive association between APM intake and hematopoietic neoplasms [Soffritti et al., 2005, 2006, 2007; Belpoggi et al., 2006]. However, the authors cautioned that, because this is the first large-scale observational human study reporting the association between diet soda and APM intake and hematopoietic cancers, and because no clear effect was seen in women, results necessarily require confirmation in other cohorts in order to rule out chance as a possible explanation for their findings. In order to address the disparity between results in men and women, the authors conjectured that it may have been due to the recognized hyper enzymatic activity of alcohol dehydrogenase type 1 (ADH1) in men (as compared to women), which may induce higher conversion of methanol into formaldehyde. Similarly, the differing results between male and female rats exposed to APM may be due to the higher activity of ADH1 in females than in males [Simon et al., 2002], which could explain the leukemogenic effect observed in females rats.

Request by the European Commission (EC) for EFSA to Urgently Re-Examine APM Safety

In March 2011, after publication of the RI mouse study results in the American Journal of Industrial Medicine [Soffritti et al., 2010], the European Commission on Health and Consumers organized a hearing on APM in Brussels where they invited speakers from European scientific institutions and the EFSA. At the hearing, the results of the APM carcinogenicity bioassays on rats and mice performed by the RI were presented and discussed.

In May 2011, as a result of the hearing, and due to the persistent controversy amongst scientists, members of the European Parliament, and consumer organizations, over the safety of APM, the Directorate-General of the EC Health and Consumers asked the EFSA to perform a full re-evaluation of APM safety. The Directorate-General requested that the re-evaluation be completed by the conclusion of July 2012, rather than the original deadline of December 2020 (set in 2010).

In May 2013 the EFSA presented their "Draft Scientific Opinion on the re-evaluation of APM" [EFSA, 2013] and the final report is still under discussion.

CONCLUSIONS

Almost 40 years after the discovery and first production of APM, studies performed by the industry responsible have finally been made available to the public, to the scientific community and to related stakeholders (such as EFSA, FDA, and various industry spokespersons). These studies have now been shown to be unsatisfactory in many respects, including their limited design, conduct and reporting.

In the early 1980s, several scientists in the US and in Europe proposed that government agencies should fund new research and testing, with the motivation that widely used compounds, especially those with considerable human exposure, should be retested using modern methods [Huff and LaDou, 2007].

At the end of the 1990s, the laboratory of the RI planned a series of experiments to test the possible carcinogenicity of APM. Such studies are referred to as mega-experiments in light of the larger number of animals per sex and group of exposure, the numerous dose levels tested, and the observation until the end of the life-span [Soffritti et al., 1999]. Between 2005 and 2007 the results of the RI studies showed that APM was carcinogenic in rats, and in 2010, APM was shown to be carcinogenic in mice [Belpoggi et al., 2006; Soffritti et al., 2006, 2007, 2010]. In 2012 the Harvard prospective epidemiological study showed a significant increased risk of non-Hodgkin's lymphoma and multiple myeloma in men who consumed diet soda containing APM [Schernhammer et al., 2012].

In response to the current data, the appropriate scientific course would be either to repeat and confirm the RI studies, or to adopt a different methodological approach (different strains, etc.), in order to resolve the controversy over APM carcinogenicity. Unfortunately we have witnessed an unproductive, endless, and acrimonious debate that, in being drawn out, is likely to have a harmful effect on public health.

Over the past few years our knowledge about the carcinogenic risks of APM has gained greater clarity, with consistent and troubling findings in both animals and

humans. The question therefore is how to best proceed in order to better protect occupational and public health.

First we must consider that, for >40 years, there has been general agreement amongst experts in chemical carcinogenesis that substances that cause significantly increased incidence of cancers in experimental animals, in well-conducted long-term bioassays, pose a presumptive and predictive carcinogenic risk to humans, even in the absence of conclusive epidemiological data [Tomatis, 1979; Maltoni et al., 1999; Maronpot et al., 2004; Cogliano, 2006; Huff, 2010].

Secondly, in light of the evidence of carcinogenic risk and the likelihood that there is little or no benefit from APM consumption in the general population, we strongly recommend that intake of APM be substantially decreased. Further cautionary steps to limit consumption of this sweetener are needed to protect the most vulnerable subgroups, especially pregnant women and children. Children are the main consumers of products containing APM, and their vulnerability to chemical hazards is distinct from that of adults [Landrigan, 1999; Soffritti et al., 2008]. Though there is no epidemiological evidence on the cancer risk in children exposed to APM prenatally, or during adolescence, such studies are vital in light of the findings from two animal studies performed by the RI, both of which included exposure at pre-natal and neo-natal stages. Until such studies are completed, consumers and regulators must bear in mind that the safety of APM remains *sub judice*, despite the controversy stoked by industrial interests. For these reasons we recommend that pregnant women and children should not consume APM and we urge all public health agencies including the International Agency for Research on Cancer, the EFSA and the FDA to re-examine their positions on APM. Finally, we urge NTP to design and conduct a two-sex, two-species bioassays on APM using their in utero exposed model, extending to at least 30-months duration.

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